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Genetic Polymorphisms and Molecular Pathogenesis of Endometriosis

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1. Introduction

Endometriosis occupies a unique position in medicine. The natural history of the disease is uncertain, its precise etiology is unknown, the clinical presentation is inconsistent, diagnosis is difficult and can be confirmed only by invasive procedures, and the treatment is poorly standardized.

It is one of the commonest benign gynaecological conditions affecting 15 – 25% of women in the reproductive age group. Yet, it displays features similar to malignancy, ranging from neo-vascularisation to local invasion and aggressive spread to distant organs (metastasis).

The most widely accepted theory to explain endometriosis is that viable endometrial cells reach the peritoneal cavity through retrograde menstruation along the fallopian tubes. Some of the cells then adhere to the peritoneal surface and proliferate in response to the ovarian hormones. However, it is well established that menstrual debris is present in the peritoneal cavity of 90% of menstruating women, suggesting that endometrial cells from only 'some women' are capable of establishing ectopic endometrial implants. Why does this happen in only 'some (10-15%) women'? There are several possible explanations for disease susceptibility, including differences in genetic predisposition (Bischoff FZ et al, 2000), increased exposure to menstrual debris, abnormal eutopic endometrium, altered peritoneal environment, reduced immune-surveillance (Sinaii N et al, 2002), and increased angiogenic capacity (Absenger Y et al, 2004).

Endometriosis, the name by itself implies an endometrial pathology but this concept has long been disputed by Thomas and Prentice in 1992. The acceptance of an endometrial origin poses the question of whether endometrium that is able to proliferate and implant at ectopic sites is in some way abnormal. The commonest site for endometriosis is the pelvis. Endometrium reaches the pelvis most commonly by retrograde menstruation which is now accepted to be an almost ubiquitous event. Endometriosis is more common in women whose normal menstrual egress is occluded by genital tract anomalies, women with an early menarche, short menstrual cycles and prolonged menses. All these factors suggest that peritoneal soiling is important but if retrograde menstruation is ubiquitous then why do not all women have endometriosis? The truth is that the presence of ectopic endometrium within the peritoneal cavity is probably universal but the association of ectopic

endometrium with either symptoms or anatomic distortion is not. It is these latter two groups of women that present with pathological endometriosis as the presence of ectopic endometrium alone is not pathological; this can clearly be seen in the case of infertility associated with minimal and mild endometriosis.

The endometrium shed at menstruation contains numerous cytokines and angiogenic growth factors. These factors undoubtedly could promote proliferation of and angiogenesis around ectopic endometrial implants, allowing survival of the shed endometrium at ectopic sites. If they are of importance in the pathogenesis of endometriosis then it would be expected that differences would exist between those women with and those without endometriosis. However, no qualitative differences exist between either eutopic and ectopic endometrium nor between endometrium from women with and those without endometriosis. This observation is true for all growth factors and cytokines studied to date, and strongly suggests that endometriosis is not an endometrial disease.

If endometriosis is not primarily an endometrial disease, then it is uncertain what permits and facilitates the survival of endometrium at ectopic sites. It is becoming increasingly clear that the peritoneal fluid and its cellular constituents are important in the pathogenesis of endometriosis.

In many ways endometriosis can be considered to be a chronic inflammatory condition of the pelvis. The endometriotic implants have many different appearances but in early stages in particular, they are surrounded by new blood vessel formation which resembles an inflammatory response. The peritoneal fluid in endometriosis contains an increased size and number of macrophages that have an increased activation status. They secrete large number of cytokines and produce growth factors that support the establishment of the ectopic implants. Peritoneal macrophages are regulated by ovarian steroids and in their activated state produce increased levels of VEGF (vascular endothelial growth factor) that supports angiogenesis around the endometriotic implants. These macrophages are less susceptible to apoptosis which is the normal consequence of activation.

However, there is convincing evidence that the disease is inherited as a complex genetic trait (Kennedy S, 1997). Genetic factors accounted for 52% of the variation in liability to endometriosis in an Australian twin study (Treloar et al, 2000). The genetic relative - recurrence risk for sibs was estimated to be 2.34 in a cohort of Australian twins and their families. This risk indicates that polygenic and multifactorial etiology is far more likely to be the cause than Mendelian inheritance. Familial aggregation has been reported in humans and non-human primates (Kennedy S et al, 1995; Zondervan et al, 2004). A genome-wide Linkage study in 1,176 affected sister pair families identified a significant susceptibility locus for endometriosis on chromosome 10q26 (Treloar SA, Kennedy SH, et al, 2005).

Initially *cytogenetic studies* of chromosomal rearrangements in affected endometriotic tissue were conducted to uncover candidate chromosomal loci. Dangel et al, (1994), found no evidence of abnormalities in any of the 42 implants studied. However, with the application of chromosome-specific probes using multicolor fluorescent in situ hybridization (FISH) technique, a significantly greater frequency of chromosome 17 aneuploidy in the endometriotic specimens was observed. It was proposed that acquired chromosome-specific alterations may be involved in endometriosis through a multistep pathway suggesting

clonal expansion of chromosomally abnormal cells. Cytogenetic R-banding studies on human endometriosis-derived permanent cell line (FbEM-1) showed numerous chromosomal aberrations, including monosomy X, 4q⁺, 5q⁺, trisomy 7,8,10 and tetrasomy of chromosomes 17, 18, 19 and 20 (Bouquet DJ et al, 1997). A caveat for these studies is that cultured cell-lines may be unstable, which reflects growth of selectively advantaged cells and is no longer representative of the original tissue. Comparative genomic hybridization (CGH) has also been used which revealed overrepresentation of chromosomes 1, 2, 3, 5, 6p, 7, 16, 17q, 20, 21q, and 22q, whereas chromosomes 6q, 9, 11p, 12, 13q, 18 and X were underrepresented. Subsequent FISH analysis confirmed gain at 6p and 17q and loss of 1p, 22q, and chromosome X (Gogusev J et al 2000).

Subsequent studies were conducted using the method of *quantitative genetic analysis*. To find the genes of endometriosis from nearly 30,000 total human genes, the technique of sibling-pair quantitative linkage analysis was used. Sibling-pair analysis obligatorily requires that informative DNA polymorphic markers exist every few centimorgans (10cM). The polymorphisms usually used are DNA variants, such as di-nucleotide, tri-nucleotide, or tetra-nucleotide repeats.

It has been argued that findings have been inconsistent because most studies have used inappropriate controls. However, confusion is also created by underpowered studies and/or studies investigating genes without sufficient evidence to support their biological plausibility. *Two examples are given here.*

First, significant association of GALT gene with endometriosis was reported with at least one allele with the N314D mutation, whereas GALT gene had been previously associated with Mullerian anomalies. The polymorphism causes reduced activity of the enzyme galactose-1-phosphate uridyl transferase, which is involved in galactose metabolism. Three subsequent studies involving more patients have failed to replicate the association (Stefansson H et al, 2001).

Second, genes that encode enzymes involved in detoxification, such as the glutathione S-transferase (GST) family, have been investigated based on the finding that the environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) induces endometriosis in the rhesus monkey. Homozygotes for a null mutation in one of the GST family genes, *GSTM1*, were more common in endometriosis cases than in controls. Subsequently, studies in UK (Hadfield RM et al, 2001), Japanese (Morizane M et al, 2004), Chinese (Ding Y et al, 2004) and Indian (Babu KA et al, 2005) populations have failed to replicate the association. No association has been found for a mutation in a similar gene, *GSTT1*. Moreover, in a critical appraisal of all the human and nonhuman primate evidence implicating dioxin exposure as a risk factor for endometriosis, Guo SW (2004) concluded that there was insufficient evidence to support the theory, about 10 years after it was first proposed! It is possible, therefore, that research groups around the world have investigated genes involved in detoxification as functional candidates based on an entirely false premise.

Although endometriosis is a benign disorder, it has also been viewed as a neoplastic process. Evidence to support this hypothesis includes molecular similarities between endometriosis and cancer; and the increased susceptibility to develop ovarian clear-cell cancer and endometrioid cancer in the presence of endometriosis. Molecular genetic

alterations in endometrioid cancers of the ovary were analyzed (Catasus L et al, 2004). Frequent alterations are seen in beta-catenin and PTEN genes, as well as MSI in low-stage ovarian carcinomas of endometrioid type. However, allelic loss studies do not provide evidence for the 'endometriosis as tumor' theory (Prowse AH et al, 2005). Ovarian endometriosis with cytological atypia has potential for malignant transformation. Endometriotic cysts seem to be monoclonal and demonstrate aggressive growth and localized invasion of the myometrium. Malignant transformation has been documented. The proposed pathway is through loss of chromosome material by causing allelic imbalance or loss of heterozygosity (LOH). Genomic instability in any form can transform a normal cell to an abnormal or malignant cell. DNA damage can cause genetic alterations and can manifest as gross DNA damage, chromosomal instability or by a more subtle genetic change like microsatellite instability.

DNA studies examining the role of LOH in endometriotic lesions have identified candidate suppressor gene loci, including 9q, 11q and 22q (Jiang X et al 1996). Alterations in chromosome arms 5q, 6q, 11q and 22q were observed in 25-30% of women with endometriosis and associated carcinoma of the ovary (Jiang X et al 1998).

Gene expression analysis by oligo-nucleotide micro-arrays indicated inflammatory immune-reactions due to up-regulation of FCER1G and PGDS mast cell specific genes which play an important role in producing fibrosis and adhesions in endometriotic lesions (Konno R et al, 2003). Downregulated elements included the tumor suppressor Tp53, genes related to apoptosis, and the gene encoding OVGPI1, a protein involved in maintenance of early pregnancy (Arimoto et al, 2003).

Eyster KM et al (2002) illustrated the use of cDNA micro-array technology by studying eutopic endometrium and endometriotic implants from three patients and reported eight genes that were over-expressed in endometriotic implants as compared to eutopic endometrium, that had roles in the cytoskeleton.

The revolutionary evolution of genetics and molecular technologies has given a new perspective to the understanding of the etiology of the perplexing disease of endometriosis. Meyer's (1919) hypothesis of coelomic metaplasia in the totipotential peritoneal cells subjected to repeated irritation by a variety of factors, as a cause of endometriosis, would explain those cases of endometriosis in primary amenorrhoea and in men on prolonged estrogen treatment. There are some unknown factors produced within the uterus which stimulate undifferentiated mesenchyme to undergo metaplastic transformation. These *unknown factors* that allow endometrial fragments to implant in the peritoneal cavity of some women and lymphatic spread to allow ectopic endometrium to develop at distant sites are presently being hypothesized to be due to genetic factors. It has been suggested that endometriosis has a genetic basis by Kennedy et al two decades back.

1.1 Definition of endometriosis

Endometriosis is defined as a disease characterized by the presence of functional endometrial cells, comprising of glands and stroma in ectopic sites outside the uterine cavity in addition to their normal presence in the innermost lining of the uterus. The ectopic endometrial tissue responds to hormones and drugs in a similar manner to eutopic endometrium.

Ectopic endometrium shares many morphological aspects with eutopic endometrium, but differs in its biological behaviour due to the fact that the cells are present in a different environment. Ectopic and eutopic endometrium are not synchronized in their histological changes; ectopic tissue implants present a maturation disorder with different degrees of maturation and organization in the same implant. Complete secretory modification is rarely found in endometriosis and this could be due to an impact on the production of progesterone dependent factors like prostaglandins.

2. Factors affecting endometriosis

There are numerous suggested etiological factors in the pathogenesis of endometriotic implants. However, only the genetic factors will be discussed here.

2.1 Genetic factors

Epidemiological data show a familial tendency of endometriosis. Patients with an affected first-degree relative have nearly a ten-fold increased risk of developing endometriosis. Concordance of twins has also been demonstrated (Bischoff FZ et al 2000). There is evidence in both, human and non-human primates that supports the theory of a genetic basis to endometriosis (Zondervan KT et al, 2001).

Attempts to explore the role of genetic and molecular factors in the etiology of endometriosis have begun in the last decade. Genetic studies also detected an association between endometriosis and polymorphic mutations of several genes, including the N-acetyltransferase 2 gene (NAT2), the glutathione S-transferase M gene (GSTM) and Estrogen Receptor alpha gene (ER- α).

2.1.1 DNA- polymorphism

Polymorphism literally means many forms and it is seen that on an average, every 1 in 500 base pairs of DNA varies between individuals. Variations in coding regions are rare because of the need to preserve function. Changes in non-coding regions of the genome are subject to very little selection pressure and generate allelic variations at a very high frequency. This variation can be an alteration in a single base, deletion or addition of bases and expansion or contraction of repeats.

DNA polymorphisms are the basis of all current *genetic markers*. A marker is any *polymorphic Mendelian* character that can be used to follow a chromosomal segment through a pedigree. Allelic human gene *expression variation* may be caused by changes in regulatory DNA, including sequences which regulate transcription and splicing. This type of sequence variation may often underlie the susceptibility to common diseases but quantitative methods to explore allelic variation in human gene expression have been developed only very recently (Yan H et al, 2002).

2.1.2 Single Nucleotide Polymorphism (SNP)

This involves a single nucleotide which is substituted by a different nucleotide. Typically, SNPs have only two alleles. Since coding DNA accounts for only about 1.5% of the human

genome, most SNPs are found in the non-coding DNA, such as within introns and intergenic sequences.

3. Endometriosis and genetic polymorphism

A number of polymorphisms in candidate genes have been studied to identify the genes responsible for the etiology of endometriosis. It was only since 2000, that reports were published establishing an association of genetic polymorphism with endometriosis.

Transforming growth factor beta (TGF- β) family members are multi-functional cytokines that play a key role in cellular growth, proliferation, and differentiation (Hsieh YY et al, 2005). It has been shown that an association of endometriosis with TGF- β 1-509 gene polymorphism exists. T homozygote and T allele for TGF- β 1 are associated with higher susceptibility to endometriosis. Arg448Gly, a common polymorphism located within nuclear receptor interacting protein 1 (NRIP1) gene, is associated with endometriosis. NRIP1 gene variants, separately or in combinations, might act as predisposing factors for human endometriosis (CaballeroV, et al., 2005).

A significant linkage on chromosome 10q26 and another region of suggestive linkage on chromosome 20p13 as susceptibility loci, has been associated with endometriosis (Treloar SA et al, 2005).

It has been hypothesized that dysregulation of the normal apoptotic process takes place in the endometrium. One of the apoptotic pathways playing a crucial role in the programmed cell death within the endometrium is the Fas-FasL system. Three polymorphisms within FAS (-1377G>A and -670A>G) and FASL (-843C>T) genes, as susceptibility factors for endometriosis have been analysed. However, the differences in the distribution of the polymorphic variants were not statistically significant (Fernandez,R.M.et.al,2005). The angiotensin I-converting enzyme (ACE) A2350G and A-240T gene polymorphism has been suggested as markers of susceptibility in endometriosis as the genotypes and alleles are associated with higher susceptibility to endometriosis and might be associated with endometriosis development (HsiehYY et al, 2005).

3.1 Environmental toxin genes

Endometriosis shows significantly elevated frequency in industrial areas and there is a possible genetic pre-disposition (Kennedy S et al. 2001). The glutathione-S-transferases (GSTs) constitute a family of xenobiotic-detoxifying phase-II enzymes catalyzing the conjugation of glutathione to a variety of electrophilic compounds including polycyclic aromatic hydrocarbons (PAH), which are widely present in the human environment and known to be carcinogenic.

Several GSTs are polymorphic and some allelic variants causing enzyme activity impairment are suspected to increase susceptibility to malignancies associated with environmental PAH, particularly colorectal cancer (Strange RC & Fryer AA, 1999). A very small portion of endometriosis develops into cancer later, but endometriosis itself is not a malignant disease. It has many characteristics similar to cancer, for example progressive growth, invasive growth, estrogen-dependent growth, recurrence and a tendency to metastasis (van Gorp T et al. 2004).

Environmental toxic compounds like *dioxin* may increase the risk of endometriosis. Previous association studies implicated GALT gene (a gene involved in galactose metabolism, located on chromosome 9), glutathione S transferases (GSTM 1), (GSTT1), cytochrome p 450 (CYP1A1) and N- acetyltransferase 2 (NAT2) genes, which encode for detoxification enzymes, as possible disease susceptibility genes (Zondervan KT et al, 2001; Hadfield RM et al, OXEGENE collaborative group, 2001; Deguchi M et al, 2005). The diversity of biological effects resulting from exposure to dioxin may reflect the ability of this environmental pollutant to alter gene expression by binding to the Aryl hydrocarbon receptor (AHR) gene and related genes (Watanabe T et al, 2001).

One of the genes previously implicated in endometriosis is CYP17; this encodes the enzyme P450c17alpha, which plays a vital role in steroid biosynthesis in the ovary. However, the CYP17 MspA1 polymorphism has not been associated with endometriosis in either the UK or the Japanese population (Asghar, T. et al, 2005).

A study of the association between endometriosis and polymorphisms in the N-acetyltransferase 1 (NAT1) and N-acetyltransferase 2 (NAT2) genes has previously demonstrated a positive association with NAT2 polymorphisms in a UK population. However, polymorphisms in NAT1 and NAT2 were not associated with an increased risk of endometriosis in the Japanese population (Deguchi M et al, 2005). A case control study suggested no association between endometriosis and NAT2 in South Indian women (Arvind-Babu, K. et al, 2005).

Glutathione S-transferases (GSTs) are enzymes involved in the metabolism of many disease-causing carcinogens and mutagens that are present in human environments. An association between the incidence of endometriosis and the GST genotypes of patients has been suggested. The study inferred that GSTM1, GSTT1 and GSTP1 genetic polymorphisms are not associated with the development of endometriosis in Korean women (Hur, S.E. et al, 2005).

3.2 Angiogenic genes

Vascular endothelial growth factor (VEGF), a major mediator of angiogenesis and vascular permeability, is known to play a key role in the pathophysiology of endometriosis. The single nucleotide polymorphisms, -460C>T and +405G>C, in the 5'-untranslated region of the VEGF gene were associated with lower promoter activity, which was significantly less common in women with endometriosis suggesting that the +405G allele may influence the likelihood of a woman developing the disease (Bhanoori M et al, 2005).

A relationship between the *alpha 2-Heremans Schmidt glycoprotein (AHSG)* gene polymorphism and endometriosis has been studied. Women not carrying the AHSG 2 allele were found to have twice the risk of endometriosis there by suggesting an association of endometriosis with the AHSG gene polymorphism in Korean women. (Kim JG et al, 2004).

3.3 Hormonal genes

3.3.1 Estrogen Receptor (ER) gene

The risk and severity of endometriosis has been associated with polymorphisms in genes coding for estradiol-synthesizing enzyme like the Ser312Gly polymorphism in 17-beta-

hydroxysteroid dehydrogenase type 1 (HSD17B1). Evidence for association between the Ser312Gly polymorphism in HSD17B1 and endometriosis was found in a Japanese population. The A-allele of HSD17B1 appears to confer higher risk for endometriosis (Tsuchia M et al, 2005).

The *AluI* polymorphism in the ER β gene is associated with an increased risk of stage IV endometriosis in a Japanese population (Wang Z et al, 2004). The *PvuII* polymorphism of the ER α gene is associated with the risk for endometriosis, adenomyosis and leiomyomata in Japanese women (Kitawaki J et al, 2001). The ER α dinucleotide repeat and cytochrome P450c17 α gene polymorphisms are associated with susceptibility to endometriosis in Taiwanese women (Hsieh.Y Y et al, 2005).

3.3.2 Androgen Receptor (AR)

Androgen receptor gene is present in the endometrial tissue and the pelvic organs, which are the targets for endometriotic implants. The AR was detected in endometriosis, adenomyosis and endometrial carcinoma (Horie K et al 1992). Endometrial cysts are monoclonal in origin and are related to the reaction with AR. The endometrioma might be formed from an independent monoclonal ovarian endometrial cell after inactivation of AR allele in the X chromosome (Fujimoto J et al, 1999). The proliferation and differentiation of the endometrium are mediated mainly by the Estrogen and Progesterone receptors. However, Androgen receptor also plays a role in modulating the cyclic change of the endometrium.

Androgen Receptor (AR) gene trinucleotide polymorphism has been associated with endometriosis (Yao-Yuan-Hsieh et al, 2001). The AR gene has a polymorphic cytosine, adenine and guanine (CAG) microsatellite in exon 1 that codes for variable length glutamine repeats in the amino-terminal domain of the AR protein (Hsieh.Y Y et al, 2004). The 21-CAG repeats may be associated with some determinants for endometriosis formation as indicated in a study by Lattuada et al, (2004) in Italian women.

3.3.3 Progesterone Receptor (PR)

Estrogen and progesterone receptors are present in the ectopic endometrium but in lower concentrations than in eutopic endometrium. Cyclical variation in the receptor population has not been observed and also there seems to be a difference in the way that estrogen is handled by the endometrium at the two sites (Vierikko P et al, 1985).

The expression of the variants of the Progesterone Receptor (PR-A and PR-B) was shown to be aberrant in endometriotic tissues, which may indicate a role of the progesterone receptor in the pathogenesis of endometriosis (Nisolle M et al, 1994; Attia GR et al 2000).

The progesterone receptor gene is located in chromosome region 11q22-23. *PROGINS* polymorphism has been studied in association with breast cancer (Wang-Gohrke S et al, 2000) and ovarian cancer (Vigano P et al, 2006). The data indicate that a mutated progesterone receptor gene contributes to the development of disease in hormone-sensitive tissues

Progesterone Receptor (PR) gene *PROGINS* polymorphism has been shown to be associated with endometriosis in Caucasian women (Weiser F et al, 2002). The secretory phase of the

menstrual cycle is controlled largely by progesterone, and this sex steroid hormone is absolutely required for normal implantation and pregnancy. PROGINS polymorphism of the progesterone receptor may be associated with an increased risk of endometriosis in Italian women (Lattuada et al, 2004).

4. Microsatellite Instability (MSI)

Microsatellites are short sequence elements that consist of mononucleotide to hexanucleotide motifs reiterated several times. This form of genomic instability is caused by defects in the DNA mismatch repair system. Genomic instability is an almost universal feature of cancer cells. Microsatellite instability is a DNA level instability seen in a number of tumors, including colon cancers. Instability is probably necessary to enable a cell to amass enough mutations and is not a chance feature, but is the result of selection. High frequency of MSI is defined as >29% of all markers and are defined as a class of MSI positive tumors (Strachanan & Read 2004).

4.1 Microsatellite (MS) markers

Genetic alterations in microsatellite marker sites among eight tumor suppressor genes in endometriosis were examined and reported that MSI is not ubiquitous in endometriosis and may be uncommon (Nakayama K et al, 2001). MSI assays reveal an allelic imbalance and loss of heterozygosity (LOH) on p16(Ink4), GALT, p53, APOA2 loci in endometriosis. The 9p21 locus may be a prognostic marker of the disease (Goumenou AG et al, 2001).

5. Gene expression

Gene expression profiles in endometriosis have been studied by two groups. In one study differentially expressed genes were investigated in epithelial and stromal cells from deep endometriosis and matched eutopic endometrium using cDNA microarrays and laser capture microdissection (Matsuzaki S et al, 2004) while Smith SK, (2003) undertook a genome-wide analysis of transcript abundance and changes in transcript level between normal endometrium in the proliferative and secretory phases of the menstrual cycle, between normal and ectopic endometrium of endometriosis and between normal and RU-486 exposed endometrium.

Gene expression profiling to identify genes involved in endometriosis has shown that Cyr61 gene, which codes for cystein-rich heparin-binding protein that promotes cell adhesion, migration and neo-vascularisation was deregulated (Absenger Y et al, 2004). Overexpression of p53 in atypical endometriosis and cancer associated with endometriosis has been reported (Sainz de la Cuesta R et al, 2004). Thymosin beta 4 (Tb4) gene expression, an actin sequestering protein, was up-regulated in uterine adenomyosis (endometriosis of myometrium) in mice (Kawahara R et al, 2003). Dysregulation of 14 genes was found to be overtly associated with endometriosis by Real-Time RT-PCR expression profiling of endometriosis (Wei Ping Hu et al, 2006).

6. Research by our group

The research work aimed to identify polymorphisms of candidate genes which increase susceptibility to endometriosis by genetic and molecular methods in 106 women who were

confirmed with the diagnosis of endometriosis and with age matched 140 normal women as controls from South India. Progesterone and Androgen Hormone Receptor *gene polymorphisms*; genomic instability at ectopic and eutopic endometrial tissues resulting in *Micro Satellite Instability (MSI)*; *gene expression* in ectopic endometrial tissue by Differential Display Reverse Transcriptase Polymerase Chain Reaction (DD-RT-PCR) to identify novel genes were studied. DNA was isolated from peripheral blood and from fresh tissue samples of both ectopic and eutopic endometrium. We subsequently assessed the polymorphisms of Estrogen Receptor, TNF-alpha and TLR 4 genes. The ongoing research work involves ICAM, IL-6, NOD-2, MMP-2, and MMP-9 genes polymorphism studies.

6.1 Microsatellite instability analysis by multiplex PCR

Allelic imbalance in selected loci was reported by microsatellite assays in endometriosis (Goumenou et al, 2001). Three different microsatellite markers were selected based on reports from previous studies (Luokola et al, 2001; Xing et al, 1999). These three microsatellite markers are as follows:

The mononucleotide adenine (A)_n repeats- (BAT-RII) and dinucleotide (CA)_n repeats- (D3S1313, D9S171).

Multiplex PCR was carried out using specific primers for the microsatellite markers selected. PCR was performed to amplify the three target sequences using specific primers. Multiplex PCR was performed on blood and tissue DNA from 12 cases and a matching number of controls. Thus 60 multiplex PCR reactions were set up using specific primers for each of the selected microsatellite markers.

6.1.1 Single strand conformation polymorphism (SSCP)

Each of the samples was analysed for all the three markers in the tissue using blood as control. MSI positivity was indicated by *mobility* difference between blood, ectopic and eutopic tissue; presence of additional band or absence of bands.

MSI is defined as a difference in length due to insertion or deletion of the amplified microsatellite markers between the normal and ectopic tissue of the same individual. MSI in the ectopic tissues is assessed by the detection of alleles of novel size that are not present in the normal tissues of the same individuals. All microsatellite markers showing instability were analyzed. Samples lacking MSI were defined as Microsatellite stable (MSS).

6.1.2 Differential display- reverse transcriptase-PCR (DD-RT-PCR)

RNA isolation and RT-PCR analysis: Biopsy material obtained from the ectopic and eutopic endometrial tissue at the time of surgery was collected in sterile normal saline and transported to the lab for storage at minus 70°C. mRNA was isolated using mRNA direct isolation kit (Qiagen, Oligotex direct mRNA micro kit, Catalogue No. 72012) according to the manufacturer's instructions. mRNA was converted to cDNA by Reverse Transcriptase (RT) step which was followed by 45 cycles of 3-stage PCR with a specific annealing

temperature for each primer set. DD RT-PCR allows identification of differentially expressed genes in various cell types and under defined physiologic conditions. DD was performed by the modified method of Hasan et al (2000). PCR was performed using selected primers to study the differential expression of the selected genes in the ectopic and eutopic endometrial tissues.

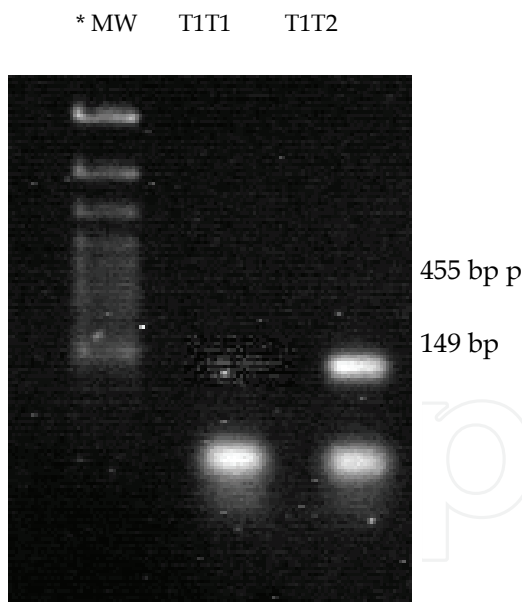
cDNA DD PCR: 5 sets of random exonic primers were used. DD-PCR was repeated with a different set of seven primers, which were selected arbitrarily.

7. Results and statistical analysis

A non-parametric statistic is opted because (1) there is no '*a priori reason*' to assume a particular disease model and (2) the assignment of the status '*unaffected*' is problematic because a surgical procedure is required to exclude endometriosis.

Allele and genotype frequencies were compared in the patient and control groups. The odds ratio (OR) was used to measure the strength of the association between the frequencies of allele and genotype and endometriosis. The software MedCalc (version 7.4.3.0) was used for statistical analyses. All 'p' values two-tailed and 95% Confidence Intervals (CI) were calculated.

7.1 PROGINS analysis



*MW- Molecular Weight ladder

Fig. 1. Photograph of a 2% agarose gel stained with ethidium bromide to resolve the 306-base pair intron G insertion polymorphism of the progesterone receptor gene. The 149-base pair band represents the wild type allele (allele T1) and the 455-base pair band represents the mutant allele (allele T2). Lane 1 indicates the molecular weight markers; lanes 2 and 3 show the homozygous wild type T1T1 and the heterozygous T1T2 patterns. There is no homozygous mutant T2T2 pattern.

7.2 Androgen receptor analysis

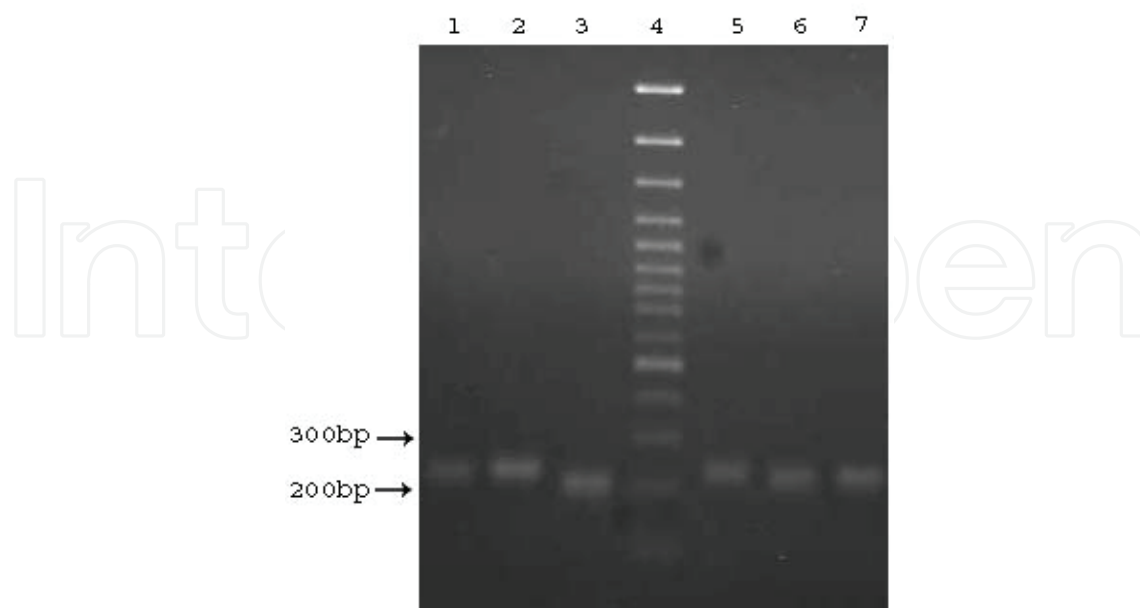


Fig. 2. Photograph of a 2% Agarose gel stained with ethidium bromide to resolve the trinucleotide CAG repeats in AR gene encompassing Exon 1. The bands appeared between 200bp - 300bp. The Lane 4 indicates the molecular weight marker.

The small repeat changes occurring between 200bp - 300bp and heterozygotic alleles could not be identified on agarose gels. Hence, analysis was done using 12% PAGE for further analysis.

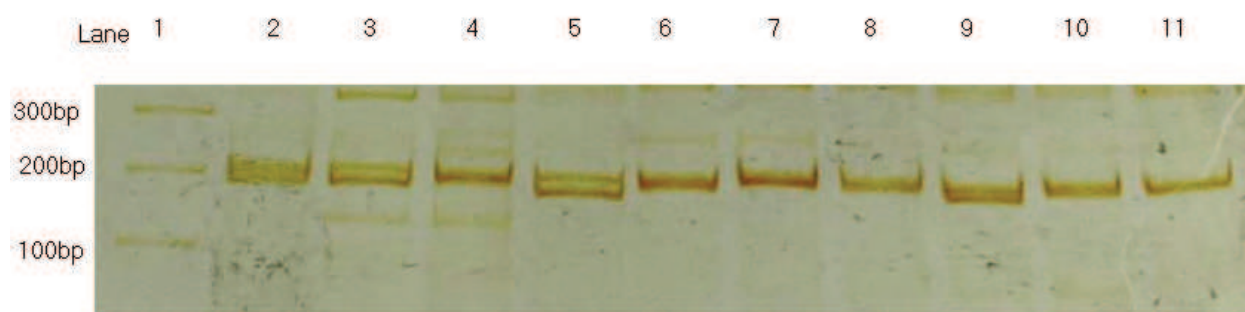


Fig. 3. Native PAGE showing heterozygous and homozygous alleles of AR gene: 200 samples processed with 12% polyacrylamide gel and silver staining showed band sizes ranging from 156 bp - 238 bp which fall within the CAG repeats range of 4-34.

7.3 MSI analysis

Multiplex PCR for 12 cases and 12 controls was carried out using blood as control. Each case had three sets of samples, viz. blood, eutopic tissue and ectopic tissue. Hence 36 multiplex PCR reactions were set up for cases. For the controls two sets of samples were set up, viz. blood and eutopic tissue. Hence 24 multiplex PCR reactions were set up for controls.

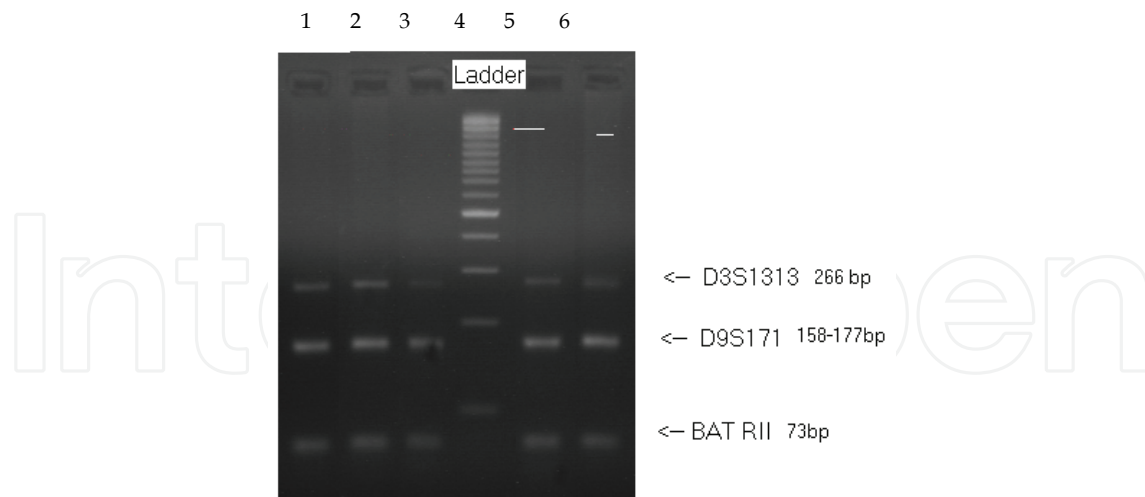


Fig. 4. 2% Agarose gel showing results of multiplex PCR both from endometriotic cases and controls with the specific MSI markers and their band sizes. Lanes 1,2,3 are of a case; Lane 4 is the DNA ladder; Lanes 5,6 are of a control

7.4 SSCP (Single Stranded Conformation Polymorphism) analysis

SSCP was carried out on 15% polyacrylamide gel to identify mobility shift, and/or additional bands at the three selected markers. SSCP PAGE gel showing mobility shift at BAT R II locus in ectopic tissue from a case of endometriosis. 2 cases of endometriosis showed mobility shift in the BAT R II locus associated with TGF beta R II receptor gene. One control showed additional band in D3 locus associated with FHIT gene.

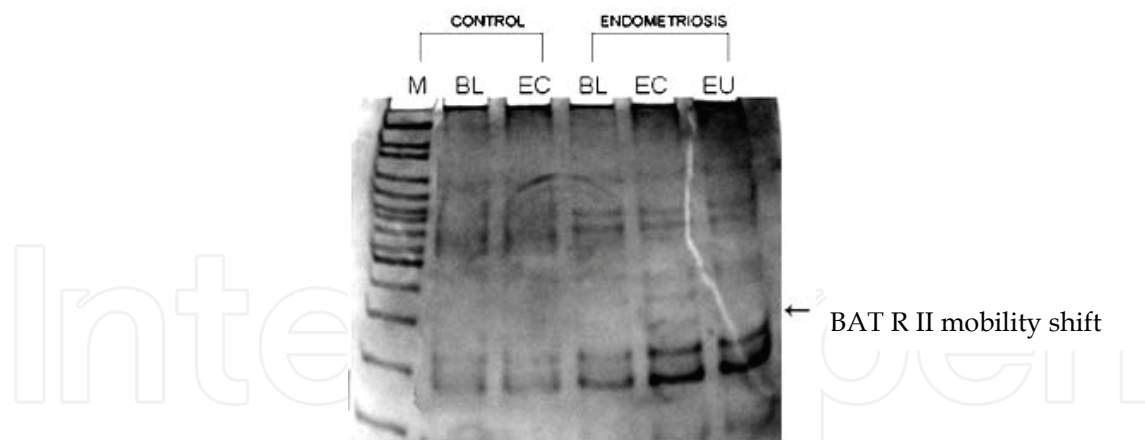


Fig. 5. Lane 1 is the DNA ladder; Lanes 2 and 3 are blood and eutopic tissue of control;

Lanes 4, 5 and 6 are blood, ectopic and eutopic tissue of a case ; Additional bands are seen in all the samples of the case but not in the control, whereas mobility shift of the band is seen in the ectopic tissue only and not in the eutopic tissue of the case.

7.5 DD – RT – PCR analysis

Differential display RTPCR helps in identifying novel genes which are expressed or suppressed in tissues with an altered pathophysiology compared to controls using two sets

of five and seven arbitrary primers. A number of common bands were seen between eutopic and ectopic samples from the same patient. However, in three cases studied differential bands of 45bp in the first set, and 350bp region in the second set were observed in the ectopic tissue of patient suffering from severe endometriosis, stage IV. DD-RT-PCR using first set of 5 primers. Fig. 6.

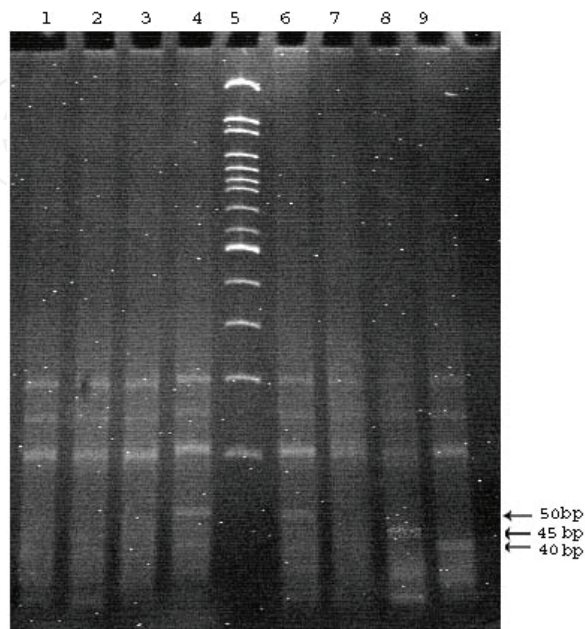
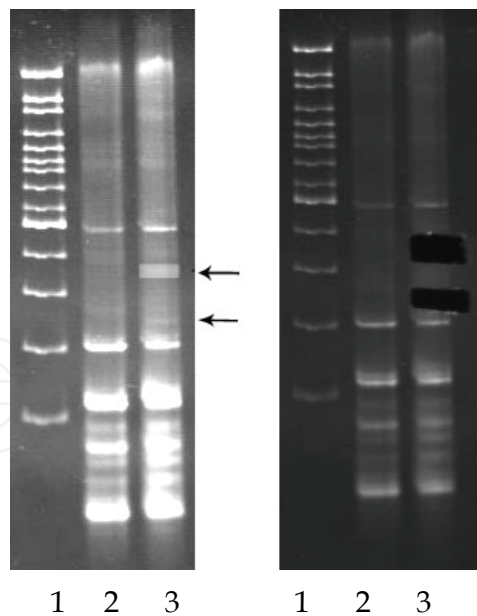


Fig. 6. DD-RT-PCR using second set of seven primers



Upper arrow: differential band of 350 bp
Lower arrow: faint differential band of 250 bp

Fig. 7. Lane 1: 100 bp ladder; Lane 2: Eutopic tissue; Lane 3: Ectopic tissue. Differentially expressed bands are seen in ectopic tissue in lane 3, as shown by the arrows (before eluting), and in lane 3 on the right side (after eluting).

The eluted bands were automatically sequenced using MWG-AG, BIOTECH, Bangalore. The sequences obtained are shown below.

Sequencing of differentially expressed bands:

- I. 350bp band showing 60/65 gene sequence showing homology with Shigella species which included *S. dysenteriae*, *S. boydii*, *S. sonnei*.
- II. 250 bp showing (a) 20/20 gene sequence showing homology with Hepatocellular carcinoma- associated antigen HCA557b, (b) 24/25 gene sequence showing homology with *S. dysenteriae*

8. Discussion

One hundred and six (106) cases of endometriosis, diagnosed at laparoscopy and/or laparotomy were staged according to the Revised American Fertility Society (rAFS) classification and were enrolled in the study. One hundred and forty (140) controls were also included in the study who comprised of women who either had no symptoms, or no endometriosis at the time of laparoscopy/laparotomy which was performed for other indications. Mild endometriosis (stages I & II) was diagnosed in 72% of cases and advanced endometriosis (stages III & IV) was diagnosed in 28% of cases in the cohort studied.

The aim of the present study was to identify individuals clinically suffering from endometriosis and establish genetic and molecular markers for understanding the cellular and molecular pathogenesis of this condition.

Few well-designed epidemiologic studies of risk factors for endometriosis exist. Eskinazi B et al, (1997) conducted a review of more than 100 published studies and found that only 6 (1 cohort and 5 case-control studies) included a surgically case confirmed group, provided clear criteria for control selection, and considered potential confounding factors in the analysis. Hence, the importance of the present study is that all the cases of endometriosis included were confirmed by laparoscopy/laparotomy and the controls were age matched women who were surgically proven to have no endometriosis or had no symptoms suspicious of endometriosis throughout their reproductive life.

Endometriosis plaques have been shown to have estrogen, progesterone and androgen receptors, and they grow in the presence of estrogen but atrophy when exposed to androgens. Since endometriosis is a hormone-sensitive disease, in the present study the role of hormonal genes polymorphism was analyzed in women with surgically confirmed diagnosis of endometriosis. This is the first Indian study to evaluate the hormonal genetic factors in the etiology of endometriosis.

The PROGINS polymorphism has been shown to be associated with endometriosis in Caucasian women (Weiser et al, 2002) including Italian women (Lattuada D et al, 2004). *Our study established 5% prevalence of PROGINS polymorphism in Indian population and showed no susceptibility to endometriosis.*(Govindan et al 2006). Estrogen receptor- α gene (T/C) Pvu II polymorphism in Endometriosis and Uterine Fibroids has been studied by our group. It was observed to be significantly associated with endometriosis in Asian Indian population. (Govindan et al. 2009)

The highly polymorphic trinucleotide repeat (CAG) in AR varies in length and methylation pattern which affects both AR expression and function. The AR gene CAG polymorphism has been associated with a number of benign and malignant conditions, eg. polycystic ovarian syndrome in women, and male infertility and prostate cancer in men. Its association with endometriosis in Italian women did not constitute an important factor of genetic predisposition (Lattuada D et al, 2004). The range of CAG repeats varies from 9-31 in Japanese population (Hsieh Y et al, 2001) and 14-32 in Italian population. However, there are no studies reported for the AR polymorphism in Indian women with endometriosis.

Our study proposes that the 19 CAG repeats may be associated with increased risk of endometriosis in our population. AR gene CAG repeat polymorphism may become a useful marker to predict the future development of endometriosis and to permit early therapeutic intervention in women at high risk of developing endometriosis (Shaik et al.2009)

Since endometriosis clinically mimics cancer with proliferation, angiogenesis and metastasis, three markers associated with carcinogenesis were selected for MSI analysis. TGF-beta II receptor gene is a putative tumor suppressor. It has been found that the TGFBR2 gene was inactivated in a subset of colon cancer cell lines exhibiting MSI. Once generated, the proliferative advantage of cells with inactivated type II receptor would drive colon tumor progression (Markowitz et al 1995). This pathway may also be operative in other human pathologies like endometriosis. Human FHIT (fragile histidine triad) protein is encoded by the FHIT putative tumor suppressor gene (Barnes et al 1996). Aberrant transcripts of the FHIT locus were found in approximately 50% of esophageal, stomach, and colon carcinomas (Ohta et al 1996). The results from several studies showed aberrant regulation of several cell cycle proteins, including CDKN (Kim et al 2005).

In endometriotic lesions the differentiation of glands and stroma is absent as the lining is attenuated, lost or replaced with granulation tissue and dense fibrous tissue. Hence it is difficult to get suitable ectopic endometrial tissue from cases for DNA isolation. Therefore MSI could be assessed in 12 cases only and tissue samples were obtained from 12 controls. Out of the 12 cases 2 (16.66%) showed microsatellite instability in the TGFbetaR2 gene. *This is the first study reporting an association of TGF-beta receptor II gene with endometriosis in Indian women.* None of the samples studied showed any instability with regards to the other two markers. This suggests that D3S1313 and D9S171 may not be important markers for endometriosis.

In the present study mRNA was isolated from ectopic and eutopic tissues and DD-RT-PCR was carried out twice using 7 different sets of arbitrary primers. **Ectopic endometriotic tissue of one case showed unique band of 45bp after DD-RT-PCR using the first set, while a band in the region of 350bp was obtained using the second set of primers.** Both the 45bp and 350bp bands were cut out, eluted and automatically sequenced. The 45bp band gave multiple errors during sequencing, whereas the 350bp band could be sequenced successfully. Sequencing was carried out twice from the latter band. Both the sequences were analyzed using the BLAST search (National Centre for Biotechnology Information-NCBI; Google Search). It revealed a 60/65bp, 96% homology with *Shigella dysenteriae*, *boydii* and *sonnei* species. This sequence also matched a smaller 20/20bp stretch of 100% homology with human Hepatocellular carcinoma associated antigen (HCAA). Hence, a simple cost-effective technique like DD-RT-PCR has enabled the identification of novel gene

sequences in ectopic tissue of endometriosis which was hitherto not associated with endometriosis.

A world-wide literature search through Medline, PubMed and Google, showed no reported work associating either the *Shigella* species nor the HCAA gene with endometriosis. These results open up new possibilities for the etiology of endometriosis.

The HCAA gene is a likely candidate for causing endometriosis, which needs to be further investigated.

Shigella are gram-negative, non-motile, aerobic and facultatively anaerobic bacilli from the family Enterobacteriaceae. *S. dysenteriae*, *flexneri*, *boydii*, and *sonnei* are highly infectious strains that can cause dysentery in humans with an ID₅₀ of only 100-200 bacteria. Bacterial diarrhoea in humans caused by *Shigella* species due to the shiga toxin is called shigellosis.

It causes inflammation in the small and large bowel, which may extend to the pelvic peritoneum. Although this organism has not been earlier associated with endometriosis, it can be hypothesized that a bacteria related to shigella which induces pelvic peritoneal inflammation, may be playing a role in the etiology of endometriosis. This may not be far-fetched after the Nobel Prize winning discovery of Warren and Marshall (Julie Parsonnet 2005), who found that inflammation caused by *Helicobacter pylori* is responsible for esophageal/gastric cancer (Forbes et al, 1994).

Studies on the pathogenesis of *Shigella* have revealed unique methods of mucosal invasion that result in the lesions seen with infection. Because most lesions are often centered on gut-associated lymphoid tissue (GALT) and spread outward, it is suspected that the bacteria make their initial entry into the body through the normally *phagocytic macrophage cells* overlying the lymphoid tissue (Salys AA et al.1994) Additional studies have revealed that through a complex process involving multiple genes found on both a large plasmid and on the *Shigella* chromosome, attachment of the bacteria to mucosal epithelial cells stimulates a structural alteration of the normally nonphagocytic epithelial cell cytoskeleton and actin filaments to cause uptake of the organism in a manner similar to phagocytosis. Once within the intracellular vacuole of the invaded cell, a hemolysin produced by *Shigella* causes release of the organism into the cytoplasm. The *Shigella* then rapidly multiply and migrate along polymerized actin filaments to reach the plasma membrane so that adjacent cells can be invaded (Keusch GT et al, 1993). Early in the course of disease, low numbers of *Shigella* organisms can be found by electron microscopy within mucosal epithelial cell vacuoles. As the disease progresses, fibrinous exudate replaces the dead epithelial cells (Brady AG et al, 1998). Death of epithelial cells and sloughing of mucosa creates the ulceration, pseudomembrane formation, hemorrhage, and inflammatory response that typifies shigellosis.

An additional aspect of virulence involves the production of an exotoxin, shiga toxin, by *S. dysenteriae*. Shiga toxin also enhances the lipopolysaccharide-mediated release of cytokines, such as interleukin-1 and tumor necrosis factor-alpha (Kodati V et.al, 2009), which likely contributes to the vascular damage leading to renal failure seen in a complication of shigellosis, hemolytic uremic syndrome. Subsequent to the identification by sequencing of shigella bacterial association with peritoneal inflammation, a hypothesis was proposed by our group to explain the molecular pathogenesis of endometriosis.

9. Hypothesis

The identification of genetic sequences homologous to shigella bacteria (Kodati V et al, 2007) in the ectopic endometriotic tissue in this study unravels an understanding of the etiopathogenesis of endometriosis, which has not yet been reported. An important element in the initiation of inflammatory responses is the activation of macrophages, resulting in the

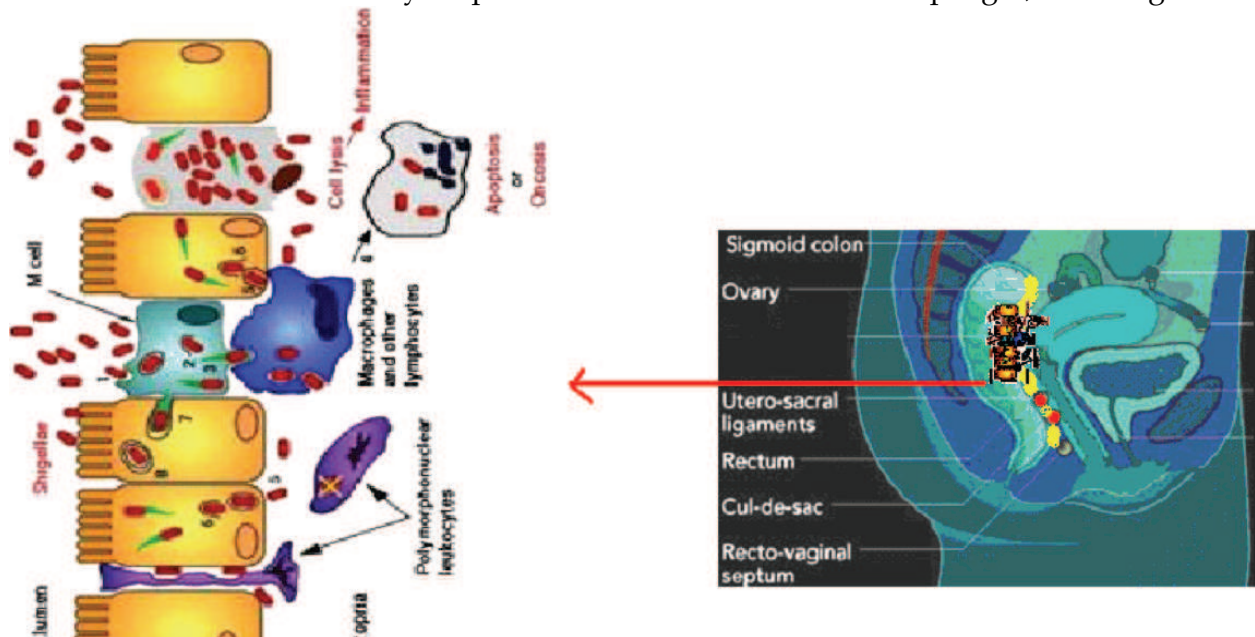


Fig. 8. Right side of the figure shows the saggittal section of the pelvis showing the route of shigella invasion from the colon to the Pouch of Douglas. Note the close proximity of the colon and Cul-de-sac/ posterior surface of uterus. Left side of the figure is the microscopic appearance of the mucous cells of the colon showing migration of the non-motile bacteria from cell to cell upto the lamina propria.

production of pro-inflammatory cytokines such as interleukins-12 (IL-12). Toll-Like Receptors (TLRs) which are expressed on macrophages, recognize microbial molecules and transmit signals that initiate transcription of cytokine genes. TLR4 recognizes the gram-negative bacterial product lipopolysaccharide (LPS). TLRs use several signaling pathways to initiate gene transcription. With the environmental toxins theory of endometriosis being now disproved, and with inconsistent results from genetic polymorphism studies amongst different ethnic groups, it could be postulated that the “infection theory” caused by Shigella or a similar bacteria may be the trigger that sets into action the immunological changes in the pelvic peritoneum resulting in the phenotype of endometriosis. While this can explain the pelvic and abdominal endometriosis, the occurrence of distant metastasis is yet unclear. Further work in this area should enhance the understanding of TLR signaling and the regulatory mechanisms controlling the inflammatory response by bacteria like shigella and their role in endometriosis.

9.1 Probable mechanism of Shigellosis to endometriosis

Commonly, shigella bacteria are known to invade the mucosa of the colon through the feco-oral route causing Shigellosis. The non-motile bacteria travel from cell to cell of the colonic epithelium through the cytoplasm by a unique mechanism called F-actin polymerization.

Thereby the bacteria reach the lamina propria of the colonic mucosa (Fig. 8). It is hypothesized that by the same mechanism the bacteria can enter the blood stream and/or travel across the colon wall to reach the outer peritoneal surface of the colon which is in close proximity to the posterior uterine surface, the site which incidentally happens to be the commonest site of early endometriosis (Cul-de-sac or Pouch of Douglas) as shown in Fig. 8. We propose that the peritoneal reaction to this bacterial invasion may be similar to any antigenic response by the host immune system resulting in the activation of macrophages and production of cytokines characteristic of acute inflammatory response. The endometrial cells that are shed during the retrograde menstruation into the cul-de-sac adhere to the inflamed peritoneal and ovarian surfaces and come under the influence of circulating ovarian hormones. The thus implanted endometrial cells in the peritoneum progress to endometriosis through angiogenesis. Our postulated bacterial hypothesis proposes that shigella or shigella-like organisms may be the trigger for the immunological changes in the pelvic peritoneum which initiate the etiopathogenesis of endometriosis. The inflammatory hypothesis is further reinforced by our subsequent research on TNF-alpha -C850T polymorphism, which showed significant association with endometriosis. (Lakshmi KV et al 2009) The bacterial hypothesis is supported by our recent research work on TLR-4 (A896G) polymorphism. Toll- Like Receptor 4 is specific for recognition of the molecular pattern of gram-negative bacteria. TLR-4 is present on the surface of endometrial cells. TLR-4 A896G is a functional polymorphism resulting in hypo-responsiveness of the receptor, causing peritoneal inflammation in the female pelvis. The molecular micro-environment of the cul-de-sac becomes favourable for initiation of endometriosis. (Latha M et al 2011)

10. Conclusion

Endometriosis remains a difficult clinical problem today and warrants more extensive research to understand the disease pathology. The future is to confirm early diagnosis by non-invasive test using a panel of potential genetic and molecular bio- markers. A long term goal is to be able to identify genetic determinants that contribute to the expression of the different phenotypes seen in endometriosis.

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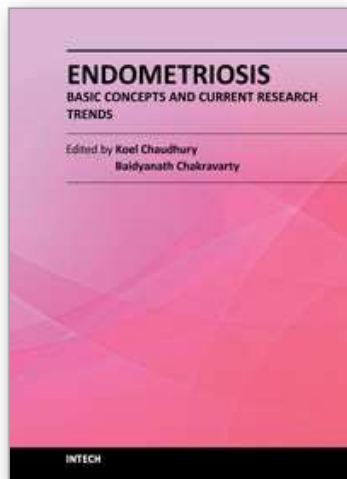
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This book provides an insight into the emerging trends in pathogenesis, diagnosis and management of endometriosis. Key features of the book include overviews of endometriosis; endometrial angiogenesis, stem cells involvement, immunological and hormonal aspects related to the disease pathogenesis; recent research reports on infertility, endometrial receptivity, ovarian cancer and altered gene expression associated with endometriosis; various predictive markers, and imaging modalities including MRI and ultrasound for efficient diagnosis; as well as current non-hormonal and hormonal treatment strategies. This book is expected to be a valuable resource for clinicians, scientists and students who would like to have an improved understanding of endometriosis and also appreciate recent research trends associated with this disease.

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