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Hypermethylation of *EBF3* and *IRX1* Genes in Synovial Fibroblasts of Patients with Rheumatoid Arthritis

Sung-Hoon Park[1], Seong-Kyu Kim[1], Jung-Yoon Choe[1], Youngho Moon[2], Sungwhan An[2], Mae Ja Park, and Dong Sun Kim*

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease of unknown origin, which exhibits a complex heterogeneity in its pathophysiological background, resulting in differential responses to a range of therapies and poor long-term prognosis. RA synovial fibroblasts (RASFs) are key player cells in RA pathogenesis. Identification of DNA methylation biomarkers is a field that provides potential for improving the process of diagnosis and prognosis of various human diseases. We utilized a genome-wide technique, methylated DNA isolation assay (MeDIA), in combination with a high resolution CpG microarray for discovery of novel hypermethylated genes in RASFs. Thirteen genes (*APEX1, EBF3, EGR2, EN1, IRX1, IRX6, KIF12, LHX2, MIPOL1, SGTA, SIN3A, TOLLIP,* and *ZHX2*) with three consecutive hypermethylated probes were isolated as candidate genes through two CpG microarrays. Pyrosequencing assay was performed to validate the methylation status of TGF-β signaling components, *EBF3* and *IRX1* genes in RASFs and osteoarthritis (OA) SFs. Hypermethylation at CpG sites in the *EBF3* and *IRX1* genes was observed with a high methylation index (MI) in RASFs (52.5% and 41.4%, respectively), while a lower MI was observed in OASFs and healthy SFs (13.2% for *EBF3* and 4.3% for *IRX1*). In addition, RT-PCR analysis showed a remarkable decrease in their mRNA expression in the RA group, compared with the OA or healthy control, and their reduction levels correlated with MI. The current findings suggest that methylation-associated down-regulation of *EBF3* and *IRX1* genes may play an important role in a pathogenic effect of TGF-β on RASFs. However, further clinical validation with large numbers of patients is needed in order to confirm our findings.

INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic inflammatory and multi-factorial disorder affecting 1% of the adult population worldwide; it cannot be cured and its personal, social, and economic costs are substantial (Firestein et al., 2008). It has a complex heterogeneity in its pathophysiological background, resulting in differential responses to a range of therapies and poor long-term prognosis (Firestein et al., 2008), suggesting that a sub-classification of RA using molecular biomarkers could be helpful in prediction of clinical outcomes of therapies. Of particular interest, there is evidence pointing to geographical difference in occurrence or outcome of RA (Carmona et al., 2010). Although the etiology of RA is not yet fully understood, environmental and genetic factors play a role in pathogenesis and they can be responsible for different clinical pictures and response to therapy (Molnar-Kimber and Kimber, 2012). RA synovial fibroblasts (RASFs) (also known as FLSs, fibroblast-like synoviocytes) exhibit abnormal behavior in RA and are thought to be the key effector cells responsible for synovial inflammation, contributing to destruction of bone and cartilage (Huber et al., 2006; Neumann et al., 2010). RASFs are also involved in production of inflammatory cytokines, molecular mediators of inflammation, and matrix-degrading enzymes.

Significant progress has been made in identification of more than 30 genes associated with RA (Bax et al., 2011), however, the precise molecular mechanisms underlying pathogenesis of RA remain unclear. Transcriptional deregulation in RASFs lacking specific genetic mutations suggests that epigenetic mechanisms may be involved. Global hypomethylation and promoter-specific hypermethylation are believed to be associated with conditions such as cancer, atherosclerosis, and autoimmune diseases (Rakyan et al., 2011). Considerable evidence of epigenetic changes, particularly altered patterns of DNA methylation, in RA has been reported (Ballestar 2011; Trenkmann et al., 2010). Of particular importance, changes in DNA methylation, leading to changes in gene expression in RASFs, shaping the aggressive phenotype of these cells has been reported (Karouzakis et al., 2009; Ospelt et al., 2011). Most recent studies have focused on hypomethylation of inflammatory cytokine genes affecting gene regulation in RA (Fu et al., 2011; Hashimoto et al., 2009; Lin et al., 2012; Nile et al., 2008). However, our under-

Department of Anatomy, School of Medicine, Kyungpook National University, Daegu 700-422, Korea, [1]Department of Internal Medicine and Arthritis and Autoimmunity Research Center, School of Medicine, Catholic University of Daegu, Daegu 705-718, Korea, [2]Genomictree Inc., Daejeon 305-510, Korea
*Correspondence: doskim@knu.ac.kr

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standing of promoter hypermethylation at multiple loci is limited to the few genes analyzed to date. Herein, in order to improve our understanding of the molecular complexity of RA and to identify epigenetic biomarkers for diagnosis, patient stratification and prognostication, we investigated a whole-genome methylation in SFs of Korean RA patients using methylated DNA isolation assay (MeDIA)-mediated CpG microarray analysis and the methylation status of two candidate methylation genes was evaluated in RASFs, SFs of osteoarthritis (OA) patients, and healthy controls (HC).

MATERIALS AND METHODS

Tissue specimens and genomic DNA isolation

Synovial tissue was obtained from seven patients with RA, four patients with OA, and two patients with joint trauma (healthy control specimens) undergoing joint replacement surgery at Daegu Catholic University Hospital. Informed consent was obtained from all patients, and RA patients fulfilled the American Rheumatism Association criteria for classification of the disease (Arnett et al., 1988). For cell culture, tissue was digested and SFs were grown as previously described (Stanczyk et al., 2008). Patients with other underlying disease that might affect the general condition of patients were excluded from the study. Mean age of patients in the RA group was 58.00 ± 6.66 years (yrs) and did not differ significantly from that of the OA group (62.00 ± 5.48 yrs) and the healthy control group (58.50 ± 2.12 yrs). A review of the baseline characteristics of recruited patients is shown in Table 1. Genomic DNA was extracted from cultured SFs using the QIAamp DNA kit and blood kit protocol (Qiagen, Germany). DNA concentration and purity was determined by measuring absorbance at 260 nm and 280 nm using spectrophotometer.

MeDIA-mediated CpG microarray analysis

MeDIA was performed as previously described (Moon et al., 2009) and all procedures are shown in Fig. 1. A minimized methyl-DNA binding domain (MBD) tagged by histidine (MBD2bt) of human MBD2b, which provides high binding sensitivity to methylated DNA was used. In brief, 2 μg of MBD2bt protein was incubated for 8 h at 4°C on a rocking platform with 500 ng of sonicated genomic DNA from RASFs and OASFs. The enriched methylated DNA was amplified using a whole genome amplification kit (Sigma, USA) as recommended by the manufacturer. The amplified products from RASFs and OASFs were labeled with Cy5-dUTP and Cy3-dUTP, respectively, using the BioPrime® Total for the Agilent® aCGH system (Invitrogen, USA). Next, the dye-labeled DNA samples were mixed and hybridized to the human 244K CpG island (CGI) microarrays (Agilent, USA) using conditions specified by the manufacturer. Two independent CpG microarray analyses were performed for RA and OA SFs. Preprocessing of raw data and normalization steps were performed using Gene-Spring 7.3.1 software. The signal intensity of the dye-labeled DNA samples was interpreted using a high resolution microarray scanner (Agilent). For selection of target genes, when comparing RA and OA SFs, it was considered methylation-positive when at least three adjacent probes allowing a one probe gap within the CpG islands (CGIs) in the promoter regions scored a fold-difference factor of ≥ 5.0.

Bisulfite treatment and pyrosequencing (PS)

The PS method was used for quantitative analysis of the methylation status of individual CpG motifs in *EBF3* and *IRX1* genes (Tost and Gut, 2007). Following treatment of genomic DNA with bisulfite using an EZ-DNA methylation Gold Kit (Zymo Research, USA), according to the manufacturer's instructions, it was amplified through performance of PCR using a forward primer and a reverse primer, enabling conversion of the PCR product to a single-stranded DNA template suitable for PS. All samples were heated to 95°C for 5 min, and then amplified for 45 cycles of 95°C (45 s), 58°C (45 s), and 72°C (45 s), followed by a final extension at 72°C (5 min). The quality of the PCR products was established on 2% agarose gel with ethidium bromide staining. After purification of PCR product using Se-pharose beads on a PyroMark Vacuum Prep Workstation (Qia-gen), PS was performed according to the manufacturer's specifications with a sequencing primer using the PyroMark Q96MD System (Qiagen). A summary of the forward, reverse, and sequencing primers used in this analysis is shown in Table 2. A mean methylation index (MI) was calculated from the mean of the methylation percentage for all observed CpG sites. To set the controls for PS, we used CpGenome™ Universal methylated and unmethylated DNA (Chemicon, USA) that were consistently positive or negative with stable levels of methylation. We measured each gene in triplicate and used the average in the statistical analyses.

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from six clinical samples using TRIzol (Invitrogen) according to the manufacturer's instructions. Residual genomic DNA was digested with RNase-free DNase (Invitrogen). First strand cDNA was reverse-transcribed from 2 μg of total RNA in a total volume of 20 μl using oligo (dT) and a SuperScript pre-amplification kit (Invitrogen). The resulting cDNA was amplified by AmpliTaq Gold (PE Applied Biosystems, USA) using gene-specific primers whose sequences are described in Table 2. Amplified products were separated on 2% agarose gels, visualized using ethidium bromide, and photographed.

Statistical analysis

SPSS for windows 18.0 (SPSS, USA) was used in performance of statistical analyses. The Linear Mixed Model test was used for comparison of intergroup differences. The chi-squared (χ^2) test was applied for identification of the correlation between the methylation index and clinical parameters. Statistical significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Genome-wide screening and identification of candidate hypermethylation targets

Two independent CpG microarray analyses were performed for RASFs and OASFs (Fig. 1A). A total of 1,714 probes were detected as hypermethylated targets with more than five-fold differences in signal intensity (represented as red color in Fig. 1B). Abundance of hypomethylated probes was much greater than that of hypermethylated probes, supporting the working hypothesis that a hypomethylating milieu in growth factors and receptors, inflammatory cytokines, matrix-degrading enzymes, and adhesion molecules, contributes to the chronicity of RA (Karouzakis et al., 2009). Moreover, promoter hypomethylation and upregulation of these genes have extensively studied in RA (Fu et al., 2011; Hashimoto et al., 2009; Lin et al., 2012; Nile et al., 2008), but our understanding of CGI hypermethylation at multiple loci is limited to the few genes analyzed to date.

Table 1. Baseline characteristics of study population

Parameters, mean (SD)	RA (n = 7)	OA (n = 4)	Healthy control (n = 2)
M/F	1/6	1/3	1/1
Age, yrs	58.00 (6.66)	62.00 (5.48)	58.50 (2.12)
RF, IU/ml	138.47 (124.0)	NA	NA
Anti-CCP, U/ml	188.39 (172.63)	NA	NA
CRP, mg/l	28.33 (21.39)	NA	NA
ESR, mm	38.43 (12.96)	NA	NA
DAS28 score	4.03 (1.04)	NA	NA
Mean disease duration, years	7.61 (4.36)	NA	NA

RA, Rheumatoid arthritis; OA, Osteoarthritis

We have therefore focused on the hypermethylated candidate targets. Thirteen genes (*APEX1, EBF3, EGR2, EN1, IRX1, IRX6, KIF12, LHX2, MIPOL1, SGTA, SIN3A, TOLLIP,* and *ZHX2*) with three consecutive hypermethylated probes were finally selected as candidate genes (Table 3). The signal intensity of the dye-labeled DNA samples at individual CpG motifs was interpreted using a high-resolution microarray scanner and visualized as a graph in Fig. 2. The methylation difference of the individual CpG motif was directly compared by ratio of signals from MeDIA-enriched DNA of RASF and OASFs. The stability of methylation patterns and its direct link to disease phenotype indicates that DNA methylation may prove to be a superior, promising biomarker for many applications (Tost, 2010). Therefore, our identification of aberrantly methylated genes can improve the clinical management of RA by facilitating earlier diagnosis of disease and providing more accurate prognostic information.

Clinical validation of DNA hypermethylation and mRNA expression of *EBF3* and *IRX1* genes in RASFs

The methylation status of *EBF3* and *IRX1* genes was quantitatively determined in seven RASFs and four OASFs through performance of PS analysis. Accurate and reproducible estimates of methylated cytosine content were obtained in 100% tested samples and their pyrograms are shown in Fig. 3. We measured each gene in triplicate and used the average in the statistical analyses. In the case of the *EBF3* gene, the MI (51.14%) of the RA group was significantly higher, compared with the OA (15.19%) or HC group (8.28%) ($p < 0.0001$), whereas no significant difference was observed between the OA and HC group ($p = 0.11$) (Fig. 4A). The MI of the *IRX1* gene was also significantly higher in the RA group (40.77%) than in the OA (4.64%) or HC group (4.05%) ($p < 0.0001$, Fig. 4A). However, hypermethylation of the *EBF3* and *IRX3* genes did not show correlation with clinicopathologic features, including age, disease duration, and DAS28 score of RA patients (data not shown), which may be due to the small size of the sample. Next, we examined the expression levels of *EBF3* and *IRX1* mRNA in six clinical samples. RT-PCR analysis showed a remarkable decrease of their mRNA expression in the RA1 and RA2 samples with higher MI, compared to the OA or HC samples with unmethylated alleles, but no noticeable reduction was found in RA3 sample with lower MI (Fig. 4B), suggesting that hypermethylation of the *EBF3* and *IRX1* genes may be closely associated with their transcriptional silencing in RASF. However, conduct of further studies to confirm the methylation and transcriptional silencing of the *IRX1* and *EBF3* genes in large numbers of samples is warranted.

Homeobox gene *IRX1* is a member of the Iroquois family of genes, involved in patterning and regionalization of embryonic tissues in both vertebrates and invertebrates (Cavodeassi et al., 2001). *IRX1* has also been identified as a tumor suppressor gene to be hypermethylated in several types of cancer (Bennett et al., 2008; Guo et al., 2010). *EBF*3 belongs to the EBF/Olf/Collier family of transcription factors, with specific roles in differ-

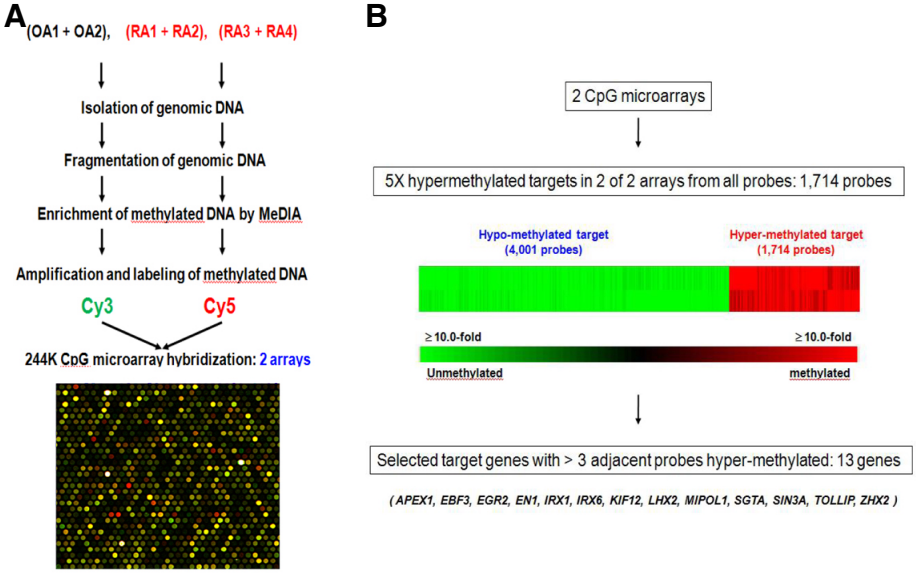

Fig. 1. Scheme of genome-wide screening in RASFs using MeDIA-mediated CpG microarray analysis. (A) Two pooled DNA samples from either two RASFs or two OASFs were amplified, and labeled with Cy5-dUTP and Cy3-dUTP, respectively. The dye-labeled DNA samples were mixed and hybridized to the human 244K CpG island (CGI) microarrays. Two independent chip microarrays (OA1 and OA2 *versus* RA1 and RA2, OA1 and OA2 *versus* RA3 and RA4) were performed. A Cy3-dUTP labeled OA DNA was represented as a green color, while Cy5-dUTP labeled RA DNA as a red color. (B) CpG microarray of genomic DNA in SFs. When comparing RASFs and OASFs, it was considered methylation-positive when at least three adjacent probes allowing a probe gap within the CGIs in the promoter regions scored a fold-difference factor of ≥5.0.

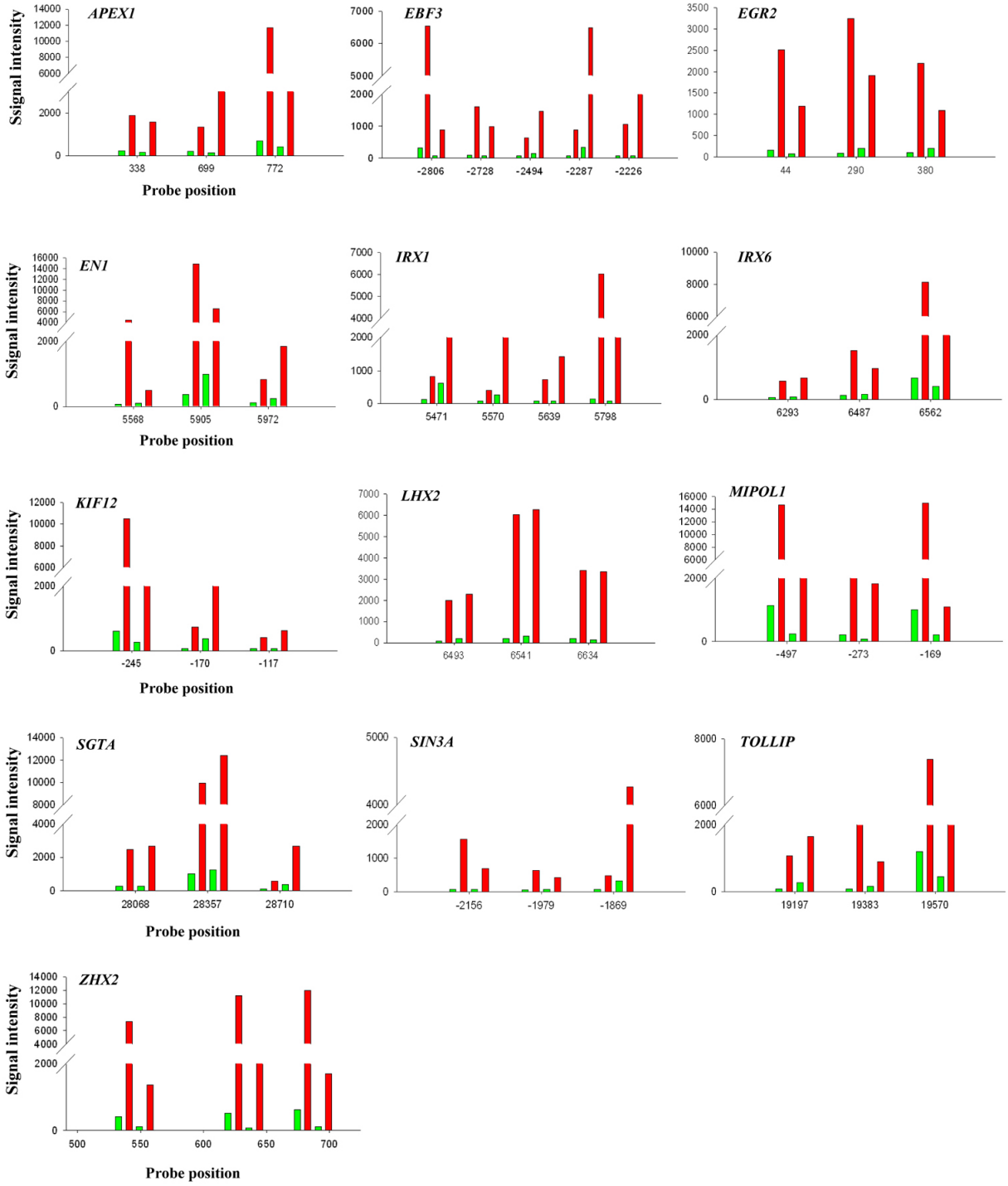

Fig. 2. Signal intensity of 13 hypermethylated genes in RASFs and OASFs. The signal intensity of the dye-labeled DNA samples at each of three adjacent probes was interpreted using a high resolution microarray scanner. Intensities of DNA of OASFs and RASFs were represented in green and red color, respectively. Numbers on X- indicate the relative probe positions from the transcription start site (TSS, +1).

entiation and maturation of several cell lineages, including B-progenitor lymphoblasts, neuronal precursors, and osteoblast progenitors (Liberg et al., 2002). In addition, aberrant DNA methylation and tumor suppressive activity of the *EBF3* gene have been observed in various cancers (Liao, 2009). Of particular interest, the most prominent feature of RA is the progressive destruction of articular cartilage and bone, which is orchestrated by activated RASFs. These aggressive cells share many char-

Table 2. Primer set for Pyrosequencing and RT-PCR analysis

Gene	Primer	Sequence (5′→ 3′)	Amplicon size (bp)
Pyrosequencing			
EBF3	Forward	GGTTGGATATTTTAGGAAGTGTGT	259
	Reverse	TTCCACTCTCRACAAACACTAAT	
	Sequencing	TGATAATAATGTTTGTTTGG	
IRX1	Forward primer	AATAYGGGAAGAAATTGGAAGGTT	142
	Reverse primer	AACCAAAACCACCTCCTCTTTCTC	
	Sequencing	AATTTTATAGATGGGTTAGT	
RT-PCR			
EBF3	Forward	CAGATATCGTGCCTCTTAGTGC	403
	Reverse	TCTCAGTGCGTCCATGTGAGC	
IRX1	Forward	GCTCTTCGGCAGCGACAC	260
	Reverse	GCTCTGGGGCCTCCTTTG	

bp, base pairs; R = A + G; Y = C + T

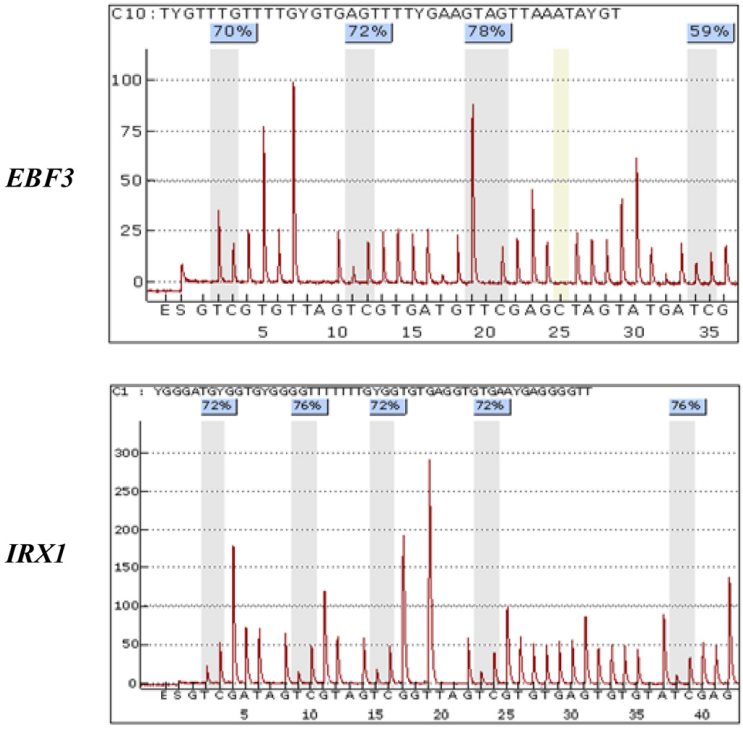

Fig. 3. Pyrograms of *EBF3* and *IRX1* genes in RASFs. The methylation status of the two candidate genes was determined quantitatively on the PyroMark Q96MD System using the PS assay. The letters on the axis represent the order of dispensation; E, enzyme mix; S, substrate; A, G, C, and T, nucleotides. Shaded bars encompassing T/C (T, conversion of unmethylated C to U; C, non-conversion of methylated C) pairs indicate individual interrogated CpG motifs. The methylation of each CpG site was calculated as a percentage of C incorporation.

acteristics with tumor cells, with up-regulated expression of proto-oncogenes and pro-migratory adhesion molecules, increased production of pro-inflammatory cytokines and matrix-degrading enzymes (Huber et al., 2006), and increased resistance to apoptosis (Baier et al., 2003). In addition, RASFs can undergo a process resembling epithelial-mesenchymal transition (EMT), a phenomenon that occurs during carcinogenesis (Steenvoorden et al., 2006). Notably, IRX1 and EBF3 are downstream targets of SMAD2 and SMAD3, components of the transforming growth factor-β (TGF-β) pathway, respectively (Amir et al., 2006; Ferguson et al., 2001). Indeed, TGF-β and its receptor are up-regulated in RASFs, compared to OASFs, resulting in significantly enhanced expression of matrix-degrading enzymes and inflammatory mediators (Pohlers et al., 2007; Rosengren et al., 2010). TGF-β is also important for induction of fibrosis associated with RA (Pohlers et al., 2009). Taken together, it is reasonable to hypothesize that hypermethylation of tumor suppressor genes *IRX1* and *EBF3* by TGF-β signaling could lead to uncontrolled proliferation of SFs, apoptosis resistance, and gain of mesenchymal characteristics during RA progression. Therefore, further study will be required in order to clarify this issue.

In summary, although the current investigation was limited by a small number of samples and a virtual lack of direct interactions at molecular levels, results of this study have demonstrated that the CGIs in the *EBF3* and *IRX1* genes are specifi-

Table 3. Genes list to show differential methylation in RASFs and their function

Gene symbol	GenBank	UniGene	Product	Description
APEX1	NM_001641	Hs.73722	APEX nuclease 1	Apurinic/apyrimidinic endonuclease 1
EBF3	NM_001005463	Hs.591374	Early B-cell factor 3	DNA-binding transcription factor COE3
EGR2	NM_000399	Hs.1395	Early growth response 2	E3 SUMO-protein ligase, Zinc finger protein Krox-20
EN1	NM_001426	Hs.271977	Engrailed homeobox 1	Homeobox protein engrailed-1
IRX1	NM_024337	Hs.424156	Iroquoishomeobox 1	Iroquois homeobox protein 1
IRX6	NM_024335	Hs.369907	Iroquoishomeobox 6	Iroquois homeobox protein 6
KIF12	NM_138424	Hs.28149	Kinesin family member 12	Kinesin-like protein 12
LHX2	NM_004789	Hs.445265	LIM homeobox 2	LIM homeobox protein 2
MIPOL1	NM_138731	Hs.660396	Mirror-image polydactyly 1	Mirror-image polydactyly gene 1 protein
SGTA	NM_003021	Hs.203910	Small glutamine-rich tetratricopeptide repeat (TPR)-containing, alpha	Small glutamine-rich TPR-containing protein α
SIN3A	NM_015477	Hs.513039	SIN3 transcriptional regulator homolog A	Histone deacetylase complex subunit Sin3 homolog A
TOLLIP	NM_019009	Hs.368527	Toll interacting protein	Toll-interacting protein, adapter protein
ZHX2	NM_014943	Hs.377090	Zinc fingers and homeobox 2	Zinc fingers and homeobox protein 2, α-fetoprotein regulator 1

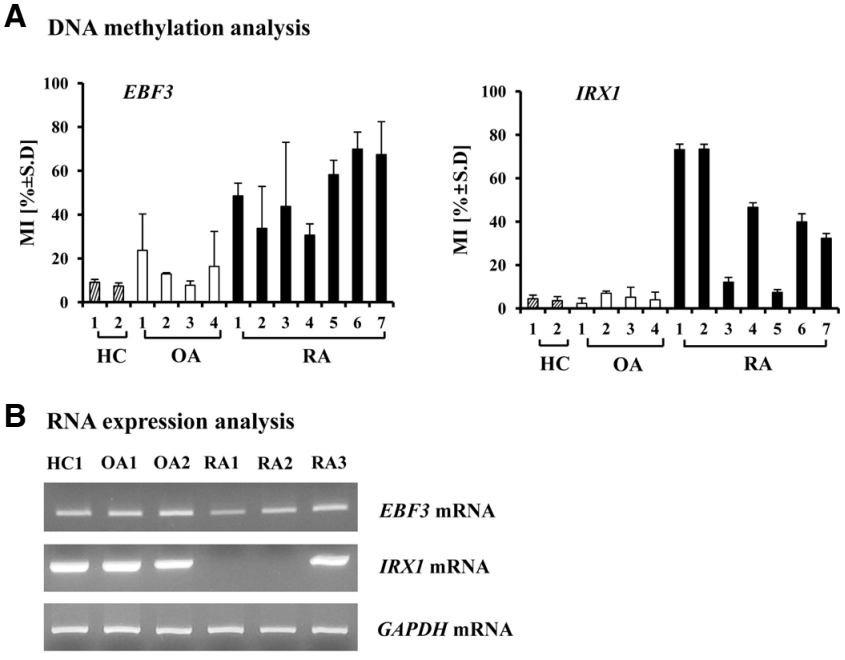

Fig. 4. Methylation index and mRNA expression of *EBF3* and *IRX1* genes in healthy control SFs, OASFs and RASFs. (A) The methylation status of *EBF3* and *IRX1* genes was determined on genomic DNA obtained from six clinical samples by PS. We measured each gene in triplicate and used the average in the statistical analyses. MI, methylation index; HC, healthy control; OA, osteoarthritis; RA, rheumatoid arthritis. (B) Expression of *EBF3* and *IRX1* mRNAs was performed by RT-PCR on total RNA obtained from some representative samples. Amplification of GAPDH was used as an internal loading control.

cally methylated to down-modulate their mRNA expression in rheumatoid synovial fibroblasts, which may shape the activated phenotype of these cells in RA pathogenesis.

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