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Research paper

Gender differences of B cell signature related to estrogen-induced IFI44L/BAFF in systemic lupus erythematosus

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ABSTRACT

Systemic lupus erythematosus (SLE) possesses a gender-dependent incidence characterized by a male/female ratio 1:9. B-cell, a vital part of the immune system, plays an important role in pathogenesis of SLE. Thus, we hypothesize that gender differences of B cells may exist in SLE and relate to the onset and the progression of SLE. Here, we showed that the genes expression pattern is similar between healthy female and male. However, SLE female and SLE male showed more upregulated genes, in which the trendline of SLE male is higher than that of SLE female. The most differentially expressed genes between SLE male patients and female patients are only on two chromosomes. While the differentially expressed genes between healthy male and female are distributed on several chromosomes. There are more differentially expressed genes in SLE male vs healthy male than these in SLE female vs healthy female. OAS3, RGS13, STAG3, IFI44L, STS-1, FERIL14, ZBTB16, USP18, USP41, RSAD2, FKBP5, IL1R2, DNAPTP6 and ILI27, which top 14 significantly upregulated mRNAs in SLE patients compared with healthy donors, showed different expression pattern in gender-based analyses. Furthermore, we revealed that this difference may be related to estrogen-induced IFI44L/BAFF. Therefore, we conclude that the diagnosis and treatment of these immune-related diseases should consider the baseline gender-related differences.

1. Introduction

Gender differences are universal in health, disease and medical research [1]. Sex differences have been shown in the development of neural systems [2], cardiac structure and function [3,4], risk of cancer [5,6], renal disease [7], and mental disorder [8]. The researches also have demonstrated naturally occurring gender differences in immune response [9–12]. Women had higher levels of CD4+ and CD8+ T cell activation and inflammation-associated gene expression [13]. Lymphocyte subsets enumeration revealed higher B cells in females [14]. Females are protected against mortality arising from severe sepsis, which may be attributed to a fundamental sex difference in phenotype of resident leukocytes [15]. These data suggest that the gender-dependent differences may have a biological base and molecular mechanisms.

As is well known, systemic lupus erythematosus (SLE) is the prototypic systemic autoimmune disease. Several lines of evidence support a role for B cells in SLE pathogenesis [16,17]. Loss of B-cell tolerance and abnormal B-cell homeostasis are hallmark features of the pathogenesis in SLE patients [18]. The B-cell depletion with rituximab improves the clinical manifestations of SLE [19]. Furthermore, defective regulation of intracellular signaling in B lymphocytes could directly lead to lupus-like autoimmunity in mouse [20]. Importantly, SLE also possesses a gender-dependent incidence characterized by a male/female ratio 1:9. Thus, it is necessary to elucidate the gender differences of B cells in SLE for understanding the onset and the progression of SLE.

In the present study, gene expression signature of B cells in sex differences was undertaken. Bioinformatics analysis of the microarray results was performed. In addition, mouse B cells were obtained to verify whether the change of some gene expression is related to estrogen. Results showed that the gender differences were existent in global gene expression of B cells. This difference may be related to estrogen-induced IFI44L/BAFF. These findings suggest that the diagnosis and treatment of these immune-related diseases should consider the baseline gender-related differences.

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2. Materials and methods

2.1. Patients and healthy subjects

The study protocol was approved by the research ethics committee of Nanjing University. After obtaining written informed consent from each patient and healthy controls (HC), phlebotomy was carried out on SLE patients and HC. All SLE patients were diagnosed according to the criteria set out by American College of Rheumatology revised criteria in 1997 [21]. Disease activity was evaluated using the SLE Disease Activity Index (SLEDAI) with a cutoff of ≥8 was used to define active disease [22]. The SLE patients aged 30 ± 6 years. For the arrays, six healthy subjects (3 males and 3 females) from local blood bank (150 mL) with a mean (±SD) age of 27 ± 8 years were recruited. Six patients with SLE (3 male and 3 female), who were hospitalized at the clinical unit of the Affiliated Drum Tower Hospital of Nanjing University Medical School, were recruited for whole blood (150 mL). Although patients were on a variety of disease modifying agents, patients on high dose immunocytotoxic therapies or steroids were excluded from the study.

2.2. Sample acquisition and isolation of human B cells

Peripheral blood samples from study subjects were collected in heparinized tubes and immediately processed. Peripheral blood mononuclear cells (PBMCs) were isolated over Lymphocyte Separation Medium (LSM 1077) gradients for immediate use. Cells were kept at 4 °C or on ice and cold buffers were employed to minimize alterations in gene expression during labeling and isolation. The purified B cells were prepared from PBMCs, using negative selection by Pan B-cell Isolation Kit II from Miltenyi Biotec (Bergisch Gladbach, Germany). Immunofluorescence labeling for flow cytometry was performed by incubating isolated B cell with anti-CD19-PE antibodies (BD Biosciences, San Jose, CA) for 30 min and washed as described [23]. Flow cytometry was performed using a FACSCalibur (BD Biosciences) with CellQuest software. Analysis was conducted with FloJo software. The purification of B cells over 95% was used in the study.

2.3. RNA isolation and microarray

The purified B cells were centrifuged in RNase-free tubes treated with Trizol Reagent (Invitrogen, USA). Total RNA was extracted by using the RNeasy kit according to the instructions of the manufacture (Qiagen, Valencia, CA) and then RNA concentration was assessed by Nanodrop 2000 spectrophotometry (Thermo). RNA quality was determined by formaldehyde denaturation electrophoresis and only those samples with a 260 nm/280 nm optical density ratio (OD260/280) >1.8 and a total RNA concentration >1 mg/ml were submitted for hybridization to generate labeled targets.

Then the total RNA was used to synthesize double-strand cDNA (ds-cDNA) using an Invitrogen SuperScript ds-cDNA synthesis kit in the presence of oligo dT primers. The ds-cDNA was cleaned and labeled using NimbleGen One-Color DNA Labeling Kit in accordance with the manufacturer's protocol (NimbleGen Systems, Inc., Madison, WI). The labeled ds-cDNA was hybridized with NimbleGen 12 × 135 K Human Gene Expression Microarray consisting of 45,033 probes for human genes (Hybridization System-NimbleGen Sys-199 tems, Inc., Madison, WI). Following hybridization, washing was performed using the NimbleGen Wash Buffer kit (NimbleGen Systems, 201 Inc., Madison, WI). After being washed in an ozone-free environment, the processed slides were scanned with the Roche-NimbleGen MS200 confocal laser scanner (CapitalBio Corporation) using the recommended settings.

2.4. Analysis of microarray data

The acquired array images were analyzed with Roche Nimblegen Nimblescan V2.5. Quality normalization and subsequent data processing were performed with the Roche Nimblegen Nimblescan V2.5. Differentially expressed mRNAs were identified through Fold Change filtering and hierarchically clustered by the Roche Nimblegen Nimblescan V2.5.

2.5. Mice

Female C57BL/6 and the sex and age-matched MRL-Fas^{lpr}/J lupus-prone mice were obtained from Model Animal Research Center of Nanjing University (Nanjing, China) and were kept in specific pathogen-free (SPF) conditions. MRL-Fas^{lpr}/J lupus-prone mice are characterized by high levels of circulating autoantibodies, systemic vasculitis (inflammation of blood vessels), skin and renal lesions, and early death due to renal dysfunction, hypertension and spontaneous haemorrhage. As seen in people with SLE, these mice develop intense vasculitis in organs such as the kidneys, salivary glands, lungs and gastrointestinal tract, and arthritis in the MRL/faslpr strain [24]. All manipulations were approved by the Nanjing University Animal Care Commission.

2.6. B cell isolation

Spleen B cells from mice were obtained by a mouse CD45R (B220) beads according to the procedures of the manufacturer. The purity of B cells measured by PE conjugated CD19 antibody was >95%. Purified B cells were used for the identification of some gene expression levels.

2.7. Cell culture

A20 B cell lines were cultured in 48-well flat-bottom plates (Corning) were cultured in phenol red-free RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% charcoal-stripped FBS (Gibco, Grand Island, NY, USA), and antibiotics (penicillin 100 μg/ml, streptomycin 10 μg/ml; Invitrogen Life Sciences) at 37 °C in a humidified atmosphere of 5% CO_2. For stimulation treatment, B cells were stimulated with IFN-α (1000 U/ml, ebiosience, USA), Estrogen(10 nM, sigma, USA)or IFN-α (1000 U/ml) plus Estrogen(10 nM)respectively or in control medium.

2.8. Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from mice spleen B cells or from the A20 B cells. qRT-PCR was used to validate the selected data from the microarray experiments and to identify the expression levels of genes after stimulation with IFN-α or Estrogen. Total RNA was reverse-transcribed using the PrimeScript TM Reagent Kit (Takara) according to the manufacturer's instructions, with GAPDH serving as an endogenous control. The genes and their primer sequences used for the qRT-PCR assays are listed in Table 1. The cDNA samples were used for quantitative qRT-PCR analysis, which was performed using a 7300 qRT-PCR System (ABI) with a standard SYBR Green PCR protocol according to the manufacturer's instructions. The thermal cycling conditions included a first stage of 8 min at 95 °C, a second stage corresponding to the 40 cycles of the PCR (for each cycle 95 °C for 15 s and 65 °C for 1 min), and a third stage(rapid heating at 95 °C, cooling at 60 °C, and heating at 95 °C). Each sample was run in triplicate alongside the endogenous control to normalize reactions. After completion of the PCR amplification, the relative foldchange was calculated based on the $2^{-\Delta\Delta Ct}$ method.