ABSTRACT

BACKGROUND: Ovarian cancer is the fifth most common cancer among women and it causes more deaths than any other type of female reproductive cancer. In the United States, approximately 22,430 new cases of ovarian cancer are diagnosed annually. Ovarian cancer is a cancerous growth arising from the ovary. Infertile women and those with a condition called endometriosis, those who have never been pregnant and those who use postmenopausal estrogen replacement therapy are at increased risk. Among the causes of cancers, epigenetic change like hypermethylation of tumor suppressor genes has been one of the main routes for the development of cancer. The aim of this study was to identify promoter hypermethylation in CpG islands of RASSF1A gene in ovarian cancer patients among the Kashmiri population.

METHODS: This was a hospital-based case-control study. We analyzed the methylation status of CpG islands in the RASSF1A gene in histopathologically confirmed ovarian cancer samples and histopathologically normal ovarian tissues using methylation specific polymerase chain reaction (MS-PCR). Chi square test with odds ratio was used to ascertain whether the results were statistically significant and a p-value <0.01 was considered statistically significant.

RESULTS: Subjects with histopathologically confirmed ovarian carcinoma (50 cases) and histopathologically confirmed normal ovaries (20 controls) were evaluated. The epigenetic analysis of the cases and controls revealed that the Kashmiri population has a different hypermethylation profile of RASSF1A gene promoter. We found that 66% of the cases had RASSF1A promoter hypermethylation as compared to normal controls in which only 10% were hypermethylated. The association of promoter hypermethylation with ovarian cancer was statistically significant (OR=17.47, 95% CI=3.62-84.32, p=0.0001).

CONCLUSION: The RASSF1A gene promoter is often methylated in ovarian cancer patients of Kashmiri origin. The hypermethylation of RASSF1A may be one of the mechanisms that derive uninterrupted cell division and growth, a hallmark of malignant cells. Whether growth rate of cancer cells is decreased by modulating RASSF1A gene expression with demethylating agents and DNA methylation inhibitors needs further study.

Key Words: Hypermethylation; Ovarian Cancer; RASSF1A gene; Kashmiri; MS-PCR

INTRODUCTION

Ovarian cancer is the fifth most common cancer among women, and it causes more deaths than any other type of female reproductive cancer [1]. In United States, approximately 22,430 new cases of ovarian cancer are diagnosed annually. Estimates indicate that 1 in 70 women will develop ovarian cancer in her lifetime, which accounts for 3.3% of all new cases of cancer [2-5]. The risk for developing ovarian cancer appears to be affected by several factors. Women
with a personal history of breast cancer or a family history of breast or ovarian cancer have an increased risk for ovarian cancer. Older women are at the highest risk for developing ovarian cancer [6]. However, some evidence suggests that the fallopian tubes can also be the source of some ovarian cancers [7, 8]. Use of combined oral contraceptive pills is a protective factor [9-11]. Ovarian cancer can also be a secondary cancer; the result of metastasis from a primary cancer elsewhere in the body (7% of ovarian cancers are due to metastases). Among the causes of ovarian cancers, aberrant promoter hypermethylation of some important genes has been one of the main routes for the development of cancer. DNA methylation is a naturally occurring event in both prokaryotic and eukaryotic organisms. In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA, and in higher eukaryotes DNA methylation functions in the regulation/control of gene expression [12]. It has been demonstrated that aberrant DNA methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis [13]. Promoter hypermethylation is one of the hallmarks of carcinogenesis associated with transcriptional silencing and loss of expression of genes encoding for diverse cellular pathways [14]. Most of the evidence exists for tumor suppressor genes [15]. Hypermethylation leads to silencing of gene transcription through a complex process involving chromatin condensation and histone deacetylation [16, 17]. The susceptible genes are those that are involved in cell cycle regulation (p16INK4a, p15INK4a, Rb, p14ARF), DNA repair (BRCA1, MGMT), apoptosis (DAPK, TMS1), drug resistance, detoxification, differentiation, angiogenesis, and metastasis. Although certain genes such as RASSF1A and p16 are commonly methylated in a variety of cancers, other genes are methylated in specific cancers. A large number of genes are modified in ovarian cancer including RASSF1A [18-21]. Hypermethylation of promoter region of this gene results in the loss of gene expression and lack of the protein product. Due to the absence of proliferation control, the cell continues to grow at an unprecedented rate. Methylation of CpG islands in promoter region of this gene is a common phenomenon in malignancies [22]. Unlike genetic modifications, epigenetic changes are reversible, and are a good therapeutic target. Experiments using cell culture have shown that demethylating drugs can reverse the silencing of genes resulting from methylation. Finding the epigenetic changes may lead to better determination of the scope of the problem as well as in identifying better treatment regimens for patients. While several studies have been performed in various other populations, the prevalence of hypermethylation in RASSF1A gene in Kashmiri population has not been studied. Therefore, we aimed to examine if an association between RASSF1A gene promoter hypermethylation and ovarian cancer exists in Kashmiri population.

**METHODS AND MATERIALS**

The Departmental Ethical Committee at the Government Medical College (GMC), Srinagar, India, approved this case-control study. Subjects with histopathologically confirmed ovarian carcinoma (50 cases) and histopathologically confirmed normal ovaries (20 controls) were evaluated. The ovarian samples were collected from the two GMC-affiliated hospitals (Lal Ded and SMHS) and SKIMS-affiliated hospital (JVC). The diagnosis of ovarian cancer was based on the standard histopathological criteria. A patient was included as a case if she had a histopathologically confirmed ovarian cancer irrespective of cancer stage and patient’s age and was of Kashmiri origin. Patients were excluded if they were suffering from any other major co-morbidity, if they had received chemotherapy or radiation therapy.

**Collection of tissue samples:** The case and control samples were collected in 50 mL plastic containers containing 10 mL of normal saline and were transported from the operation room to the laboratory on ice and immediately stored at -80 °C until further analysis. A part of the specimen underwent histopathological examination before any further sample processing was performed.

**Epigenetic analysis:**

**Extraction of genomic DNA and modification:** For the isolation of genomic DNA, we used Quick- g DNA™ MiniPrep supplied by ZYMO RESEARCH. The integrity of the genomic DNA was examined by gel electrophoresis using 1 % agarose gel. The extracted genomic DNA was modified by EZ DNA Methylation-Direct™ Kit supplied by ZYMO RESEARCH. The modification results in the conversion of unmethylated cytosines to uracil. Methylated cytosines, however, remain unmodified. This
modification helps to differentiate between methylated and unmethylated DNA using specific primers in methylation specific polymerase chain reaction (MS-PCR).

**Methylation Specific Polymerase Chain Reaction (MS-PCR):** MS-PCR is a novel and sensitive way for detection of hypermethylation in CpG islands of DNA. The principle of this PCR method lies in the amplification of the hypermethylated and non-methylated DNA of the same gene by different primer sequence; one for hypermethylated version of the gene and the other for non-methylated version of the same gene. Thus, by visualising the PCR product we can easily determine whether amplification is by hypermethylated or non-methylated primers, hence determine whether CpGs were hypermethylated or unmethylated. The methylated cytosine pairs with guanine and unmethylated cytosine modified to uracil pairs with adenine during annealing.

For MS-PCR, we took modified DNA into two PCR vials in equal quantity and added the same amount of all reagents to both vials except that methylated primers were added in one vial and non-methylated primers were added in the other. The primers used are described by Shohei et al., 2008 and shown in table 1. PCR amplification was achieved using Thermal cycler (Gradient thermal cycler from EPPENDORF MASTERCYCLER PRO). Reactions were hot-started at 95°C for 5 minutes followed by addition of Taq Polymerase. This was followed by 35 cycles of melting (95°C for 45 sec.), annealing (59°C for 45 sec.) and extension (72°C for 45 sec.) followed by final extension step at 72°C for 4 min. Universal Methylated Human DNA Standard and Control with primers (Supplied by ZYMO RESEARCH) was used as positive control, and water was used as negative control. Each PCR product (10 μl) was directly loaded onto non denaturing 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination. The amplified DNA were of the same base pairs in length. Both the methylated and the unmethylated products were 169bp which were then visualized under UV light for the presence of a 100 bp DNA ladder run parallel to the amplified PCR products.

**Statistical analysis:** The $\chi^2$-test with odds ratio was used to examine the association between promoter hypermethylation of RASSF1A gene and ovarian cancer in a case-control study. ORs with 95% CIs were computed using univariable unconditional logistic regression using Graph Pad Prism Software Version 5.0 by Graph Pad Software 2236, Avenida de la Playa, La Jolla, CA 92037, USA.

**RESULTS**

Most women with ovarian cancer were married (n=37). Of the 50 ovarian cancer cases, DNA from 33(66%) was amplified with methylated primers and the rest (34%) with unmethylated primers (Figure 1). Normal ovarian samples (n=20) were taken from subjects in whom oophorectomy was performed for non-malignant diagnoses and resected ovaries were submitted for histopathological confirmation. DNA from only two (10%) controls was amplified by methylated primers during MS-PCR. Presence of methylation in controls may either be normally present or may indicate a premalignant stage at which symptoms had not appeared. The promoter hypermethylation of** RASSF1A gene was significantly associated with ovarian cancer (OR = 17.5, 95% CI = 3.6 to 84.3, p<0.001).

**DISCUSSION**

We found a significant association of hypermethylation of the promoter region of RASSF1A gene and presence of ovarian cancer in Kashmiri women. Our findings confirm that the pathophysiological mechanisms underlying the development of ovarian cancer in Kashmiri women are similar to women in other parts of the
world. We further found a very high prevalence of RASSF1A promoter methylation suggesting that presence of this epigenetic modification may play a large role in the development of ovarian cancer in Kashmiri women.

Ovarian cancer is a common malignancy of the female genital tract. Common risk factors include family history of cancer, personal history of cancer (breast, uterus, colon, or rectum), age > 55 and never pregnant and menopausal hormone therapy. Several genetic and epigenetic modifications have been identified as important mediators in the development of ovarian cancer.

Of these, modifications in the Ras-association domain family (RASSF) proteins, a family of polypeptides initially discovered as Ras-GTP binding proteins and soon thereafter described as products of tumor suppressor genes, have been well-studied [23-26]. RASSF1A gene codes for a protein that binds to cyclin D1 and stops cell cycle at G1/S check point. Loss of RASSF1A gene expression due to methylation allows unhindered cell division potentially resulting in development of malignancy. RASSF1A is silenced through CpG promoter methylation in a wide range of tumors [27, 28]. The role of RASSF1A is confirmed by the presence of increased tumorigenesis in aged mice [28, 29]. RASSF1A, like all members of the RASSF family, contains both a Ras-association (RA) domain and a Salvador/Rassf/Hippo (SARAH) domain located at its carboxyl terminus [30, 31]. The RA domain enables binding to activated ras proteins [32], whereas the SARAH domain allows for interaction with Mst1/2 [33]. Hypermethylation of promoter region of this gene results in the loss of gene expression and the lack of a protein product. We found 2 controls who had hypermethylation in the promoter region of the RASSF1A gene. Studies have shown that hypermethylation can be detected well before subjects developed symptoms or full blown disease. For example, detection of hypermethylation in breast lesion before the clinical diagnosis of breast cancer or in sputum before the clinical diagnosis of lung cancer highlights the potential utility of RASSF1A in early diagnosis [34, 35]. Detection of RASSF1A methylation in peritoneal fluid and serum from ovarian cancer patients with all histological types, grades, and stages of disease, suggests its potential role as a diagnostic marker [36].

CONCLUSION

In conclusion, we found that the RASSF1A promoter hypermethylation is strongly associated with ovarian cancer in Kashmiri women. The promoter hypermethylation of RASSF1A could be or may be one of the reasons that derive uninterrupted cell division and growth, a hallmark of malignant cells. The data gives a clue that whether growth rate of cancer cells is decreased by modulating RASSF1A gene expression with demethylating agents and DNA methylation inhibitors needs further clinical investigation.

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