Molecular Genetics and Metabolism 100 (2010) 88–95

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Genes regulated by Nkx2-3 in siRNA-mediated knockdown B cells: Implication of endothelin-1 in inflammatory bowel disease

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ARTICLE INFO

Article history:
Received 1 February 2010
Accepted 1 February 2010
Available online 6 February 2010

Keywords:
Nkx2-3
Gene regulation
EDN1
IBD
cDNA microarray

ABSTRACT

Nkx2-3 gene variants are strongly associated with inflammatory bowel disease (IBD) and its expression is up-regulated in Crohn's disease (CD). However, the nature of its role underlying IBD pathogenesis is unknown. We investigated the genes regulated by Nkx2-3 using cDNA microarray. A small interfering RNA (siRNA)-mediated knockdown of Nkx2-3 in a B cell line from a CD patient was generated. Gene expression was profiled on high-density cDNA microarrays representing over 25,000 genes. Ingenuity pathway analysis (IPA) was used to identify gene networks according to biological functions and associated pathways. Expression profiling analysis by cDNA microarray showed that 125 genes were regulated by Nkx2-3 knockdown (fold change $\geqslant 3.0$, $p < 0.01$), among which 51 genes were immune and inflammatory response genes. Microarray results were validated by RT-PCR and further confirmed in a B cell line expressing siRNA of Nkx2-3 from an additional CD patient. The results showed that Nkx2-3 was up-regulated ($p < 0.05$) and EDN1 was down-regulated ($p < 0.05$) in B cell lines from CD patients. mRNA expression levels of Nkx2-3 were negatively correlated with those of EDN1 ($r = -0.6044$, $p < 0.05$). EDN1 was also down-regulated in intestinal tissues from UC patients ($p < 0.05$). Our present results demonstrate that a decrease in Nkx2-3 gene expression level can profoundly alter the expression of genes and cellular functions relevant to the pathogenesis and progression of IBD, such as EDN1.

Introduction

IBD consists of two major forms, Crohn's disease (CD) and ulcerative colitis (UC). Genetics plays an important role in the development of inflammatory bowel disease (IBD) [1]. Nkx2-3 has recently been strongly associated with IBD by genome-wide association studies (GWAs) [2,3]. The functional role of Nkx2-3 in IBD pathogenesis is currently unknown. Recently, we observed that the expression of Nkx2-3 is up-regulated in intestinal tissues and B cells from CD patients [4].

Nkx2-3 is a member of the Nkx family of homeodomain transcription factors. Homeobox transcription factors regulate cell-specific expression of genes involved in tissue differentiation and development [5,6]. Nkx2-3 is principally expressed in subsets of cells of mesodermal origin in the gastrointestinal tract and spleen [7,8]. It also has an important role in spleen and Peyer patch development, affecting B cell maturation and T cell-dependent immune responses [9,10]. Within the gastrointestinal tract, Nkx2-3 is expressed in microvascular endothelial and smooth muscle cells of the lamina propria and submucosa.

Evidence indicates that one of the Nkx family members, Nkx2-5, regulates heart development by regulating over 20 genes, such as Cx40, Cx43, Dio2, and Rae28 [11–13]. Nkx2-3, similarly to Nkx2-5 in the heart, may significantly influence gene expression within the intestine, and thereby play an important role in IBD pathogenesis, Nkx2-3-deficient mice have shown that Nkx2-3 is required for expression of MAdCAM-1, an endothelial cell adhesion molecule, which is critical for lymphocyte homing to the gut [9,10]. In this context, Nkx2-3 likely influences migration of antigen-responsive lymphocytes to the gut and thus affects the intestinal inflammatory response. Expression of MAdCAM-1 has been shown to be dramatically up-regulated within focal sites of intestinal inflammation in both animal models of IBD [14] and in human tissue samples from patients with CD and UC [15].

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doi:10.1016/j.ymgme.2010.02.001

Since transcription factors can regulate downstream genes, we performed a gene expression microarray analysis on an Nkx2-3 siRNA knockdown B cell line from a CD patient. Using this technique, we have identified genes differentially expressed in Nkx2-3 knockdown cells as compared to control cells, many of which are inflammation-related. Our further study on EDN1 (endothelin-1) regulation in IBD patients suggests possible pathways by which Nkx2-3 may contribute to the pathogenesis of human IBD.

Materials and methods

Cell lines

B cell lines were established by infection with Epstein–Barr virus (EBV) [4]. CD-1 is a B cell line from a CD patient. This patient is a 50 year old woman with terminal ileum Crohn's disease first diagnosed at age 18. She underwent an ileocecetomy for fibrous stricture of the ileum after recent repetitive bouts of disease flares (Montreal Classification: A2, L1, B2). CD-2 is a B cell line from a 27 years old female CD patient. All B cell lines used in this study were obtained from an established Central Pennsylvania IBD patient registry. Cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in an atmosphere of 5% CO_2.

Patients and intestinal tissue samples

Intestinal tissues were obtained from UC patients undergoing surgery at the Penn State Hershey Medical Center. An informed consent was signed by the patients before surgery. Macroscopically normal areas of intestine and areas of intestine with obvious disease were classified by a pathologist. The intestinal tissues were immediately submerged in RNA*later* Solution (Ambon, CA, USA) and stored at 4 °C overnight. Tissues were then stored frozen at −70 °C until total RNA extraction.

Establishment of a stable Nkx2-3 siRNA cell line

Small hairpin RNA (shRNA) vector targeting human Nkx2-3 was generated using the pSUPER vector system as described previously [16]. Briefly, oligonucleotide pairs encoding shRNA against human Nkx2-3 were designed according to established guidelines using OligoEngine software. The 19-nucleotide sequence within Nkx2-3 targeted by the shRNA oligonucleotide pair was 5′-AGGAACATGAAGAGGAGCC-3′. Forward and reverse primers were synthesized containing this sequence in sense and antisense orientations with an intervening linker. Primer pairs were designed to generate single-strand overhangs upon annealing that would allow the annealed duplex oligonucleotide to be cloned into BglII and HindIII sites in the pSUPER.retro.puro vector. Forward and reverse primers were annealed and ligated into the pSUPER.retro.puro vector according the manufacturer's instruction (OligoEngine, WA, USA).

Oligonucleotide pairs encoding the siRNA for human Nkx2-3 ligated into the pSUPER.retro.puro vector were transfected into Phoenix-Ampho cells with Lipofectamine Plus (Invitrogen, CA, USA). Forty-eight hours posttransfection, CD-1 and CD-2 cells were infected with the viral supernatant. Forty-eight hours later, the infected cells were switched into a medium containing 0.4 μg/ml puromycin to start selection. To confirm the suppression of expression of Nkx2-3, mRNA and cell lysates from individual clones of infected cells were isolated for further examination.

Microarray and data analysis

Illumina Human HT12 v 3 Expression BeadChips (Illumina, CA) were used in this study. This BeadChip targets >25,000 genes with >48,000 probes derived from the RefSeq (Build 36.2, rel22) and UniGene (Build 99) databases. Total RNA (300 ng) collected from two independent cultures of parental and shNkx2-3-expressing CD-1 cells was reverse transcribed into cRNA and biotin-UTP labeled using the Illumina Total Prep RNA Amplification Kit (Ambion, Austin, TX). cRNA was quantified using a Nanodrop spectrophotometer and the cRNA quality (size distribution) was further analyzed on a 1% agarose gel. Microarray hybridization, data collection, and analysis were performed at the Genomics Core of the Cleveland Clinic Lerner Research Institute. Gene expression profiles were compared between parental and shNkx2-3-expressing samples. The parental samples were used as the baseline. cRNA was hybridized to the BeadChip using standard Illumina protocols.

Raw data filtering and quantile normalization were performed using the Bioconductor package *lumi*, a Beadarray specific software package for Illumina microarray data. Due to the small sample size, the moderated *t*-statistic implemented in the Bioconductor *LIMMA* package was used to detect differentially expressed genes. This statistic has the same interpretation as the standard *t*-statistic; however, standard errors were calculated to shrink toward a common value by empirical Bayes model to borrow information across all genes [17]. The *p*-values from moderated *t*-tests were adjusted by Benjamini and Hochberg's method to control false discovery rate.

RNA isolation and reverse transcription PCR

Total RNA was extracted from B cell lines and intestinal tissues using the RNeasy mini Kit (QIAGEN Sciences, MD, USA) according to the manufacturer's instructions. cDNA was synthesized from 1.5 μg of total RNA using a Superscript III 1st Strand Synthesis Kit (Invitrogen, CA, USA).

Primer sequences are listed in Supplementary Table 1. Primers were designed using Primer3 software. PCR amplifications were performed at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 45 s for 35 cycles (all genes except GAPDH) or 28 cycles (GAPDH), followed by a final extension at 72 °C for 5 min. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

RT-PCR product bands were scanned by densitometry and results were normalized by GAPDH for each cell line. Fold change results of normalized Nkx2-3 or EDN1 were summarized as a ratio of medians (CD:non-IBD sibling) for each sibling pair.

Quantitative real-time PCR (qPCR) for EDN1

mRNA expression of EDN1 was measured in comparison to GAPDH using primers GAPDH-F 5′-GAAGGTGAAGGTCGGAGTC-3′, GAPDH-R 5′-GAAGATGGTGATGGGATTTC-3′, EDN1-F 5′-CCAAGGAGCTCCAGAAACAG-3′, EDN1-R: 5′-AGTTCTTTTCCTGCTTGGCA-3′. Assays were performed in triplicate with a QuantiTect SYBR Green PCR Kit (Qiagen Inc.) in an ABI 7900HT sequence detection system (Applied Biosystems) at the Penn State Hershey Functional Genomics Core Facility.

Western blots

Western blotting was performed as described previously [4]. Briefly, cells were lysed with RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Roche, Applied Science). Protein (30 μg/lane) was separated using SDS–15% PAGE and transferred onto nitrocellulose membranes. The membranes were