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Demethylation within the proximal promoter region of human estrogen receptor alpha gene correlates with its enhanced expression: Implications for female bias in lupus

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ABSTRACT

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease primarily affecting women. Previous studies have indicated that sex hormone estrogens contribute to the female predilection of SLE. Estrogen regulates gene expression by translocating estrogen receptors (ER) α and β into the nucleus where they induce transcription by binding to estrogen response elements of target genes. We have previously observed that expression of ERα gene and protein in lupus patients is significantly higher than in healthy controls and that estradiol up-regulates calcineurin expression via over-expression of ERα gene in SLE. However, the pathogenesis of over-expression of ERα gene is unknown. Here we report that enhanced expression of ERα mRNA and protein in SLE and rheumatoid arthritis is associated with DNA demethylation within the proximal promoter region located between −232 and +81 base pair relative to transcription start site of human ERα gene (GenBank Accession no. AL356311.6). The frequency of DNA demethylation was comparable between male and female. These findings suggest that estrogen and demethylated ERα promoter associated up-regulated ERα genes are two critical factors in the gender biased development of autoimmune diseases besides genetic factor.

1. Introduction

Systemic lupus erythematosus (SLE) is a female predominant autoimmune disease characterized by autoantibody formation and immune complex deposition. A large mass of evidence has suggested that the preponderance of SLE in women is due in part to estrogen. Estrogen functions as a transcriptional regulator by activating estrogen receptors (ERs) ER alpha (ERα) and ER beta (ERβ) which then translocate to the nucleus, where they bind to estrogen response element of target gene promoters. Although both ERs are expressed in most immune cells, ERα is shown to be predominantly expressed (Erlandsson et al., 2001). Results from human observation indicate that estrogen enhances lupus development,

Abbreviations: SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; ERα, estrogen receptor alpha; PBMCs, peripheral blood mononuclear cells; PBLs, peripheral blood lymphocytes; SLEDAI, SLE disease activity index; TE buffer, Tris–EDTA buffer; gDNA, genomic DNA; TSS, transcriptional start site; 5-azaC, 5-aza-2′-deoxycytidine; SssI, CpG methylase; TSA, tricostatin; NE buffer, New England Biolabs buffer; MSP, methylation specific polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; IFN, interferon; ITGAL, integrin alpha L; PRF1, perforin 1; TNFSF, tumor necrosis factor superfamily.

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Table 1
Demographic and clinical characteristics of the nine SLE patients.

Patient no.	Gender	Age at onset (y)	ACR criteria	Disease duration (y)	Prednisolone dose (mg/day)	SLEDAI
1	F	27	Ar, Ph, DNA, ANA	7	0	2
2	F	28	Mr, Ou, Ar, ANA	5	5	4
3	F	43	Ph, Ar, Rd, DNA, ANA	4	5	4
4	F	59	Ar, Hd, DNA, ANA	9	5	6
5	F	17	Mr, Ou, Ph, Ar, DNA	16	10	11
6	F	16	Mr, Hd, DNA, ANA	25	0	5
7	F	24	Mr, Ou, Ar, Ph, Rd, ANA	18	0	1
8	F	15	Se, Hd, DNA, ANA	4	10	9
9	F	13	Mr, Ou, Rd, DNA, ANA	20	10	4

Mr, malar rash; Ph, photosensitivity; Ou, oral ulcer; Ar, arthritis; Se, serositis; Rd, renal disorder; Hd, hematologic disorder; DNA, anti-ds DNA antibody; ANA, anti-nuclear antibody. SLEDAI, systemic lupus erythematosus disease activity index.

but concrete evidence implicating ERα in promoting SLE is lacking. A recent murine study (Li and McMurray, 2007) using ER-selective agonists in NZB × NZW F1 female mice has suggested that activation of ERα but not ERβ promotes SLE. In contrast, ER-deficient NZB × NZW F1 and NZM2419 mice with disruption of ERα attenuate glomerulonephritis and increase survival (Svenson et al., 2008; Bynote et al., 2008). One of these studies (Bynote et al., 2008) further indicates that ERα promotes lupus by inducing interferon-gamma (INF-γ), an estrogen-regulated cytokine that impacts this disease. These data suggest that ERα plays a major role in mediating the effects of endogenous estrogens by promoting both loss of tolerance and the development of pathogenic autoantibodies. In a recent human study (Inui et al., 2007), expression of ERα, but not ERβ, is increased in peripheral blood mononuclear cells (PBMCs) from SLE patients compared with normal controls. The pathogenesis of enhanced expression and activation of ERα gene in SLE patients is unknown.

Epigenetic mechanisms including DNA methylation and histone modification are known to play key roles in transcriptional regulation. DNA methylation occurs at cytosines within CpG dinucleotides that are clustered frequently in regions of ~1–2 kb in length, called CpG islands, in or near promoter and first exon regions of genes (Esteller and Herman, 2002). In mammals, 60–70% of CpG sites are methylated in the genomic DNA (gDNA) (Boyes and Bird, 1992). CpG methylation can down-regulate gene expression by preventing the binding of transcription factors or by recruiting repressor molecules (Boyes and Bird, 1992; Ballestar and Wolffe, 2001). Accumulating evidence has indicated that increased methylation level of the CpG islands within the ERα promoter region is highly negatively associated with ERα expression in a variety of diseases including neoplastic and atherosclerotic lesions (Yoshida et al., 2000; Bird, 1992). On the contrary, demethylation of promoter C region in the ERα is in part responsible for the enhanced expression of ERα gene in long-term estrogen deprivation cells (Sogon et al., 2007). Hypomethylation in the promoter region of ERα gene in uterine leiomyoma may be associated with high ERα mRNA expression (Asada et al., 2008).

The present study is undertaken to investigate whether demethylation of CG pairs within the ERα promoter region is associated with enhanced ERα gene expression in peripheral blood lymphocytes (PBLs) of lupus patients.

2. Materials and methods

2.1. Human subjects and cell culture

Approval for this study was obtained from Kaohsiung Medical University Hospital Human Ethics Committee. Taiwanese patients who met the 1982 revised American College of Rheumatology Criteria for classification of SLE (Tan et al., 1982) were recruited randomly from outpatient and inpatient services. PBMCs were isolated by using ficoll-paque cushion centrifugation gradient. Cells were harvested and cultured in Petri dish for 2 h. Non-adherent cells (92–95% lymphocyte-rich cells; PBLs) were harvested and resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel) until use.

For study of specificity of demethylated ERα promoter in SLE, we collected a total of 44 DNA specimens from lupus patients (39 females and 5 males). Among them, 21 were collected from DNA bank of our laboratory and 14 (1 male and 13 females) aged between 8 and 24 years-old were obtained from pediatrics. Twenty-one healthy controls were selected from the volunteers aged between 23 and 37 years (mean 28.4 ± SD 5.1 years) without history of collagen vascular disease. In addition, we recruited 33 patients (26 females and 7 males) who met the American College of Rheumatology criteria for classification of rheumatoid arthritis (RA) (Arnett et al., 1988) as autoimmune disease controls, and 10 patients with osteoarthritis (OA) and 8 patients with gout from our outpatient service as non-autoimmune disease controls.

For study of correlation between demethylation of proximal promoter in ERα gene and expression of ERα mRNA and protein levels, 9 SLE patients and 11 healthy controls were recruited. Table 1 showed the demographics and clinical characteristics of these 9 SLE patients who were between 13 and 59 years of age (mean 29.1 ± SD 16.8 years). SLE status was assessed using the SLE Disease Activity Index (SLEDAI) (Costedoat-Chalumeau et al., 2006), and active SLE was defined as a SLEDAI score of ≥6. Most of them were in remission state with SLE disease activity index (SLEDAI) score between 1 and 11 (mean 5.1 ± SD 3.2). Three patients were not taking prednisolone. Three were receiving prednisolone 5 mg/day; one was receiving prednisolone 5 mg/day and cyclophosphamide 50 mg/day and the other three were receiving 10 mg/day. Eleven healthy controls were selected from the volunteers aged between 24 and 35 years (mean 27.7 ± SD 4.9 years) without history of collagen vascular disease.

2.2. DNA extraction

Cells were collected and suspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1.2% sodium dodecylsulfate, 150 mM NaCl, 0.2 mg/ml proteinase K), followed by incubation at 37 °C overnight. Cellular DNA was isolated by phenol extraction. The DNA samples were suspended in TE buffer and incubated with RNAs for 1 h at 37 °C. The DNA purity and quantity were assessed by spectrophotometric analysis and the amount was measured spectrophotometrically at 260 nm.

2.3. Methylation-specific PCR (MSP)

Bisulfite treatment of gDNA was done as described previously (Herman et al., 1996; Trinh et al., 2001). The gDNA 2–5 μg in 100 μl of water was denatured in 0.2 N NaOH at 50 °C for 10 min and