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Identification of Two Genetic Loci Associated with Leukopenia after Chemotherapy in Breast Cancer Patients

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Author contributions

PAF, DL, SS, DJS, LH; RMW, LW have made substantial contributions to the conception or design of the work; PAF, DL, SS, JNI, PCLJ, BR, AH, ABE, AR, AS, HT, TNF, GH, MWB, MR, HH, DL, EM, JS, JR, KD, GDJ, EEC, LL, BLF, JMC, WJ, ANAM, DJS, LH; RMW, LW have made substantial contributions to the acquisition, analysis, or interpretation of data; PAF, DL, SS, JNI, PCLJ, BR, AH, ABE, AR, AS, HT, TNF, GH, MWB, MR, HH, DL, EM, JS, JR, KD, GDJ, EEC, LL, BLF, JMC, WJ, ANAM, DJS, LH; RMW, LW have drafted the work or substantively revised it.

All authors have approved the submitted version and have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

Conflict of interests

Dr Fasching reports personal fees from Novartis, grants from Biontech, personal fees from Pfizer, personal fees from Daiichi-Sankyo, personal fees from AstraZeneca, personal fees from Eisai, personal fees from Merck Sharp & Dohme, grants from Cepheid, personal fees from Lilly, personal fees from Pierre Fabre, personal fees from seagen, personal fees from Roche, personal fees from Agendia, personal fees from Sanofi Aventis, and personal fees from Gilead outside the submitted work. Drs. Wang and Weinshilboum are co-founders of and stockholders in OneOme, LLC, a pharmacogenomic decision support company. Dr. Tesch, Prof. Dr. reports personal fees and non-financial support from Novartis, personal fees and non-financial support from Roche, personal fees and non-financial support from Astra Zeneca, personal fees and non-financial support from Pfizer, personal fees and non-financial support from Amgen, outside the submitted work; Dr. Schneeweiss reports grants from Celgene, grants from Roche, grants from AbbVie, grants from Molecular Partner, personal fees from Roche, personal fees from AstraZeneca, personal fees from Celgene, personal fees from Roche, personal fees from Roche, personal fees from Celgene, personal fees from Pfizer, personal fees from AstraZeneca, personal fees from Novartis, personal fees from MSD, personal fees from Tesaro, personal fees from Lilly, personal fees from Pfizer, other from Roche, outside the submitted work; all other authors have declared that they have no conflicts of interest.

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Abstract

Purpose: To identify molecular predictors of grade 3/4 neutropenic or leukopenic events (NLEs) after chemotherapy using a genome-wide association study (GWAS).

Patients and Methods: A GWAS was performed on patients in the phase III chemotherapy study SUCCESS-A (n = 3322). Genotyping was done using the Illumina HumanOmniExpress-12v1 array. Findings were functionally validated with cell culture models and the genotypes and gene expression of possible causative genes were correlated with clinical treatment response and prognostic outcomes.

Results: One locus on chromosome 16 (rs4784750; NLRC5; P = 1.56E-8) and another locus on chromosome 13 (rs16972207; TNFSF13B; P = 3.42E-8) were identified at a genome-wide significance level. Functional validation revealed that expression of these two genes is altered by genotype-dependent and chemotherapy-dependent activity of two transcription factors. Genotypes also showed an association with disease-free survival in patients with an NLE.

Conclusions: Two loci in NLRC5 and TNFSF13B are associated with NLEs. The involvement of the major histocompatibility complex I regulator NLRC5 implies the possible involvement of immuno-oncological pathways.

Keywords

chemotherapy; leukopenia; GWAS; *NLRC5* ; *TNFSF13B*

INTRODUCTION

Chemotherapy remains one of the main options in the treatment of many cancers. Due to its adverse effects and limited efficacy in some cancers, however, its use should be limited to patients who have an excellent risk–benefit ratio. Myelotoxicity is the most relevant side effect, resulting in anemia, thrombopenia, and leukopenia. Severe neutropenic or leukopenic events (NLEs) may be complicated by life-threatening infections (febrile neutropenia, FN), requiring hospitalization and antibiotic therapy (1).

Dose reductions and treatment delays in patients with NLEs were considered as a possible reason for a worse prognosis (2). More recently, effects of chemotherapy on the immune system that consequently affect cancer therapy have been explored (3,4). In breast cancer (BC), for example, the importance of immunoregulatory genes for prognosis and treatment efficacy has been shown in several studies, and a PD-L1 antibody has been approved for the treatment of advanced BC (5).

Apart from clinical predictors for FN (6), very few molecular markers have been reported to be associated with either FN or NLEs. Our group previously published a report based on a genome-wide association study in lymphoblastoid cell lines that identified genetic variants in *PIGB* (phosphatidylinositol glycan anchor biosynthesis, class B) as a predictor for NLEs (7). Other, mostly retrospective, studies have described genetic risk factors for chemotherapy-induced leukopenia, neutropenia, or FN in BC patients (8–10). The largest fluorouracil, epirubicin, and cyclophosphamide (FEC) chemotherapy study, including around 1000 BC patients, concluded that adding single nucleotide polymorphisms (SNPs) to clinical predictors of FN might improve prediction of the events (8,9). A smaller report from a genome-wide association study (GWAS) in 270 Asian patients with various solid tumor histologies (11) found that SNPs in *MCPH1* were predictive for chemotherapy-induced neutropenia or leukopenia.

In the present study, we conducted a GWAS embedded in a large prospective and randomized chemotherapy study in patients with early BC, investigating associations with the occurrence of grade 3/4 NLEs. Two genome-wide significant ($P < 5E\text{-}8$) SNP signals were identified. One, rs4784750, mapped to the NLR family CARD domain containing 5 gene (*NLRC5*) and the other, rs16972207, to tumor necrosis factor superfamily member 13 beta (*TNFSF13B,* also known as *BAFF*, B-cell activating factor). The role of these genetic variants was also investigated in relation to prognosis and drug efficacy.

METHODS

Patients and Treatment

The multicenter SUCCESS-A study (12,13) included a prospective subprotocol concerned with the influence of germline genetic variants on side effects and efficacy of the chemotherapy. Patients were eligible if they had a histologically confirmed invasive BC with an increased risk for recurrence. Inclusion and exclusion criteria and patient characteristics are provided in Supplementary Tables S4A and S4B. The SUCCESS-A study was conducted in 251 study centers in all regions of Germany. The main study and all prespecified translational research projects, including the one reported here, were approved by all the ethics committees responsible and conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent.

Patients in the SUCCESS-A study were treated with three cycles of fluorouracil–epirubicin–cyclophosphamide (500/100/500 mg/m^2, FEC) followed by three cycles of docetaxel (100 mg/mg^2) every 3 weeks (q3w) versus three cycles of FEC followed by three cycles of gemcitabine (1000 mg/m^2 d1,8)–docetaxel (75 mg/m^2) q3w. HER2 positive patients were additionally treated with a 12-month treatment of adjuvant trastuzumab. After completing chemotherapy, the patients were further randomized to receive either 2 or 5 years of zoledronic acid. Premenopausal hormone receptor–positive women received tamoxifen alone or in combination with goserelin for 2 years if they were under 40 years of age. Postmenopausal patients were treated with tamoxifen for 2 years, followed by anastrozole for 3 years.

Primary surgery consisted of either breast conservation or mastectomy, leading to R0 resection in all cases. Sentinel-node dissection (SND) was performed in all cN0 patients (with SND as the only axillary intervention), followed by complete axillary node dissection in patients with positive sentinel nodes. The cN1 patients primarily received axillary node dissection. Radiotherapy was performed in accordance with national guidelines.

Clinicopathologic Information and Follow-Up

During the treatment phase blood cell counts were required at least twice per week. Hematological toxicity was documented according to NCI-CTCAE Version 3.0 at the end of every three weekly therapy cycle. The patients were followed at the study sites at 3-month intervals for the first 3 years and every 6 months thereafter. Follow-up included clinical examinations (each visit), mammography (every 6 months), and symptom-driven examinations if necessary. Disease-free survival was defined as the time from randomization to censoring without event or to a local recurrence, a distant recurrence or death of any cause, whichever occurred first. All data were obtained from the SUCCESS-A study electronic case record forms. The quality of the data was ensured through electronic data management, including automated plausibility checks and regular monitoring visits to the study site by an independent clinical research organization (Alcedis GmbH, Giessen, Germany) and a data monitoring committee (DMC).

Biomaterial Sampling and Patient Selection

A total of 3754 patients were randomized between September 2005 and March 2007. Whole blood samples were retrieved from 3584 patients (initial biomarker cohort) at the time of randomization. An initial quality check with 2% agarose gel electrophoresis of all samples showed that 1751 of the samples were not good enough for genotyping. The patients were therefore recalled, and blood was again drawn from 1102 patients. A total of 493 samples with DNA quality assessed as good enough were restored using the Illumina FFPE restoration kit, resulting in 3428 patients for genotyping, which was successful in 3328 individual patients, of which 2 had withdrawn consent and 4 were unexpected duplicates (all removed). Five patients were removed because they were related, nine patients of non-European heritage were also removed. DNA samples from 3308 patients (final biomarker cohort) were therefore used for GWAS analyses. Patient data for the final biomarker cohort and all randomized patients did not differ from each other (Supplementary Tables S4B and S5).

SNP Genotyping, Quality Control, and Imputation

The Illumina HumanOmniExpress-12v1 G FFPE array and the Infinium HD assay, in accordance with the manufacturer's recommendations, were considered as the best option for the restored DNA samples. Therefore, all samples were genotyped for 693,543 SNPs using the HumanOmniExpress-12v1 G FFPE array (genome build 37) regardless of whether they were restored or not.

For calling, the algorithm GenomeStudio, RRID:SCR_010973, version 2011.1, Genotyping Module 1.9.4, and GenTrain version 1.0 were used. Hardy Weinberg equilibrium was tested using two sets of unrelated subjects. Autosomal SNPs deviated from expectation at about 0.01 and the X-chromosome SNPs showed deviations between 0.01 and 0.001. SNPs were excluded using a filter threshold of 0.0001. Quality assurance and quality control were performed in accordance with Laurie et al. (14). As a consequence of this QC process the following number of SNPs were excluded in hierarchical order: 9,400 SNP assays failed; 17,134 SNPs had a MAF of zero; 26,652 SNPs had a missing call rate of >2%; for 160 SNPs, mendelian errors were observed in more than one HapMap trio/duo; 1,330 SNPs were excluded with HWE p-values < 1E-4, and 46 SNPs had more than one discordant call in 46 pairs of duplicated stidy samples, resulting in 638,837 SNPs remaining after the QC SNP filters. Finally, SNPs with a MAF < 0.01 were excluded resulting in genotyped 604,785 SNPs.

Median missing call rates per sample were 0.12% for genotypes from the original samples, 0.09% for new blood draws and 0.42% for the restored samples. No sample had a missing call rate >5%, and no sample was excluded because of post genotyping release QC failure. There were no statistically significant different call rates comparing patients with and without a NLE.

Variants were imputed from the 1000 Genomes Project, RRID:SCR_008801, data using the v3 April 2012 release35 as the reference panel. Imputation was based on the 1000 Genomes Project data with singletons removed. Genotype data (≈12.66 million SNPs) were imputed in a two-step procedure, with prephasing using SHAPEIT software and imputation of the phased data in the second step with IMPUTEv2. SNPs with MAF<0.01 and SNPs with the IMPUTEv2 “info” metric <0.3 were excluded, resulting in ≈8.86 million SNPs for further analysis. The “info” metric is highly correlated with the squared correlation r^2 from BEAGLE, RRID:SCR_001789, and MARCH, and for convenience will be denoted r^2 here too.

Statistical Analysis

The primary end point was grade 3/4 NLEs in the first three cycles of chemotherapy (yes vs. no), during which all patients were treated uniformly with 5-FU, epirubicin, and cyclophosphamide. Multiple logistic regression models were fitted for each SNP (ordinal; count of minor alleles) with age (continuous), body surface area (BSA, continuous), DNA type (restored vs. not restored), estrogen-receptor status, and HER2 status as additional predictors. Covariables were selected to account for general population differences (age, BSA), treatment differences (trastuzumab for HER2 positive patients), and possible differences concerning the influence of inherited genotypes on the molecular biology of breast cancer (e.g, estrogen receptor status). Adjusted odds ratios (ORs) per minor alleles and *P* values from likelihood ratio tests for each SNP were obtained from these logistic regression models.

A principal component analysis (PCA) was done using the R package SNPRelate. In order to avoid a strong influence of SNP clusters on the PCA, we used a LD-based pruned SNP set (ld.threshold = 0.2). The variance proportion was below 0.01% for each of the first ten principal components (PCs) indicating that the PCs have hardly any influence on the data variation. Therefore, PCs were not used as predictors for the logistic regression analyses. As a sensitivity analysis, however, the first and second PC were added to the regression models for the top SNPs and the ORs were recalculated.

The GWAS SNPs with a *P* value below the commonly accepted threshold of 5E-8 were regarded as having genome-wide significance (15). Only individuals with complete observations were considered (3276 of 3308 patients). Statistical analyses were conducted using the R statistical computing package. The Q–Q plot is shown in Supplementary Fig. S3.

As an exploratory study aim, the influence of the top SNP and NLE on disease-free survival was analyzed using the Kaplan-Meier method.

Cell Culture

The HL-60 and Jurkat cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The “Human Variation Panel” of lymphoblastoid cell lines (LCLs) was obtained from the Coriell Institute (Camden, NJ). DNA from these 287 LCLs had been genotyped in the Coriell Institute using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA), and in our laboratory using Illumina HumanHap 550K and HumanExon 510S-Duo BeadChips (Illumina, San Diego, CA). Imputation was then performed using 1000 Genomes data (http://www.1000genomes.org/data). We also generated gene expression data for these LCLs with Affymetrix U133/2.0/Plus GeneChip expression arrays, as described previously (16). Jurkat cells were cultured in Roswell Park

Memorial Institute (RPMI) media with 10% fetal bovine serum (FBS), and the HL-60 and LCL cells were cultured in the same media with 15% FBS.

***NLRC5* and *TNFSF13B* Knockdown and qRT-PCR**

Green fluorescent protein (GFP)-labeled vectors that contained short hairpin RNA (shRNA) and small interfering RNA (siRNA) for *NLRC5* as well as scrambled controls were obtained from OriGene Technologies, Inc. (Rockville, MD). For transfection of HL-60, Jurkat, and LCL cell lines, the Lonza (Basel, Switzerland) Anexa Nucleofector II Electroporation System was utilized. The knockdown efficiency was determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). mRNA was isolated with the DNA-free RNA kit (ZYMO Research Inc., Irvine, CA) and 100 ng/well total RNA was added for qRT-PCR assay using the Power SYBR® Green RNA-to-CT™ 1-Step Kit (Life Technologies, Carlsbad, CA) and predesigned PrimeTime primers obtained from Integrated DNA Technologies (Coralville, IA).

Cytotoxicity, Proliferation and Apoptosis assays

5-Fluorouracil and epirubicin were purchased from Sigma-Aldrich (St. Louis, MO). Mafosfamide (MFF) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MFF can spontaneously decompose to 4-hydroxycyclophosphamide, the active metabolite of cyclophosphamide, when added in culture media. To assay drug cytotoxicity, varying concentrations were added at 2–10-fold dilutions based on the half-maximal effective concentration (EC_{50}) values for each cell line. Cell viability was determined by MTS proliferation assay performed after drug treatment with the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) Reagent (Promega, Madison, WI), followed by analysis with an Infinite® M1000 PRO microplate reader (Tecan Systems Inc., San Jose, CA). Detailed methods for the cytotoxicity assay can be found in our previously publications (16–18). Briefly, cells were seeded in 96 well plates (Corning, Corning, NY) at a density of 5×10^5 cells/mL (100 μL/well). 10 μL of 5-FU (500–0.01 μM), epirubicin (10–0.0005 μM), or mafosfamide (100–0.005 μM) were added into the wells and incubated at 37°C for 72 hrs. 20 μL of MTS buffer was then added and plates were read in an Infinite M1000 PRO plate reader (Tecan AG, Switzerland) after incubation for 3 hrs. Relative cell viability was then plotted against drug concentration to derive cytotoxicity curves and EC_{50} values using GraphPad Prism (RRID:SCR_001789. GraphPad Software, La Jolla, CA). The drug concentrations (10 μM of 5-FU, 0.5 μM of EPI, and 5 μM of MFF) that were used to treat LCLs for TNFSF13B mRNA quantification were chosed based on their EC_{50} values determined by the cytotoxicity assay. For apoptosis assays, APC annexin V was purchased from BD Bioscience and propidium iodide (PI) from ThermoFisher Scientific. Samples were run on a BD FACSCanto™ flow cytometry system (San Jose, CA).

Western Blot Analyses

For Western blot experiments, cells were centrifuged at 200×g, washed with phosphate-buffered saline (PBS), and lysed with a hypotonic buffer that consisted of 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 2 mM ethylenediamine tetraacetic acid (EDTA), and 0.5% Triton X-100, to which "complete Mini EDTA free" tablets (Roche Applied Science, Indianapolis, IN) had been added. The buffer was maintained at 4°C and was added to the cells to initiate lysis. The mixture was incubated on ice for 15 minutes, and the lysed cell suspension was then centrifuged at 4°C at 12,000×g for 5 minutes. Protein concentrations in the supernatant were measured using the "Protein Assay Dye" Reagent (Bio-Rad, Hercules, CA) with bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as a standard. Samples of the supernatant were then denatured with 4× Lamelli buffer (Bio-Rad), heated for 3 minutes, and cooled to 4°C before loading onto Mini-PROTEAN gels (Bio-Rad). Gels were transferred using the Turboblot system (Biorad), and blots were incubated with appropriate antibodies. NLRC5 (Anti-NOD4, produced in a rabbit) was purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal vinculin antibody was purchased from Sigma-Aldrich. Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Chemiluminescence was determined using Pierce SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL) and was assayed using a Geldoc XR+ system (Biorad, Hercules, CA).

Chromatin Immunoprecipitation (ChIP) Assays

ChIP assays were performed using the "Epitect ChIP OneDay Kit" (Qiagen) procedure, with the following modifications: as LCLs are nonadherent, they were centrifuged at 200×g and washed with PBS. Fresh 1% formaldehyde was added to cross-link proteins to DNA, and cell lysis was performed. Chromatin shearing by sonication was performed using a Misonix XL sonication system (Qsonica L.L.C., Newtown, CT). Protein/DNA immunoprecipitation, DNA isolation and purification, ChIP DNA detection, and data analysis were then performed. All antibodies used were ChIP-grade and were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primers used for ChIP were purchased from IDT and had the following sequence: 5′-CAGGGCCTCATCTCCCA-3′ was the forward primer and 5′-TCCGAGCTCCTTCAGAAA-3′ was the reverse primer for the *NLRC5* rs4784751 SNP site to which SRF was bound; 5′-GGGTGAGGAAGGG-AAAGAAAT-3′ was the forward primer and 5′-CCTACCCATGTCTGCAATGT-3′ was the reverse primer for the *TNFSF13B* rs16972207 SNP site to which PXR was bound.

Patients for Testing the Effect of *NLRC5* Leukocyte Expression on the Efficacy of Neoadjuvant Chemotherapy

In order to explore possible roles of NLRC5 in the context of breast cancer treatment, we included an additional neoadjuvant study in which the therapeutic response of the tumors to chemotherapy could be assessed in relation to leukocyte NLRC5 expression. To test the effect of *NLRC5* leukocyte expression on the rate of pCR after neoadjuvant chemotherapy, a patient cohort was selected from the iMODE-B/TilGen Study (19). The first consecutive triple-negative BC (TNBC) patients treated with carboplatin and paclitaxel were selected, as well as healthy control individuals. Patient and tumor characteristics, including therapy and surgery results, were documented prospectively (Supplementary Table S6). A pCR was defined as complete disappearance of all tumor cells (pT0/pN0). The ethics committee of the medical faculty of Friedrich-Alexander-University, Erlangen, approved the study and all patients provided written informed consent.

RT-PCR of *NLRC5* from Leukocyte RNA for Predicting Pathological Complete Response

Full blood samples were collected in PaxGene® tubes from control individuals (n=21) and TNBC patients (n=21). All patient samples were collected before primary diagnosis. RNA was isolated according to the Maxwell® RSC miRNA tissue kit (Promega, Mannheim, Germany) with minor modifications. After centrifugation, the cell pellet was homogenized with 1-thioglycerol. Samples were denatured for 5 minutes at 80°C and treated with proteinase K for 10 minutes at 56°C. Lysates were centrifuged with QIAshredder® tubes (QIAGEN, Hilden, Germany) at full speed for 3 minutes. The flow-through of the sample lysates was taken for the Maxwell® extraction. After the automated run, samples were centrifuged at full speed for 5 minutes and supernatants were taken and incubated for 5 minutes at 65°C. RNA concentrations and purity were determined with the QuantiFluor® RNA Sample Kit (Promega, Mannheim, Germany) and PicoDrop® (Biozym, Hessisch Oldendorf, Germany).

cDNA synthesis (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Darmstadt, Germany) was performed in a thermal cycler (ABI2720, Applied Biosystems, Darmstadt, Germany) for 2 h at 37 °C. Gene expression of *NLRC5* (TF 5′-AGCAGTGCAAGAAGCAGCAGC-3′; BR 5′-GCTGATGCCGCGGGCAGTG-3′) was measured with SYBR Green®-based technology (Applied Biosystems, Darmstadt, Germany). The internal standards OAZI, Calm2, and RPL37 had also been determined in order to achieve semiquantitative results for gene expression. For data evaluation, the CT values were transformed into ratios using the 2-ΔΔ-CT method.

The nonparametric Mann–Whitney *U* test for independent samples was performed. A *P* value below 0.05 was considered as statistically significant.

Data Availability

Data of this GWAS is available under the dbGaP (Study Accession: phs000547.v1.p1).

RESULTS

GWAS for NLEs in Breast Cancer

A total of 1679 patients (51.3%) had a grade 3 or 4 NLE at any time during FEC chemotherapy. Two loci were associated with grade 3/4 NLEs at a genome-wide significance level ($P < 5E-8$) in women with BC after chemotherapy. One (rs4784750) mapped to the *NLRC5* gene on chromosome 16q12.2, and the other (rs16972207) to the *TNFSF13B* gene on chromosome 13q33.3 (Fig. 1A). The 10 SNPs with the lowest *P* values are shown in Table 1.

13q33.3 Locus

On chromosome 13, two imputed SNPs (rs16972207 and rs17564816) showed a genome-wide significant association with grade 3/4 NLEs (Figs. 2A and 2B). Both SNPs are located in intron 2 of *TNFSF13B* (Fig. 2B, red arrows). A SNP cluster in high linkage disequilibrium (LD) with the *TNFSF13B* rs16972207 SNP also showed low *P* values ($P<1E-6$). These SNPs mapped across this locus, which includes two other genes, *LIG4* and

ABHD13 (Fig. 2B). None of these SNPs is a nonsynonymous or nonsense SNP for either of these two genes. Frequencies of grade 3/4 NLEs according to rs16972207 are shown in Fig 1C.

Functional Analysis of the 13q33.3 Locus

To identify a set of credibly causal SNPs operating at the locus, we retrieved all SNPs in high ($r^2 \geq 0.8$) linkage disequilibrium (LD) with rs16972207 using LDlink (https://ldlink.nci.nih.gov/) in CEU (Utah residents from north and west Europe), generating a list of 32 additional SNPs located in genic (*TNFSF13B*, *ABHD13*, and *LIG4*) and intergenic regions (Supplementary Table 1). Interestingly, 17 SNPs in the set showed significant expression-quantitative trait locus (eQTL) associations with *LIG4*, including the two SNPs with the lowest (3a) RegulomeDB score (rs61972007 and rs61971985) (Supplementary Table 1). In addition, several SNPs showed interactions between enhancers and promoters, as identified by GeneHancer (which links enhancers to genes using tissue co-expression correlation between genes and enhancer RNAs, as well as enhancer-targeted transcription factor genes; expression quantitative trait loci for variants within enhancers; and capture Hi-C) and ChIA-PET (Chromatin Interaction Analysis by Paired-End Tag Sequencing) — suggesting that *TNFSF13B, LIG4,* and *ABHD13* constitute plausible target genes at the locus (Supplementary Table 1; Fig. 2B).

It has been reported that patients with late-onset neutropenia after rituximab therapy have a very high level of BAFF in serum (20,21). We therefore set out to determine whether expression of the *TNFSF13B* gene changes after therapy with the drugs used to treat patients enrolled in the SUCCESS trial (13). To determine whether the *TNFSF13B* SNPs affect gene expression after drug treatment, LCLs that were homozygous reference (n=4) and homozygous variant (n=4) for the *TNFSF13B* "top" SNP, rs16972207 (C>G), were used. These LCLs are B-lymphocytes in origin and they highly express *TNFSF13B*. After 48 hours of epirubicin alone and epirubicin plus 5-fluorouracil (5-FU) treatment, the *TNFSF13B* mRNA level was increased in LCLs that were homozygous variant (G/G), in comparison with the reference genotype (C/C) for rs16972207 (Fig. 3A). TNFSF13B mRNA levels were more highly induced after treatment with the 5-FU/EPI combination than after EPI alone, indicating a synergistic effect on TNFSF13B induction by 5-FU/EPI combined treatment (Fig. 3A). No differences in *TNFSF13B* mRNA levels were observed after treatments with 5-FU or mafosfamide (MFF) alone or in combination (data not shown). Since the TNFSF13B protein is cleaved and is present in the extracellular milieu as a cytokine, we sought to determine whether levels of BAFF in cell media differ in an SNP-dependent manner by performing an enzyme-linked immunosorbent assay (ELISA). The BAFF levels did not differ in an allele-specific fashion at baseline after 48 hours when charcoal-stripped conditioned media incubated with LCLs was analyzed. However, after drug exposure for 24 and 48 hours, there was an allele-specific difference in the level of BAFF released by LCLs with the homozygous variant genotype (G/G) in comparison with LCLs with the reference genotype (C/C) for rs16972207 (Fig. 3B). To determine the possible mechanism for this difference, we analyzed the DNA sequences of *TNFSF13B* SNPs in the TRANSFAC database. These *in silico* analyses indicated that the rs16972207 SNP was located in a binding site for the pregnane X receptor (PXR), a ligand-activated transcription factor that can be activated by xenobiotics, including chemotherapy drugs (22) (Fig. 3C). To test the possibility that the SNP altered PXR binding to the nearby DNA sequence, a ChIP assay using anti-PXR antibody was performed in LCLs with homozygous variant or WT SNP genotypes after combined epirubicin and 5-fluorouracil treatment. The variant SNP sequence showed a 4.5-fold increase in the PXR bound (Fig. 3D), which might explain the higher *TNFSF13B* transcription in variant LCLs after combined epirubicin and 5-fluorouracil treatment.

16q13 Locus

At chromosome 16, the most strongly associated SNP (rs4784750, P=1.56E-8) was imputed (imputation r^2=0.99), while the second most strongly associated one was an originally genotyped SNP (rs4784751, P=4.07E-8) (Fig. 2C). These SNPs, including the two additional SNPs at 16q12.2 (rs12444396 and rs12445252), are in tight linkage disequilibrium (LD). All SNPs with the lowest P values mapped to introns in *NLRC5* (Fig. 2D).

With regard to rs4784751, Grade 3/4 NLEs occurred in 47.2% of patients with the common genotype during the FEC-containing chemotherapy cycles, increasing to 54.6% in heterozygous patients and 62.9% in patients who had two minor alleles. Corresponding odds ratios were 1.34 (95%CI: 1.16–1.55) and 1.89 (95%CI: 1.43–2.49) when heterozygous or homozygous variant patients were compared with patients carrying two reference alleles (Fig. 1B). Adding the first and second principal component to the regression model as a sensitivity analysis did not change those results.

As per protocol, prophylactic granulocyte colony-stimulating factor (G-CSF) was not required in the first chemotherapy cycle. Prophylactic G-CSF is usually given in the first week after chemotherapy regardless of white blood cell counts. After the occurrence of a grade 3 or 4 NLE, prophylactic G-CSF use is recommened during all subsequent chemotherapy cycles. While not being required in the first chemotherapy cycle, 10.9% of the patients received prophylactic G-CSF during the first chemohtherapy cycle and 16.7% and 19.8% inc cycles 2 and 3 respectively. To examine the effects of G-CSF on our results, we performed a sensitivity analysis for rs4784751, and restricted the outcome measure of grade 3/4 NLEs to the first chemotherapy cycle, and introduced G-CSF as a covariate. A total of 39.2% had a grade 3/4 NLE in the first chemotherapy cycle. rs4784751 maintained its predictive value both in patients who received G-CSF (10.9% of the patient population) and patients who did not. Nor were there any differences in effect size between the two groups (data not shown).

Functional Analysis of the 16q13 Locus

To identify a set of credible causal SNPs operating at the locus, we retrieved all SNPs in high (r^2≥0.8) linkage disequilibrium with rs4784750 using LDlink (https://ldlink.nci.nih.gov/) in CEU (Utah residents from north and west Europe), generating a list of four additional SNPs, all located in an intronic region of *NLRC5*. This 3-kb region shows evidence of regulatory activity in blood (Haploreg v4.1) (Supplementary Table 1). Interestingly, chromatin interaction analysis paired-end tags (ChIA-PET) data from ENCODE/GIS-Ruan for RNA polymerase II in K562 cells show that three SNPs (rs4784751, rs12444396, and rs12445252) interact with the promoter region of *NLRC5*, while rs11644171 interacts with an intronic region of *CPNE2* (Fig. 2D). Notably, all five SNPs displayed eQTL associations to *NLRC5.* Taken together, functional assessment of the credible causal SNPs in the locus strongly suggests that *NLRC5* is the likely target gene in this locus.

As NLEs are mainly caused by the effect of chemotherapy on hematopoietic cells, functional studies were conducted on cell lines from the hematopoietic system to understand the role of *NLRC5* in NLEs. We used three leukemia cell lines expressing *NLRC5,* representing different origins such as promyeloblasts, B lymphocytes and T lymphocytes.

We first performed cytotoxicity assays to determine whether silencing of *NLRC5* might influence the cell response to the chemotherapy drugs used in the SUCCESS-A trial, including epirubicin (EPI), cyclophosphamide, and 5-fluorouracil (5-FU). Since cyclophosphamide is a prodrug that requires metabolic activation, mafosfamide (MFF) — which spontaneously decomposes to form the cyclophosphamide active metabolite (4-hydroxycyclophosphamide) when added in culture media (23) — was used in the cytotoxicity assay. After 72 hours of drug treatment, no significant changes were observed in the cytotoxicity of these drugs in HL-60, LCL, or Jurkat cells after *NLRC5* knockdown (Supplementary Fig. 1). These results suggested that *NLRC5* expression in these cell lines does not directly dramatically affect the cytotoxicity of the chemotherapy drugs used in the SUCCESS-A trial.

Next, we investigated whether *NLCR5* silencing might affect cell viability in HL-60 cells — a cell line of promyelocytic origin that most closely represents hematopoietic stem cells. An immediate effect on cell viability after *NLRC5* silencing was observed. Apoptosis increased after *NLRC5* silencing, as analyzed by flow cytometry with staining of apoptotic markers by APC annexin-V (APC-A) and propidium iodide (PI) or PI alone (Fig. 4A). Cells that were in the process of undergoing apoptosis displayed annexin-V and PI, and cells that had already undergone apoptosis only displayed PI alone (Fig. 4A).

Since we observed that silencing of *NRLC5* promotes cell apoptosis, we further determined the drug effects on *NLRC5* expression and how the SNPs might contribute to the changes. To do this, the LCLs from which the genome-wide SNP genotype data were generated were used. Based on the genotype of the *NLRC5* rs4784750 SNP, four each reference and variant LCLs were treated with the chemotherapy drugs at concentrations equivalent to their EC_{50} values. After 72 hours of drug exposure, we observed a genotype-dependent difference in *NLRC5* mRNA levels that corresponded to a difference in *NLRC5* protein levels in the same LCLs (Figs. 4C and 4D). Specifically, LCLs homozygous for the variant alleles (T/T) showed a greater decrease in *NLRC5* mRNA and protein after drug exposure than did LCLs homozygous for the reference alleles (G/G) alleles, and this was statistically significant after 72 hours (Fig. 4D).

To determine the possible cause of this genotype-dependent and drug exposure–dependent difference in expression of *NLRC5* mRNA and protein, we analyzed the DNA sequences of the top 5 *NLRC5* SNPs with low *P* values in the GWAS using the TRANSFAC database. TRANSFAC suggested that the rs4784751 (C>T) SNP variant allele disrupted a serum response element (SRE) motif to which the serum response factor (SRF) transcription factor was predicted to bind (Fig. 4E). The rs4784751 SNP is in linkage disequilibrium (r^2 = 0.99 in Caucasians, based on the 1K Genomes data) with the rs4784750 SNP that shows the lowest *P* value in the GWAS. To test the TRANSFAC prediction, a ChIP assay was performed with anti-SRF antibody, and the wild-type sequence showed 3.8-fold greater SRF binding than did the variant sequence (Fig. 4F).

These functional studies suggested a possible mechanism in which the chemotherapy dramatically decreased *NLRC5* expression in patients with a variant genotype for the rs4784751 SNP, which might result from decreased SRF binding and *NLRC5* transcription; thus, the decrease in *NLRC5* expression was associated with increased cell apoptosis. Further mechanistic studies need to be pursued in order to understand how decreased levels of *NLRC5* may lead to apoptosis.

eQTL Analysis in Whole Blood Samples for both loci

To determine whether the two loci on chromosome 16 and chromosome 13 are expression-quantitative trait loci (eQTLs), we associated the genotypes of the “top” SNPs in both loci (rs4784750 in *NLRC5* and rs16972207 in *TNFSF13B*) with gene expression in a publicly available GTEx (v7) database (http://www.gtexportal.org/home/). As our phenotype, NLE, occurs in whole blood, we examined gene expression in the tissue “whole blood.” All sufficiently expressed genes (n = 23,076) were tested for eQTL analysis. The “top” ten genes with expression mostly associated with rs4784750 and rs16972207 SNP genotypes are shown in Supplementary Tables 2 and 3, respectively. The rs4784750 SNP genotype is most significantly associated with *NLRC5* expression, with a beta value of –0.26 and a *P* value of 2.2E-7 in whole blood samples (Supplementary Table 2). The variant genotype was associated with decreased *NLRC5* expression (Supplementary Fig. 2A). The rs4784750 SNP is a *trans*-eQTL for other genes in whole blood samples, such as *PANX1*, expression of which correlated positively with the variant genotype of rs4784750 (β=0.28, *P*=7.9E-6) (Supplementary Fig. 2B).

The rs16972207 SNP was an eQTL for *TNFSF13B* mRNA expression (Z-score=4.60, *P*=4.22E-6) in whole blood samples from a study with large cohort (n=5,311) while it was not associated with *TNFSF13B* mRNA expression in GTEx in which a relatively small sample size was tested (n=407) (24,25). Our functional study demonstrated that rs16972207 was significantly associated with *TNFSF13B* mRNA expression after drug exposure, a situation which has been referred to as a “pharmacogenomic-eQTL” (26–28). The rs16972207 genotype was associated with *LIG4* gene expression (β=0.13, *P*=4E-4), which mapped centromerically to *TNFSF13B* (Fig. 2A and Supplementary Table FIg3).

Combined effects of *NLRC5* and *TNFSF13B* SNPs

Since SNPs in both the *NLRC5* and *TNFSF13B* genes were strongly associated with NLEs, we investigated possible correlations of mRNA expression for these two genes in our “Human Variation Panel” and other datasets (Fig. 5A–5C). It was found that *NLRC5* and

TNFSF13B mRNA expression was highly correlated in the LCLs (r^2=0.51, P<1E-21) (Fig. 5B) and in BC datasets listed in Oncomine (r^2=0.645, n=160 and r^2=0.578, n=55) (29,30). We also assessed the “combined effect” of SNPs in these two genes relative to the risk for NLEs. This analysis showed that the difference between odds ratio (OR) values for patients who were homozygous for both risk alleles was 4.8, compared to patients homozygous for both protective alleles (Fig. 5B). It is interesting to note that the trend observed at baseline in healthy untreated LCLs as well as untreated BCs from Oncomine suggest that these genes may be under a similar type of regulation at baseline. However, upon treatment with chemotherapy and induction of these transcription factors, these genes become regulated in an inverse way (Fig. 5C).

Results for Clinical Outcome Parameters

Association with prognosis in the SUCCESS study.—In an exploratory analysis, we associated the genotypes and NLE with disease-free survival in the SUCCESS-A study (Fig. 6A). Median follow up time was 5.2 years and the number of events was 414. There appeared to be an effect on the prognosis in the group of women who had a homozygous variant effect. The group of women with NLEs and a genotype associated with down-regulation of *NLRC5* after chemotherapy appeared to perform worst, while women without NLE after chemotherapy performed better.

Influence of white blood cell *NLRC5* expression on neoadjuvant chemotherapy responsiveness.—As the eQTL analysis showed that *NLRC5* genotype could have an influence on NLRC5 expression in whole blood (Supplementary Figure 2), we tested the possible influence of leukocyte *NLRC5* expression on chemotherapy responsiveness. To do that, we conducted a small neoadjuvant study in which white blood cell RNA was collected prospectively before the start of chemotherapy. Blood was also collected from healthy control individuals. Although no differences were detectable between healthy individuals and triple-negative BC patients before chemotherapy (Fig. 6B), patients who did not achieve a pathological complete response (pCR) after chemotherapy had significantly lower (P=0.02) *NLRC5* expression than patients who did achieve a pCR after chemotherapy (Fig. 6B).

DISCUSSION

We identified two chromosomal loci associated with grade 3/4 neutropenic or leukopenic events after chemotherapy with epirubicin, cyclophosphamide, and 5-fluorouracil. At the 13q33.3 locus, *ABHD13*, *LIG4,* and *TNFSF13B* (also known as *BAFF,* B cell activating factor) emerged as plausible target genes after functional annotation using publicly available data. *ABHD13* has been associated through GWAS with the monocyte count (31), and *LIG4* is essential for V(D)J recombination and DNA double-strand break (DSB) repair through nonhomologous end joining (NHEJ) — processes known to affect the response to therapeutic drugs. *TNFSF13B* is expressed by many cells such as antigen-presenting cells (B cells, macrophages, dendritic cells), neutrophils, epithelial cells, T lymphocytes, and stromal cells (32,33). Most functional knowledge of this gene relates to its role as a survival factor for peripheral B cells.

Impaired B cell maturation, decreased immunoglobulin levels, decreased T cell dependent and T cell independent immune responses have been observed in *Tnfsf13b* knockout mice (34). On the other hand, transgenic *Tnfsf13b* mice develop B cell hyperplasia, glomerulonephritis, and destruction of the salivary glands, as well as expansion of the effector and regulatory T cell compartments (35,36). Our top SNP in *TNFSF13B* (rs16972207) was observed to be an eQTL for TNFSF13B mRNA expression in a large cohort of subjects. We also demonstrated that this SNP is significantly associated with TNFSF13B expression after drug exposure, which appeared to be related to an influence on the binding of transcription factor PXR with the variant genotype, which showed greater affinity for the transcription factor than did the wild type.

There have been reports of ‘late-onset’ neutropenia after treatment with rituximab, with high levels of BAFF being found in patients with neutropenia. The neutropenia might be the consequence of hematopoietic lineage competition due to excessive B cell recovery in the bone marrow (20,21). Taken together, the data suggest that *TNFSF13B* is a likely target gene at this locus, but a contribution of *ABHD13* or *LIG4* cannot be ruled out.

At the 16q13 locus, *NLRC5* emerged as the most likely target gene. The top SNPs at 16q13 showed an eQTL association with *NLRC5* expression in whole blood samples, supporting the hypothesis that *NLRC5* is the target gene. *NLRC5* has been described as a key regulator of major histocompatibility complex (MHC) class I gene expression (37–40) as well as other genes in the antigen-presenting system (37,38). In contrast to MHC class II molecules, which are mainly expressed on hematopoietic cells, MHC class I molecules are expressed in all cells that contain a nucleus (41). This is observed in all immune tissues and organs such as the spleen, lymph nodes, bone marrow, and thymus. Although the transcription of *NLRC5* has been described as being increased by interferon gamma (IFN-γ) and activation of *STAT1* (42,43), our *in silico* analysis of the locus in *NLRC5* implied a change in a region that might serve as a binding site for the transcription factor SRF. SRF has indeed been shown to indirectly regulate type I interferon signaling in macrophages (44) without interfering with the classic JAK/STAT pathway (44). It has also been demonstrated that in macrophages, lipopolysaccharide administration induces high levels of *NLRC5* through the type I interferon pathway (45,46). The interaction between the genotype discovered and the effect of the chemotherapy might be mediated by an SRF-dependent effect after the interferon type I pathway. Recently, SRF has been described as an essential transcription factor in hematopoiesis (47). The present functional *in vitro* assessment showed that chemotherapy modulates the expression of *NLRC5* and *TNFSF13B* in an allele-specific manner, down-regulating *NLRC5* after chemotherapy and up-regulating *TNFSF13B.* Both *NLRC5* and *TNFSF13B* are known genes with functions in innate and adaptive immune responses.

A more toxic effect of chemotherapy on the white blood cell count in patients with the *NLRC5* variant genotype may be mediated through *PANX1*, which was the top *trans*-eQTL finding for the SNP rs4784750. Although formally not having a false discovery rate of ≤0.05 as required by the GTEx project (48,49), *PANX1* has been reported to drive inflammation (50) and facilitate apoptosis, pyroptosis, and autophagy (50,51).

An interesting aspect of the association between these two genes and chemotherapy-related neutropenia or leukopenia is their relation to recent immuno-oncological findings. Particularly because *NLRC5* regulates MHC class I gene expression, its role in immune evasion by cancer cells has been analyzed (52,53). For several histologies, high *NLRC5* expression is associated with a favorable prognosis (52). It has also been shown that *NLRC5* expression is associated with increased activation of CD8^{+} cytotoxic T cells (52). This makes *NLRC5* an interesting target for possible cancer therapies, as well as an interesting prognostic marker. The present analysis in relation to the clinical outcome in the SUCCESS-A study did not show that rs4784750 was associated with prognosis. Nor was the occurrence of grade 3/4 NLEs associated with the prognosis. However, when the analysis for neutropenia and rs4784750 genotype was stratified, there was some indication that patients who suffer neutropenia after chemotherapy in the variant genotype group have an unfavorable prognosis. This effect may correlate with *NLRC5* expression in white blood cells, but could also be a consequence of differential *NLRC5* expression in the tumor. Our small neoadjuvant chemotherapy study also showed that chemotherapy responsiveness correlates with *NLRC5* expression in white blood cells — implying a possible interaction of *NLRC5* with immuno-oncological mechanisms and chemotherapy response. Of note, the effects of genotypes on prognosis and the effect of NLRC5 gene expression on chemotherapy responsiveness do not validate the GWAS findings. However, they shed light on the possible role of this gene in relation to breast cancer treatment.

With regard to treatment implications, one possible clinical application might be up-regulation of *NLRC5* during chemotherapy — e.g., with interferon. For tumor cells *in vivo*, it has already been demonstrated that increasing *NLRC5* activity restores tumor immunogenicity and stimulates antitumor immunity (54).

This study has both strengths and limitations. It is the first study to examine neutropenia and leukopenia as part of a prospective phase III chemotherapy study. This ensures high data quality, with on-site monitoring and auditing as well as pre-specified data management and statistical analysis procedures. Cumulative NLE events were available as a variable. While documentation according to NCI-CTCAE criteria as in our study is a standard for capturing this phenotype, more detailed data such as time to NLE were not available. With NLE grade 3/4 occurring in more than 40% of patients, the phenotype is also frequent enough to provide adequate statistical power. Having more than 3300 patients, the sample size should, therefore, have been sufficient to discover relevant genetic variants. Despite extensive *in vitro* functional validation of the findings, we acknowledge the need for in depth mechanistic studies. Although the functional experiments provide a degree of confidence for accurate findings, empirical validation would be desirable. At the time when the study was conducted (2005–2007), combined treatment with epirubicin, cyclophosphamide, 5-fluorouracil, docetaxel, and gemcitabine was reasonable, but gemcitabine never became a standard treatment in the adjuvant setting for BC. 5-FU, which appeared to play a role at least in molecular effects in relation to *TNFSF13B,* is no longer administered to BC patients, as its effectiveness was not confirmed (55). With regard to generalization of the data, it is noteworthy that minor allele frequencies differ widely among Caucasian, Han Chinese, and African-American individuals. The minor allele frequencies (MAFs) for the SNPs in our two genes were compared with the HapMap data. The variant alleles for rs4784751 in *NLRC5* and rs16972207 in *TNFSF13B* were most prevalent in the Caucasian population, with MAFs of 0.32 and 0.18, respectively. In our study these MAFs were 0.27 and 0.19 respectively. In comparison with other ethnicities, the Han Chinese population had MAFs of 0.04 and 0.00, and the African-American population had MAFs of 0.02 and 0.192, respectively. While there have been reports on ethnicity-specific differences in the occurrence of neutropenia after chemotherapy (56), it is unclear whether genotypes might play a major role in these differences, in part because other factors might play a role like pre-chemotherapy baseline white blood cell count (57,58). Also, studies of this question are scarce. With regard to the clinical meaning of our results, the top SNPs could differentiate patient groups with 47–49% to 63–68% of patients experiencing grade 3 or 4 neutropenie/leukopenia. While these differences might seem clinically relevant, it has to be noted, that even with the protective alleles a large proportion of patients still experiences NLE, warranting further studies designed to examine the reasons for NLE after chemotherapy.

In summary, this study provides evidence that genetic variants of the key regulator of MHC class I expression may be involved in chemotherapy-induced neutropenia through genotype-dependent down-regulation of the gene. In addition, *NLRC5* genotypes may be involved in differences in the efficacy of chemotherapy and in the prognosis in BC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of translational relevance

Grade 3 or 4 neutropenic or leukopenic events are the most relevant side effects after chemotherapy but molecular factors associated with the occurrence are unclear. This study identifies loci in NLRC5 and TNFSF13B that are associated with post-chemotherapy neutropenia and leukopenia. Genotypes also showed an association with the prognosis in patients with a neutropenic or leukopenic event. Thus, NLRC5 is suggested as a prognostic and predictive marker for breast cancer patients receiving chemotherapy. With regard to treatment implications, one possible clinical application might be up-regulation of NLRC5 during chemotherapy — e.g., with interferon. For tumor cells *in vivo*, it has already been demonstrated that increasing NLRC5 activity restores tumor immunogenicity and stimulates antitumor immunity.

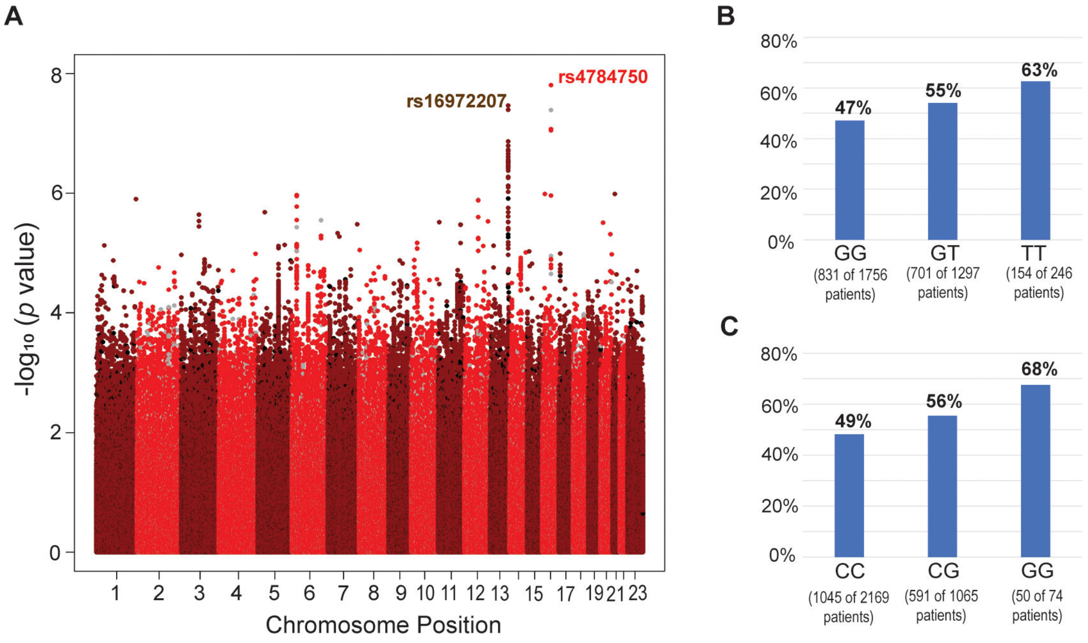


Figure 1.

(A) Manhattan plot for the association between neutropenic or leukopenic events (NLEs) and the genotypes from genome-wide genotyping for SNPs with a minor allele frequency > 0.01 (imputed: red/dark red; genotyped: black/grey). (**B and C)** Distribution of genotypes and neutropenic and leukopenic events (NLEs) among all genotyped patients for the top SNPs in the 16q13 (rs4784751; P = 1.56E-8) locus (**B**) and 13q33.3 (rs16972207; P = 3.42E-8) locus (**C**).

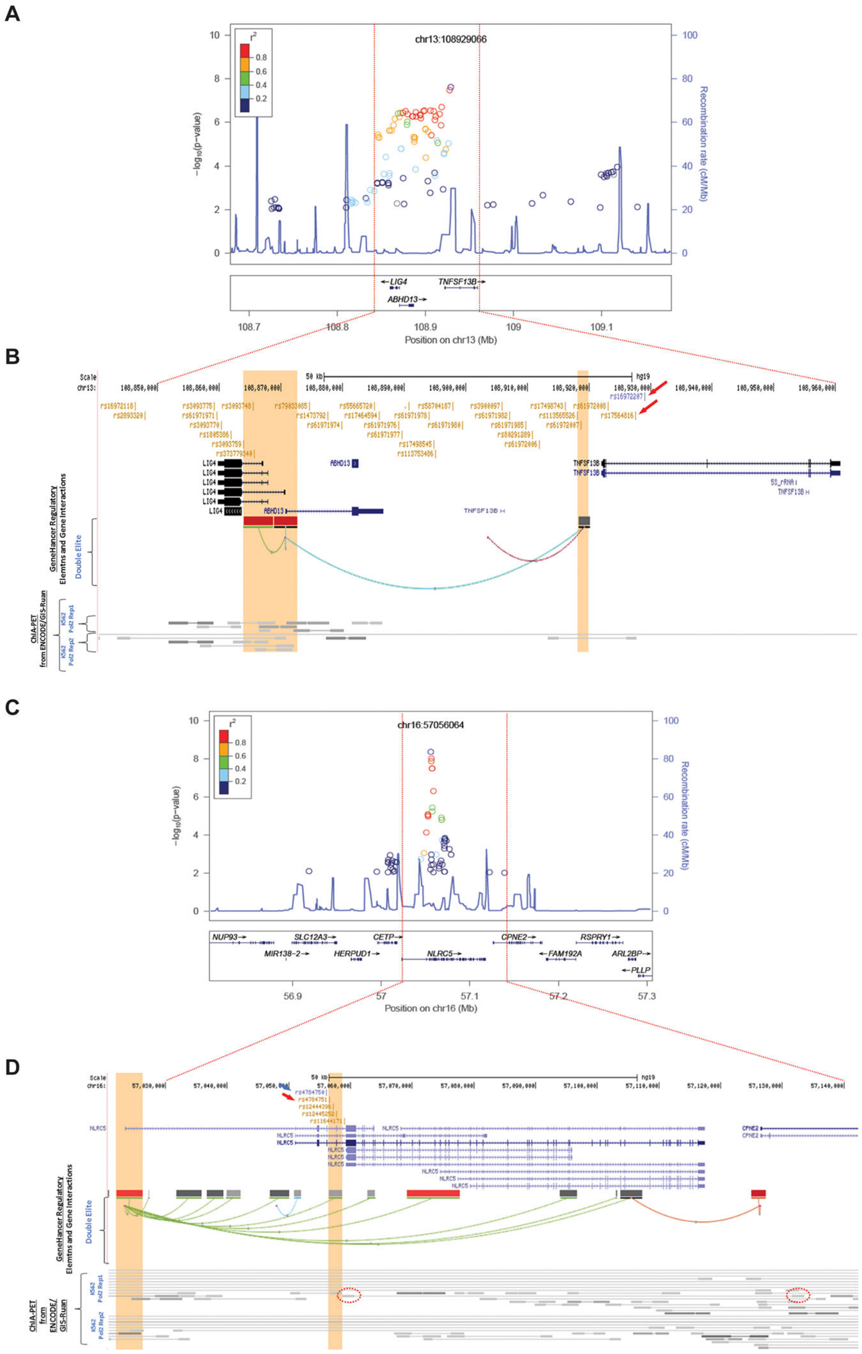


Figure 2.

(A and **B)** Architecture of the 13q33.3 NLE susceptibility locus; (**C** and **D**) Architecture of the 16q13 NLE susceptibility locus.

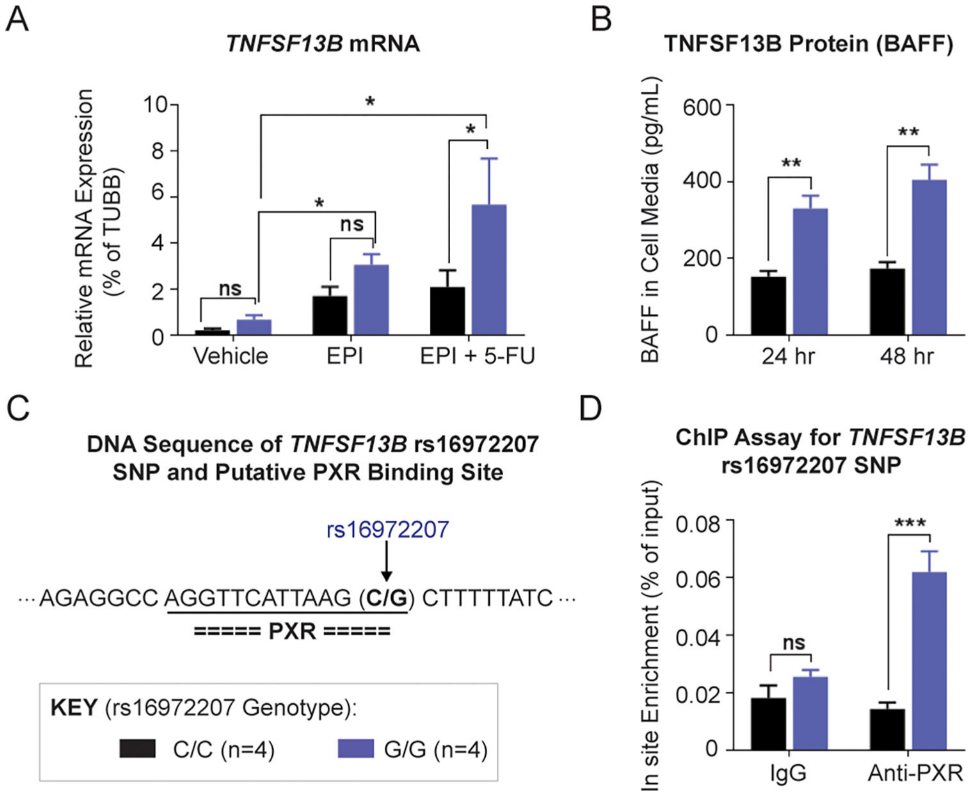


Figure 3. Functional studies for the *TNFSF13B* signal.

(**A**) *TNFSF13B* mRNA levels in lymphoblastoid cell lines (LCLs) with WT (C/C) and homozygous variant (G/G) rs16972207 SNP genotype after 48 h of EPI and EPI plus 5-FU treatment. Concentrations of 5-FU and EPI that were used were 10 μM and 0.5 μM, respectively, concentrations which are approximately equal to their EC_{50} values. (**B**) Secreted TNFSF13B protein (BAFF) in LCL cell media after EPI plus 5-FU treatment. (**C)** DNA sequence near the *TNFSF13B* rs16972207 SNP and putative transcriptional factor binding sites predicted by the TRANSFAC. The rs16972207 SNP was predicted, which maps to a site that binds pregnane X receptor (PXR). (**D)** ChIP assay with anti-PXR antibody for the rs16972207 SNP site in LCLs with WT (C/C) and homozygous variant (G/G) genotypes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant.

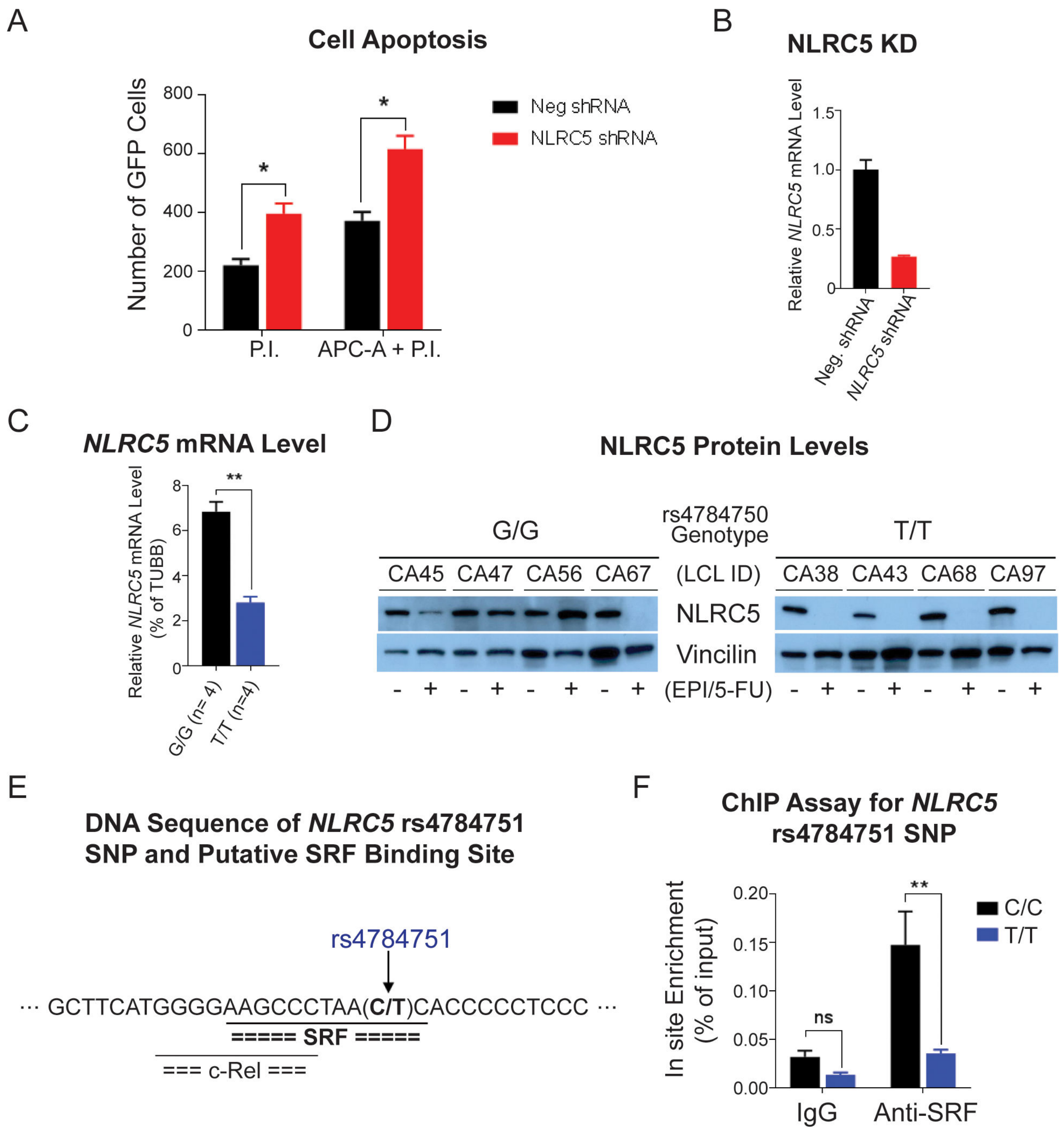
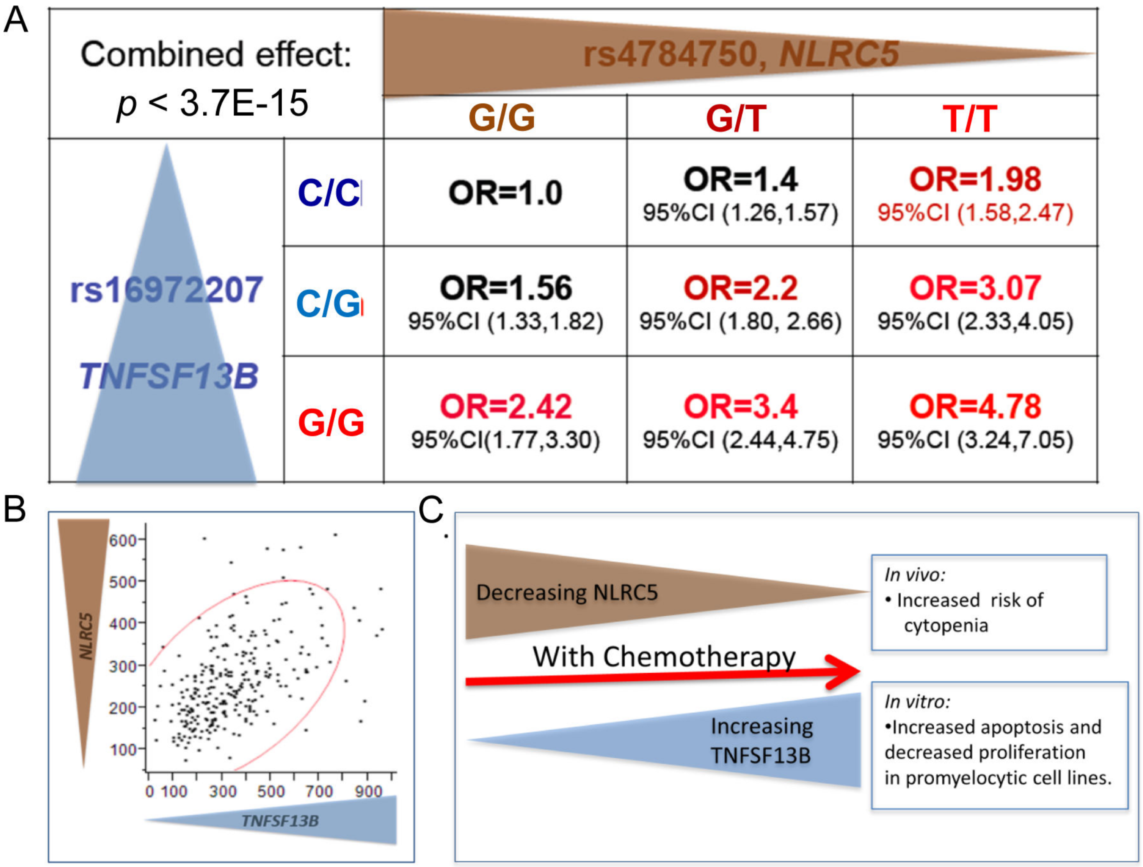


Figure 4. Functional studies for the *NLRC5* signal.

(**A**) HL-60 cell apoptosis measured by flow cytometry. The bar graph shows the numbers of cells that were stained with apoptosis markers by propidium iodide (P.I.) and P.I. plus APC annexin V (APC-A) 12 h after transfection of shRNAs. (**B**) Relative *NLRC5* mRNA level in HL-60 cells after 12 h of shRNA transfection. (**C**) The *NLRC5* mRNA level in lymphoblastoid cell lines (LCLs) with WT (G/G) and homozygous variant (T/T) rs4784750 SNP genotypes after 48 h of EPI and 5-FU combined treatment. (**D**) Western blot for NLRC5 protein in LCLs with WT and homozygous variant rs4784750 SNP genotypes after 72 h of EPI and 5-FU combined treatment. (**E**) DNA sequence near the *NLRC5* rs4784751

SNP and putative transcriptional factor binding sites predicted by the TRANSFAC. The rs4784751 SNP, which is in LD ($r^2 = 0.99$) with the rs4784750, was predicted in a serum response element that binds serum response factor (SRF). (**F)** ChIP assay with anti-SRF antibody for the rs4784751 SNP site in LCLs with WT (C/C) and homozygous variant (T/T) genotypes. * $P < 0.05$, ** $P < 0.01$, ns = not significant.



Combined effect: *p* < 3.7E-15		rs4784750, *NLRC5* G/G	G/T	T/T
rs16972207 *TNFSF13B*	C/C	OR=1.0	OR=1.4 95%CI (1.26,1.57)	OR=1.98 95%CI (1.58,2.47)
	C/G	OR=1.56 95%CI (1.33,1.82)	OR=2.2 95%CI (1.80, 2.66)	OR=3.07 95%CI (2.33,4.05)
	G/G	OR=2.42 95%CI(1.77,3.30)	OR=3.4 95%CI (2.44,4.75)	OR=4.78 95%CI (3.24,7.05)

(CI, confidence interval; M, major allele; m, minor allele; OR, odds ratio.)

Figure 5.

(A) Combined effect of *NLRC5* and *TNFSF13B* top SNP*s* calculated as the odds ratio for developing cytopenia/toxicity with chemotherapy. (**B**) Correlation of the gene expression between NLRC5 and TNFSF13B in the Mayo Human Variation Panel of lymphoblastoid cell lines (LCLs) derived from 287 individuals. (**C**) Putative model of the interaction of NLRC5 and TNFSF13B and their influence on the risk concerning cytopenia.

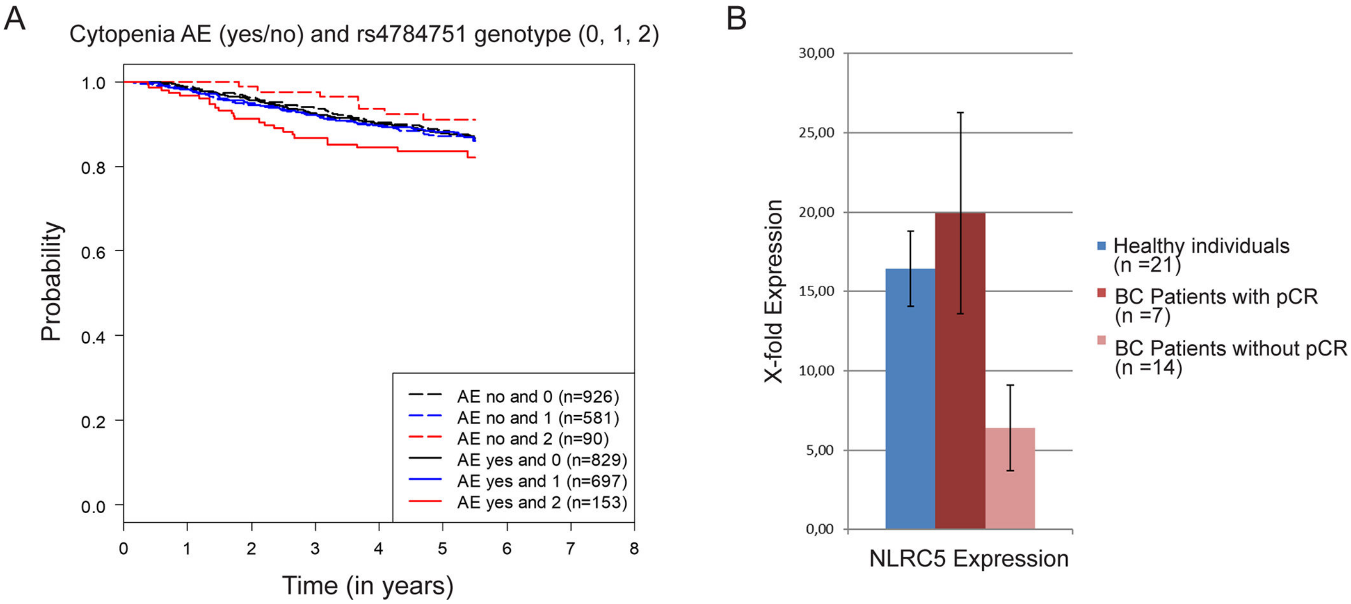


Figure 6.

(A) Effect of *NLRC5* genotype on the *prognosis* in the SUCCESS-A study: Kaplan–Meier curves for disease-free survival relative to neutropenic or leukopenic events (AE) and the *NLRC5* rs4784751 genotype (0, zero minor alleles; 1 one minor allele; 2, two minor alleles). (**B**) Effect of *NLRC5* leukocyte expression on the *pathological complete response* (pCR) in a group of triple-negative breast cancer (TNBC) patients: comparison of white blood cell *NLRC5* expression between healthy individuals, patients who achieved a pCR after neoadjuvant chemotherapy, and patients who did not achieve a pCR.

Table 1.

Genetic variants with the lowest *P* values

SNP	Chr	Position	MAF	Gene	Common allele	Minor allele	Type	Odds ratio (95% CI)	*P* value
rs4784750	16	57056064	0.273	*NLRC5*	G	T	I	1.38 (1.23–1.54)	1.56E-8
rs16972207	13	108929066	0.185	*TNFSF13B*	C	G	I	1.54 (1.32–1.79)	3.42E-8
rs17564816	13	108927503	0.185	*TNFSF13B*	G	A	I	1.52 (1.31–1.77)	4.01E-8
rs4784751	16	57056574	0.270	*NLRC5*	C	T	O	1.36 (1.22–1.52)	4.07E-8
rs12444396	16	57057194	0.264	*NLRC5*	G	A	I	1.36 (1.21–1.52)	8.43E-8
rs12445252	16	57057679	0.264	*NLRC5*	C	T	I	1.36 (1.21–1.52)	8.90E-8
rs61972007	13	108918701	0.191	*TNFSF13B*	A	G	I	1.42 (1.25–1.62)	1.37E-7
rs61971976	13	108889127	0.216	*TNFSF13B*	G	A	I	1.39 (1.23–1.58)	1.60E-7
rs61971980	13	108899416	0.186	*TNFSF13B*	G	A	I	1.40 (1.23–1.60)	1.87E-7
rs3900097	13	108905819	0.186	*TNFSF13B*	C	T	I	1.40 (1.23–1.60)	1.88E-7

Abbreviations: Chr, chromosome; I, imputed single nucleotide polymorphism; MAF, minor allele frequency; O, originally genotyped single nucleotide polymorphism.