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Research Article

Dienogest exerts its anti-endometriotic effect through the direct suppression of matrix metallopeptidases

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Abstract

Background: Endometriosis, which affects up to 10% women of reproductive age, is defined by the presence of ectopic endometrial tissue outside the uterus. The current key drug of hormonal therapies for endometriosis is dienogest, which is a progestin with high specificity for the progesterone receptor. Although many findings about the anti-endometriotic effect of dienogest on endometriosis have been reported, the precise mechanisms of dienogest's anti-endometriotic effect remain unknown.

Methods: To investigate the direct anti-endometriotic effect of dienogest on endometriotic cells, we determined and compared the genome-wide gene expression profiles of endometriotic stromal cells treated with dienogest (Dienogest group) and those not treated with dienogest (Control group) and then performed a pathway analysis using these data. To test the microarray data, we performed real-time RT-PCRs for matrix metalloproteinase (MMP)-1, MMP-3, MMP-10, and TIMP-4.

Results: Six-hundred forty-seven genes were revealed to be differentially expressed between the Dienogest and Control groups. Of them, 314 genes were upregulated and 333 genes were downregulated in the Dienogest group compared to the Control group. We identified 20 canonical pathways that are significantly distinct in the Dienogest group versus the Control group. Among the 20 canonical pathways, MMPs including MMP-1, -3, and -10 were found to be the most involved genes.

Conclusions: Our results suggest that dienogest may exert its anti-endometriotic effect through the direct suppression of MMPs.

Key words: endometriosis, dienogest, microarray, gene expression profile, ingenuity pathways analysis, MMP

Background

Endometriosis, which is defined by the presence of ectopic endometrial tissue outside the uterus, is a common chronic disease affecting up to 10% women of reproductive age [1]. Infertility and chronic pelvic pain are the main symptoms. For chronic pelvic pain, mainly hormonal therapies have been provided in the past decades, and a gonadotropin-releasing hormone (GnRH) analogue has been used as the key drug of the hormonal therapies. However, a GnRH analogue cannot be used for a long period because of its side effects such as bone loss. It has thus been difficult for individuals with chronic pelvic pain due to endometriosis to obtain relief from pain over a long period by using a GnRH analogue.

Dienogest is classified as a 4th-generation progestin with high specificity for the progesterone receptor. It has an effect on endometriosis-induced pain that is equivalent to that of a GnRH analogue. However, unlike GnRH analogues, dienogest can be used for long periods, and it has brought endometriosis patients long-term relief from the pain [2]. Dienogest has replaced GnRH analogues as the key drug of the hormonal therapies for endometriosis. The anti-endometriotic effect of dienogest is thought to be due mainly to its ability to suppress ovulation [3], and several studies have shown that dienogest directly inhibits the inflammatory responses or aromatase expression in endometriotic cells [4,5,6]. Another investigation indicated that dienogest directly inhibits the proliferation of endometriotic stromal cells [7]. Although many studies have attempted to reveal the biological mechanisms that underly the effects of dienogest on endometriosis, the precise mechanisms remain unknown.

Genome-wide gene expression profiling using a microarray and its subsequent pathway analysis sometimes reveal novel biological cell-related findings for a disease.

1 There has been no report of genome-wide gene expression profiling conducted to
2 investigate the biological actions of dienogest in endometriotic cells.

3 The present study analyzed the genome-wide gene expression profile of
4 endometriotic stromal cells treated with dienogest. We also performed a pathway
5 analysis using the data of the gene expression profile. The results demonstrated that in
6 the endometriotic stromal cells, matrix metalloproteinases (MMPs) were the most
7 closely involved genes among the genes that are directly modified by dienogest.

10 **Methods**

11 **Patients and samples**

12 The protocol of the present study was approved by the Ethical Committee of Hiroshima
13 City Asa Hospital, and all experiments were carried out in accordance with relevant
14 guidelines and regulations. Tissue specimens of ovarian endometriotic cysts (n=5) were
15 obtained during gynecological surgeries from five patients with stage III–IV
16 endometriosis evaluated according to the American Society for Reproductive Medicine
17 classification of endometriosis. Before the surgeries, the patients provided their written
18 informed consent. All five patients were of reproductive age and had not received any
19 hormonal therapies. All specimens were collected at the proliferative phase of the
20 menstrual cycle and were pathologically confirmed as endometriotic cyst tissues after
21 the surgeries.

22 The isolation of endometriotic stromal cells (ESCs) was performed as described
23 by Honda et al [8]. Briefly, Endometriotic tissue layers were scraped from the inner wall
24 of the cyst, minced into small pieces, and enzymatically dissociated by incubation with

0.25% collagenase (Sigma-Aldrich, St. Louis, MO, USA) and 0.02% DNase I (Sigma-Aldrich) in phenol-red-free Dulbecco's modified Eagle's Medium/Ham's F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% charcoal-stripped fetal bovine serum (FBS) (Invitrogen) for 1 hr at 37°C in an atmosphere of 5% CO₂. Enrichment of the ESCs was performed by serial filtration using 100-µm and 40-µm nylon sieves (BD Falcon, Franklin Lakes, NJ), and equally collected onto two 6-cm culture dishes per sample. After incubation at 37°C for 30 min to allow the ESCs to attach to the dishes, the media were removed to remove the floating endometriotic epithelial cells in the supernatant.

The ESCs were cultured in DMEM/Ham's F-12 medium supplemented with 10% charcoal-stripped FBS and 1% penicillin and streptomycin (100 mg/ml) (Invitrogen) under the conditions described above. The medium was changed every other day, and, when the cells reached 80% confluence, the cultures were serum-starved in serum-free DMEM/Ham's F-12 medium before hormone treatments. After 24 hr of culture, the medium was replaced with either serum-free DMEM/Ham's F-12 medium with estradiol (10⁻⁸ M; Sigma-Aldrich) alone or estradiol (10⁻⁸ M) + dienogest (10⁻⁶ M; Santa Cruz Biotechnology, Dallas, TX). The ESCs treated without dienogest were used as a control (Control group), and those treated with dienogest were named the Dienogest group. After these hormonal treatments for 48 hr, the ESCs were directly lysed on the culture dishes with TRIzol™ reagent (Invitrogen), immediately snap-frozen, and stored at -80°C until further processing.

RNA isolation

Total RNA was purified from the cell lysates of ESCs using the RNeasy Mini Kit

(Qiagen, Valencia, CA) according to the manufacturer's instructions. The quantity and quality of the purified RNA were measured and assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) before cRNA amplification and labeling.

The following processes of cRNA amplification and labeling, sample hybridization, and data analysis of the microarray were performed according to the protocol described by Yokoi et al [9]. with minor modification.

cRNA amplification and labeling

Total RNA was amplified and labeled with cyanine 3 (Cy3) using the Agilent Low Input Quick Amp Labeling Kit, one-color (Agilent Technologies) following the manufacturer's instructions. Briefly, 100 ng of total RNA was reversed transcribed to double-strand cDNA with the use of a poly dT-T7 promoter primer. The primer, template RNA, and quality-control transcripts of known concentration and quality were first denatured at 65°C for 10 min and incubated for 2 hr at 40°C with 5X first strand Buffer, 0.1 M DTT, 10 mM dNTP mix, and AffinityScript RNase Block Mix. The AffinityScript enzyme was inactivated at 70°C for 15 min.

The cDNA products were then used as templates for in vitro transcription to generate fluorescent cRNA. The cDNA products were mixed with a transcription master mix in the presence of T7 RNA polymerase and Cy3 labeled-CTP and incubated at 40°C for 2 hr. Labeled cRNAs were purified using Qiagen's RNeasy mini-spin columns and eluted in 30 µl of nuclease-free water. After amplification and labeling, the cRNA

1 quantity and the incorporation of cyanine were determined using the Nanodrop ND-
2 1000 spectrophotometer and the Agilent Bioanalyzer.

3 4 **Sample hybridization**

5 For each hybridization, 0.60 µg of Cy3-labeled cRNA was fragmented and then
6 hybridized at 65°C for 17 hr with an Agilent SurePrint G3 Human GE v2 8x60K
7 Microarray (Design ID: 039494). After washing, the microarrays were scanned using an
8 Agilent DNA microarray scanner.

9 10 **Data analysis of the microarray**

11 The intensity values of each scanned feature were quantified using Agilent feature
12 extraction software ver. 10.7.3.1, which performs background subtractions. We used only
13 features that were flagged as 'no errors' (present flags), and we excluded features that
14 were not positive, not significant, not uniform, not above the background, saturated, or
15 population outliers (marginal and absent flags). Normalization was performed using
16 Agilent GeneSpring GX ver. 11.0.2 software (per chip: normalization to the 75th
17 percentile shift; per gene: normalization to the median of all samples). There are a total
18 of 50,599 probes on the Agilent SurePrint G3 Human GE v2 8x60K Microarray (Design
19 ID: 039494) without control probes. The RNA samples of the Control group were used
20 as the total RNA reference.

21 Differentially expressed genes between the Dienogest and Control groups with
22 corrected p-values <0.05 and an absolute fold change >1.5 were considered to be
23 significantly differentially expressed. The microarray data were also used for an ingenuity
24 pathway analysis (IPA, Ingenuity® Systems, www.ingenuity.com) for the analysis of

canonical pathways. Fisher's exact test was used to calculate a p-value to determine the significance of each canonical pathway, and p-values <0.05 were accepted as significant.

Quantitative RT-PCR

Total RNA purified for microarray was also used for quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) to validate the microarray data. One microgram of total RNA was reverse transcribed into first-strand cDNA by using a first-strand cDNA synthesis kit, ReverTra Ace- α (Toyobo, Osaka, Japan) with random primers. The cDNA was stored at -20°C until used.

Of the genes that were differentially expressed between the Dienogest and Control groups, those for MMP-1, MMP-3, MMP-10, and TIMP-4 were selected for the PCR reactions, which were performed in an ABI 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA) using the KAPA SYBR[®] FAST qPCR kit (Nippon Genetics, Tokyo) under thermal cycling conditions according to the manufacturer's instructions. The primer sequences of the MMPs and TIMP-4 used in the analysis were the same as those designed by Pennington [10]. A real-time RT-PCR for the housekeeping gene GAPDH was performed in all of the samples to evaluate the quality of the cDNAs used in the study. The primers used for the GAPDH quantification were designed by Wang et al [11].

Results

Identification of genes differentially expressed between the Dienogest and Control groups

Under the condition of a fold change of at least ± 1.5 with corrected p-values of

<0.05, 647 genes were revealed to be differentially expressed between the Dienogest and Control groups. Of these 647 genes, 314 genes were upregulated and 333 genes were downregulated in the Dienogest group compared to the Control group. Tables 1 and 2 show the top 20 genes upregulated or downregulated in the Dienogest group, respectively.

Ingenuity pathways analysis (IPA)

The comparison analysis performed by IPA for the Dienogest and Control groups identified 20 significant canonical pathways based on these 647 differentially expressed genes, which were extracted under the same conditions. Figure 1 shows the top 10 pathways of those 20 canonical pathways. The most frequently associated genes in the 20 canonical pathways were MMPs (MMP-1, -3, -10, -12, -25, and -27). Table 3 shows the eight canonical pathways with which these MMPs are associated among the 20 canonical pathways.

Quantitative RT-PCR for the validation of the microarray data

Of the genes that were revealed to be differentially expressed between the Dienogest and Control groups, we selected MMP-1, -3, and -10 and TIMP-4 for validation of the microarray data. The data illustrated in Figure 2 demonstrated that MMP-1, -3, and -10 were significantly decreased in the the Dienogest group compared to the Control group, and that TIMP-4 was significantly increased in the Dienogest group compared to the Control group.

1 Discussion

2 Although dienogest has already been used for the main hormonal therapy of
 3 endometriosis, its precise anti-endometriotic mechanism has been unclear. In the present
 4 study, we comprehensively investigated the direct effects of dienogest on the
 5 endometriotic stromal cells, and our findings revealed that the MMPs were the most
 6 frequently involved genes in the 20 canonical pathways that are significantly different
 7 between the Dienogest and Control groups. Our results thus suggest that MMPs have
 8 central roles in the anti-endometriotic effect of dienogest.

9 Surprisingly, although several investigations have described an anti-
 10 endometriotic effect of dienogest, there has been no report such as the present study that
 11 analyzed differences in genome-wide gene expression profiles between untreated
 12 endometriotic cells and endometriotic cells treated with dienogest. Bruner et al [12].
 13 reported that the establishment of ectopic human endometrium in nude mice was
 14 inhibited by the suppression of MMPs caused by progesterone treatment. In addition,
 15 abnormal expressions of specific MMPs have been shown in endometriotic lesions
 16 compared to eutopic endometrium. Several research groups have observed that
 17 endometriotic cells have significantly higher expressions of MMP-1, -2, -3, -7, or -9
 18 compared to eutopic endometrial cells in in vitro or in vivo experiments, or in patients'
 19 tissues[13, 14, 15, 16, 17, 18, 19]. We thus believe that MMPs play crucial roles in the
 20 establishment and progress of endometriotic lesions.

21 In the present study, the expressions of MMP-1 and MMP-3 in ESCs were
 22 downregulated by the addition of dienogest. Earlier studies noted that MMP-1 and
 23 MMP-3 were inhibited by progesterone or progestin in endometrial explants or
 24 endometriotic cells [20, 21, 22]. The downregulations of MMP-1 and MMP-3 by

1 dienogest are thus likely to be reliable. The expression of MMP-7, which in
2 endometrium or endometriotic tissues is expressed only in epithelial cells, has been
3 shown to be inhibited by progesterone or progestin in two studies [21 22], but our
4 present investigation did not reveal the similar effect of dienogest on the endometriotic
5 cells. This discrepancy between the present and past studies regarding the effect of
6 dienogest is probably due to the use of endometriotic stromal cells in the present study.
7 It therefore seems that dienogest possibly has an inhibitory effect on MMP-7 expression
8 in endometriotic epithelial cells.

9 Our present analyses did not reveal any inhibitory effect of dienogest on the
10 expression of MMP-2 or MMP-9. Regarding the inhibitory effect of progesterone on
11 MMP-2 or MMP-9 expression, conflicting results have been reported. Some studies
12 indicated that progesterone inhibited MMP-2 or MMP-9 expression in endometriotic
13 cells, whereas other studies indicated that progesterone did not change the MMP-2 or
14 MMP-9 expression [23, 24]. It was also reported that the MMP-10 expression in the
15 endometriotic tissues of ovarian endometriosis was significantly increased compared to
16 the expression in eutopic endometrial tissues without endometriosis [25], and our
17 present results demonstrated that dienogest directly inhibited the MMP-10 expression in
18 the endometriotic stromal cells. Taken together, the previous and present findings
19 indicate that the suppression of MMP-10 expression (as well as those of MMP-1 and
20 MMP-3) by dienogest might be one of dienogest's direct anti-endometriotic effects.

21 We also observed changes in the expressions of MMP-12, -25, and -27 by the
22 addition of dienogest, but the involvement of these MMPs in the pathogenesis of
23 endometriosis has not been well studied. It remains unknown whether the changes in the
24 expressions of these MMPs are related to the anti-endometriotic effect of dienogest.

1 Tissue inhibitors of metalloproteinases (TIMPs) have also been revealed to be
2 abnormally expressed in endometriotic tissues compared to normal eutopic
3 endometrium. The expressions of TIMP-1, -2, and -3 were reported to be significantly
4 lower in endometriotic tissues than normal eutopic endometrium [26, 27], and an
5 experimental study showed that the addition of TIMP protein to the peritoneal cavity of
6 nude mice prevented the establishment of endometriosis [12]. These findings indicate
7 that TIMPs are also likely to be involved in the pathogenesis of endometriosis.

8 However, there are few studies describing a change in the expression of TIMP-1,
9 -2, or -3 by an addition of progesterone, and our present findings did not provide any
10 information about the changes of TIMP expressions by the addition of dienogest, with
11 the exception of TIMP-4. TIMP-4, which is also expressed in human endometrium, has
12 affinity for MMP-1, -2, -3, -7, -9, and -12 and shows the highest inhibitory capacity for
13 MMP-26 [28, 29, 30, 31]. TIMP-4 is coordinately expressed with MMP-26, which is an
14 effective activator of pro-MMP-9 [32]. The results of our present analyses showed that
15 dienogest increased the expression of TIMP-4 in endometriotic stromal cells. Although
16 there has been no report about the relationship between endometriosis and TIMP-4, the
17 increase in TIMP-4 expression by treatment with dienogest may be related to the anti-
18 endometriotic effect of dienogest.

19 This study has some limitations to consider. Endometriotic tissues consist of
20 various types of cells such as ectopic endometrial epithelial and stromal cells and
21 immune cells. Inflammatory cytokines, which are produced mainly by immune cells in
22 endometriotic tissues, are thought to play important roles in the pathogenesis of
23 endometriosis [33]. However, the experimental system of the present study lacked
24 immune cells, and it was thus not possible to assess the anti-endometriotic effect of

dienogest through the inflammatory cytokines in this study, which used only endometriotic stromal cells.

It is also not yet possible to recreate the exact biological environment of endometriotic tissues in an in vitro experiment such as that in the present study; however, our findings revealed for the first time the direct effect of dienogest on endometriotic stromal cells by genome-wide gene expression profiling and a pathway analysis. MMPs were most frequently involved in the canonical pathways distinct to the dienogest-treated endometriotic stromal cells. Our results strongly suggest that MMPs play important roles in the therapeutic action of dienogest against endometriosis. Although our findings demonstrated the direct suppression of MMPs by dienogest in endometriotic stromal cells, we were not able to discern the precise molecular pathways that link dienogest to MMPs. It is certain that dienogest is useful and effective for endometriosis, but it is not used for patients who want to conceive, because of its anti-ovulatory effect. Patients with severe endometriosis who want to conceive more than once are likely to experience disease progression during under the period without dienogest. A substrate that suppresses the expressions of MMPs at a region downstream from dienogest and that does not suppress ovulation is desired, as such a substrate could conceivably be used as a drug to treat patients with endometriosis who want to conceive.

Conclusions

It is clear from many earlier investigation that MMPs play important roles in the pathogenesis of endometriosis and that their expressions are regulated mainly by inflammatory cytokines [12–19, 33]. We speculated that dienogest might indirectly

suppress the expressions of MMPs through the inhibition of inflammatory cytokines [33]. Our present findings suggest that in addition to its indirect suppression, dienogest may exert its anti-endometriotic effect through the direct suppression of MMPs.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Abbreviations

MMP: matrix metalloproteinases

GnRH: gonadotropin-releasing hormone

ESCs: endometriotic stromal cells

IPA: Ingenuity pathways analysis

TIMPs: Tissue inhibitors of metalloproteinases

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Contributions

H.H designed the study, carried out most of the experiments and wrote the paper. N.N. assisted with some experiments. M.Y., Y.A., H.T., M.T. and H. Teramoto provided the patients' samples and clinical information. Y.Y. contributed to the data analysis and interpretation. All of the authors reviewed and revised the manuscript.

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Ethics declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Hiroshima City Asa Hospital, and all experiments were carried out in accordance with relevant guidelines and regulations. All participants provided their written informed consent.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Figure legends

Fig. 1. The top 10 canonical pathways that are significantly distinct in the Dienogest group compared to the Control group. The *transverse line* indicates the threshold of the distinct pathways in the Dienogest group under the condition of $p < 0.05$.

Fig. 2. Validation of the microarray data and the pathway analysis by real-time RT-PCR. MMP-1, MMP-3, and MMP-10 were significantly decreased and TIMP-4 was significantly increased in the Dienogest group compared to the Control group.

Table 1. Top 20 genes upregulated in the Dienogest group

Probe ID	Gene Symbol	Fold change
A_23_P8801	CYP3A5	5.96
A_33_P3422499	Not identified	5.48
A_33_P3327673	COBL	4.84
A_19_P00318561	LOC100506516	4.70
A_24_P127691	DNAH14	4.40
A_21_P0008435	XLOC_011016	4.27
A_23_P390958	PCDHB18	4.23
A_33_P3290748	LOC648044	4.10
A_33_P3212269	MAP2K3	4.09
A_24_P7965	ESRRG	3.95
A_21_P0010916	XLOC_l2_001548	3.92
A_33_P3409518	TUBBP5	3.77
A_23_P120504	C20orf46	3.76
A_21_P0014652	LOC100507411	3.65
A_19_P00330814	HOTAIR	3.51
A_33_P3272614	PPP1R26	3.41
A_33_P3268129	Not identified	3.37
A_33_P3333156	C11orf70	3.33
A_21_P0014780	LOC100652747	3.20
A_33_P3424062	KCNF1	3.18

Table 2. Top 20 genes downregulated in the Dienogest group

Probe ID	Gene symbol	Fold change
A_23_P1452	NPFFR1	-6.53
A_23_P313550	SLC25A41	-5.84
A_23_P420281	PRKCB	-5.71
A_23_P329768	GREB1	-5.61
A_33_P3376273	GK	-4.77
A_33_P3409266	GAFA2	-4.16
A_32_P32905	Not identified	-3.67
A_21_P0010584	XLOC_l2_000696	-3.51
A_33_P3410659	CLEC12B	-3.49
A_24_P265088	PDZD4	-3.45
A_23_P302681	FIGNL1	-3.42
A_33_P3262376	OTUD7A	-3.31
A_24_P395814	CGB	-3.21
A_33_P3281171	LOC100130987	-3.08
A_33_P3270197	DIS3L2	-3.06
A_33_P3260342	NFASC	-2.94
A_21_P0001141	XLOC_000869	-2.91
A_21_P0010120	XLOC_013781	-2.78
A_21_P0001404	XLOC_000511	-2.74
A_21_P0010200	XLOC_014056	-2.72

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Table 3. Eight pathways to which MMPs are associated among the 20 canonical pathways

↑ : upregulated in the Dienogest group ↓ : downregulated in the Dienogest group

Pathway	Number of associated genes	Associated genes
Inhibition of Matrix Metalloproteases	7	MMP-1↓ MMP-3↓ MMP-10↓ MMP-12↑ MMP-25↓ MMP-27↓ TIMP-4↑
Granulocyte Adhesion and Diapedesis	13	C5AR1↓ CCL7↑ CCL11↑ CCL25↑ CLDN3↓ CXCL14↑ MMP-1↓ MMP-3↓ MMP-10↓ MMP-12↑ MMP-25↓ MMP-27↓ VCAM1↑
Atherosclerosis Signaling	11	APOA1↑ APOB↓ CCL11↑ CSF1↓ IL6↑ MMP-1↓ MMP-3↓ PLA2G2A↑ PLA2G4E↓ PON1↑ VCAM1↑
Bladder Cancer Signaling	7	FGF13↑ MMP-1↓ MMP-3↓ MMP-10↓ MMP-12↑ MMP-25↓ MMP-27↓
HIFα Signaling	6	MMP-1↓ MMP-3↓ MMP-10↓ MMP-12↑ MMP-25↓ MMP-27↓
Leukocyte Extravasation Signaling	9	CLDN3↓ CXCL14↑ MMP-1↓ MMP-3↓ MMP-10↓ MMP-12↑ MMP-25↓ MMP-27↓ VCAM1↑
Role of Macrophages, Fibroblast and Endothelial Cells in Rheumatoid Arthritis	12	C5AR1↓ CAMK2B↓ CSF1↓ GNAO1↓ IL6↑ MMP-1↓ MMP-3↓ PLCE1↑ PLCL2↓ SOST↓ TLR3↑ VCAM1↑
Hepatic Fibrosis /Hepatic Stellate Cell Activation	8	CSF1↓ HGF↓ IGF1↑ IL6↑ IL4R↓ MMP-1↓ SERPINE1↑ VCAM1↑

Figures

Figure 1

The top 10 canonical pathways that are significantly distinct in the Dienogest group compared to the Control group. The transverse line indicates the threshold of the distinct pathways in the Dienogest group under the condition of $p < 0.05$.

Figure 2

Validation of the microarray data and the pathway analysis by real-time RT-PCR. MMP-1, MMP-3, and MMP-10 were significantly decreased and TIMP-4 was significantly increased in the Dienogest group compared to the Control group.