Association between absolute neutrophil count and variation at *TCIRG1*: the NHLBI Exome Sequencing Project

Elisabeth A. Rosenthal[1], Vahagn Makaryan[2], Amber A. Burt[1], David R. Crosslin[3], Daniel Seung Kim[3], Joshua D. Smith[3], Deborah A. Nickerson[3], Alex P. Reiner[4], Stephen S. Rich[5], Rebecca D. Jackson[6], Santhi K. Ganesh[7,8], Linda Polfus[9], Lihong Qi[10], David C. Dale[2], UW Center for Mendelian Genomics[11,12], Gail P. Jarvik[1,3] on behalf of the NHLBI GO Exome Sequencing Project[12]

1. Division of Medical Genetics, School of Medicine, University of Washington, Seattle, WA, USA
2. Division of General Internal Medicine, School of Medicine, University of Washington, Seattle, WA, USA
3. Department of Genome Sciences, University of Washington, Seattle, WA, USA;
4. Department of Epidemiology, School of Public Health, University of Washington, Seattle, WA, USA
5. Center for Public Health Genomics, School of Medicine, University of Virginia, Charlottesville, VA, USA
6. Division of Endocrinology, Diabetes and Metabolism, Department of Internal Medicine, The Ohio State University, Columbus, OH, USA
7. Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI, USA
8. Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA
9. Human Genetics Center, University of Texas Health Science Center, Houston, TX, USA
10. Division of Biostatistics, Department of Public Health Sciences, School of Medicine, University of California, Davis, CA, USA
11. University of Washington, Seattle, Washington, USA
12. Authorship banner is included in supplemental materials

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Corresponding author:
Elisabeth A. Rosenthal
erosen@uw.edu
University of Washington Medical Center
1705 NE Pacific St, Box 357720
Seattle, WA 98195

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Abstract
Neutrophils are a key component of innate immunity. Individuals with low neutrophil count are susceptible to frequent infections. Linkage and association between congenital neutropenia and a single rare missense variant in *TCIRG1* have been reported in a single family. Here, we report on nine rare missense variants at evolutionarily conserved sites in *TCIRG1* that are associated with lower absolute neutrophil count (ANC)($p=0.005$) in 1058 participants from three cohorts: the Atherosclerosis Risk in Communities (ARIC), Coronary Artery Risk Development in Young Adults (CARDIA) and Jackson Heart (JHS) studies of the NHLBI Grand Opportunity Exome Sequencing Project (GO ESP). These results validate the effects of *TCIRG1* coding variation on ANC and suggest that this gene may be associated with a spectrum of mild to severe effects on ANC.

Key words: Neutropenia, absolute neutrophil count, rare variant replication, next generation sequence data

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Introduction

White blood cells are critical components of innate and adaptive immune responses. About two-thirds of blood leukocytes are neutrophils, the cells which first respond to bacterial or fungal infection. Normal neutrophil counts, in adults, are in the range of 1.8-7.7 X 10^9/L(Dale 2010). Severely neutropenic individuals (neutrophil count < 0.5 X 10^9/L) suffer from frequent and often severe infections. Severe congenital neutropenia (SCN) (MIM 202700) is characterized by low absolute neutrophil count (ANC) and bacterial infections beginning early in childhood, and is most often inherited as a dominant disorder (Dale and Link 2009, 3-5). However, there are recessive and X-linked forms of congenital neutropenia. Cyclic Neutropenia (CN) (MIM 162800) is an autosomal dominant disease characterized by episodic neutropenia, also beginning in childhood. Variation at *ELANE* can cause both forms of neutropenia, and variation at other genes (e.g., *GFI1*, *WAS*, *CXCR4*, *HAX1*, and *SDBS*) causes SCN (Germeshausen et al. 2013, 905-914; Germeshausen, Ballmaier, and Welte 2007, 93-99; Person et al. 2003, 308-312; Devriendt et al. 2001, 313-317; Ancliff et al. 2006, 2182-2189; Klein et al. 2007, 86-92; Faiyaz-Ul-Haque et al. 2010, 661-666; Smith et al. 2009, 762-770; Germeshausen et al. 2008, 4954-4957; Xia et al. 2009, 535-542; Hauck and Klein 2013, 596-606; Newburger and Dale 2013, 198-206). Individuals of African American (AA) ancestry and some other ethnic groups tend to have lower ANC than individuals with European American (EA) ancestry (Reiner et al. 2011, e1002108). In addition to these known genetic causes, environmental factors correlate with ANC. Medications, radiation therapy, alcohol, and immuno-compromising viruses also affect the ANC.

We previously reported a novel *TCIRG1* coding single nucleotide variant (SNV; NG_007878.1:c.2206C>A, p.Arg736Ser, rs587779413) linked and associated with congenital neutropenia (Makaryan et al. 2014, 824-827) (PMID 24753205). Homozygous or compound

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heterozygous mutations in *TCIRG1* are also known to cause congenital osteopetrosis, a rare autosomal recessive lethal pediatric disorder (Sobacchi et al. 2013, 522-536). *TCIRG1*, through alternative splicing, gives rise to two major isoforms, iso-a and iso-b. Iso-a is a full-length isoform (20 exons) and encodes an a3 subunit of vacuolar H+-ATPase. Iso-b is a shorter isoform, lacking the first five exons of the longer isoform. Iso-a is highly expressed in bone marrow, particularly in osteoclasts (Frattini et al. 2000, 343-346). Iso-b is the major isoform expressed in T-lymphocytes and plays an essential role for T-lymphocyte activation and immune response (Jiang et al. 2013, e58599). Additional splice variants of *TCIRG1* with unknown functional effects are expressed in numerous human tissues (heart, liver, kidney, lung, and pancreas) (Susani et al. 2004, 225-235; Smirnova et al. 2005, 943-949). Rs587779413 is a missense variant in the 18th exon of *TCIRG1* which is included in both major isoforms; thus, it is expected to affect both major isoforms.

In this current work, we attempt to validate the association of ANC with rs587779413 and other rare coding variation in *TCIRG1* in a cohort that is unselected for neutrophil count or infection. We identified rare, conserved coding SNVs in 1058 participants from three cohort studies who had ANC data. In addition to providing replication in an independent dataset, we evaluated evidence for addition functional *TCIRG1* SNVs affecting ANC.

Materials

Phenotype, genotype, and ancestry principal components (PCs) data were collected from the NHLBI Grand Opportunity Exome Sequencing Project (NHLBI GO Exome Sequencing Project (ESP)). Unrelated participants with both percent neutrophils (%NC) and white blood count (WBC) were included in the study. Permissions to access genotype data was restricted to *TCIRG1*, *DARC* SNVs rs2814778 and rs12075, which associate with white blood counts in

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African Americans, and the most common neutropenia pathologic genes *ELANE*, *CSF3R*, *GFI1*, *WAS*, *CXCR4*, *HAX1*, and *SDBS*(Xia et al. 2009, 535-542; Reiner et al. 2011, e1002108; Crosslin et al. 2012, 639-652).

Methods and Results

Study Samples

Three cohort studies collected differential blood counts: the Atherosclerosis Risk in Communities (ARIC) Study, Coronary Artery Risk Development in Young Adults (CARDIA) and Jackson Heart Study (JHS) (Anonymous1989, 687-702; Friedman et al. 1988, 1105-1116; Sempos, Bild, and Manolio 1999, 142-146). These study sites ascertained participants through different vascular phenotypes. The CARDIA study contains a younger cohort, whereas the JHS study includes exclusively AA participants. The demographics and ANC summary data are shown by cohort in **Table I**.

Adjusted absolute neutrophil count

Participants with WBC $\geq$ 11 x 10^9/L (N=69) were excluded due to possible infection, leaving 3723 participants. Only 381 EA and 343 AA participants were genotyped at rs2814778, the *DARC* SNV which associates with neutrophil count in AA. However, the first 7 ancestry PCs (PC1-7) explained 92% variation in the rs2814778 genotype (linear regression F-test p< 2.2e-16) and were used as a proxy for this SNV as well as for ancestry. Only 1060 unrelated participants had both %NC and WBC. ANC was calculated as %NC*WBC/100. Of these individuals, 82 had low neutrophil count (ANC < 2 X 10^9/L). ANC was square root transformed and then adjusted (ANCadj) for age, sex, PC1-7, study site, and *DARC* SNV rs12075 using linear regression (model summarized in **Table II**). Two participants were removed from the analysis, leaving 1058 participants; one participant was determined to be

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an outlier (Cook's distance for ANCadj was 28 standard deviations from the mean) and one individual had a sex discrepancy; These individuals do not appear in the Tables or Figure.

Genotype Data

No known pathologic variants at the 7 most common genes (*ELANE*, *CSF3R*, *GFI1*, *WAS*, *CXCR4*, *HAX1*, *SDBS*) associated with neutropenia were found in this data set. Variants in *TCIRG1* were assessed for several characteristics which would indicate possible pathology: known to cause autosomal recessive osteopetrosis (ARO), frameshift, early stop, splice, or rare evolutionarily conserved missense, where rare is defined as ESP minor allele frequency (MAF) < 0.005. Copy number variation was not captured in the available data. Of these, only 9 rare missense variants with the Genomic Evolutionary Rate Profiling score (GERP)>2.95 were found (Cooper et al. 2005, 901-913). These variants, listed in **Table III**, were considered candidates for causing altered TCIRG1 function, and were identified in 18 participants. All 18 participants were heterozygous for one of the 9 variants. No participant was a homozygote or compound heterozygote for any of these 9 variants. Three variants were carried by multiple (3-5) participants.

Association between TCIRG1 and ANC

As a group, the 18 participants who were heterozygous for one of these 9 candidate functional variants listed in **Table III** had lower ANCadj than those who were not (one-sided t-test p=0.005). These variants explained 0.5% of the variation in ANCadj. **Figure 1** compares the distribution of ANCadj for these 18 heterozygotes to the rest of the participants. Additionally, the mean ANCadj was observed to be lower for each candidate variant. The 6 candidate variants that were each carried by a single participant were observed to be associated with lower ANCadj (between 0.5 and 2.4 standard deviations below the mean) than the 3 variants that were carried by multiple participants (between 0.17 and 0.3 standard

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deviations below the mean). Additionally, 8 individuals without one of these 9 candidate functional SNVs had lower ANCadj (between 2.4 and 3.3 standard deviations below the mean) than the heterozygotes for one of the 9 candidate functional SNVs. All analyses were performed with the statistical software package R using general linear models(R Core Team 2012).

Discussion

We recently reported a strong linkage and association of a heterozygous mutation in *TCIRG1* and the occurrence of neutropenia in a single large family of European American descent with 11 affected individuals (Makaryan et al. 2014, 824-827). Homozygous mutations in this gene have been shown to be the most common cause for autosomal recessive osteopetrosis, with an estimated frequency of 1 in 250,000 people (Sobacchi et al. 2013, 522-536). We have not found reports indicating that parents of autosomal recessive osteopetrosis patients have been evaluated for neutropenia. We also have not found *TCIRG1* mutations in 20 neutropenic patients (all sequenced for most common neutropenia associated genes) enrolled in the severe chronic neutropenia international registry (Dale et al. 2003, 82-93). To further understand *TCIRG1* associated neutropenia, we sought to determine if there is a correlation between potentially pathogenic, rare *TCIRG1* variants at conserved sites and blood neutrophil count.

The results of this study provide supportive evidence for a role of *TCIRG1* variation in determining ANC. Having previously shown that a single *TCIRG1* variant co-segregated and was associated with ANC (Makaryan et al. 2014, 824-827), we now additionally demonstrate an association between rare, coding variants at conserved sites in *TCIRG1* and ANC. Notably the more rare of these variants were associated with lower ANC than the ones seen in more than one individual, suggesting a spectrum of effect sizes may be associated with different

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variants. Variation at *TCIRG1* explains a small portion of the variation in ANC, consistent with the hypothesis that ANC is a complex trait controlled by many biological pathways. Even with this small sample size, the approach of considering only candidate variants in a single gene, reducing multiple testing, allowed detection of statistically significant effects on ANC.

There are several limitations to this study. First, it is possible that other types of variation at *TCIRG1* correlate with ANC. There were no *TCIRG1* frameshifts, splice variants, or early stops identified in these cohorts; the effects of these more disruptive variants would be of particular interest. Additionally, copy number variation data was not available. It also possible that regulatory variants that are not in the ESP database have an effect. Rare variation at evolutionarily conserved bases is expected to have a larger effect on phenotype than common variation (Gorlov et al. 2008, 100-112). Second, the sample size in our study is under-powered to detect the expected relatively small effects of common variants. However, a larger study would be useful in assessing the correlation between common variants and ANC. Third, the samples were not available to allow Sanger validation. It is unlikely that calling error obscures the results, as exome sequencing of rare variants has been found to be of high quality (Tennessen et al. 2012, 64-69) and misclassification would be expected to reduce power.

The pathological mechanisms for *TCIRG1*-associated neutropenia are not yet known. This gene encodes multiple proteins affecting hydrogen ion transport and T-cell immune response(Jiang et al. 2013, e58599). In our prior large family with *TCIRG1*-associated neutropenia, all of the heterozygotes for the pathogenic variant had neutropenia, with variable severity. Some members have severe neutropenia and associated infections whereas others

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are mildly affected. The data in this report suggest that there may be other patients and families with neutropenia attributable to variants in *TCIRG1*.

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Table I: Demographics and summary neutrophil data, by cohort.

Site	N	%Male	%AA	Min Age	Mean Age	Max Age	Min ANC	Mean ANC	Median ANC	Max ANC
ARIC	603	47	46	44	54.0	65	0.55	3.5	3.4	8.42
CARDIA	132	58	39	18	26.4	33	0.69	3.5	3.3	7.17
JHS	323	42	100	24	53.8	85	0.31	3.2	2.9	8.40
Total	1058	47	62	18	50.5	85	0.31	3.4	3.2	8.42

Abbreviations: AA, African Ancestry (All other participants are of European ancestry); Min, minimum; Max, maximum; ANC, absolute neutrophil count

Table II: Variables and model from linear regression used to adjust square root of the absolute neutrophil count, before assessing significance of variation at *TCIRG1*.

Covariate	Beta	Std. error	p-value
Age	4.3e-03	0.012	0.73
Sex(Male)	0.03	0.21	0.90
rs12075	-0.34	0.2	0.09
PC1-7	NA	NA	<2e-16
Site	NA	NA	0.01

Abbreviations: PC1-7, Ancestry Principal Components 1 through 7

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Table III: The 9 coding variants with GERP > 2.95 and MAF <0.005 considered in the analyses.

GRCh37.hg19 Position on Chr. 11	EXON (of20)	RSID	GERP	MAF% EA	MAF% AA	MAF% ALL	N EA	N AA	N ALL	Mean ANCadj
67809250	3	rs370319355	3.72	0	0.02	0.008	0	1	1	-3.1
67810304	4	rs377377656	4.93	0	0.03	0.009	0	1	1	-7.8
67810477	5	rs34227834	3.46	0.11	0.05	0.09	1	0	1	-5.5
67810963	6	rs372826788†	3.84	0.04	0	0.02	1	0	1	-2.3
67811114	7	rs116001129	3.85	0	0.16	0.05	0	1	1	-4.8
67814943	11	rs140191063	4.07	0.10	0.09	0.10	2	1	3	-1.0
67814983	11	rs140963213	4.07	0.49	0.11	0.36	4	1	5	-0.57
67816589	15	rs115854062	4.53	0.01	0.16	0.06	0	3	4‡	-0.70
67817635	18	rs141095902	3.89	0.01	0	0.008	1	0	1	-1.7
Overall	NA	NA	NA	1.35	0.69	1.13	12	8	21	-1.9

†: This SNV may affect splicing, ‡: One participant is of indeterminate ancestry

Minor allele frequency (MAF) is from the entire exome sequence project. Abbreviations: GERP, Genomic Evolutionary Rate Profiling score; EA, European Ancestry; AA, African Ancestry; N, count of participants heterozygote for the variant; ANCadj, ANC adjusted for covariates.

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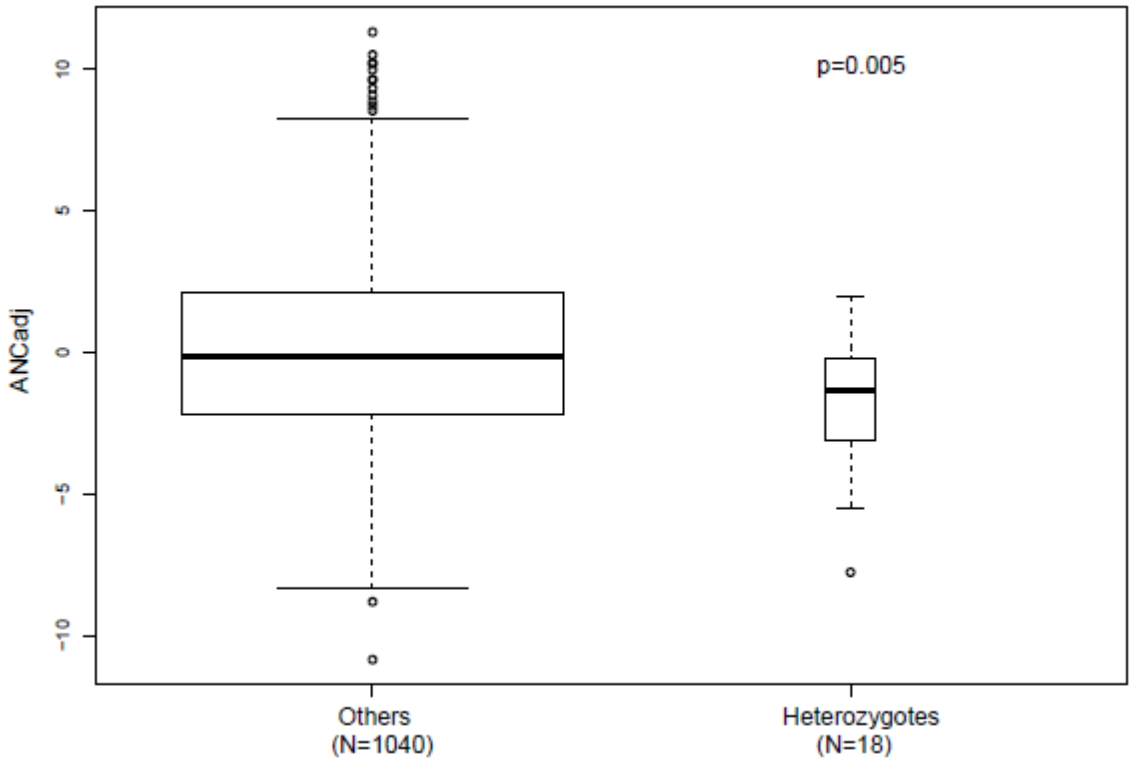

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