Lack of Evidence That Male Fetal Microchimerism is Present in Endometriosis

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Abstract

Introduction: Fetal microchimerism has been implicated in the etiology of autoimmune diseases. This study was done to test the hypothesis that male fetal microchimerism is present in eutopic and ectopic endometrium (EM) obtained from women with endometriosis but not in eutopic EM from women without endometriosis. **Methods:** A total of 31 patients were selected, including women with endometriosis (paired eutopic and ectopic EM; n = 19) and women without endometriosis (eutopic EM; n = 12). Tricolor interphase fluorescence in situ hybridization analysis was performed by cohybridization of CEP Y SpectrumAqua and CEP X SpectrumGreen (SG)/CEP Y SpectrumOrange probes. **Results:** Ectopic EM from women with endometriosis had 75% XX chromosomes (double SG signals) and 25% X chromosomes (single SG signal). Y chromosomes were not observed in any of the eutopic/ectopic endometrial tissues from cases or controls. **Conclusions:** We were unable to confirm our hypothesis that male fetal microchimerism is present in eutopic and/or ectopic EM obtained from women with endometriosis.

Keywords

endometrium, FISH, male fetal microchimerism

Background

Extrauterine endometrial implants are the hallmark of endometriosis and occur in 15% of reproductive-aged women resulting in dysmenorrhea, menstrual irregularities, chronic pelvic/back pain, and reduced fecundity. Theories on etiology of disease include retrograde menstruation, coelomic metaplasia, altered immunity, stem cells, and genetics.¹ Retrograde menstruation of viable endometrial cells that implant and grow at ectopic sites is Sampson's postulation for the histogenesis of endometriosis² and the most widely accepted theory. Since retrograde menses³ and exfoliated endometrial cells⁴ in peritoneal fluid can be found in most cycling women, there must be supplementary factors that predispose some women to perseverance of endometriotic lesions within their peritonea. The pathogenesis of endometriosis is complex and likely represents the interaction and combined effects of several pathways: hormonal, cell adhesion, angiogenic, and immunological mechanisms. Approximately 20 years ago, Weed and Arquembourg⁵ observed immunoglobulin G and complement deposits in the eutopic endometrium (EM) of women with endometriosis. These investigators hypothesized that an autoimmune endometrial mechanism might explain poor reproductive outcomes in

some women with this syndrome. A decade later, Mathur et al⁶ were the first to describe autoantibodies in patients with endometriosis that recognize endometrial antigens that are candidates for the autoantigens responsible for this immune response. Gleicher and colleagues⁷ noted that a significant proportion of women with endometriosis

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(40%-60%) has elevated autoantibody titers when tested against a panel of autoantigens. Thus, in addition to the development of specific anti-endometrial antibodies, a generalized polyclonal B-cell autoimmune activation may be associated with some cases of endometriosis,⁸ supporting clinical observations that women with endometriosis may have an increased risk to develop autoimmune diseases like systemic lupus erythematosus.⁹ Endometriosis shares the following characteristics with common autoimmune diseases such as rheumatoid arthritis, Crohn's disease, and psoriasis: increased inflammation, elevated levels of tissue remodeling components, altered apoptosis, and increased local levels of cytokines.¹⁰ Increased numbers of activated macrophages and lymphocytes can be found in women with endometriosis^{11,12} and may contribute to the implants' pathophysiology.^{13,14} It has been proposed that soluble secreted factors from these cells play a role in the pathogenesis of endometriosis and its sequelae.

Various fetal cell types can be found in the maternal circulation: fetal lymphocytes, granulocytes, nucleated red blood cells, and trophoblast cells.¹⁵ In fact, fetal cells persist in the maternal circulation for decades after pregnancy transfer.¹⁶ Fetal microchimerism, the migration of fetal cells into maternal tissue during pregnancy, has been implicated in the etiology of autoimmune diseases. The prevalence of DNA microchimerism Y-chromosome analyses has been reported to be higher in women with autoimmune diseases like scleroderma¹⁷ and Hashimoto's thyroiditis^{18,19} than in healthy controls (Table 1).

Fetal microchimerism may be involved in the pathogenesis of endometriosis by initiating a graft-versus-host reaction. Fetal DNA microchimerism may also play a fundamental role in the recruitment and activation of peritoneal macrophages and lymphocytes into the peritoneal milieu. Moreover, the transferred fetal cell may have progenitor properties and thus be a stem cell for future population in the EM. Scientists have studied the possibility that bone marrow-derived cells may also differentiate into endometrial cells,^{1,30} and pertinently, may be implicated in the development of ectopic endometrial implants.¹ This stem cell theory can also explain how ectopic tissue can occur in locations outside the peritoneal cavity such as the lung and central nervous system.¹

This study was done to test the hypothesis that male fetal microchimerism is present in eutopic and ectopic EM obtained from women with endometriosis but not in eutopic EM from women without endometriosis. This hypothesis was based on data from a prior small pilot study in 7 women where we had observed that 1 of the 3 women with endometriosis revealed Y-chromosome microchimerism in an endometriotic lesion, Y positive patient: XX(419) X(80) Y(1) whereas Y chromosomes were absent in peritoneal or endometrial biopsies from 4 controls without endometriosis.³¹ A representative image of fluorescence in situ hybridization (FISH) performed on an endometrial implant specimen is depicted in Figure 1.³¹

Materials and Methods

Sample Selection

Male fetal microchimerism can be detected by the presence of a Y chromosome in tissue samples from women with and without endometriosis who previously delivered a son prior to surgery. Samples stored at the biobank of the Department of Histopathology were selected according to the following criteria: from women who previously delivered a son prior to surgery and the histological confirmation of endometriosis. In total, 50 samples from 31 patients were selected, including women with endometriosis (cases; paired eutopic and ectopic EM; $n = 19$ women, and 38 samples) and women without endometriosis (controls; eutopic EM; $n = 12$ women, and 12 samples). Of 31 women, 23 women (17 cases and 6 controls) were on hormonal treatment. Sample demographics are shown in Table 2. Control samples were included in each FISH experiment. Male bowel sections were used as positive controls (XY). Endometrial slides without XY probes were used as negative controls. The slides were blinded for Dr Fassbender who analyzed the slides.

Fluorescence In Situ Hybridization

To assess microchimerism, paraffin-embedded specimens were cut into 3- μ m sections and put on positively charged Superfrost plus slides (Menzel-Gläser, Braunschweig, Germany). Tricolor interphase FISH analysis was performed using CEP Y (DYZ1, satellite III) SpectrumAqua (SA) and CEP X (DXZ1) SpectrumGreen (SG)/CEP Y (DYZ3; α satellite) SpectrumOrange (SO) probes (Abbott Molecular Inc, Des Plaines, Illinois; Table 3), using standard procedures. The cohybridization of probes combined in 1 mixture enabled the detection of both chromosomes, X and Y, in 1 experiment. In addition, adding 2 differentially labelled chromosome Y probes to the hybridization mixture allows more reliable detection of the Y chromosome, excluding mistakes due to artifacts associated with possible nonspecific probe hybridization.

In short, first the slides were kept at 37°C overnight, followed by placement in xylene twice for 10 minutes, and consecutive 3 hydration steps (96% ethanol twice for 10 minutes, then 70% ethanol for 5 minutes, and finally distilled H₂O for 5 minutes). After drying on a shaker for 5 minutes, slides were incubated for 1 hour with SPOT LIGHT TISSUE PRETREATMENT (Sanbio, Mountain View, California) solution at 95°C to 100°C, followed by double washes in phosphate-buffered saline (PBS) for 5 minutes. Afterwards, slides were dried again and sections were digested with enzyme reagent (SPOT-LIGHT tissue pretreatment kit [Sanbio] in humidified boxes at 37°C for 30 minutes. After 2 subsequent washes in PBS for 5 minutes, slides were dehydrated in ethanol series (70%, 90%, and 96% for 2 minutes each), and air dried on a shaker. The X and Y probes were applied on tissue sections, which were covered with appropriate cover slips and sealed with rubber cement. Probes denaturation was performed on the heating plate at 85°C for 4 minutes. Hybridization was carried out overnight at 37°C in a humidified box. On the

Table 1. Summary of Fetal Microchimerism Presence (Positive Y Chromosomes) in Women With Autoimmune Diseases.

Reference	Disease —Assay—	Tissue/Blood	N Cells Counted	Y in Cases	Y in Controls	P
Artlett et al ¹⁷	Systemic sclerosis —PCR—	Blood (n = 69) Skin biopsy (n = 19)	—	32/69 (46%) 11/19 (58%)	1/25 (4%) 0/68 (0%)	<.001 <.001
Miyashita et al ²⁰	Systemic sclerosis (SSc), systemic lupus erythematosus (SLE), Sjögren's syndrome (SS) —PCR—	Peripheral blood SSc (n = 20), SLE (n = 21), SS (n = 18), controls (n = 41)	—	10/20 SSc (50%), 6/18 SS (33%) 0/21 (0%) SLE	8/41 (20%)	.017
Vietor et al ²¹	Intrauterine transfusion (IUT) —PCR and FISH—	Peripheral blood mononuclear cells (n = 9)	3000	6/7 PCR (0.1%) FISH (n = 7)	0	—
Klitschar et al ¹⁸	Hashimoto's thyroiditis —PCR—	Thyroid gland (n = 17)	—	8/17 (47%)	1/25 (4%)	<.05
Srivatsa et al ¹⁹	Thyroid disorders —FISH—	Thyroid gland (n = 29)	—	16/29 (55%)	0/8 (0%)	0.005
Kuroki et al ²²	Sjögren's syndrome —PCR and FISH—	Peripheral blood mononuclear cells (PBMC; n = 27) Labial salivary glands (LSG; n = 42) Bronchoalveolar lavage fluid (BALF; n = 56)	3000	PCR 10/28 (36%) LSG 2/9 (22%) BALF 0/22 PBMC FISH 3/3 LSG 19/28 (68%)	PCR 0/10 LSG 0/15 BALF FISH 0/1 LSG 6/18 (33%)	.028 (PCR) .034
Filho et al ²³	Systemic lupus erythematosus (SLE) —PCR—	Peripheral mononuclear blood cells n = 28 Healthy controls (n = 18) Skin punch biopsies (n = 5) Healthy female controls (n = 10)	—	FISH 1/5 (20.0%) affected skin 4/5 (80%) unaffected skin PCR 4.73 cells/100 µg DNA affected skin vs unaffected skin 12.49 cells/100 µg DNA	FISH and PCR 0/10 (0%)	.0037 (unaffected vs controls; FISH) 0.4 (unaffected vs affected; PCR)
Sawaya et al ²⁴	System sclerosis (SSc) —PCR and FISH—	Liver samples (n = 27) Male controls (n = 4)	—	FISH 1/4 fulminant hepatitis 2/10 chronic hepatitis 5/6 acute liver disease PCR 2/4 fulminant hepatitis 7/10 chronic hepatitis 5/6 acute liver disease	PCR results 2/7 with normal liver	—
Guettier et al ²⁵	Woman's age comparison —PCR and FISH—	Thyroid tumor tissue (n = 63)	Up to 2 million	PCR 19/40 (47.5%) FISH 1.8 to 7.8 per section (n = 4)	PCR 0/23 FISH 0 to 1.5 per section	.02 (FISH)
Cirello et al ²⁶	Papillary thyroid cancer —PCR and FISH—	Synovial tissue (ST) Skin fibroblast (SF; n = 10)	—	5/13 (38.5%) ST 4/10 (40%) SF	—	.77
Hromadnikova et al ²⁷	Rheumatoid arthritis —PCR—	Peripheral blood lymphocytes RS (n = 12) PBC (n = 11) SSc (n = 12) Controls (n = 24) Rheumatoid nodule (RN; n = 33)	—	RS 2/12 PBC 4/11 SSc 2/12 6/33 RN (18%) 6/29 patients (21%)	Controls 7/24	No statistical difference
Svryrd et al ²⁸	Reynolds syndrome (RS), primary biliary cirrhosis (PBC), systemic sclerosis (SSc) —PCR—	Rheumatoid arthritis —PCR—	—	—	—	—
Chan et al ²⁹	Rheumatoid arthritis —PCR—	—	—	—	—	—

Abbreviations: PCR, polymerase chain reaction; FISH, fluorescence in situ hybridization.

Figure 1. Fluorescence in situ hybridization (FISH) is used to detect XY cells in endometrial implant of 1 woman having endometriosis. The nuclei are stained blue with 4',6-diamidino-2-phenylindole (DAPI), hybridization to the X and Y chromosomes appears as green and red signals, respectively (100× magnification). Arrow indicates red dot (presence of Y chromosome). (The color version of this figure is available in the online version at <http://rs.sagepub.com/>.)

Table 2. Clinical Characteristics of the Study Population.

	Control	Endometriosis
Sample size		
Eutopic endometrium	12	19
Ectopic endometrium	—	19
Age, mean $\pm$ SD	32 $\pm$ 3.13	35 $\pm$ 4.86
Medication	6	17
Stages	—	
I		4
II		4
III		4
IV		7
Cycle information		
Regular	8	7
Irregular	2	1
Absent	—	6
Unknown	2	5
Pain	10	17
Infertility		
No	1	5
Secondary	10	10
Unknown	1	4
Endometrioma	—	2

Abbreviation: SD, standard deviation.

Table 3. Detailed Description of the Probes.

Probe Name	Probe Location	Fluorophore
CEP X	Xp11.1-q11.1 Alpha Satellite DNA	Spectrum Green
CEP Y	Yp11.1-q11.1 Alpha Satellite DNA	Spectrum Orange
CEP Y	Yq12 Satellite III DNA	Spectrum Aqua

second day, the rubbercement and the cover slips were carefully removed to avoid damage of the tissue. Washes were performed in $0.4 \times$ saline-sodium citrate (SSC)/0.3% nonyl phenoxypolyethoxyethanol (NP40) solution at 73°C for 5 minutes, followed by $2 \times$ SSC/0.1% NP40 solution at room temperature for 1 minute and $2 \times$ SSC at room temperature for 5 minutes, and finally air dried on the shaker. 4',6-Diamidino-2-phenylindole of 30 μ L was added onto each slide and covered with a cover slips and left on heating plate at 37°C for a few minutes. The slides were kept in the fridge till analysis.

Quantification of XY Chromosomes

Five hundred cells were counted for each specimen on Zeiss Axioplan 2 imaging microscope with 100× oil immersion objective using CytoVysion (version 4.5.3.9, Leica, Germany). Meta-systems Isis software (version 5.4.9 V3.10) was used for imaging. Total numbers of XX and XY cells were quantified manually. The entire tissue section was scanned for male fetal microchimerism using different fluorescence (SA, SO, and SG) filters.

Results

By FISH analysis, the male bowel control had 92% XY (SG + SO + SA signals), 7% X (single SG), and 1% XX (double SG) chromosomes (Figure 2 and Table 4). In comparison, ectopic EM from women with endometriosis had 75% XX chromosomes (double SG signals) and 25% X chromosomes (single SG signal; Figure 3 and Table 4). Y chromosomes were not observed in any of the eutopic EM tissues from cases or controls.

Discussion

In this study, we were unable to confirm our hypothesis that male fetal microchimerism is present in eutopic and ectopic EM obtained from women with endometriosis. Our hypothesis that male fetal microchimerism can play a role in the pathogenesis of endometriosis can be challenged because of the propensity for endometriosis women being nulliparous and thus prior transfer of fetal cells would be unlikely without previous exposure to pregnancy. However, 4 reasonable explanations for this conundrum of microchimerism in the setting of infertility or teenage endometriosis are as follows: (1) an unrecognized miscarriage could be a source of fetal cells in a woman³²; (2) transfer of foreign DNA from sexual intercourse^{33,34}; (3) maternal microchimerism wherein allogenic cells were transferred at the time of fetal life from the participant's mother,^{35,36} and (4) the endometriosis seen in these women may have a different etiology. Interestingly, significant fetal cell transfer has been observed during miscarriage and termination of pregnancy by Peterson and coworkers.³² Studies exploring fetal microchimerism in autoimmune diseases such as thyroid, rheumatoid arthritis, systemic sclerosis, and systemic lupus erythematosus have shown the presence of fetal microchimerism^{17-19,20,22-24,26,27,29,37} except in 1 study exploring patients with systemic lupus erythematosus (Table 1).²⁰

Figure 2. FISH is used to detect XY cells in male bowel tissue. The nuclei are stained blue with 4',6-diamidino-2-phenylindole (DAPI), hybridization to the X and Y chromosomes appears as green (X) and red (DYZ3) as well as aqua blue (DYZ1) signals, respectively (63× magnification). A, Phase contrast image without DAPI. B, Image including DAPI. Arrow indicates red and aqua blue hybridization signals (presence of Y chromosome). (The color version of this figure is available in the online version at <http://rs.sagepub.com/>.)

Table 4. Results of the Male Fetal Microchimerism.

Groups	XX	XX (%)	X	X (%)	XYY	XYY (%)	XY	XY (%)
Average male bowel tissue control	5	1	33	7	460	92	2	0
Ectopic lesion (EL) Stage I	305	61	195	39	0	0	0	0
EL Stage I	386	77.2	114	22.8	0	0	0	0
EL Stage I	392	78.4	108	21.6	0	0	0	0
EL Stage I	358	71.6	142	28.4	0	0	0	0
EL Stage II	383	76.6	117	23.4	0	0	0	0
EL Stage II	363	72.6	137	27.4	0	0	0	0
EL Stage II	386	77.2	114	22.8	0	0	0	0
EL Stage II	425	85	75	15	0	0	0	0
EL Stage III	296	59.2	204	40.8	0	0	0	0
EL Stage III	369	73.8	131	26.2	0	0	0	0
EL Stage III	433	86.6	67	13.4	0	0	0	0
EL Stage IV	364	72.8	136	27.2	0	0	0	0
EL Stage IV	379	75.8	121	24.2	0	0	0	0
EL Stage IV	332	66.4	168	33.6	0	0	0	0
EL Stage IV	401	80.2	99	19.8	0	0	0	0
EL Stage IV	312	62.4	188	37.6	0	0	0	0
EL Stage IV	434	86.8	66	13.2	0	0	0	0
EL Stage IV	409	81.8	91	18.2	0	0	0	0
Average EL Stage I-IV	374	75	126	25	0	0	0	0

When critically reading the articles on autoimmune diseases and fetal microchimerism, one can conclude that there is evidence for fetal microchimerism in autoimmune diseases. However, further studies are needed with larger cohorts to confirm these results since most of the published studies had small samples size^{18,22,23,24,27,21,25,28} and/or did not show positive results.^{20,27,29,28} Interestingly, in 1 study FISH results could only partially confirm the results of the PCR.²⁵

In our study, we did not find any male fetal microchimerism present in women with endometriosis compared to controls. From our study results, there is lack of evidence to confirm the involvement of male microchimerism as a nidus for autoimmunity or the stem cell theory in the pathogenesis of endometriosis.

The strengths of our study were the well-defined study population, optimized FISH procedure (optimization through a small pilot study on probe concentration, washing steps, and incubation time), scanning entire tissue for male fetal microchimerism, blinded study design, and use of positive and negative controls during FISH. In our male control, we observed 92% of XY and 1% false positive (double X chromosomes; possible artifact) as well as 7% loss of 1 chromosome (possible “cut-off” effect). Our observation of 1% false positive XX and 7% X chromosome in male bowel and 25% X (0 presumably) in endometriosis cases (summarized in Table 4) is probably related to technical aspects of our methodology. Some targets (X or Y or both) for hybridization were likely cut off during sectioning of the blocks (cut-off

Figure 3. Fluorescence in situ hybridization (FISH) is used to detect XY cells in an endometrial implant of 1 woman having endometriosis. The nuclei are stained blue with DAPI, hybridization to the X and Y chromosomes appears as green and red (DYZ3) as well as aqua blue (DYZ1) signals, respectively (63× magnification). A, Phase contrast image without 4',6-diamidino-2-phenylindole (DAPI). B, Image including DAPI. The pictures indicate only X and XX chromosomes present (see arrow, green dots; 63× magnification). (The color version of this figure is available in the online version at <http://rs.sagepub.com/>.)

effect), especially since our sections were as thin as 3 µm, explaining why it is impossible to have 100% of cells with complete XY or XX hybridization pattern in our experimental setting.

A limitation of our study was the lack of an extra negative control using Y chromosome FISH probes on sections from women who never had a male offspring or other pregnancy. Another limitation of our study is that microchimerism could be missed since a limited number (500) of cells was counted, particularly because fetal cells have been reported to be present in very low numbers in adult tissues.³² In other studies, most researchers did not specify how many cells exactly were counted (Table 1), with a range varying between 1000 up to 2 million cells. For example, in 1 report the authors admit the limitation of their analysis only to 4 of 63 specimens in patients with thyroid tumors (Table 1) since it was too time consuming to quantify XY cells in all samples.²⁶ Finally, microchimeric retention of foreign XX (daughter, mother, or grandmother) cells may be better tolerated immunologically than heterologous XY cells.

Conclusions

We were unable to confirm our hypothesis that male fetal microchimerism is present in eutopic and ectopic EM obtained from women with endometriosis.

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Authors' Note

Van Bree prepared and blinded the slides. Fassbender analyzed all slides and wrote the manuscript. Fassbender, Debiec-Rychter,

Meuleman, Tomassetti, Peeraer, Lebovic, and D'Hooghe, Vermeesch were involved in study design, interpretation, and manuscript writing. In addition, Debiec-Rychter acquired FISH images. All authors read and approved the final manuscript. Thomas D'Hooghe, and Dan Lebovic are Joint last authors.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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