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Common chromosomal imbalances and stemness-related protein expression markers in endometriotic lesions from different anatomical sites: the potential role of stem cells

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BACKGROUND: Endometriosis is a multifactorial gynecological disease characterized by the presence of functional endometrium-like tissue in ectopic sites. Several studies have focused on elucidating the immunological, endocrine, environmental and genetic factors involved in endometriosis. However, its pathogenesis is still unclear.

METHODS: High-resolution comparative genomic hybridization was applied to screen for genomic imbalances in laser microdissected stromal and epithelial cells from 20 endometriotic lesions and three samples of eutopic endometrium derived from eight patients. The expression of seven stemness-related markers (CD9, CD13, CD24, CD34, CD133, CD117/c-Kit and Oct-4) in endometrial tissue samples was evaluated by immunohistochemistry.

RESULTS: Samples of eutopic endometrium showed normal genomic profiles. In ectopic tissues, an average of 68 genomic imbalances was detected per sample. DNA losses were more frequently detected and involved mainly 3p, 5q, 7p, 9p, 11q, 16q, 18q and 19q. Many of the genomic imbalances detected were common to endometriotic stroma and epithelia and also among different endometriotic sites from the same patient. These findings suggested a clonal origin of the endometriotic cells and the putative involvement of stem cells. Positive immunostaining for CD9, CD34, c-Kit and Oct-4 markers was detected in isolated epithelial and/or stromal cells in eutopic and ectopic endometrium in the majority of cases.

CONCLUSIONS: The presence of shared genomic alterations in stromal and epithelial cells from different anatomical sites of the same patient and the expression of stemness-related markers suggested that endometriosis arises as a clonal proliferation with the putative involvement of stem cells.

Key words: endometriosis / high-resolution comparative genomic hybridization / chromosomal imbalances / protein expression / stem cells

Introduction

Endometriosis is a chronic, progressive and complex gynecological disease affecting 6–10% of women of reproductive age. The major symptoms include severe dysmenorrhea, dyspareunia, chronic pelvic pain and subfertility (McLeod and Retzloff, 2010).

Endometriotic lesions are characterized by the presence of functional endometrium-like tissue (glandular epithelia and/or stroma)

outside of the uterus. Although benign, this disease could be invasive and comprises a wide spectrum of ectopic lesions in different sites (mainly in peritoneal cavity, ovaries, retrocervical region, bladder, ureter and bowel) varying in severity and histopathological aspects (Giudice, 2010).

It is well known that genetic abnormalities may play a role in endometriosis development modifying stromal-epithelial interactions, peritoneal environment, immune surveillance, cell adhesion and proliferation, apoptosis and angiogenesis (Viganò et al., 2006; Silveira et al., 2009). Comparative genomic hybridization (CGH) is a molecular cytogenetic tool useful for the identification of chromosomal imbalances and has been successfully applied for the detection of alterations in solid tumors, including ovarian and endometrial carcinomas (Kallioniemi et al., 1992). However, CGH data on endometriosis remain controversial (Gogusev et al., 1999; Mhawech et al., 2002).

In addition to the genetic aspects, an increasing interest has been given to the potential role of stem cells in endometriosis development. Stem cells are rare undifferentiated cells present in all adult tissues and organs. These cells retain high proliferative, self-renewal and differentiation potential (Raff, 2003). The number of stem cells in adult tissues is actively regulated through a strict balance between cell proliferation, differentiation and death (Roobrouck et al., 2008). The presence of adult stem cells in the normal endometrium has been reported. Chan et al. (2004) described clonogenic stromal and epithelial cells in the human endometrium, suggesting the involvement of stem cells. Other studies also suggested the presence of stem cells in endometrium by the label-retaining cell methodology (Chan and Gargett, 2006; Cervelló et al., 2007), investigation of clonogenic properties (Schwab et al., 2005), differentiation ability (Kato et al., 2007) and analysis of stemness-related surface cell markers (Forte et al., 2009; Pacchiarotti et al., 2011).

The presence of aberrant stem cells has been associated with the pathogenesis of several tumors and proliferative disorders in the endometrium (Sasson and Taylor, 2008). In particular, some evidence has suggested the involvement of endometrial stem cells in endometriosis development: (i) the monoclonal origin described in epithelial cells from ovarian and peritoneal endometriotic lesions (Jimbo et al., 1997; Tamura et al., 1998; Wu et al., 2003); (ii) the possibility of establishing primary cultures from cell clones derived from endometriotic lesions (Tanaka et al., 2003); (iii) the presence of progenitors cells with high proliferation and differentiation potential in menstrual fluid (Meng et al., 2007).

In the present study, we successfully combined laser capture microdissection (LCM) in formalin-fixed paraffin-embedded samples (FFPE) and high-resolution CGH (HR-CGH) analysis to identify chromosomal imbalances in stromal and epithelial cells from endometriotic lesions located at different anatomical sites. Based on these findings, a set of stemness-related markers was targeted in the immunohistochemical analysis to investigate whether these markers are expressed in ectopic endometria, suggesting the putative role of stem cells in endometriosis pathogenesis.

Materials and Methods

Patients and tissues specimens

Ectopic ($n = 20$) and eutopic ($n = 3$) endometrial tissue samples were obtained from eight patients who underwent laparoscopy at Sirio-Libanês Hospital for diagnostic and treatment purposes. The Human Research Ethics Committee of the Institution has approved the study. The lesions were morphologically classified according to Hoeger and Guzick (1999), based on the variations in the ectopic endometrial structures. The endometriotic lesions were derived from different anatomical sites including the bowel ($n = 10$), ovary ($n = 3$), bladder ($n = 3$), peritoneum ($n = 1$), uterosacral ligament ($n = 1$), Fallopian tube ($n = 1$) and retrocervical region ($n = 1$). With the exception of two patients (Cases 4 and 8), two or more endometriotic lesions located at different anatomical sites were included (Table I).

All patients were evaluated for clinical symptoms and CA-125 serum levels and examined by transvaginal pelvic ultrasound with bowel preparation to evaluate ovarian and deep endometriosis (Abrao et al., 2007). The inclusion criteria were: reproductive age (26–43 years), absence of hormonal therapy for at least 3 months previously to the surgery and absence of any malignancy or clinical signs of rheumatologic or immunologic diseases. The symptoms reported included dysmenorrhea (moderate to severe) ($n = 8$), infertility ($n = 6$), chronic pelvic pain ($n = 8$) and dyspareunia ($n = 7$). All cases presented disease in Stage IV according to the American Society for Reproductive Medicine (ASRM, 1996) revised classification of endometriosis. Clinical information of patients and histopathological characteristics of selected lesions are shown in Table I.

Chromosomal imbalances analysis

Endometrial cells were obtained from stromal and epithelial layers by LCM (Pix Cell II™ system with CapSure™ Macro LCM caps, Arcturus, Inc., CA, USA). After cell digestion, genomic DNA was obtained using QIAamp DNA Micro Kit (Qiagen, GmbH, Germany). HR-CGH was applied using the DNA test and reference amplified and labeled by PCR-based protocols (SCOMP, Single Cell Comparative Genomic Hybridization), as described by Stoecklein et al. (2002). The hybridization and washes were performed as described previously (Ojopi et al., 2002). Chromosomal imbalances were detected by standard reference intervals, as described in Kirchhoff et al. (1998). Superposed chromosomes and heterogeneous hybridization patterns were excluded from the analysis. A library with differentially labeled normal samples (15 health volunteers, 140 metaphases) was constructed to select the upper and lower limits for chromosomal gains and losses (standard reference intervals). The standard reference interval was scaled automatically to fit the individual test case. The description of HR-CGH copy number imbalances was based on the ISCN recommendation (Shaffer et al., 2009).

Immunohistochemistry

To our knowledge, no specific marker has been established for endometrial progenitor cells. Therefore, CD9, CD13, CD24, CD34, CD117/c-kit, CD133 and Oct-4 were selected as markers, based on their involvement in stemness preservation (Satterthwaite et al., 1992; Yin et al., 1997; Belicchi et al., 2004; Cho et al., 2004; Singh et al., 2004; Kim et al., 2011) and their association with mesenchymal-originated cells (Ashmun and Look, 1990; Park et al., 2000).

Protein expression of these stemness-related markers was investigated on 17 ectopic and all three eutopic endometrial tissue samples evaluated by HR-CGH. Coated glass slides containing FFPE tissues were constructed with 3-μm cross-sections for immunohistochemical staining. Exclusively for CD9 marker, slides were treated with 1 mM Tris-EDTA, pH 9.0, buffer for heat-induced epitope recovery. After 18 h of incubation with the primary antibodies (Supplementary data, Table SI), sections were washed in PBS, followed by antibody detection using the streptavidin-biotin system (Advance™ HRP Link and HRP Enzyme-Dako, Carpinteria, CA, EUA). Reactions were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB, *Sigma*, D-5637), and slides were counter-stained with

Table I Clinical and histopathological data of the endometriotic lesions evaluated by HR-CGH and IHC.

Patients	Age at surgery (years)	Menstrual phase at surgery	Anatomical sites	Sample IDs	Histological features	Depth of lesions	ASMR stage
1	39	Secretory	Sigmoid Ovary	1-S 1-O	S + E/DM S + E/DM	Infiltrative Superficial	IV
2	33	Secretory	Rectosigmoid Bladder Rectal	2-RS 2-B 2-R	S + E/DM S + E/D S + E/D	Infiltrative Superficial Superficial	IV
3	43	ND	Sigmoid Ovary	3-S 3-O	S + E/DM S	Infiltrative Superficial	IV
4	28	Menstrual	Peritoneum	4-P	S + E/DM	Superficial	IV
5	26	ND	Bladder Sigmoid	5-B 5-S	S S + E/DM	Infiltrative Infiltrative	IV
6	40	Secretory	Ovary Rectosigmoid (three distinct foci) Uterosacral Fallopian tube	6-O 6-RS (I, II and III) 6-I 6-F	S + E/DM S + E/DM S + E/DM S + E/DM	Superficial Infiltrative Superficial Superficial	IV
7	31	Proliferative	Rectosigmoid Bladder Retrocervical	7-RS 7-B 7-RC	S + E/DM S + E/D S + E/D	Infiltrative Superficial Superficial	IV
8	33	Proliferative	Sigmoid	8-S	S + E/DM	Infiltrative	IV

ND, not defined; S, stromal pattern; E, glandular epithelia; D, differentiated cells; DM, differentiated and undifferentiated cells; ASMR, American Society for Reproductive Medicine.

hematoxylin. Positive and negative controls were included in all assays in accordance with the manufacturer's recommendations. The intensity score was calculated based on staining intensity (0, negative; 1, weak; 2, moderate; and 3, strong immunostaining intensity) and extension (1, ≤1/3; 2, 1/3 to 2/3; 3, >2/3 of the total area). Immunohistochemistry (IHC) scoring analysis was blinded to patient and clinical data.

Results

The eutopic endometrium specimens (stromal and epithelial cells) from three patients showed normal HR-CGH profiles. In contrast, ectopic tissues presented, on average, 68 chromosomal regions involved in gains or losses per lesion. These chromosomal imbalances were not associated with the histological type or the site of endometriotic lesion. Chromosomal losses were more frequent than gains in both stromal (31 chromosomal regions of gains; 45 regions of losses) and epithelial (11 chromosomal regions of gains; 32 regions of losses) components.

A large number of chromosomal imbalances were detected in more than 40% of cases, in both cell components. The comparison between stromal and epithelial chromosomal profiles showed the prevalence of common genomic alterations (Fig. 1 and Table II). DNA losses involved mainly 3p24-p25 (16/20), 5q34 (15/20), 7p14-p21 (15/20), 9p21 (17/20), 11q23-q24 (16/20), 16q22-q23 (16/20), 18q12-q21 (15/20), 18q22-q23 (17/20) and 19q13 (18/20), which were observed in more than 75% of lesions. Common gains were also detected but in lower frequency involving mainly 1q21-q23 (18/20) and 11q12-q21 (19/20).

Although in lower frequency, each cell component also presented exclusive chromosomal alterations. Stromal cells showed gains on 1p, 2q, 3p, 4q, 5, 6, 8q, 9p, 10p, 12 and X and losses on 1p, 3q, 4q, 5p, 5q, 8p, 13q, 14q, 19p and X. Endometriotic epithelial cells showed gains on 4p and losses on 6p and Xq (Fig. 1 and Table II).

Endometriotic lesions located at different anatomical sites from the same patient were compared in a subset of patients with multiple lesions (6 patients, 18 lesions). Common genomic alterations were detected in stromal and epithelial cells from different endometriotic lesions of the same patient in all cases with multiple lesions. A representative case (Case 6), showing the genomic alterations in multiple lesions, is depicted in Fig. 2. The common genetic alterations observed in both cell types, i.e. glandular epithelium and mesenchymal cells (stroma) and among different endometriotic sites (e.g. retrocervical, rectosigmoid and bladder lesions), suggested a clonal origin of the endometriotic cells. In addition, the involvement of stem cells in pathogenesis of endometriosis is one plausible possibility to explain these findings. Thus, to better investigate this hypothesis, stemness-related markers were selected for immunohistochemical analysis in the same ectopic and eutopic endometrial tissue samples (Table III).

Protein expression of CD13, CD24 and CD133 was negative in all the endometriotic and eutopic endometrial tissues evaluated. Particularly, CD13 expression was totally absent in ectopic and eutopic endometrial tissues, although positive membrane immunostaining was observed in positive control samples (human tonsils; data not shown).

In contrast, the positive immunostaining of CD9 and CD34 was detected in the majority of endometriotic lesions as well as in eutopic endometrium. Mild-to-moderate CD9 expression (intensity and extension varying from 1 to 2) was observed in all endometriotic lesions (13/13 samples with appropriate cellular morphology for analysis), specifically on basal cells of glandular epithelia (Table III and Fig. 3). Only one lesion (Case 5-B) presented CD9 positivity in

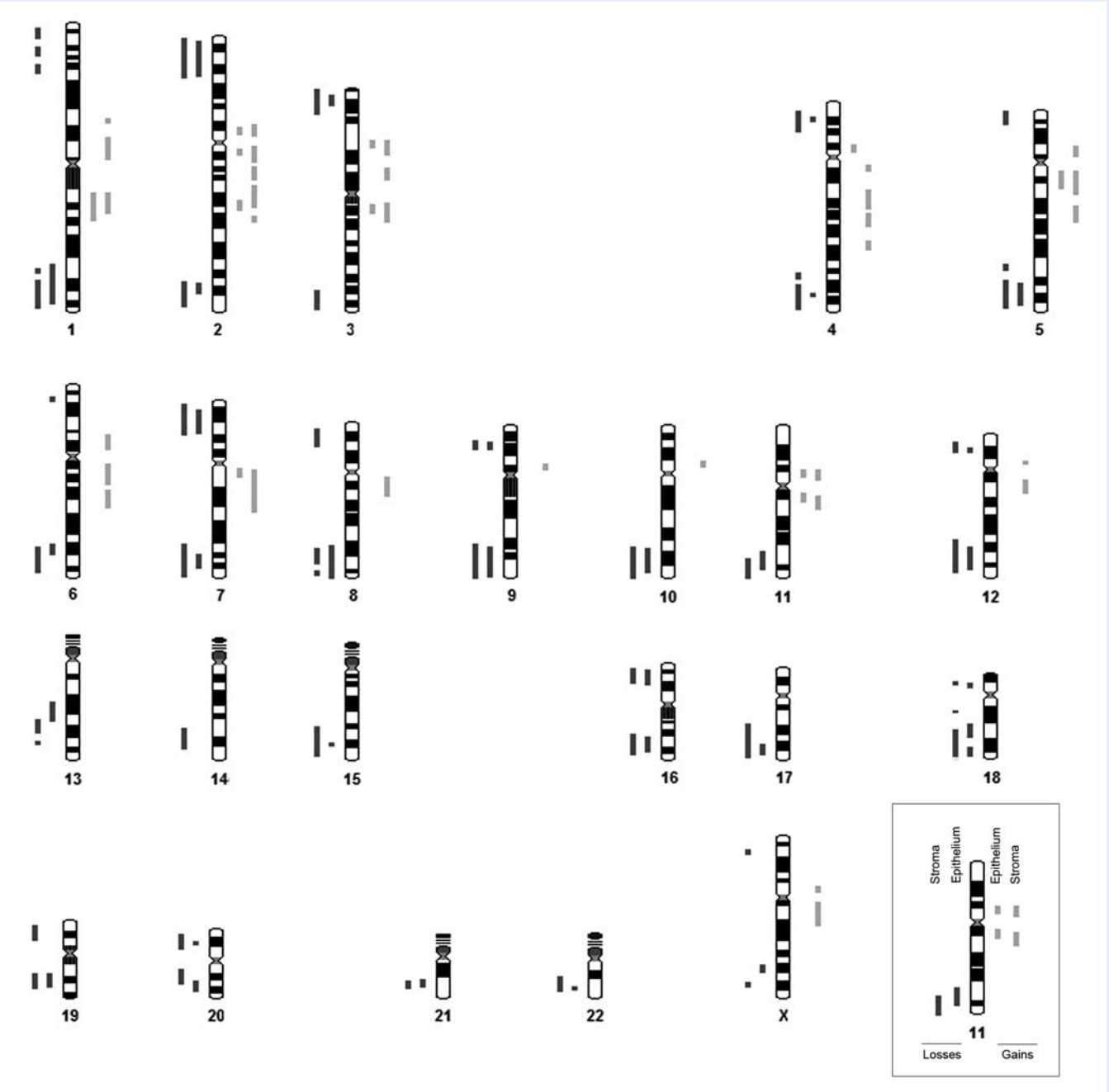

Figure 1 Ideogram representative of minimal chromosomal imbalances detected by HR-CGH in stromal and epithelia cells from all endometriotic lesions evaluated. Dark gray vertical bars on the left side indicate losses and light gray bars on the right side show gains. The bars nearest and most distant to each chromosome represent epithelial and stromal cell components, respectively.

stromal cells from lymph nodes. The positive expression of CD34 was detected in all endometriotic and eutopic endometrium samples evaluated (Table III and Fig. 3C and D). CD34 positivity was observed in isolated cells from the basal layer in all endometriotic lesions with representative and appropriate epithelial component (12/12 samples). In endometriotic stroma, CD34 expression was detected in endothelial cells and showed significantly increased vascular density near to endometriotic foci for all specimens (16/16 samples). A similar pattern of CD34 staining was detected in epithelial (3/3 samples) and stromal (2/3 samples) cell components of eutopic endometrium tissue sections. Interestingly, 12 endometriotic lesions and all three eutopic endometrium samples were positive for both CD9 and CD34 markers (Table III).

CD117/c-Kit and Oct-4 expression were evaluated in six and five endometriotic lesions, respectively (there was no tissue available on other blocks for this analysis) and in two eutopic endometrial samples (Table III). For CD117/c-Kit, positive membrane and cytoplasmatic immunostaining were observed in three of six samples in

Table II Genomic alterations (chromosomal gains and losses) detected in stroma and epithelia derived from different endometriotic lesions evaluated by HR-CGH.

Alterations	Stroma	Epithelia
Gains	**1p12-p21**; **1p22**; 1q21-q23; 2p11.2-p12; 2q11.2-q13; **2q14.1-q14.3**; 2q21-q23; **2q24**; **3p12-p13**; 3p14-p21; 3q12-q13.3; **4q12-q13**; **4q21-q22**; **4q24-q26**; **4q27-q28**; **5p12-p13**; 5q11.2-q13; **5q14-q21**; **6p12-p21.1**; **6q12-q15**; **6q16-q21**; 7q11.2-q22; **8q11.2-q13**; **9p12-p13**; **10p11.2**; 11p11.2-p12; 11q12-q13; **12p11.2**; **12q12-q13**; **Xp11.2**; **Xq12-q21**	1q21-q24; 2p11.2-p12; 2q11.2-q12; 2q22-q23; 3p21; 3q12-q13.1; **4p12-p13**; 5q11.2-q13; 7q11.2; 11p11.2-p12; 11q12-q13
Losses	**1p32-p33**; **1p34.2-p35**; 1p36.1-p36.3; 1q32; 1q41-q44; 2p21-p24; 2q35-q37; 3p23-p26; **3q26.3-q29**; 4p15.1-p16; **4q31.3**; 4q32-q35; **5p15.1-p15.3**; **5q31**; 5q32-q35; 6q24-q27; 7p14-p22; 7q32-q36; **8p21-p23**; 8q23-q24.1; 8q24.2-q24.3; 9p21-p23; 9q31-q34; 10q24-q26; 11q23-q25; 12p12-p13; 12q22-q24.3; 13q22-q31; **13q32**; **14q24-q32**; 15q23-q26; 16p12-p13.3; 16q21-q24; 17q21-q25; 18p11.2-p11.3; 18q12; 18q21-q23; **19p13.1-p13.3**; 19q13.1-q13.4; 20p11.2-p13; 20q11.2-q13.1; 21q22; 22q12-q13; **Xp22.1**; **Xq27**	1q32-q43; 2p21-p25; 2q35-q36; 3p24-p25; 4p15.3; 4q33; 5q32-q35; **6p23**; 6q24; 7p14-p21; 7q33-q35; 8q23-q24.3; 9p21-p22; 9q31; 9q34; 10q24-q26; 11q23-q24; 12p12; 12q23-q24.3; 13q21-q22; 15q25; 16p12-p13.2; 16q22-q24; 17q24-q25; 18p11.2-p11.3; 18q12-q21; 18q22-q23;19q13.1-q13.3; 20p12; 20q13.1-q13.2; 21q22; 22q13; **Xq25**

Chromosomal alterations presented in ≥40% of samples were considered significant and included in the table. The regions altered exclusively in one or the other cell component are in bold.

isolated cells of basal epithelia (Scores 2–3) and rarely in stromal cells (Fig. 3E). The nuclear protein expression of Oct-4 was detected in four of five tissue sections in epithelial component, with moderate-to-strong immunostaining in basal and apical epithelial layers, and on stromal cells of all five endometriotic samples evaluated (Fig. 3F). Three lesions presented positive expression for both markers. In eutopic endometrium, CD117/c-Kit and Oct-4 expression was not observed in any cell component.

Discussion

The novelty of this study is the detection of genomic alterations in endometriosis common to the two cellular components (stroma and epithelium) of multifocal endometriotic lesions derived from a particular patient. In contrast to two other reports showing the low level of chromosomal alterations by conventional CGH (Gogusev *et al.*, 1999; Mhawech *et al.*, 2002), we detected an increased number of chromosomal alterations in both cell components. Indeed, data obtained from CGH assays strictly depend on the composition of the selected biological sample and is greatly enhanced by including a preprocessing step, such as LCM, as described herein.

In agreement with previous reports (Gogusev *et al.*, 1999; Guo *et al.*, 2004), losses predominated over gains in different lesions, suggesting that tumor suppressor gene inactivation is a critical event in endometriosis pathogenesis. It is also noteworthy that these chromosomal imbalances were observed in both endometriotic cell components, suggesting that the perturbation of stromal-epithelial interactions may play a critical role in endometriosis development and maintenance. The interactions between these cell components and their microenvironments are essential for cell growth and differentiation in normal and pathological endometrial tissues (Cooke *et al.*, 1986; Donjacour and Cunha, 1991; Arnold *et al.*, 2001; Witz, 2002; Griffith *et al.*, 2010).

Overall, recurrent chromosomal abnormalities observed in both cell components comprised mainly losses at 3p24-p25 (80%), 5q34 (75%), 7p14-p21 (75%), 9p21 (85%), 11q23-q24 (80%) and 19q13 (90%) and gains at 1q21-q23 (90%). These genomic alterations have been previously described as involved in endometriosis pathogenesis (Jiang *et al.*, 1998; Bischoff and Simpson, 2000; Gogusev *et al.*, 2000; Campbell and Thomas, 2001; Guo *et al.*, 2004; Zondervan *et al.*, 2007; Uno *et al.*, 2010; Painter *et al.*, 2011) and include potential candidate genes associated with cell proliferation control (*GIRK3*, *ATF6* and *DUSP12*, mapped at 1q21-q23; *TGF-β*, on 19q13) and transcriptional regulation (as *PPARG*, mapped at 3p25.1). Allelic losses at 11q23-q24 were formerly reported in endometriosis (Jiang *et al.*, 1998; Campbell and Thomas, 2001) and might be related to reduced progesterone receptor (PGR, mapped at 11q23) activity, which might lead to attenuated or deregulated progesterone response and decreased expression of progesterone responsive genes in eutopic endometrium (Cakmak and Taylor, 2010). In addition, gains at 11q12-q21 detected in 90% of our samples might alter the expression of *CCND1* (Hui *et al.*, 2005) and *CCTN* (Ambrosio *et al.*, 2011) genes, which are important promoters of cell cycle frequently associated with invasion and tumor progression.

An interesting and unexpected finding of the present study was the detection of high frequency of common genetic alterations observed in both cell components of endometriotic lesions as well as among different anatomical sites from the same patient. This finding was observed in all patients with multiple lesions, suggesting a recurrent nonrandom pattern of genomic alterations and an evidence of clonal origin. Although HR-CGH is not the gold standard technique to evaluate clonality, it is well established that the detection of similar genomic profiles in tumors strongly support a clonal relationship among multiple lesions from the same patient (Teixeira and Heim, 2011). In fact, the frequency of chromosome alterations associated with the invasive behavior of endometriosis suggests parallels between

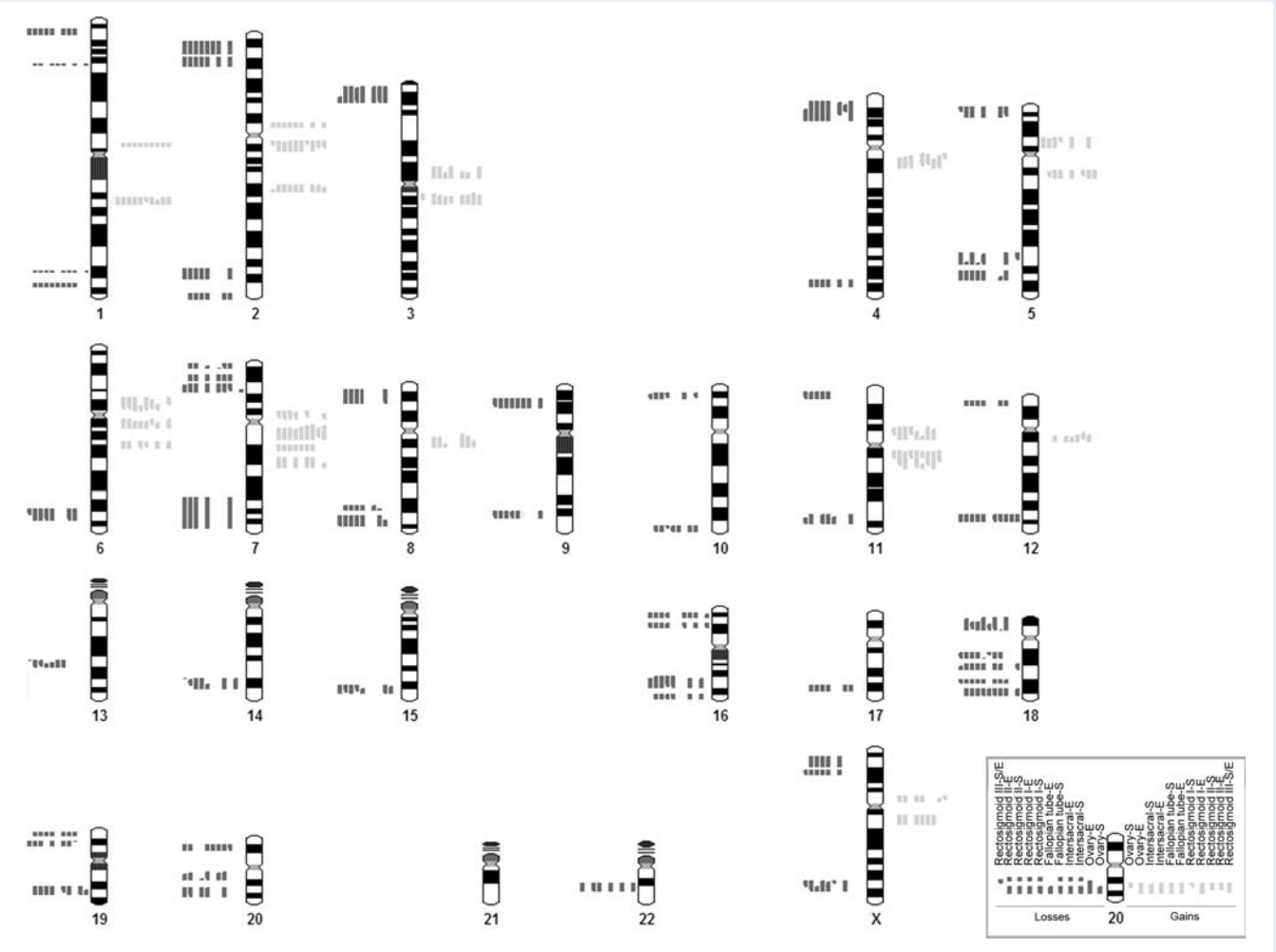

Figure 2 Ideogram representative of minimal chromosomal imbalances detected by HR-CGH in stroma and epithelia from distinct endometriotic lesions from Case 6. Dark gray vertical bars on the left side indicate losses and light gray bars on the right side show gains. Sample disposal per chromosome are depicted. S, stroma; E, epithelia.

endometriosis and neoplasia (Viganò *et al.*, 2006; Silveira *et al.*, 2009). The clonal origin of endometriotic lesions has been long debated and conflicting results have been reported from X chromosome inactivation studies (Nilbert *et al.*, 1995; Jiang *et al.*, 1996; Jimbo *et al.*, 1997, 1999; Tamura *et al.*, 1998; Mayr *et al.*, 2003; Nabeshima *et al.*, 2003; Wu *et al.*, 2003). Nevertheless, potential limitations associated with this approach are well known (i.e. few and different polymorphic markers used) and consequently its findings may be misinterpreted by multiple independent investigators (Teixeira and Heim, 2011). An alternative explanation for our findings is that these alterations could be developed in response to a particular microenvironment, and those cells with advantageous genomic changes could be selected (Koninckx *et al.*, 1998; Bischoff and Simpson, 2000). However, numerous identical abnormalities acquired repeatedly and independently by several cells and at distant lesions from the same patient are highly unlikely to be solely originated by clonal selection.

An emerging hypothesis in endometriosis pathogenesis suggests that stem/progenitor cells can be the primary source of the ectopic endometrium (Sasson and Taylor, 2008; Gargett and Masuda, 2010; Maruyama *et al.*, 2010; Figueira *et al.*, 2011). Therefore, we speculated the involvement of stem cells as a hypothesis to explain the presence of common genomic imbalances detected in endometriotic lesions from different anatomical sites in the same patient. Although there is no direct evidence of the role of stem/progenitor cells in endometriosis, data from experimental studies and the detection of cells with stemness-related properties in both eutopic and ectopic endometrium indicate that this hypothesis cannot be discarded (Sasson and Taylor, 2008; Gargett and Masuda, 2010). Thus, we investigated the expression pattern of stemness-related markers in eutopic and ectopic endometrium tissue samples primarily evaluated by HR-CGH. Since there are no specific known markers for endometrial progenitor stem cells, CD13, CD24, CD34, CD133, CD117/c-Kit and Oct-4 were selected for analysis by being expressed in embryonic and/or adult stem cells. Some of them were previously reported in endometrial cells (Gargett *et al.*, 2007; Gargett and Masuda, 2010; Figueira *et al.*, 2011).

In accordance with previous findings in eutopic endometrial cells (Park *et al.*, 2000; Cho *et al.*, 2004), we detected basal epithelial

Table III Expression pattern of stemness-related markers evaluated by IHC (intensity + extension)[a] in both cell components of endometriotic lesions ($n = 17$) and eutopic endometrium ($n = 3$) derived from seven patients with endometriosis.

Patients	Endometrial tissue samples	Markers							
		CD9		CD34		CD117/c-kit		Oct-4	
		E	S	E	S[b]	E	S	E	S
1	Sigmoid	1 + 2	0	1 + 3	3	–	–	–	–
	Ovary	ICM	ICM	1 + 3	1	0	0	3 + 2	2 + 1
2	Rectosigmoid	ICM	0	1 + 3	1	0	1 + 1	3 + 2	2 + 1
	Bladder	1 + 1	0	1 + 2	3	–	–	–	–
	Rectal	ICM	0	ICM	2	0	0	0	1 + 1
3	Sigmoid	1 + 2	0	1 + 3	3	2 + 2	1 + 1	3 + 3	2 + 3
5	Bladder	1 + 3	1 + 1[c]	1 + 1	1	2 + 2	0	–	–
	Sigmoid	2 + 1	0	1 + 2	3	–	–	–	–
6	Ovary	2 + 1	0	ICM	ICM	–	–	–	–
	Rectosigmoid: focus I	1 + 1	0	ICM	3	–	–	–	–
	Rectosigmoid: focus II	ICM	ICM	3 + 1	2	–	–	–	–
	Rectosigmoid: focus III	1 + 2	0	3 + 2	3	–	–	–	–
	Intersacral	1 + 1	0	3 + 2	3	–	–	–	–
	Fallopian tube	2 + 2	0	ICM	2	–	–	–	–
	Eutopic endometrium	2 + 2	0	3 + 2	2	–	–	–	–
7	Rectosigmoid	1 + 2	0	1 + 3	3	–	–	–	–
	Retrocervical	1 + 1	0	ICM	1	–	–	–	–
	Eutopic endometrium	1 + 1	0	3 + 3	1	0	0	0	0
8	Sigmoid	2 + 1	0	2 + 1	1	2 + 1	1 + 1	3 + 3	2 + 3
	Eutopic endometrium	1 + 2	0	3 + 3	3	0	0	0	0

E, epithelial cells; S, stromal cells; ICM, inappropriate cellular morphology; –, not tested.
[a]Intensity (0, negative; 1, weak; 2, moderate; 3, strong immunostaining) and extension (1, ≤1/3; 2, 1/3 to 2/3; 3, >2/3 of total area).
[b]In this cell component, only CD34 staining intensity was evaluated.
[c]CD9 detected in stromal lymph node cells.

cells expressing CD9 and CD34 markers in endometriotic lesions (13 and 12 lesions, respectively) and endometrium tissue samples independently of menstrual phases. Particularly, CD9 expression has been identified in endometrial progenitor cells isolated from the menstrual fluid (Meng *et al*., 2007) and associated with the endometrial cell progeny that differentiates into epithelial lineage (Kato *et al*., 2007). Similar to our findings in endometriotic lesions, the classic human stem cell marker CD34 (which was also detected in all eutopic and ectopic endometrial stroma analyzed in this study) has been found in the stroma and basal epithelia of endometrium co-expressing with other potential stemness-related cell surface markers (including c-Kit), suggesting the existence of progenitor cells that retain the potential for mesenchymal differentiation in endometrium (Cho *et al*., 2004).

The proto-oncogene c-Kit encodes a tyrosine kinase receptor that interacts with its ligand stem cell factor, also known as Kit ligand, which has multiple functions including stem cell maintenance (Cho *et al*., 2004). Previous studies showed positive c-Kit expression in endometriotic lesions and peritoneal fluid of women with endometriosis and its association with stem cell involvement (Osuga *et al*., 2000; Cho *et al*., 2004). In this study, c-Kit expression was observed in stromal and epithelial cells of three endometriotic samples which also expressed Oct-4, a well-known molecular marker for pluripotent cells (Matthai *et al*., 2006) identified in several cancers and in epithelial cells of normal endometrium (Du *et al*., 2001; Chan *et al*., 2004; Schwab *et al*., 2005; Kato *et al*., 2007; Lynch *et al*., 2007). Interestingly, Pacchiarotti *et al*. (2011) also detected high levels of both c-Kit and Oct-4 markers in ectopic epithelial cells from ovarian and peritoneal implants, further supporting the hypothesis that stem cells might be involved in endometriosis pathogenesis.

In contrast, CD13, CD24 and CD133 markers were not found in any eutopic and ectopic endometrium sections evaluated here. Indeed, the expression patterns of these markers in endometrial cells are not completely known and some related studies have not provided precise data. Although there is one report showing increased CD24 expression in endometrial carcinoma, there is no study evaluating its expression in endometriosis (Kim *et al*., 2009). In addition, CD13 expression in endometrial progenitor cells remains controversial (Park *et al*., 2000; Seli *et al*., 2001; Kato *et al*., 2007; Di Matteo *et al*., 2011). Similarly, the discordant expression pattern of CD133 has been observed in cancer stem cells from different tumors (Bidlingmaier *et al*., 2008; Hermansen *et al*., 2011), though it has been speculated that CD133 may be involved in maintaining stem cell properties (Bauer *et al*., 2008). Thereby, the use of these molecules as stemness-related markers in the eutopic and ectopic endometrium should be cautiously evaluated and further investigated.

Our results suggest that stem cells could be the primary source of ectopic endometrium. However, an intriguing result of this study was the absence of genomic alterations detected in all three eutopic

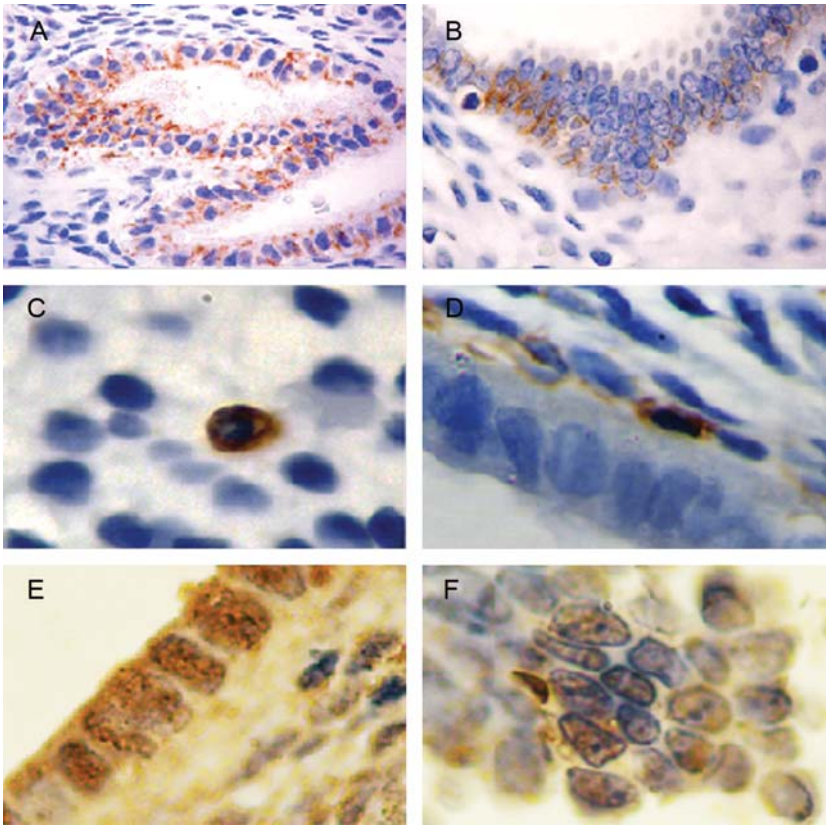

Figure 3 Expression of stemness-related markers detected in endometriotic lesions, varying in intensity (I) and extension (E) according to the marker and/or lesion. (**A** and **B**) CD9 expression in the epithelial layer. Isolated CD34-positive cells (**C**) in stroma and (**D**) epithelia. (**E**) Nuclear/cytoplasmatic expression of CD117/c-kit in the epithelial layer, I2, E2. (**F**) Nuclear expression of Oct-4 in stromal cells, I2, E3 (1000×-IMH).

samples, which also presented positivity to stemness-related markers. This result may give rise to various interpretations. Actually, the involvement of stem cells in endometriosis is compatible with all theories of the cellular origin of ectopic endometrial cells (Sasson and Taylor, 2008).

One possible explanation is that the apparent normal genomic profile in eutopic endometrium observed in our study could be due to the technical limitation of the HR-CGH in detecting those imbalances existing in a restricted number of cells from a tested sample, since the number of stem/progenitor cells is presumably limited compared with ectopic endometrial cells. Indeed, it is reasonable to consider that endometriosis might particularly arise from specific stem cells that possess higher ability to reattach, migrate and invade in an ectopic location. Thus, the ectopic endometrial stem cells may behave differently from eutopic stem cells concerning the cell migration and invasion. In accordance, Kao *et al.* (2011) recently demonstrated that ectopic endometrial mensenchymal stem cells retain a significantly higher migration and invasive abilities *in vitro* and *in vivo,* in comparison with a non-selected population of eutopic stem cells. Despite these differences related to cell migration and invasion, the authors have detected that the phenotypes of eutopic and ectopic endometrial stem cells were largely similar, such as the expression pattern of cell surface markers including CD9 and CD34, also found in our study. From these results, the authors pointed out that these similarities could indicate a common origin of the two types of endometrial stem cells (Kao *et al.*, 2011).

Alternatively, extra-uterine stem cells derived from bone marrow or other sources (e.g. stem cells persisted in the remnants of the Müllerian system) could be involved in the ectopic endometrium origin, supporting the theory of celomic metaplasia. Conversely, it is also likely that normal endometrial stem cells can simply be implanted on an abnormal peritoneal mesothelium (Sasson and Taylor, 2008). Moreover, clones derived from endometrium could acquire genomic alterations during menstrual or peritoneal passage increasing the abilities to spread and attach in an ectopic site resulting in endometriotic lesions in different anatomical sites. This hypothesis has been speculated in tumor metastatic event, especially in those cases with delayed clinical relapse (Korkaya *et al.*, 2011).

In conclusion, the present study showed a high frequency of non-random genomic alterations in endometriotic lesions suggesting a common origin for multiple endometriotic lesions found in the same patient. Recurrent genomic gains and losses were detected in regions of putative oncogenes and tumor suppressor genes associated with cell proliferation, apoptosis, inflammatory response and stromal–epithelial interactions; such genes may thus play a role in growth and surveillance of ectopic endometrium. In addition, it was shown the

presence of stem cell markers in multiple endometriotic lesions, suggesting the involvement of stem cells in endometriosis pathogenesis.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

C.G.T.S. was involved in the study conception and design, collection and assembly of data, data analysis and interpretation and manuscript writing. M.S.A. and J.A.D. were responsible for sample collection and follow-up of the patients. R.A.C., F.A.S and M.A.C.D. played a role in histopathological analysis, laser capture microdissection procedures and data analysis. S.A.D. played a role in data analysis and interpretation and in critical review of the manuscript. S.R.R. was involved in the study conception and design, study supervision, administrative support, data analysis and interpretation as well as manuscript writing. All authors have approved the final version of the manuscript.

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Conflict of interest

None declared.

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