RNA-Seq for Gene Expression Profiling of Human Necrotizing Enterocolitis: a Pilot Study

Kyuwhan Jung,[1]* InSong Koh,[2]*
Jeong-Hyun Kim,[3] Hyun Sub Cheong,[4]
Taejin Park,[5] So Hyun Nam,[6]
Soo-Min Jung,[7] Cherry Ann Sio,[8†]
Su Yeong Kim,[6] Euiseok Jung,[9]
Byoungkook Lee,[9] Hye-Rim Kim,[9]
Eun Shin,[10] Sung-Eun Jung,[11]
Chang Won Choi,[9] Beyong Il Kim,[9]
Eunyoung Jung,[12] and Hyoung Doo Shin[3,13]

[1]Department of Surgery, Jeju National University
Hospital, Jeju, Korea; [2]Department of Physiology,
College of Medicine, Hanyang University, Seoul,
Korea; [3]Research Institute for Basic Science, Sogang
University, Seoul, Korea; [4]Department of Genetic
Epidemiology, SNP Genetics, Inc., Seoul, Korea;
[5]Department of Surgery, Gyeongsang National
University Hospital, Jinju, Korea; [6]Department of
Surgery, Donga University Hospital, Busan, Korea;
[7]Department of Surgery, Konkuk University Medical
Center, Konkuk University School of Medicine,
Seoul, Korea; [8]Department of Surgery, Seoul
National University Bundang Hospital, Seongnam,
Korea; [9]Department of Neonatology, Seoul National
University Bundang Hospital, Seongnam, Korea;
[10]Department of Pathology, Seoul National
University Bundang Hospital, Seongnam, Korea;
[11]Department of Pediatric Surgery, Seoul National
University Children's Hospital, Seoul, Korea;
[12]Department of Surgery, Gyemyoung University
Dongsan Hospital, Daegu, Korea; [13]Department of
Life Science, Sogang University, Seoul, Korea

*Kyuwhan Jung and InSong Koh contributed equally
to this work.

†Current address: Department of Surgery, University
of Santo Tomas Hospital, España Boulevard, Manila,
Philippines

Received: 6 December 2016
Accepted: 27 January 2017

Address for Correspondence:
Hyoung Doo Shin, PhD, DVM
Department of Life Science, Sogang University,
35 Baekbeom-ro, Mapo-gu, Seoul 04107, Korea
E-mail: hdshin@sogang.ac.kr

Funding: This study was supported by grants from the Basic
Science Research Program through the National Research
Foundation of Korea (NRF), funded by the Ministry of Science,
ICT and Future Planning (NRF-2014R1A1A1005096 and NRF-
2012M3A9D1054450) and by the Ministry of Education,
Science and Technology (2009-0093822).

Necrotizing enterocolitis (NEC) characterized by inflammatory intestinal necrosis is a major cause of mortality and morbidity in newborns. Deep RNA sequencing (RNA-Seq) has recently emerged as a powerful technology enabling better quantification of gene expression than microarrays with a lower background signal. A total of 10 transcriptomes from 5 pairs of NEC lesions and adjacent normal tissues obtained from preterm infants with NEC were analyzed. As a result, a total of 65 genes (57 down-regulated and 8 up-regulated) revealed significantly different expression levels in the NEC lesion compared to the adjacent normal region, based on a significance at fold change ≥ 1.5 and $P \leq 0.05$. The most significant gene, *DPF3* ($P < 0.001$), has recently been reported to have differential expressions in colon segments. Our gene ontology analysis between NEC lesion and adjacent normal tissues showed that down-regulated genes were included in nervous system development with the most significance ($P = 9.3 \times 10^{-7}$; $P_{corr} = 0.0003$). In further pathway analysis using Pathway Express based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, genes involved in thyroid cancer and axon guidance were predicted to be associated with different expression ($P_{corr} = 0.008$ and 0.020, respectively). Although further replications using a larger sample size and functional evaluations are needed, our results suggest that altered gene expression and the genes' involved functional pathways and categories may provide insight into NEC development and aid in future research.

Keywords: Necrotizing Enterocolitis; RNA-Seq; Gene Expression

INTRODUCTION

Necrotizing enterocolitis (NEC) characterized by intestinal ischemia and necrosis is one of the most common gastrointestinal emergencies in premature infants with very low birth weights (VLBWs), i.e., those who weigh less than 1,500 g (1-3). NEC affects about 5%–14% of VLBW neonates. Since NEC is a life-threatening gastrointestinal disease and an unpredictable surgical emergency, the overall mortality from NEC is high, ranging from 25% to 40% (4,5). Many researchers have tried to determine the precise pathophysiology of NEC by examining the various mechanisms that influence NEC development, such as the interaction between intraluminal microbiology and enteral nutrition and the change in inflammatory response by proinflammatory cytokines (6-11). Although several studies have been conducted and their associated hypotheses tested, substantial evidence to confirm risk factors (and effective therapies) for NEC has yet to be determined (12,13), other than prematurity and low birth weight (14).

Although several studies suggest that genetic factors affect NEC development in preterm infants with a potential susceptibility to NEC (15,16), the mechanisms underlying NEC are not fully understood. Previously, an NEC mice model has been used in many studies to establish the causes of or risk factors for human NEC due to several limitations in using human subjects, such as necrosis of human intestinal tissues and non-specific inflammatory changes (17). However, although the animal models contribute to our understanding of disease mechanisms, limitations of reliability and reproducibility make the use of such models controversial (18,19).

Since transcripts are crucial intermediaries between the genome and the proteome, the detection of global gene expression is an important method for understanding molecular mechanisms of diseases and specific functions of particular tissues. Although microarray-based gene expression analysis has become the primary high-throughput platform in the past decade, this method has certain limitations, including high background noise and low resolution (20). Recently, RNA sequencing (RNA-Seq), using high-throughput next-generation sequencing methods, has become a powerful technology providing robust quantification of gene expression levels with a low background signal and high resolution (21,22).

MATERIALS AND METHODS

Study subjects

Study subjects were collected from Seoul National University Bundang Hospital, Gyemyoung University Dongsan Hospital, National Gyeongsang University Hospital, Donga University Hospital, and CHA University Bundang Hospital in Korea. The study protocol was approved by the institutional review board of the hospital, and written informed consent was provided by guardians of all patients. Premature infants at less than 32 weeks of gestational age and less than 1,500 g birth weight were enrolled

in this study.

Two tissue sections (NEC lesion and adjacent normal regions) from the resected small bowel segment were collected as follows: 1) an NEC lesion that showed perforation or necrosis; and 2) adjacent normal tissue. Next, the tissues were immediately stored in liquid nitrogen until RNA extraction at −80°C; a portion of the tissues was evaluated by histological examination. Before RNA extraction, the mucosal layers of each tissue section were collected by the pediatric surgeon who performed the operation on the infants.

RNA isolation and quality control

Total RNA was extracted from the dissected tissue sections (NEC lesion and adjacent normal regions) with the Nucleo-Spin-RNA-II-Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. RNA integrity and purity were analyzed with the Experion™ automated electrophoresis system (Bio-Rad, Hercules, CA, USA) with the Experion RNA StdSens chip. The mRNA was extracted and purified from the total RNA with the TruSeq stranded mRNA HT sample preparation kit (Illumina, Inc., San Diego, CA, USA), and this was followed by a purity check with Qubit 2.0 fluorometer (Life Technologies, Waltham, MA, USA) before proceeding to cDNA synthesis.
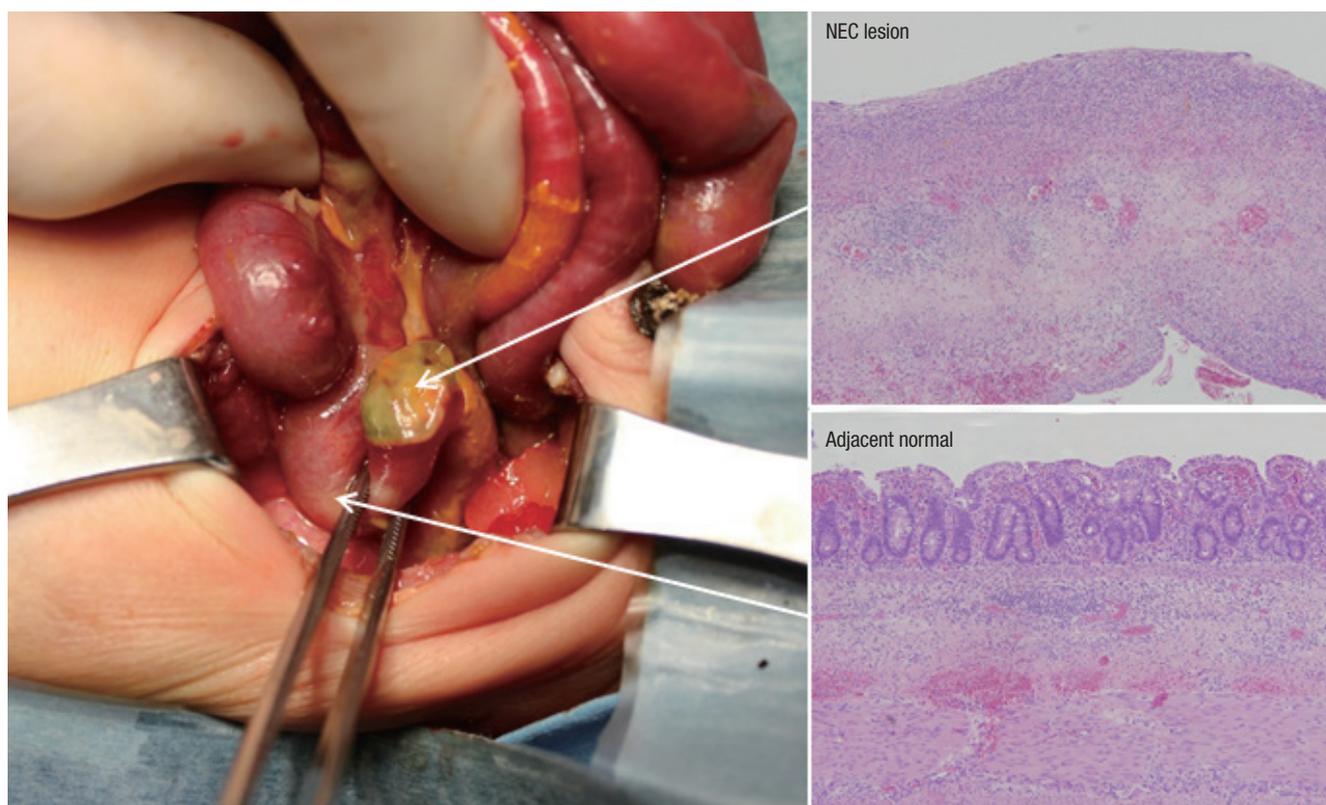
Fig. 1. NEC tissues for experiment and histological features. Arrows indicate 2 tissue sections (NEC lesion and adjacent normal regions) from the resected small bowel segment and each histological examination.
NEC = necrotizing enterocolitis.

Table 1. Down-/up-regulated genes in comparison of NEC lesion and adjacent normal tissues

No.	Down-/up-regulation	Gene	Locus	Expression level (FPKM, average)		Fold change (NEC lesion/adjacent normal)	P value
				NEC lesion (n = 5)	Adjacent normal (n = 5)		
1	Down	HTR3A	Chr11:113845796-113861034	0.71	2.43	0.29	0.030
2	Down	CPLX2	Chr5:175223609-175311023	0.38	1.09	0.35	0.040
3	Down	PCP4	Chr21:41239346-41301322	12.98	36.34	0.36	0.050
4	Down	PCSK2	Chr20:17206751-17465222	0.39	1.08	0.36	0.030
5	Down	ST8SIA6	Chr10:17362675-17496254	0.53	1.39	0.38	0.050
6	Down	SLC5A7	Chr2:108602994-108630443	1.52	3.98	0.38	0.050
7	Down	PCDHGA6	Chr5:140753650-140892546	0.44	1.11	0.40	0.050
8	Down	TMIE	Chr3:46742822-46752413	0.45	1.13	0.40	0.050
9	Down	DNAJC6	Chr1:65730376-65881552	1.69	4.18	0.40	0.030
10	Down	ELAVL4	Chr1:50513685-50667540	5.64	13.81	0.41	0.050
11	Down	SULT4A1	Chr22:44220386-44258378	2.24	5.44	0.41	0.040
12	Down	ATRNL1	Chr10:116853123-117708496	0.59	1.38	0.43	0.050
13	Down	RET	Chr10:43572516-43625797	3.38	7.74	0.44	0.040
14	Down	SPOCK2	Chr10:73818791-73848790	4.04	9.01	0.45	0.007
15	Down	PTGES3L	Chr17:41120104-41132545	1.65	3.66	0.45	0.040
16	Down	PTPRR	Chr12:71031852-71314584	1.71	3.76	0.45	0.030
17	Down	CHODL	Chr21:19289656-19639687	0.79	1.73	0.46	0.010
18	Down	NRXN3	Chr14:78636715-80334633	2.96	6.47	0.46	0.050
19	Down	PIRT	Chr17:10725791-10741418	0.73	1.58	0.46	0.050
20	Down	SCUBE1	Chr22:43599228-43739394	0.57	1.21	0.47	0.020
21	Down	BZRAP1-AS1	Chr17:56402810-56431088	1.07	2.25	0.48	0.020
22	Down	CAMK4	Chr5:110559946-110820748	1.90	3.99	0.48	< 0.001
23	Down	ACTG2	Chr2:74120092-74146780	155.56	322.23	0.48	0.050
24	Down	FAM226B	ChrX:72161567-72163589	0.54	1.11	0.49	0.040
25	Down	LOC151174	Chr2:239133753-239140318	0.64	1.31	0.49	0.050
26	Down	MBNL1-AS1	Chr3:151980404-151987415	2.53	5.05	0.50	0.050
27	Down	IQCH-AS1	Chr15:67695948-67814182	1.46	2.90	0.50	0.020
28	Down	CDK5R1	Chr17:30814104-30818271	1.13	2.23	0.51	0.010
29	Down	FOXN3-AS1	Chr14:89883697-89886137	0.94	1.85	0.51	0.030
30	Down	DPF3	Chr14:73136659-73360809	0.53	1.04	0.51	< 0.001
31	Down	PTPRZ1	Chr7:121513158-121702090	2.98	5.70	0.52	0.020
32	Down	SH3GL2	Chr9:17578952-17797122	0.73	1.37	0.53	0.050
33	Down	SEPT6	ChrX:118749687-118827333	14.02	26.02	0.54	0.050
34	Down	CTNND2	Chr5:10971951-11904110	0.76	1.41	0.54	0.020
35	Down	BAI3	Chr6:69345631-70099403	1.35	2.48	0.54	0.040
36	Down	RUNDC3A	Chr17:42385926-42396038	4.46	8.18	0.55	0.040
37	Down	ARNT2	Chr15:80696691-80890277	1.91	3.42	0.56	0.030
38	Down	SEMA4F	Chr2:74881354-74910981	0.71	1.26	0.56	0.050
39	Down	FAM161B	Chr14:74399694-74417117	1.15	2.04	0.56	0.050
40	Down	RPS6KL1	Chr14:75370656-75389145	0.63	1.11	0.57	0.020
41	Down	GRIP1	Chr12:66741210-67072925	0.89	1.56	0.57	0.020
42	Down	MACROD2	Chr20:13976145-16033841	0.47	0.82	0.57	0.010
43	Down	VAMP1	Chr12:6571403-6579843	3.88	6.76	0.57	0.040
44	Down	C3orf70	Chr3:184795837-184870802	5.02	8.49	0.59	0.040
45	Down	RBM38	Chr20:55966453-55984386	12.64	20.81	0.61	0.020
46	Down	TSPAN11	Chr12:31079837-31149537	0.68	1.11	0.61	0.020
47	Down	LRRC4C	Chr11:40135750-41481186	1.73	2.81	0.62	0.030
48	Down	BCL11A	Chr2:60678301-60780633	2.83	4.47	0.63	0.050
49	Down	MANEAL	Chr1:38259773-38267278	1.49	2.33	0.64	0.050
50	Down	MOXD1	Chr6:132617193-132722664	5.43	8.39	0.65	0.030
51	Down	JAZF1	Chr7:27870192-28220437	7.10	10.87	0.65	0.050
52	Down	TENM3	Chr4:183245136-183724177	1.61	2.43	0.66	0.010
53	Down	PCDHB2	Chr5:140474236-140476964	1.10	1.65	0.67	0.050
54	Down	PODXL2	Chr3:127348001-127391653	4.85	7.29	0.67	0.040

(Continued to the next page)

Table 1. Continued

No.	Down-/up-regulation	Gene	Locus	Expression level (FPKM, average)		Fold change (NEC lesion/adjacent normal)	P value
				NEC lesion (n = 5)	Adjacent normal (n = 5)		
55	Down	KLHL23	Chr2:170590355-170608396	8.80	13.15	0.67	0.050
56	Down	GPC4	ChrX:132435063-132549205	13.27	19.31	0.69	0.030
57	Down	PRKCE	Chr2:45879042-46415129	3.62	5.26	0.69	0.050
58	Up	WT1	Chr11:32409321-32457081	1.48	0.39	3.82	0.050
59	Up	SPAG4	Chr20:34203808-34208965	1.65	0.84	1.97	0.030
60	Up	ADAMTS14	Chr10:72432558-72522195	3.87	2.05	1.89	0.040
61	Up	GHRLOS	Chr3:10322635-10335133	1.02	0.55	1.85	0.030
62	Up	DSTNP2	Chr12:6993845-6994950	4.34	2.35	1.85	0.020
63	Up	ZNF503-AS2	Chr10:77161285-77168740	1.07	0.68	1.57	0.030
64	Up	GBAP1	Chr1:155183615-155197325	5.97	3.91	1.53	0.030
65	Up	MCOLN1	Chr19:7587495-7598895	9.50	6.22	1.53	0.020
		GAPDH*	Chr12:6643584-6647537	1,320.16	1,255.20	1.05	-

"-" in the fold change indicates down regulation.
NEC = necrotizing enterocolitis, Chr = chromosome.
*GAPDH indicates a housekeeping gene.

RNA-Seq and data analysis

RNA-Seq was carried out in order to identify differentially expressed genes in the NEC lesion and adjacent normal tissues. The isolated mRNA from 4 µg total RNA using oligo-dT magnetic beads was fragmented and primed at 94°C for 8 minutes, and then prepared for sequencing according to the protocol of the TruSeq stranded mRNA HT sample preparation kit (Illumina, Inc.). The resulting complementary DNA (cDNA) libraries were sequenced on the Illumina MiSeq system with 75 bp paired-end reads using the Illumina MiSeq sequencing kit v3 (150 cycles; Illumina, Inc.) following the manufacturer's instructions. Image processing and base calling were performed using the Illumina Real Time Analysis Software RTA v1.9.35. FastQ sequences were aligned to the human genome database (NCBI37/hg19) using TopHat v.2.0.12 with default parameters. The reads were mapped using the gene models as provided in the annotation GTF file (GRCh37.75). Gene expression values were determined using Cufflinks 2.2.1 release (http://cole-trapnell-lab.github.io/cufflinks/), and the FPKM (fragments per kilobase of exon per million fragments mapped) values were calculated for each transcript. Cufflinks default settings were adopted, and FPKM values were computed by summing the values of different transcripts of the same gene. To identify differentially expressed genes, a fold change over 1.5 between any pairwise comparisons was applied. For statistical analysis, data were examined by t-test.

Ethics statement

The present study protocol was reviewed and approved by the Institutional Review Board of Seoul National University Bundang Hospital (IRB No. B-1404-245-008). Informed consent was submitted by guardians of all patients.

RESULTS

Patients

A total of 5 NEC patients, with median gestational ages of 26 weeks and 2 days, birth weight of 922 g, and birth height of 34.2 cm, were enrolled in this study. None of the patients showed other congenital anomalies in the perinatal period, and the mothers had no antenatal/perinatal problems except for premature delivery. The exploratory laparotomy was performed in the NEC patients at a median gestational age of 29 weeks and 1 day. The operations included a segmental resection with temporary ileostomy or primary anastomosis. All patients recovered from NEC and survived without complications. Following surgery, a histological examination of NEC lesion and adjacent normal tissues from the patients was performed (Fig. 1).

RNA-Seq analysis and gene expression comparison

To investigate the gene expression profiles for NEC development in preterm infants, RNA-Seq analysis using the Illumina MiSeq system was performed. Mapping of sequences resulted in an average read count of 11.75×10^6 ($\pm 4.36 \times 10^6$) in 10 RNA samples composed of those from 2 paired small-bowel sections (NEC lesion and adjacent normal tissues) from each of 5 NEC patients. Of the 23,972 tested genes, a total of 65 genes (57 down-regulated and 8 up-regulated) were observed to have significantly different expression levels in the comparison between NEC lesion and adjacent normal tissues, based on a significance at fold change ≥ 1.5 and $P \leq 0.05$ (Table 1). As a housekeeping gene, GAPDH was measured as 1,320.16 in NEC lesion and 1,255.20 in adjacent normal tissue (fold change = 1.05).

Among the differentially expressed genes in NEC lesions compared to the adjacent normal region, *double PHD fingers 3* (*DPF3*, *P* < 0.001) and *calcium/calmodulin-dependent protein kinase*

Table 2. Gene ontology analysis of differentially expressed genes in comparison of NEC lesion and adjacent normal tissues

Down-/up-regulation	Category	Gene ontology category	Observed genes	Observed genes number	Expected genes number	Ratio of enrichment	Significance of enrichment	
							P value	Corrected P value
Down	Biological process	Nervous system development	DPF3, LRRC4C, PCSK2, MACROD2, RET, PCP4, PCDHB2, CDK5R1, BCL11A, NRXN3, SEMA4F, SH3GL2, PTPRR, PTPRZ1, CPLX2, ARNT2, SPOCK2	17	4.71	3.61	9.3×10^{-7}	< 0.001
Down	Biological process	Neuron cell-cell adhesion	CTNND2, CDK5R1, RET	3	0.03	91.44	4.1×10^{-6}	0.001
Down	Biological process	System development	DPF3, LRRC4C, PCSK2, MACROD2, RET, CAMK4, PCP4, PCDHB2, CHODL, CDK5R1, RBM38, BCL11A, NRXN3, BAI3, SH3GL2, SEMA4F, SCUBE1, PTPRR, CPLX2, PTPRZ1, ARNT2, TMIE, SPOCK2	23	9.63	2.39	6.0×10^{-6}	0.002
Down	Biological process	Synaptic transmission	NRXN3, SH3GL2, VAMP1, HTR3A, CPLX2, CAMK4, GRIP1, SLC5A7, PCDHB2, CTNND2	10	1.78	5.62	7.2×10^{-6}	0.003
Down	Biological process	Single-multicellular organism process	DPF3, LRRC4C, PCSK2, MACROD2, RET, CAMK4, SLC5A7, PCP4, PCDHB2, CHODL, ACTG2, CDK5R1, RBM38, BCL11A, NRXN3, BAI3, SH3GL2, VAMP1, SEMA4F, SCUBE1, PTPRR, HTR3A, CPLX2, PTPRZ1, GRIP1, ARNT2, TMIE, SPOCK2, CTNND2	29	15.34	1.89	1.2×10^{-5}	0.004
Down	Biological process	Multicellular organismal process	DPF3, LRRC4C, PCSK2, MACROD2, RET, CAMK4, SLC5A7, PCP4, PCDHB2, CHODL, ACTG2, CDK5R1, RBM38, BCL11A, NRXN3, BAI3, SH3GL2, VAMP1, SEMA4F, SCUBE1, PTPRR, HTR3A, CPLX2, PTPRZ1, GRIP1, ARNT2, TMIE, SPOCK2, CTNND2	29	15.43	1.88	1.4×10^{-5}	0.005
Down	Biological process	Anatomical structure development	DPF3, LRRC4C, PCSK2, MACROD2, RET, CAMK4, PCP4, PCDHB2, CHODL, CDK5R1, RBM38, BCL11A, NRXN3, BAI3, SH3GL2, SEMA4F, SCUBE1, PTPRR, CPLX2, PTPRZ1, ARNT2, TMIE, SPOCK2, CTNND2	24	11.02	2.18	1.7×10^{-5}	0.006
Down	Biological process	Multicellular organismal development	DPF3, LRRC4C, PCSK2, MACROD2, RET, CAMK4, PCP4, PCDHB2, CHODL, CDK5R1, RBM38, BCL11A, NRXN3, BAI3, SH3GL2, SEMA4F, SCUBE1, PTPRR, CPLX2, PTPRZ1, ARNT2, TMIE, SPOCK2, CTNND2	24	11.15	2.15	2.1×10^{-5}	0.007
Down	Biological process	Transmission of nerve impulse	NRXN3, SH3GL2, VAMP1, HTR3A, CPLX2, CAMK4, GRIP1, SLC5A7, PCDHB2, CTNND2	10	2.01	4.98	2.1×10^{-5}	0.007
Down	Biological process	Multicellular organismal signaling	NRXN3, SH3GL2, VAMP1, HTR3A, CPLX2, CAMK4, GRIP1, SLC5A7, PCDHB2, CTNND2	10	2.05	4.87	2.5×10^{-5}	0.009
Down	Biological process	Neurotransmitter secretion	NRXN3, SLC5A7, VAMP1, CPLX2	4	0.27	14.78	< 0.001	0.030
Down	Biological process	Cell-cell signaling	NRXN3, SH3GL2, SEMA4F, VAMP1, HTR3A, CPLX2, CAMK4, GRIP1, SLC5A7, PCDHB2, CTNND2	11	3.00	3.66	< 0.001	0.030
Down	Cellular component	Synapse	SEMA4F, VAMP1, SEPT6, HTR3A, CPLX2, GRIP1, CDK5R1, CTNND2	8	1.20	6.64	2.2×10^{-5}	0.002
Down	Cellular component	Synapse part	GRIP1, SEMA4F, VAMP1, SEPT6, CTNND2, CDK5R1, HTR3A	7	0.91	7.68	3.0×10^{-5}	0.002
Up	Biological process	Extracellular structure organization	ADAMTS14, WT1	2	0.06	34.50	0.001	0.030
Up	Biological process	Extracellular matrix organization	ADAMTS14, WT1	2	0.06	34.67	0.001	0.030

Gene ontology categories with corrected P value of enrichment significance below 0.05 are shown.
NEC = necrotizing enterocolitis.

Table 3. Potential pathways affected by differentially expressed genes in comparisons of NEC lesion and adjacent normal tissues

Pathway name	Differentially regulated genes	Input genes in pathway, %	Impact factor	Corrected P value
Thyroid cancer	RET	1.587	6.914	0.008
Axon guidance	LRRC4C, SEMA4F	3.175	5.740	0.020

Corrected P value is obtained using the classical hypergeometric model (32).
NEC = necrotizing enterocolitis.

IV (CAMK4, P < 0.001) showed relatively robust association signals of upregulation, whereas downregulated genes showed weak signals (Table 1). In addition, 3 genes (PCP4, PTPRR, and WT1), which were recently reported as potential genes of NEC, were also observed to be differentially expressed in the NEC lesion (Table 1).

Ontology and pathway analyses of differentially expressed genes in NEC

To assess the biological functions of the differentially expressed genes in NEC lesions and adjacent normal tissues, this study performed a gene ontology analysis using the WEB-based GEne SeT AnaLysis Toolkit (http://bioinfo.vanderbilt.edu/webgestalt/). As a result, 16 gene ontology categories (14 in biological processes and 2 in the cellular component) were predicted to affect NEC development in humans (Table 2), with the most significant signal at nervous system development ($P = 9.3 \times 10^{-7}$; $P_{corr} = 0.0003$). In additional pathway analysis using Pathway Express (http://vortex.cs.wayne.edu/projects.htm) based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, genes involved in thyroid cancer and axon guidance showed significant associations (Table 3, $P_{corr} = 0.008$ and 0.02, respectively).

DISCUSSION

Acquired conditions of diffuse necrotic injury to the intestinal segments are known to affect NEC development. Abnormal bacterial colonization and formula feeding have also been implicated as predisposing factors for NEC in humans (23,24). In addition, potential associations between NEC and environmental factors (such as microbiome, microbiome-intestinal reaction to breast milk or formula milk feeding, vaginal or cesarean section mode of delivery, and antibiotics) have been reported (10,11,23-25). Interestingly, a significant reduction of NEC in infants who were fed breast milk, compared to those who were fed formula, has been reported (26). Thus, many neonatologists have gone to great effort to manage the microbiome to prevent NEC development. Many neonatologists in Korea have changed their management protocols for preterm infants and observed a decreased incidence of NEC during the last few years.

NEC development may be multifactorial with the interplay between intrinsic and extrinsic factors. In addition, the main

risk factor for NEC development in premature infants is thought to be intestinal immaturity (23,27), suggesting that intrinsic risk factors may be more important because premature infants have had a short exposure time to external environments. In this study, we hypothesized that global gene expression profiling may reveal distinct genetic differences between NEC lesion and adjacent normal region. Although candidate genes in this study did not reach great values of significance, several potential genes (such as DPF3 and CAMK4) with relatively robust association signals were identified (P < 0.001). These markers may have a role in NEC development. However, further replication and evaluation studies are needed.

As noted, this study showed relatively robust association signals at DPF3 and CAMK4. DPF3, which is known as an epigenetic key factor for the development of heart and muscle tissue, has been reported to play a role in the neuronal differentiation process and also to take part in the disassembly of muscular fibers (28). In the different colon segments of Hirschsprung's disease, the gene product of DPF3 has been observed to be lowly expressed in a stenotic segment, whereas it is highly expressed in proximal anastomosis (29), suggesting that DPF3 may be dysregulated in colonic diseases such as NEC. In the case of CAMK4, although a direct association between CAMK4 and NEC has not been reported, several connections in the literature related to necrosis can be found. In particular, CAMK4 was observed to be involved in the necrosis factor (NF)-kappaB mediated signaling pathway in human endothelial cells (30). These previous results and our findings suggest that dysregulated expressions of genes identified in this study may contribute to NEC development.

Recently, the first RNA-Seq for gene expression profiling in NEC was reported (31). This first RNA-Seq study used ileum tissues from preterm patients with other diseases for the control, and several genes associated with immune functions (in particular, genes associated with Crohn's disease) were identified as contributing factors to NEC development, together with other candidate genes. When compared to our results, PCP4 and PTPRR were overlapped; however, no connections in the literature related to NEC or related cellular functions (such as necrosis) could be found. Therefore, further studies are required to elucidate the association between these potential genes and NEC development.

So as to remove the heterogeneity of genetic background, this study excluded non-Korean parents. However, the study also has several limitations, such as insufficient sample size and lack of functional evaluation. The small sample size was due to the overall decreased incidence of NEC. In addition, normal tissues from the small bowel segment in infants without NEC or related diseases would have been ideal for the comparison analysis; however, it was limited to obtain these normal tissues. Although the first RNA-Seq analysis study of NEC used the ileum for the nor-

mal control (31), this study used adjacent normal tissues, and we do not rule out the possible effect of congenital diseases (such as small intestinal perforation, intestinal atresia, etc.).

In conclusion, despite study limitations, our preliminary results have identified potential involvements of certain genes (such as *DPF3* and *CAMK4*) in NEC development, suggesting that these genetic factors, perhaps together with epigenetic factors such as microbiomes and breast milk, may have a role in NEC development in humans. Further validation studies are needed to determine clinical applications of these potential targets.

DISCLOSURE

The authors have no potential conflicts of interest to disclose.

AUTHOR CONTRIBUTION

Conceptualization: Jung K, Koh I, Shin HD. Data curation: Jung K, Park T, Nam SH, Jung SM, Sio CA, Kim SY, Jung E, Lee B, Kim HR, Jung SE, Choi CW, Kim BI, Jung E. Funding acquisition: Investigation: Jung K, Koh I, Shin HD. Koh I, Kim JH, Cheong HS, Shin E. Writing - original draft: Jung K, Koh I, Shin HD.

ORCID

Kyuwhan Jung http://orcid.org/0000-0001-5997-4975
InSong Koh http://orcid.org/0000-0001-6896-9748
Jeong-Hyun Kim http://orcid.org/0000-0003-4879-0075
Hyun Sub Cheong http://orcid.org/0000-0003-1749-6172
Taejin Park http://orcid.org/0000-0002-8508-2353
So Hyun Nam http://orcid.org/0000-0003-3757-4684
Soo-Min Jung http://orcid.org/0000-0002-2636-8028
Cherry Ann Sio http://orcid.org/0000-0003-0273-8239
Su Yeong Kim http://orcid.org/0000-0001-6463-0210
Euiseok Jung http://orcid.org/0000-0003-0693-5627
Byoungkook Lee http://orcid.org/0000-0001-5435-606X
Hye-Rim Kim http://orcid.org/0000-0001-6445-5934
Eun Shin http://orcid.org/0000-0003-0624-8149
Sung-Eun Jung http://orcid.org/0000-0003-3155-2900
Chang Won Choi http://orcid.org/0000-0003-1911-0253
Beyong Il Kim http://orcid.org/0000-0003-0408-9279
Eunyoung Jung http://orcid.org/0000-0002-8884-1642
Hyoung Doo Shin http://orcid.org/0000-0003-1732-7838

REFERENCES

1. Bisquera JA, Cooper TR, Berseth CL. Impact of necrotizing enterocolitis on length of stay and hospital charges in very low birth weight infants. *Pediatrics* 2002; 109: 423-8.
2. Blakey JL, Lubitz L, Campbell NT, Gillam GL, Bishop RF, Barnes GL. Enteric colonization in sporadic neonatal necrotizing enterocolitis. *J Pediatr Gastroenterol Nutr* 1985; 4: 591-5.
3. Hintz SR, Kendrick DE, Stoll BJ, Vohr BR, Fanaroff AA, Donovan EF, Poole WK, Blakely ML, Wright L, Higgins R; NICHD Neonatal Research Network. Neurodevelopmental and growth outcomes of extremely low birth weight infants after necrotizing enterocolitis. *Pediatrics* 2005; 115: 696-703.
4. Holman RC, Stoll BJ, Clarke MJ, Glass RI. The epidemiology of necrotizing enterocolitis infant mortality in the United States. *Am J Public Health* 1997; 87: 2026-31.
5. Lemons JA, Bauer CR, Oh W, Korones SB, Papile LA, Stoll BJ, Verter J, Temprosa M, Wright LL, Ehrenkranz RA, et al. Very low birth weight outcomes of the National Institute of Child health and human development neonatal research network, January 1995 through December 1996. NICHD Neonatal Research Network. *Pediatrics* 2001; 107: E1.
6. Hunter CJ, Upperman JS, Ford HR, Camerini V. Understanding the susceptibility of the premature infant to necrotizing enterocolitis (NEC). *Pediatr Res* 2008; 63: 117-23.
7. Morowitz MJ, Poroyko V, Caplan M, Alverdy J, Liu DC. Redefining the role of intestinal microbes in the pathogenesis of necrotizing enterocolitis. *Pediatrics* 2010; 125: 777-85.
8. Neu J, Mihatsch W. Recent developments in necrotizing enterocolitis. *JPEN J Parenter Enteral Nutr* 2012; 36: 30S-35S.
9. Sharma R, Hudak ML. A clinical perspective of necrotizing enterocolitis: past, present, and future. *Clin Perinatol* 2013; 40: 27-51.
10. Sim K, Shaw AG, Randell P, Cox MJ, McClure ZE, Li MS, Haddad M, Langford PR, Cookson WO, Moffatt MF, et al. Dysbiosis anticipating necrotizing enterocolitis in very premature infants. *Clin Infect Dis* 2015; 60: 389-97.
11. Cortese R, Lu L, Yu Y, Ruden D, Claud EC. Epigenome-Microbiome crosstalk: a potential new paradigm influencing neonatal susceptibility to disease. *Epigenetics* 2016; 11: 205-15.
12. Christensen RD, Lambert DK, Baer VL, Gordon PV. Necrotizing enterocolitis in term infants. *Clin Perinatol* 2013; 40: 69-78.
13. Stoll BJ. Epidemiology of necrotizing enterocolitis. *Clin Perinatol* 1994; 21: 205-18.
14. Kanto WP Jr, Wilson R, Breart GL, Zierler S, Purohit DM, Peckham GJ, Ellison RC. Perinatal events and necrotizing enterocolitis in premature infants. *Am J Dis Child* 1987; 141: 167-9.
15. Chan KY, Leung KT, Tam YH, Lam HS, Cheung HM, Ma TP, Lee KH, To KF, Li K, Ng PC. Genome-wide expression profiles of necrotizing enterocolitis versus spontaneous intestinal perforation in human intestinal tissues: dysregulation of functional pathways. *Ann Surg* 2014; 260: 1128-37.
16. Ng PC, Chan KY, Poon TC. Biomarkers for prediction and diagnosis of necrotizing enterocolitis. *Clin Perinatol* 2013; 40: 149-59.
17. Tian R, Liu SX, Williams C, Soltau TD, Dimmitt R, Zheng X, De Plaen IG. Characterization of a necrotizing enterocolitis model in newborn mice. *Int J Clin Exp Med* 2010; 3: 293-302.
18. Hackam DG, Redelmeier DA. Translation of research evidence from animals to humans. *JAMA* 2006; 296: 1731-2.
19. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, Macleod MR. Can animal models of disease reliably inform human studies? *PLoS Med* 2010; 7: e1000245.
20. Ioannidis JP, Allison DB, Ball CA, Coulibaly I, Cui X, Culhane AC, Falchi M, Furlanello C, Game L, Jurman G, et al. Repeatability of published microarray gene expression analyses. *Nat Genet* 2009; 41: 149-55.
21. Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPher-

son A, Szcześniak MW, Gaffney DJ, Elo LL, Zhang X, et al. A survey of best practices for RNA-seq data analysis. *Genome Biol* 2016; 17: 13.

22. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009; 10: 57-63.

23. Lin PW, Stoll BJ. Necrotising enterocolitis. *Lancet* 2006; 368: 1271-83.

24. Lucas A, Cole TJ. Breast milk and neonatal necrotising enterocolitis. *Lancet* 1990; 336: 1519-23.

25. Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, Sun J, Antonopoulos DA, Chang EB, Claud EC. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *ISME J* 2009; 3: 944-54.

26. Repa A, Thanhaeuser M, Endress D, Weber M, Kreissl A, Binder C, Berger A, Haiden N. Probiotics (Lactobacillus acidophilus and Bifidobacterium infantis) prevent NEC in VLBW infants fed breast milk but not formula [corrected]. *Pediatr Res* 2015; 77: 381-8.

27. Claud EC. Neonatal necrotizing enterocolitis -inflammation and intestinal immaturity. *Antiinflamm Antiallergy Agents Med Chem* 2009; 8: 248-59.

28. Lange M, Kaynak B, Forster UB, Tönjes M, Fischer JJ, Grimm C, Schlesinger J, Just S, Dunkel I, Krueger T, et al. Regulation of muscle development by DPF3, a novel histone acetylation and methylation reader of the BAF chromatin remodeling complex. *Genes Dev* 2008; 22: 2370-84.

29. Liu H, Luo Y, Li S, Wang S, Wang N, Jin X. Expression profiles of HA117 and its neighboring gene DPF3 in different colon segments of Hirschsprung's disease. *Int J Clin Exp Pathol* 2014; 7: 3966-74.

30. You J, Peng W, Lin X, Huang QL, Lin JY. PLC/CAMK IV-NF-kappaB involved in the receptor for advanced glycation end products mediated signaling pathway in human endothelial cells. *Mol Cell Endocrinol* 2010; 320: 111-7.

31. Tremblay É, Thibault MP, Ferretti E, Babakissa C, Bertelle V, Bettolli M, Burghardt KM, Colombani JF, Grynspan D, Levy E, et al. Gene expression profiling in necrotizing enterocolitis reveals pathways common to those reported in Crohn's disease. *BMC Med Genomics* 2016; 9: 6.

32. Draghici S, Khatri P, Tarca AL, Amin K, Done A, Voichita C, Georgescu C, Romero R. A systems biology approach for pathway level analysis. *Genome Res* 2007; 17: 1537-45.