

Examination of *HFE* associations with childhood leukemia risk and extension to other iron regulatory genes

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Abstract

Hereditary hemochromatosis (*HFE*) variants correlating with body iron levels have shown associations with cancer risk, including childhood acute lymphoblastic leukemia (ALL). Using a multi-ethnic sample of cases and controls from Houston, TX, we examined two *HFE* variants (rs1800562 and rs1799945), one transferrin receptor gene (*TFRC*) variant (rs3817672) and three additional iron-regulatory gene (IRG) variants (*SLC11A2* rs422982; *TMPRSS6* rs855791 and rs733655) for their associations with childhood ALL. Being positive for either of the *HFE* variants yielded a modestly elevated odds ratio (OR) for childhood ALL risk in males (1.40, 95% CI = 0.83 to 2.35), which increased to 2.96 (95% CI = 1.29 to 6.80) in the presence of a particular *TFRC* genotype for rs3817672 ($P_{interaction} = 0.04$). The *TFRC* genotype also showed an ethnicity-specific association, with increased risk observed in non-Hispanic Whites (OR = 2.54, 95% CI = 1.05 to 6.12; $P_{interaction}$ with ethnicity = 0.02). The three additional IRG SNPs all showed individual risk associations with childhood ALL in males (OR = 1.52 to 2.60). A polygenic model based on the number of variant alleles in five IRG SNPs revealed a linear increase in risk among males with the increasing number of variants possessed (OR = 2.0 per incremental change, 95% CI = 1.29 to 3.12; $P = 0.002$). Our results replicated previous *HFE* risk associations with childhood ALL in a US population and demonstrated novel associations for IRG SNPs, thereby strengthening the hypothesis that iron excess mediated by genetic variants contributes to childhood ALL risk.

1. Introduction

The hereditary hemochromatosis gene, *HFE*, has shown multiple associations with cancer susceptibility [1-7], including risk for childhood acute lymphoblastic leukemia (ALL) [8], which has been replicated [9]. In multiple cancers [1,2], including childhood ALL [9], the association of *HFE* variants with cancer risk gets stronger in interaction with a polymorphism in the transferrin receptor gene (*TFRC*). Since *HFE* and *TFRC* proteins biologically interact in iron transfer across membranes such as intestinal mucosa and placenta, the observed statistical interaction supports the notion that the involvement of *HFE* variants in cancer risk modification is mediated via their effect on body iron levels [10]. Body iron levels have long been known to be positively correlated with general cancer risk as several cohort studies have shown [11-14],

and iron's carcinogenic effect has been well documented [15]. Thus, *HFE* associations with cancer have strong biological plausibility.

Recent genome-wide association studies (GWAS) have identified the *HFE* variant C282Y as a major determinant of body iron levels [10]. The mediation of iron homeostasis by genetic variants extends beyond the *HFE* gene, with the strongest association being *TMPRSS6* rs855791 [16]. We reasoned that if *HFE* associations are due to their effect on iron levels rather than linkage disequilibrium with nearby polymorphisms, other iron regulatory gene (IRG) polymorphisms should show similar associations with childhood ALL risk. To test our hypothesis that previously observed *HFE* associations are mediated via their effect on body iron levels, we aimed to expand our study beyond *HFE* variants to additional IRG polymorphisms, first by confirming previously shown *HFE* SNP associations with childhood ALL, and assessing novel SNPs in *TMPRSS6* and *SLC11A2* for their associations. To further test our hypothesis that *HFE* variants modify the risk for childhood ALL via their effects on iron levels, we also included the *TFRC* polymorphism that is known to interact with *HFE* variants in the genotyping scheme to test whether this interaction occurs. The additional IRGs do not interact with *TFRC* biologically, so we did not predict any other interaction. To test our hypothesis, we used a new case-control set, which was first validated by replicating known childhood ALL associations [17].

2. Subjects & Methods

2.1 Subjects

Institutional Review Boards of Baylor College of Medicine (BCM) and Florida International University approved the study protocol. The case-control sample was from Houston, TX, consisting of 161 incident cases with childhood (<18 years at diagnosis) ALL diagnosed at Texas Children's Hospital from 2007 to 2012, and 231 healthy controls with the same age range (<18 yr) contemporaneously and locally recruited. Thus, all subjects were less than 18 years of age, and exclusion criteria for both cases and controls were refusal to participate in the study and the diagnosis of any other cancer or disease. Representing the age peak typical of childhood ALL, 73% of cases were 1 to 5 years old. Subjects and their parents were approached to obtain informed consent for provision of epidemiological data with a questionnaire and a biological sample. The DNA samples were extracted from saliva or

peripheral blood samples at BCM. The sample was multiethnic to allow us to examine effect modification of childhood ALL risk by ethnicity. Ethnicity was determined by the responses provided on the questionnaire by the children's parents. Our main interest was the contrast between non-Hispanic Whites (NHW) and Hispanic Whites (HW), since childhood ALL is very rare in African-Americans, and we had a very small number of African-Americans (n=17) in the case group. Information on clinical subtype of ALL was collected from medical records, and 88% of the cases were diagnosed with early precursor B (early pre-B) ALL subtype.

2.2 SNP selection

We included two *HFE* variants known to influence body iron levels commonly known as C282Y (rs1800562) and H63D (rs1799945), as well as the *TFR3* variant S142G (rs3817672), which is known to interact with *HFE* variants in previously reported cancer associations [1,2,9]. As other IRG variants, we included the GWAS-identified iron-related SNP *TM6SP1* rs855791 [16], as well as two additional SNPs we selected also from the *TM6SP1* gene (rs733655) and the *SLC11A2* gene (rs422982) involved in the non-transferrin receptor-related iron transfer across membranes. The last two SNPs were selected as the promoter region haplotype tagging SNPs for these two genes. The selected SNP from *TM6SP1* (rs733655) is 32kb away and not in linkage disequilibrium with the GWAS-identified marker (rs855791) in the same gene according to the HapMap project European population data ($r^2 = 0.29$). Two more SNPs were included as ancestry-informative markers (AIMs) to adjust for the ethnic heterogeneity in the multiethnic sample to supplement the self-reported ethnicity data. The two SNPs were rs285 and rs2891, which were identified as AIMs in previous studies due to their largely different allele frequencies in major ancestral human populations [18,19]. Characteristics of each SNP are given in Table I.

2.3 Genotyping

TaqMan allelic discrimination assay was the choice of method for genotyping. All SNPs were genotyped by TaqMan assays purchased from Life Technologies (Foster City, CA) on CFX96 real-time PCR instrument (Bio-Rad, Hercules, CA). The assay ID of each assay is given in Table I.

2.4 Statistical analysis

Genetic associations (both crude and adjusted) were evaluated by logistic regression using Stata v.11 (StataCorp, College Station, TX). Two-way statistical interactions were also analyzed by logistic regression by including an interaction (product) term for the genetic variant

and the potential effect modifier (age, gender or ethnicity) in the explanatory variables in multivariable analysis. All statistical tests were two-tailed and threshold for statistical significance was set at $P \leq 0.05$. All genetic associations, except the *TFRC* locus, were assessed by using the dominant genetic model which corresponds to variant allele positivity and coded as 1 for heterozygote and variant allele homozygote genotypes, and 0 for the common allele homozygosity (referent). Due to the low frequency of their variant alleles, the two *HFE* SNPs were pooled together by creating a new variable for the number of cumulative variant alleles at both SNPs (0 for no variant allele, 1 for variant allele at either SNP, 2 for heterozygosity at both SNPs (compound heterozygosity) or variant allele homozygosity at either SNP). To be consistent with the previous studies, *TFRC* SNP was analyzed in recessive model (by coding variant allele homozygosity as 1 and the other genotypes as 0). A similar approach was used for a polygenic risk model using the total number of variant alleles at two *HFE* variants and three variants in non-*HFE* (*TMPRSS6*, *SLC11A2*) IRGs (0 for no variant allele at any SNP, 1 for one or two variant alleles at any SNP, 2 for three or more variant alleles at any of the five SNPs). All statistical associations in the overall group were adjusted for the ethnicity variable which had four categories (NHW, HW, African-Americans, and others). The efficiency of statistical adjustment for ethnicity was double-checked by adjustment for each of the AIMs separately. Before proceeding to the statistical analysis of genetic associations, Hardy-Weinberg disequilibrium was ruled out in controls as a test for gross genotyping errors. Given the number of associations examined, we used a slightly more stringent statistical significance threshold of $P \leq 0.01$ in the interpretation of our results.

3. Results

3.1 HFE C282Y and H63D frequencies in the sample population

As expected, *HFE* C282Y mutation was more common in the NHW subjects: variant allele frequencies were 0.113, 0.030 and 0.011 in NHWs, HWs, and African-Americans, respectively. The H63D variant positivity also had some variation across ethnic groups with frequencies of 0.254, 0.151 and 0.032 in NHWs, HWs, and African-Americans, respectively. These variations were similar to those observed in HapMap project population samples. Only two cases and two controls (all NHWs) were compound heterozygotes for C282Y and H63D.

3.2 Univariable genetic marker analyses in the overall group

Genotype frequencies for each SNP were in Hardy-Weinberg equilibrium in the control group when analyzed for each ethnicity group. All associations reported below for the whole group were adjusted for self-reported ethnicity. Replacing the ethnicity variable by either AIM did not appreciably alter the results. As shown in Table II, neither C282Y nor H63D showed an overall association with childhood ALL risk. The *TFRC* SNP, which was included in the study to assess its interaction with *HFE* SNPs did not show any individual association in the overall group. The three IRG variants all yielded high ORs in the overall analysis, but only one (rs733655) reached statistical significance in the overall analysis (Table II).

3.3 Gender- and ethnicity-specific analyses and statistical interactions

For the two *TMPRSS6* SNPs, males had risk associations with ORs of 1.91 and 2.60 (Table III), but statistical interaction with gender did not reach statistical significance. Despite yielding greater ORs for males, the male-specific *HFE* associations did not reach statistical significance in individual analysis. Results from pooling of the two *HFE* variants in one variable (as described in the Methods) are shown in Figure I. The bars depict the risk genotype frequencies in the case and control groups, and ORs for childhood ALL risk are provided. There was no statistically significant association in the overall group (OR = 1.46, $P = 0.17$; Graph A in Figure I), but the pooled variable revealed a promising result in males (OR = 2.09, $P = 0.04$; Graph B in Figure I), which reached statistical significance in interaction with the *TFRC* variant (OR = 4.92, $P = 0.002$; Graph D in Figure I). Graph C depicts the frequencies and OR for female cases/controls, and Graph E shows the frequencies and OR for males with the wild type allele for *TFRC*.

We explored the *TFRC* and *HFE* gene-gene interactions previously observed in multiple cancers [1,2], including childhood ALL [9]. In the overall sample, there was no interaction (data not shown). Our main group of interest was males because of the previous findings in childhood ALL, and also because of generally higher ORs in males in univariable analysis of *HFE* variants. Since the small sample size would not allow a reliable assessment of interactions for rare *HFE* variants, especially C282Y, we used the *HFE* pooled variant variable for this analysis. In the two groups of males with and without the *TFRC* homozygous genotypes, the ORs were 0.59 (CI = 0.24 to 1.45) and 4.92 (CI = 1.29 to 6.80), yielding an interaction ($P_{interaction} = 0.04$). We also examined interactions of non-*HFE* SNPs with *TFRC*. This analysis did not reveal any interaction

($P = 0.33, 0.46,$ and 0.96) as expected from the lack of biological interaction between these IRGs and *TFRC*.

There was no ethnicity-specific association of *HFE* variants. Ethnicity-specific analyses revealed a high OR (2.54, CI= 1.05 to 6.12, P value = 0.04) for the *TFRC* rs3817672 allele A homozygote genotype in NHWs, while the OR was less than 1.0 (non-significant) in Hispanics ($P_{interaction} = 0.02$ for ethnicity). Thus, the largest ethnicity-specific difference was the NHW-specific association of *TFRC* with borderline statistical significance. The association of the *TMPRSS6* promoter region tagging SNP rs733655 was equally strong in both ethnicities that could be examined in this study (ORs 2.32 and 2.55; Table III).

3.4 Polygenic risk model

We constructed a polygenic risk variable consisting of five SNPs in three IRGs as described in the Methods section. Analysis using this variable showed that for stepwise increase in the number of variant alleles, there was a linear increase in childhood ALL risk in the overall group (OR = 1.63, 95% CI = 1.18 to 2.26, $P = 0.003$), and in males (OR = 2.00, 95% CI = 1.29 to 3.12, $P = 0.002$; shown in Figure II), but not in females (OR = 1.26, 95% CI = 0.77 to 2.08, $P = 0.36$) in stratified analyses. In ethnicity-specific analysis, the association remained statistically significant in NHW (OR = 2.19, 95% CI = 1.18 to 4.06, $P = 0.01$), but not in HW (OR = 1.42, 95% CI = 0.89 to 2.27, $P = 0.14$).

None of the associations were lost when we restricted the analysis to early pre-B subtype or the cases within the age peak. All associations observed in the whole group were also statistically significant in these subgroups sometimes with greater effect size (data not shown).

4. Discussion

We examined previously reported *HFE* associations and interactions with *TFRC* with the risk of childhood ALL. In this multi-ethnic sample, we observed associations similar to previously reported ones with gender effect, and extended the observations to additional iron regulatory gene polymorphisms to provide further support for our hypothesis that *HFE* and *TFRC* association in childhood ALL is due to their effect on iron homeostasis. The only statistically significant gender-specific associations with IRG variants and childhood ALL risk were in males, and we also noted a novel ethnicity-specific difference in the association of the *TFRC* variant.

To increase statistical power, we pooled the two *HFE* variants to be able to detect their associations with childhood ALL risk. The ORs for the pooled variables were always in the risk direction for individual SNPs, and were greater in males. When the interaction with the *TFRC* genotype and the gender effect was considered, a more robust association was found, as in a previous study [9]. The same interaction was also observed in multiple cancers [1,2] and in childhood ALL. While interaction analysis is usually seen as a challenge in terms of statistical power, as happened in the present study, the increase in the effect size may compensate for the loss of statistical power due to comparison of smaller subsets of the sample. Like any statistical association, our results should be considered cautiously. However, similarities with previous observations provide sufficient credibility to the cumulative results, which now strongly suggest that iron excess, whether environmentally- or genetically-induced, increases the risk for cancer in general, and in particular for childhood ALL. As previously postulated [9,20], the mechanism of the childhood ALL risk association with *HFE* variants known to elevate body iron levels may include increased materno-fetal iron transport through placenta. This process is mediated by *HFE* and *TFRC* [21,22], and these genetically-mediated alterations in fetal iron homeostasis may also have implications on the developmental origins of health and disease [23].

Another novel finding of the present study was the risk associations of previously unexamined IRG SNPs (rs422982 and rs733655) with childhood ALL. Together with the association of rs855791, a GWAS identified marker for iron levels [16], which reached statistical significance only in males, these new findings lend support to our hypothesis that iron homeostasis related risk modification in childhood ALL extend beyond *HFE/TFRC* polymorphisms. We do not yet know whether the SNPs selected by us and used in any association study for the first time have any correlation with body iron levels. Their locations in the promoter regions of crucial IRGs suggest that they will be somewhat involved in some aspect of gene function and subsequently in iron homeostasis, but only functional studies can confirm their roles. We were not surprised by the lack of interaction between *SLC11A2 / TMPRSS6* and *TFRC* since they do not interact biologically. The lack of statistical interaction between these additional SNPs and the *TFRC* SNP suggested the specificity of the observed *HFE* and *TFRC* interaction.

The present study highlights the benefits of explorations of effect modification by gender or ethnicity. Although such explorations are usually reserved for well powered studies, when

backed up by previous observations or strong biological hypotheses, stratified analyses complemented by statistical interaction analyses are powerful approaches to unravel otherwise masked associations. It is only natural that in a multigenic disorder like cancer, effect modification will be operational. Researchers usually shy away from analysis of effect modification or statistical interaction to avoid performing multiple comparisons and subsequent chance findings, but there are ways to rule out chance findings by additional replication studies.

Besides the limitations already mentioned such as sample size and statistical power, our study has another limitation, which has stemmed from one of its strengths. Examination of these associations in a multi-ethnic cohort has benefits, but also brings about heterogeneity, which should be accounted for during analysis. We had self-reported ethnicity data, but adjustment of the results by these data may still leave some residual confounding. We also used two AIMs to make sure that the heterogeneity in the population would not result in spurious findings. The current practice in well-resourced GWAS studies is to use thousands of AIMs to adjust for genetic ancestry, which is particularly crucial when the sample includes recently admixed populations such as African Americans or Hispanics. We could not do that, but could include two AIMs to control for population heterogeneity. Another limitation of using a multi-ethnic sample was the constraints it adds on checking genotyping error. We followed the usual safeguards of genotyping error avoidance at the experimental phase, and checked for errors at the analysis phase by using Hardy-Weinberg equilibrium testing. This test, however, has to be done in each ethnicity subgroup separately. This practice further reduces the statistical power of this test and may have caused inefficiency of genotyping error checking. Genotyping errors have the potential to cause both false positives and false negatives. Our results basically replicated previously observed associations, and there is no overwhelming reason to consider genotyping errors as an alternative explanation.

By mainly replicating the *HFE* and *TFRC* interaction in childhood ALL risk association and revealing new associations with IRGs, we provided further support for the hypothesis on the iron connection in childhood ALL susceptibility. These findings have far reaching implications beyond childhood leukemia in the cancer field. We hope that our results will stimulate interest in secondary analyses of existing GWAS data on multiple cancers to explore the pathways involved in iron homeostasis. We also report novel associations with gender or ethnicity specificity. These associations should be explored in already existing datasets for replication. Beyond genetic

association studies, functional work on whether these polymorphisms indeed mediate placental iron transport and whether this effect differs by gender will provide ultimate confirmation of our results with translational implications.

Conflict of interest statement

The authors disclose no conflicts of interest.

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Table I. Main features of SNPs analyzed

Gene	SNP	Chromosome nucleotide position*	Minor allele and frequency[†]	SNP Type	Assay ID
<i>HFE</i>	rs1800562	chr6: 26093141	(A) 0.053	Transition substitution, missense mutation	C__1085595_10
<i>HFE</i>	rs1799945	chr6: 26091179	(G) 0.179	Transversion substitution, missense mutation	C__1085600_10
<i>TFRC</i>	rs3817672	chr3: 195800811	(G) 0.383	Transition substitution, missense mutation	C__3259537_10
<i>SLC11A2</i>	rs422982	chr12: 51406354	(A) 0.246	Transversion substitution, intragenic	C___570333_10
<i>TMPRSS6</i>	rs733655	chr22: 37495051	(C) 0.221	Transition substitution, intragenic	C__3289858_1_
<i>TMPRSS6</i>	rs855791	chr22: 37462936	(T) 0.412	Transition substitution, missense mutation	C__3289902_10
<i>LPL</i>	rs285	chr8: 19815189	(T) 0.500	Transition substitution, intragenic	C_12104266_10
<i>ITGAE</i>	rs2891	chr17: 3705526	(G) 0.496	Transition substitution, intragenic	C__3211308_20

*Genome Reference Consortium Human Build 37 patch release 10 (GRCh37.p10) used for nucleotide position (<http://www.ncbi.nlm.nih.gov/SNP>)

†Minor allele frequencies are from a reference Caucasian population (U.S. residents of northern and western European ancestry) genotyped in HapMap project

Table II. Univariable analyses of associations with childhood ALL risk*

SNP	OR (95% CI)	P value
<i>HFE</i> rs1800562	1.37 (0.52 to 3.60)	0.52
<i>HFE</i> rs1799945	1.33 (0.74 to 2.38)	0.35
<i>TFRC</i> rs3817672	0.8 (0.52 to 1.23)	0.31
<i>SLC11A2</i> rs422982	1.55 (1.01 to 2.37)	0.04
<i>TMPRSS6</i> rs733655	2.06 (1.33 to 3.20)	0.001
<i>TMPRSS6</i> rs855791	1.41 (0.91 to 2.18)	0.12

*Adjusted for self-reported ethnicity (non-Hispanic White, Hispanic White, African-Americans, and others)

Table III. HFE and non-HFE associations with childhood ALL in gender and ethnicity groups (ORs and 95% CIs)

	Females (n=176)	Males (n=216)	Non-Hispanic Whites (n=115)	Hispanic Whites (n=170)
<i>HFE</i> rs1800562	0.40 (0.08 to 1.89) <i>P</i> = 0.25	3.41 (0.86 to 13.52) <i>P</i> = 0.08	1.78 (0.51 to 6.14) <i>P</i> = 0.36	0.91 (0.15 to 5.58) <i>P</i> = 0.92
	<i>P</i> _{interaction} = 0.09		<i>P</i> _{interaction} = 0.55	
<i>HFE</i> rs1799945	1.01 (0.41 to 2.48) <i>P</i> = 0.98	1.51 (0.69 to 3.31) <i>P</i> = 0.30	1.26 (0.53 to 2.99) <i>P</i> = 0.60	0.87 (0.37 to 2.08) <i>P</i> = 0.76
	<i>P</i> _{interaction} = 0.78		<i>P</i> _{interaction} = 0.56	
<i>TFRC</i> rs3817672	1.66 (0.71 to 3.86) <i>P</i> = 0.24	0.7 (0.31 to 1.57) <i>P</i> = 0.38	2.54 (1.05 to 6.12) <i>P</i> = 0.04	0.69 (0.37 to 1.27) <i>P</i> = 0.23
	<i>P</i> _{interaction} = 0.21		<i>P</i> _{interaction} = 0.02	
<i>SLC11A2</i> rs422982	1.58 (0.83 to 2.99) <i>P</i> = 0.17	1.52 (0.86 to 2.68) <i>P</i> = 0.15	1.47 (0.70 to 3.10) <i>P</i> = 0.31	1.91 (0.99 to 3.68) 0.055
	<i>P</i> _{interaction} = 1.00		<i>P</i> _{interaction} = 0.61	
<i>TMPRSS6</i> rs733655	1.56 (0.81 to 3.03) <i>P</i> = 0.19	2.6 (1.44 to 4.70) <i>P</i> = 0.002	2.35 (1.07 to 5.16) <i>P</i> = 0.03	2.52 (1.26 to 5.04) <i>P</i> = 0.009
	<i>P</i> _{interaction} = 0.19		<i>P</i> _{interaction} = 0.90	
<i>TMPRSS6</i> rs855791	1.12 (0.58 to 2.14) <i>P</i> = 0.74	1.91 (1.04 to 3.51) <i>P</i> = 0.04	1.71 (0.78 to 3.72) <i>P</i> = 0.18	1.22 (0.62 to 2.38) <i>P</i> = 0.57
	<i>P</i> _{interaction} = 0.40		<i>P</i> _{interaction} = 0.52	

*Adjusted for self-reported ethnicity (non-Hispanic white, Hispanic white, African-Americans, and others)

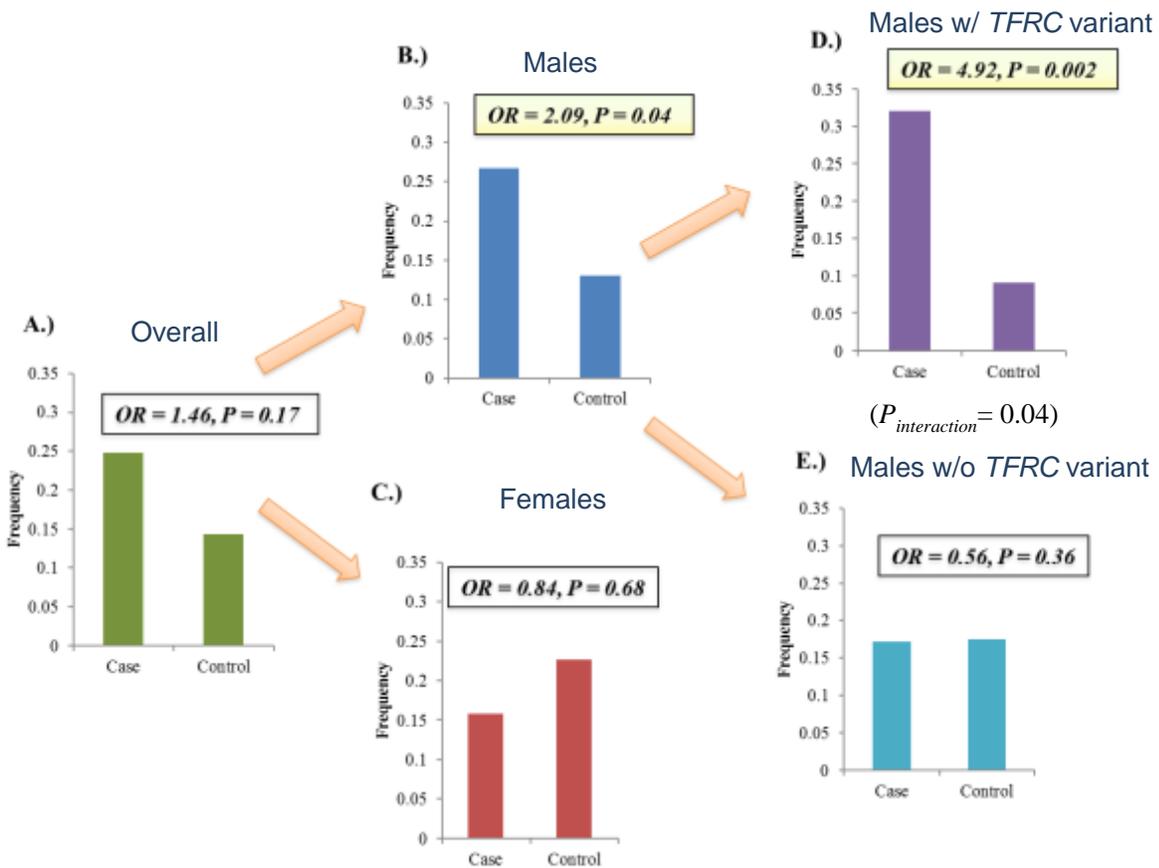


Figure I: Risk genotype frequencies and pooled *HFE* association in childhood ALL in case and control groups, by gender and *TFRC* genotype group.

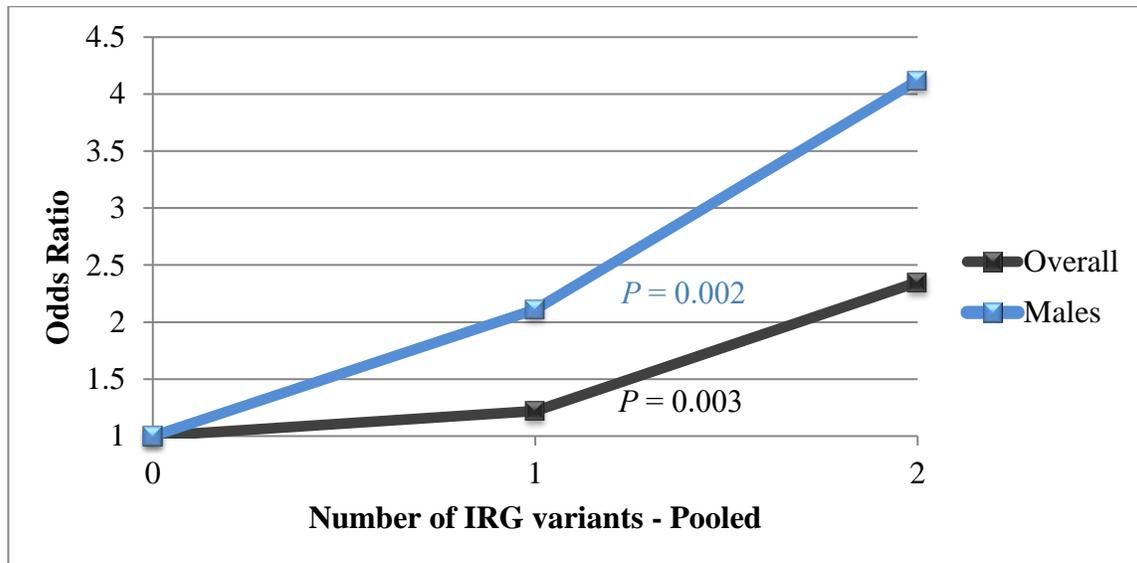


Figure II: Polygenic risk variable consisting of five IRG SNPs