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INVESTIGAÇÃO DO PERFIL DE METILAÇÃO DE GENES CANDIDATOS A BIOMARCADORES NA ENDOMETRIOSE

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Se...

Se eu pudesse deixar algum presente a você,

deixaria aceso o sentimento de amar a vida dos seres humanos.

A consciência de aprender tudo o que foi ensinado pelo tempo afora...

Lembraria os erros que foram cometidos para que não mais se repetissem.

A capacidade de escolher novos rumos.

Deixaria para você, se pudesse, o respeito àquilo que é indispensável:

Além do pão, o trabalho.

Além do trabalho, a ação.

E, quando tudo mais faltasse, deixaria um segredo:

O de buscar no interior de si mesmo

a resposta e a força para encontrar a saída."

Mahatma Gandhi

SUMÁRIO

RESUMO

A endometriose é uma doença crônica multifatorial, inflamatória, estrógeno-dependente, que afeta entre 8 a 10% das mulheres em idade reprodutiva e caracterizada por tecido similar ao endométrio (glândula e estroma) fora da cavidade uterina. Embora a sua etiologia seja pouco conhecida, evidências recentes indicam que alterações epigenéticas estão envolvidas na sua patofisiologia. Esta hipótese é apoiada pelos achados envolvendo padrões alterados de metilação do DNA em genes específicos, bem como pelos níveis alterados de expressão de componentes da maquinaria epigenética nas lesões endometrióticas, quando comparadas ao endométrio eutópico da mesma paciente ou ao endométrio de mulheres que não apresentam a doença. Assim, um melhor entendimento do papel dos mecanismos epigenéticos na patogênese e progressão da endometriose tem se tornado necessário, podendo contribuir para a identificação de marcadores diagnósticos e para o delineamento de novas abordagens terapêuticas, que trariam benefícios e melhor qualidade de vida para as mulheres que apresentam esta condição. Neste contexto, este estudo buscou a identificação de genes diferencialmente metilados candidatos a biomarcadores na endometriose. Os resultados obtidos foram organizados em dois artigos científicos. O primeiro artigo teve como objetivo investigar o perfil diferencial de metilação do DNA na endometriose intestinal em comparação com amostras pareadas de endométrio eutópico da mesma paciente. Para isso, foi empregada uma abordagem em larga escala baseada em microarranjos contendo 27.800 ilhas CpG, que resultou na identificação de 546 genes hipermetilados e 871 genes hipometilados. A análise *in silico* para a classificação funcional dos genes diferencialmente metilados permitiu identificar conjuntos de genes com funções de fatores de transcrição, remodeladores da cromatina entre outras classes funcionais. Após a realização de uma avaliação comparativa com dados disponíveis na literatura de outros estudos em larga escala, foi possível a identificação de alterações recorrentes envolvendo 227 genes hipermetilados e 322 genes hipometilados. Dentre estes, 52 genes hipermetilados e 53 hipometilados foram correlacionados com níveis diferenciais de expressão. Após uma análise dos processos biológicos envolvidos por estes genes, foi possível identificar vias biológicas específicas que podem ser importantes no desenvolvimento das lesões endometrióticas como as relacionadas à mecanismos moleculares do câncer e pluripotência de células-tronco embrionárias e vias de sinalização Notch, relaxina e TGF-beta. Uma avaliação pelas metodologias de *Methylation-Specific Restriction Enzyme – real time PCR* (MSRE-PCR) e sequenciamento do DNA modificado pelo bissulfito de sódio permitiu a confirmação do ganho de metilação das ilhas CpGs localizadas na região promotora

dos genes *HOXA7 (homeobox A7)* em 5/10 casos e *HOXD4 (homeobox D4)* em 8/10 casos corroborando os achados obtidos. Dois genes adicionais, *BCOR (BCL6 co-repressor)* e *AKT1S1 (AKT1 substrate 1 (proline-rich))* não foram confirmados como diferencialmente metilados. O segundo artigo científico teve como objetivo identificar o padrão de metilação do DNA dos promotores alternativos dos genes codificadores de receptores esteróides *ESR1 (estrogen receptor 1), ESR2 (estrogen receptor 2)* e *PGR (progesterone receptor)* na endometriose intestinal, pela metodologia de *Methylation-Specific PCR* (MSP-PCR). Para os genes *ESR1* e *ESR2*, não foi identificada metilação diferencial. Ganhos de metilação do DNA na endometriose foram identificados em 39% (17/44 casos) e 19% (7/37 casos) para os promotores associados aos transcritos *PGRA* e *PGRB*, respectivamente. Em 3 casos, a metilação anormal foi detectada em ambos os promotores do gene *PGR*. No conjunto, nossos resultados permitiram uma melhor caracterização molecular da endometriose, pela identificação de um perfil epigenético consistente e robusto entre diferentes estudos, sugerindo um conjunto de genes diferencialmente metilados potencialmente relevantes como novos biomarcadores da doença.

ABSTRACT

The endometriosis is a multi-factorial and chronic disease affecting 5% to 10% of women in reproductive age characterized by endometrium-like tissue (gland and stromma) outside uterine cavity. Although its etiology is poorly understood, recent evidences have indicated that epigenetic alterations are implicated in the pathophysiology. This hypothesis has been supported by findings surrounding altered DNA methylation pattern of specific genes, as well as by altered levels of expression of epigenetic machinery components in endometriotic lesions compared to eutopic endometrium from the same patient or endometrium of women free of disease. Thus, a better understanding of the role of epigenetic mechanisms in the pathogenesis and progression of endometriosis has become necessary and may contribute to the identification of diagnostic markers and for designing new therapeutic approaches that could benefit and improve the quality of life of women with this condition. In this context, this study aimed to identify differentially methylated genes as candidate biomarkers in endometriosis. The results could be organized in two papers. The first study aimed to investigate the profile of differential DNA methylation in intestinal endometriosis compared to eutopic endometrium of paired samples from the same patient. For this, we carried out a large-scale approach based on microarray containing 27,800 CpG islands, resulting in the identification of 546 genes as hypermethylated and 871 genes as hipomethylated. In silico analysis for the functional classification of differentially methylated genes enabled us to identify sets of genes with functions of transcription factors, chromatin remodeling, besides other functional classes. After conducting a comparative assessment with data available in other large-scale studies of literature, it was possible to recognize recurrent alteratons evolving 227 hypermethylated and 322 hypomethylated genes. Of these, 52 hypermethylated and 53 hypomethylated genes were correlated with differential levels of gene expression. After an analysis of the biological processes involved in these genes, it was possible to identify specific biological pathways that could be important for the development of endometriotic lesions such as related to molecular mechanisms of cancer, human embryonic stem cell pluripotency, Notch signaling, relaxin signaling and TGF-beta signaling. Further assays by *Methylation-Specific Restriction Enzyme – real time PCR* (MSRE-PCR) and bisulfite sequencing confirmed the gain of methylation of CpG islands mapped in the promoter region of genes *HOXD4* (homeobox D4) in 5/10 cases and *HOXA7* (homeobox A7) in 8/10 cases corroborating those findings. Two additional genes, *BCOR* (BCL6 corepressor) and *AKT1S1* (BCL6 corepressor), were not confirmed as differentially methylated. The second scientific paper aimed to identify the pattern of DNA

methylation of alternative promoters of genes encoding steroid receptors *ESR1* (estrogen receptor 1), *ESR2* (estrogen receptor 2) and *PGR* (progesterone receptor) in intestinal endometriosis by Methylation-Specific PCR (MSP-PCR) methodology. The differential methylation was not identified for the *ESR1* and *ESR2* genes. Gains of DNA methylation in endometriosis were identified in 39% (17/44 cases) and 19% (7/37 cases) for transcripts-linked promoters *PGRA* and *PGRB* isoforms, respectively. In 3 cases, abnormal methylation was detected in both promoters of *PGR* gene. Altogether, our results had allowed a better molecular characterization of endometriosis by identifying a consistent and robust epigenetic profile between different studies, suggesting a set of differentially methylated genes potentially relevant as novel biomarkers of disease.

CAPÍTULO 1

1. INTRODUÇÃO

1.1 Endometriose

A endometriose constitui-se de uma doença ginecológica considerada enigmática e que afeta de 8 a 10% das mulheres em idade reprodutiva. Caracteriza-se pela presença de tecido similiar ao endométrio (glândula e estroma) fora da cavidade uterina, sendo encontrada primariamente na cavidade e órgãos abdominais e pélvicos, como peritônio, ovários, tubas uterinas, superfície externa do útero e membranas e ligamentos do útero e pelve. Menos comumente, as lesões endometrióticas podem invadir a parede do reto, bexiga, intestino ou apêndice. Raramente, a endometriose se desenvolve em sítios externos ao trato reprodutivo, tais como pulmões, cérebro e tecidos periféricos como o nervo ciático (Giudice e Kao, 2004).

Os implantes ectópicos de tecido endometrial permanecem responsivos à estímulos hormonais (Kitawaki et al., 2002), estimulam a angiogênese (Girling e Rogers, 2005), podem ser altamente invasivos e, ainda, podem resultar em adesões e distorções da cavidade pélvica, fatores que podem explicar as principais queixas clínicas apresentadas pelas pacientes com endometriose: dor pélvica crônica, dismenorréia (dor pélvica durante menstruação), dispareunia (dor durante relação sexual) e infertilidade (Wu et al., 2007a); esta última ocorrendo em 30% a 50% das mulheres com endometriose. Ainda, há sintomas decorrentes da localização da lesão como alterações intestinais e urinárias, principalmente durante o período menstrual.

A apresentação e evolução da doença é variável; em alguns casos pode permanecer como mínima ou leve, ou pode, inclusive, desaparecer. Outros casos, entretanto, podem apresentar sintomatologia mais severa decorrentes da invasão e infiltração tecidual, crescimento de endometriomas ou cistos ou adesões pélvicas severas que podem afetar outros órgãos (Acién e Velasco, 2013). Em mulheres assintomáticas, a prevalência de endometriose é de 2% a 22%. Essa incidência aumenta para 25% a 50% em mulheres com infertilidade e para 40% a 60% em mulheres que apresentam dismenorréia (Farquhar, 2007).

Além disso, a severidade dos sintomas e a probabilidade do diagnóstico aumentam com a idade, sendo que o pico de incidência se dá ao redor de 40 anos de idade (Farquhar, 2007). Entre os principais fatores de risco dessa doença podem-se citar a menarca precoce e menopausa tardia, ciclos menstruais curtos e intensos e nuliparidade (Campbell e Thomas, 2001). Geralmente, a doença é resolvida quando a menopausa ocorre, devido à diminuição nos níveis de estrógeno. Entretanto, esta doença pode ser reativada em mulheres após a menopausa quando hormônios endógenos ou iatrogênicos estão presentes (Acién e Velasco, 2013).

Apesar dos avanços na compreensão da doença, a endometriose permanece significativamente de difícil diagnóstico, já que não apresenta sinais ou sintomas patognomônicos. A hipótese diagnóstica inicialmente é elaborada baseando-se na história clínica da paciente. Ultrassonografia transvaginal e ressonância magnética (RMI) podem também auxiliar; entretanto, o diagnóstico mais acurado é alcançado após cirurgia laparoscópica seguido da análise histopatológica das lesões. De acordo com Abrao et al. (2007), a ultrassonografia transvaginal é capaz de detectar 98% das lesões localizadas na região retosigmóidea e 95% das lesões retrocervicais. Da mesma forma, a RMI mostra 83% de sensibilidade para a detecção da endometriose retosigmóidea e 76% para as retrocervicais. Embora as concentrações de CA125 (*Cancer Antigen 125*, uma glicoproteína com expressão elevada em carcinomas ovarianos e outras doenças) possam estar levemente aumentadas em mulheres com endometriose, a dosagem desse antígeno não é considerada útil no estabelecimento do diagnóstico (Farquhar, 2007).

O tratamento atual inclui terapia medicamentosa e/ou cirúrgica, sendo a última o procedimento de escolha. Entretanto, o risco de recorrência após a cirurgia é elevado: 7-30% após três anos. Este risco aumenta para 40-50% cinco anos após a cirurgia. Assim, terapia medicamentosa adjuvante pós-cirúrgica torna-se também necessária (Guo, 2009).

Como a endometriose é uma doença dependente de estrógeno, o tratamento atual tem como objetivo a modulação do controle hormonal do ciclo menstrual para produzir uma pseudo-gravidez, pseudo-menopausa ou anovulação crônica, criando um ambiente hipoestrogênico, acíclico e, consequentemente, a atrofia dos implantes endometrióticos. Para isso, a terapia padrão pode incluir agentes androgênicos, progestágenos, antiestrogênicos ou agonistas do GnRH (*Gonadotropin-Releasing Hormone*) em combinação com analgésicos e antiinflamatórios não esteroidais. Entretanto, tais tratamentos são controversos porque, embora possam melhorar a dor ou infertilidade, não curam a doença (Acién e Velasco, 2013). Além disso, podem ter uma duração curta devido aos efeitos colaterais hipoestrogênicos substanciais como fogachos, vagina ressecada e perda da densidade mineral óssea. Além disso, a recorrência de dor pélvica ocorre em 30-70% das pacientes entre 6 a 18 meses depois da descontinuação do tratamento (Hassan et al., 2009).

A endometriose é classificada em quatro estadiamentos, de acordo com a *American Society for Reproductive Medicine* (1997): forma mínima (estadio I), leve (estadio II), moderada (estadio III) e severa (estadio IV). Essa classificação mundialmente aceita baseia-se em um sistema de pontos aplicados após a avaliação de critérios como: diâmetro, localização, aparência e quantidade de lesões, presença de processos aderenciais, tempo de recorrência, taxa de efeitos adversos ao tratamento, predição sobre a probabilidade de gravidez, facilidade da intervenção cirúrgica (ranqueada como fácil, moderada, difícil ou muito difícil), entre outros.

Uma classificação histológica adicional das lesões endometrióticas foi proposta por Abrao et al. (2003), de acordo com o grau de diferenciação do epitélio glandular e quanto à presença de estroma:

A) Padrão glandular – presença de epitélio superficial ou constituindo espaços glandulares ou císticos, associado a estroma e/ou sinais de hemorragia prévia, caracterizada pela presença de hemossiderófagos, fibrose e angiogênese. Este

padrão é ainda subclassificado segundo a similaridade com o epitélio endometrial eutópico nas seguintes formas:

A.1 Bem diferenciado – células epiteliais apresentam morfologia indistinguível do endométrio eutópico nas diferentes fases do ciclo;

A.2 Indiferenciado – composto por células epiteliais não endometrióides, podendo apresentar-se como epitélio pavimentoso, assemelhando-se ao mesotélio do revestimento peritoneal, ou com epitélio mülleriano distinto do endometrióide, como seroso ou mucinoso

A.3 Diferenciação mista – presença, na mesma localização, de padrões glandulares diferenciados e indiferenciados.

B) Padrão estromal – presença de estroma morfologicamente similar ao do endométrio eutópico em qualquer fase do ciclo;

Segundo os autores (Abrao et al., 2003) o padrão glandular indiferenciado foi mais frequentemente associado aos estadios III e IV e em relação a dor, as pacientes que apresentaram padrão bem diferenciado ou estromal responderam melhor ao tratamento. Adicionalmente, Kamergorodsky et al. (2009) avaliaram a correlação entre o padrão de diferenciação histológica e o grau de infiltração da lesão. Os autores demonstraram que as lesões endometrióticas com padrão indiferenciado apresentam características mais invasivas.

1.1.1 Endometriose profunda intestinal

Embora a endometriose possa acometer diferentes regiões externas ao útero, ao microscópico, todas pertencem a uma única entidade, ou seja, glândulas endometriais e estroma de localização extra-uterina (Fauconnier e Chapron, 2005). Esse tecido ectópico pode apresentar alterações cíclicas, nos quais as glândulas mostram uma atividade proliferativa mínima ou uma transformação secretória inadequada. Esta atividade é decorrente da expressão

de receptores específicos de estrógeno e progesterona, com uma distribuição similar ao endométrio eutópico, embora em menor concentração e com ausência da expressão dos receptores de progesterona beta (PGRB). Sua resposta à estimulação hormonal é, assim, variável (Acién e Velasco, 2013).

Macroscopicamente, as lesões endometrióticas podem apresentar-se em duas entidades clássicas, bem diferenciadas: adenomiose (ou endometriose interna) e endometriose (ou endometriose externa). A adenomiose ocorre quando focos endometrióticos infiltram a parede muscular externa do útero. O adenomioma, por sua vez, corresponde a uma área da adenomiose que é encapsulada no tecido miometrial. A endometriose externa, ou simplesmente endometriose, está presente quando focos ectópicos endometriais estão localizado em outras regiões fora do útero, sendo a pélvica, a mais comum (Acién e Velasco, 2013). A endometriose pélvica pode ser subdividida, ainda, em três apresentações diferentes: endometriose peritoneal superficial, endometriose ovariana e endometriose profunda.

A doença peritoneal se caracteriza pela presença de implantes superficiais no peritônio; a ovariana inclui os implantes superficiais de ovário e os cistos típicos da doença, os endometriomas. A endometriose profunda é caracteriza pela presença de lesões que penetram 5mm ou mais na superfície peritoneal e acomete as regiões retro-cervical e para-cervical, o septo reto-vaginal, o retossigmóide, os ureteres e a bexiga, sendo considerado o tipo mais severo e incapacitante (Cornillie et al, 1990; Koninckx and Martin 1992; Koninckx et al, 1994; Kamergorodsky et al, 2009).

A frequência da endometriose intestinal pode variar entre 3,7 e 35% dos casos de endometriose (Remorgida et al., 2007). Quanto à localização, acomete geralmente a porção retossigmóide do intestino; no entanto, múltiplos nódulos podem ser encontrados no ceco e no íleo distal. Nestes casos, a endometriose pode causar obstrução intestinal ou intussuscepção

devido à estenose da válvula ileocecal, sendo necessário cirurgia de emergência (Indraccolo et al, 2010).

Adicionalmente, o diagnóstico clínico e cirúrgico é complexo, sendo os nódulos endometrióticos da parede intestinal de difícil visualização por exames de imagem, devido principalmente aos altos níveis de motilidade da região (Kinkel et al, 2006). Os principais sintomas deste tipo de endometriose são dores pélvicas, constipação e/ou diarréia recorrentes, flatulência, distensão abdominal e tenesmo. Nos casos em que há acometimento da mucosa ou submucosa intestinal ocorre hematoquezia (hemorragia) periódica de intensidade variável, principalmente acompanhando o período menstrual, podendo também ocorrer sangramento anal isolado (Garcia et al, 2006).

1.1.2 Origem dos implantes endometrióticos: das teorias às bases moleculares

A origem dos implantes endometriais e a patogênese da doença têm sido uma área de constante investigação. Embora, isoladamente, nenhuma das teorias propostas possam explicar todos os casos de endometriose, a teoria de Sampson (1927a) ou implantação metastática é a de melhor aceitação. Esta teoria propõe que fragmentos de endométrio menstrual passariam, por um fluxo menstrual retrógrado, através das tubas uterinas e se implantariam na cavidade abdominal. Esta teoria é apoiada por achados nos quais mulheres com endometriose apresentavam grandes volumes de refluxo menstrual em relação ao grupo controle sadio (Giudice e Kao, 2004). No entanto, considerando-se que: (i) a menstruação retrógrada ocorre em 76 a 90% das mulheres, (ii) células endometriais viáveis são detectadas no fluido peritoneal (Kruitwagenn et al, 1991) e, (iii) a prevalência da doença é muito inferior a estes números, acredita-se que possam existir outros mecanismos envolvidos na viabilidade dos implantes (Seli et al, 2003).

Uma derivação desta teoria, também proposta por Sampson (1927b) e chamada de teoria da metástase linfovascular, argumenta que o tecido menstrual da cavidade endometrial poderia se dirigir a outros sítios pelos vasos sanguíneos e linfáticos. Em contraste, a hipótese da metaplasia celômica (descrita em 1919 por Meyer) propõe que as lesões endometrióticas dentro da cavidade peritoneal se originariam pela diferenciação de células mesoteliais em tecido semelhante ao endométrio (Gruenwald, 1942). Finalmente, uma última hipótese propõe que células-tronco circulantes, originadas na medula óssea e que contribuiriam para a repopulação das células endometriais no útero, seriam as responsáveis pelo desenvolvimento de implantes endometrióticos ectópicos (Sasson e Taylor, 2008). Neste contexto, Figueira et al. (2011) sugeriram que células-tronco progenitoras ou adultas residentes na cavidade uterina e responsáveis pela alta capacidade regenerativa do endométrio, seriam capazes de gerar tecido endometrial fora da cavidade uterina, quando as mesmas alcançassem a cavidade pélvica após o refluxo menstrual.

Alguns autores também têm considerado uma associação entre alterações endócrinas, imunológicas e inflamatórias do peritônio pélvico, as quais podem ocasionar liberação de prostaglandinas e permitir a ocorrência de processos aderenciais com variados graus de distorção (Crosera et al., 2010). No entanto, os mecanismos moleculares envolvidos permanecem pouco conhecidos. Alguns estudos têm demonstrado que a origem dos implantes endometrióticos pode ser resultado da interação entre fatores genéticos e ambientais, após observação da frequência aumentada da doença em mulheres que foram expostas à toxinas ambientais ou desreguladores endócrinos como o dietiletilbestrol (Bulun, 2009). Neste contexto, alguns estudos apontaram anormalidades nas enzimas de detoxificação, as quais poderiam aumentar a suscetibilidade aos estímulos ambientais (Giudice e Kao, 2004).

Quanto ao envolvimento genético, alguns trabalhos têm mostrado que a incidência da endometriose entre parentes de mulheres afetadas é até sete vezes maior que a incidência em

parentes de mulheres não afetadas (Bulun, 2009). No entanto, estudos de associação genética e envolvendo gêmeos têm relatado poucas alterações em loci cromossômicos específicos, ou envolvendo genes candidatos com potencial biológico (Burney et al., 2013).

Atualmente, diferenças moleculares específicas entre o tecido endometriótico e o endométrio eutópico, tais como os elevados níveis de estrógeno, prostaglandinas e citocinas no tecido endometriótico vêm sendo descritas com frequência. Vários estudos (Eyster et al., 2002; Arimoto et al., 2003; Kao et al., 2003; Absenger et al., 2004; Matsuzaki et al, 2004, 2005; Wu et al., 2006a; Burney et al., 2007; Chand et al., 2007; Flores et al., 2007; Hever et al., 2007; Borghese et al., 2008; Hull et al., 2008; Sherwin et al, 2008; Zafrakas et al., 2008; Kusakabe et al., 2010; Aghajanova et al., 2011) empregando abordagens de análise genômica em larga escala mostraram perfis diferenciais de expressão gênica entre endométrio eutópico e ectópico de mulheres com endometriose.

Mais recentemente, componentes epigenéticos também têm sido reconhecidos como exercendo importantes papéis na etiologia e patofisiologia da doença (Wu et al., 2005, 2006b; Xue et al., 2007a, 2007b; Jichan et al., 2010 Ren et al., 2012; Fambrini et al., 2013; Xue et al., 2014, Lu et al., 2013; Andersson et al., 2014).

1.2 Alterações epigenéticas na endometriose

As modificações epigenéticas abrangem mecanismos reversíveis e herdáveis de regulação da expressão de genes endógenos. Os principais fatores epigenéticos compreendem radicais químicos inseridos nas caudas das histonas ou nas moléculas de DNA, as quais promovem sinalização e recrutamento de complexos remodeladores da cromatina, resultando em conformações repressivas ou permissivas à transcrição. Em adição, mecanismos de ação mediando a regulação da expressão gênica por RNAs não codificantes também foram recentemente considerados como epigenéticos (Guil e Esteller, 2009).

A metilação do DNA é a modificação epigenética melhor caracterizada até o momento exercendo grande importância no silenciamento e regulação gênica, em particular no *imprinting* genômico, na inativação do cromossomo X e no silenciamento de transposons. Em eucariotos, o radical metil (CH_3) é encontrado predominantemente no carbono da posição 5 da citosina seguida por uma guanina (dinucleotídeo CpG). Esta modificação é biologicamente relevante quando ocorre em regiões ricas em dinucleotídeos CpG conhecidas como ilhas CpG (Herman et al., 1996). Em mamíferos, também foi descrita a metilação em citosinas não-CpG, mas esta é exclusivamente encontrada em células-tronco embrionárias (Lister et al., 2009).

Em média, 60% dos genes humanos apresentam uma ilha CpG na sua região promotora, sendo que algumas estendem-se até o primeiro éxon. Atualmente, uma ilha CpG é definida como um fragmento de DNA maior do que 500pb, com um conteúdo de CG maior do que 55% e onde a razão entre o número de sítios CpG observado/esperado é superior a 0,65 (Takai e Jones, 2002). Estima-se que o número total de ilhas CpG no genoma humano seja de 37 729, sendo que aproximadamente 35% destas são mapeadas em regiões promotoras (Han e Zhao, 2009).

No geral, a alta densidade de metilação do DNA nas ilhas CpGs presentes nas regiões promotoras resulta em silenciamento gênico devido à inibição estérica da ligação dos complexos de transcrição à suas regiões regulatórias (Bernstein et al., 2007) ou pelo recrutamento de proteínas que se ligam à sequências CpG metiladas iniciando modificações epigenéticas coordenadas na cromatina (Esteller, 2007). Em genes transcricionalmente ativos, estas ilhas são normalmente não metiladas. Entretanto, elementos repetitivos (DNA satélite, LINES, SINES e transposons) e a região promotora de alguns genes caracterizados por ausência de expressão tecido-específica são geralmente metiladas (Espada e Esteller, 2010). É importante destacar que a metilação do DNA resultando em repressão transcricional pode também ser observada em regiões conhecidas como *shores* de ilhas CpG localizadas a até 2Kb

de uma ilha CpG típica e que apresentam uma densidade de dinucleotídeos CpG bem menor (Irizarry et al., 2009).

Como a metilação do DNA é uma marca epigenética herdável, a manutenção e a transmissão fidedigna desses padrões às células filhas é um processo essencial do ciclo celular. As enzimas responsáveis pela metilação do DNA nos dinucleotídeos CpG são membros de uma família de proteínas DNA metiltransferases (DNMTs) que utilizam a S-adenosil metionina (SAM) como doadora de grupos metil. Cinco membros de DNMTs foram identificados em mamíferos: DNMT1, DNMT2, DNMT3A, DNMT3B e DNMT3L. A DNMT1, a mais abundante em células somáticas, possui uma forte preferência por DNA hemimetilado e está associada com a forquilha de replicação formada na fase S do ciclo celular. Assim, é a enzima responsável por copiar e manter os padrões de metilação do DNA durante a replicação. DNMT3A e 3B são expressas principalmente em células embrionárias e não diferenciadas. São críticas durante o desenvolvimento embrionário quando ocorrem ondas sequenciais de metilação *de novo* no genoma. As duas são assim, conhecidas como DNMTs *de novo*. Entretanto, alguns relatos indicam que DNMT1 assim como DNMT3A e 3B podem ter ambas as funções de manutenção e *de novo in vivo*, cooperando para a geração de um padrão global de metilação. DNMT2 e 3L não contêm domínios regulatórios compartilhados pelas outras enzimas, assim possuem atividade de metiltransferase muito baixa (Espada e Esteller, 2010).

As metilcitosinas estabelecidas pelas DNMTs agem como sítios de ligação para proteínas com domínios de ligação a grupos metil (MBDs) (Dhasarathy e Wade, 2008) e estas interagem com histonas desacetilases, histonas metiltransferases e demais enzimas remodeladoras da cromatina promovendo a compactação da cromatina que é repressiva à transcrição (Bird e Wolffe, 1999).

Ultimamente, alterações epigenéticas têm sido observadas mediando os padrões de expressão diferenciais de genes alvo na etiopatogenia da endometriose (Wu et al., 2005, 2006b;

Xue et al., 2007a, 2007b) ou secundárias à expressão anormal das DNA metiltransferases (Wu et al., 2007c; van Kaam et al., 2011). Na tabela 1 encontra-se a lista das publicações que formam o conjunto de evidências que apoia a hipótese de que a endometriose é uma doença epigenética.

Tabela 1. Alterações epigenéticas na endometriose identificadas por metodologias locus específicas.

GENE*	GRUPO AMOSTRAL	PRINCIPAIS ACHADOS	REFERÊNCIA
CDKN2A	46 casos de endometriose	Hipermetilação em uma amostra	Martini et al., 2002
hMLH1	46 casos de endometriose	Hipermetilação em 4 amostras	Martini et al., 2002
	3 grupos amostrais: 1) 29 casos de carcinoma ovariano associado à endometriose, compreendendo 25 tecidos neoplásicos, 9 endometrioses atípicas, 13 endométrios ectópicos e 15 endométrios eutópicos; 2) 26 casos de endometriose ovariana, compreendendo 20 endométrios ectópicos e 17 eutópicos; 3) 16 casos de endométrio controle (eutópico)	Frequência de hipermetilação mais alta, bem como de expressão proteica mais baixa, no grupo 1, seguido do grupo 2 e 3	Ren et al., 2012
HOXA10	6 amostras de tecido endometrial de mulheres com endometriose, 4 biópsias endometriais de mulheres sem endometriose e 5 amostras de sangue menstrual de mulheres sem endometriose	Hipermetilação associada a baixos níveis de expressão gênica nas amostras de mulheres com endometriose	Wu et al., 2005
	Endometriose foi induzida experimentalmente em 6 fêmeas de babuínos pela inoculação de endométrio menstrual	Metilação deste gene em endométrio eutópico de babuínos com endometriose	Kim et al., 2007
	Endométrio eutópico de camundongos com endometriose induzida experimentalmente (grupo de oito animais)	Hipermetilação confirmada nas amostras de endométrio	Lee et al., 2009
	11 amostras de endométrio eutópico de mulheres com endometriose ovariana e 11 amostras de endométrio de mulheres sem endometriose	Nível médio de metilação mais elevado no grupo de endométrio de mulheres com endometriose	Fambrini et al., 2013
	12 amostras de endométrio de mulheres sem endometriose e 12 amostras de endométrio eutópico de mulheres com endometriose. Deste último grupo, 6 amostras de células estromais foram mantidas em culturas celulares	Expressão gênica e proteica reduzida no endométrio eutópico de mulheres com endometriose. Ambos os níveis de expressão foram aumentados após tratamento das células com o agente desmetilante 5-aza-2´desoxicitidina (5-aza)	Lu et al., 2013
	18 amostras de endométrio eutópico e ectópico de mulheres com endometriose peritoneal e retovaginal e 12 amostras de endométrio de mulheres sem a doença	Endométrio eutópico de mulheres com endometriose mais metilado do que endométrio ectópico e endométrio de mulheres sem a doença	Andersson et al., 2014

Tabela 1. (continuação)

GENE*	GRUPO AMOSTRAL	PRINCIPAIS ACHADOS	REFERÊNCIA
PGR	8 amostras de endometriose, 6 amostras de endométrio eutópico de pacientes com endometriose e 4 controles (endométrio de pacientes sem endometriose)	Hipermetilação no componente epitelial da endometriose (*PGRB*)	Wu et al., 2006b
	Tecido endometrial ectópico de 9 mulheres com adenomiose e tecido endometrial de 8 mulheres sem endometriose, mantidos em cultura	Hipermetilação nas células estromais endometrióticas associada com redução na expressão gênica e proteica, as quais foram aumentadas após tratamento com combinação de TSA e 5-aza.	Jichan et al., 2010
	44 amostras de tecido endometriótico e 7 amostras pareadas de endométrio eutópico	Metilação de PGRB em 17 das 44 amostras de endometriose e de PGRA em 7 de 37 amostras de endometriose	Meyer et al., 2014
ESR2	8 amostras de endométrio eutópico e 8 amostras de endometriose não pareadas	Perda da metilação da região promotora deste gene detectada na endometriose	Xue at al., 2007a
NR5A1 *(SF1)*	8 amostras de endométrio de mulheres sem endometriose e 8 amostras de endométrio eutópico de mulheres com endometriose	Hipermetilação associada com a ausência de expressão deste fator no estroma de endométrio eutópico, mas não na endometriose	Xue et al., 2007b
		Metilação aumentada na ilha CpG abrangendo o éxon II e íntron III no endométrio ectópico, associada com aumento de expressão do gene	Xue et al., 2011
		Metilação aumentada na ilha CpG mapeada no íntron I no endométrio ectópico, associada com aumento de expressão do gene	Xue et al., 2014
CDH1	Linhagens celulares	Hipermetilação associada ao silenciamento gênico	Wu et al., 2007b
CYP19A1	Tecido endometrial de 12 pacientes sem endometriose e amostras de endometriose ovarianas de 18 pacientes	Níveis de expressão da aromatase significativamente maiores após o tratamento das células endometriais com o agente desmetilante 5-aza	Izawa et al., 2008
PTGS2 *(COX-2)*	60 amostras de tecido endometrial de mulheres com endometriose e 20 amostras de tecido endometrial de mulheres sem endometriose	Frequência de metilação mais baixa nas amostras provenientes de mulheres com endometriose, associada com expressão elevada	Wang et al., 2012

**Símbolo oficial do gene conforme recomendações do* Guidelines for Human Gene Nomenclature *(http://www.genenames.org/guidelines.html):* CDKN2A, *cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) também conhecido por p16; MLH1, mutL homolog 1, colon cancer, nonpolyposis type 2; HOXA10, homeobox A10; PGR, progesterone receptor*; *ERS2*, *estrogen receptor 2* ou *ER beta*; *NR5A1 - nuclear receptor subfamily 5, group A, member 1 também conhecido por SF-1*, *steroidogeneic factor 1*; *CDH1, cadherin 1, type 1, E-cadherin (epithelial)*; *CYP19A1*, *cytochrome P450*, *family 19, subfamily A, polypeptide 1 ou aromatase; PTGS2, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase), também conhecido por COX-2.*

Desde o estudo pioneiro de Martini et al. (2002), o qual descreveram ganho de metilação dos genes *hMLH1 (mutL homolog 1, colon cancer, nonpolyposis type 2)* e *CDKN2A [cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)]* em quatro e um de 46 casos de endometriose, respectivamente, após análise de MSP (*Methylation Specific Polymerase Chain Reaction*), outros autores também descreveram padrões de metilação aberrantes em regiões promotoras de genes específicos. A hipermetilação do gene *hMLH1* também foi identificada por Ren et al. (2012) em amostras de endométrio ectópico provenientes de grupos amostrais compreendendo casos de endometriose ovariana e carcinoma ovariano associado à endometriose quando comparadas ao endométrio controle, após microdissecção a laser e MSP (*Methylation Specific – Polymerase Chain Reaction*). De forma interessante, os autores detectaram que a frequência de metilação se apresentava mais alta no grupo de casos de carcinoma, com a diminuição gradual no grupo de casos de endometriose seguido do grupo de amostras de endométrio eutópico. Esses dados também foram correlacionados com a expressão proteica. Da mesma forma que Martini et al. (2002), os autores sugeriram que a expressão reduzida deste gene pode estar envolvida na evolução da endometriose para fenótipos agressivos, uma vez que este gene está envolvido em mecanismos de reparo do DNA.

Outro gene amplamente investigado por estratégias integradas de avaliação dos padrões de metilação e de expressão gênica é o *HOXA10 (homeobox A10),* um membro de uma família de fatores de transcrição expressos no endométrio (Kim et al., 2007) e regulados pela ação do estrógeno e progesterona (Taylor et al., 1998; Gui et al., 1999). Wu et al. (2005) demonstraram que a hipermetilação da região promotora deste gene poderia explicar os níveis diminuídos de expressão na endometriose. A hipermetilação foi observada em seis amostras de endométrio eutópico de mulheres com endometriose por MSP (*Methylation Specific – Polymerase Chain Reaction*) e subsequente confirmação pelo sequenciamento do DNA modificado pelo bissulfito de sódio.

O gene *Hoxa10* também foi identificado como metilado em amostras de endométrio eutópico obtidos de fêmeas de babuínos com endometriose induzida experimentalmente após sequenciamento do DNA modificado por bissulfito de sódio, cujo resultado foi corroborado pela detecção da diminuição nos níveis de expressão proteica deste gene. Além disso, os autores identificaram também a diminuição na expressão gênica de *ITGB3 (integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)),* um gene normalmente induzido por *HOXA10*, enquanto a expressão do gene *EMX2 (empty spiracles homeobox 2),* normalmente reprimido por *HOXA10,* apresentava expressão aumentada (Kim et al., 2007). Similarmente, na endometriose induzida experimentalmente em camundongos, o gene *Hoxa10* apresentou hipermetilação e expressão reduzida no endométrio destes animais (Lee et al., 2009).

Recentemente, os estudos de Fambrini et al. (2013) e Lu et al. (2013) também corroboraram os achados anteriores. O primeiro detectou níveis médios de metilação na região promotora do gene *HOXA10* mais altos em 11 amostras de endométrio eutópico provenientes de mulheres com endometriose, comparado ao nível médio detectado no grupo de amostras controle, após pirosequenciamento do DNA modificado por bissulfito de sódio; enquanto o último relatou que os níveis reduzidos de expressão gênica e protéica deste gene em lesões endometrióticas foram decorrentes da metilação do DNA, determinada após tratamento de endométrio eutópico endometriótico com o agente desmetilante 5-aza. Além destes, o estudo de Andersson et al. (2014), o primeiro a avaliar o endométrio ectópico quanto à metilação do gene *HOXA10*, identificou que o endométrio eutópico de mulheres que possuem endometriose apresenta níveis mais altos de metilação deste gene comparado ao das lesões e ao do endométrio de mulheres que não possuem a doença.

A presença de hipermetilação na região promotora do gene *PGR (progesterone receptor)* foi descrita por Wu et al. (2006b) em seis de oito amostras de endométrio ectópico empregando uma combinação de microdissecção a *laser* (LCM), MSP e sequenciamento após

modificação pelo bissulfito de sódio. Os autores sugeriram que a metilação anormal poderia ser responsável pelos baixos níveis de expressão de receptores de progesterona, o que explicaria o fato de 9% das mulheres com endometriose não responderem ao tratamento com progestina.

Concordante com o estudo anterior, Jichan et al. (2010) relataram aumentos na expressão gênica e proteica deste gene em células estromais ectópicas após o tratamento com uma combinação de tricostatina A (TSA), um inibidor de desacetilase de histonas, e o agente desmetilante 5-aza-2´desoxicitidina (5-aza). Este tratamento também resultou em uma redução na viabilidade celular e na progressão do ciclo celular.

Nosso grupo também obteve achados interessantes envolvendo a metilação dos genes *PGRB* e *PGRA* (Meyer et al., 2014). Empregando a metodologia de MSP, a metilação dos genes *PGRB* e *PGRA* foi detectada em 17/44 e em 7/37 casos de endometriose intestinal profunda investigados, respectivamente. Para o gene *PGRB*, foram avaliados 7 amostras pareadas de endométrio eutópico, as quais se mostraram não metiladas, sugerindo o potencial deste gene como um biomarcador da doença (Capítulo 3).

Xue et al. (2007a) demonstraram que a metilação da ilha CpG na região promotora do gene *ESR2* (*estrogen receptor 2, ER beta*) representa o mecanismo primário da expressão diferencial deste gene no endométrio e na endometriose. Ao contrário dos estudos anteriores, a presença de metilação na região promotora do gene *ESR2,* observada nas oito amostras de endométrio eutópico analisadas, foi associada aos baixos níveis de expressão deste receptor no endométrio eutópico e a perda de metilação foi correlacionada com os níveis elevados de expressão na endometriose. A hipermetilação na ilha CpG presente na região promotora deste gene foi detectada pelo sequenciamento do DNA modificado pelo bissulfito de sódio e confirmada após a verificação do aumento na expressão do receptor após o tratamento das células endometriais normais com o agente desmetilante 5-aza.

Um achado semelhante foi descrito para o gene *NR5A1* (também denominado de *SF1* ou *steroidogenic factor 1*) que codifica um fator de transcrição essencial para a ativação de vários genes das vias de biossíntese de estrógenos. Xue et al (2007b) relataram que a hipometilação da ilha CpG deste gene está correlacionada com a sua ativação transcricional na endometriose. A metilação nas células normais foi também observada após sequenciamento do DNA pelo bissulfito de sódio em oito amostras de endométrio eutópico de mulheres sem endometriose. O tratamento com 5-aza-2'-desoxicitidina, análises de metilação *in vitro* e imunoprecipitação da cromatina foram também realizados e confirmaram os resultados obtidos. Posteriormente, o mesmo grupo identificou que a metilação de duas ilhas CpGs mapeadas no corpo do gene *NR5A1,* uma localizada no íntron I e a outra abrangendo do éxon II ao íntron III, poderiam regular positivamente a expressão deste gene na endometriose (Xue et al., 2011; 2013). A metilação foi detectada após sequenciamento do DNA pelo bissulfito de sódio nas mesmas 8 amostras de endométrio ectópico investigadas no estudo anterior.

Por outro lado, Wang et al. (2012) relatou que a hipometilação do promotor do gene *PTGS2 (*também denominado de *COX-2* ou *prostaglandin-endoperoxide synthase 2 - prostaglandin G/H synthase and cyclooxygenase)*, que codifica uma enzima-chave na biossíntese de prostaglandinas, pode estar relacionado com o aumento da expressão deste gene no endométrio eutópico de mulheres com endometriose. A metilação foi detectada após MSP em 25 de 60 amostras de endométrio eutópico de mulheres com endometriose e em 15 de 20 amostras de endométrio de mulheres sem a doença.

Em conjunto, estas alterações epigenéticas promoveriam uma desregulação da via esteroidogência nas células endometrióticas: a perda da metilação do DNA no promotor estaria associada com a ativação do gene *PTGS2*, com consequente aumento de expressão da ciclooxigenase 2 e o aumento de expressão dos receptores nucleares nas células estromais endometrióticas. Já que *NR5A1* está envolvido na indução da produção de estradiol dependente

de prostaglandina e ER beta suprime o receptor de estrógeno alfa (ER alpha) e receptores de progesterona (PRs), estas alterações epigenéticas poderiam culminar em resistência à progesterona, aumento da sobrevivência celular e inflamação (Bulun, 2009).

No entanto, a principal evidência para o envolvimento das alterações epigenéticas na endometriose foi dada pelos padrões de expressão anormais das DNA metiltransferases. Wu et al. (2007c) estudaram o padrão de expressão das DNA metiltransferases após a microdissecção a laser dos tecidos em 13 amostras de endométrio ectópico, 10 amostras de tecido endometrial eutópico de mulheres com endometriose e oito endométrios de mulheres sem endometriose. Foi observado um aumento na expressão das DNMT1, DNMT3A e DNMT3B na endometriose em relação aos demais tecidos. De acordo com estes autores, van Kaam et al. (2011) também encontraram níveis de expressão mais baixos das DNMTs, bem como das MBDs (*methyl-CpG-binding domain proteins*) no endométrio ectópico comparado ao endométrio eutópico de mulheres com e sem endometriose, confirmados por imunohistoquímica.

De forma contrária aos relatos anteriores, Vestergaard et al. (2011) sugeriram que eventos envolvendo a hipermetilação do DNA são raros na endometriose, já que não foi detectada nenhuma alteração no perfil de metilação dos genes *APC (adenomatous polyposis coli), CDKN2A, PYCARD (PYD and CARD domain containing), RARB (retinoic acid receptor, beta), RASSF1A (Ras association (RalGDS/AF-6) domain family member 1)* e *ESR1 (estrogen receptor 1, ER alpha)* no endométrio ectópico de 23 mulheres com endometriose.

Recentemente, quatro estudos realizaram o *screening* do perfil de metilação do DNA global na doença por metodologias em larga escala e identificaram um grande número de genes diferencialmente metilados na endometriose (Borghese et al., 2014; Dyson et al., 2014; Naqvi et al., 2014; Yamagata et al., 2014) (Tabela 2).

Tabela 2. Alterações epigenéticas na endometriose identificadas por metodologias em larga escala

REFERÊNCIA	METODOLOGIA	RESOLUÇÃO	GRUPO AMOSTRAL	PRINCIPAIS ACHADOS
Borghese et al., 2010	Imunoprecipitação do DNA metilado e hidridação na plataforma *GeneChip Human Promoter 1.0R* (Affymetrix)	25.500 promotores	5 amostras de endometriose superficial, 10 amostras de endometriomas, 5 amostras de endometriose profunda e 20 amostras de endométrio eutópico correspondentes	383 genes diferencialmente metilados na endometriose 35 genes correlacionados com expressão
Dyson et al., 2014	Modificação do DNA por bissulfito de sódio e hibridação na plataforma *Infinium Human-Methylation450 Beadchips* (Illumina)	485.000 dinucleotídeos CpG	Culturas de células estromais endometriais de mulheres sem endometriose e culturas de células estromais de endometriomas	6.349 genes hipermetilados e 5.559 genes hipometilados na endometriose 403 genes correlacionados com expressão
Naqvi et al., 2014	Modificação do DNA por bissulfito de sódio e hibridação na plataforma Infinium *HumanMethylation27 Beadchip* (Illumina)	27.578 dinucleotídeos CpG	7 amostras de endométrio eutópico de mulheres com endometriose e 6 amostras de endométrio de mulheres sem endometriose	120 genes diferencialmente metilados na endometriose
Yamagata et al., 2014	Modificação do DNA por bissulfito de sódio e hibridação na plataforma *Infinium HumanMethylation27 BeadChip* (Illumina)	27.578 dinucleotídeos CpG	Culturas de células estromais endometriais de endométrio eutópico de mulheres com e sem endometriose e de cistos endometriais ovarianos	329 genes hipermetilados e 441 genes hipometilados na endometriose 75 genes correlacionados com expressão

No primeiro estudo (Borghese et al, 2010), os autores realizaram um *screening* do *status* de metilação do DNA de 25 mil promotores em diferentes *pools* de lesões endometrióticas (5 amostras de endometriose superficial, 10 amostras de endometrioma e 5 amostras de endometriose profunda infiltrativa) que foram comparados ao perfil obtido do *pool* contendo as 20 amostras de endométrio eutópico correspondentes, após a imunoprecipitação do DNA metilado e hibridação em plataformas de *microarray* da *Affymetrix*. Esses dados revelaram que, de acordo com a teoria atual sobre a origem endometrial da endometriose (Sharpe-Timms, 2001; Bulun, 2009), o perfil global de metilação em promotores gênicos foi altamente similar entre endométrio e lesões endometrióticas. Apesar dessas semelhanças, foram identificados 35 potencias genes candidatos relevantes para a patogênese da doença, os quais apresentaram alterações na metilação do DNA e consequente, alterações na expressão gênica.

Posteriormente, Dyson et al. (2014), Naqvi et al. (2014) e Yamagata et al. (2014), empregando metodologias baseadas na modificação do DNA por bissulfito de sódio e hibridação em plataformas que interrogam dinucleotídeos CpG da Illumina, identificaram entre 120 e 770 genes diferencialmente metilados em amostras de endométrio de mulheres com e sem endometriose (Naqvi et al., 2014) ou em culturas de células estromais de endométrio de mulheres com e sem endometriose e de endometriomas (Dyson et al., 2014; Yamagata et al., 2014).

Como evidenciado por estes últimos estudos, sendo a metilação do DNA parte de um processo de regulação epigenética que altera a conformação da cromatina, ela pode afetar vários *loci* simultaneamente, sendo que alguns podem ser cruciais para o início e/ou progressão da endometriose.

De acordo com o exposto, embora estes estudos evidenciem a necessidade de investigações adicionais para um maior entendimento e caracterização de potenciais marcadores da doença, no conjunto, estes achados fortemente sugerem o envolvimento de padrões anormais de metilação do DNA na patogênese e desenvolvimento desta condição.

2. JUSTIFICATIVA

Atualmente, a endometriose representa um problema de saúde pública mundial devido à sua alta prevalência, impactos promovidos na qualidade de vida e encargos econômicos decorrentes dos custos diretos relacionados aos cuidados com a saúde e da perda de produtividade das mulheres que apresentam esta doença (Nnoaham et al., 2011).

Neste cenário, Rogerst et al. (2013) definiram algumas prioridades baseadas em um consenso internacional que resultou em 56 recomendações para encorajar e facilitar o desenvolvimento de pesquisas envolvendo esta debilitante doença. Estas recomendações foram agrupadas em algumas necessidades prioritárias como o desenvolvimento de novas ferramentas diagnósticas e prognósticas, novas abordagens terapêuticas, além de um maior entendimento de aspectos epidemiológicos e patofisiológicos da endometriose. Neste contexto, é possível observar que os estudos epigenéticos apresentam um grande potencial de contribuir para que esses objetivos sejam atingidos.

3. OBJETIVOS

Gerais

Baseado no exposto, este estudo propôs a identificação de genes diferencialmente metilados em amostras pareadas de endometriose intestinal e endométrio eutópico e que possam ser caracterizados como potenciais biomarcadores dessa doença.

Específicos

(1) Identificar genes diferencialmente metilados na endometriose intestinal em comparação ao endométrio eutópico baseado nos dados de análise em larga escala após hibridação em microarranjos de ilhas CpG;

(2) Avaliar *in silico* a lista de genes diferencialmente metilados, incluindo a classificação funcional e avaliação de redes de interação e vias biológicas nos quais os mesmos estão envolvidos;

(3) Selecionar e validar o status de metilação de alguns genes candidatos empregando diferentes abordagens como MSRE-*real time* PCR e sequenciamento do DNA modificado pelo bissulfito de sódio.

(4) Identificar se os promotores alternativos dos genes *ESR1, ESR2* e *PGR* apresentam metilação diferencial na endometriose intestinal em comparação ao endométrio eutópico pela metodologia de *Methylation-Specific PCR* (MSP-PCR), com a posterior identificação do perfil de expressão dos receptores estrógenos codificados por esses genes pela análise de imunohistoquímica.

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CAPÍTULO 2

Este artigo será submetido à revista *Epigenetics*.

RESEARCH PAPER

Genome-wide screening of differentially methylated CpG island in deep infiltrating endometriosis

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Keywords: global DNA methylation, intestinal endometriosis, epigenetics, CpG island, microarray

Abbreviations

bp base pair

BCOR BCL6 co-repressor

CpG cytosine-guanine dinucleotide

DIE deep infiltrating endometriosis

DNA deoxyribonucleic acid

DNMT DNA methyl transferase

HDAC1 histone deacetylase 1

HDAC2 histone deacetylase 2

HOX homeobox gene

HOXA7 homeobox A7 gene

HOXD4 homeobox D4 gene

IPA Ingenuity Pathway Analysis

MBD methyl-CpG-binding domain protein

MSRE-PCR Methylation-sensitive restriction enzyme - Polymerase Chain Reaction

PCR polymerase chain reaction

Abstract

Recent evidence indicates that endometriosis may be considered an epigenetic disease. This hypothesis was essentially based on the abnormal DNA methylation patterns observed in the promoter region of specific genes and on the higher expression levels of DNMTs in endometriotic lesions in comparison with normal endometrium. Based on these, we hypothesized that endometriosis is a polygenic multifactorial disease where epigenetic changes accumulate throughout its development and progression. In this study, we contribute to elucidate the epigenetic mechanisms underlying the development of deep infiltrating endometriosis (DIE) by a genome wide microarray-based approach, with focus in DNA methylation, by performing a direct comparison between eutopic endometrium and endometriotic lesions from the same patient. We have notice an enrichment of homeobox transcription factors, of which *HOXA7* and *HOXD4* genes were identified as potential markers of the disease. A comparative analysis with data available in recent large-scale studies enable us to recognize a consistent number of 52 hypermethylated and 53 hypomethylated recurrently genes. In addition, the results had suggested that epigenetic events could potentially modulate specific biological pathways that could be important for the development of endometriotic lesions such as Notch signaling, relaxin signaling and TGF-beta signaling, besides others process involved to molecular mechanisms of cancer and human embryonic stem cell pluripotency. In conclusion, we have contributed to further characterization of this disease through the identification of recurrently differentially methylated genes that have shown important biological functions for the stablishment and maintenance of endometrial implants in the ectopic sites.

Introduction

Endometriosis, characterized by the presence and growth of endometrium-like tissue (gland and stromma) outside the uterine cavity, is a multi-factorial disease affecting women in reproductive age and associated to dysmenorrhea, pelvic pain, and subfertility.[1] Currently, this estrogen-dependent disease represents a public health problem worldwide due to its prevalence, impact on health-related quality of life and economic burden arising of direct health care costs and loss of productivity.[2] Clinically, the endometriotic lesions are classified in three subtypes: superficial endometriosis when located on the surface of peritoneum, ovarian endometriomas or deeply infiltrating endometriosis (DIE) when invading the septum rectovaginal and bladder. Despite of recent advances in the diagnosis and clinical management, its etiology and pathophysiology remains poorly understood. This enigmatic disease presents some features such as immunological alterations[3] and aggressiveness and capability of invasion,[4] but it is not considered a malignant disease. These aspects have been described as the most relevant in the rectosigmoid endometriosis, a particular deeply infiltrating form of the disease.[5]

In the last decade, some studies have attempted to establish a link between epigenetic mechanisms and the pathogenesis of endometriosis. In addition to the abnormal DNA methylation patterns observed in locus-specific promoter regions,[6-12] altered expression of components of the epigenetic machineries, i.e. enzymes that add, remove or recognize chromatin modifications, were observed in endometriotic lesions in comparison with normal endometrium. These studies suggest that disrupted expression of DNA methyltransferases,[13,14] histone deacetylases HDAC1 and HDAC2,[15-17] and methyl-CpG-binding domain proteins (MBDs)[14] could affect the genome as a whole explaining, at least partially, the differential gene expression profiles observed in eutopic and ectopic endometrium.[18-29]

Indeed, epigenetic mechanisms most likely underlie the initiation and progression of this common gynecological disorder. In support to this hypothesis, the study of Saare et al.[30]

provide evidence that no endometriosis specific somatic copy number alterations (SCNAs) or events of copy neutral loss of heterozygosity (cn-LOH), characterized by deletion of one copy and compensatory duplication of the other allele, are present in either eutopic or ectopic endometrium when compared with blood DNA of the same patient. Based on these preliminary evidences, we hypothesized that endometriosis is a polygenic multifactorial disease where epigenetic changes, rather than chromosomal rearrangements, accumulate throughout its development and progression. Thus, the present study was conducted to identify differentially methylated CpG islands in rectosigmoid deep endometriosis compared to matched eutopic endometrium, employing a microarray-based genome-wide approach.

Results

Differentially methylated CpG islands in deeply infiltrating endometriosis (DIE)

Given the reported epigenetic aberrations in endometriosis, we design the present study in order to identify genome-wide differentially methylated regions in nine deep intestinal endometriosis samples compared to matched eutopic endometrium, simultaneously collected from the same patients, by profiling DNA methylation changes of 27,800 CpG islands. The study used a microarray-based approach after the enrichment of methylated and unmethylated fractions with the use of specific restriction enzymes and hybridization to Human CpG Island 244K chip (Agilent Technologies). Our design would be useful as a means of override the individual epigenetic background besides of characterize DNA methylation changes potentially involved in the genesis and development of endometriosis.

Each hybridization experiment was conducted in replicates. We first generate a correlation coefficient and observed that the global methylation patterns correlated well between each pair of replicates (Figure S1). After the bioinformatics analysis, we generate a list of differentially methylated genes for each of the cases investigated in both fractions (Table

1) and after that; we carried out a comparative strategy to find differentially concordant genes between the total cases.

After the comparative analysis, we have found that the CpG islands identified as differentially methylated were representative of 1164 genes: 546 genes with significant gains of methylation and 871 genes with significant losses of methylation in at least two cases. A complete list of differentially methylated genes can be found in Table S1.

Subsequently, we interrogated the CpG islands probe distribution from the Human CpG Island 244K platform and identified that 31% were located in promoter regions, whereas 59% were mapped in the bodies of genes and 4% downstream of genes. The same proportion have been noticed after the analysis of our data: 31% of the hypermethylated probes were mapped at the promoter regions and 57% inside the genes; whereas the hypomethylated probes had been located at a proportion of 32% in the promoter and 62% at the bodies of genes.

Gene distribution and functional clustering of differentially methylated CpG islands

By observing the total number of genes represented in the platform for each chromosome, we have noted that the genes were distributed homogeneously for all chromosomes but there have been slight increases in the number of methylated probes detected at chromosome 7, 17 and 19 and hypomethylated probes at chromosomes 10 and 19 (Fig. S2A). In turn, the differentially methylated genes distributed by chromosome region p and q denotes a uniform arrangement, with some slights differences (Fig. S2B).

In addition, a nonsupervised gene functional clustering was performed using DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov/home.jsp) for the same gene list identified as differentially methylated in endometriosis. DAVID was able to group these genes as enriched for transcription factors and chromatin remodelers, which are involved at gene

expression function. One notably finding of this analysis was the significant high representation of homeobox genes, with special emphasis to HOXA and HOXD clusters.

Validation of differentially methylated regions by PCR and bisulfite sequencing

In this step, we selected the *HOXA7* (homeobox A7), *HOXD4* (homeobox D4) and *BCOR* (BCL6 co-repressor) to be evaluated by a locus-specific method, i.e., methylation-sensitive restriction enzyme real-time PCR (MSRE-qPCR) and bisulfite sequencing. The choice of these loci was based on the large number of probes identified as differentially methylated (and many of which presenting high values of log ratio) as well as the potential role of the selected genes in the regulation of gene expression.

After the design of the locus-specific primers in the site of promoter region mapped with the probes identified as differentially methylated, the analysis had been followed with *HOXA7* and *HOXD4* genes by MSRE-PCR methodology with nine matched samples were investigated: seven samples from the same experimental group employed in the microarray-based strategy (116, 169, 190, 207, 214, 255 and 256) and two additional samples from an independent set (121 and 185). In parallel, the bisulfite sequencing methodology was carried out for the *HOXA7* and *BCOR* genes with three matched samples: one from the same experimental group employed in the microarray-based strategy (256) and two additional samples from an independent set (185 and 218). After the first methodology, the gain of methylation of *HOXD4* and *HOXA7* genes was confirmed in 89% (8/9) and 56% (5/9) samples, respectively (Fig. 1A, B). In addition, after the bisulfite sequencing only the *HOXA7* gene validate de microarray results, which one out of three cases analyzed showing an extensive gain of methylation in ectopic endometrium compared to eutopic endometrium. Interestingly, this case was that have shown the higher value of gains of methylation for this gene after the MSRE-PCR approach (Fig.1C).

Recurrent differentially methylated genes associated with endometriosis

After the identification of the differentially methylated genes in a subset of deeply infiltrating endometriosis in the present study, we attempt to search for recurrent genes showing DNA methylation changes in the few previous reported genome-wide studies,[31-33] of which the data were available.

A comparison between the data available for those three studies resulted in a number of just 74 hypermethylated and 92 hypomethylated genes. After that, we have found that 227 genes identified as hypermethylated and 322 hypomethylated in our study were concordant with those of previous studies. It is noteworthy that of above mentioned genes, 222 of hypermethylated and 312 hypomethylated genes were uniquely in accordance with the study conducted by Dyson et al. [32] Of the total of genes recurrently reported as epigenetically aberrant, a set of 105 genes (52 hypermethylated and 53 hypomethylated) showed a correlation with the expression levels according the same study.[32] So, we choose to focused in that group comprised of 105 recurrent epigenetic aberrantly genes, including the 52 hypermethylated and 53 hypomethylated genes, to identify the main biological process altered in endometrioses by IPA (Ingenuity Pathways Analysis, http://www.ingenuity.com/) tool analysis. It is important to highlight that the main biological pathways were that involved in the molecular mechanisms of cancer (p-value 1,26E-02), Notch signaling (p-value 7,65E-05), human embryonic stem cell pluripotency (p-value 1,31E-03), relaxin signaling (p-value 7,04E-03) and TGF-beta signaling (p-value 1,36E-02) (Fig.2) indicating that this specific pathways can be important in the modulation of development of endometriotic lesions.

Discussion

In the present study, we identify differentially methylated genes in deeply infiltrating endometriosis in comparison to the eutopic endometrium obtained from the same patient. At

first, we presume that if the most widely accepted theory for the origin of endometriotic implants is correct, i.e., the retrograde menstruation or implantation theory of Sampson, it should be expected that the epigenetic changes are most likely involved in the maintenance of the endometrial cells in the ectopic sites. Thus, our data are in accordance with the hypothesis that epigenetic changes could provide the plasticity necessary for the maintenance and progression of endometriosis.

Evidences suggesting that endometriosis may be an epigenetic disease came from the demonstration that the promoter region of the gene *HOXA10* is hypermethylated in endometrium from women with endometriosis compared to the endometrial samples of women without endometriosis.[7,33] These finding were supported by both "in vitro" studies with the demethylating agent 5-aza-2'deoxicytidine[34] and "in vivo" studies of induced endometriosis in baboon[35] and mice,[36] which showed that *Hoxa10* promoter hypermethylation correlates with reduced mRNA expression levels. Furthermore, a recent study described that the eutopic endometrium presented a higher frequency of methylation than ectopic lesions and endometrium from women without endometriosis.[12]

Since aberrant expression of components of the epigenetic machinery, specially the overexpression of the three genes encoding DNA methyltransferases involved in de "novo" (*DNMT3A* and *DNMT3B*) as well as in the maintenance of DNA methylation (*DNMT1*) was detected in endometriosis,[13] it is reasonable to expect a genomic widespread effect. Thus, based on recent data indicating that endometriosis could be an epigenetic disease triggered by the aberration of expression of key enzymes involved to the dynamics of epigenetic modifications such as DNMTs, HDACs and MBD[13-17], it would be expect that this cellular microenvironment could promote a huge of epigenetic instability at the ectopic lesions. Consistent with this view, and after the analysis of nine samples of deeply infiltrating intestinal endometriosis, we identified a total of 1164 differentially methylated genes: 546 hypermethylated and 871

hypomethylated. Furthermore, we confirmed that *HOXA7* and *HOXD4* genes were hypermethylated in DIE samples using a locus-specific MSRE-PCR analysis and bisulfite sequencing.

Still, among these 1164 genes, we were not able to confirm the hypermethylation of the genes *HOXA10*[7,12,34] and *PGR,*[8,11,37] neither the hypomethylation of the genes encoding the steroidogenic factor-1,[10,38,39] estrogen receptor beta,[9] and cyclooxigenase-2.[39] To evaluate whether this fact could be due to the number and sequence of the gene probes in the Human CpG Island 244K chip, we performed a comprehensive analysis by annotating the position of each probe plotted in the array and the annealing sites of the primer sequences employed in those studies devoted to investigate gene-specific DNA methylation patterns in endometriosis. We find that these previous studies have not interrogated the same regions (data not showed); only the study of Wu et al[7] interrogated CpG dinucleotides that overlap with the location of probes in the Human CpG Island 244K chip for the *HOXA10* gene, however this study was conducted with a different sample set (endometrium from women with endometriosis compared to endometrium from women without endometriosis).

Interestingly, the first whole genome profiling of methylated regions in superficial endometriosis, endometriomas, and DIE showed promoter regions consistently hypo or hypermethylated in all three subtypes of endometriosis as well as other methylation changes specific to one given anatomical location.[31] The authors also observed that the overall methylation profiles were highly similar between endometrium and endometriosis besides of hypermethylated regions preferentially located at the ends of the chromosomes and hypomethylated regions equally distributed along of the chromosomes. Also, it was suggested that this distribution of methylation changes between endometrium and endometriosis could collaborate to the chromosomal stability and protection against malignant transformation of endometriotic cells.

In fact, genome-wide studies focusing on genomic alterations in endometriotic lesions have indicated some chromosomal alterations, but no consistent genomic gains and losses were observed.[40-43] According to Saare et al.,[30] the absence of "de novo" somatic genomic alterations in eutopic endometrium, endometriotic cells harvested from endometriotic lesions by laser capture microdissection, and DNA from peripheral blood from the same patients suggested that it is possible that many of the described genomic alterations reported previously in eutopic or ectopic endometrium actually represent copy number variations that are present in all tissues of studied individuals, rather than endometriosis lesion-specific somatic copy number alterations (SCNAs).

Thus assuming that SCNAs are rare events in endometriosis and in order to override the individual epigenetic background, our study confronted matched eutopic and ectopic endometrium from de same patient using a co-hybridization strategy of methylated or unmethylated enriched fractions after clivage with restriction enzymes. This strategy have been succeeded as noticed by the good concordance between our and the data from recent genome wide studies that aimed to interrogate the methylation status in endometriosis using distinct strategies, such as methylated DNA immunoprecipitation[31] and bisulfite conversion[32,33] prior to hybridization in the selected microarray platform.

In this context, we hypothesized that recurrently differentially methylated genes between those different studies would be an interesting strategy in order to identify alterations on the DNA methylation strongly retained in the endometriotic lesions. Further, to identify recurrently differentially methylated regions in endometriosis, we compared the methylation profiles previously described with our data, even considering the above mentioned differences in methodological approaches, platform coverage and resolution of the analysis. Besides this, it is important to note that different sample sets were investigated: pools of endometriotic lesions (superficial, ovarian, or DIE compared to a pool of endometrial biopsies),[31] endometrial

stromal cells isolated and cultured from disease-free women compared to endometriomas[33] or compared with eutopic endometrium from women with the disease,[34] while we employed a unique set of samples of DIE and compared each one with your corresponding eutopic endometrium.

An analysis of the biological relevance of the set of 105 recurrent genes epigenetically altered has provided some additional insights regarding interesting interacting pathways which could be involved in this intriguing disease, particularly the Notch and TGF beta signaling pathways, involved in proliferation, differentiation and survival of stem cells, as well as related frequently as aberrantly altered in a variety of cancer, including endometrial cancer.[44,45] This data also corroborate the finding that the recurrently methylated genes are involved in molecular mechanisms of cancer and human embryonic stem cell pluripotency after the same analysis. It is interesting to notice that many of these pathways were identified as enriched in our gene list, and remained after the analysis of the recurrently methylated genes (Figure 2).

Still corroborating the study with the higher level of concordance with our data, we also have notice an enrichment of the *GATA* family and *HOXA* and *HOXD* genes clusters, of which the *HOXA1, HOXA3, HOXA5, HOXA7, HOXA13, HOXD4, HOXD8, HOXD9* and *HOXD13* genes were identified as differently methylated. The homeobox genes are families of transcription factors carrying large roles during the embryonic development and differentiation of female reproductive tract, particularly the *HOXA* genes.[46] In female adult, have been noted that some of these genes remain actives and to modulating its expression during the menstrual cycling, such as *HOXA10, HOXA11, HOXA13* and *HOXD13*.[47] Based on our results, we can speculate the role of *HOXA7* gene on development of endometriosis, since this gene is apparently not affected by changes in menstrual cycling. Until now, this gene mapped at chromosome 7, have not been described as involved on endometriosis, but have been reported as higher expression[48] or lower methylation levels[49] on ovarian carcinoma. However, although

this is an interesting find, additional experiments need to be performed in order to confirm this gene as a powerful marker of the disease. Besides this, it noteworthy the key regulation identified by Dyson et al.[32] for the GATA family, especially for GATA2 and GATA6 genes, the former also identified as methylated in the recurrently genes.

We acknowledge that the lack of validation of our findings in an independent group of endometriotic lesions is a limitation of the present study. The same criticism is valid to the absence of correlated expression analysis of the genes indicated as differentially methylated; however, this could be explained by the very small size of the endometriotic lesions and sparse stromal cells in the intestinal tissue surrounding. Thus, the availability of endometriotic lesions is a critical factor for multiple analyses in the same sample set. Despite this, recent studies applied an integrative genome approach in order to correlate epigenetic changes and gene expression levels in endometriosis.[31-33] The correlation between both data was discrete, reaching up to 15-20%. In the present study, we are able to identify 105 genes epigenetically altered and potentially aberrantly expressed in endometriotic lesions, highlighting the key role for some biological pathways and functions enriched.

The search of epigenetic changes in endometriosis has been considered a promising area for the monitoring of the onset and progression of the disease, as well as for prediction of therapy response. In conclusion, altogether our results add information in order to characterize the target genes to aberrant DNA methylation in endometriosis potentially associated with abnormal regulation of important pathways and biological processes.

Material & Methods

Tissue samples

Matched samples of deeply endometriosis compromising the rectum and eutopic endometrium were obtained from 12 patients, age 28-58 years (35.2 ± 8.1 years), after

laparoscopy at the Division of Endometriosis of the Gynecology Clinic of the Faculty of Medicine, The University of Sao Paulo - USP, Brazil. Eutopic endometrium was simultaneously collected from each patient by curettage. The institutional Human Research Ethics Committee has approved the study (protocol 0531/08) and informed consent was obtained from all subjects. All patients were clinically evaluated and both the CA-125 serum levels and transvaginal/pelvic ultrasound were tested. Disease status was classified according to the American Society for Reproductive Medicine (ASRM, 1996)[50] revised classification of endometriosis and histological features was based on the glandular and stromal patterns.[51] Among 12 patients included in the study, the main features detected were infertility (n = 6), dysmenorrhea (moderate to severe) (n = 8), acyclic pelvic pain (n = 5) and dyspareunia (n = 7).

Microarray-based methylation analysis

The tissue samples were manually macrodissected according Pires et al.[52], with modifications. Briefly, according to the haematoxylin/eosin staining, the regions containing the lesions were identified and marked under light microscopy. Using this section as a template, the endometriotic lesions were macrodissected. HE analyses of the tissue slices were also performed after the macrodissection as a quality control of the procedure. Genomic DNA was obtained by manual extraction and evaluated for concentration and quality in a spectrophotometer (NanoDrop 1000; Thermo Scientific).

Following, the samples was carried out on an approach based on MMASS (microarray-based methylation assessment of single samples) as described previously,[53] with modifications. An overview of experimental approach is provided in Fig. S3. Briefly, 1 to 3µg of genomic DNA was digested with 20U of *MseI* (New England Biolabs), in a volume of 30µl at 37°C overnight. Then, the enzyme was inactivated at 65°C for 10 min. Adaptors were ligated to the fragment ends in a total volume of 60µl, containing 30µl of digested DNA, 16µM of annealed

linkers (H-14 5'TACTCCCTCGGATA3' and H-24 5'AGGCAACTGTGCTATCCGAGGGAG3'),[53] 50mM Tris-HCl, 10mM $MgCl_2$, 1mM ATP, 10mM DTT – pH7.5, PEG 6000 6%, 400U of T4 DNA ligase and 10U of *MseI* at 16°C overnight. After, the DNA fragments were purified with QiAquick Purification Kit (Qiagen) and eluted in 40µl of sterile water.

For the unmethylated sequences enrichment, half of the volume (20µl) was digested with the methylation-dependent restriction enzyme *McrBC* (New England Biolabs, MA, USA) in a volume of 40µl, containing 10mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$, 1mM DTT - pH7.9, 10X GTP, 10X BSA and 20U of *McrBC* at 37°C for 4 hours, followed by enzymatic inactivation of 65°C for 10 min. For the methylated sequences enrichment, the other half of the initial volume (20µl) was digested with a cocktail containing the methylation-sensitive restriction enzymes *AciI*, *HinP1I*, *HpyCH4IV* and *HpaII* (New England Biolabs), in a total volume of 70µl, containing 10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM DTT - pH 7.0, 10X BSA and 20U of each enzyme. After 4 hours of incubation at 37°C, a further 10U of each enzyme were added and the incubation continued for a two additional hours, follow by 10 min at 65°C. The digestion reactions were purified and eluted in 50µl of sterile water.

DNA concentration was determined by spectrophotometry. Purified DNA (50ng) was amplified by Polymerase Chain Reaction (PCR) in a total volume of 50µl, containing 0,1µM of linker H-24, 200µM of dNTPs, 1X *buffer* 1 (1,75mM of $MgCl_2$) and 2U of *Expand Long Template* (Roche). The amplification condition was 5 min at 72ºC; 25 cycles of 1 min at 94ºC, 1 min at 65ºC and 3 min at 72ºC; 1 cycle of 10 min at 72ºC. The amplified samples were purified and eluted in 50µl of sterile water. After, the amplified products were evaluated in the Bioanalyzer equipment (Agilent Technologies) with the DNA 1000 kit and the presence of a diffuse smear between 200-2000bp indicated the success of the protocol.

The amplified DNA fragments from ectopic and eutopic endometrium were labeled with dyes Cy5 and Cy3, respectively, (ULS Labeling Kit, Agilent Technologies), mixed and co-hybridized to the Agilent 244K Human CpG Island microarray. After, the slides were scanned using an Agilent's High Resolution scanner. This procedure was performed in duplicate for each sample.

Microarray information and data analysis

The Human CpG Island 244K chip (Agilent Technologies) contains 195 K CpG island probes covering 21Mb relative to 27,800 CpG islands annotated by hg18 (NCBI *build* 36.1, *March* 2006) and 50 K non-CpG island probes. The average spacing between probes is about 100bp. Data were extracted with Agilent Feature Extraction 9.1 software and analyzed by Agilent Technologies Genomic Workbench (DNA Analytics 5.0) (Agilent Technologies) and subjected to Blank substraction, Inter-array median, Intra-array (dye-bias) median and Intra-array Lowess (intensity-dependent) normalizations. Methylation-specific sites were called incorporating the Whitehead Error Model, which has a false-positive rate of approximately 0.5% and a false-negative rate of approximately 20%. This analysis generates files that contain the intensities for all probes on the arrays, which are expressed as log ratios (estimating the Cy5/Cy3 signals in Log2 scale) and as P values (probabilities that measure the significance of the enrichment). The probes with a threshold of log ratio ≥ 2.0, corresponding to a differential signal of 4X, and P values < 0.05 were selected as statistically significant. The subsequent analysis was performed with data that the R values (between 0.87 and 0.98) had showed consistent in the replicates (Fig. S1). The microarray data is available in ArrayExpress database (https://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2630.

The genes were categorized in molecular and cellular functions by *DAVID Bioinformatics Resources* (http://david.abcc.ncifcrf.gov/home.jsp) and Ingenuity Pathways

Analysis (Ingenuity® Systems, www.ingenuity.com). The Fischer's exact test was used to calculate p-values relative to the probability that each biological function and/or disease assigned to that dataset was due to chance alone.

Methylation-sensitive restriction enzyme - Polymerase Chain Reaction (MSRE-PCR)

The methylation status of the promoter region of *HOXA7* (homeobox A7, CpG:28, hg18:chr7:27164708-27165039) and *HOXD4* (homeobox D4, CpG:18, hg18:chr2:176723195-176723460) genes was evaluated by methylation-sensitive *HpaII*-PCR assay,[54,55] with modifications. Briefly, 600ng of genomic DNA was digested with 5U of methylation-sensitive restriction enzyme *HpaII* (New England Biolabs), containing 10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM DTT - pH 7.0 in total volume of 30µl. The reactions were incubated overnight at 37°C, followed by heat inactivation for 10 min at 65°C. In parallel, the genomic DNA was also sham-treated under identical conditions. In this case, an equivalent volume of water replaced the *HpaII*. Both *HpaII*-digested and sham-treated reactions from the same sample were analyzed by quantitative real-time PCR employing two pairs of locus-specific PCR primers: either produced amplicon spanning 3 (*HOXA7*-MS) and 1 (*HOXD4*-MS) *HpaII* sites (Fig. S4) or produced amplicon spanning no *HpaII* sites (*HOXA7*-Ctrl and *HOXD4*-Ctrl). The control primers were design producing amplicon with the same size (200bp for *HOXA7* and 206bp for *HOXD4*) and CG percentage (> 50% for both primers) of methylation-targeted primers. Primer sequences were: 5'TCTGTCTAGACTCAAGCGACTGAAG3' (forward) and 5'TGCGTCCTGCCAGAACCT3' (reverse) for *HOXA7*-MS; 5'CCAGTCGCATCGCCTTCT3' (forward) and 5'CCCTTCAGTCGCTTGAGTCTAGA3' (reverse) for *HOXA7*-Ctrl 5'GGTGGTGGCTCAGAGGAAGA3' (forward) and 5'TGGTCTCTGGCTATTCCGAAGA3' (reverse) for *HOXD4*-MS; 5'GCCGCCTCGTTCTGTTTTATT3' (forward) and 5'GCATCCATGCAACCAATTAGG3'

(reverse) for *HOXD4*-Ctrl. Reactions were performed in triplicates in a StepOne Real Time PCR System equipment (Life Technolgies) using the QuantiFast SYBR Green PCR Kit (Qiagen). Quantitative PCR was carried out with 10ng of digested or sham-treated DNA and 80nM each primer to a final volume of 10µl. PCR cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s; and 60°C for 1 min, followed by a plate read. The melt curve range was 60°-95°C, at increments of 0.3°C for product detection. The Ct (threshold cycle) value of *HpaII*-digested samples was subtracted from the Ct value of sham-treated sample to determine the ΔCt of each locus. The proportion of template remaining after the MSRE treatment (region of interest) is an indication of the amount of densely methylated DNA that is computed by $2^{-\Delta Ct}$. The acceptable variability of the ΔCt values for control region from eutopic endometrium samples was < 0.4 cycles. The samples that fall within this range were selected and the amount of densely methylated DNA was calculated. The mean of these values was used as a reference for comparison of amount of densely methylated DNA from ectopic endometrium samples.

Sequencing of bisulfite-modified DNA

Initially, the DNA was converted by sodium bisulfite employing the EpiTect Bisulfite kit (Qiagen). After, the *HOXA7* (CpG:28, hg18:chr7:27164708-27165039) (Fig. S4) and *BCOR* (CpG:1121, hg18:chrX:39838379-39854191) genes was amplified with the primer sequences 5'AGGGGTTAATAGAGTTGGTGTTTAAA3' (forward) and 5'CCCAATTAACTAACTTAAATCCATAAAAA3' (reverse) and 5' GGGGAGGAGTAGTTAGGTAG3' (forward) and 5' TCAATAAACTACACAACTCATTAAC3' (reverse), respectively. The reactions were carried out with a final volume of 25µL, containing 0.32µM of each primer, 200µM of dNTP, 4mM of MgCl2, 15mM of pH 8.0 Tris-HCl, 50mM of KCl and 2U of AmpliTaq Gold (Applied

Biosystems). The amplification conditions were 95ºC for 5 min, followed by 35 cycles at 95ºC for 45 s, 60°C for 1 min and 72ºC for 1 min, followed by 72ºC for 7 min. After, the amplicon was cloned with the pGEM-T Easy Vector System (Promega) and a total varying between 3 and 11 clones were randomly selected for DNA sequencing using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) in an ABI 3500 DNA sequencer (Applied Biosystems). The software BIQ Analyzer[56] (http://biq-analyzer.bioinf.mpi-inf.mpg.de/) was used to evaluate the quality of the sequences and to generate a diagram of the methylation profile for each individual assay.

Descriptive correlation with data available at literature

Data from studies of gene candidate approaches and gene lists available from genome wide-based studies were obtained and directly compared to our final data in order to identify recurrent differentially methylated genes associated with endometriosis. After that, the recurrent genes had been also evaluated regarding the biological relevance using the *DAVID Bioinformatics Resources* and Ingenuity Pathways Analysis tools.

Conflict of interest

None of the authors have financial or any other conflict of interests that might prejudice the interpretation or presentation of the data contained within this manuscript.

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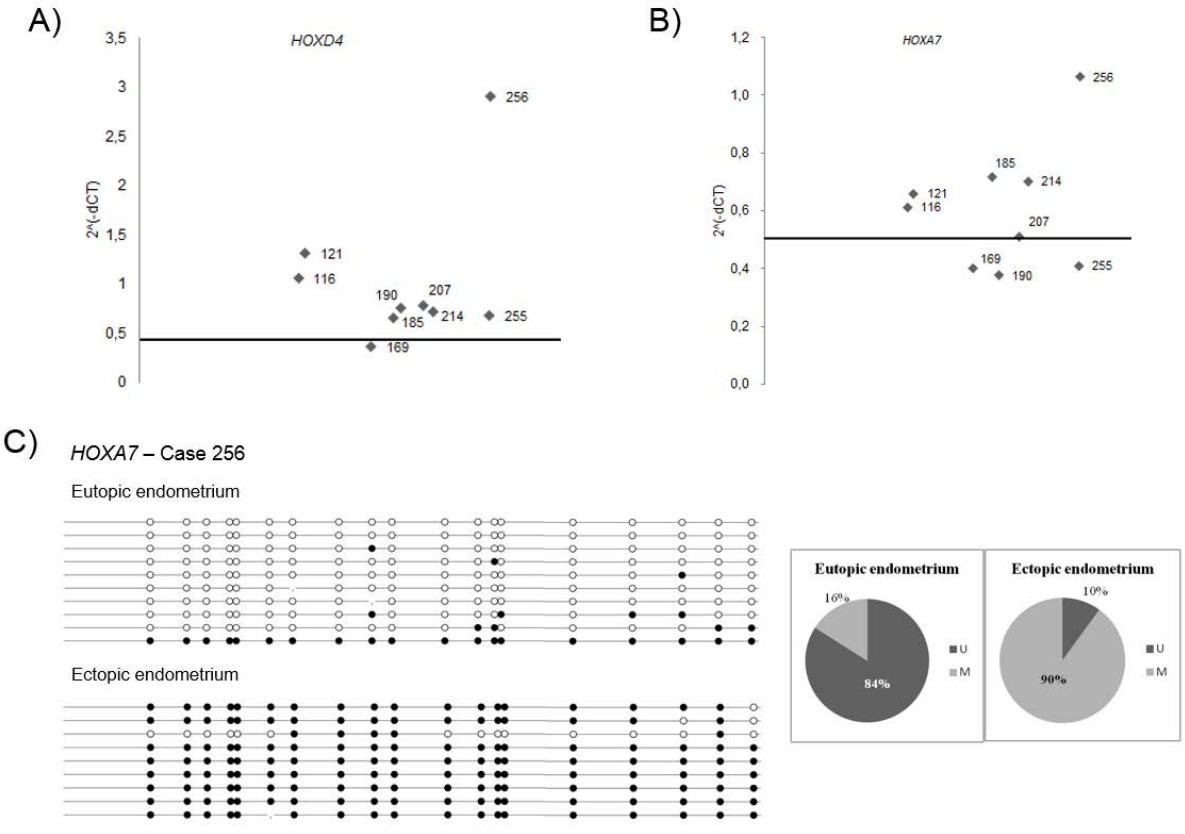
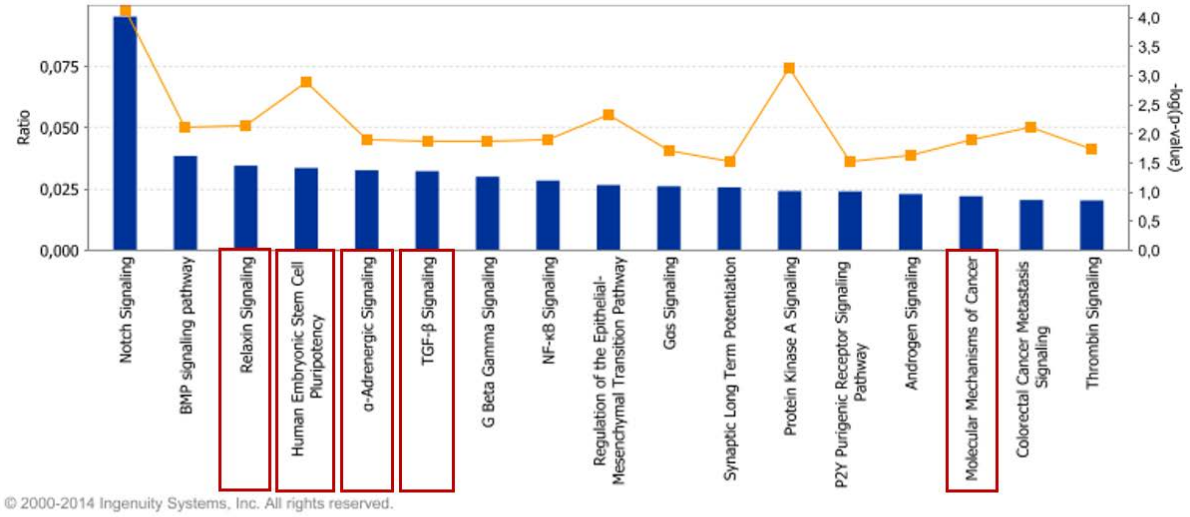

A)
HOXD4
2^(-dCT)
3,5
3
2,5
2
1,5
1
0,5
0
256
121
116
190
207
185
214
255
169
B)
HOXA7
2^(-dCT)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
256
185
214
121
116
207
169
190
255
C)
HOXA7 – Case 256
Eutopic endometrium
Ectopic endometrium
Eutopic endometrium
16%
84%
U
M
Ectopic endometrium
10%
90%
U
M



IPA Canonical Pathways	***p-value***	**Molecules altered**
Molecular Mechanisms of Cancer	1,26E-02	*BMP2,NFKBIE,PRKAG2,LEF1,NOTCH1,RALBP1,BCL2L11, PRKCZ*
Human Embryonic Stem Cell Pluripotency	1,31E-03	*FGFR3,BMP2,PDGFRA,LEF1,WNT2*
Relaxin Signaling	7,04E-03	*PDE9A,NFKBIE,PRKAG2,PRKCZ,GNG7*
NF-κB Signaling	1,25E-02	*FGFR3,BMP2,NFKBIE,PDGFRA,PRKCZ*
Notch Signaling	7,65E-05	*DLL1,CNTN1,JAG2,NOTCH1*
TGF-β Signaling	1,36E-02	*ZNF423,PIAS4,BMP2*
BMP signaling pathway	7,70E-03	*ZNF423,BMP2,PRKAG2*

Figure Legends

Figure 1. Real time PCR analysis of *HOXD4* (A) and *HOXA7* (B) genes. The cut-off calculated by the methylation level of eutopic endometrium samples is demonstrated by the black line. All samples plotted above this line represented gain of methylation compared to eutopic endometrium, and below this line represent loss of methylation. For *HOXA7* gene, the extensive gain of methylation of the case 256 after the sequencing of DNA modified by sodium bisulfite can also be visualized (C). U, unmethylated; M, methylated.

Figure 2. The most significant canonical pathways generated after IPA analysis for the recurrent differently methylated genes. The red box highlights the pathways that were the most significant considering just the data generated by our study.

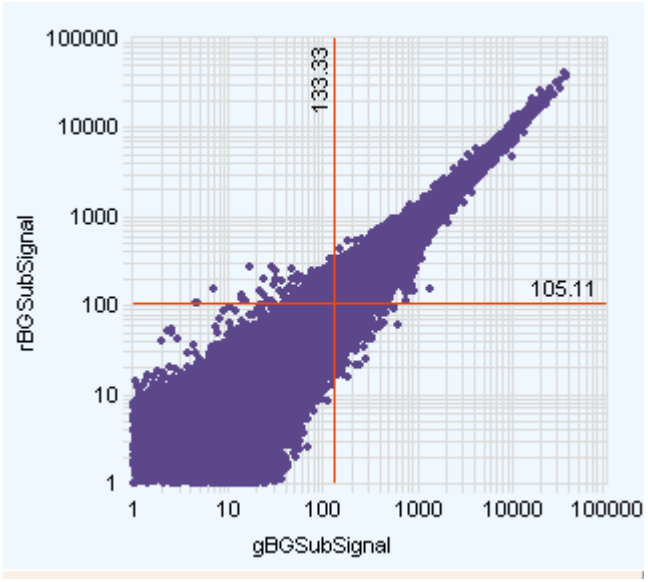
Table 1. Clinical and histopathological data of the patients selected to study and results obtained after the micrarra-based approach, MSRE-qPCR and bisulfite sequencing.

Patient	Age	Features				Phase of menstrual cycle	CA125 test	ASMR stage [17]	Histological classification [18]	Microarray-based approach		MSRE-qPCR		*Bisulfite sequencing*	
		Infertility	Dyspareunia	Dysmenorrhea	Acyclic pelvic pain					Hypermethylated genes	Hypomethylated genes	*HOXD4*	*HOXA7*	*HOXA7*	*BCOR*
116	35	-	+	-	-	Proliferative	33.7	III	E + M	139	545	Y	Y	-	-
121	29	-	+	+	-	Proliferative	0	III	M, U	-	-	Y	Y	-	-
169	30	NA	+	-	+	Proliferative	0	III	M	161	-	N	N	-	-
185	33	+	-	-	-	Proliferative	11	I	E + M	-	-	Y	Y	N	N
190	36	+	+	+	-	Proliferative	177	IV	E + M	189	188	Y	N	-	-
192	39	+	-	+	+	Proliferative	11.6	II	E + M	86	-	-	-	-	-
199	28	NA	+	+	+	Proliferative	70	III	E + M	225	-	-	-	-	-
207	39	+	-	+	-	Secretory	0	I	M	134	254	Y	N	-	-
214	29	+	-	+	-	Proliferative	69	III	E + M	151	613	Y	Y	-	-
218	58	-	+	+	+	Proliferative	0	IV	E + M	-	-	-	-	N	N
255	34	-	-	+	+	Proliferative	0	I	E + M	109	294	Y	N	-	-
256	32	+	+	-	-	Proliferative	0	IV	E + M	103	-	Y	Y	Y	N

-, absent; +, present; E, strommal pattern; ND, undetermined; NA, no attempt; M, glandular pattern; U, undifferentiated cells; Y, gain of methylation confirmed; N, gain of methylation non confirmed

SUPPLEMENTARY DATA

A)



B)

Cases

Cases	116	169	190	192	199	207	214	255	256
116	0,9758 (M) 0,9537 (U)								
169		0,8677 (M)							
190			0,9663 (M) 0,8975 (U)						
192				0,9215 (M)					
199					0,9570 (M)				
207						0,9130 (M) 0,9366 (U)			
214							0,9708 (M) 0,9273 (U)		
255								0,9746 (M) 0,9021 (U)	
256									0,9822 (M)

Figure S1. Scatter plot exemplifying the correlation of both channels (Cy3 and Cy5) after the extraction by Feature Extraction software (Agilent Tehnologies) (A) and the R values between replicates showing the correlation (B). M, methylated fraction; U, unmethylated fraction.

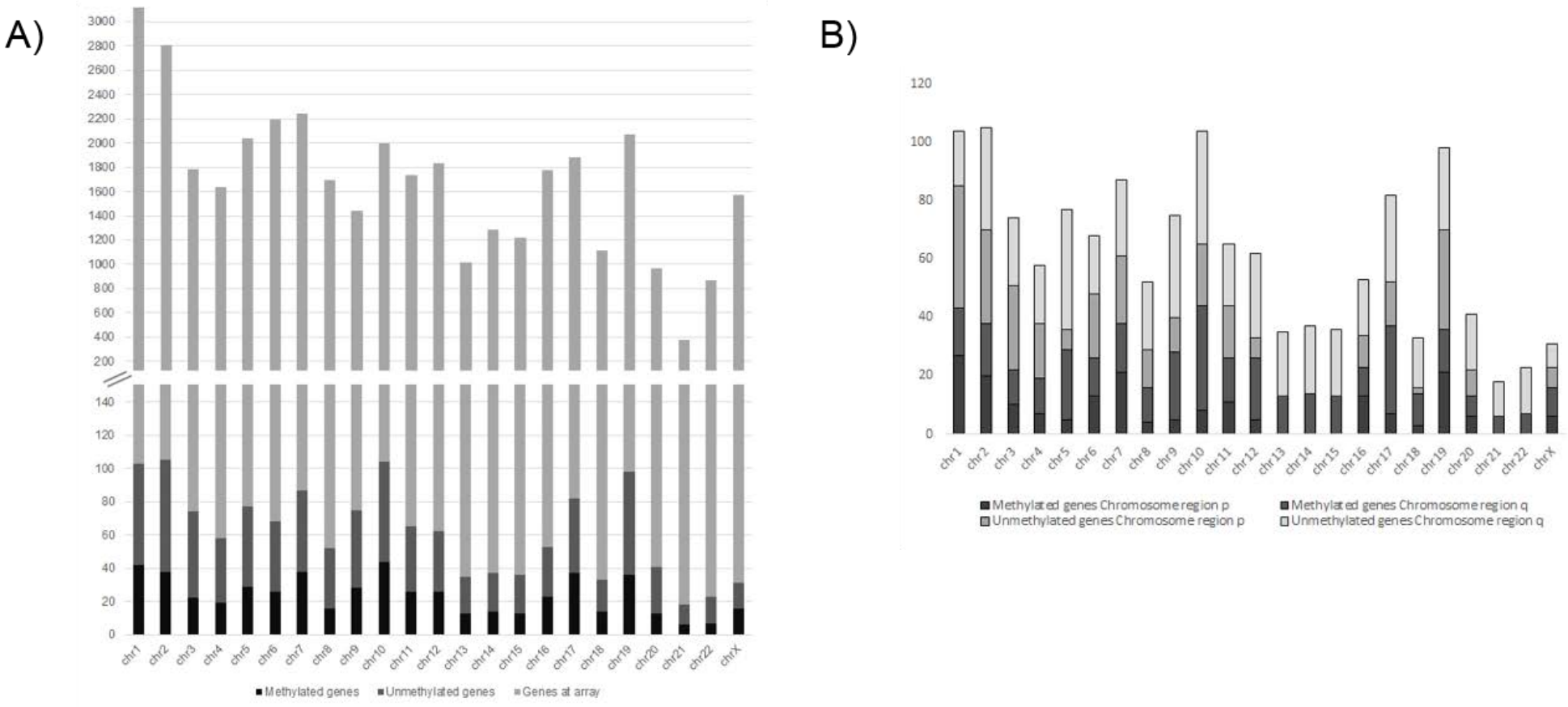


Figure S2. Number of genes identified as differently methylated by chromosome relatively to the number of genes represented at Agilent Human CpG Island ChIP-on-Chip Microarray 244K (A) and number of genes identified as differentially methylated by chromosome region p and q (B).

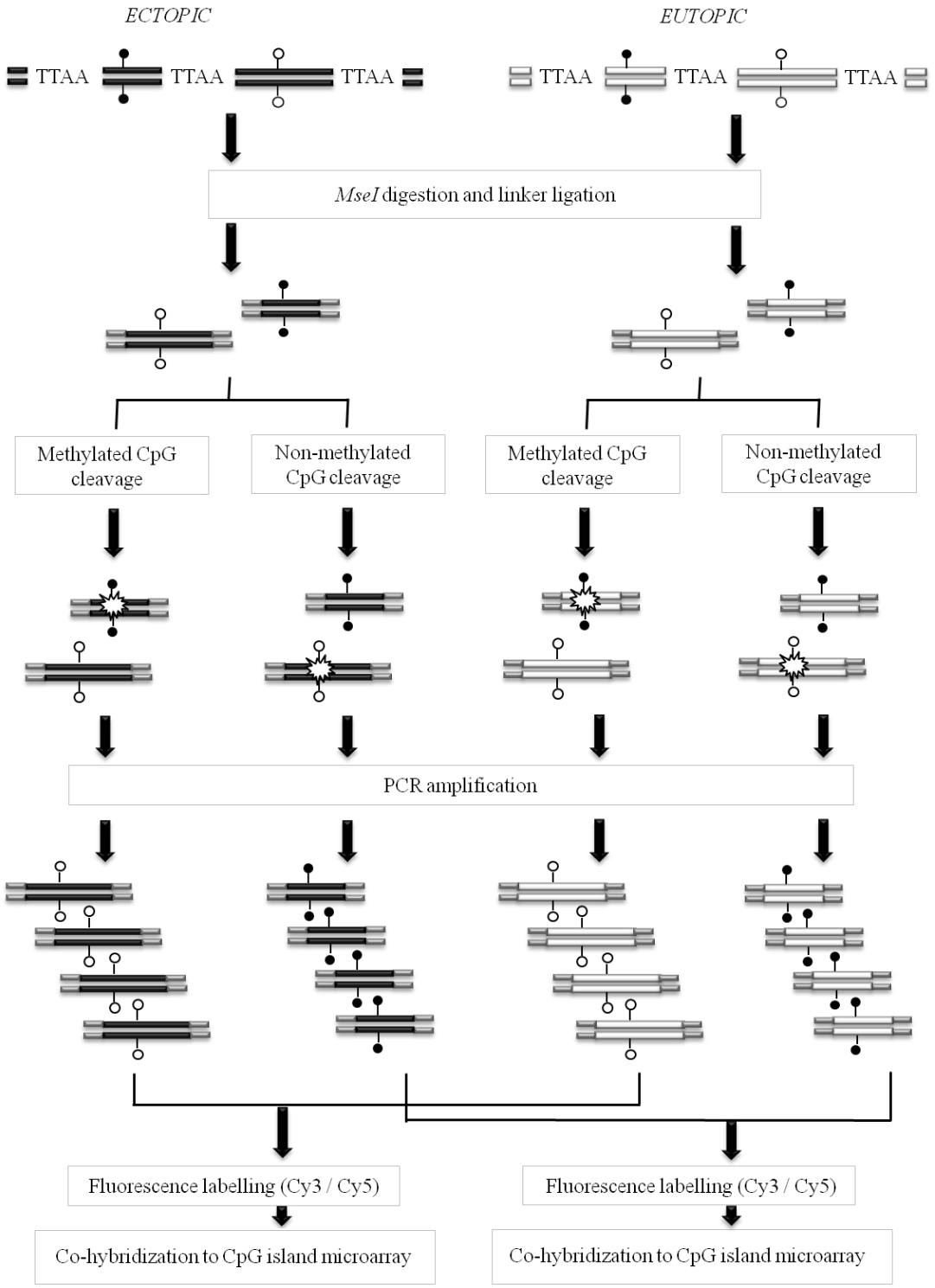


Figure S3. Experimental design of investigation strategy of differentially methylated CpG islands by a genome-wide analysis with CpG islands microarray.

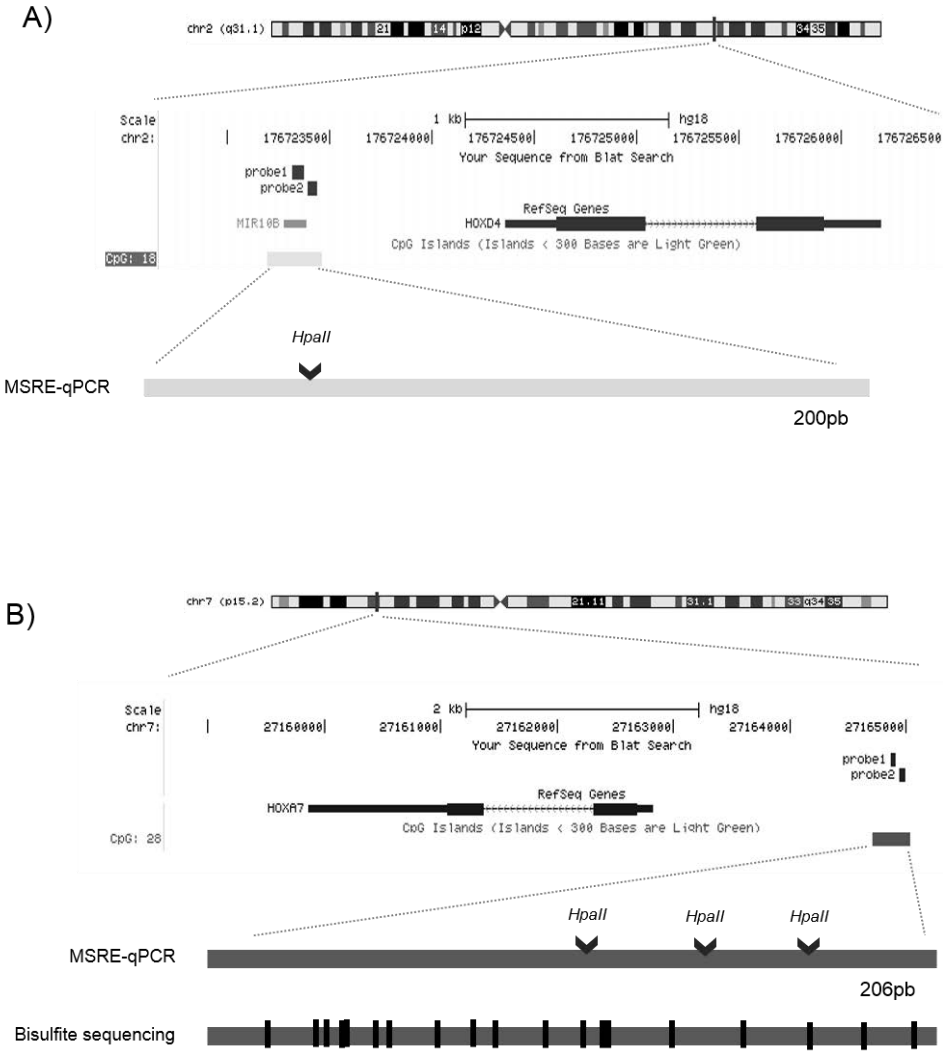


Figure S4. UCSC Genome browser indicating the CpG island of *HOXD4* (A) and *HOXA7* (B) genes selected to be evaluated by locus-specific methods. The HpaII sites on the region amplified by MSRE real-time PCR (one site for *HOXD4* at amplicon of 200bp and 3 sites for *HOXA7* at amplicon of 206bp) are indicated, corresponding to the location of probes identified as differentially methylated after the microarray-approach (probe 1 and probe 2). At B also is shown the location of CpGs (black lines) investigated by sequencing of DNA modified by sodium bisulfite of *HOXA7* gene.

As ONLINE SUPPLEMENTARY TABLEs não foram anexadas a este documento em razão da grande extensão dos arquivos. Ambas estarão disponíveis somente na versão on-line do artigo.

CAPÍTULO 3

Dados parciais deste artigo foram obtidos por Meyer JL. Avaliação do padrão de metilação de regiões promotoras dos genes *ESR1, ESR2* e *PGR* na endometriose profunda intestinal. Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas-Genética, IBB, Unesp, Botucatu-SP, 2011.

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RESEARCH PAPER

DNA methylation patterns of steroid receptor genes (ESR1, ESR2, and PGR) in deep endometriosis compromising the rectum

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Abbreviations: ASRM, American Society for Reproductive Medicine*; COX-2*, cyclooxygenase-2; *CYP19A1,* cytochrome P450, family 19, subfamily A, polypeptide 1; DNMTs, DNA methyltransferases; *ESR1* or ERα, estrogen receptor 1 or α; *ESR2* or ERβ, estrogen receptor 2 or β; *hMLH1,* mutL homolog 1, colon cancer, nonpolyposis type 2; HE, hematoxylin; *HOXA10*, transcription factor homeobox 10A; MS-PCR, Methylation-Specific Polymerase Chain Reaction; *NR5A1* or SF-1, nuclear receptor subfamily 5 or steroidogenic factor-1; *PGR* or PgR, progesterone receptor; *TP16*, cyclin-dependent kinase inhibitor 2A; UCSC, University of California Santa Cruz.

Key words: DNA methylation, endometriosis, epigenetics, CpG island, steroid hormone receptors, biomarkers

Abbreviated running title: DNA methylation of steroid receptor genes in intestinal endometriosis

Abstract

Endometriosis is characterized by the presence of endometrial-like tissue localized outside the uterine cavity. Recent evidence has suggested that endometriosis may be an epigenetic disease, besides an estrogen-dependent disease. Based on the unique steroid hormone receptors expression profile observed in endometriotic lesions compared to eutopic endometrium**,** this study tried to gain further insight into the DNA methylation patterns of alternative promoters of the steroid receptor genes *ESR1*, *ESR2,* and *PGR* in deep intestinal endometriosis, one of the most aggressive forms of endometriosis. The DNA methylation patterns was evaluated by Methylation-Specific Polymerase Chain Reaction (MS-PCR) after bisulfite modification in 44 endometriotic tissues as well as in seven matched eutopic endometrium. No differences in the DNA methylation were observed for the *ESR1* and *ESR2* genes. Methylation of the *PGR* gene was observed in 39% (17 out of 44 cases) and 19% (7 out of 37 samples) for promoter regions B (*PGRB*) and A (*PGRA*), respectively. Both PGR promoter regions were methylated in three cases. *PGRB* methylated alleles were detected exclusively in endometriotic lesions when compared to eutopic endometrium obtained from the same patient. The effect of DNA methylation in inhibiting *PGR* gene expression was corroborated by immunostaining for PgR protein in a subset tissue samples. The present study demonstrated that epigenetic changes occur in both promoter regions of *PGR* gene in intestinal endometriosis. Since eutopic and ectopic tissues do not respond sufficiently to progesterone in women with endometriosis, new studies are necessary to evaluate the effect of the epigenetic changes in the progesterone-resistance in this enigmatic disease.

Introduction

In the past two decades, epigenetic mechanisms have been recognized as key factors in the development of complex human disorders, including cancer, neurodegenerative, neurological and autoimmune diseases (1). Changes in chromatin conformation constitute the basis of epigenetic effects since they can alter gene expression without depending on DNA sequence changes. Chromatin epigenetic mechanisms include DNA methylation and post-translational covalent modifications of histones. These modifications comprise heritable changes that regulate essential biological processes, such as gene and microRNA expression, cellular differentiation, X-chromosome inactivation and genomic imprinting. DNA methylation is the most widely studied and best characterized epigenetic modification of the human genome (2). This epigenetic modification is catalyzed by DNA methyltransferases (DNMTs), which add a methyl group to the carbon 5 of cytosines that are followed by a guanine. Thus, DNA methylation occurs almost exclusively in the context of a 5′-CpG-3′ dinucleotide, especially in dense CpG regions termed CpG islands. Promoter-specific CpG island hypermethylation is known to be associated with gene silencing due to their transcription repression effect (3).

Recent evidence indicates that endometriosis, an enigmatic disease in which endometrial-like tissue is detected outside the uterus, is also an epigenetic disease (4-6). This hypothesis was based on the abnormal DNA methylation patterns observed in the promoter regions of specific genes (7-12) and the higher expression levels of DNMTs in endometriotic lesions in comparison with normal endometrium (13). The first evidence of increased levels of DNA methylation of a specific gene came from the pioneering study by Martini et al. (7), which reported aberrant DNA methylation in the tumor suppressor genes *TP16* (cyclin-dependent kinase inhibitor 2A) and *hMLH1* (mutL homolog 1, colon cancer, nonpolyposis type 2) in one and four samples of endometriosis, respectively. Subsequently, by using a candidate gene approach, others studies have described aberrant methylation patterns in the promoter region of

genes encoding the transcription factor homeobox 10A (*HOXA10*) (8), progesterone receptor – isoform B (*PGR*) (9), estrogen receptor 2, ERβ (*ESR2*) (10), nuclear receptor subfamily 5 (*NR5A1,* primed named SF-1 or steroidogenic factor-1) (11) and cyclooxygenase-2 (*COX-2*) (12) in endometriotic lesions derived from different anatomical sites. Furthermore, endometriotic cells treated *in vitro* with the demethylating agent 5-aza-2'deoxycytidine showed an increase in the expression of aromatase mRNA, suggesting that DNA methylation of the *CYP19A1* aromatase gene also occurs in stromal cells from endometriotic lesions (14).

Endometriosis is believed to be an estrogen-dependent disease characterized by the loss of progesterone-protective signaling in endometriotic cells. Xue et al. (10) demonstrated that this effect could be related to hypomethylation of the *ESR2* gene in stromal endometriotic cells, which could lead to an increase in the expression of estrogen receptor β (ERβ). These authors also demonstrated the presence of DNA methylation in a specific region of *ESR2* that confers promoter activity and co-localizes with a CpG island: *in vitro* methylation was correlated with inactivation of this promoter. Subsequently, it was proposed that the increased expression of ERβ, due to the hypomethylation of its coding gene, could inhibit the expression of the *PGR* gene (15). In addition, hypermethylation of the *PGR* gene, particularly at promoter B, was also observed in the epithelial component of the endometriotic lesions (9), with consequent decrease in the expression of its transcript.

Overall, steroid hormone receptor genes show a complex pattern of regulation evolving multiple promoters and alternative mRNA isoforms. *ESR1* gene has two proximal promoters (transcripts A and B) located within ~2kb of the translation start site and an upstream promoter C (16, 17). Similarly, the *PGR* gene has two alternative transcripts regulated by two promoter specific regions for the *PGRA* and *PGRB* isoforms, which are associated with well-characterized CpG islands (18). The *ESR2* gene also presents alternatively spliced transcript

variants and distinct promoters regulated by DNA methylation (19), although not associated to the presence of classic CpG islands.

Potentially, aberrant DNA methylation can disrupt the effects of hormone steroid due to changes in the expression levels of its receptors and may influence endometriosis origin and progression. Thus, the present study was delineated to investigate the methylation patterns of the alternative promoter regions of *ESR1*, *ESR2* and *PGR* genes in deep intestinal endometriosis, which accounts for 8-12% of all endometriosis. This condition is characterized by multifocal infiltrating and aggressive lesions associated with pelvic pain and infertility (20).

Materials and methods

Biological samples. Fresh endometriotic tissues were collected from 44 premenopausal patients (mean age 35.1 ± 6.8 years) who underwent laparoscopy for diagnosis and surgical resection (nodule resection or segmental resection of the rectum). The laparoscopy was indicated after a clinical evaluation and imaging methods (transvaginal ultrasound with bowel preparation). Matched eutopic endometrial tissue samples were collected by curettage simultaneously from seven of these patients. No patient has clinical diagnosis of immunological diseases or cancer. The classification system followed the American Society for Reproductive Medicine's recommendations for staging (I-IV) (Revised American Fertility Society classification of endometriosis - ASRM, 1997) (21). After histopathological confirmation of the diagnosis, lesions were morphologically classified as well-differentiated glandular pattern, stromal pattern, glandular pattern of mixed differentiation and undifferentiated glandular pattern according to previously described criteria (22). In addition, the phase of menstrual cycle was also estimated by histological evaluation. The clinical and histopathological data are provided in Table I. Approval for the study was obtained from the Institutional Review Board of the

Hospital das Clínicas and the Faculdade de Medicina da Universidade de São Paulo (CAPPesp – USP). All samples were collected after each patient provided informed consent.

All tissue samples were kept on dry ice immediately after surgical resection, stored in a -80°C freezer. Subsequently, the frozen tissue samples were manually macrodissected by using custom-built needles (23).

DNA extraction and methylation analysis. Genomic DNA was obtained by standard sodium dodecyl sulfate/proteinase K digestion, followed by phenol/chloroform extraction and ethanol precipitation.

DNA conversion by sodium bisulfite was performed using an established protocol (24). Methylation-Specific Polymerase Chain Reaction (MS-PCR) was used to evaluated the DNA methylation patterns of the alternative promoter regions A and B of *PGR* and *ESR1* genes as well as a promoter region of the *ESR2* gene. Methylated and unmethylated sequences of each gene were detected using specific oligonucleotides as previously described by Sasaki et al. (25). The reactions were performed in a total volume of 25µL containing, 200µM of each dNTP, 15mM of Tris-HCl pH 8.0, 50mM of KCl and 1U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA). Amplification and additional reactions conditions are listed in Table II. The amplified products were visualized after electrophoresis on a 6% polyacrylamide gel, followed by silver nitrate staining. Water blanks were included in each assay.

For the standardization of the DNA methylation analysis, MS-PCR assays were firstly performed in three breast cancer cell lines showing a known hormone receptor expression profile (26). The cell lines selected were DA-MB-231, classified as negative for ER and PGR protein expression, and T47D and MCF-7 cells which are positive for both receptors. Furthermore, gene-specific methylation data for these cell lines, available in the Cancer

Methylome System (27), were obtained *in silico* and used as reference in the optimization step of the MS-PCR assays in the present study.

Immunohistochemistry analysis. ERα, ERβ and PgR protein levels were analyzed in formalin-fixed and paraffin-embedded tissues from seven selected endometriotic tissue samples (cases 2, 3, 28, 35, 36, 40, and 42). Sections were freshly cut (3μm) and mounted on slides with organosilane (3-aminopropyl triethoxy-silane) (Sigma-Aldrich Co, St, Louis, MO). Slides were deparaffinized in xylene, gradually rehydrated through a series of alcohol rinses, and washed in phosphate-buffered saline. Intrinsic peroxidase activity was blocked with hydrogen peroxidase and the sections were incubated with the primary antibodies: the RTU-ER-6F11 antibody (Novocastra, Newcastle, UK) (dilution 1:50), monoclonal anti-human estrogen receptor β1 antibody (DAKO Cytomation, Glostrup, Denmark) (dilution 1:60), and the monoclonal anti-human progesterone receptor 1A6 antibody (DAKO, Carpinteria, CA) (dilution 1:50). After incubation for 1 hour, the sections were washed in phosphate-buffered saline, incubated for 30 minutes with secondary biotinylated antibody, and for additional 30 minutes with streptavidin peroxidase complex (LSAB, DAKO, Carpinteria, CA). Color development was obtained with 3,3′-diaminobenzidine and counterstaining with hematoxylin-eosin (HE). Positive and negative controls for each marker were routinely performed during experiments. In areas of well-preserved tissue, the staining intensity of endometriotic lesions and the percentage of cells showing antibody reactivity were scored as +1 (< 25%), +2 (>25% and < 50%), +3 (> 50% and <75%), and +4 (>75%).

Statistical analysis. Descriptive mean and percentage statistics were used to summarize patient data and gene hypermethylation status. Associations between DNA methylation and clinical

and histological parameters were evaluated via Fisher's exact test with a 5% level of significance.

Results

The set of biological samples investigated in the present study was comprised of 44 cases of intestinal deep endometriosis. In this group of women, the most frequent symptoms were moderate to severe dysmenorrhea (n=39, 89%), dyspareunia (n=29, 66%) and infertility (n=24, 54%). Moreover, the majority of endometriosis specimens was classified as advanced stages III and IV (n=31, 70%). The histological classification could be assessed in 36 cases indicating a predominance of glandular and stromal pattern (n=26, 72%) and an undifferentiated glandular pattern in nine cases (25%). In addition, the menstrual cycle stage was determined as proliferative in almost all the tissue samples evaluated for this parameter (34/39 cases, 87%).

As expected, the presence of methylation determined by the MS-PCR assays was inversely correlated with the expression at protein level in the three breast cell lines investigated: methylated alleles for *PGRA, PGRB, ESR1A* and *ESR1B* were detected in MDA-MB-231 cell line (ER and PgR negative) and only unmethylated alleles were found in T47D breast cancer cells (ER and PgR positive). Similarly, in the MCF-7 cells (ER and PgR positive) only unmethylated alleles were detected for the *ESR1A, PGRA* and *PGRB* promoter regions, while both methylated and unmethylated alleles were found to *ESR1B* gene. These methylation pattens were also confirmed in the Cancer Methylome System, which present the methylation profile generated by immunoprecipitation using the methyl-CpG binding domain of the MBD2 protein followed by high-throughput sequencing (27, available at http://cbbiweb.uthscsa.edu/KMethylomes/) (data not shown).

Subsequently, the *PGRB* and *ESR2* promoter methylation patterns were obtained in the 44 cases of infiltrating intestinal. Unmethylated and methylated alleles were observed for the

ESR2 gene in all samples analyzed. *PGRB* promoter methylation was observed in 39% (17/44 cases) of endometriotic lesions. In seven of the 44 samples, it was also possible to determine the methylation pattern in matched endometrium obtained from the same patients. No difference was observed in the *ESR2* methylation pattern in these cases; however, only unmethylated alleles were observed in the *PGRB* promoter region. Thus, direct comparison of the *PGRB* promoter region methylation between matched endometrium and endometriosis samples revealed a differential pattern in four pairs since hypermethylated alleles were specifically detected in the endometriotic tissue (Figure 1).

In an attempt to remove the adjacent intestinal tissue in the fragments containing the endometriotic infiltrating lesions, it was performed the macrodissection of the frozen tissue specimens, which resulted in a limited quantity of DNA for multiple MS-PCR tests. Thus, the MS-PCR analysis of the *ESR1A*, *ESR1B* and *PGRA* promoters was possible in a subset of only 37 out of 44 endometriotic lesions. While methylated and unmethylated alleles were simultaneously detected for both *ESR1* promoters in all of these samples, *PGRA* methylated alleles were only detected in seven lesions (19%) (Figure 1).

Differences in the DNA methylation frequencies in endometriotic lesions were observed for the two promoter regions of the *PGR* gene: 14 samples were methylated in the *PGRB* promoter, seven were methylated at the *PGRA* promoter, and three were methylated at both promoters (cases 11, 13 and 14). However, no statistically significant differences were observed between the presence/absence of DNA methylation of *A* and *B* promoter regions of *PGR* gene and stage of the lesions, histological classification, or cycle phase (Table III).

The immunostaining of the ERα, ERβ and PgR proteins in endometriotic lesions evidenced a heterogeneous pattern with the presence of both positive and negative epithelial and stromal cells (Figure 2). Interestingly, some endometriotic samples showed positive staining to PgR just in the stromal component, while the epithelial component were negative

(Figure 1, case 3 and Figure 2, H). In these cases, were identified both methylated and unmethylated alleles, probably due the epigenetic silencing mediated by DNA methylation of the *PGR* gene in the epithelial fraction of endometriotic lesions.

Discussion

The major finding of our study is that promoter region *B* of the *PGR* gene differs in methylation status between eutopic endometrium and deep endometriosis compromising the rectum: methylated alleles were specifically detected in endometriotic lesions, while endometrium samples showed only unmethylated alleles. These data suggest that this epigenetic change may be a possible biomarker for endometriosis. Using the same MS-PCR primer set, Wu et al. (9) reported the presence of partial methylation in this promoter in the epithelial component of peritoneal and ovarian endometriotic implants, but not in the promoter region that controls the expression of the A isoform. In addition, the authors reported low levels of *PGRB* expression in endometriotic epithelial cells. In the present study, we have demonstrated that both A and B promoter regions of *PGR* genes are methylated in a subset of infiltrating intestinal endometriosis.

It has been proposed that the *PGRB* isoform is crucial for mediating the inhibitory effects of progesterone on cell growth and invasion, while *PGRA* has a suppressor function (28, 29). Thus, the results of the present study indicates the importance of DNA methylation changes of *PGR* gene in a subset of infiltrative deep endometriosis, which is characterized by multifocal lesions sites, increased aggressiveness and severe clinical symptoms compared to pelvic endometriosis, usually investigated by the previous epigenetic studies (7, 9-11, 14).

Taken together, these data support the assumption that endometriotic lesions show a trend of partial methylation of the *PGR* gene, which not completely inactivate the gene expression in the entire lesion focus, but in some extent, is responsible by a decrease at

expression of PgR receptor restricted to some areas or specific cells. These findings were enforced in the present study by the heterogeneous pattern observed after PgR immunohistochemistry (Figure 2, H), which showed positive and negative staining the PgR receptor coexisting in the same endometriotic focus. In some way, this observation could explain the lower expression level of progesterone receptors in endometriosis and could be associated with to the fact that approximately 9% of women with endometriosis do not respond to treatment with progestin (5).

Currently, endometriotic lesions has been described as estrogen-dependent endometrium-like tissue consisting of glands and stroma. This abnormal tissue shows a unique expression profile of steroid hormone receptors compared to matched eutopic endometrium or endometrium from women without endometriosis. Previous studies have detected lower levels of ERα and higher levels of ERβ in endometriosis (30, 31). ERα appears to be the primary mediator of estradiol-induced progesterone action in this tissue (32), and progesterone exerts its functions in the endometrium by binding to the nuclear receptors PgR-A and PgR-B.

Epigenetic mechanisms modulate the dynamic regulation of the estrogen receptor genes and their functions (32). Promoter-specific DNA methylation could be causally related to the differential expression of the ERα and ERβ receptors in endometriosis. These receptors have differential affinity for ligands, are differentially expressed in a tissue-specific manner and may function antagonistically (33). Both ER isoforms exist as several splice variants. The alternative transcripts are ubiquitous, although their biological significance remains poorly understood (34). Furthermore, alternative promoter usage leads to pre-mRNAs with a variable length non-coding 5' untranslated region (35-37).

In breast cancer cell lines, *ESR1* expression was suppressed by promoter DNA methylation (38, 39) and histone hypoacetylation (40) and was derepressed by DNA methyltransferase (5-aza-2'-deoxycytidine) and histone deacetylase (trichostatin A) inhibitors

(41, 42). Promoter region hypermethylation has also been shown to be inversely associated with *ESR2* expression levels (43). In line with this, the methylation pattern at the *ESR1* gene was investigated in the endometriosis in the present study, for the first time, and methylated alleles at both promoters A (*ESR1A*) and B (*ESR1B*) were found. The consequences of DNA methylation in the gene expression have been better understood at CpG island-containing promoters, however, two promoters regions investigated in this study, *ESR1B* and *ESR2,* are CpG island free (Figure 1). However, this also represents a promising gene region to further DNA methylation studies in endometriosis, since it has been proved differentially methylated in studies investigating other diseases, as prostate or breast cancer (25, 44), suggesting that the regulation of this gene by DNA methylation overlaps other complex molecular mechanisms as described recently (45, 46).

To the best of our knowledge, only one study has evaluated the methylation status of the *ESR2* gene in eight ovarian endometriomas by bisulfite-modified DNA sequencing (10). The authors described high levels of *ESR2* transcripts associated with hypomethylation of the respective promoter region in comparison with endometrium. Contrarily, in the present study, MS-PCR analysis targeting a different region of an alternative promoter of *ESR2* (Figure 1) in endometria and intestinal deep endometriosis matched samples showed a more complex pattern, with methylated and unmethylated alleles in both tissues. Notwithstanding, the region investigated in this study represents the start of many new transcripts identified as expressed at premenopausal endometrium or endometriosis (GenBank, available at http://www.ncbi.nlm.nih.gov/nuccore, accession numbers AF074598 and AF074599, respectively). In this context, it is tempting to speculate whether the *ESR2* gene could generate multiple transcripts by triggering transcription initiation at alternative sites that could be inactivated by tissue-specific methylation. Therefore, interpretation of differential DNA methylation patterns has proven difficult, in part because the functional consequences depend

on the genomic region involved, the specific CpG dinucleotides, and inter- and intratissue heterogeneity. In line with this, the immunostaining performed in this study was able to indicate the heterogeneity inherent to the endometriotic lesions, due to the different staining pattern between epithelial and stromal cells, or even inside of the same cellular component (stromal or epithelial) only. Further, this heterogeneity is increased due to the additional presence of other kind of infiltrative cells, such as inflammatory cells (Figure 2, A e B).

From a translational research perspective, gene-specific methylation patterns may be useful biomarkers for detection because DNA methylation changes potentially provide a positive signal for endometriotic cell detection. MS-PCR is a very sensitive PCR-based method, and it has been used to detect hypermethylated genes in limited quantity samples derived from patients with various types of malignancies, such those obtained from fine needle aspiration, biopsies, and corporal fluids (47). In this context, epigenetic studies could lead to the identification of the target genes of aberrant DNA methylation in endometriosis, thus representing a promising area for the monitoring of the onset and progression of the disease. In the present study, we have shown that DNA methylation in the promoter of *PGRB* gene was a biomarker of the endometriotic lesions as compared to endometrium of the same patient. Also, aberrant *PGRB* methylation could be identified at the entire lesion focus, without the necessity of isolation the glandular and stromal components. Thus, this epigenetic change has the potential to be considered as a useful biomarker to the pathogenesis of a subset of endometriosis comprised by intestinal deep endometriosis.

In summary, these data indicate that abnormal methylation patterns occur in deep endometriosis compromising the rectum. Further studies are clearly necessary to elucidate the possible relationship between DNA hypermethylation of the A and C promoter regions of *PGR* gene and progesterone resistance. In addition, future epigenetic studies involving the evaluation of steroid receptor methylation in endometriosis should consider the histological pattern and

each component separately (stromal or epithelial) in order to verify the effect of DNA methylation on the expression of each alternative steroid receptor transcript in this complex disorder.

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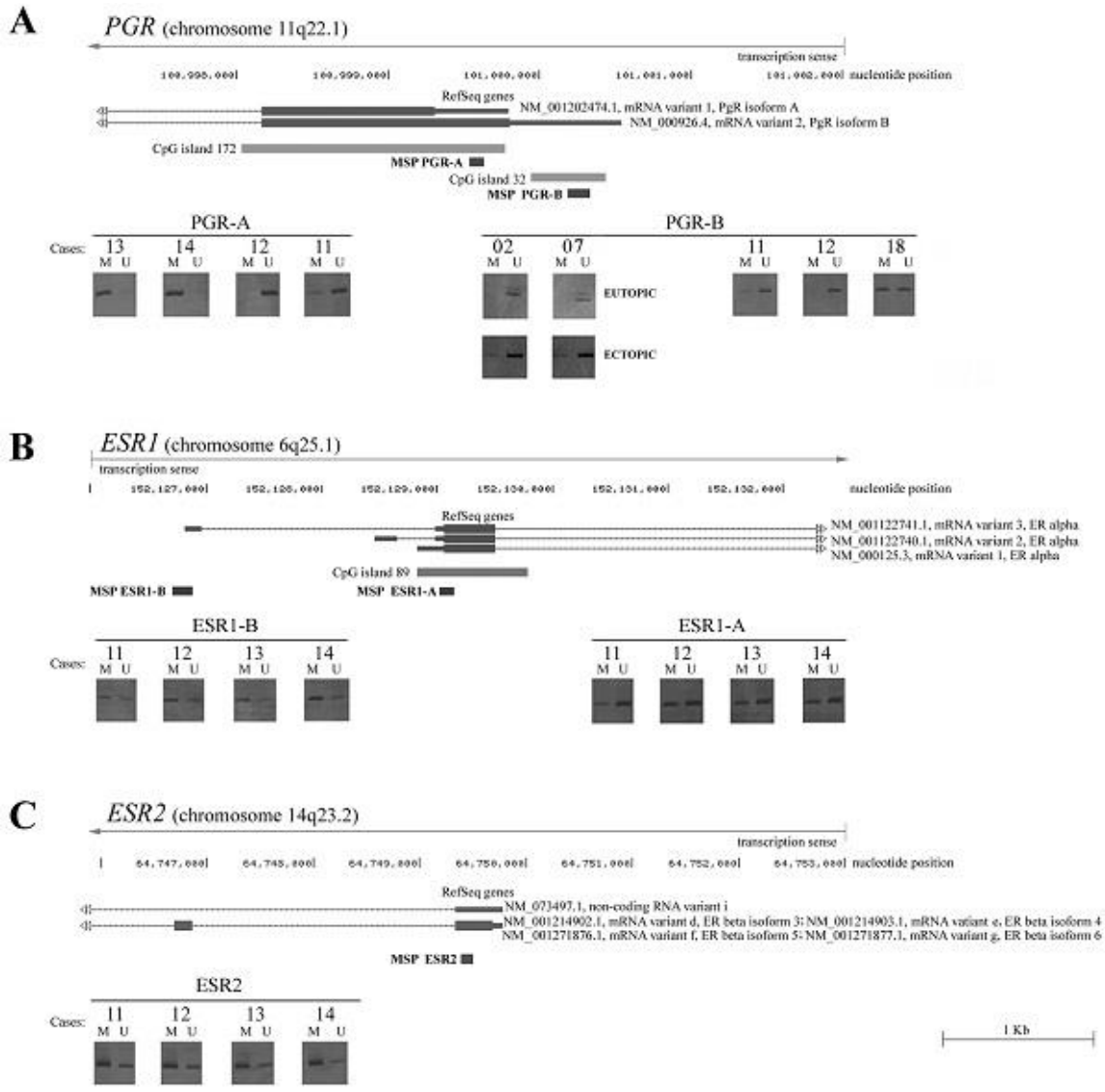
Conflict of interest

None of the authors have financial or any other conflict of interests that might prejudice the interpretation or presentation of the data contained within this manuscript.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101,001,000l
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MSP PGR-A
CpG island 32
MSP PGR-B
PGR-A
PGR-B
Cases:
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02 07 11 12 18
M U
EUTOPIC
ECTOPIC
B
ESR1 (chromosome 6q25.1)
transcription sense
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ESR1-A
Cases:
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C
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NM_001271876.1, mRNA variant f, ER beta isoform 5; NM_001271877.1, mRNA variant g, ER beta isoform 6
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ESR2
Cases:
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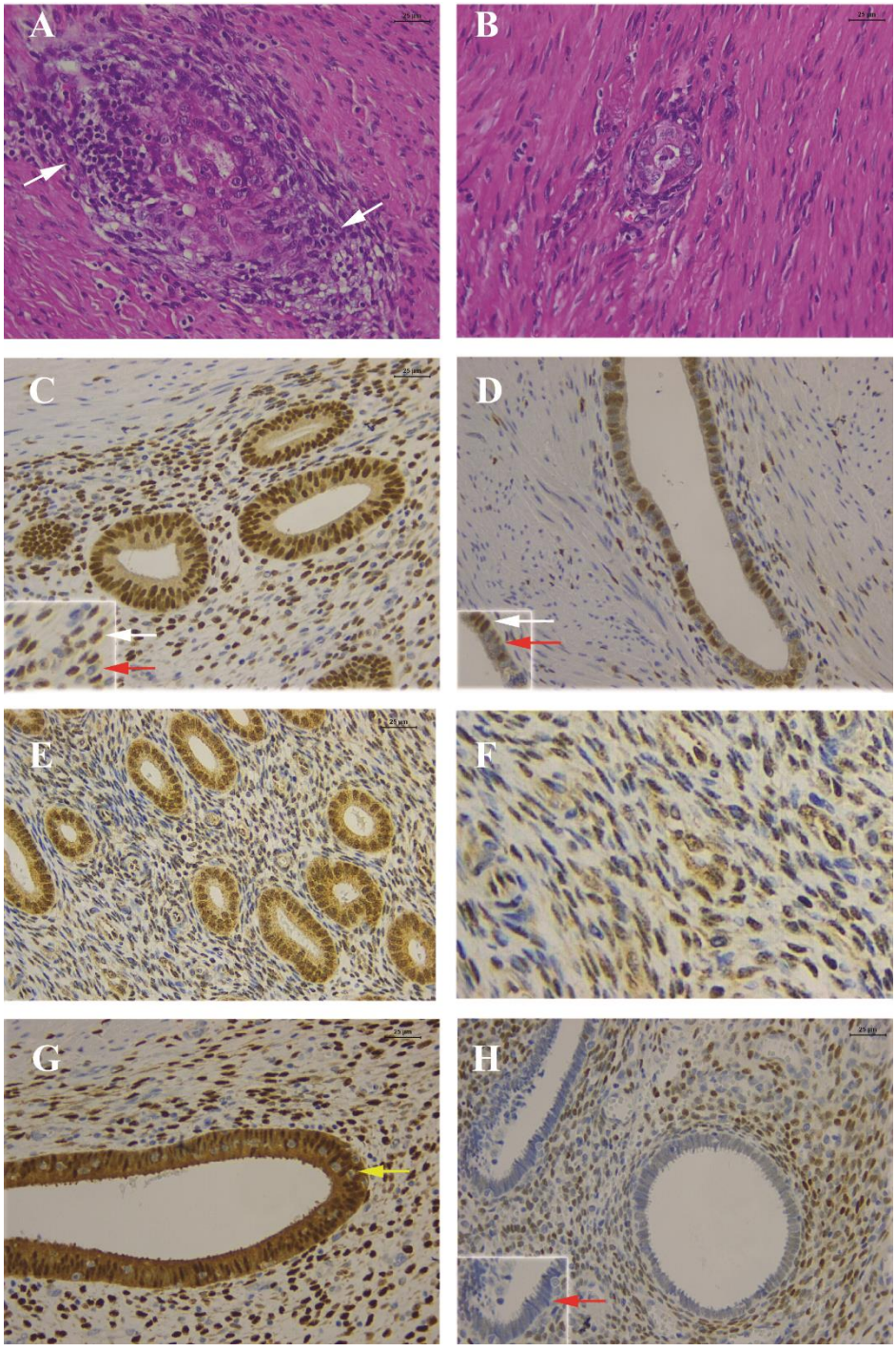
M U
1 Kb


A
B
C
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G
H

Figure 1. MS-PCR analysis of steroid receptor genes in deep endometriosis compromising the rectum. A, B, and C) UCSC browser representation of the alternative transcripts of *PGR*, *ESR1* and *ESR2* genes, associated CpG islands and respective MS-PCR assays (UCSC Genome Browser on Human, Feb. 2009 (GRCh37/hg19) Assembly, available at http://genome.ucsc.edu/). **A**) MS-PCR results of *PGR* gene showing methylation of promoter A (cases 11, 13, and 14) and of promoter B (cases 02, 07, 11, and 18). Methylated alleles in cases 02 and 07 were detected only in the deep endometriotic lesions. **B** and **C**) Methylated and unmethylated alleles were detected evenly after MS-PCR analysis of *ESR1* (promoters A and B) and *ESR2* genes in all cases. (M – methylated alleles; U – unmethylated alleles).

Figure 2. HE and immunostaining of ERα, ERβ and PgR (**A**) Note the moderate inflammatory infiltrate (white arrow) in the stroma of the endometriosis, HE; (**B**) Endometrial gland with scattered stroma and few inflammatory cells, HE; (**C**) Estrogen receptor alpha (ERα) in the epithelial glandular cells (all cells are positive) and some stromal cells positive. Inset: detail of positive (white arrow) stromal cells and negative (red arrow) stromal cells for ERα (epithelium: score +4; stroma: score +3); (**D**) ERα. Endometrial gland of an endometriotic intestinal focus, with positive and negative nuclear ERα, and few positive cells in the stroma. Inset: detail of positive (white arrow) stromal cells and negative (red arrow) epithelial cells for ERα (epithelium: score +4; stroma: score +3); (**E**) Estrogen receptor beta (ERβ). Positive nucleus in all epithelial cells and in most of the stromal cells (epithelium and stroma: score +4); (**F**) ERβ. Higher magnification of the stromal component of the endometriosis focus, with positive and negative cells (epithelium and stroma: score +4); (**G**) Progesterone receptor (PgR). Note that all the nucleus of the epithelial cells are positive for PgR and some of the stromal cells. Inside the gland there are some inflammatory cells, negative for PgR (yellow arrow) (epithelium and stroma: score +4); (**H**) PgR of case 03. All epithelial cells from the endometriosis focus are negative, and the majority of the stromal cells are positive (epithelium: negative; stroma: 3+). Inset: Details of the negative (red arrow) epithelial cells for PgR. Bar: 25 μm.

Table I. Clinical and histopathological data of intestinal deep endometriosis patients.

	Number of Cases	Percentage
Clinical Features/ Symptoms		
Infertility	24/44	54%
Dyspareunia	29/44	66%
Dysmenorrhea	39/44	89%
ASMR stage* (n=44)		
I	7	16%
II	6	14%
III	3	7%
IV	28	63%
Histological classification** (n=36)		
Well-differentiated glandular pattern	1	3%
Glandular of mixed differentiation + stromal pattern	26	72%
Undifferentiated glandular + stromal pattern	9	25%
Phase of menstrual cycle (n=39)		
Proliferative	34	87%
Secretory	2	5%
Menstrual	3	8%

*The staging was determined according to the Revised American Society for Reproductive Medicine classification of endometriosis (21).

**The histological classification was determined according Abrão et al (22).

Table II. Primer sequences, reaction and amplification conditions used in MS-PCR analysis.

Primer	Oligonucleotide Sequence	Reaction Conditions	Amplification Conditions	Amplicon
PGRA-UF	5'-ATGGGTTATTTTTTTTTTG-3'	[$MgCl^2$] 2.5 mM	1 cycle: 95°C – 10min; 35 cycles: 95°C – 1min, 51°C – 1min, 72°C – 1min; 1 cycle: 72°C – 1min	99bp
PGRA-UR	5'-TAAAATATACACCCTCCACA-3'			
PGRA-MF	5'-ACGGGTTATTTTTTTTTCG-3'	[Primers] 0.08 µM		
PGRA-MR	5'-TAAAATATACGCCCTCCACG-3'			
PGRB-UF	5'-TGATTGTTGTTTGTAGTATG- 3'	[$MgCl^2$] 4.0 mM	1 cycle: 95°C – 10min; 35 cycles: 95°C – 1min, 50°C – 1min, 72°C – 1min; 1 cycle: 72°C – 1min	200bp
PGRB-UR	5'-CAACAATTTAATAACACACA- 3'			
PGRB-MF	5'-TGATTGTCGTTCGTAGTACG- 3'	[Primers] 0.24 µM		
PGRB-UR	5'-CGACAATTTAATAACACGCG- 3'			
ESR1A-UF	5'-GGATATGGTTTGTATTTTGTTTGT-3'	[$MgCl^2$] 2.5 mM	1 cycle: 95°C – 10min; 35 cycles: 95°C – 1min, 54°C (U)/50°C (M) – 1min, 72°C – 1min; 1 cycle: 72°C – 1min	123bp 121bp
ESR1A-UR	5'-ACAAACAATTCAAAAACTCCAACT-3'			
ESR1A-MF	5'-GATACGGTTTGTATTTTGTTCGC-3'	[Primers] 0.16 µM		
ESR1A-MR	5'-CGAACGATTCAAAAACTCCAACT-3'			
ESR1B-UF	5'-TTTATTGTTATTTATTTAGT-3'	[$MgCl^2$] 4.0 mM	1 cycle: 95°C – 10min; 35 cyles: 95°C – 1min, 47°C (U)/49°C (M) – 1min, 72°C – 1min; 1 cycle: 72°C – 1min	180bp
ESR1B-UR	5'-AAAAATATACTCACATATACA-3'			
ESR1B-MF	5'-TTTATTGTTATTTATTTAGC-3'	[Primers] 0.16 µM		
ESR1B-MR	5'-AAAAATATACTCGCATATACG-3'			
ESR2-UF	5'-TTTGGAAGGTGGGTTTGGTT-3'	[MgCl2] 3.0 mM	1 cycle: 95°C – 10min; 35 cycles: 95°C – 1min, 54°C – 1min, 72°C – 1min; 1 cycle: 72°C – 1min	109bp
ESR2-UR	5'-CACATACAAATATAATAACTAACA-3'			
ESR2-MF	5'-TTTGGAAGGTGGGTTTGGTC-3'	[Primers] 0.16 µM		
ESR2-MR	5'-CGCATACAAATATAATAACTAACG-3'			

MF = Methylated Foward Primer; MR = Methylated Reverse Primer; UF = Unmethylated Foward Primer; UR = Unmethylated Reverse Primer; bp = base pair.

Table III. Clinical and histopathological features and DNA methylation of A and B promoter regions of the *PGR* gene.

Parameter	*PGRA*			*PGRB*		
	M	U	p-value	M	U	p-value
ASMR stage (*PGRA*, n=37 and *PGRB*, n=44)						
I + II	1	6	0.64	6	7	0.52
III + IV	9	21		11	20	
Histological classification (*PGRA*, n=29 and *PGRB*, n=36)						
Differentiated glandular pattern + stromal pattern	4	16	1.0	9	18	1.0
Undifferentiated glandular + stromal pattern	1	8		3	6	
Phase of menstrual cycle (*PGRA*, n=29 and *PGRB*, n=36)						
Proliferative	6	22	1.0	12	0	0.54
Secretory	0	1		22	2	

M = methylated alleles; U = unmethylated alleles.

5. CONCLUSÕES

Diante dos resultados obtidos no presente estudo, pode-se concluir que:

CAPÍTULO 2

- Alterações no perfil de metilação do DNA em ilhas CpG foram identificadas em 1164 genes na endometriose profunda intestinal, sendo 546 hipermetilados e 871 hipometilados, o que pode indicar um papel importante da modulação epigenética na manutenção da lesão no sítio ectópico;

- Destes genes, um grupo de 227 hipermetilados e 322 hipometilados foram recorrentes entre o nosso e diferentes estudos;

- Deste grupo, um subgrupo de 105 genes diferencialmente metilados (52 hipermetilados e 53 hipometilados) foram correlacionados com dados de expressão gênica e apresentaram um enriquecimento para vias biológicas envolvidas em mecanismos moleculares do câncer e pluripotência de células-tronco embronárias, bem como das vias de sinalização de Notch, relaxina e TGF-beta;

- Estas vias, com exceção da via de sinalização de Notch, foram também identificadas como enriquecidas no grupo de 1164 genes identificados como diferencialmente metilados em nosso estudo, reforçando a relevância biológica destas vias para a modulação da endometriose;

- Em adição, um enriquecimento de genes *homeobox* foi também identificado, dos quais os genes *HOXA7* e *HOXD4* mostraram-se como candidatos promissores a biomarcadores para a doença.

CAPÍTULO 3

- O gene PGR foi identificado como um potencial biomarcador de lesões endometrióticas intestinais;

- O padrão de metilação deste gene pode ser detectado sem a necessidade de isolamento dos componentes epitelial e estromal da lesão.

ANEXO

HOSPITAL DAS CLÍNICAS
DA FACULDADE DE MEDICINA
DA UNIVERSIDADE DE SÃO PAULO

APROVAÇÃO

A Comissão de Ética para Análise de Projetos de Pesquisa - CAPPesq da Diretoria Clínica do Hospital das Clínicas e da Faculdade de Medicina da Universidade de São Paulo, em sessão de 24/09/2008, **APROVOU** o Protocolo de Pesquisa nº **0531/08**, intitulado: "**IDENTIFICAÇÃO DE PERFIS DIFERENCIAIS DE METILAÇÃO DO DNA NA ENDOMETRIOSE**" apresentado pelo Departamento de **OBSTETRÍCIA E GINECOLOGIA**, inclusive o Termo de Consentimento Livre e Esclarecido.

Cabe ao pesquisador elaborar e apresentar à CAPPesq, os relatórios parciais e final sobre a pesquisa (Resolução do Conselho Nacional de Saúde nº 196, de 10/10/1996, inciso IX.2, letra "c").

Pesquisador (a) Responsável: **Mauricio Simões Abrão**
Pesquisadores Executantes: **Daniela Zimbardi, Cláudia Aparecida Rainho, Silvia Regina Rogatto**

CAPPesq, 25 de Setembro de 2008

Prof. Dr. Eduardo Massad
Presidente da Comissão de Ética para Análise de Projetos de Pesquisa

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