RESEARCH ARTICLE

Oral Contraceptive Use May Modulate Global Genomic DNA Methylation and Promoter Methylation of APC1 and ESR1

Mostafa Moradi Sarabi[1], Parvin Ghareghani[2], Fatemeh Khademi[3], Fatemeh Zal[3,4]*

Abstract

Background: There are challenging reports in the public health sphere regarding associations between oral contraceptive (OC) use and cancer risk. **Methods:** To evaluate possible effects of OCs on cancer susceptibility, we quantified of global 5-methyl cytosine (5-mC) levels and assessed methylation patterns of CpG islands of two key tumor suppressor genes, APC1 and ESR1, in serum of users by enzyme-linked immunosorbent assay and methylation specific PCR methods, respectively. **Results:** Our results indicated that OCs significantly decrease the level of global DNA methylation in users relative to control non-users. However, our data revealed no significant differences between CpG island methylation patterns for ESR1 and APC1 in healthy control and OC-treated women. However, we did find a trend for hypermethylation of both tumor suppressor genes in OC users. **Conclusion:** Our data suggest that the level of 5-mC but not individual CpG island patterns is significantly influenced by OCs in our cross-section of adult users.

Keywords: Oral contraceptives- global methylation- CpG islands- APC1- ESR1

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Introduction

Oral contraceptives (OCs) are exogenous synthetic sex hormones that used primarily to inhibit pregnancy through disrupt endogenous endocrine function. The association between OCs use and cancer risk is a key challenge in public health and has been controversial (Bethea et al., 2015; Burkman et al., 2004; Gierisch et al., 2013a; Marchbanks et al., 2012). Some studies indicated that the use of OCs is related to decreases in colorectal and endometrial cancers, whereas, other studies claimed that widespread use of OCs appears to be increased risk of certain cancers including breast, cervical and liver cancer (Beaber et al., 2014; Burkman et al., 2004; Cogliano et al., 2005; Gierisch et al., 2013a; Hunter et al., 2010; Rosenberg et al., 2009; Urban et al., 2012). The precise mechanism between OC use and cancer risk have not been cleared, but, it has been found that OCs contain estrogens and progestins which could cause progression of cancer through applicable mechanisms such as increase circulating levels of estradiol, estrogen and progesterone, and promote angiogenesis (Bethea et al., 2015; Gupta et al., 2007; Isaksson et al., 2001; Iversen et al., 2011; Merki-Feld et al., 2012). Other studies represent epigenetic properties of OCs and suggested another potential mechanism that oral contraceptives alter cancer susceptibility through epigenetic mechanisms such as alteration in DNA methylation (Bredfeldt et al., 2010; Campesi et al., 2012b; Starlard-Davenport et al., 2010; Tao and Freudenheim, 2010). DNA methylation is generated by a family of three active DNA methyltransferases (DNMTs) such as DNMT1, DNMT3A and DNMT3B (Sarabi and Naghibalhossaini, 2015). Many studies have indicated that DNMTs are under the regulation of estrogen and progesterone, therefore, DNA methylation may be influenced by oral contraceptives (Yamagata et al., 2009). Furthermore, other new data showed that exposure to synthetic estrogens induce epigenetic modification in the mammary gland and germ cells (Hilakivi-Clarke et al., 2013). These studies also reported that in utero estrogenic exposures modify the epigenome through alteration in DNA methylation (de Assis et al., 2012; Sato et al., 2006, 2009). Alteration in DNA methylation of tumor suppressor genes is a well-established epigenetic mechanism that plays an integral role in cancer etiology and drive tumorgenesis (Baylin, 2005; Belinsky et al., 1998; Esteller et al., 2001; Esteller et al., 2000b; Prosperi and Goss, 2010; Wu et al., 2012). It has been found that in comparison with normal cells, several classical tumor suppressor genes such as adenomatous polyposis coli (APC), and estrogen receptor α (ESR1) to be hyper methylated in cancer (Esteller et al., 2001; Jin et al., 2001; Lehmann et al., 2002; Tao and Freudenheim, 2010). For example, APC promoter hypermethylation has been linked to approximately 70% and 7% of inflammatory and metaplastic human breast carcinomas, respectively (Hayes et al., 2008; Van der Auwera et al., 2008). Furthermore, it has been observed that APC hypermethylation significantly correlate with

*[1]Biochemistry and Genetics Department, Faculty of Medicine, Lorestan University of Medical Sciences, Khorramabad, [2]Islamic Azad University, Science and Research Branch, [3]Biochemistry Depatment, Medical School, [4]Infertility Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. *For Correspondence: fatemehzal@yahoo.com*

cancer survival and its epigenetic silencing, contributes significantly to ER+ and HER2+ breast cancer progression (Prasad et al., 2008; Swift-Scanlan et al., 2011). ESR1 is a key molecular marker to prognosis and prediction of response to endocrine therapy in cancer patients (Martinez-Galan et al., 2014). It is proposed that OC may trigger DNA methylation of the ER genes resulting in impaired estrogen signaling and cancer (Strifert, 2015). Many studies reported a significant association of OC use with increased risk of estrogen receptor positive (ER+) and estrogen receptor negative (ER–) subtypes of breast cancer (Dolle et al., 2009; Ritte et al., 2013; Rosenberg et al., 2010; Rosenberg et al., 2009), while some studies did not observe significant associations of OCs use with either subtype and overall breast cancer risk (Bao et al., 2011; Cotterchio et al., 2003; Hunter et al., 2010; Ma et al., 2010). In breast cancer ER- cases and endometrial cancer tissues, hypermethylation of ESR1 is observed in up to 50% and 94% of cases, respectively, and this cases exhibit a develop resistance to endocrine treatment and will favor to metastasis and death (Johnston et al., 1992; Martinez-Galan et al., 2014; Platet et al., 2004; Sasaki et al., 2001). Therefore, DNA methylation of specific genes is a potential biomarker for many applications and DNA methylation patterns can be used as a surrogate marker for the detection of hidden carcinoma, cancer screening and can also precede tumor formation. In addition to specific gene locus DNA hypermethylation, global genomic DNA hypomethylation was associated with different types of cancer risk through different molecular mechanisms such as microsatellite instability, increased chromosome breakage, loss of imprinting and activation of oncogenes (Choi et al., 2009; Dumitrescu, 2012; Sarabi and Naghibalhossaini, 2015; Szyf et al., 2004; Wu et al., 2012). For this reason and in given the role of OCs on DNA methylation and following previous our studies on OC side effects (Torkzahrani et al., 2014; Zal et al., 2012) in the current study, we have investigated the influence of OCs on APC and ESR1 gene specific and global genomic DNA methylation in serum of OCs exposed mothers.

Materials and Methods

subjects

60 healthy adult women were enrolled and stratified in to two groups: thirty women who did not use oral contraceptives (control) and thirty women who were regular OC users as prevention of pregnancy. The average duration of OC use was 2 to 5 years. All the OC users were taking a contraceptive pill containing 0.03 mg ethinylestradiol and 0.15 mg levonorgestrel 21 days on and 7 days off. The study was approved and performed under the guidelines of the Ethics Committee of Shiraz University of Medical Sciences, and informed consent was and informed consent was obtained from each of the subjects before blood sampling. Baseline characteristics of the subjectsare listed in Table 1.

Genomic DNA preparation

Genomic DNA was extracted from serum of control and OCs users by the standard method of proteinase K digestion, phenol-chloroform extraction and ethanol precipitation.

Methylation specific polymerase chain reaction (MS-PCR)

We determined the status of promoter methylation of APC and ESR1 tumor-related genes in serum by sodium bisulfite chemical treatment of genomic DNA followed by MS-PCR as described (Herman et al., 1996). Briefly, in this technique all un-methylated, but not methylated, cytosines convert to uracil by bisulfite modification. The modified DNA samples were amplified using MS-PCR with specific primers for either the methylated (M) or unmethylated (U) DNA. The primers and PCR conditions for MS-PCR analysis are listed in Table 2. Water without DNA template were included as a control for contamination for each PCR set. In all sodium bisulfite conversion reactions, the universal human methylated DNA standards from Zymo Research (ZYMO Research, Freiburg, Germany) was used as positive methylated controls and DNA from normal lymphocytes was used as negative control for methylated alleles of APC and ESR1. The PCR products were analyzed by electrophoresis on a 1·5% of agarose gel, stained with GelRed (Biotium, Belgium) and visualized under ultraviolet illumination.

Analysis of global DNA methylation

We measured global methylation in DNA isolated from serum of healthy control and OCs users using 5-mC DNA enzyme-linked immunosorbent assay (ELISA) kit (Zymo Research, Germany), as per the manufacturer's instructions. Briefly, 100ng of genomic DNA extracted from serum of OCs users and controls provided by the kit were denatured and used to coat the wells of the plate with 5-mC coating buffer. After incubation at 37 °C for 1h, the wells were washed with 5-mC ELISA buffer and then an antibody mix consisting of anti-5-mC and the horseradish peroxidase (HRP)-conjugated secondary antibody was added to each well. The plate was incubated at 37°C for 1h in the dark. After washing the antibody mix from the wells with the 5-mC ELISA buffer, an HRP developer was added to each well and incubated at room temperature for 1h. The amount of methylated DNA was proportional to the optical density (OD) intensity measured in an ELISA plate reader at 450 nm. The standard curve was constructed with negative and positive controls supplied with the kit, and the 5-mC percentage was calculated using the following equation: 5-mC%= [(OD sample - OD negative control/100ng) / (OD positive control - OD negative control) ×2/5ng]×100; 2 is a factor for normalization of 5-mC in the positive control to 100%, 100ng is the amount of input genomic DNA, and 5ng is the amount of positive control DNA, which contains 50% 5-mC. Three replicates of each sample were measured in duplicate.

Statistical analysis

SPSS 18 analytic software (SPSS, Inc., Chicago) and GraphPad Prism (Version 6·01) were used for data analysis. Depending upon the sample size, associations between clinical, biological and genotypic features were evaluated using either the Chi square test or the Fisher's

exact test. The correlation between OCs user and global DNA methylation was analyzed by Pearson correlation test. Differences with p value ≤0·05 were set as the level of significance.

Results

Analysis of global DNA methylation

In order to measure the levels of global methylation, we quantified the amount of 5-mC in genomic DNA from serum of healthy control and oral contraceptive treated women using ELISA method. Our data showed that the levels of methylated cytosine in healthy control women DNA were significantly higher (1.007) as compared with oral contraceptive treated women (0.979) (p<0·01) (Figure 1). On the other hand, our results indicated that oral contraceptive users had a significantly lower level of genomic global DNA methylation.

CpG islands hypermethylation status of APC1 and ESR1

In addition to the measurement of the global DNA methylation status, CpG island promoter hypermethylation of APC1 and ESR1 genes was analyzed by MS-PCR as described in the section on Materials and Methods. Illustrative examples are shown in Figure 2. Our results showed that 3 of 30 (10.0%) of healthy adult women (control) had APC1 hypermethylation while 4 of 30 (13.33%) of women treated with oral contraceptive had APC1 promoter hypermethylation. Also, 9 of 30 (30.0%) of the healthy adult women (control) had ESR1 hypermethylation and 11 of 30 (36.7%) of oral contraceptive treated women had methylation of ESR1. Although there were no significant differences (p>0.05) between methylation pattern in the healthy control and oral contraceptive treated women for both genes, but ESR1 and APC1 were found to be more methylated (36.7% and 13.33%, respectively) in oral contraceptive treated women compared to control women (30.0% and 10.0% unmethylated, respectively).

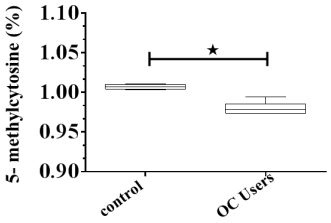

Figure 1. Global DNA Methylation Levels in Serum of Healthy and Oral Contraceptive Treated Women. Percentage of 5-mC was evaluated using ELISA assay. Mean values ±SE of three experiments are given. The asterisk represents significantly different between samples as verified by Mann Whitney t test honestly significant difference comparison test (p<0·05)

Table 1. Baseline Characteristics of Healthy Participant Women

Variable	Control	OC users
age	31.21 (5.3)	30.36 (6.1)
Body mass index (Kg/m^2)	21.6 (2.8)	22.54(3.4)
Blood pressure		
Systolic (mm/Hg)	114 (12)	118 (13)
Diastolic (mm/Hg)	63 (6)	62 (6)

Values are means (SD)

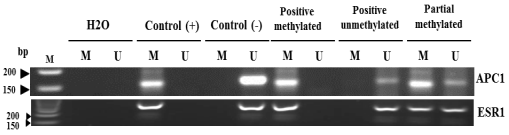

Figure 2. Representative Examples of MSP Products for Ppromoter Methylation Analysis of APC1 and ESR1 Genes in Serum of Healthy Adult and Oral Contraceptive Treated Womens. Universal methylated DNA and un-methylated lymphocytes DNA were used as positive and negative controls, respectively. U, un-methylated genes; M, methylated genes; M, 50 bp DNA size marker

Discussion

Among different contraceptive options, oral contraceptives (OCs) are the most commonly used reversible method of con¬traception in the USA due to effectiveness, convenience and tolerability (Daniels et al., 2013). The relationship between OCs and cancer is a key challenge in public health due to the high prevalence of use of OCs and the serious consequences of cancer as a leading cause of death worldwide. Aberrant methylation, including gene-specific DNA hypermethylation and global genomic hypomethylation can lead to genomic instability, alter gene transcription, and increase mutation rates (Chen et al., 1998) which may impact normal cell growth and increase the likelihood of tumorigenesis (Terry et al., 2011). However, these epigenetic alterations are accepted as playing a fundamental role in cancer, we are still in the early stages of understanding the timing of epigenetic alterations. While an increasing number of studies have

Table 2. Primers'sequence and the Annealing Temperature Used for Methylation-Specific PCR

Gene	Primer sequence (5′ → 3′)	Annealing T (˚C)	Product size (bp)
APC1	UF: 5′-GTGTTTTATTGTGGAGTGTGGGTT-3′ UR: 5′-CCAATCAACAAACTCCCAACAA-3′ MF: 5′-TATTGCGGAGTGCGGGTC-3′ MR: 5′-TCGACGAACTCCCGACGA-3′	63	U: 112 M: 97
ESR1	UF: 5'-ATGAGTTGGAGTTTTTGAATTGTTT-3' UR: 5'- ATAAACCTACACATTAACAACAACCA- 3' MF: 5'- CGAGTTGGAGTTTTTGAATCGTTC- 3' MR: 5'- CTACGCGTTAACGACGACCG- 3	58	U: 151 M: 158

reported the presence of whole genome or gene-specific aberrant methylated DNA in matched samples from tumor tissue and plasma in patients with different types of cancer but not in normal control tissues (Goessl et al., 2000; Sanchez-Cespedes et al., 2000; Silva et al., 1999; Silva et al., 2002), Palmisano et al have found that in lung cancer patients aberrant DNA methylation is detectable as early as 3 years prior to diagnosis in the sputum of subjects exposed to carcinogens (uranium miners and smokers) (Palmisano et al., 2000). Furthermore, in a study of hepatocellular carcinoma, Santella et al. detected changes in serum methylation patterns of RASSF1A, p16, and p15 as much as 9 years prior to diagnosis (Zhang et al., 2007). Therefore, detection of aberrant methylation in serum/plasma DNA of cancerous patients could be a marker of disease (an early neoplastic effect) and in healthy subjects may reflect chronic exposure to carcinogenic factors (Hoque et al., 2006) and these results can support the use of methylation status as a screening biomarker for detection of high risk cases or a diagnostic biomarker for early tumor detection. Among the studies on the global methylation status, Fraga et al demonstrated that in the mouse skin cancer progression model the earliest decrease in global methylation status occurred during the early stages of benign tumor growth and normal mouse skin showed the highest level of methylation (Fraga et al., 2004). In contrast to studies that reported the absence of this epigenetic alteration in normal control samples, our results showed that global DNA methylation was significantly decreased under the effect of OCs even in normal adult users. In close agreement with our results Campesi et al. observed that genome-wide methylation was significantly lower in women treated with OCs for at least 3 months (Campesi et al., 2012a). A possible explanation for genome-wide methylation alterations seen in normal cases is that there may be a threshold for methylation to affect gene expression and lead to a growth advantage. Tumor-suppressor genes are known to be frequently methylated in different malignancies and among these are two important ones APC and ESR1 (Esteller et al., 2000a; Issa et al., 1996; Li et al., 2000; Müller et al., 2003; Xu et al., 2011). Our results showed that there were no significant methylation changes in APC and ESR1 genes between the normal adult OCs users and the control non users. Although we found no significant differences between methylation pattern in the healthy control and oral contraceptive users for both genes, but there were a trend for promoter methylation of ESR1 and APC1 tumor suppressor genes in oral contraceptive users relative to control non users. Since our cases were normal women, our results are in line with previous studies, but several studies have reported the presence of significant methylation in several genes in a small proportion of control subjects (Hoque et al., 2006) or in nonmalignant tissues and serum DNA of smokers (Palmisano et al., 2000). One reason for these conflicting results may be due to the differences between the sensitivity of detection techniques because detection of methylation in circulating DNA depends on the ability of the assay to detect methylated DNA in a background of wild type DNA. Another possible reason for this contradictory may be related to difficulties in assessing the risk of cancer associated with the use of OCs including the long latency period of cancer disease, variations in OCs formulations available on the market, as well as duration and patterns of OCs use over a woman's lifetime. Furthermore, the use of OCs may be influenced by gravidity, parity, breastfeeding and these factors also affect cancer risks (Gierisch et al., 2013b; Moorman et al., 2008). To our best knowledge; this is the first experimental study to examine the association between OCs exposure and methylation status of serum DNA and the most remarkable result to emerge from our data is that OCs usage can affect genome-wide status of methylation. Therefore, based on the fundamental role of epigenetic alterations in cancer, this result may point to the possible involvement of OCs exposure in tumorigenesis. More extensive research with more sensitive DNA methylation detection techniques will require exploring the possible gene-specific methylation changes under the effect of oral contraceptives.

Conflict of interest

The authors have declared that there is no conflict of interest.

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