Genome-wide microarray gene expression, array-CGH analysis, and telomerase activity in advanced ovarian endometriosis: A high degree of differentiation rather than malignant potential

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Abstract. The aim of the present study was to investigate whether endometriosis and cancer share common molecular characteristics. Tissue samples were collected prospectively during diagnostic laparoscopy of patients with primary infertility. Using high-density oligonucleotide microarrays, (Affymetrix Gene Chip HG-U133 Set) the genome-wide gene expression profile of advanced ovarian endometriosis was analyzed compared with matched normal endometrium. Expression of *TERT*, the gene encoding the telomerase reverse transcriptase subunit, and telomerase activity were analyzed in eutopic and ectopic endometrium. Genome-wide, high-resolution array-CGH was used to screen for genomic aberrations in endometriosis. Expression microarray data were validated quantitatively with RT-PCR. The genes *RARRES1* and *RARRES2* (*retinoic acid receptor responder 1* and *2*) were found to be up-regulated in endometriosis, suggesting a high degree of differentiation. Consistently, down-regulated genes included those involved in the cell cycle, cell metabolism and homeostasis. Expression of *TERT* and telomerase activity were present in eutopic but absent in ectopic endometrium. Array-CGH revealed a normal genomic pattern without gross amplifications and deletions. In conclusion, these data suggest that advanced ovarian endometriosis represents a highly differentiated tissue with minimal or no malignant potential.

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Introduction

Endometriosis is the presence of endometrial tissue, including endometrial glandular and stromal cells, outside the uterine cavity. It is usually confined to the pelvis, where it may be associated with pelvic pain and infertility, and in rare cases it can be found in distant sites, such as the thorax and skin (1,2). It is estimated that endometriosis affects 5-15% of 'normal' and up to 60-80% of women with pelvic pain and/or infertility (3).

The most widely accepted theory on the etiopathogenesis of endometriosis is the theory of ectopic transplantation of endometrium, mainly through reflux menstruation (4). Similarly, endometriosis in distant sites can be explained by migration of endometrial cells through lymphatic and blood vessels (5). In this way, endometriosis represents a paradigm of benign dissemination and metastasis. At the cellular level it is characterized by monoclonal growth and can reveal features of malignant behaviour including local invasion and metastasis (6). Therefore, endometriosis might serve as a model to study the molecular and genomic requirements needed for dissemination of non-malignant cells.

The association of endometriosis with cancer is unclear. Although it is not neoplastic and does not create a cachectic or catabolic state, certain processes characterizing metastasis and carcinogenesis are also seen in endometriosis. These include cell motility, adhesion, homing and invasion, immunologic factors, maintenance of the original structure and tissue architecture at the ectopic site, angiogenesis and metaplasia (6). Moreover, evidence exists that endometriotic lesions present genetic changes similar to certain malignancies (7,8). Furthermore, a direct association of ovarian cancer and endometriosis has been previously described in numerous

case reports and case series (9-12). Like any other tissue, endometriotic lesions may well undergo malignant transformation. On the other hand, large retrospective, epidemiologic studies show that women with endometriosis have an increased risk of ovarian and other types of cancer compared with the normal population (13,14).

Given the multitude of cellular and molecular mechanisms involved in endometriosis formation and maintenance and the gap of knowledge concerning the association of endometriosis with tumorigenesis and metastasis, we conducted a large-scale gene expression analysis of ovarian endometriotic lesions and corresponding eutopic endometrium. To examine for genomic variations which might influence the gene expression profile, we performed an array-CGH analysis on selected endometriotic tissue samples, including samples analyzed with gene expression microarrays.

Materials and methods

Tissue samples. Tissue samples were collected prospectively from patients with primary infertility, who underwent laparoscopy during the proliferative phase of the menstrual cycle, between day 4 and 10 after the last menstrual period. All patients tested negative on a urine pregnancy test, had an otherwise healthy medical history, and were not under any hormone treatment. A total of 14 endometriotic ovarian cysts were excised, with operative time ranging between 30 and 45 min in all cases. All patients had moderate to severe endometriosis - stages III and IV according to the Revised American Fertility Society Classification for Endometriosis. Biopsies of normal endometrium were possible in four of these cases. Diagnosis was confirmed with histopathological examination in all cases. Biopsy material was stored in liquid nitrogen immediately after surgery (10-30 min after excision). An independent academic board approved the protocol prior to the study, and all specimens were obtained in accordance with the Declaration of Helsinki of 1975 on Human Experimentation. For microarray studies, only endometriotic lesions with matched eutopic endometrium available were used (i.e. four cases). For array-CGH analysis, 10 endometriosis samples were used including the 4 cases from the gene expression analysis.

RNA preparation. Tissue specimens were homogenized in liquid nitrogen using pestle and mortar. Total RNA was prepared by lysing specimens in Trizol reagent according to the manufacturer's instructions (Invitrogen GmbH, Karlsruhe, Germany). Total RNA was cleaned-up using RNeasy Mini Kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany).

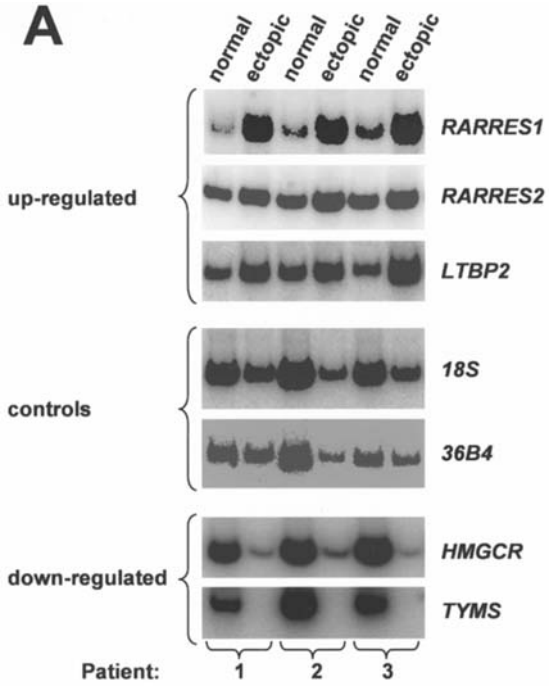
DNA preparation. All cryopreserved tissue samples were homogenized, and genomic DNA was isolated using the Qia-DNA Isolation Kit (Qiagen GmbH) according to the manufacturer's instructions.

Gene expression profiling. For all experiments, high-density oligonucleotide microarrays, screening for >40,000 human genes and expressed sequence tags ESTs (44,928 probe sets) were used (GeneChip HG-U133 Set; Affymetrix, Santa Clara, CA). The targets for DNA-microarray analysis were prepared as described by the manufacturer. In brief, after RNA extraction, reverse transcription, cDNA clean-up, *in vitro* transcription and cRNA-labelling with biotin and fragmentation, cRNA was hybridized on the microarrays. The amount of total RNA used for cDNA-synthesis was 10 μg for each reaction. GeneChip microarrays were hybridized with the targets for 16 h at 45˚C, and then washed and stained using the Affymetrix Fluidics Station according to the GeneChip Expression Analysis Technical Manual. Microarrays were scanned with a Hewlett-Packard-Agilent GeneChip scanner, and the signals were processed using the GeneChip expression analysis algorithm ver. 2 (Affymetrix). To compare samples and experiments, the trimmed mean signal of each array was scaled to a target intensity of 200 for A-Arrays and 80 for B-Arrays. Absolute and comparison analyses were performed with Affymetrix MAS 5.0 and DMT software using default parameters. In order to identify genes positively or negatively regulated, genes that were increased or decreased at least 2-fold compared to the baseline (eutopic endometrium) were selected (15). Annotations were further analyzed with interactive query analysis at www.affymetrix.com. Pathways and other functional groupings of genes were evaluated for differential regulation using the visualization tool GenMAPP (UCSF) as described previously (16,17).

Radioactive RT-PCR. First-strand cDNA synthesis was performed using 1 μg total RNA with the ThermoScript Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) in the presence of 100 ng oligo-dT_{20} in a volume of 20 μl under the following conditions: 1 h at 50˚C followed by 15 min at 75˚C, followed by 20 min at 37˚C in the presence of 2 units of RNase H to digest the RNA. cDNA (2 μl) was used for the PCR analysis in a total volume of 50 μl containing 0.2 μM specific primers, 10% DMSO, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 unit of Taq polymerase (Eppendorf, Hamburg, Germany). The sequences of primers used were: *RARRES1*, 5'-GAG CGC TAC AAC CCA GAG TC-3' (forward) and 5'-GAA AGC CAA ATC CCA GAT GA-3' (reverse); *RARRES2*, 5'-GAC TGG AAG AAA CCC GAG TG-3' (forward) and 5'-CTT GGA GAA GGC GAA CTG TC-3' (reverse); *LTBP2*, 5'-CTG AAT CCG CCC AGT GAT AG-3', (forward) and 5'-ACA TTG GAA ATC TGG CTG CT-3' (reverse); *TYMS*, 5'-TTC AGG ACA GGG AGT TGA CC-3' (forward) and 5'-CAT GTC TCC CGA TCT CTG GT-3' (reverse); *HMGCR*, 5'-TCT CCG CAG GCT ATT TGT TC-3' (forward) and 5'-TAA GGT CCC AGT CTT GCT TG-3' (reverse); *hTERT*, 5'-TCT GGA TTT GCA GGT GAA CAG CC-3' (forward) and 5'-GGG TGG CCA TCA GTC CAG GAT GG-3' (reverse); *36B4*, 5'-GTG GGA GCA GAC AAT GTG GGC TCC-3' (forward) and 5'-ATG GAT CAG CCA AGA AGG CCT TGA C-3' (reverse); *18Sr RNA*, 5'-GTT GCA AAG CTG AAA CTT AAA GG-3' (forward) and 5'-CCG ATC CGA GGG CCT CAC TAA ACC-3' (reverse). For radioactive PCR analysis, 2.5 μCi of [α-^{32}P] dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech, Freiburg, Germany) was added to the reaction. Amplification products were analyzed on 4% (*36B4*, *18Sr RNA* and *hTERT*) or 6% (*RARRES1*, *RARRES2*, *LTBP2*, *TYMS* and *HMGCR*) non-denaturing polyacrylamide gels, respectively.

Table I. Up-regulated genes and ESTs in all 16 comparisons.

Fold-up	GenBank ID	Gene description	Gene symbol
45.10	NM_022844.1	Myosin, heavy polypeptide 11, smooth muscle	MYH11
17.37	S55735.1	Hypothetical protein MGC27165	MGC27165
09.85	AW009747	*Homo sapiens* cDNA FLJ20767 fis, clone COL06986	
07.54	NM_003186.2	Transgelin	TAGLN
07.24	NM_002888.1	Retinoic acid receptor responder (tazarotene induced) 1	RARRES1
06.01	NM_031311.1	Carboxypeptidase, vitellogenic-like	CPVL
05.98	AW404894	Immunoglobulin κ constant	IGKC
05.66	AI669229	Retinoic acid receptor responder (tazarotene induced) 1	RARRES1
04.76	AI634580	Synaptopodin 2	SYNPO2
04.57	AV733266	Ig J polypeptide, linker protein for immunoglobulin α and μ polypeptides	IGJ
03.72	NM_000428.1	Latent transforming growth factor ß binding protein 2	LTBP2
02.42	AV706522	Hypothetical protein DKFZp761G058	DKFZp761G058
02.41	BG538564	*Homo sapiens* PRO2743 mRNA, complete cds	
02.15	AA628586	Phosphatidic acid phosphatase type 2B	PPAP2B



B

	up-regulation
RARRES1	15-fold
RARRES2	9-fold
LTBP2	7-fold

C

	down-regulation
HMGCR	3,5-fold
TYMS	≥ 3-fold

Figure 1. RT-PCR analysis of selected genes confirming microarray data. (A) Total RNA was isolated from eutopic and ectopic endometrium of 3 patients and reverse transcribed. Amplification of the cDNAs was performed in the presence of trace amounts of [α-^{32}P] dCTP in addition to non-radioactive dNTPs in the reaction mixture (see Materials and methods). For radioactive PCR, the linear range of amplification was determined beforehand and amplification (94˚C, 30 sec; 55˚C, 30 sec; 72˚C, 1 min) was performed for 18 cycles for *36B4* and 20 cycles for *18S rRNA*, 22 cycles for *RARRES2*, 25 cycles for *LTBP2* and 27 cycles for *RARRES1*, *TYMS* and *HMGCR*, respectively. (B and C) Signals from RT-PCR results were detected by Phosphoimager screens, quantified with TINA 2.0 program (Raytest, Straubenhardt, Germany), and normalized to the mRNA levels of the *36B4* and *18S rRNA* genes.

Array-CGH. Array-CGH was carried out as described previously (18). In brief, endometriotic DNA and reference normal male DNA were labelled by random priming (BioPrime DNA labelling system, Invitrogen, Breda, The Netherlands) to incorporate Cy3-dCTP and Cy5-dCTP (Perkin-Elmer Life Sciences, Zaventem, Belgium), respectively. Non-incorporated nucleotides were removed using ProbeQuant G-50 Microcolumns (Amersham Biosciences, Roosendaal, The Netherlands). Hybridization was carried out with labelled DNA mixed with Cot-1 DNA (Invitrogen) for 2 days to the in-house-developed Bacterial Artificial Chromosome array containing 5,659 clones spotted in triplicate, which provides an ~1.0-Mb resolution across the human genome. Log2 ratios of Cy3 and Cy5 intensities for each spot were calculated. Data were processed as described (18) and plotted relative to the position of the clones.

Table II. Down-regulated genes and ESTs in all 16 comparisons.

Fold-down	GenBank ID	Gene description	Gene symbol
12.18	NM_018123.1	asp (abnormal spindle)-like, microcephaly associated (*Drosophila*)	ASPM
10.83	AL561834	Topoisomerase (DNA) II α 170 kDa	TOP2A
10.60	NM_014750.1	Discs, large homolog 7 (*Drosophila*)	DLG7
08.32	AL524035	Cell division cycle 2, G1 to S and G2 to M	CDC2
08.32	NM_014736.1	KIAA0101 gene product	KIAA0101
07.21	NM_018131.1	Chromosome 10 open reading frame 3	C10orf3
06.06	BC001886.1	Ribonucleotide reductase M2 polypeptide	RRM2
05.28	NM_001827.1	CDC28 protein kinase regulatory subunit 2	CKS2
05.14	NM_001071.1	Thymidylate synthetase	TYMS
04.95	AK027006.1	Trinucleotide repeat containing 9	TNRC9
04.78	AB032983.1	KIAA1157 protein	KIAA1157
04.78	NM_014791.1	Maternal embryonic leucine zipper kinase	MELK
04.54	NM_001786.1	Cell division cycle 2, G1 to S and G2 to M	CDC2
04.25	BE407516	Cyclin B1	CCNB1
04.12	NM_024629.1	Hypothetical protein FLJ23468	FLJ23468
03.29	NM_016359.1	Nucleolar protein ANKT	ANKT
03.11	BF439316	Transmembrane protein with EGF-like and two follistatin-like domains 1	TMEFF1
03.10	AF213033.1	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	CDKN3
03.07	AL518627	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	HMGCR
03.03	BC000323.1	Flap structure-specific endonuclease 1	FEN1
02.82	NM_006486.1	Fibulin 1	FBLN1
02.25	AF037448.1	NS1-associated protein 1	NSAP1

Smoothing with data was performed using the aCGHsmooth program (http://www.few.vu.nl/~vumarray/) to detect genomic aberrations.

Determination of telomerase activity (TRAP assay). In order to determine the enzymatic activity of telomerase in extracts from endometriotic and normal endometrium tissue samples, the Telomeric Repeat Amplification Protocol (TRAP) with the Trapeze Kit (Serologicals Corp., Norcross, GA, USA) was used, according to the manufacturer's instructions.

Results

Microarray data analysis. For data analysis, gene expression of the endometriotic lesion of each patient was compared to that of the eutopic endometrium of all 4 patients. In this way, the expression profiles of all 4 endometriotic lesions were compared to 4 different reference probes, leading to a total of 16 comparisons. Using this strategy, two groups of genes were identified. The first included 14 up-regulated (Table I), and the second included 22 down-regulated genes and ESTs in all 16 comparisons (Table II). Furthermore, 56 additional genes and ESTs were up-regulated in 15 out of 16 comparisons (Table III), and an additional 75 genes and ESTs were down-regulated in 15 out of 16 comparisons (Table IV).

Validation of microarray data. Expression of the following genes was used for initial *in silico* validation: *MAGE-A1* and *2*, *MAGE-B1-4*, *MAGE-C1*, *BAGE* and *GAGE1-4* and *6-7*. These genes are expressed only in malignancies but not in normal tissues, with some rare exceptions (e.g. normal testis and placenta) (19). Consistent with their tumor-associated expression pattern, all of these genes exhibited an 'absent call' in all samples tested with high-density oligonucleotide microarrays.

Five genes were selected for microarray data validation with RT-PCR. Four of these genes, *RARRES1*, *LTBP2*, *TYMS*, and *HMGCR,* showed significant variation in all 16 comparisons. There were two probe sets corresponding to *RARRES1*. The first (Affymetrix 221872_at) showed a 7.24-fold-up, and the second (206392_s_at) a 5.66-fold-up variation. *LTBP2* showed a 3.72-fold-up, *TYMS* a 5.14-fold-down, and *HMGCR* a 3.07-fold-down variation. The fifth gene, *RARRES2*, was up-regulated in 14 out of 16 comparisons (21-fold-up). *RARRES1* and *2* were selected as members of the same gene family, while *LTBP2*, *TYMS*, and *HMGCR* were randomly selected among genes showing significant variation.

Radioactive RT-PCR was used to detect and quantify expression of these genes (Fig. 1). For this purpose, total RNA was freshly isolated from the eutopic (normal) endometrium and corresponding ectopic tissue from 3 patients (no material was left for RNA isolation from patient 4 for these studies). The signals were quantified and normalized to the average signal values obtained from two different ubiquitously expressed genes, *36B4* and *18S rRNA*, to minimize tissue-specific variation in the expression of the control genes (Fig. 1A). We found up-regulation of *RARRES-1* (in average 15-fold), *RARRES-2* (in average 9-fold) and *LTBP-2* (in average 7-fold) (Fig. 1), and down-regulation of *HMGCR* (in average 3.5-fold) and *TYMS* (in average 3-fold). Thus, RT-PCR

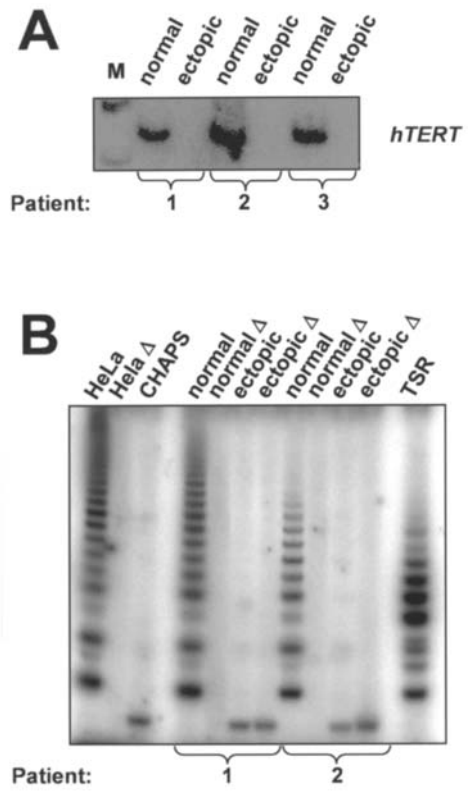


Figure 2. *hTERT* gene expression and telomerase activity are down-regulated in ectopic endometrium. (A) RT-PCR was performed for the mRNA detection of the *hTERT* gene and (B) TRAP assay was performed to detect enzymatic activity of telomerase with tissue samples obtained from patients 1 and 2. RT-PCR and TRAP assays were performed as described in Materials and methods. For radioactive PCR, the same cDNA was used as in Fig. 1 and amplification (94˚C, 30 sec; 55˚C, 30 sec; 72˚C, 1 min) was performed for 28 cycles for *hTERT*. The loading controls (*18S rRNA* and *36B4* mRNA levels) are shown in Fig. 1. M, 100 base pair ladder. One microgram of the 100 base pair ladder was end-labelled according to standard procedures, and ~1000 cps was loaded per lane.

results confirmed the microarray data. Differences in signal intensities between RT-PCR and microarray results were probably due to the different numbers of patients analyzed with the two methods, and inherent methodological differences. The expression of these genes was also analyzed by RT-PCR in endometriotic lesions from 10 additional patients, and the results were in line with those described above, with negligible variations between patients (data not shown).

Lack of telomerase activity and hTERT gene expression in ectopic endometrium. Telomerase activity is repressed in most human somatic tissues during differentiation processes, but it is strongly up-regulated in the vast majority of tumors. Telomerase activity has been previously detected in normal endometrium (20), but it has not been examined in endometriosis. Regulation of telomerase activity occurs primarily at the level of transcriptional initiation of the *hTERT* (human telomerase reverse transcriptase) gene (21,22). Thus, the presence or absence of *TERT* gene mRNA, and consequently telomerase activity, may be indicative of the proliferative capacity of a given cell, a prerequisite for malignancy. *hTERT* exhibited an 'absent call' on the microarray analysis in all 8 probes tested (4 ectopic and 4 eutopic endometrium probes). We reasoned that this might be due to very low levels of *hTERT* mRNA (23). For this reason, a very sensitive method, radioactive RT-PCR, was used to detect low levels of mRNA. *hTERT* mRNA was detectable in all normal endometrium samples, but completely absent in endometriosis samples (Fig. 2A). Moreover, telomerase activity was detectable in samples from normal endometrium but absent in endometriosis samples (Fig. 2B).

Array-CGH analysis of endometriotic lesions. Array-CGH was performed on selected endometriotic tissues (including the samples used in the gene expression analysis) in order to screen for genomic aberrations, which could possibly influence gene expression. Identification of such aberrations could also provide indications of the possible association between endometriosis and neoplastic disease, and endometriosis dissemination. Genomic information obtained with conventional CGH is limited to a resolution of ~10 Mb, since the target DNA is highly condensed and supercoiled within the chromosome. Thus, array-based CGH was used in order to achieve a resolution approaching 1 MB. For automated identification of breakpoints and smoothing of array-CGH data the aCGHsmooth program was used as described previously (24). Reliability of array-CGH results was confirmed through hybridization of the female endometriotic DNA with male reference DNA. As expected, DNA gain was detected on chromosome X (numbered as chromosome 23), while DNA loss was detected on chromosome Y (chromosome 24). Representative examples are shown in Fig. 3A and B. No genomic aberrations were detected in any of the analyzed endometriotic lesions (Fig. 3).

Discussion

Large-scale gene expression analyses of endometriosis and normal endometrium have been previously reported (25-28), but only a subset of the human genome was analyzed in these studies with various methodological approaches. In the present study, gene expression profiles of advanced ovarian endometriotic lesions and corresponding normal endometrium were analyzed with high-density oligonucleotide microarrays, covering for the first time almost the entire human genome by screening for >40,000 human genes and ESTs. Notably, all biopsies in this study were taken in the proliferative phase, since gene expression profiles in the eutopic endometrium differ between the two phases of the menstrual cycle (29). Furthermore, the microarray method used allowed robust data analysis, since signal intensities of each sample could be compared to those of any other sample generated under the same experimental conditions, and thus more than one normal endometrium sample could be used as reference.

Moreover, normal and ectopic endometrium samples were taken from the same patients, allowing gene expression analysis with minimal genetic background heterogeneity. Genetic heterogeneity between individuals confounds the comparison of gene expression profiles between individual patients. CGH with parallel gene expression microarray analyses have shown that individual genetic alterations can directly lead to gene expression dysregulation (30). These

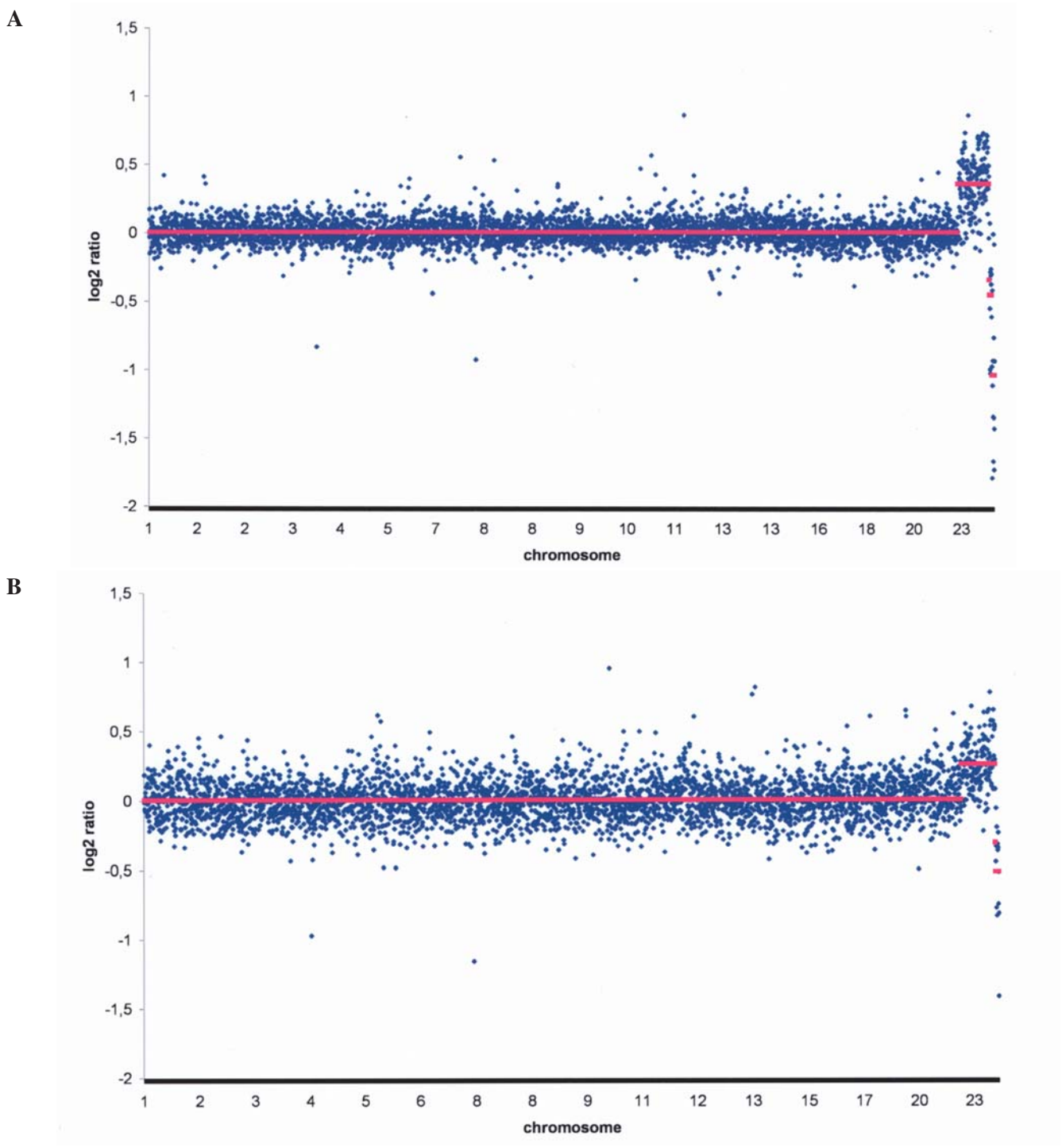


Figure 3. No genomic aberrations were detected in DNA of endometriotic lesions. Array-CGH from two patients (A and B) was carried out as described. The log2 ratios are plotted on individual chromosomes. Blue representing the log2 ratios of the hybridization values and violet representing the breakpoints after the smoothing with the aCGHsmooth program are used to facilitate the detection of genomic aberrations.

limitations can be eliminated only in rare cases in which the patient and the healthy control are genetically identical, as is the case with identical twins; this approach has been previously used in two microarray studies, involving identical twins discordant for type I diabetes (31) and multiple myeloma (32). In the present study, microarray data were analyzed with a minimal genetic background approach, comparing in essence four sets of 'identical twin' probes, as previously described (31,32).

Though studies with conventional CGH with human metaphase chromosomes (33,34), allowing a resolution of 10 MB, and high-resolution CGH (35) have previously described certain aberrations in endometriotic epithelial cells, our analysis by array-CGH to a much higher resolution of 1.0 Mb did not show any specific genomic aberrations in endometriosis samples. This contradiction is probably due to different experimental settings, patient characteristics, and disease stages. Moreover, genome variations such as segmental

Table III. Up-regulated genes and ESTs in 15 out of 16 comparisons.

Fold-up	GenBank ID	Gene description	Gene symbol
30.25	AA707199	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2
21.76	NM_012134.1	Leiomodin 1 (smooth muscle)	LMOD1
19.53	NM_022870.1	Myosin, heavy polypeptide 11, smooth muscle	MYH11
12.72	S67238.1 R[3]C	Myosin, heavy polypeptide 11, smooth muscle	MYH11
12.55	NM_001442.1	Fatty acid binding protein 4, adipocyte	FABP4
11.81	AL359062.1	*Homo sapiens* mRNA full length insert cDNA clone EUROIMAGE 1913076	
08.32	NM_000300.1	Phospholipase A2, group IIA (platelets, synovial fluid)	PLA2G2A
06.96	NM_005525.1	Hydroxysteroid (11-ß) dehydrogenase 1	HSD11B1
06.50	AI807950	ESTs, weakly similar to hypothetical protein FLJ20489 [*Homo sapiens*]	
05.76	NM_005965.1	Myosin, light polypeptide kinase	MYLK
05.61	BE551451	ESTs	
05.12	X57812.1	Immunoglobulin λ joining 3	IGLJ3
04.86	M63438.1	Immunoglobulin κ constant	IGKC
04.52	NM_003246.1	Thrombospondin 1	THBS1
04.42	M87789.1	Immunoglobulin heavy constant γ 3 (G3m marker)	IGHG3
04.34	NM_002084.2	Glutathione peroxidase 3 (plasma)	GPX3
04.18	AF288391.1	Chromosome 1 open reading frame 24	C1orf24
04.16	NM_000362.2	Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	TIMP3
04.03	AL577531	Caldesmon 1	CALD1
03.97	BC005332.1	Immunoglobulin κ constant	IGKC
03.93	NM_013281.1	Fibronectin leucine-rich transmembrane protein 3	FLRT3
03.91	AW027333	Hypothetical protein LOC253827	LOC253827
03.86	NM_007203.1	A kinase (PRKA) anchor protein 2	AKAP2
03.85	AA706788	Hs mRNA; cDNA DKFZp434C1915 (from clone DKFZp434C1915); partial cds	
03.81	AA680302	Immunoglobulin λ joining 3	IGLJ3
03.78	AW151360	ESTs	
03.73	M87790.1	Immunoglobulin λ joining 3	IGLJ3
03.70	NM_001613.1	Actin, α 2, smooth muscle, aorta	ACTA2
03.67	AV726673	Thrombospondin 1	THBS1
03.66	NM_022083.1	Chromosome 1 open reading frame 24	C1orf24
03.57	NM_014840.1	KIAA0537 gene product	ARK5
03.57	BG540494	A kinase (PRKA) anchor protein 2	AKAP2
03.48	BG485135	Immunoglobulin κ constant	IGKC
03.45	NM_001299.1	Calponin 1, basic, smooth muscle	CNN1
03.41	BC002416.1	Biglycan	BGN
03.36	AA845258	Biglycan	BGN
03.31	NM_003289.1	Tropomyosin 2 (ß)	TPM2
03.23	AI658662	*Homo sapiens* cDNA FLJ20767 fis, clone COL06986	
03.17	NM_005195.1	CCAAT/enhancer binding protein (C/EBP), Δ	CEBPD
03.01	NM_004342.2	Caldesmon 1	CALD1
02.99	AW978896	*Homo sapiens* hAWMS1 mRNA, complete cds.	
02.87	AI754423	Colon carcinoma-related protein	LOC51159
02.82	AB046817.1	Synaptotagmin-like 2	SYTL2
02.64	AF132202.1	PRO1073 protein	PRO1073
02.61	NM_000014.3	α-2-macroglobulin	A2M
02.56	AF164622.1	Golgin-67	GOLGIN-67
02.52	AL583520	Caldesmon 1	CALD1
02.49	NM_001912.1	Cathepsin L	CTSL
02.39	AW005982	PRO1073 protein	PRO1073
02.38	AL136842.1	CDC42 effector protein (Rho GTPase binding) 3	CDC42EP3
02.29	BF221852	LIM domain containing preferred translocation partner in lipoma	LPP
02.29	AW513612	Hypothetical protein LOC119587	LOC119587
02.21	AI123567	Formin homology 2 domain containing 2	FHOD2
02.14	AA524053	Splicing factor, arginine/serine-rich 7, 35 kDa	SFRS7
02.06	NM_014128.1		
02.06	AB000889.1	Phosphatidic acid phosphatase type 2B	PPAP2B

Table IV. Down-regulated genes and ESTs in 15 out of 16 comparisons.

Fold-down	GenBank ID	Gene description	Gene symbol
14.74	NM_021127.1	Phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1
12.83	NM_002160.1	Tenascin C (hexabrachion)	TNC
12.07	NM_006101.1	Highly expressed in cancer, rich in leucine heptad repeats	HEC
08.88	NM_004456.1	Enhancer of zeste homolog 2 (*Drosophila*)	EZH2
08.28	NM_001168.1	Baculoviral IAP repeat-containing 5 (survivin)	BIRC5
06.70	NM_001255.1	CDC20 cell division cycle 20 homolog (*S. cerevisiae*)	CDC20
06.28	Y11339.2	GalNAc α-2, 6-sialyltransferase I, long form	ST6GalNAcI
06.17	NM_003318.1	TTK protein kinase	TTK
05.78	BF589515	Hypothetical protein FLJ34221	FLJ34221
05.63	D88357.1	Cell division cycle 2, G1 to S and G2 to M	CDC2
05.30	NM_001211.2	BUB1 budding uninhibited by benzimidazoles 1 homolog ß (yeast)	BUB1B
05.21	NM_001996.1	Fibulin 1	FBLN1
05.10	AL574096	Tissue factor pathway inhibitor 2	TFPI2
04.88	NM_004701.2	Cyclin B2	CCNB2
04.82	AK025084.1	Trinucleotide repeat containing 9	TNRC9
04.80	BG054916	Patched homolog (*Drosophila*)	PTCH
04.76	NM_002358.2	MAD2 mitotic arrest deficient-like 1 (yeast)	MAD2L1
04.72	NM_001809.2	Centromere protein A, 17 kDa	CENPA
04.72	AK023208.1	Anillin, actin binding protein (scraps homolog, *Drosophila*)	ANLN
04.38	AL547946	Progesterone receptor membrane component 1	PGRMC1
04.21	NM_003981.1	Protein regulator of cytokinesis 1	PRC1
04.02	AI419030	Glycoprotein M6B	GPM6B
03.98	AI346350	Polymyositis/scleroderma autoantigen 1, 75 kDa	PMSCL1
03.78	NM_012259.1	Hairy/enhancer-of-split related with YRPW motif 2	HEY2
03.78	BC005978.1	Karyopherin α 2 (RAG cohort 1, importin α 1)	KPNA2
03.78	NM_004702.1 .1	Cyclin E2	CCNE2
03.62	NM_004900.1	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	APOBEC3B
03.59	U36189.1	Chromosome 5 open reading frame 13	C5orf13
03.53	NM_003524.1	Histone 1, H2bh	HIST1H2BH
03.42	D13889.1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1
03.15	NM_021968.1		
03.11	AI431730	A disintegrin-like and metalloprotease (reprolysin type)with thrombospondin type 1 motif, 9	ADAMTS9
03.07	NM_004415.1	Desmoplakin (DPI, DPII)	DSP
02.99	NM_003878.1	γ-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	GGH
02.90	AK026161.1	Ca^{2+}-dependent endoplasmic reticulum nucleoside diphosphatase	SHAPY
02.88	NM_002266.1	Karyopherin α 2 (RAG cohort 1, importin α 1)	KPNA2
02.87	NM_004219.2	Pituitary tumor-transforming 1	PTTG1
02.85	NM_002105.1	H2A histone family, member X	H2AFX
02.84	AL563283	Androgen-induced basic leucine zipper	AIBZIP
02.83	NM_012177.1	F-box only protein 5	FBXO5
02.72	AU153848	Rac GTPase activating protein 1	RACGAP1
02.72	NM_002402.1	Mesoderm-specific transcript homolog (mouse)	MEST
02.72	NM_004772.1	Chromosome 5 open reading frame 13	C5orf13
02.69	M69148.1	Midkine (neurite growth-promoting factor 2)	MDK
02.69	NM_015677.1	Likely ortholog of mouse Sh3 domain YSC-like 1	SH3YL1
02.56	NM_006452.1	Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	PAICS
02.52	NM_006451.1	Olyadenylate binding protein-interacting protein 1	PAIP1
02.48	D87292.1	Thiosulfate sulfurtransferase (rhodanese)	TST
02.47	NM_002592.1	Proliferating cell nuclear antigen	PCNA
02.45	NM_006667.2	Progesterone receptor membrane component 1	PGRMC1
02.44	AB000888.1	Phosphatidic acid phosphatase type 2A	PPAP2A
02.43	AA534817	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 3	EDG3

Table IV. Continued.

Fold-down	GenBank ID	Gene description	Gene symbol
02.41	NM_018204.1	Cytoskeleton associated protein 2	CKAP2
02.40	NM_020987.1	Ankyrin 3, node of Ranvier (ankyrin G)	ANK3
02.34	AF014403.1	Phosphatidic acid phosphatase type 2A	PPAP2A
02.34	Z95331	Fibulin 1	FBLN1
02.31	M96789	Gap junction protein, α4, 37 kDa (connexin 37)	GJA4
02.31	AI970157	Glycine cleavage system protein H (aminomethyl carrier)	GCSH
02.31	NM_006334.1	Olfactomedin 1	OLFM1
02.29	NM_006117.1	Peroxisomal D3,D2-enoyl-CoA isomerase	PECI
02.29	NM_005496.1	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	SMC4L1
02.25	NM_002915.1	Replication factor C (activator 1) 3, 38 kDa	RFC3
02.19	NM_001070.1	Tubulin, γ 1	TUBG1
02.18	NM_001123.1	Adenosine kinase	ADK
02.18	AA112507	LSM4 homolog, U6 small nuclear RNA associated (*S. cerevisiae*)	LSM4
02.13	L04636.1	Complement component 1, q subcomponent binding protein	C1QBP
02.13	NM_005815.1	Kruppel-type zinc finger (C2H2)	ZK1
02.11	AA902652	Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	PAICS
02.10	S81916.1	Phosphoglycerate kinase {alternatively spliced} [human, phosphoglycerate kinase deficient patient with episodes of muscl, mRNA partial mutant, 307 nt]	
02.08	AW235061	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	SLC1A1
02.07	NM_004999.1	Myosin VI	MYO6
02.05	AW237404	Glycine cleavage system protein H (aminomethyl carrier)	GCSH
02.04	NM_002482.1	Nuclear autoantigenic sperm protein (histone-binding)	NASP
02.03	NM_001954.2	Discoidin domain receptor family, member 1	DDR1
02.03	BC004439.1	Translocase of inner mitochondrial membrane 17 homolog A (yeast)	TIMM17A

duplications might have been discovered by the conventional CGH methods depending on the reference DNA used. We excluded measurement of genome variations by using male reference DNA of a mixed pool of different individuals. Importantly, in the endometriosis samples analyzed with gene expression microarrays no genomic alterations were found by array-CGH. These findings confirm that genetic background heterogeneity in our gene expression analysis was indeed minimal, and were in line with our gene expression data demonstrating the minimal malignant potential of endometriosis.

Up-regulated candidate genes identified with gene expression microarray analysis in the present study included genes encoding proteins involved in immunologic processes (*LTBP2*, *IGJ*, *IGKC*, *IGLJ3*, *MSR1*, *FCGR2B*, *IGHG3*, *IGL@*, *IGLV*, *Ig κ light chain mRNA*, *CCL18*, *TNFSF13B*, *CXCL12*, *CRLF3*), and genes involved in cancer (*TAGLN*, *GLTSCR2*, *WISP21*), including putative tumor-suppressor genes (*RARRES1* and *2*, *ARHI* and *CREBL2*) (Tables I and III). Down-regulated candidate genes included genes involved in cell metabolism and homeostasis (*RRM2*, *H2AFX*, *CENPA*, *HMGCR*, *APOBEC3B*), genes involved in the cell cycle (c*yclins B1*, *B2* and *E2*, *CDKN3*, *CKS2*, *CDC2*, *TOP2A*, *MAD2L1*), and *PTTG1*, a gene with transforming activity *in vitro* and tumorigenic activity *in vivo* (Tables II and IV). These genes have not been identified by previous microarray analyses of endometriosis, apparently due to different methodological approaches, lower number of genes analyzed in previous studies, and different subsets of patients, as discussed above.

Altogether, our results showing up-regulation of putative tumor-suppressor genes such as *RARRES1* and *RARRES2* and down-regulation of genes involved in the cell cycle suggest that advanced ovarian endometriosis is a highly differentiated tissue with minimal proliferative capacity. Moreover, these conclusions are further supported by the absence of both *hTERT* gene expression and telomerase activity with a distinct methodological approach. This state of high tissue differentiation does not allow definitive conclusions to be made with respect to the etiopathogenesis of endometriosis, but overall, these data suggest that advanced ovarian endometriosis is unlikely to be a precursor lesion of cancer. The lack of detectable genomic aberrations in endometriosis with high-resolution array-CGH analysis is in line with these findings. Modulated genes identified in this setting should be further evaluated individually in order to define their role in endometriosis.

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