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*ITGB2 (*Integrin β2) immunomodulatory gene variants in premature infants with necrotizing enterocolitis

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Abstract

Aberrant Toll Like receptor (TLR) activation is central to necrotizing enterocolitis (NEC) pathogenesis. β2 integrins regulate TLR signaling, and Integrin β2 (ITGB2) deficiency causes TLR hyper-responsiveness. To test the hypothesis that *ITGB2* genetic variants modulate NEC susceptibility, we sequenced the exonic *ITGB2* locus to compare the prevalence of deleterious variants among 221 preterm infants with and without NEC. *ITGB2* variants were not associated with NEC in our entire cohort [NEC (9/56) *vs*. controls (16/165), p=0.19)], or in extremely low birthweight infants [ELBW, controls (7.9%) vs. NEC (18.2%); p=0.11)] but was increased compared to the populace (4.5%, gnomad.broadinstitute.org). Combined Annotation Dependent Depletion (CADD)-predicted deleterious *ITGB2* variants increased proportionately with increasing NEC severity in ELBW infants [controls (6.7%) *vs*. medical NEC (16.7%) *vs*. surgical NEC (19%), p=0.03]. While *ITGB2* variants were not associated with NEC in our preterm cohort, sub-group analysis showed a trend towards enrichment with NEC severity in ELBW infants.

What is known

- Genetic variation in immunomodulatory genes can alter susceptibility to Necrotizing enterocolitis (NEC)
- Toll Like Receptor (TLR) - mediated immune responses play a critical role in NEC pathogenesis
- Deficiency of Integrin β2 (ITGB2) causes hyper-responsiveness to TLR stimulation.

What is new

- First study to explore role of *ITGB2* variants in NEC susceptibility and severity
- The entire *ITGB2* exonic locus was sequenced to identify whether rare or common *ITGB2* variants are enriched in NEC infants
- The prevalence of CADD-predicted deleterious *ITGB2* variants increased with NEC severity in ELBW infants, supporting its potential role as modulator of NEC severity in ELBW infants.

Introduction

The pathogenesis of NEC is multifactorial, involving complex interactions between intestinal microbiota and the immature immune system[1]. While several risk factors have been identified, factors implicated cannot account for significant variability in incidence and severity of NEC. This has led to the recognition that genetic factors might modulate NEC susceptibility[2]. However, the genetic basis of altered immune responses in NEC remains incompletely understood[2]. Studies have shown that variants in immune genes can alter the risk of NEC[3]. Toll Like Receptors (TLRs) regulate immune responses to bacteria and are critical for maintenance of intestinal bacterial tolerance. TLR4 recognizes lipopolysaccharide (LPS) in Gram-negative bacteria. There is increased TLR4 expression in human NEC, and activation of TLR4 by enteric bacteria leads to epithelial barrier injury and inflammation [3]. Studies have shown that mice with loss of intestinal TLR4 signaling are protected against experimental NEC[3]. Prior work from our group has identified TLR pathway variants as potential modulators of preterm NEC[4,5].

β2 integrins are molecular mediators of cell-cell interactions that are known to regulate TLR-mediated inflammatory responses[6]. ITGB2, also known as CD18, a key member of the integrin family, has established roles in T-cell development and function, and plays a crucial role in host defense by regulating neutrophil recruitment and apoptosis[6]. Mutations in the *ITGB2* gene are an established cause of Leucocyte Adhesion Deficiency [7]. ITGB2 has also been linked to chronic colitis in human and animal models, and *ITGB2* variants are reported to play a role in Hirschsprung's associated enterocolitis[7]. Moreover, *Itgb2*$^{-/-}$ mice demonstrate hyper-responsiveness to TLR stimulation[6]. As TLR activation plays a central role in NEC pathogenesis, we hypothesized that *ITGB2* genetic variants would predispose preterm infants to NEC. To test this hypothesis, we sequenced all the exons of *ITGB2* to determine the relationship between *ITGB2* variants and NEC susceptibility in premature infants.

Methods

Data and samples were obtained from infants at the level IV NICU, and the outpatient clinics at Children's Mercy Kansas City (Kansas City, MO), neonatal nurseries at Children's hospitals and clinics of Minnesota (Minneapolis, MN) and three hospitals in Atlanta, GA (Emory University Hospital Midtown, Grady Memorial Hospital and Northside Hospital) after institutional review board approval. Informed consent was obtained from parents. Clinical data was de-identified and entered into a password protected database.

Eligibility criteria: Premature infants born at < 36 weeks of gestation with stage II+ NEC and their gestational age matched controls were eligible. Infants with major congenital anomalies and known genetic diagnoses were excluded. Diagnosis and staging of NEC were based on modified Bell's criteria[8]. We included infants with Stage IIA+ NEC who were treated with bowel rest and antibiotics for ≥7 days. Infants with spontaneous intestinal perforation (SIP) and stage 1 NEC were excluded.

DNA extraction and sequencing: Blood, buccal swabs, or autopsy tissue specimens were collected for DNA isolation. DNA was extracted using the Flexigene kit (Qiagen Inc., Valencia, CA). A custom amplicon exome sequencing panel was designed to sequence all 16 coding exons of the *ITGB2* gene (TruSeq Custom Amplicon, Illumina). Targeted sequencing of the entire exonic *ITGB2* gene locus at 100x coverage was performed using bar-coded, multiplexed, high-throughput sequencing (MiSeq, Illumina Inc.).

Variant annotation and interpretation: Genetic variants were annotated using Rapid Understanding of Nucleotide variant Effect Software (RUNES)[9,10.11]. Potentially deleterious variants were identified using VIKING software[12] (Variant Integration and Knowledge Interpretation in Genomes). Only deleterious or potentially deleterious variants (American College of Medical Genetics (ACMG) class 1-3), were included in our analysis. Variants were assigned to 5 categories for analysis: category 1 variants have been previously reported in association with human disease, category 2 variants are expected to be deleterious (loss of initiation, premature stop codon, frameshift deletion/insertion etc.), and category 3 variants are potentially deleterious (non-synonymous substitution, in-frame deletion/insertion etc.)[11]. Category 4 and 5 variants, not expected to be deleterious, were excluded. Identified variants were further analyzed using Combined Annotation-Dependent Depletion (CADD), which predicts deleteriousness of variants by providing scaled CADD scores for each *ITGB2* variant [13].

Statistical Analysis: This was a prospective case control study. To calculate power, a catalogue of known missense and loss of function (LOF) *ITGB2* variants (ACMG class 1-3) was collated using the ExAC population database (http://exac.broadinstitute.org/), which has exome sequencing data from 60,706 unrelated individuals. Data from ExAC estimated that potentially deleterious, missense, and LOF variants of the *ITGB2* gene have a prevalence of about ~ 6.0% in the general population (database accessed on 6/6/2016). Based on this data, it was estimated that 55 infants with NEC and 160 gestational age matched controls would give us > 80% power to detect a 3-fold increase in prevalence of *ITGB2* variants in premature infants with NEC. Continuous variables were compared using one-way analysis of variance, categorical variables were compared using chi-square or Fisher's exact test. A p value of <0.05 was considered significant. For genetic analysis, the proportion of infants with and without stage II+ NEC who had ≥ 1 ACMG class 1- 3 variants were compared. A priori was to examine *ITGB2* variants across infants with no NEC, medical NEC, and surgical NEC using the Cochran Armitage (CA) trend test in extremely low birthweight (ELBW) infants.

Results

NEC demographics: We enrolled 221 premature infants <36 weeks (23-35 weeks), 56 with stage II+ NEC (medical NEC=21; surgical NEC=35) and 165 controls. 70.1% of infants were delivered at gestational age < 29 weeks. Infants with NEC were less likely to be inborn, have received antenatal steroids or be Caucasian. There were no differences in gestational age, birthweight, sex, 5-minute Apgar scores, proportion that received prenatal care, clinical chorioamnionitis, or feed type between the groups (Table 1).

ITGB2 analysis: Among the 221 infants sequenced, we identified 25 infants with ≥ 1 ACMG class 1-3 *ITGB2* variants, with minor allele frequency (MAF) of individual variants varying from 0.000008 to 0.008. The number of variants varied from 1-5 variants per infant, with a total of 33 variants detected (Supplement table 1 , http://links.lww.com/MPG/B966). Potentially deleterious *ITGB2* variants were found in 16/165 (9.7%) controls and 9/56 (16.1%) NEC infants (OR=1.78, 95% CI: 0.74 - 4.3, p=0.19). Cochran Armitage (CA) tests revealed a non-significant trend towards enrichment of *ITGB2* variants with increasing severity of disease (no NEC vs. medical NEC vs. surgical NEC; p=0.18) (Fig.1A). Interestingly, the prevalence of *ITGB2* variants among NEC infants (16.1%) and controls (9.7%) was higher compared to the population distribution of similar *ITGB2* variants in gnomAD (4.3%, 10282/239,243; accessed May 2020) or ExAC (6%; accessed June 2016) databases.

Subgroup analysis of infants < 1000 gm (ELBW, n=122) revealed similar demographic/clinical risk factors for NEC (Supplementary table 2 , http://links.lww.com/MPG/B967). ACMG class 1-3 variants were found in 7/89 (7.9%) of controls and 6/33 (18.2%) of NEC infants (OR=2.6, 95% CI; 0.8 – 8.4, p=0.11). CA tests revealed a non-significant enrichment of *ITGB2* variants (p=0.10) with increasing NEC severity (Fig.1B).

ITGB2 analysis based on CADD scores: To incorporate software-based prediction of variant deleteriousness in our analysis we calculated CADD scores[13]. Among the 33 variants, 28 variants had a scaled CADD score of >10 [top 10% of all deleterious variants in human genome] and was used for further analysis (Supplement table 1 , http://links.lww.com/MPG/B966). Based on this, predicted deleterious *ITGB2* variants were found in 14/165 (8.5%) *vs*. 2/21 (9.5%) *vs*. 5/35 (14.3%) of control *vs*. medical NEC *vs*. surgical NEC infants (CA trend; p=0.15). In ELBW infants, we noted a significant enrichment of ITGB2 variants with increasing NEC severity [Control vs. medical NEC vs. surgical NEC; 6/89 (6.72%) *vs*. 2/12 (16.7%) vs. 4/21 (19%), CA trend; p =0.032].

Discussion

While there is increasing acceptance that NEC susceptibility is genetically determined, very few genetic loci have been validated. We have previously published data on *TLR* genetic variants[4,5] and reported that *SIGRR* variants may increase susceptibility to NEC through loss of inhibition of TLR4- mediated inflammation. Hartel et al. found that while no single *NOD2* (Nucleotide binding oligomerization domain containing protein 2) variant was associated with NEC, the presence of 2 or more *NOD2* variants was associated with increased NEC risk [14]. A major limitation of most published studies includes not examining rare variants (MAF< 1%), which while individually rare, contribute to > 80% of genetic variation in human population[15]. Jilling et al. performed the first genome wide association study (GWAS) in NEC and identified a cluster of SNPs in chromosome 8 that have the strongest association for NEC[16].

We targeted *ITGB2*, a known regulator of TLR signaling, as aberrant TLR activation is implicated in NEC. β2 integrins can limit TLR signaling by inhibiting the activation of NFκB[6]. Yee et al. demonstrated that β2 integrin-deficient macrophages are hyper-responsive to TLR stimulation, and *Itgb2*$^{-/-}$ mice have increased production of LPS-induced cytokines[6]. To identify rare variants in *ITGB2* gene that could impact NEC risk, we sequenced all the 16 exons and splice sites in 221 infants. We found non-significant enrichment of potentially deleterious *ITGB2* variants in surgical NEC (almost two-fold). CADD-predicted *ITGB2* variants proportionately increased with NEC severity in ELBW infants in sub-group analysis. Our study was powered based on the expected prevalence of deleterious/potentially deleterious *ITGB2* variants being ~ 6%. The prevalence of deleterious/potentially deleterious variants in *ITGB2* was 16.1% (x 3 times population prevalence) in NEC infants. However, the prevalence of ACMG 1-3 variants among controls in our study population was almost twice as planned (9.7%). Although the study was adequately powered based on prevalence in the general population, we were underpowered to

detect a significant difference in our study population in view of the higher than expected prevalence of variants in controls. The higher prevalence among controls could also be from enrichment of *ITGB2* variants in premature infants. While we did find a significant trend towards enrichment of *ITGB2* variants with NEC severity in ELBW infants, suggesting a potential role in modulating NEC severity in most vulnerable population, the small N limits the generalizability of our results till replication in an independent cohort.

The primary pathophysiologic process in NEC is aberrant activation of mucosal innate immune signaling with subsequent breakdown of the mucosal barrier, and neutrophil-mediated injury is a secondary insult. This could be another potential reason why *ITGB2*, that plays a very significant role in neutrophil recruitment and apoptosis, does not seem to have a significant effect on NEC susceptibility but might play a role in modulating the severity of disease. While we compared the prevalence of ACMG class 1-3 variants between case and controls, we did not evaluate functionality of class 3 variants. Analysis based on CADD-predicted thresholds of top 10% of deleterious variants in the human genome suggested enrichment of *ITGB2* variants with NEC severity in ELBW infants. A larger cohort of ELBW infants and in vitro functional analysis would have clarified the relationships between *ITGB2* variants and NEC severity better.

The known role of *ITGB2* gene in immunomodulatory pathways and colonic inflammation support a potential role for *ITGB2* variants in NEC pathogenesis. In our study, known epidemiological variables associated with NEC, such as African American race, and lack of prenatal steroids were confirmed[17,18]. Although we did not find associations between *ITGB2* variants and NEC in our cohort, we found a non-statistically significant enrichment in surgical NEC in ELBW infants, and a significant association with increasing NEC severity when only CADD-predicted deleterious variants were analyzed. This study had several strengths including a comprehensive sequencing strategy that allows for querying of all rare and common variants, use of standardized guidelines for classifying variants, analysis based on predicted deleteriousness scores (CADD), prospective case-control design, and powered study. Limitations include lack of adequate power for sub-group analysis and lack of functional analysis of identified variants. Despite these limitations, this is one of the largest studies to date that has used a comprehensive sequencing-based targeted approach. Considering that NEC susceptibility is likely to be polygenic, approaches incorporating *ITGB2* variants to other putative NEC susceptibility loci such as *NOD2* and *SIGIRR* may enhance risk estimates attributable to genetic factors.[5,14,19,20]

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Figure legend

Figure 1 - Distribution of ITGB2 variants by NEC severity in the entire cohort (1A), and among the infants with birthweight <1000 gm (1B) : [A] The proportion of infants who had ACMG class 1-3 variants in the ITGB2 gene are compared among infants with no NEC 16/165 (9.7%), medical NEC 3/21 (14.3%) and surgical NEC 6/35 (17.1) (p=0.18). [B] Subgroup analysis of infants with birthweight < 1000 gm shows ITGB2 variants in 7/89 (7.9%) of infants with no NEC, 2/12 (16.7%) in infants with medical NEC, and 4/21 19% in infants with surgical NEC (p=0.105). Cochran Armitage Trend test.

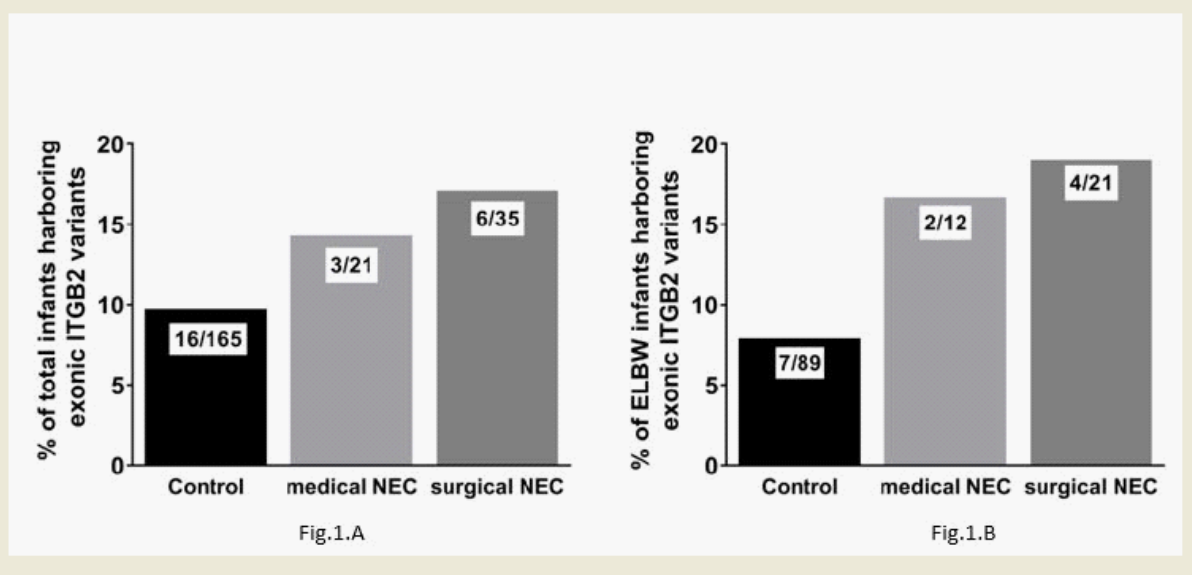

Table 1. Demographics

	NEC severity			P-Value
	Medical n = 21	**Surgical n = 35**	**None n = 165**	
Significant variant	3 (14.3%)	6 (17.1%)	16 (9.7%)	0.181
Birthweight	999.5 ± 458.6	965.3 ± 399.7	972.9 ± 331.0	0.937
Weight < 1000 gm	12 (57.1%)	21 (60.0%)	89 (53.9%)	0.793
Gest Age in wks.	27.6 ± 3.7	27.1 ± 2.9	27.1 ± 2.7	0.740
Gest Age < 29 wks.	16 (76.2%)	25 (71.4%)	114 (69.1%)	0.786
Inborn	13 (61.9%)	13 (37.1%)	146 (88.5%)	< 0.001
Race B W	 10 (55.6%) 8 (44.4%)	 16 (50.0%) 16 (50.0%)	 53 (32.9%) 108 (67.1%)	0.048
Received prenatal care	17 (89.5%)	31 (91.2%)	158 (95.8%)	0.265
Received ANS	13 (68.4%)	25 (78.1%)	145 (87.9%)	0.021
Mat. chorioamnionitis	2 (9.5%)	7 (20.0%)	25 (15.2%)	0.169
Sex F M	 10 (47.6%) 11 (52.4%)	 12 (34.3%) 23 (65.7%)	 76 (46.1%) 89 (53.9%)	0.423
5 min APGAR	6.8 ± 2.2	7.0 ± 2.2	6.9 ± 2.0	0.902
Feed type BM BOTH FORM NONE Missing	 16 (76.2%) 1 (4.8%) 3 (14.3%) 1 (4.8%)	 14 (46.7%) 6 (20.0%) 9 (30.0%) 1 (3.3%) 5	 89 (53.9%) 38 (23.0%) 37 (22.4%) 1 (0.6%)	0.079
Continuous variables compared using one-way analysis of variance. Categorical variables compared using chi-square or Fisher's exact test.				

ANS = Antenatal steroids, Mat= Maternal, BM = Breast milk, FORM = formula