Mutagen sensitivity as a susceptibility marker for endometriosis

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BACKGROUND: The mutagen sensitivity assay has been well established and widely used as a good independent risk predictor for developing cancers. Although endometriosis is considered a benign disorder, it exhibits several features similar to malignancy. The objectives of this study were to evaluate whether mutagen sensitivity can predict the risk of endometriosis development. METHODS: The subjects were women undergoing different surgical procedures due to different stages of endometriosis. Bleomycin was used as a mutagen, and the mutagen sensitivity of peripheral lymphocytes from women with and without endometriosis was determined by measuring chromatid breaks induced by bleomycin in short-term culture using cytogenetic analysis. RESULTS: The mean ± SD (range) number of chromatid breaks per cell in women with and without endometriosis was 0.68 ± 0.12 (0.50±0.94) and 0.52 ± 0.10 (0.35±0.68), respectively. There was a significant difference with regard to mean chromatid breaks per cell between women with and without endometriosis (P < 0.001). On logistic regression analysis, the odds ratio (95% confidence interval) of chromatid breaks per cell was 5.80 (2.19±15.37, P < 0.001) for cases compared with controls. Yet, variables of interest including age, dysmenorrhea, previous induced abortion and smoking in the home and workplace were not statistically correlated with chromatid breaks per cell. CONCLUSIONS: These preliminary data suggest that sensitivity to bleomycin-induced chromatid breaks in lymphocytes is associated with the risk of endometriosis development.

Key words: bleomycin/chromatid/cytogenetics/endometriosis susceptibility/mutagen sensitivity

Introduction

Endometriosis is a common disease characterized by the growth of endometrial tissue outside the uterine cavity, usually resulting in dysmenorrhea, dyspareunia, pelvic pain and infertility. Retrograde menstruation has traditionally been considered as a common underlying mechanism. However, most women exhibit retrograde menstruation, but relatively few women develop endometriosis. Development of endometriosis in only certain types of women implies individual disease susceptibility. In turn, susceptibility usually indicates genetic factors, one or more of which could act in isolation or interact either with ubiquitous (reflux) factors or exogenous (environmental) factors (Bischoff et al., 2002).

Studies from family investigations have demonstrated a higher incidence of endometriosis among familial cases compared with women without family history (Malinak et al., 1980; Simpson and Bischoff, 2002). Familial tendency for endometriosis suggests an individual genetic susceptibility. Recent studies from molecular cytogenetic analysis have detected abnormal chromosomes including chromosomal gains or losses, loss of heterozygosity (LOH), clonal genetic changes and allelic imbalances in endometriotic cells (tissue) and endometriosis-derived cell lines (Jiang et al., 1998; Gogusev et al., 2000a,b; Sato et al., 2000). These findings indicate that endometriosis can be considered as a polygenic/multifactorial disease with a possible genetic predisposition and with the involvement of environmental toxins, especially dioxins (TCDD) and polychlorodiphenyl compounds (PCBs), in its pathogenesis (Baranova et al., 1997). In turn, research into the association of genetic susceptibility with environmental toxins may be useful to elucidate the undefined aetiology and pathogenesis of endometriosis.

Although chromosome breakage syndromes reflect extreme examples of the susceptibility syndrome, there may be, within the general population, individuals with latent genetic instability that can be unmasked by mutagen challenge in vitro. On the basis of this hypothesis, the mutagen sensitivity challenge assay has been used as a measure of constitutional genetic instability (net results of DNA repair capability and initial genetic instability) (Wu et al., 1998b). Numerous studies have shown that mutagen sensitivity is a good independent risk predictor for developing cancers (Spitz et al., 1993; Wu et al., 1995a). Yet, no correlation between mutagen sensitivity and risk of endometriosis, which exhibits some characteristics reminiscent of malignancy, has been reported.
Materials and methods

Study subjects

Sixty-five subjects who were recruited from lists of upcoming scheduled laparoscopies or laparotomies from December 1999 to April 2002 at the Women’s Hospital, Zhejiang University School of Medicine, Hangzhou, China were included in this case-control study for mutagen sensitivity analysis of endometriosis risk. The case group consisted of patients (n = 46) diagnosed with different stages of endometriosis by clinical, laparoscopic and histological examinations. They had no previous history of any cancer or exposure to hazardous agents, and no family history of endometriosis. Also, they were non-smokers (active smoking) and did not use any sex hormone drugs including oral contraceptive pills. Endometriosis was staged according to the revised American Fertility Society classification (American Fertility Society, 1985), and the endometriosis group was divided into two groups: a minimal/mild group (n = 21) = AFS Stage I plus II, and moderate/severe group (n = 25) = AFS Stage III plus IV. The control subjects (n = 19) comprised 14 women with ectopic pregnancy and five women with ovarian simple cyst, and all had no previous history of any cancer or endometriosis. All control subjects were admitted to the same hospital for surgical resection and had no malignancy or endometriosis confirmed pathologically. These control subjects were matched to the endometriosis patients with respect to active smoking, drug intake, history of exposure to hazardous agents and family history of endometriosis. All study subjects (n = 65) were Han Chinese originated from Zhejiang province of China, and completed a 10 min interviewer-administered questionnaire in which information on personal history, smoking habits, menstrual history, education, place of residence, health and occupational history was assessed. Drug intake, obstetric history, marital history and family history of cancer and endometriosis were also obtained. Consent forms were signed by all participants and the study protocol was approved by the ethnic committee of the Women’s Hospital, Zhejiang University School of Medicine, China.

Cytogenetic analysis

In order to avoid subjective bias, a special technician at the genetics laboratory blind to the status of subjects was required to collect blood from all study subjects and conducted the standard procedure of cytogenetic analysis. First, blood was collected into heparinized tubes, transferred to the genetics laboratory and processed within 24 h. Afterwards, standard lymphocyte cultures were established as described previously (Zych et al., 2000). Briefly, 0.5 ml of whole blood was added to the growth medium consisting of 4.5 ml of RPMI 1640 medium (Gibco) supplemented with 15% fetal calf serum (Gibco), 1.5% phytohaemagglutinin (Gibco) and 100 U/ml each of penicillin and streptomycin (Shanghai Pharmaceutical Co., China). After the cells had been cultured for 3 days at 37°C with 5% CO2, they were incubated for 5 h with 30 ml/ml bleomycin (Chemical and Pharmaceutical Co. Ltd, Japan). To arrest the cells at metaphase, 100 μl of 50 μg/ml colcemid (Sigma, St Louis, MO) was added to the cultures 1 h before harvesting. This yielded cells in metaphase that were damaged by the bleomycin in the late S-G2 phase of the cell cycle. The red blood cells were removed, and the lymphocytes were swollen in hypotonic solution (0.06 M KCl) and fixed in Carnoy’s fixative [3:1 (v/v) methanol:acetic acid]. After dropping the cells onto wet slides, the metaphase spreads were air dried and stained with Giemsa (Merck). All slides were number-matched to all study subjects and read by another experienced technician blind to the status of the subjects. For each subject, chromatid breaks were counted in 50 metaphases and expressed as chromatid breaks per cell. In the process of counting, only frank chromatid breaks or exchanges were recorded; chromatid gaps or attenuated regions were disregarded (Figure 1).

Statistical analysis

Analyses of demographic characteristics, including age, menstrual history, previous abortion, dysmenorrhea, smoking, place of residence and education level, were made using logistic regression in endometriosis cases and controls. The odds ratio (OR) and 95% confidence interval (95% CI) were presented as estimates of the relative risk and the range. The mean age and the mean chromatid breaks per cell were compared using the independent samples test between cases and controls. The correlation between age and chromatid breaks per cell was made using Pearson’s correlation. The effects of previous abortion, dysmenorrhea and smoking in the home and workplace on chromatid breaks per cell were also analysed using independent samples test. All data were analysed statistically using the statistical software package SPSS 10.0. P < 0.05 was considered as statistically significant.

Results

Demographic characteristics of study participants

The demographic characteristics of study subjects are summarized in Table I. Mean age in cases and controls was 40.2 ± 9.0 and 39.8 ± 9.9 years, respectively. There was no significant difference with respect to mean age between cases and controls (t = 0.16, P = 0.876). All study participants were non-smokers, but they were subjected to passive smoking either in the home or in the workplace. Furthermore, nine cases
and five controls were subjected to smoking both in the home and in the workplace. Education level and residence condition were considered as a reflection of the socio-economic difference between cases and controls included in this study. On logistic regression analysis, the differences in all variables of interest including age, menstrual characteristics, previous abortion, smoking, place of residence and education level were not statistically significant, except dysmenorrhoea between the cases and controls ($P = 0.037$, OR (95% CI) = 8.612 (1.140–65.058)).

Chromatid breaks in cases and controls

Chromatid breaks induced by bleomycin in peripheral blood lymphocytes from all the study participants were mostly seen on chromosomes 4 and 5 (Figure 1), although some were also seen on chromosomes 1, 2, 6, 16 and 17. For each subject, chromatid breaks were expressed as the mean number of chromatid breaks per cell. The distribution of individual chromatid breaks per cell in cases and controls is summarized in Figure 2. The mean ±SD (range) number of chromatid breaks per cell in cases and controls was 0.68 ± 0.12 (0.50–0.94) and 0.52 ± 0.10 (0.35–0.68), respectively. There was a significant difference with respect to the mean chromatid breaks per cell between cases and controls ($t = 5.05$, $P < 0.00$). Furthermore, the mean number of chromatid breaks per cell in women with minimal/mild endometriosis (0.65 ± 0.10) and moderate/severe endometriosis (0.72 ± 0.13) was significantly higher than in the control group ($t = 3.85$, 5.30; $P < 0.001$, <0.001). Yet, no statistical difference with regard to mean chromatid breaks per cell was found between women with moderate/severe endometriosis and with minimal/mild endometriosis ($t = 1.94$, $P = 0.059$). On logistic regression model analysis, the OR (95% CI) of chromatid breaks per cell was 5.80 (2.19–15.37) for cases compared with controls ($P < 0.001$).

Effects of variables of interest including smoking, age, dysmenorrhoea and previous abortion on chromatid breaks per cell

In order to confirm whether variables of interest including smoking, age, dysmenorrhoea and previous induced abortion affected chromatid breaks per cell, we used Pearson correlation to analyse the correlation between age and chromatid breaks per cell, and used the independent samples test to compare the differences in mean chromatid breaks per cell between the presence and absence of dysmenorrhoea, previous induced abortion, and passive home and workplace smoking. On

![Figure 1. Bleomycin-induced chromatid breaks in cases and controls. (A) Controls: the arrow indicates the 3q21 break. (B) Cases: arrow 1, a 6q11 break; 2, 4q25; 3, 5q31; 4, 16q22; and 5, 2q33.](image-url)
Pearson correlation analysis, age was not statistically correlated with chromatid breaks per cell ($r = 0.16$, $P = 0.218$). On independent samples test analysis, dysmenorrhea, previous induced abortion, and smoking in the home and workplace were not significantly correlated with chromatid breaks per cell ($t = 0.61, 0.23, 0.87, 1.29; P = 0.541, 0.821, 0.389, 0.201$).

**Discussion**

The objective of the present study was to evaluate whether mutagen sensitivity, determined by measuring the chromatid breaks induced by bleomycin in peripheral blood lymphocytes, can predict the risk of development of endometriosis. The results showed that the mean number of chromatid breaks per cell was significantly higher in endometriosis patients than in controls. On grouping, the mean numbers of chromatid breaks per cell in minimal/mild and moderate/severe endometriosis were also both statistically higher than the controls. This indicates that differences in host factors related to the predisposition to chromosome breakage, or the capacity for DNA repair, or both may be involved in endometriosis development by influencing the susceptibility to environmental toxins (Wu et al., 1995a). Therefore, assessing such differences between individuals may provide new insights into endometriosis research.

There is increasing evidence that endometriosis, like neoplasias, may require the acquisition of somatic genetic alterations for its development. Molecular cyogenetic studies on endometriotic tissues and established endometriosis-derived cell lines have provided new evidence that acquired chromosome-specific alterations may be involved in endometriosis, possibly reflecting clonal expansion of chromosomally abnormal cells (Wu et al., 1995b). Molecular DNA studies examining the role of LOH in endometriotic lesions have identified that candidate tumor suppressor gene loci, including 5q, 6q, 9p, 11q and 22q, may play a role in the malignant transformation of endometriotic implants to endometrioid ovarian cancers (Bischoff and Simpson, 2000). Recent evidence of mutations in the tumour suppressor gene in the endometrioid subtype of epithelial ovarian cancer has shown that somatic genetic alterations represent early events in the transformation of benign endometriotic cells (Thomas and Campbell, 2000). In present study, we found that chromatid breaks induced by bleomycin in peripheral blood lymphocytes were seen mostly on chromosome 4 and 5, although some were seen on chromosome 1, 2, 6, 16 and 17. This result was in agreement with the reported bleomycin sensitivity test (Wu et al., 1995a). It is implied that chromosome breakage depends not only on induced exogenous mutagen but also on individual inheritable susceptibility to environmental toxins.

**Table II.** The results of logistic regression analysis on all variables of interest in cases and controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>B</th>
<th>SE</th>
<th>Wald</th>
<th>df</th>
<th>Sig</th>
<th>Exp(B)</th>
<th>95% CI for Exp(B)</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking in the home</td>
<td>-0.904</td>
<td>1.074</td>
<td>0.709</td>
<td>1</td>
<td>0.400</td>
<td>0.405</td>
<td>0.049</td>
<td>3.323</td>
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<tr>
<td>Education level</td>
<td>0.140</td>
<td>0.561</td>
<td>0.062</td>
<td>1</td>
<td>0.803</td>
<td>1.150</td>
<td>0.383</td>
<td>3.453</td>
<td></td>
</tr>
<tr>
<td>Previous abortion</td>
<td>-0.557</td>
<td>0.697</td>
<td>0.638</td>
<td>1</td>
<td>0.424</td>
<td>0.573</td>
<td>0.146</td>
<td>2.245</td>
<td></td>
</tr>
<tr>
<td>Dysmenorrhea</td>
<td>2.153</td>
<td>1.032</td>
<td>4.356</td>
<td>1</td>
<td>0.037*</td>
<td>8.612</td>
<td>1.140</td>
<td>65.058</td>
<td></td>
</tr>
<tr>
<td>Regularity of cycle</td>
<td>-1.309</td>
<td>1.329</td>
<td>0.970</td>
<td>1</td>
<td>0.325</td>
<td>0.327</td>
<td>0.020</td>
<td>3.655</td>
<td></td>
</tr>
<tr>
<td>Amount of flow</td>
<td>0.042</td>
<td>0.159</td>
<td>0.002</td>
<td>1</td>
<td>0.969</td>
<td>1.043</td>
<td>0.131</td>
<td>8.316</td>
<td></td>
</tr>
<tr>
<td>Chromatid breaks per cell</td>
<td>1.758</td>
<td>0.497</td>
<td>12.517</td>
<td>1</td>
<td>&lt;0.001*</td>
<td>5.802</td>
<td>2.191</td>
<td>15.368</td>
<td></td>
</tr>
<tr>
<td>Age at menarche</td>
<td>-1.530</td>
<td>1.142</td>
<td>1.795</td>
<td>1</td>
<td>0.180</td>
<td>0.217</td>
<td>0.023</td>
<td>2.030</td>
<td></td>
</tr>
<tr>
<td>Length of cycle</td>
<td>0.240</td>
<td>0.897</td>
<td>0.072</td>
<td>1</td>
<td>0.789</td>
<td>1.272</td>
<td>0.219</td>
<td>7.383</td>
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<tr>
<td>Length of flow</td>
<td>1.485</td>
<td>1.988</td>
<td>0.558</td>
<td>1</td>
<td>0.455</td>
<td>4.413</td>
<td>0.090</td>
<td>217.159</td>
<td></td>
</tr>
</tbody>
</table>

*Exp(B) = OR.

*Statistically significant.

Figure 2. Distribution of chromatid breaks per cell in cases and controls.
genes encoding polymorphic metabolic detoxification enzymes including glutathione S-transferase M1 (GSTM1) (Poli et al., 1999; Norppa, 2001), N-acetyltransferase 2 (NAT2) (Norppa, 2001) and CYP 1A1/1A2 (Poli et al., 1999; Abdel-Rahman et al., 2000). In cultured human lymphocytes, the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced a significantly higher level of chromosome aberration in cells with the CYP2E1 WT/*5B genotype compared with cells with the CYP2E1 WT/WT (Abdel-Rahman et al, 2000). The individuals who lack the GSTM1 gene have a higher level of DNA adducts and chromosome damage detected in lymphocytes, and the GSTT1-null individuals have an increased ‘baseline’ level of sister chromatid exchanges (SCEs) in lymphocytes. In addition, subjects having the NAT2 slow acetylator genotype also have an increased baseline frequency of lymphocyte chromosome aberrations (Norppa, 2001). As a result, genetic polymorphisms may be important in explaining individual variation in genotoxic response observed in genetic toxicology tests with human cells. Baranova et al. (1997) reported that endometriosis patients had a higher percentage of null GSTM1 genotype and NAT2 slow acetylator genotypes compared with controls. A recent study of genetic polymorphism analysis has found that the CYP19 VNTR (TTTA)(10) allele as well as the combined genotype CYP1A1 m1 polymorphism and GSTM1 null deletion were associated with the risk of endometriosis development (Arvanitis et al., 2003). In this context, it is speculated that gene polymorphic metabolic detoxification enzymes GSTM1, NAT2 and CYP1 may affect bleomycin sensitivity in short-term cultured lymphocytes from our study participants. Interestingly, Kocabas et al. (2000) reported that null GSTM1 genotype, null GSTT1 and NAT2 slow acetylator genotype associated with the elevated risk of endometriosis development does not influence bleomycin sensitivity. In contrast, genetic polymorphisms of X-ray repair cross-complementing 1 (XRCC1), a DNA repair gene, affect cell sensitivity to bleomycin (Tuimala et al., 2002). Consequently, whether gene polymorphic metabolic detoxification enzymes GSTM1, NAT2 and CYP1 impact on bleomycin sensitivity in patients with endometriosis warrants further investigation. Also, it is indicated that sensitivity to bleomycin may have high heritability, reflecting individual capacity to repair DNA lesions, and endometriosis may arise as result of somatic DNA alterations occurring in a multistep process, analogous to the origin of neoplasias. Recent studies have shown that individuals who smoke heavily and have a history of tobacco dust exposure have an increased percentage of chromosome aberrations in lymphocytes compared with healthy non-smokers (Kao-Shan et al., 1987; Umadevi et al., 2003). In short-term cultured lymphocytes, the mean number of chromatid breaks per cell induced by bleomycin was significantly higher in smokers than in non-smokers (Strom et al., 1995). However, in our current study, all participants were non-smokers, and we did not find any statistical correlations between passive smoking in the home and workplace and chromatid breaks per cell. The possibility that the small sample size and light passive smoking in this study might affect our results should be considered. Since ageing has an important influence on DNA damage and repair due to a gradual accumulation of aberrant cells in both the stem cells of bone marrow and peripheral lymphocytes resulting from a decreased efficiency in the recognition and repair of induced damage or an increase in accumulated exposure to environmental clastogenic agents, the cells from older individuals should exhibit an increase in the amount of chromosome aberrations (Carbonell et al., 1996). However, our data showed no significant correlation between chromatid breaks per cell and age. This discrepancy may be relative to the age distribution selected in our participants. Since endometriosis may arise as a result of somatic DNA alterations occurring in a multi-step process analogous to the origin of neoplasia, it is suggested that patients with late-stage endometriosis might be more sensitive to bleomycin than those with early-stage endometriosis. However, our data did not support this hypothesis. Of course, one possibility is that bleomycin sensitivity may represent an effect rather than a cause of endometriosis. Therefore, further studies are necessary to determine these correlations.

Although some personal lifestyle factors including menstrual characteristics, dysmenorrhoea, previous abortion, age and smoking have been reported to be associated with endometriosis risk (Cramer and Missmer, 2002), only dysmenorrhoea in our study was found to have a higher incidence in cases than in controls when using logistic regression analysis. On further analysis, dysmenorrhoea was not correlated with chromatid breaks per cell when we categorized it as presence or absence. To date, endometriosis is only diagnosed pathologically, and thus it is difficult to recruit control participants. In this study, we used 14 patients with an ectopic pregnancy and five patients with an ovarian simple cyst (cysts with a thin wall and clear liquid) as the control group, and these might not be the ideal control subjects. However, if we use healthy participants as the control group, we cannot exclude endometriosis patients among them without undergoing surgery. Which healthy population participants are most appropriate as the non-endometriosis control group needs further investigation. In any case, our result with regard to mean chromatid breaks per cell in controls agreed with previous reported results (Carbonell et al., 1996). In summary, this was the first study, to our knowledge, to evaluate the correlation between mutagen sensitivity and the risk of development of endometriosis. The preliminary data suggested that sensitivity to bleomycin-induced chromatid breaks in lymphocytes correlated with the risk of endometriosis development. Although the differences between cases and controls were statistically significant, the small sample size necessitates that this finding be validated in a larger study. More data are also needed to determine whether this sensitivity applies to the general endometriosis population.

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References


