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Review

Complementary Participation of Genetics and Epigenetics in Development of NSAID-exacerbated Respiratory Disease

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ABSTRACT

Nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (NERD) has attracted a great deal of attention because of its association with severe asthma. However, it remains widely underdiagnosed in asthmatics as well as the general population. Upon pharmacological inhibition of cyclooxygenase 1 by NSAIDs, production of anti-inflammatory prostaglandin E2 and lipoxins ceases, while release of proinflammatory cysteinyl leukotrienes increases. To determine the underlying mechanisms, many studies have attempted to elucidate the genetic variants, such as single nucleotide polymorphisms, responsible for alterations of prostaglandins and leukotrienes, but the results of these genetic studies could not explain the whole genetic pathogenesis of NERD. Accordingly, the field of epigenetics has been introduced as an additional contributor to genomic alteration underlying the development of NERD. Recently, changes in CpG methylation, as one of the epigenetic components, have been identified in target tissues of NERD. This review discusses *in silico* analyses of both genetic and epigenetic components to gain a better understanding of their complementary roles in the development of NERD. Although the molecular mechanisms underlying NERD pathogenesis remain poorly understood, genetic and epigenetic variations play significant roles. Our results enhance the understanding of the genetic and epigenetic mechanisms involved in the development of NERD and suggest new approaches toward better diagnosis and management.

Keywords: NSAID hypersensitivity; asthma; genetics; epigenetics

INTRODUCTION

Oral aspirin challenge is the best method of diagnosing nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (NERD), but this is a time-consuming procedure that results in serious complications in some cases. Therefore, the development of noninvasive biomarkers for easy diagnosis has been attempted to confirm the diagnosis of NERD and prevent unexpected complications of nonsteroidal anti-inflammatory drugs (NSAIDs)/aspirin use in susceptible patients. Using several genetic approaches, including

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There are no financial or other issues that might lead to conflict of interest.

biologically plausible candidate genes and genome-wide association studies (GWAS), more than 100 genetic variants have been identified in association with NERD. Among them, the best mechanistic evidence supports intrinsic dysregulation of the leukotriene (LT)/prostaglandin (PG) pathway, leading to increased recruitment of eosinophils and immune effector cells into the target tissues. These effects are mainly mediated by single nucleotide polymorphisms (SNPs) of the genes that regulate mRNA and protein expression responsible for PG and LT metabolism, i.e., *LTC4S*,[1,2] *ALOX5*,[3,4] *CYSLTR1*,[5-7] *CYSLTR2*,[5,6] *TBX*,[6,8] *EP2*,[9] and *COX2*[10,11] (**Table 1**).

Table 1. NERD-associated single nucleotide polymorphisms in the genes for cysteinyl LTRs, PG, and thromboxane synthesis and their receptors

Gene	Locus	rsnumber	Genotype	Race	No. of study subjects (AIA/ATA)	MAF (AIA/ATA)	*P* value	OR (95% CI)	AF	Reference (PMID)
LTC4S	Promoter	rs730012	−444 A>C	Polish	76/110	0.39/0.27	0.01	2.61 (1.38–4.98)	0.50 (0.35–0.66)	10970818
				Japanese	60/100	0.19/0.11	0.042	1.88 (0.91–3.87)	0.26 (0.14–0.42)	12063521
				American	51/33	0.27/0.33	Negative	-		10887308
				Korean	93/181	0.14/0.18	Negative	-		14749922
ALOX5	Promoter	-	VNTR	American	6/25	-	< 0.05 (Luc activity)	-		9062372
				Japanese	55/63		Negative			12063521
	HT1	-	(G-C-G-A)	Korean	93/181	0.69/0.36	0.01	5.0 (1.54–17.9)	0.77 (0.51–0.92)	14749922
CysLTR1	Promoter	rs321029	−634 C>T	Korean	105/110	-	0.02 in male	2.89 (1.14–7.28)		16630147
		Novel	−475 A>C							
		Novel	−336 A>G							
	Exon1	Novel	927 T>C	UK	341 asthmatic families	-	-	-		16776674
		-		Spanish	87 (41 asthma with atopic dermatitis)	0.47/0.08	< 0.008 in male	9.78 (1.73–55.30)	0.82 (0.44–0.96)	16846449
	Exon3	Novel	899 G>A	Tristan da Cunha	52/60 (atopic/non-atopic asthma)	0.11/0.03	< 0.0001	6.28 (2.2–17.7)	0.40 (0.19–0.66)	17558309
CysLTR2	Exon	rs41347648	601 G>A	Caucasian	1st 359, 2nd 384 asthma families	0.03	0.04	-		15475736
	Promoter	Novel	−1220 A>C	Japanese	137 asthmatic families	0.93	0.0066	-		15454733
	Promoter	rs7324991	−819 T>G	Korean	66/134	0.51/0.43	0.031	2.04 (1.06–3.85)	0.51 (0.35–0.66)	
	3'UTR	Novel	+2078 C>T			0.44/0.30	0.013	2.28 (1.19–4.40)	0.50 (0.34–0.66)	15970796
		rs912278	+2534 A>G			0.47/0.39	0.031	2.02 (1.07–3.84)	0.48 (0.33–0.64)	
PTGER1	3'UTR	rs2241363	−500 G>C	Korean	268/137	0.42/0.36		0.37 (0.19–0.73)	0.13 (0.07–0.23)	
PTGER2	5'-up-stream	Novel	−12813 G>A	Japanese	198/282/274	0.31/0.22/0.22	0.0017	3.21 (1.53–6.75)	0.50 (0.32–0.67)	15898979
			−10814 T>A			0.49/0.39	0.0025			
	Promoter	-	−6179 A>G	Korean	108/93	0.37/0.45	0.0199	-		
		rs2075797	−616 C>G			0.34/0.44	0.039	0.64 (0.42–0.98)	0.17 (0.12–0.25)	17496729
		rs1353411	−166 G>A			0.45/0.38	0.023	2.60 (1.14–5.92)	0.53 (0.33–0.72)	
PTGER3	Promoter	rs7551789	−1709 T>A	Korean	108/93	0.38/0.30	0.043	3.02 (1.04–8.80)	0.53 (0.28–0.77)	
	Intron	rs7543182	A>C		243/919	0.29/0.24	0.02	1.31 (1.04–1.66)	0.27 (0.23–0.32)	15632198
	3'UTR	rs959	A>G			0.44/0.38	0.005	1.36 (1.10–1.68)	0.37 (0.33–0.42)	17496729
PTGER4	Promoter	Novel	−1254 A>G	Korean	108/93	0.28/0.20	0.018	1.90 (1.12–3.22)	0.34 (0.23–0.47)	17496729
PTGIR	3'UTR	rs1126510	+1915 T>C	Korean	108/93	0.06/0.13	0.015	0.37 (0.17–0.83)	0.02(0.01–0.04)	21449675
PTGDR	Diplotype	-	CCCT/CCCC (−613CC, −549CC, −441CC, −197TC)	Spanish	75/51	0.11/0.10	-	-		23101307
TBXAS1	Intron 9	rs692291	+141931 T>A	Korean	115/270	0.38/0.49	0.04	0.27 (0.13–0.57)	0.09 (0.04–0.18)	
TBXA2R	Exon3	rs11085026	+795 T>C	Korean	93/172	0.41/0.36	0.009	-		15898979
	Promoter	rs4807491	−4684 C>T		108/93	0.46/0.43	0.032	2.57 (1.09–6.09)	0.54 (0.33–0.73)	17496729
						0.39/0.47	0.027	0.42 (0.19–0.91)	0.14 (0.06–0.26)	

NERD, nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; LTR, leukotriene; PG, prostaglandin; AF, attributable fraction; AIA, aspirin-induced asthma; ATA, aspirin-tolerance asthma; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; LTC4S, leukotriene C4 synthase; ALOX5, arachidonate 5-lipoxygenase; CysLTR1, cysteinyl leukotriene receptor 1; CysLTR2, cysteinyl leukotriene receptor 2; PTGER2, prostaglandin E receptor 2; PTGER3, prostaglandin E receptor 3; PTGER4, prostaglandin E receptor 4; PTGIR, prostaglandin I2 receptor; TBXAS1, thromboxane A synthase 1; TBXA2R, thromboxane A2 receptor; RGS7BP, regulator of G protein signaling 7-binding protein.

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An LTC4S−444A/C promoter SNP (rs730012) is among the most widely reported variants associated with NERD, although its association with NERD across studies is inconsistent.[12,16] Three ALOX5 promoter variants have been shown to be associated with NERD and/or its severity of hyperresponsiveness.[4,17,18] However, some results have not been replicated due to small sample sizes, ethnic differences between study populations, or epigenetic changes, for the latter of which there is compelling evidence for a role in NERD.[19,20]

CLINICAL EVIDENCE OF EPIGENETIC COMPONENTS IN NERD

The prevalence of NSAID/aspirin hypersensitivity in adult asthmatics varies depending on the method used for diagnosis. In a recent meta-analysis,[21] the prevalence was highest when determined by oral provocation test (adults 21%, children 5%), as compared to verbal history (adults 3%, children 2%). In Korea, 6.2% of 836 adult asthmatics showed a positive responses to oral aspirin challenge tests,[22] and 5.8% of 1,173 adult asthmatics showed a positive response for NSAID/aspirin hypersensitivity n histories and/or provocation tests.[23] NERD shows less familial aggregation compared with asthma, although the European Network on Aspirin-Induced Asthma found that 6% of NERD patients had a family history of aspirin hypersensitivity.[24];a study of 1,344 Turkish patients revealed a 3.7% family history of NSAID/aspirin hypersensitivity,[25] suggesting an intermediate genetic background for this condition.

NSAID/aspirin hypersensitivity is more common in women than in men, beginning in adulthood at an average age of 30 years. Once developed, it remains throughout life, although sporadic disappearance of intolerance has been reported.[26] However, considerable discordance in clinical manifestations of the disease has been noted in identical twin sisters, suggesting the greater influence of environmental factors.[27,28] As NERD usually develops in middle age after relatively long-term exposure to NSAIDs, and with a low level of familial aggregation,[24,29] epigenetic mechanisms may make greater contributions than genetic variations.

GLOBAL CHANGES IN CPG METHYLATION OF NASAL POLYPS FROM SUBJECTS WITH NERD

Epidemiological studies have shown that regular use of NSAIDs reduces the risk of development of at least some cancers.[30,31] via the well-known targets, including *COX-1* and *COX-2*, and other intracellular pathways, including cell cycle, cell differentiation, apoptosis, and regulation of transcription factors (TFs).[32] In our previous *in vitro* study, *DNMT3a* and 3b mRNAs were elevated in a mucoepidermoid cell line (NCI-H292) within 1 day after stimulation with medium and high doses of aspirin, while *DNMT1* and *MeCP2* showed no significant changes in expression (**Fig. 1**). *DNMT3b* mRNA was enhanced by stimulation for 2 hours with 0.2 and 2 mM aspirin. *DNMT3a* mRNA level was increased progressively for up to 3 days by treatment with high-dose aspirin. These data suggest that aspirin may induce global CpG methylation, which may affect gene expression. In contrast, NSAIDs are known induce promoter demethylation of Secreted Protein Acidic and Cysteine Rich (*SPARC*) by repressing DNMT expression.[33] In agreement with the experimental data, an epidemiological study showed that chronic aspirin use may be associated with a lower prevalence of E-cadherin (*CDH1*) promoter methylation in non-neoplastic gastric mucosa.[34]

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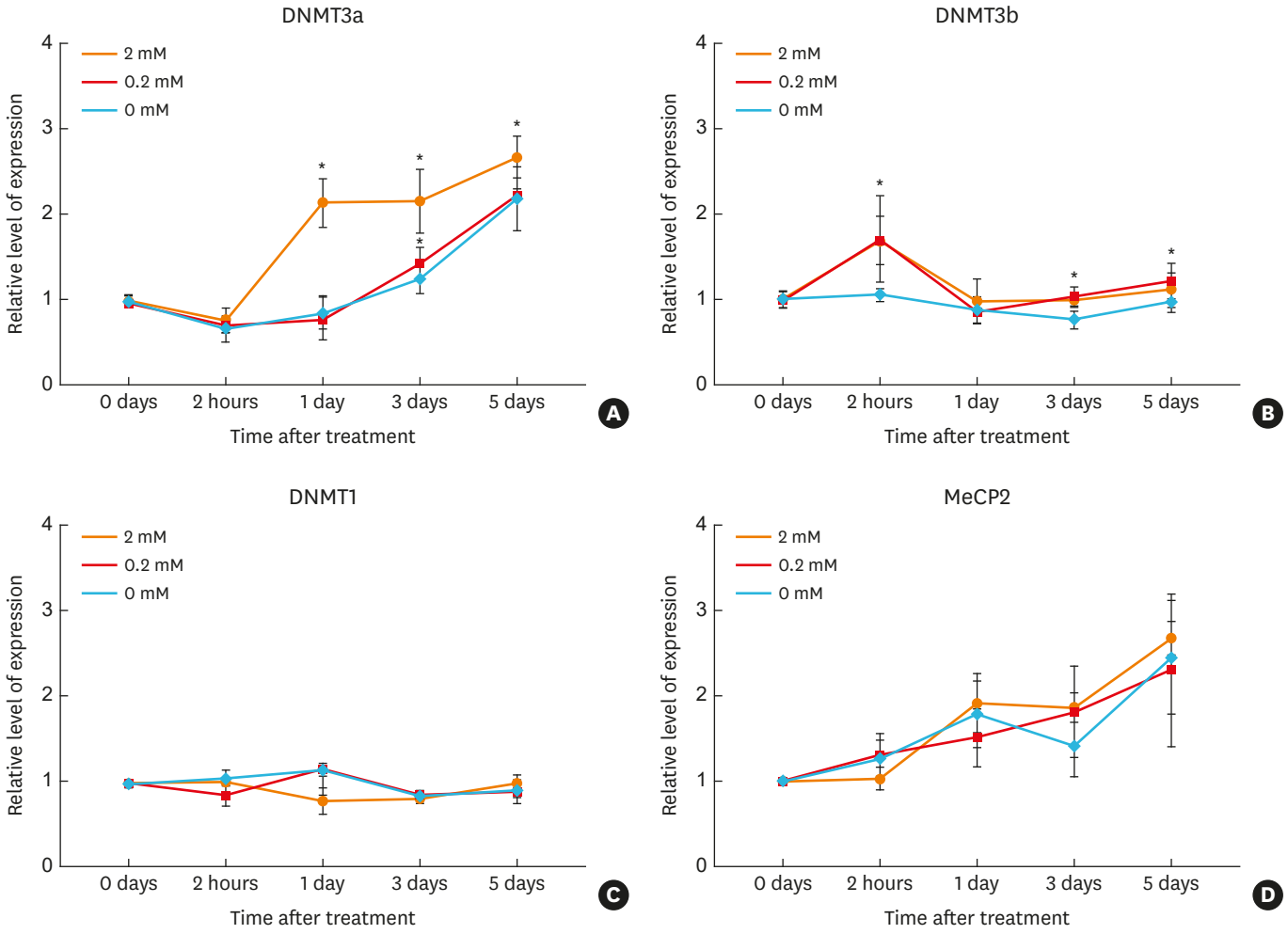

Fig. 1. Effects of aspirin on DNA methyltransferase genes in the mucoepidermoid NCI-H292 lung cell line. Quantitative real-time polymerase chain reaction assay was conducted in a Smart Cycler instrument, and the relative levels of DNMT3a, DNMT3b, and DNMT1 mRNAs were normalized relative to that of peptidylprolyl isomerase A. The data are representative of 3 consecutive experiments.
*$P < 0.05$ vs. 0 mM aspirin.

There have been few studies on global DNA CpG methylation in NERD. In a genome-wide CpG methylation study of nasal polyps in subjects with NERD and aspirin-tolerant asthma (ATA) patients,[35] 332 CpG sites on 296 genes were hypomethylated and 158 sites on 141 genes were hypermethylated in NERD (**Fig. 2**). Thus, the NERD-associated proportion of global differential methylated CpG (DMC) was 1.78% (490/27,587 CpGs analyzed in the test kit), which was about 10 times higher than the proportion of DMC in the bronchial epithelium of patients with atopic asthma (0.19%, 53/27,578 CpGs).[36] The 490 DMCs were located on 437 genes and, thus, the global proportion of differentially methylated genes (DMGs) was 3.02% (437/14,457 genes analyzed in the test kit). In silico analysis of the 490 DMCs indicated that 409 CpG sites (83.5%) were on promoter regions with 130 hypermethylated CpGs and 279 hypomethylated CpGs (**Fig. 3**). In general, hypermethylation of cytosines within CpG islands of promoters causes gene silencing, and hypomethylation triggers active transcription.[37,38] Thus, differential promoter CpG methylation may affect gene expression levels in nasal polyps of NERD patients, as compared to ATA patients. Ontological classification of the 36,127 genes in AmiGo2 (http://amigo.geneontology.org/amigo/search/ontology) indicated 259 genes in the arachidonate pathways (**Table 2**). Among them, 66 genes were differentially

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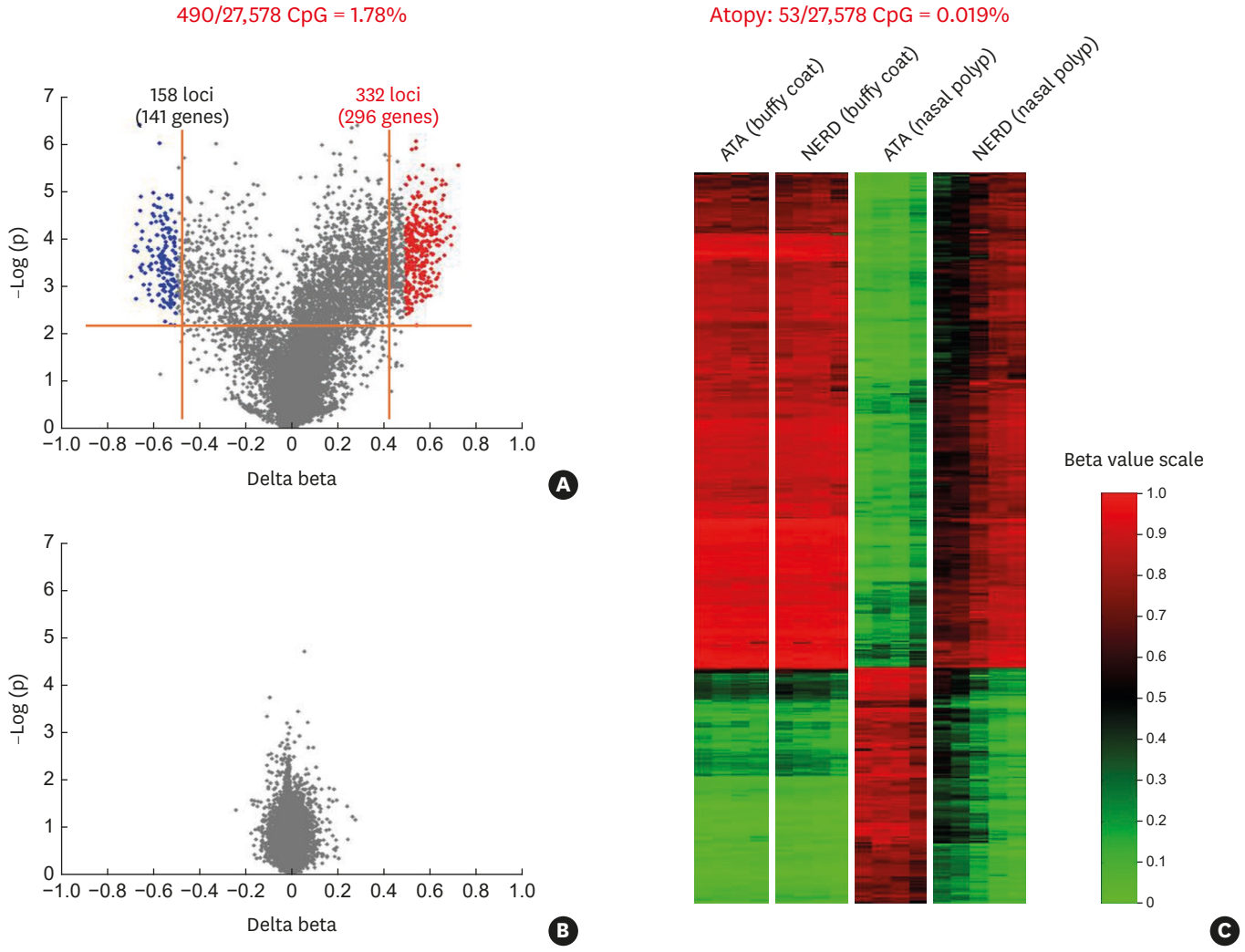

Fig. 2. CpG DNA methylation patterns of nasal polyps and peripheral blood mononuclear cells obtained from subjects with NERD and ATA. Volcano plot of differential methylation levels between NERD and ATA in nasal polyp tissues (A) and buffy coat samples (B). Red dots, delta beta ≥ 0.5 and $P \leq 0.01$; blue dots, delta beta ≤ −0.5 and $P \leq 0.01$; gray dots, −0.5 ≤ delta beta ≤ 0.5 and $P > 0.01$. Delta beta, difference in DNA methylation level (subtracting DNA methylation level of ATA from NERD). −log (p), log-transformed *t*-test *P* values. (C) Heat map of 490 differentially methylated CpGs between NERD and ATA in buffy coat and nasal polyps. Reproduced with permission from *Allergy Asthma Immunol Res* 2013;5:258-76[19] (license number: EU826007151).
NERD, nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; ATA, aspirin-tolerant asthma.

Table 2. Proportions of differently methylated and SNP genes in arachidonic acid pathways in NERD compared to those in ATA

Ontology	No. of genes	DMG	SNP-gene	DMG & SNP	cgSNPs
Arachidonic acid binding	5	3 (60)	2 (40)	1 (20)	1 (20)*
Arachidonic acid metabolism	62	34 (54.9)	11 (17.8)	4 (6.5)	0 (0)
Prostaglandin biosynthetic process	25	6 (25)	5 (20)	0 (0)	0 (1)
Leukotriene biosynthetic process	20	3 (15)	4 (20)	1 (5)	0 (2)
Arachidonic acid products	93	13 (14)	13 (14)	0 (0)	0 (3)
Leukotriene products	38	5 (13)	9 (24)	1 (2.6)	0 (4)
Lipoxygenase	16	2 (12.5)	5 (31.3)	0 (0)	0 (5)
Total	259	66 (22.5)	49 (18.9)	7 (2.7)	1 (0.4)

Values are presented as number (%). The 259 genes were recruited from 36,127 genes in the AmiGo2, the web-based set of tools for searching and browsing the Gene Ontology database. Number in parenthesis is proportion of genes among the total gene.
NERD, nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; DMG, differentially methylated gene; SNP, single nucleotide polymorphism; ATA, aspirin-tolerance asthma; SNP-gene, number of genes having single nucleotide polymorphism associated with nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; cgSNPs, CpG site related single nucleotide polymorphisms.
*S100A9 (S100 Calcium Binding Protein A9); This protein is a calcium- and zinc-binding protein which plays a prominent role in the regulation of inflammatory processes and immune response.

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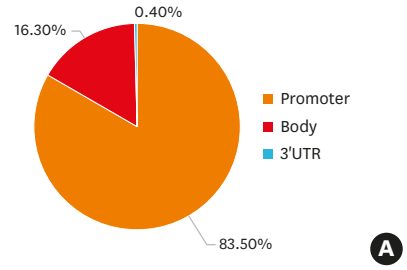
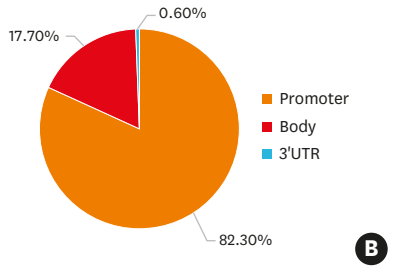
Total-methylation (490 loci on 437 genes)

FGD	CpGs	Subgroup	CpGs
Promoter	409	TSS200	84
		TSS1500	146
		5'UTR	93
		1stExon	86
Body	79		
3'UTR	2		



Hyper-methylation (158 loci on 141 genes)

FGD	CpGs	Subgroup	CpGs
Promoter	130	TSS200	26
		TSS1500	47
		5'UTR	31
		1stExon	26
Body	27		
3'UTR	1		



Hypo-methylation (332 loci on 296 genes)

FGD	CpGs	Subgroup	CpGs
Promoter	279	TSS200	58
		TSS1500	99
		5'UTR	62
		1stExon	60
Body	52		
3'UTR	1		

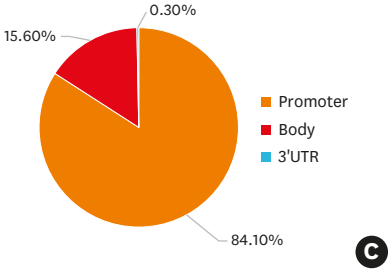


Fig. 3. In silico analysis of the 490 DMCs between NERD and ATA indicated that 409 CpG sites (83.5%) were on promoter regions with 130 hypermethylated CpGs and 279 hypomethylated CpGs.
DMC, differential methylated CpG; NERD, nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; ATA, aspirin-tolerant asthma; TSS200, 0–200 bases upstream of the transcription start site; TSS1500, 200–1,500 bases upstream of the transcription start site.

methylated (25.5%), which was 10 times higher than the global proportion of DMG (3.02%). These observations indicated that the genes in the arachidonate pathways are much more labile to CpG methylation in NERD compared to ATA.

GENOME-EPIGENOME INTERACTION VIA CPG SITE-RELATED SNPS (CGSNPS)

Some SNPs influence the presence of CpG sites, where DNA modification, such as methylation and hydroxymethylation, occurs.[39] These SNPs can lead to gain or loss of CpG sites and are defined as cgSNPs. For example, a C-to-T transition on the "C" of CpG dinucleotides leads to loss of a CpG site. A human genome study demonstrated a considerable proportion of cgSNPs (23.0%) among 4097556 common variants.[40] In the nasal polyps of NERD,[35] 42 cgSNPs were present in about 11% of the 409 DMC sites on the promoter and one cgSNP was located in the body of the gene (**Table 3**). Forty-seven TFs were predicted to bind to the DNA sequences of these sites (**Fig. 4**) on PROMO search (http://alggen.lsi.upc.edu/). Among the TFs, XBP-1, GR-alpha, and EKNTF-1 bind to more than 10 CpGs sites. The XBP-1 protein is a TF that regulates the expression of genes important to the immune system and the cellular stress response.[41] In addition, the expression of this protein is required for transcription of a subset of class II major histocompatibility genes.[42] GWAS studies performed by our group and other investigators have revealed that SNPs on HLA-DPB1 (rs1042151 and rs3128965) show the most significant association with NERD susceptibility.[43] The rs1042151 acts as a potential cis regulator of the expression of *HLA-DPB1* with an expression quantitative trait loci (eQTL) score of 36.83, as calculated using the eQTL browser (http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/). In silico analysis of SNP function indicated that the rs1042151 SNP is located in an exonic splicing enhancer region.[44] Thus, XBP-1 is thought to be one of the key TFs binding to the DMCs in the development of NERD, which will be validated in future studies.

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Table 3. List of 42 cgSNPs of 409 differentially methylated CpG sites between NERD and ATA and their transcription binding factors

Gene name	Description	Chromosome	CpG coordinate	Position	DeltaBeta (AIA-ATA)	*P* value	SNP	Genotype	Transcription factor
PIK3CG	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma	7	106293008	TSS200	−0.58	8E-05	rs189643115	C/T	GCF, TFII-I
SLA	Src like adaptor	8	134141793	TSS200	−0.56	5E-04	rs193193464	A/G/T	TFIID, GCF
DOK2	Docking protein 2	8	21827392	TSS1500	−0.55	2E-04	rs116745856	A/G	ENKTF-1, AP-2alphaA
EVL	Enah/vasp-like	14	99601344	TSS200	−0.55	2E-04	rs142509033	A/G	RXR-alpha, ENKTF-1, E2F-1, Pax-5, p53, sp1, WT1
ARHGAP15	Rho GTPase activating protein 15	2	143602842	TSS1500	−0.54	4E-05	rs145004467	C/T	XBP-1, ENKTF-1, E2F-1, AhR:Arnt
GPR77	Complement component 5a receptor 2	19	52530972	TSS1500	−0.54	1E-04	rs190777242	A/G	TFII-I, PEA3, XBP-1
PLD4	Phospholipase D family member 4	14	104462063	TSS200	−0.53	3E-04	rs138509471	A/G	GR-alpha, AP-2alphaA, ENKTF-1, Pax-5, p53, E2F-1
LILRA2	Leukocyte immunoglobulin like teceptor A2	19	59776830	TSS1500	−0.52	6E-05	rs73612405	C/T	XBP-1
EGFL7	EGF like domain multiple 7	9	138676375	TSS1500	−0.52	9E-05	rs111978941	A/G/T	AR, TFII-I
ITGAM	Integrin subunit alpha M	16	31178510	TSS1500	−0.52	1E-03	rs184907050	A/G	USF2, GCF, AP-2alphaA
TYROBP	TYRO protein tyrosine kinase binding protein	19	41091889	TSS1500	−0.51	3E-04	rs146068432	C/T	XBP-1
TSPAN32	Tetraspanin 32	11	2279317	Body	−0.51	9E-05	rs74048220	C/G/T	ENKTF-1, GR-alpha, AP-2alphaA
NALP12	NLR family pyrin domain containing 12	19	59019592	TSS200	−0.5	7E-05	rs187459687	A/G	E2F-1, c-Ets-1, STAT1beta, NF-AT1, IRF-1
PUM2	Pumilio RNA binding family member 2	2	20391116	TSS1500	0.5	7E-04	rs114135728	C/T	XBP-1, ENKTF-1, AR
METTL4	Methyltransferase like 4	18	2562965	5'UTR	0.5	2E-03	rs200286634	C/T	GR-beta, C/EBPbeta, TFIID
KLK11	Kallikrein related peptidase 11	19	56223296	TSS1500	0.5	6E-05	rs183746430	A/G	GR-alpha, TFIID, RXR-alpha
TSPAN8	Tetraspanin 8	12	69838603	TSS1500	0.5	2E-04	rs77641542	A/G	GR-beta, AP-1, XBP-1
MYOZ3	Myozenin 3	5	150020473	TSS200	0.5	1E-03	rs188156985	A/G	GR-alpha, RXR-alpha
CHRNA10	Cholinergic receptor nicotinic alpha 10 subunit	11	3649733	TSS1500	0.5	3E-03	rs190940880	A/G	FOXP3, IRF-1, TFII-I, STAT4, c-Ets-1
LGALS8	Galectin 8	1	234746847	TSS1500	0.5	2E-03	rs192328273	A/G	
UGT1A6	UDP glucuronosyltransferase family 1 member A6	2	234264888	TSS200	0.51	5E-05	rs45594938	A/C/G/T	ENKTF-1, c-Myb, RXR-alpha
SYCP2	Synaptonemal complex protein 2	20	57940627	TSS200	0.52	1E-06	rs140425806	C/G	GR-alpha, GATA-1, HNF-1C, HNF-1B
SERPINB13	Serpin family B member 13	18	59405541	5'UTR	0.52	3E-03	rs73468602	C/T	AhR:Arnt,XBP-1
SH2D4B	SH2 domain containing 4B	10	82287377	TSS1500	0.53	1E-04	rs137943319	C/T	FOXP3, HNF-3alpha, GR-beta, XBP-1
NID1	Nidogen 1	1	234304366	TSS1500	0.53	5E-04	rs187127213	A/C/G	GR-alpha, EBF, T3R-beta1, RXR-alpha
VTCN1	V-Set domain containing T cell activation inhibitor 1	1	117555488	TSS1500	0.53	4E-04	rs146060859	A/G	GR-alpha, YY1
PCK1	Phosphoenolpyruvate carboxykinase 1	20	55569418	TSS200	0.53	1E-04	rs146925480	A/G	PR B, PR A, p53, Pax-5, AhR:Arnt
TECTA	Tectorin alpha	11	120478484	TSS200	0.54	6E-04	rs79614045	C/T	XBP-1, c-Jun, YY1, Ik-1
LAMB3	Laminin subunit beta 3	1	207892479	TSS200	0.54	4E-04	rs191233438	C/T	RXR-alpha, E2F-1, Pax-5, p53, GR-alpha, EBF
RIPK1	Receptor interacting serine/threonine kinase 1	6	3021353	TSS1500	0.54	1E-04	rs186932816	C/T	c-Ets-1, c-Myb, IRF-1, YY1
SLC39A2	Solute carrier family 39 member 2	14	20537113	TSS200	0.54	8E-07	rs141598581	A/G	GATA-1
TMEM184A	Transmembrane protein 184A	7	1562743	TSS200	0.57	6E-06	rs73287493	C/T	GR-alpha, AP-2alphaA, ENKTF-1
LIPC	Lipase C, hepatic type	15	56510949	TSS1500	0.58	3E-04	rs200997055	A/G	XBP-1, c-Jun, ATF3
CDH26	Cadherin 26	20	57966838	TSS200	0.59	2E-04	rs188408166	A/G	E2F-1, c-Ets-1, Elk-1
TNKS1BP1	Tankyrase 1 binding protein 1	11	56846646	5'UTR	0.59	2E-05	rs113688171	G/T	GR-beta, NFI/CTF
PFDN2	Prefoldin subunit 2	1	159355726	TSS1500	0.6	2E-04	rs200278216	C/T	RAR-beta:RXR-alpha,YY1, XBP-1
IL22RA1	Interleukin 22 receptor subunit alpha 1	1	24342378	TSS200	0.6	7E-05	rs138275016	A/C/G	c-Ets-2, TFII-I, c-Ets-1, Elk-1
PAQR6	Progestin and AdipoQ receptor family member 6	1	154484566	TSS200	0.6	7E-05	rs144755038	A/C	GR-alpha, C/EBPbeta
GCNT4	Glucosaminyl (N-acetyl) transferase 4	5	74363037	TSS1500	0.61	5E-05	rs73122571	A/G	GR-alpha, PR B, PR A, AP-2alphaA, SRF, Pax-5, p53
ACKR2	Atypical chemokine receptor 2	3	42825852	TSS200	0.63	3E-05	rs117154133	C/T	XBP-1, AR, AP-1, c-Jun
CCN4	Cellular communication network factor 4	8	134271552	TSS1500	0.64	1E-04	rs138160356	C/T	c-Myc, c-Jun, USF1, XBP-1, ATF3
RDH5	Retinol dehydrogenase 5	12	54400422	1stExon	0.66	2E-04	rs185215037	C/T	
SLC28A1	Solute carrier family 28 member 1	15	83227915	TSS1500	0.7	6E-05	rs139316942	C/T	C/EBPbeta, GR

cgSNP, CpG site-related single nucleotide polymorphism; NERD, nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; AIA, aspirin-induced asthma; ATA, aspirin-tolerant asthma; SNP, single nucleotide polymorphism.

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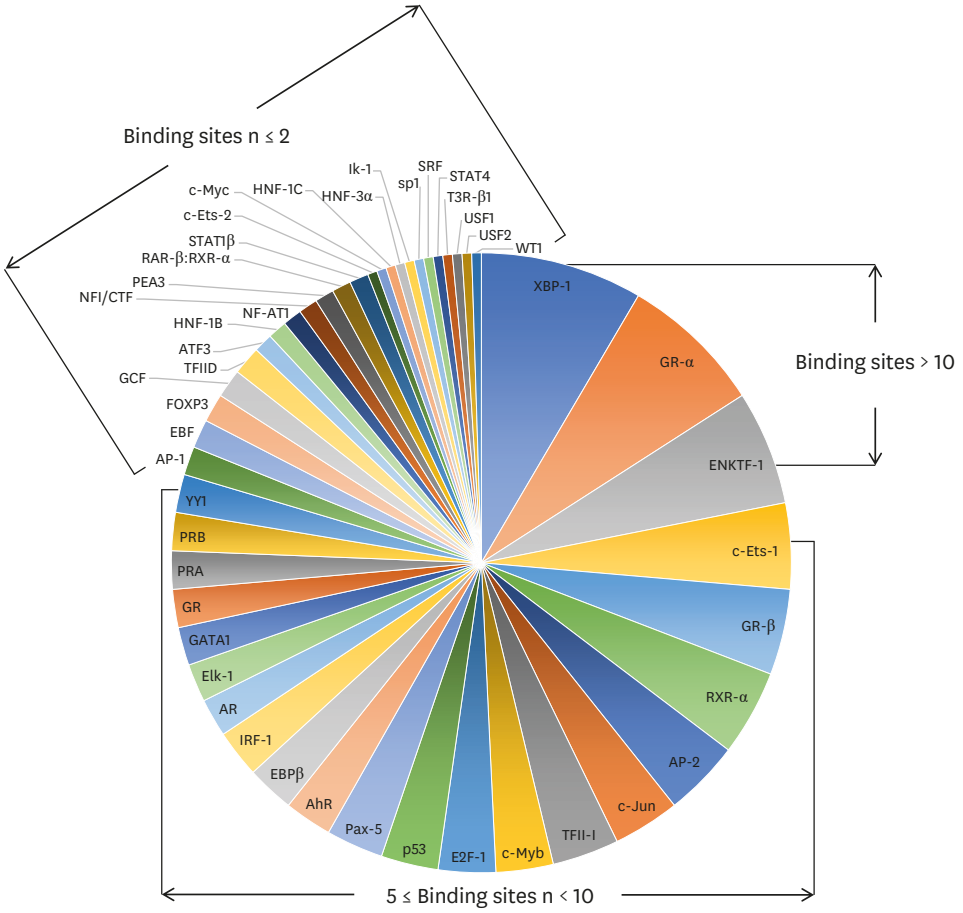

Fig. 4. Diagram of transcription factors binding to 43 CpG site-related single nucleotide polymorphisms.

Interestingly, the frequency of cgSNPs was much lower in the arachidonate pathways. Among the total of 259 genes involved in these pathways, the number of genes with SNPs associated with NERD was 49 (18.9%), which was slightly less than the proportion of DMG (25.5%) (**Table 3**). Seven genes had both DMCs and SNPs. Among them, only one SNP (rs19980990 of S100A9) was related to a CpG site (i.e., a cgSNP). Thus, among the 259 genes, gene expression may be regulated by CpG methylation on 59 genes, by SNPs on 42 genes, and by both CpG methylation and SNPs on 7 genes.

DIFFERENTIALLY METHYLATED CPGS OF THE GENES INVOLVED IN THE PG AND LTR BIOSYNTHESIS PATHWAYS

Among 37 CpGs on 19 genes found in the PG and LT biosynthesis pathways (**Supplementary Table S1**), there were 14 DMCs on 11 genes, i.e., *ALOX12* (*cg03760483*, *cg08946332*), *ALOX12B* (*cg03742272*), *ALOX15* (*CG15843823*), *ALOX15B* (*CG15799267*, *CG12343777*), *ALOX5AP* (*CG08529529*), *LOXHD1* (*cg17903316*), *PGDS* (*cg12554857*), *PTGDS* (*CG00563932*), *PTGES* (*cg26672426*, *cg17683775*), *PTGIS* (*CG07612655*), and *TBXAS1* (*CG14116569*) (**Fig. 5**). Among them, *PGDS* (*cg12554857*) and *ALOX5AP* (*CG08529529*) were highly hypomethylated (delta beta: −0.67 and −0.522, respectively),

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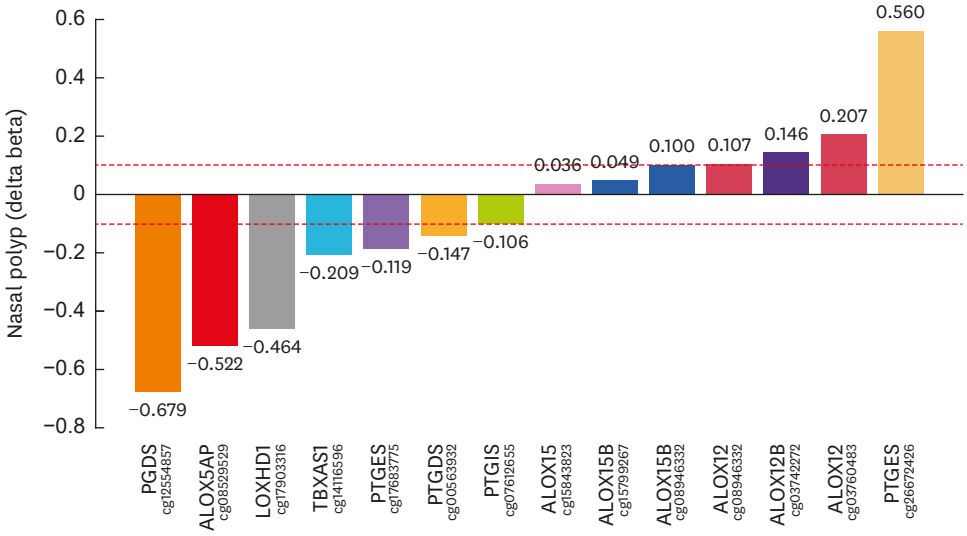

Fig. 5. Delta beta values of 16 DMCs ($P < 0.05$) on 11 genes involved in the prostaglandin and leukotriene pathways between NERD and ATA. Delta beta was calculated by subtracting the beta value of ATA from that of NERD at each CpG site.
DMC, differential methylated CpG; NERD, nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; ATA, aspirin-tolerant asthma.

while *PTGES* (*cg26672426*) was robustly hypermethylated (delta beta: 0.56) in NERD compared to ATA (**Fig. 5**). Although the genomic impact of DMCs in NERD has not been analyzed with regard to changes in their genes or metabolites, prostanglandins, cysteinyl LTs, and thromboxanes are expected to be elevated, while PGE may be downregulated by varying the extent of CpG methylation of the relevant genes of NERD patients.

Therapeutic concentrations of aspirin mainly inhibit cyclooxygenase 1 (COX-1). COX-1, also known as PGG/H synthase 1, PG-endoperoxide synthase 1, or PGH2 synthase 1, is an enzyme encoded in humans by the *PTGS1* gene. Therefore, blocking of COX-1 results in a reduction of multifunctional PGH2 level, which is a common precursor substrate for PGD2, PGE2, PGF2, PGI2, and TXB2 (**Fig. 6A**). Accordingly, the levels of all of these end products are expected to be decreased after aspirin challenge or even in the basal state in NERD. However, systemic PGF2[45] and PGD2 levels[46] and the ratio of local PGD2/PGE2 levels were increased in NERD[47] compared to ATA (**Fig. 6B-E**). These data are in good agreement with the methylation changes of these genes.

Although systemic basal TBX2 levels were significantly higher in NERD than ATA, the levels were downregulated after aspirin challenge,[48] while PGF2 and PGD2 levels were persistently elevated.[45,46] The different responses to aspirin challenge may be due to genetic differences in these genes between NERD and ATA. A genetic variant study of a Korean population demonstrated that the frequency of the minor allele *+141931T>A* (rs6962291) in intron 9 of *TBXAS1* was significantly lower in the NERD group than the ATA group (**Table 1**). Taken together, these observations suggest that DNA CpG methylation may exert a regulatory role in synthesis of PG metabolites, especially *PGD2* and *PGE*, while the genetic variants and hypomethylation of *TBX2* may be responsible for that of thromboxane B2.[49]

The other important mechanistic evidence for NERD pathogenesis supports intrinsic dysregulation of the activity of the *5-LO/LTC4S* pathway, i.e., *LTC4S*[4] and *ALOX5*[4] (**Table 1**).

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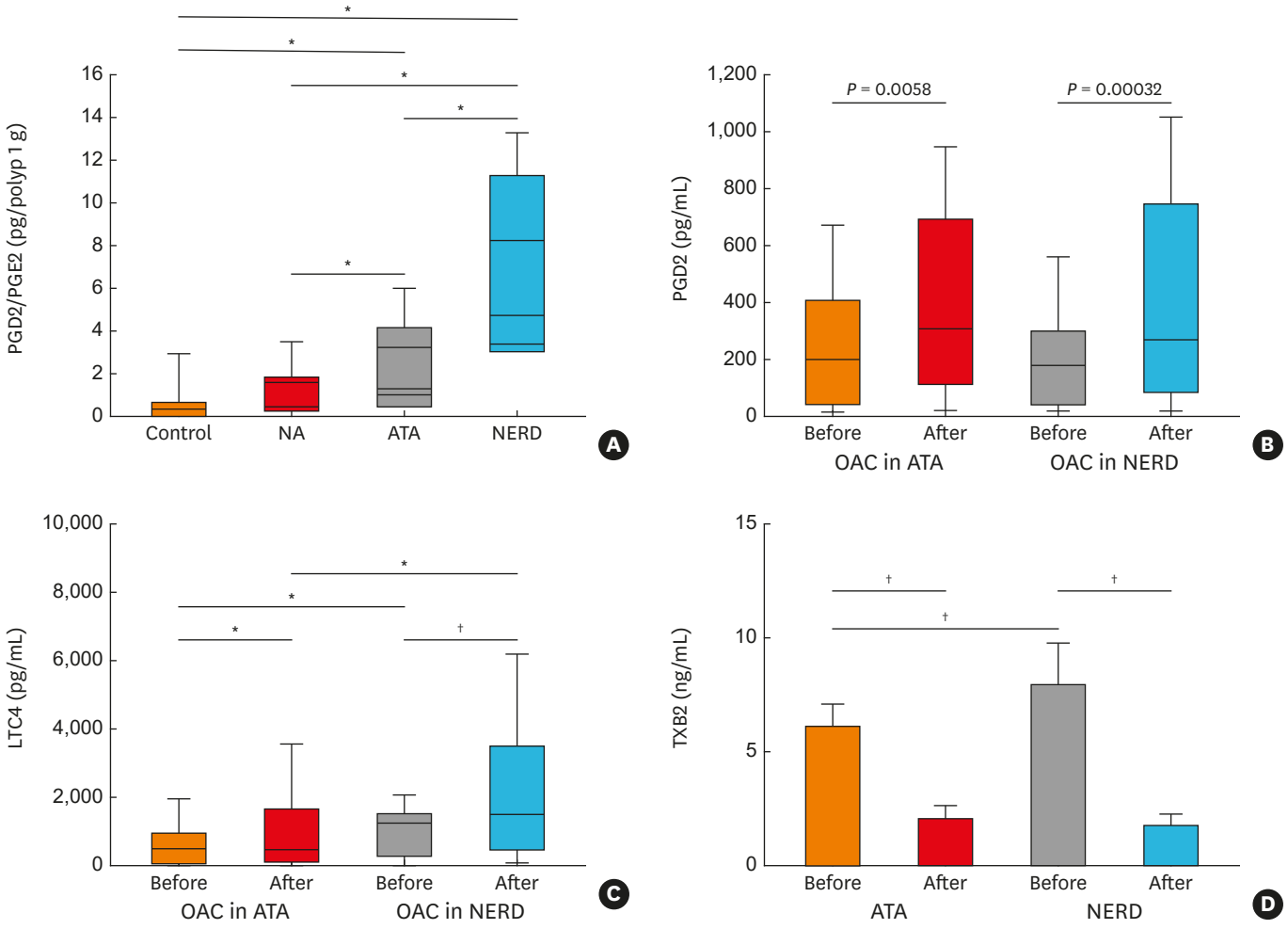

Fig. 6. Effects of aspirin and NSAID on prostaglandin and leukotriene metabolites. Theoretical hanges in PGDs followed by inhibition of COX-1 and actual results (A).[47] Changes in PGD2 (B), LTC4 (C), and TXB2 (D) levels in plasma after aspirin challenge in patients with NERD and in those with ATA (license number: 4483431214000).[46,48]

NSAID, nonsteroidal anti-inflammatory drug; PGD, prostaglandin D; NERD, nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; ATA, aspirin-tolerant asthma.

*$P < 0.05$; †$P < 0.005$.

LTC4S is among the most widely reported variants associated with NERD, although its association with NERD across studies is inconsistent.[50,51] A recent meta-analysis of 13 case–control studies of asthma revealed significantly increased risk in asthmatic patients carrying the CC or AC genotype vs. the AA genotype.[16] Three ALOX5 promoter variants are associated with NERD[4,17,18] (**Table 1**). Interestingly, ALOX5AP showed hypomethylation with delta beta of −0.52, which may affect gene expression. Thus, cysteinyl LTs may be elevated by both differences in the extent of methylation of ALOX5AP and genetic variants of ALOX5 and LTC4 synthase. Among the 14 receptor genes involved in the PG and LTR pathways, 5 genes showed 6 DMC (**Supplementary Table S2**). However, *LTB4R* and *LXA4 Receptor* (*FPRL1*) are significantly hypomethylated with delta beta > 0.2 (**Fig. 7**). As shown in **Table 1**, *CysLTR1* and *CysLTR2*, and *PTGER1*, *PTGER2*, *PTGER3*, and *PTGER4*, *PTGDR*, *PTGD2R* (*CRTH2*), *PTGIR* and *TBXA2R* have different frequencies of SNPs on their respective genes in patients with NERD or those with nasal polyps,[52] indicating that the receptors for PG and LTR metabolites have genetic effects due to SNPs except for LTB4R and FPRL1, which are modulated by differences in methylation (**Fig. 7**).

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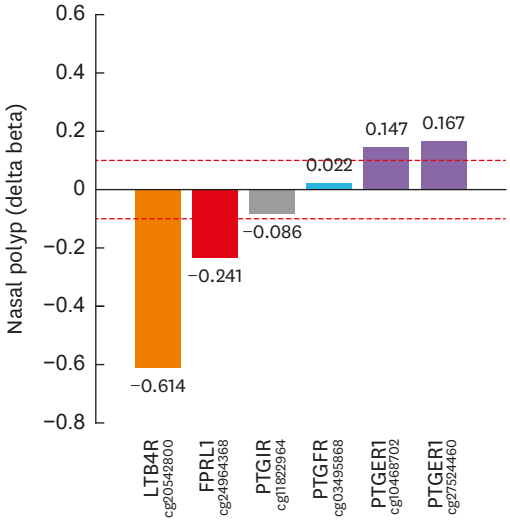

Fig. 7. Delta beta values of 6 DMCs ($P < 0.05$) on 5 genes of receptors involved in the prostaglandin and leukotriene pathways between NERD and ATA. Delta beta was calculated by subtracting the beta value of ATA from that of NERD at each CpG site.
DMC, differential methylated CpG; NERD, nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; ATA, aspirin-tolerant asthma.

CONCLUSION

PGE synthesis may be decreased due to hypermethylation of PGEs, while PGD and thromboxane synthesis may be elevated by hypomethylation of PTDS and TBXAS. The imbalance of PGE/PGD may be accentuated by genetic variants of PGERs (EP2–EP4) and PTGDR. In the LT pathways, cysteinyl LTR synthesis may be elevated by hypomethylated ALOX5AP and genetic variants of ALOX5 and LTC4S. Furthermore, the effects of cysteinyl LT may be maximized by genetic variants of CysLR1 and 2 (**Fig. 8**). Accordingly, the imbalance of PG/cysteinyl LT synthesis may be dominantly regulated by the changes in methylation and may be complemented by the SNPs of LTC4S and ALOX5, and the effects of the imbalance may be accentuated by the genetic variants of PGERs, CYSLTR1, and CYSLTR2 (**Fig. 8**). Taken together, these observations indicated that subjects with NERD may have genetically distorted and epigenetically susceptible arachidonate pathways to environmental factors, including exposure to NSAIDs or other agents. In addition, DNA methylation may indeed be tightly regulated by genetic factors such as SNPs that affect NERD development (**Fig. 8**). However, the precise mechanisms by which NSAIDs induce dysregulation of CpG methylation in NERD are still unknown. It remains to be determined when and how the epigenetic effects of NSAIDs begin and the initial mechanisms underlying the changes in CpG methylation. Other possible actions of NSAID may involve modification of other epigenetic components, including histone proteins and miRNA, or altered metabolism of methylating nutrients. Although the molecular mechanisms underlying NERD pathogenesis remain poorly understood, genetic and epigenetic variations play significant roles. Our results enhance the understanding of the genetic and epigenetic mechanisms involved in NERD development and suggest new approaches toward the diagnosis, treatment, and management of NERD.

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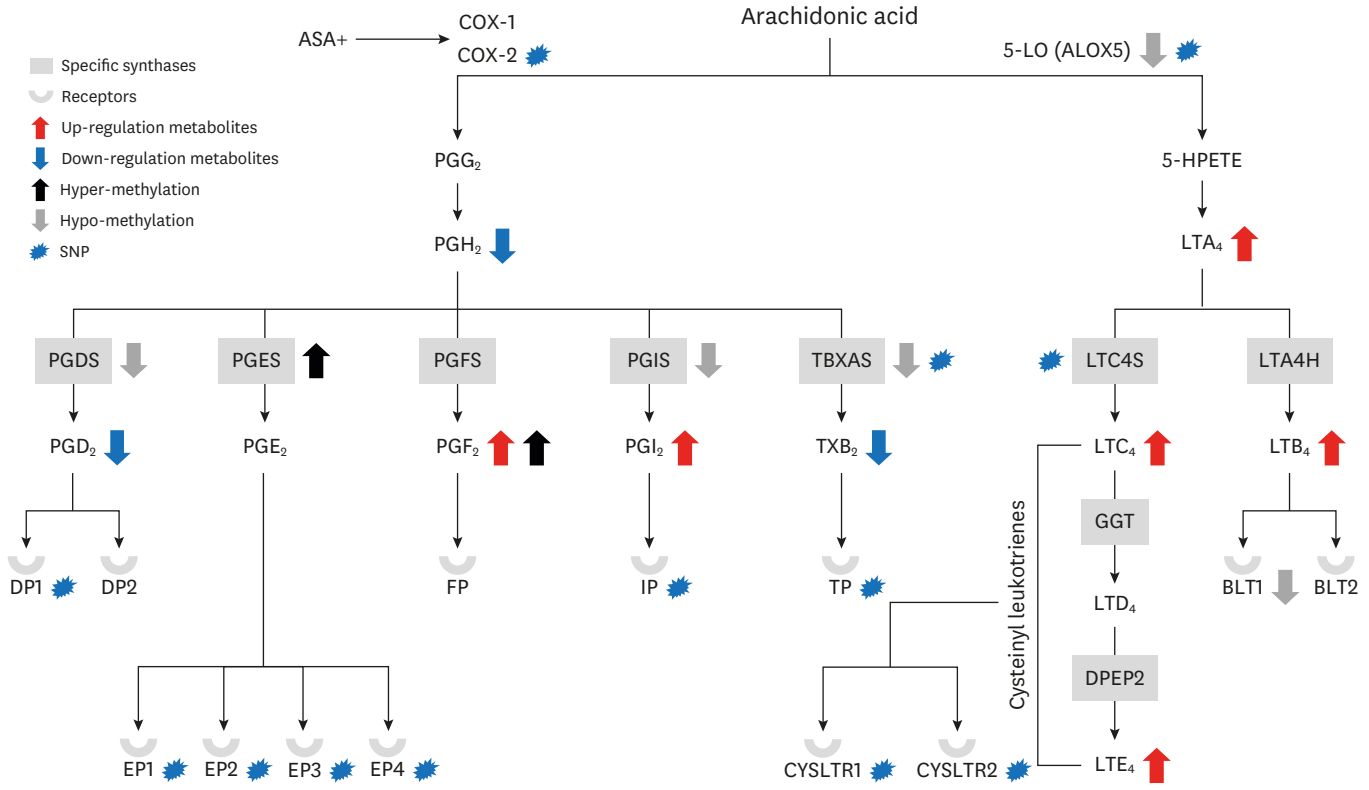

Fig. 8. NERD severity and its clinical phenotypes are affected by DNA methylation and genetic variation within genes of associated with multiple pathways for arachidonic acid metabolism.
NERD, nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; PG, prostaglandin; SNP, single nucleotide polymorphism; ASA, acetylsalicylic acid; DP, prostaglandin D (PGD) receptors; EP, prostaglandin E (PGE) receptors; FP, prostaglandin F receptor; IP, prostaglandin I2 receptor; TP, thromboxane receptors; LT, leukotriene; BLT, leukotriene B4 receptor; ALOX5, arachidonate 5-lipoxygenase; 5-HPETE, arachidonic acid 5-hydroperoxide; CysLTR1, cysteinyl leukotriene receptor 1; CysLTR2, cysteinyl leukotriene receptor 2; LTC4S, leukotriene C4 synthase; LTA4H, aeukotriene A4 hydrolase.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1

Delta beta levels of differentially methylated CpGs in prostaglandin and leukotriene pathways between NERD and ATA

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Supplementary Table S2

DNA methylation level of differentially methylated CpGs in receptor genes of arachidonic acid pathways in AIA compared to those in ATA

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