MICA polymorphism identified by whole genome array associated with NKG2D-mediated cytotoxicity in T-cell large granular lymphocyte leukemia

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Large granular lymphocyte leukemia is a semi-autonomous clonal proliferation of cytotoxic T cells accompanied by immune cytopenias and various autoimmune conditions. Due to the rarity of this disease and its association with autoimmune diseases, a theoretical germline or somatic mutation might have significant penetrance, thus enabling detection, even from samples of suboptimal size, through genome-wide association studies.

Design and Methods

To investigate a non-mendelian genetic predisposition to large granular lymphocyte leukemia, we used a step-wise method for gene discovery. First, a modified 'random forests' technique was used for candidate gene identification: this was followed by traditional allele-specific polymerase chain reaction, sequencing modalities, and mechanistic assays.

Results

Our analysis found an association with *MICA*, a non-peptide-presenting, tightly regulated, stress-induced MHC-like molecule and cognate receptor for NKG2D, found abundantly on large granular lymphocyte leukemia cells. Sequencing of germline DNA revealed a higher frequency of MICA*00801/A5.1 in patients with large granular lymphocyte leukemia than in matched controls (64% *versus* 41%, $P<0.001$, homozygous 40% *versus* 15%, $P<0.001$). Flow cytometry was employed to determine the expression of MICA within hematologic compartments, showing that the signal intensity of MICA was increased in granulocytes from neutropenic patients with large granular lymphocyte leukemia in comparison with that in controls ($P=0.033$). Furthermore, neutrophil counts were inversely correlated with MICA expression ($R^2=0.50$, $P=0.035$). Finally, large granular lymphocyte leukemia cells were able to selectively kill MICA$^+$ Ba/F3 lymphocytes transfected with human MICA*019 in a dose-dependent manner compared to naïve cells ($P<0.001$), an effect mitigated by administration of an anti-NKG2D antibody ($P=0.033$).

Conclusions

Our results illustrate that MICA-NKG2D played a role in disease pathogenesis in the majority of patients in our cohort of cases of large granular lymphocyte leukemia and further investigation into this signaling axis may provide potent therapeutic targets.

Key words: MICA, genome-wide association study, GWAS, SNP, LGL, neutropenia.

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Introduction

Immunogenetic factors are likely to modulate various aspects of physiological cellular immune responses and have been implicated in the pathogenesis of T-cell-mediated autoimmune diseases.[1-3] Such disorders are often accompanied by clonal lymphoproliferations and the propensity toward clonal dominance/skewing may be rooted in immunogenetic causes. Potential genetic factors include HLA background, immune cytokine promoter and receptor polymorphisms, as well as polymorphisms in KIR and other cytotoxic T-cell-specific genes. While these associations have been well described and validated, the mechanistic principles behind these factors remain largely elusive. Furthermore, the currently proposed molecular markers of exaggerated immune response appear ubiquitous among various autoimmune T-cell-mediated syndromes, despite the heterogeneous clinical phenotypes observed, implying the presence of additional factors contributing to pathological clonal responses. T-cell large granular lymphocyte (LGL) leukemia, a clonal malignant proliferation of cytotoxic T cells, which serves as a natural monoclonal model of polyclonal T-cell responses,[4,5] is closely linked to various autoimmune conditions,[6-9] thus a shared immunogenetic background encoded by complex genetic traits may be involved in driving over-exuberant T-cell expansions.

Cytopenias are the most common autoimmune sequelae of LGL leukemia[10] in which, unlike many clonal neoplasms, the T-cell expansion seems to be initiated and sustained by extrinsic antigen-driven triggers.[11] Clonal T cells have been suggested to mediate an immune attack on hematopoietic targets in LGL leukemia and, depending on their specificity or effector mechanisms exerted, produce highly specific pathology (e.g. lineage-specific cytopenias).[10] As for various classical autoimmune diseases, several immunogenetic causes have been proposed for the pathogenesis of LGL leukemia, all of which have been postulated to have a role in autoimmunity. Such factors include HLA,[12] KIR/KIR-ligand interaction,[13] minor histocompatibility antigens, natural killer receptors,[14] transforming growth factor-β high secretor genotype,[13] and immunoproteosome abnormalities. Due to its well-defined phenotype, LGL leukemia represents a good target for genomic analysis in order to determine additional immunogenetic factors driving aberrant lymphocytic clonal expansion.

Improved resolution of genomic features through unbiased molecular technologies has the potential to further clarify the inherited predisposition towards exaggerated T-cell responses and the connection between clonal dominance and autoimmune disease. Furthermore, we speculated that due to the rarity of this disease, a theoretical germline or somatic mutation may have significant penetrance even within a polygenic predisposition, thus enabling detection despite suboptimal sample size inherent to rare conditions. The use of single nucleotide polymorphism (SNP) arrays as a hypothesis-generating tool allows for the identification of candidate genes that can be further studied by molecular methods. In this work we present the SNP array results examining genetic susceptibility toward clonal lymphoproliferations seen in LGL leukemia as well as corresponding functional consequences that reveal a novel mechanism driving both polyclonal and clonally skewed T-cell responses.

Design and Methods

Patients

Patients with LGL leukemia seen at Cleveland Clinic Taussig Cancer Institute between 2002 and 2009 were studied. Informed consent was obtained from patients according to protocols approved by the Institutional Review Board. The diagnosis of LGL leukemia was based on modified diagnostic criteria described by Semenzato *et al.*[4] Overall, 82 patients and 162 controls were studied. For the purposes of this study, as previously described, at least three of four of the following parameters had to be fulfilled for a diagnosis of T-cell LGL leukemia: the presence of LGL (>1×10^9/L) in peripheral blood for more than 6 months, the presence of an abnormal population of cytotoxic T lymphocytes (CTL) expressing CD2, CD56, CD57, and lacking CD28, rearrangement of TCR-γ, and/or restricted TCR Vβ expansion greater than two standard deviations (SD) from the reference range in the $CD8^+$ T-cell population.[4,15] Immunophenotype was determined by flow cytometric analysis using monoclonal antibodies for CD3, CD4, CD8, CD19, CD20, CD23, CD56, and CD57. The Vβ flow cytometric analysis was performed on fresh peripheral blood as previously described.[16] Antibodies used included CD4, CD8, Vβ1-5, Vβ7-9, Vβ11-14, Vβ16-18, Vβ20-23, and Vβ6.7 (IOTest Beta Mark kit, Beckman-Coulter, Fullerton, CA, USA). The individual contribution of each of the 25 Vβ-subfamilies detectable by a specific monoclonal antibody was determined and results are expressed in percentages of α/β $CD4^+$ or $CD8^+$ cells. Vβ over-representation was established when the contribution of a particular Vβ family was greater than the mean plus two SD of values found in healthy volunteers.[16,17] Clonal TCR-γ rearrangements were analyzed by polymerase chain reaction (PCR) using previously described methods.[18] Clonality was determined based on relative intensity of the rearranged bands compared to the germline band.

DNA extraction

DNA was extracted from whole bone marrow with the Puregene DNA Purification System Blood Kit (Gentra Systems, Minneapolis, MN, USA). Red blood cell lysis solution was added to whole bone marrow at a 3:1 ratio and incubated with shaking for 10 min. The cells were pelleted and the DNA extracted according to the manufacturer's instructions. The concentration of the DNA was determined using a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and the quality established by gel electrophoresis. To study the germline genotype, granulocytes were isolated using Percoll density-gradient separation.

Candidate gene identification

To identify novel genetic targets for investigation, a large cohort of patients with LGL were subjected to analysis using two SNP array platforms and underwent two independent statistical methodologies to identify candidates for downstream biological studies. The cohort of patients was divided into a training set and an external validation cohort to remove SNP that might be selected based on technical bias, selection bias, or random chance. The training set comprised 33 patients studied using the Illumina NS-12 array and 52 patients studied with the Affymetrix 250K array. An external validation cohort of 49 patients was excluded from these analyses for technical and biological validation.

We used two independent statistical strategies to identify SNP predictive of LGL disease status. First, a novel, multivariable, non-parametric regression method, 'random forests', was applied[19] for candidate gene identification (*Online Supplementary Appendix*).

MICA sequencing

Using oligonucleotide primers for each of the *MICA* exonic transcripts, PCR-based sequencing was performed for 28 patients and 12 independent controls. An approximately 2201 bp fragment of the *MICA* gene (including exons 2, 3, 4 and 5) was amplified using primers described by Shao *et al.*[20]: MICAF 5'-TCCTCGTTCTTGTCCCTTTGCCCG-3' and MICAR 5'-CTGCCCCCATTCCCTTCCCAAATT-3'. The PCR conditions used were those described by Zou *et al.*[21] PCR products were purified using PCR Cleanup Filter Plates (Millipore, Ballerica, MA, USA). Primers MEX2F 5'-CACCTGTGATTTCCTCTTCCCCA-GAGC-3' and MEX2R 5'-TGCCGACTCTCACCTTCTTT-3' were used for sequencing exon 2 in both directions. Exon 3 was sequencing with primer MEX3F 5'-TTCCTCACTTGGGTG-GAAAG-3'. Primers MEX4F 5'-AAGAGAAACAGCCCTGTTC-CTCTCC-3' and MICAR 5'-CTGCCCCCATTCCCTTCC-CAAATT-3' were used for sequencing exons 4 and 5. Approximately 100 ng of cleaned PCR products were sequenced using an ABI PRISM Big Dye Terminator cycle sequencing kit run on a 3100 AVANT genetic analyzer (Applied Biosystems, Foster City, CA, USA).

MICA genotyping was performed for 42 additional patients and 118 healthy bone marrow donors using the PCR–based reverse-sequence-specific oligonucleotide probe method to investigate exons 2, 3, 4 and 5 of the *MICA* gene by using the Luminex platform and a commercial kit (LABType® MICA, One Lambda, CA, USA). MICA alleles were assigned on the basis of their hybridization patterns predicted from known MICA alleles using HLA Fusion™ Software version 1.0 (One Lambda).

MICA/NKG2D flow cytometric analysis

Staining using 10 μL anti-MICA PE monoclonal antibody or 10 μL anti-NKG2D monoclonal antibody (eBioscience Inc., San Diego, CA, USA) was performed on 100 μL peripheral blood with CD8-FITC, CD15-PC5, and CD20-ECD (Beckman-Coulter, Fullerton, CA, USA). Flow cytometry was used to identify cell surface expression of MICA and NKG2D in the T-cell, neutrophil, and B-cell compartments.

Cytotoxicity assay

MICA-NKG2D mediated cytotoxicity was measured using a co-culture assay and flow cytometry for membrane-incorporated $DIOC_{18}$ target cell dye and propidium iodide (LIVE/DEAD cell-mediated cytotoxicity kit, Invitrogen, Carlsbad, CA, USA). Patients' neutrophils, extracted by Ficoll gradient isolation, were co-cultured with autologous LGL at target:effector ratios of 10:1 and 25:1, and untreated for 24 h, after which cell viability was measured by flow cytometric analysis. Differential cytotoxicity for each reaction was calculated by adjusting for cytotoxicity measured at baseline. The neutrophil population was confirmed prior to co-culture by evaluating $CD15^+$ staining by flow cytometry. Comparison assays were done using wild-type Ba/F3 murine B-lymphocytes and a cell line stably transfected with human MICA 019 as previously described[22] (generously donated by the Lewis L. Lanier Laboratory, Department of Microbiology and Immunology, University of California, San Francisco). Specificity was determined by addition of 20 μL anti-NKG2D blocking antibody at a stock concentration of 10 μg/mL (R&D Bioscience, USA).

Statistical analysis

Nominal and continuous values for blood counts, diagnostic criteria, SNP and allele genotyping, and cohort composition were compared using χ^2 and Wilcoxon's rank-sum tests, respectively. t-tests were used to compare flow cytometry results (CD57, NKG2D, and MICA) among groups (LGL patients and normal subjects). For the cell-line cytotoxicity experiments, the effects of cell-line (+/-), antibody (+/-) and effector:target ratio (5:1, 25:1, and untreated) were modeled with respect to differentiated cytotoxicity. Significant interactions were included in the model. Contrasts of interest were tested with multiple partial F-tests. Similar models (excluding interactions) were developed for the neutrophil cytotoxicity experiments and relevant contrasts were tested with partial F-tests. For all tests the level of statistical significance was set at 0.05. Computations were performed using either JMP 8.0 or SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

Results

Study cohort

We identified 70 patients with T-LGL leukemia for this study under an Institutional Review Board-approved protocol (Table 1). Of these 70 patients, 69 displayed TCRα/β and one TCRδ/γ. Ninety percent of cases (n=63) expressed CD8, three cases showed a CD4 phenotype, and four were positive for both CD4 and CD8. The median age at diagnosis was 64 years (range, 28-81 years) and 52% of the patients were female. Rheumatoid arthritis was the most common co-existing autoimmune condition, being present in 15 patients (21%). Bone marrow failure disorders were present in 12 patients (10 with myelodysplastic syndrome (14%), one with aplastic anemia, and one with paroxysmal nocturnal hemoglobinuria). Splenomegaly, confirmed by ultrasound or computed tomography scans, was found in 30 patients. Hematologic complications included neutropenia, anemia, and, in rare cases, thrombocytopenia. Neutropenia, defined as an absolute neutrophil count (ANC) below 1.5×10^9/L, was found in 49 (70%) patients. Severe neutropenia, defined as an ANC below 0.5×10^9/L, was found in 27 (39%) patients. Anemia, defined as a hemoglobin concentration below 10.0 g/dL, was found in 29 (41%) patients. Thrombocytopenia, defined as a platelet count below 150×10^9/L, was present in 11 (16%) patients. A decrease in CD4:CD8 ratio was identified in the majority of patients, with the CD4:CD8 ratio being less than 1.0 in 59 (84%) patients. We intended to divide our cohort into two equal training and test sets; however, due to the additional accrual of patients, our test set is slightly larger. The clinical characteristics of both groups were compared, showing that there was no difference with regard to the clinical composition of the cohorts (Table 1).

Analysis of predisposing immunogenetic factors in large granular lymphocyte leukemia

Based on the hypothesis that predisposing genetic factors may play a role in the pathogenesis of LGL leukemia we applied an Illumina NS-12 array, which captures a large number of non-synonymous SNP and designed a two-stage strategy for analysis of the data. First, we evaluated the training set of T-LGL leukemia patients (n=33) and controls (n=56) applying the 'random forest' method, as described in the *Design and Methods* section. Five genes at two distinct loci, *ATP6V1B2* and *SLC18A1* at 8p21.3, and *MICA, C6orf10*, and *LOC442200* at 6p21.32-33, were identified as candidates for further analysis (Table 2). Four other *MICA* SNP were identified as having moderate predictability when compared to healthy controls and 48 disease controls with aplastic anemia. Similar results were generated with

Table 1. Characteristics of the cohort of patients with LGL leukemia.

	Training Set	Test Set	*P* value
Total number in cohort (female)	37 (17)	33 (15)	0.96
Median age in years (range)	63.5 (37-81)	66 (28-78)	0.71
Median ANC x10^9/L (range)	1.125 (0-4.710)	0.780 (0-5.047)	0.67
Median WBC x10^9/L (range)	5.2 (0.5-27.4)	5.4 (1.6-18.7)	0.76
Median hemoglobin g/dL (range)	11.6 (7.6-15.5)	10.0 (7.4-16.4)	0.23
Median platelet count x10^9/L (range)	189 (25-751)	194 (8-594)	0.63
Median LGL count (range)	1105 (283-20575)	1733 (98-7410)	0.46
Median CD4:CD8 (range)	0.34 (0.03-3.0)	0.40 (0.05-3.0)	0.65
Positive for TCR rearrangement (%)	36 (97)	31 (94)	0.49
Median % Vβ expansion (range)	65 (11-98)	60 (16-93)	0.89

Diagnosis based on the presence of atypical LGL on peripheral blood(n=70, 100%), LGL count smear > 1000/μL for >6 months (n=50, 71%), TCR-γ rearrangement (n=67/70, 97%), Vβ expansion > 2SD (n=65/70, 93%). Features of the cohort include TCRα/β (n=69, 99%), TCRγ/δ (n=1, 1%), CD4:CD8 ratio < 1.0 (58, 83%), cytopenia (n=60, 86%), multiple lineage cytopenia (n=29, 41%).

Table 2. Illumina platform 'random forest' results for patients with LGL leukemia *versus* healthy and disease controls.

SNP	Cytoband	VIMP	Related gene
rs11136300	8q24.3	0.3815	C8orf31
*rs1063635	*6p21.33*	0.1572	*MICA*; *LOC442200*
rs4832524	2p24	0.1492	KCNS3
rs9263871	6p21.33	0.1346	HCG27
rs7863265	9q33.3	0.109	STRBP
*rs17092104	8p21.3	0.0937	ATP6V1B2; SLC18A1
*rs926070	6p21.32	0.0919	C6orf10; LOC401252
*rs2844573	6p21.33	0.081	*MICA*; LOC442200
rs740842	12p13.31	0.0678	PLEKHG6
*rs9265797	*6p21.33*	0.0623	LOC442200
rs2275592	14q22.1	0.0677	ATP5S
rs3128982	*6p21.33*	0.0564	*MICA*; *LOC442200*
rs1943330	18q22-q23	0.0578	CDH20

*Italics indicates SNP within MICA, *indicates overlap between Random Forests and Exemplar methodologies. For Random Forests analysis, number of trees: 1000, number of variables tried at each split: 6850, number of replicates: 1000. VIMP= variable importance.*

Table 3. Illumina platform 'exemplar' results for patients with LGL leukemia *versus* healthy and disease controls.

SNP	Cytoband	Score	Related Genes
rs2844573	*6p21.33*	*0.3698531*	*MICA; LOC442200*
*rs926070	6p21.32	0.3614393	C6orf10; LOC401252
rs526680	12p13.31	0.3111844	CLEC2B; FLJ46363
rs2073044	6p21.32	0.2812237	C6orf10; BTNL2
rs1131896	*6p21.33*	*0.0311511*	*MICA; LOC442200*
rs9265797	6p21.33	0.0311511	LOC442200
rs2280090	20p13	0.0265487	SIGLEC1; ATRN; GFRA4
*rs17092104	8p21.3	0.0265206	ATP6V1B2; SLC18A1
rs1063635	*6p21.33*	*0.0264369*	*MICA; LOC442200*

**indicates overlap between Random Forests and Exemplar methodologies.*

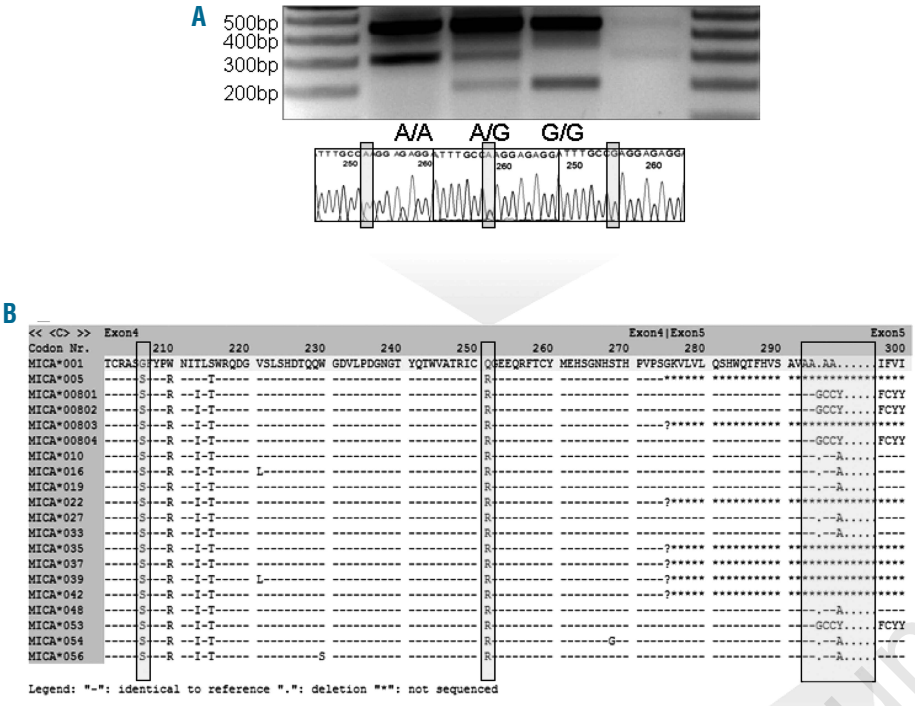
Exemplar automated analysis (Table 3). Due to its biological function, our subsequent analysis focused on MHC class-I related-chain A gene (*MICA*) polymorphisms. As the most predictive SNP within the *MICA* gene, rs1063635 was found in GG allele homozygosity in 50% of patients and 15% of controls (*P*=0.02). The prevalence of the GG allele of an additional SNP within *MICA*, rs1131896, was found to be higher in patients (86%) than in controls (45%; *P*=0.02). Using PCR-based genotyping for rs1063635, allele calls were confirmed for 93% of calls from array-based platforms (Figure 1A). Mistaken calls were identified in two patients who were called heterozygous for rs1063635 but were later demonstrated to have homozygous GG alleles.

To be assured that initial screening produced reliable association results of MICA alleles a validation set of patients (n=37) and controls (n=28) was analyzed. Prediction statistics calculated for the validation cohort were similar to those in the training cohort (positive predictive value=64%, negative predictive value=86%). Combining the two cohorts yielded a G-allele frequency of 74% in patients and 44% in controls. Our results stand in contrast to public domain registry data from HapMap-CEU, in which the G-allele frequency was reported to be 64%. However, the general applicability of the HapMap *MICA* genotyping results as a control group are questionable as the research was performed on a largely consanguineous population.

Sequence-based genotyping of MICA

Both as further technical validation and as biological validation in an external cohort, sequencing of the *MICA* gene was completed for 28 patients and 12 controls. This sequencing confirmed allele-specific PCR results for both patients and controls. Using the known increased frequency at SNP rs1063635 and rs1131896, we accessed the NCBI major histocompatibility complex database. Both SNP were found to be non-synonymous with the corresponding amino acid changes R251Q and S206G, respectively. Both were located in exon 4, the extracellular domain of the protein. MICA alleles described with these amino acid changes were identified in 18 of the 65 known MICA alleles described in dbMHC (Figure 1B). Using the sequenced gene product we were able to identify MICA allele genotypes for both the patients and a control populations (Figure 1D). MICA*00801/A5.1 was identified at a higher frequency in patients than in controls: 68% and 29%, respectively (*P*=0.001). Moreover, 39% of patients were homozygous for this allele compared to 8% of controls (*P*=0.050).

An additional 42 patients and 118 controls were genotyped using the reverse sequence specific oligonucleotide



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MICA Allele	Patients Sequenced	Controls Sequenced	Patients SSOP	Controls SSOP†	p-value
002/A9	6/56 (11%)	2/24 (8%)	8/84 (10%)	19/236 (8%)	0.516
004/A6	2/56 (4%)	3/24 (13%	3/84 (4%)	15/236 (6%)	0.619
0701/A4	1/56 (2%)	0/24 (0%)	2/84 (2%)	5/236 (2%)	0.882
008/A5.1	38/56 (68%)	7/24 (29%)	52/84 (62%)	101/236 (43%)	<0.001*
009 or 049/A6	1/56 (2%)	2/24 (8%)	2/84 (2%)	23/236 (10%)	0.005*
010/A5	1/56 (2%)	3/24 (13%)	3/84 (4%)	2/236 (1%)	0.548
011/A6	2/56 (4%)	1/24 (4%)	0/84 (0%)	10/236 (4%)	0.132
016 or 019/A5	0/56 (0%)	5/24 (13%)	1/84 (1%)	25/236 (11%)	<0.001*
017/A9	0/56 (0%)	1/24 (4%)	2/84 (2%)	12/236 (5%)	0.073
018/A4	3/56 (5%)	0/24 (0%)	5/84 (6%)	15/236 (6%)	0.975
027 or 048/A5	2/56 (4%)	0/24 (0%)	2/84 (2%)	2/236 (1%)	0.101

Figure 1. Workflow diagram for identification of SNP rs1063635 and association with MICA 00801/A5.1. (A) Allele-specific PCR for rs1063635 and confirmatory sequencing of PCR product. (B) dbMHC repository of MICA alleles with mutations S206G and R251Q and proximity to trinucleotide repeat region. (C) Sequencing of the MICA trinucleotide repeat region of exon 5. (D) Results of MICA allele genotyping. MICA allele 027/A5 and 048/A5 cannot be distinguished based on the sequencing method applied in this study. Homozygosity for the 00801/A5.1 allele was found in 27/70 patients and 19/130 controls (39% *versus* 15%, $P<0.001$). †:Trinucleotide region genotyping was not available on sequence-specific oligonucleotide probe controls.

probe platform. Overall, 83% of patients genotyped harbored at least one copy of the MICA*00801/A5.1 allele and 17 (40%) were homozygous. The allele frequency was significantly higher in LGL patients than in controls (62% *versus* 43%; $P<0.001$). By comparison, MICA alleles 009/049/A6 and 016/019/A5 were significantly more frequent in the control population. No other MICA alleles were found to be statistically significant different between the two populations and no association of allele affinity, as defined by the M129V variant, was identified.[23] Of note, two variant mutations were identified in the leukemic cohort which were not identified on dbMHC. Each was a variant of a known allele, one in 002/A9 and one in 008/A5.1. Sequences of these two alleles were reported to the WHO Nomenclature Committee For Factors of the HLA System and were officially assigned the names MICA*00203 and MICA*058, respectively, in April 2009.

MICA cellular expression

To investigate whether MICA has any pathophysiological importance in the evolution of T-LGL leukemia, we used flow cytometry with anti-MICA and anti-NKG2D antibodies to examine cell-surface expression of these markers. NKG2D, the ligand for MICA, belongs to the type II C-type lectin-like family of transmembrane proteins and functions both as an activating and co-stimulatory receptor. This NK receptor is encoded by a gene on human chromosome 12, mapping within the NK gene complex. NKG2D is expressed as a homodimer on NK, γδ T cells as well as on subsets of $CD4^+$ and $CD8^+$ T cells including LGL. Flow cytometry was performed on samples from 47 well annotated patients with T-cell LGL leukemia and 18 independent healthy controls. Our analysis showed that while expression of the activation marker CD57 on $CD8^+$ T-lymphocytes was expectedly higher in patients than in controls ($P<0.001$), there was no difference in the level of expression of NKG2D within the leukemic and normal $CD57^+$ LGL (Figure 2a, $P=0.576$). When we examined MICA expression using flow cytometry, $CD15^+$ granulocytes derived from patients with LGL leukemia and clinical neutropenia showed increased signal intensity of MICA compared with control granulocytes (Figure 2A, mean fluorescent intensity 3.88 *versus* 2.26; $P=0.033$), although no significant difference was found when all LGL leukemia patients, including

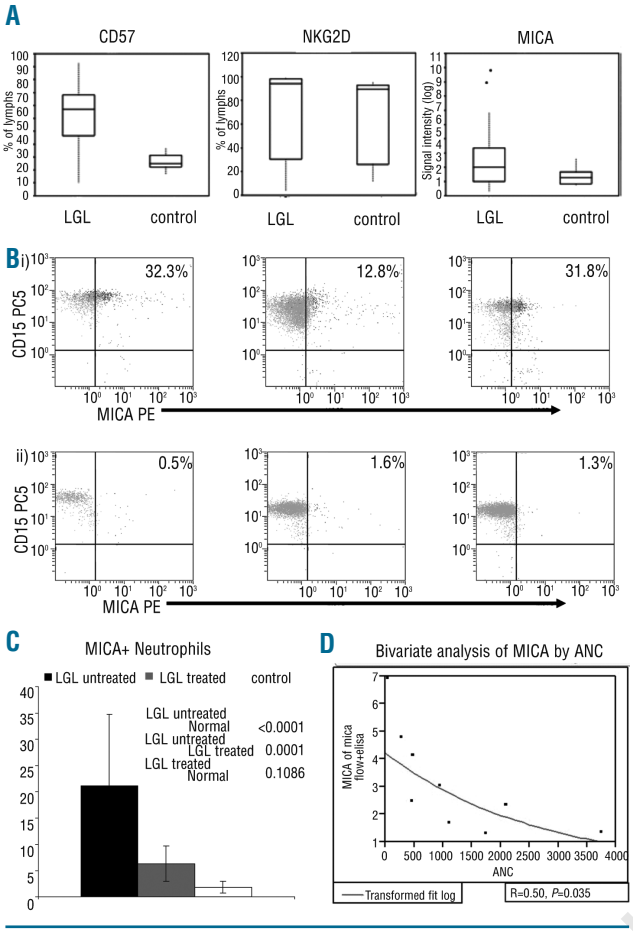

Figure 2. (**A**) **Flow cytometry results. CD57: *P*<0.01; NKG2D: *P*=0.93; MICA: *P*=0.020.** (**B**) **MICA flow cytometry based on treatment status. (i) untreated LGL patients. (ii) control.** (**C**) **Summary of flow cytometry results based on treatment status. LGL untreated *versus* control (*P*<0.0001), LGL untreated *versus* LGL treated (*P*=0.0001), LGL treated *versus* control (*P*=0.1086).** (**D**) **Bivariate analysis of MICA expression and absolute neutrophil count R=0.50, *P*=0.035.**

those in remission or patients with anemia as a hematologic presentation, were investigated (*P*=0.102). Flow cytometry data were then combined with several clinical parameters identified in our patients. Using bivariate analysis, ANC was found to be inversely correlated with cell surface MICA expression (Figure 2D, R^2=0.50, *P*=0.035). Stratification by *MICA* genotype revealed that patients with at least one copy of the MICA 008/A5.1 allele had higher absolute lymphocyte counts than those of patients without this allele (*P*=0.034). However, no prognostic value and no specific clinical course was identified when comparing these groups. No other clinical parameter, including hemoglobin concentration, platelet count, LGL count, or extent of Vβ expansion, was found to correlate with *MICA* genotype.

To determine whether clinical course correlated with level of MICA expression, we compared LGL leukemia patients who were either untreated or who had poorly controlled disease with patients treated with immunosuppression (cyclosporine A, n=3; methotrexate, n=2; prednisone, n=2; or cytoxan, n=1), and 18 healthy controls. A statistically significant relationship was observed, with untreated patients expressing MICA on the highest percentage of neutrophils, immunosuppressed patients showing a lower percentage of expression, while MICA expression was virtually absent in healthy controls (Figure 2B and C, untreated *versus* control, *P*<0.001; untreated *versus* treated, *P*=0.001; treated *versus* control, *P*=0.1086).

Cytotoxicity assay to determine the functional significance of the MICA-NKG2D signaling axis

A killing assay was developed to determine the functional consequence of MICA overexpression on granulocytes and whether NKG2D LGL could result in autoimmune attack. Patients' granulocytes and a murine B-lymphocyte cell line were used as target cells. The cell line Ba/F3 was stably transfected with human MICA*019, an allele of similar affinity for NKG2D. Target cells labeled with $DiOC_{18}$ could be differentiated from effector cells based on flow cytometry and propidium iodine counterstaining allowed for cell viability to be measured. In three patients previously genotyped to be heterozygous for the MICA*008/A5.1 allele, a dose-response relationship was shown for cellular cytotoxicity. A similar response was found when patients' LGL were co-cultured with a $MICA^+$ cell line, Ba/F3+. However, by comparison, LGL did not evoke a dose-response in cytotoxicity when co-cultured with a wild-type cell line, Ba/F3- (Figure 3A). When the dose-responses were compared, there were statistically significant differences between both transfected cells and wild type Ba/F3- cells (Ba/F3+ *versus* Ba/F3-, *P*<0.001). The addition of anti-NKG2D antibody to the cytotoxicity reaction produced a blunted cytotoxic effect of LGL on MICA-transfected Ba/F3+ cells, while the antibody had no effect on the wild-type cells. A statistically significant knockdown of measured cytotoxicity identified the specific involvement of the MICA-NKG2D interaction (*P*=0.033). This assay was repeated using patients' LGL co-cultured with autologous neutrophils and allogeneic healthy neutrophils. Dose-dependent killing was observed in both settings (*P*=0.672). Addition of an anti-NKG2D antibody failed to produce a statistically significant difference in blockade of cytotoxicity (*P*=0.683); however, due to the logistical difficulty of extracting neutrophils in the setting of severe neutropenia, only two reactions could be performed. The resulting cytotoxicity curves do, however, suggest a trend may exist whereby alloreactivity is independent of MICA-NKG2D-mediated killing whereas autologous immune neutropenia in the setting of LGL leukemia may potentially be driven by NKG2D reactivity (Figure 3B).

Discussion

Our study strongly suggests that genetic polymorphisms within the *MICA* gene are associated with LGL leukemia and, furthermore, likely play a role in disease pathogenesis although the precise mechanism remains to be elucidated. A meticulous method for gene candidate identification was used to combat the prevalence of high α-error in the context of SNP arrays. Thus, hypothesis generation was based on five genes at two distinct loci: *ATP6V1B2* and *SLC18A1* at 8p21.3, and *MICA*, *C6orf10*, and *LOC442200* at 6p21.32-33. *MICA* was chosen as the gene for further analysis based upon its biological function as a MHC class-I-like non-peptide-presenting molecule and as a ligand for NKG2D, a stimulatory receptor found exclusively on NK-cells, γδ-T cells, and LGL.[24,25]

MICA molecules are products of a polymorphic gene; they signal cellular stress and are involved in immune sur-

veillance.[24-26] MICA and the related MICB are tightly regulated and restricted to intestinal epithelium under normal conditions. However, through a poorly defined mechanism, certain conditions such and cancer, infection, and heat shock induce MIC expression. The transcriptional regulation of MICA and MICB is complex, involving several upstream protein signals including Sp1, Sp3, Sp4, and NF-Y.[27,28] Abnormalities of promoter interactions with these molecules may explain the proportion of MICA008/A5.1 null patients. Furthermore, though not reaching statistical significance, all patients with LGL leukemia were found to have a high proportion of low-affinity extracellular domains, suggesting that modulated binding of MICA to NKG2D may be a feature of the pathophysiology of this leukemia.[24,29,30] Pathological activity of this pathway has been implicated in various autoimmune conditions and several MICA alleles have been associated with particular autoimmune diseases[31-34] as well as a genetic predisposition to certain types of tumor.[35-38] To date, the inciting antigen driving T-cell clonal expansions in LGL leukemia has not been found, but certainly either the breakdown of self-tolerance or the addition of a tumor burden could be postulated to be operative in LGL leukemia. Another possibility is chronic viral infection. Of note, within exon 5 of the MICA transcript, there is a trinucleotide repeat with the number of trinucleotide repeats designated by A4, A5, A6, and A9. A single base pair insertion within this region leads to the A5.1 polymorphism, which is significant for yielding a premature stop codon within the exon resulting in the lack of a cytoplasmic tail (Figure 1C). MICA lacking a cytoplasmic tail has been suggested to have evolved out of resistance to a cytomegalovirus-specific immune escape mechanism.[39] Other viruses have also been shown to have immune escape mechanisms that operate through breaching the MICA-NKG2D signaling pathway.[40,41] Alternatively, a propensity for MICA*008/A5.1 may itself be involved in driving a polarized CTL response directly through overexpression of MICA on the cell surface. A single report has suggested that the MICA*008/A5.1 allele leads not only to variations in structure, but also to altered protein trafficking.[42] This study indicates that the allele may be shuttled to the apical surface aberrantly because of the absence of a sorting motif normally found on the cytoplasmic tail. In the context of additional somatic events such as the common anti-apoptotic abnormalities of Fas-FasL, PI3K-AKT, and STAT3 previously described in LGL leukemia,[43-46] MICA may simply represent a functional predisposition to autoimmunity.

A clinical correlation of this finding was made through

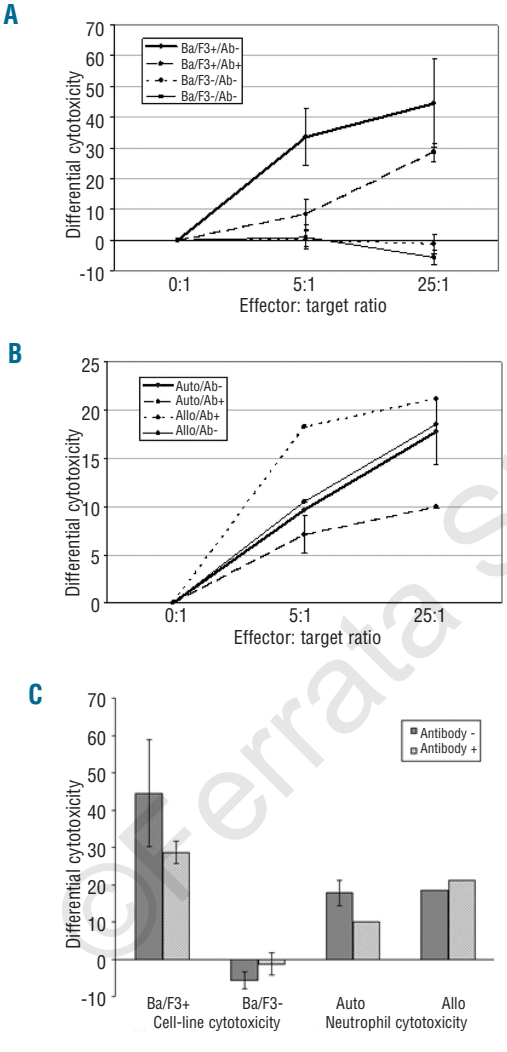

Figure 3. Cytotoxicity assay results. (A) Ba/F3 cell line *versus* LGL. Results for patients' LGL co-cultured with Ba/F3 lymphocytes with and without stable transfection of full length MICA 019. Mean differential cytotoxicity for co-culture of both cell lines with and without anti-NKG2D antibody. (B) Neutrophils *versus* LGL. Results for patients' LGL co-cultured with autologous neutrophils compared to allogeneic normal healthy neutrophils. Mean differential cytotoxicity for co-culture of both cell lines with and without anti-NKG2D antibody. (C) Comparison of differential cytotoxicity at a 25:1 effector to target ratio with and without anti-NKG2D administration. Error-bars represent standard deviation.

Table 4. MICA SNP genotypes.

Cohort	LGL	Control	HapMap	Framingham	Affy Controls
rs1063535					
AA	8 (12.3%)	23 (27.4%)	16 (13.6%)	N/A	N/A
AG	22 (33.8%)	48 (57.1%)	52 (44.1%)	N/A	N/A
GG	39 (60.0%)	13 (15.4%)	50 (42.4%)	N/A	N/A
Total	65	84	118	N/A	N/A
rs1131898					
AA	51 (81.0%)	25 (46.3%)	N/A	N/A	N/A
AG	10 (15.9%)	21 (38.9%)	N/A	N/A	N/A
GG	2 (3.1%)	8 (14.8%)	N/A	N/A	N/A
Total	63	54	N/A	N/A	N/A
rs3128982					
AA	11 (30.6%)	32 (56.1%)	46 (38.3%)	1732 (51.2%)	1793 (51.3%)
AG	15 (41.7%)	22 (38.6%)	66 (55.0%)	1367 (40.4%)	1411 (40.4%)
GG	10 (27.8%)	3 (5.3%)	10 (6.7%)	284 (8.4%)	289 (8.3%)
Total	36	57	120	3383	3493
rs2844573					
GG	11 (30.6%)	31 (54.4%)	14 (11.7%)	1003 (59.5%)	1065 (59.4%)
GT	10 (27.8%)	23 (40.4%)	60 (50.0%)	523 (31.0%)	567 (31.6%)
TT	15 (41.7%)	3 (5.3%)	46 (38.3%)	160 (9.5%)	165 (9.2%)
Total	36	57	120	1686	1797

flow cytometric analysis using anti-MICA antibodies and was performed on the CD15+ granulocyte compartment. Although no significant difference in MICA expression was found when all patients with LGL leukemia, including those in remission or those with anemia as a hematologic presentation, were investigated (P=0.102), when this dataset was stratified based on the clinical presentation of neutropenia, the signal intensity of MICA on CD15+ granulocytes derived from LGL leukemia patients with neutropenia was greater than that of control granulocytes (mean fluorescent intensity 3.88 *versus* 2.26; P=0.033). Moreover, ANC was inversely correlated with MICA expression (R=0.50, P=0.035). The specificity of over-expression of MICA only in granulocytes from patients presenting with neutropenia suggests that the MICA-NKG2D signaling axis may be operative in the observed clinical outcome, although we cannot completely rule out the possibility of MICA up-regulation as a down-stream effect of autoimmune destruction. However, in other experiments not described here, we failed to find MICA up-regulation in any hematologic compartment in patients with either aplastic anemia or idiopathic thrombocytopenic purpura.

This clinical correlate was further substantiated through our cytotoxicity assay. Patients' LGL had a dose-dependent cytotoxic effect on MICA-transfected Ba/F3 cells while wild-type cells were not killed (P<0.001). Further specificity of this cytotoxicity to the MICA-NKG2D pathways was suggested by the fact that the reaction was mitigated by the administration of anti-NKG2D antibody (P=0.033). Clearly, MICA is polymorphic within the human population, and thus varying binding affinities as well as intracellular signal machinery may result in differing degrees of cytotoxicity. We are continuing to investigate the effect of the low-affinity MICA 008/A5.1 allele in modulating overexuberant CTL expansion.

In this study we demonstrate that even in the research of orphan diseases, whole genome analysis using SNP arrays is a powerful and useful tool despite limitations of sample size. Using rigorous initial filters on SNP data we successfully identified a candidate gene, which has subsequently been shown to have pathophysiological implications. Despite our contention that predisposition to disease through *MICA* genotype plays a role in a non-mendelian polygenic disease that likely involves germline predisposition, somatic mutation, and environmental exposure, the strictness of the initial analysis likely excluded additional genes that may similarly be involved. Undoubtedly, this rare disease cannot be completely explained by a single genetic polymorphism, particularly as the frequency of the MICA*008/A5.1 allele has been reported to be as high as 37-42% in other studies.[47,48] Environmental exposure in the context of this predisposing allele may, however, be pathogenic. As previously mentioned, the allele leading to a lack of the cytoplasmic tail has been suggested to alter the cellular stress response to cytomegalovirus. While many studies have been unsuccessful in identifying a causative virus in LGL leukemia, perhaps an elusive known or unknown virus exists undetected due to the ability of the virus to survive, incompletely cleared because of impaired immune surveillance.

While the etiology of cytopenias in LGL leukemia is multifactorial, the data presented here help to elucidate the role of MICA/NKG2D interactions in the context of this disease and point towards new pathophysiological clues regarding disease mechanisms. The importance of the discovery of MICA over-expression in mediating neutropenia in LGL leukemia also lies in translation of this information into the clinic. Currently a transgenic anti-NKG2D antibody is under development for rheumatic diseases: this agent may also be useful in the treatment of patients with LGL leukemia.

Authorship and Disclosures

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