Characterization of a weakly expressed KIR2DL1 variant reveals a novel upstream promoter that controls KIR expression

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Members of the human KIR (killer cell immunoglobulin-like receptor) class I major histocompatibility complex receptor gene family contain multiple promoters that determine the variegated expression of KIR on natural killer cells. In order to identify novel genetic alterations associated with decreased KIR expression, a group of donors was characterized for KIR gene content, transcripts and protein expression. An individual with a single copy of the KIR2DL1 gene but a very low level of gene expression was identified. The low expression phenotype was associated with a single-nucleotide polymorphism (SNP) that created a binding site for the inhibitory ZEB1 (Zinc finger E-box-binding homeobox 1) transcription factor adjacent to a c-Myc binding site previously implicated in distal promoter activity. Individuals possessing this SNP had a substantial decrease in distal KIR2DL1 transcripts initiating from a novel intermediate promoter located 230 bp upstream of the proximal promoter start site. Surprisingly, there was no decrease in transcription from the KIR2DL1 proximal promoter. Reduced intermediate promoter activity revealed the existence of alternatively spliced KIR2DL1 transcripts containing premature termination codons that initiated from the proximal KIR2DL1 promoter. Altogether, these results indicate that distal transcripts are necessary for KIR2DL1 protein expression and are required for proper processing of sense transcripts from the bidirectional proximal promoter.

INTRODUCTION

Natural killer (NK) cells are lymphocytes of the innate immune system that have an important role in the elimination of neoplastic or virus-infected cells.1 NK cell cytotoxic activity is controlled in part by cell surface receptors for class I major histocompatibility complex that survey potential target cells for aberrant class I expression.2 Human NK cells use the killer cell immunoglobulin-like receptor (KIR) family3 to interrogate class I heavy chain-related Ly49 family.4,5 KIR and Ly49 genes are expressed in a probabilistic fashion, generating subsets of NK cells with distinct class I major histocompatibility complex-recognition properties.4,5 The selectivity expressed human KIR gene family provides an interesting model system for the study of stochastic activation of gene expression. The proximal promoter of most KIR genes is kept in a silent state by promoter methylation.6,9 An AML/RUNX (acute myeloid leukemia/Runt-related transcription factor) transcription factor binding site in the proximal promoter region has been suggested to have a role in the demethylation of the proximal promoter, as loss of this site in the non-transcribed KIR2DL5*002 allele is associated with non-expression, even though loss of the AML/RUNX site does not abrogate proximal promoter activity in luciferase reporter assays.10,11 Furthermore, treatment of NK cells with the demethylating agent 5-azacytidine leads to KIR2DL5*002 transcription, supporting a role for AML/RUNX in catalyzing promoter demethylation. Studies of a distal KIR promoter have indicated a role for distal transcripts in gene activation, as distal transcription precedes gene expression, and increased distal transcripts are associated with a higher frequency of gene expression.12,13 A c-Myc-binding site in the distal promoter was shown to be important for promoter activity, and overexpression of c-Myc in developing NK cells led to increased distal transcript levels and an increased frequency of NK cells expressing KIR. A model of KIR gene activation has been proposed in which the distal promoter generates transcripts that traverse the proximal promoter region, leading to an opening of this region and allowing access of key transcription factors involved in promoter demethylation, such as AML/RUNX.14

The variegated expression of Ly49 genes is controlled by a bidirectional promoter that acts as a probabilistic switch.15 The generation of forward transcripts from this upstream element (Pro1) in immature NK cells leads to activation of the downstream proximal promoter. The expression of a Ly49 gene from the proximal promoter is dependent on distal transcription, as Pro1 deletion eliminates Ly49 transcription.16 The probabilistic activation of KIR genes has been linked to the bi-directional nature of the KIR proximal promoter.17 Variation in the relative strength of transcription factor binding sites that promote either sense or antisense transcription from the proximal promoter in specific KIR genes or alleles results in bi-directional promoters with differing probabilities of generating a forward transcript.18 KIR genes or alleles with a high ratio of sense to antisense proximal promoter activity are expressed by a greater percentage of NK cells than KIR with a low sense-to-antisense ratio. The KIR antisense transcript has been associated with the production of a 28 base piwi-RNA that may lead to re-silencing of KIR loci that produce the antisense transcript.19 Although numerous KIR alleles have been identified that are non-expressed due to the presence

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of stop codons in the coding region, only one gene (KIR2DL5)\(^{10}\) has been identified that contains non-transcribed alleles. In the current study, we sought to determine whether there were additional non-transcribed KIR alleles to obtain further information with regard to the genomic regions controlling gene expression.

RESULTS
Identification of a weakly expressed KIR2DL1 allele
In order to identify novel genetic alterations associated with a lack of KIR transcription, healthy donors were screened for individuals that possessed but did not show significant expression of KIR genes. A group of 182 National Marrow Donor Program (NMDP) donors were characterized for KIR gene transcription by quantitative reverse transcriptase–PCR (qRT-PCR), KIR gene content and fluorescence-activated cell sorting (FACS) analysis of KIR surface expression. An individual was discovered that possessed the KIR2DL1 gene but had barely detectable gene expression by either FACS or qRT-PCR (Figure 1).

Identification of single-nucleotide polymorphisms (SNPs) specific to the poorly expressed KIR2DL1 allele
Complete sequencing of the KIR2DL1 gene from the individual with low KIR2DL1 expression revealed the presence of a single allele containing an intact coding region. Analysis of the three known promoter regions as well as the complete 2 kb intergenic region preceding the first exon revealed a cluster of three SNPs in the distal promoter region approximately 1 kb upstream from the start of KIR2DL1 translation (Figure 2). These three SNPs, together with the presence of an exon 4 segment derived from the KIR2DL1*002 allele, constituted the only sequence differences between the KIR2DL1 gene in this donor and the previously reported complete sequence of a KIR2DL1*00401 allele (GenBank accession GU182347). The three SNPs present in the novel KIR2DL1 allele match the sequence normally found in the distal promoter region of the non-transcribed KIR2DL5*002 allele as well as the KIR3DP1 pseudogene (Figure 2). However, the well-characterized KIR2DL5*002 SNP that disrupts the proximal promoter AML/RUNX site was not present, indicating that the distal promoter variant was able to inhibit KIR expression by a novel mechanism. This also raises the possibility that the KIR2DL1 SNPs identified here could be involved in the silencing of KIR2DL5*002.

The KIR2DL1 distal SNP creates a ZEB1 (Zinc finger E-box-binding homeobox 1)-binding site
Examination of the distal SNPs associated with non-expression of KIR2DL1 revealed that the SNP at position –1206 created a

![Figure 1](image)

**Figure 1.** Identification of a poorly expressed KIR2DL1 allele. (a) qRT-PCR performed on NK cell RNA from a NMDP donor. (b) KIR gene content typing for this individual. (c) FACS analysis performed with a KIR2DL1-specific antibody on donor peripheral blood mononuclear cells (PBMCs) in the left panel and PBMCs from the corresponding hematopoietic transplant recipient in the right panel, demonstrating an identical pattern of KIR2DL1 expression in reconstituting donor cells.
| Characteristic | WT Allele | Mut Allele |
|---------------|-----------|------------|
| Promoter 2DL1*004 | CTCGAAACCACATTACCCAGGTTGTAGGATGTCACTACGTTTTACCTCAATTTAATAAATCTTACAAAAATGAC | AACTAGATTTTAGCTTATGTGAGTTAGATTATTTTCTAGGTTACACCATAGTTTAAAGTTTAGAT |
| KIR2DP1 | | |
| ZEB1 | G | A |
| Myc/USF | G | A |

**Figure 2.** A non-transcribed KIR2DL1 allele is generated by recombination between KIR2DL*004 and KIR3DP1. The sequence of the region from −1353 to −1093 relative to the translational start of the KIR2DL1*004 gene (2DL1*004) is shown, and only nucleotide differences are shown for the novel KIR2DL1 allele (2DL1-new) and the non-transcribed KIR3DP1 gene (3DP1). The KIR3DP1-derived region of the novel KIR2DL1 allele is shown in bold, and the underlined flanking sequences indicate the regions where recombination may have occurred to generate the novel KIR2DL1 allele. The boxed sequences represent the consensus ZEB1-binding half sites in opposing orientations, followed by a Myc/USF site previously shown to have a role in distal promoter activity. The upstream ZEB1 site is created in the 2DL1-new and 3DP1 sequences due to the A to G SNP.

**Figure 3.** The KIR2DL1 SNP creates a ZEB1-binding site. An oligonucleotide probe containing the wild-type (wt) or mutant (mut) distal promoter region was combined with nuclear extract from YT cells. The left panel shows the appearance of a novel band associated with the mutant SNP. The right panel shows the ability of several commercial anti-ZEB antibodies (ZEB1-1 to ZEB1-4, ZEB2), together with anti-C-Myc or MZF-1 controls, to inhibit the formation of the novel complex.

Known KIR2DL5 alleles contain the SNP that generates the ZEB1-binding site, which may account for the low frequency of expression observed for KIR2DL5A*001.22 Some KIR2DP1 alleles also contain this SNP; however, the complete absence of KIR2DP1 transcripts precludes any observable effect of the ZEB1 SNP on the transcription of this gene.
Figure 4. The KIR2DL1 SNP is associated with weak expression. Donors were screened by PCR with primers specific for the ZEB1 SNP. The upper panel shows three donors with 0, 1 or 2 copies of the wild-type KIR2DL1 gene (WT 2DL1) stained with an NK marker (CD56) on the horizontal axis and a KIR2DL1-specific antibody on the vertical axis. The number shown in the upper right quadrant represents the percentage of NK cells reacting with the 143211 anti-KIR2DL1 antibody. The lower panel shows three donors found to possess the ZEB1 SNP. Lack of expression is evident in the individual containing only a mutant KIR2DL1 gene (1 copy mut 2DL1). The presence of either KIR2DS5 or KIR2DS3 genes in two of the donors is also indicated, as these KIR gene products have been shown to react with the 143211 anti-KIR2DL1 antibody.23

Figure 5. The KIR2DL1 SNP is associated with decreased distal transcript expression. Purified NK cell RNA from purified donor NK cells was subjected to qRT-PCR with KIR2DL1-specific primers detecting distal (a) or proximal (b) transcripts. Expression relative to actin is shown on the y axis, and KIR2DL1/KIR2DS1 genotype of individual donors is listed on the x axis. Donors were: lacking both KIR2DL1/KIR2DS1 (negative); two copies of wild-type KIR2DL1 (2Wt); one copy of WT KIR2DL1 (1Wt); one WT, one ZEB1-mutant KIR2DL1 copy (Wt/Mut); one copy of a mutated KIR2DL1 (1 Mut); and one copy of mutated KIR2DL1 and one copy of KIR2DS1 (Mut/2DS1). (c) Transcription start sites (TSS) mapped by 5’ RACE are shown for the 365 bp region immediately 5’ of the KIR2DL1 start codon (ATG). TSS are indicated by an asterisk. The underlined sequences represent forward PCR primers used for the detection of distal and proximal transcripts in panels (a) and (b).
of the KIR2DL1 gene containing the ZEB1 SNP. The proximal transcript levels actually increased in the ZEB1 mutant, possibly due to the loss of competition from the upstream promoter. Furthermore, the levels of distal transcript paralleled the level of protein expression observed in Figure 4, whereas proximal transcript did not. As previous studies had demonstrated distal KIR transcripts initiating approximately 800 bp upstream of the KIR start codon were not translatable, S’ RACE (Rapid Amplification of cDNA Ends) was performed with primers just upstream of the proximal promoter to look for additional distal transcription start sites. Two novel distal transcript start sites 230 and 273 nucleotides upstream from the major proximal promoter start site were identified in RNA from individuals with wild-type KIR2DL1 alleles, indicating the presence of an intermediate promoter (Figure 5c). The lack of correlation between proximal promoter activity and protein expression suggests that transcripts initiated from the novel intermediate promoter may be responsible for KIR2DL1 expression by mature NK cells. To investigate whether or not these novel transcripts are capable of expressing protein, a full-length cDNA originating from the intermediate promoter (GenBank accession number KJ699235) was cloned into an expression vector and transfected into the human 293T cell line. Expression of KIR2DL1 from the intermediate transcript was comparable to expression from the proximal transcript, indicating that the additional 230 base 5’ untranslated region of the intermediate transcript did not inhibit expression, consistent with the association of the novel promoter with KIR expression by mature NK cells. A KIR3DL1 cDNA originating from the intermediate promoter was also tested and found to be capable of producing KIR3DL1 expression (data not shown).

Proximal KIR2DL1 transcripts contain alternative exons

The lack of correlation between KIR2DL1 expression and proximal transcription was perplexing, as the major start site of KIR2DL1 transcription in NK cells has been mapped to the proximal promoter.12-25 However, RT-PCR analysis of individuals with reduced KIR2DL1 distal transcript revealed the presence of two alternative exons containing stop codons (Figure 6), suggesting that proximal promoter transcripts might be alternatively spliced and potentially incapable of producing translatable KIR2DL1 mRNA. To further investigate this possibility, qRT-PCR was performed with antisense primers specific for the alternate exons together with forward primers from either the intermediate or proximal transcripts. Efficient amplification of alternative exon-containing transcripts was only observed with the forward primer from the proximal transcript, suggesting that the alternative exons are specific to the proximal transcript (Figure 6c). However, the alternative exon-containing transcripts were not as abundant in NK cells that contained only wild-type KIR2DL1 genes, suggesting that the presence of distal transcript either inhibits alternative splicing or ZEB1 binding to the distal promoter region affects alternative splicing of the proximal transcript. S’ RACE performed using primers from the alternative exons generated 5’ ends mapping to the proximal promoter region. No start sites upstream of the proximal promoter were detected with alternative exon primers (data not shown).

DISCUSSION

Studies of the Pro1 distal bidirectional promoter present in the murine Ly49 genes have indicated that forward transcription from the distal promoter is required for activation of the proximal Ly49 promoter (Pro2) that is responsible for Ly49 expression in mature NK cells. This was demonstrated by both naturally occurring deletions of the distal promoter as well as a direct deletion of the Pro1 element in Ly49a transgenic mice.16 The distal KIR promoter has been shown to be transcriptionally active in NK precursors that lack KIR expression, suggesting that it may also have a role in the initial opening of the proximal KIR promoter analogous to the murine Ly49 Pro1 promoter.17 The weakly expressed KIR2DL1 allele reported herein has revealed the presence of a novel KIR intermediate promoter 230 bp upstream of the proximal promoter, and transcription from this novel promoter is associated with protein expression. For the purposes of this discussion, we will refer to this new intermediate promoter as Pro-I. The bi-directional proximal promoter will be referred to as the probabilistic switch, or Pro-S. The previously described distal promoter will be referred to as Pro-D. The presence of a ZEB1-binding site in Pro-I was correlated with a low level of KIR2DL1 distal transcription in peripheral individuals. The ZEB1 SNP was also associated with decreased in vitro KIR2DL1 Pro-I activity (data not shown) and decreased Pro-I transcripts in NK cells, indicating an enhancer function of the Pro-D element for Pro-I transcription. Pro-S activity was unchanged, indicating an association of Pro-I transcription with KIR2DL1 protein expression. The results obtained in this study necessitate a revision of the model of probabilistic KIR gene activation. The previous observation that CD56dim, KIR16 NK cells contain antisense transcripts from Pro-S indicated that these cells represented the phase of NK development where the probabilistic Pro-S element was active; however, these cells also contain forward Pro-S transcripts but no KIR protein expression. The discovery of a novel promoter associated with KIR expression indicates that the Pro-S transcriptional activity is not sufficient for protein expression, and it is Pro-I that drives expression in mature NK cells either directly or by facilitating proper splicing of proximal transcripts. This also reveals another parallel with the murine Ly49 system: forward transcripts from bidirectional promoters active in immature NK cells are non-translatable. Therefore, the process of KIR gene activation should proceed through three stages as defined by the activity of the Pro-D, Pro-S and Pro-I elements (Figure 7). NK precursors have an active Pro-D element that is associated with the activation of the Pro-S region in developing NK cells. NK cells undergoing the CD56bright to CD56dim transition represent the probabilistic transcription phase marked by Pro-S activity and the presence of either antisense transcripts or forward non-translatable transcript. Mature KIR-positive NK cells should exhibit Pro-I activity driving the expression of KIR protein or preventing alternative splicing of proximal transcripts. The inability of previous studies of KIR transcript initiation sites to detect Pro-I transcripts may be due to a high concentration of proximal Pro-S transcripts masking the presence of upstream start sites. The PCR and subsequent cloning steps performed in S’ RACE create a bias toward smaller products, making it difficult to identify start sites that are further upstream. There is also a bias toward more abundant transcripts, and Pro-S transcripts are approximately fivefold higher than the Pro-I transcripts detected in purified peripheral NK cell populations.18 As a result, the Pro-I transcription start site could only be mapped by S’ RACE using primers upstream of the Pro-S start site.

The proposed ability of Pro-I to drive KIR protein expression in mature NK cells and the non-translatable Pro-S transcripts associated with low Pro-I activity suggests that SNPs affecting the level of KIR expression should map to the Pro-I element and the upstream enhancer Pro-D as described in this study, whereas SNPs affecting the percentage of NK cells expressing a given KIR should map to the Pro-S element, as previously demonstrated for the KIR3DL1 gene.18 To test the prediction that Pro-I SNPs might affect the intensity of KIR3DL1 expression, the KIR3DL1 Pro-I region was compared among the KIR3DL1 alleles represented in GenBank. A single base pair deletion was found in the KIR3DL1*005 and KIR3DL1*004 alleles but not in any of the other KIR3DL1 alleles or KIR3DS1. This deletion affects a consensus AP-1 transcription factor binding site and would be predicted to result in

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in decreased Pro-I promoter activity, consistent with the reported low expression phenotype of KIR3DL1*005.

The analysis of a KIR2DL1 allele associated with low expression revealed a novel KIR variant, but, in addition, has resulted in a significant change in our understanding of KIR gene regulation. The promoter previously considered to be responsible for KIR protein expression (Pro-S), may function primarily as a probabilistic switch that governs the frequency of gene activation in developing NK cells. This novel paradigm of KIR gene regulation necessitates a shift away from analysis of the proximal KIR promoter when considering polymorphisms that affect the intensity of KIR expression. Mature NK cells require KIR2DL1 Pro-I transcripts to express the protein, Pro-I transcripts have been identified for the KIR3DL1 gene (data not shown) and the Pro-I element is conserved among all KIR genes except for KIR3DL3, a gene that is not expressed by mature circulating NK cells.

Figure 6. KIR2DL1 proximal promoter transcripts contain additional exons that disrupt the KIR2DL1 open reading frame. (a) A schematic of the KIR2DL1 gene structure is shown. Numbered rectangles represent KIR2DL1-coding exons with the start codon in exon 1 labeled 'ATG', whereas the shaded rectangles marked 'A' and 'B', represent alternative exons containing stop codons indicated by 'TGA'. Intermediate and proximal transcript initiation sites are marked by vertical lines with arrows attached. Lines below the KIR2DL1 gene diagram represent cDNA clones isolated from purified NK cell RNA obtained from an individual with a single copy of the KIR2DL1 gene containing the ZEB1 SNP. Three distinct isoforms of the KIR2DL1 proximal transcript were identified (Prox1–3), each containing different combinations of the alternative exons. (b) The nucleotide sequence of the Prox1 transcript is shown, with the KIR2DL1 open reading frame indicated by the amino-acid sequence listed above. Exon boundaries are marked by vertical lines, and each exon is defined by labeled brackets. The stop codons in alternative exons A and B (Alt-exon A/B) are underlined in bold. (c) qRT-PCR of alternative exon-containing transcripts using either a distal (2DL1-Distal-Alt-Exon) or proximal (2DL1-proximal-Alt-Exon) KIR2DL1 forward primer together with an alternate exon B reverse primer.

Expression relative to actin is shown on the y axis, and KIR2DL1/KIR2DS1 genotype of individual donors is listed on the x axis as described in Figure 5b.
This suggests that most KIR genes use Pro-I for the expression of KIR protein in mature NK cells. Of particular interest is the divergence of the KIR2DL3/2DL2/2DS2 Pro-I elements that only share 84% homology with the 3' half of the KIR2DL1 Pro-I element and possess no significant homology within the 5' half of the promoter. This may explain the previous observation that NK cells derived from hematopoietic progenitor cells generally acquire the promoter. This may explain the previous observation that NK cells derived from hematopoietic progenitor cells generally acquire the 5' specific inhibitory KIR2DL2/3 receptors at earlier time points compared with the KIR2DL1 promoter.

The expression of the proximal KIR promoter is regulated by specific SNPs; however, there is no significant difference in KIR2DL2/3 Pro-I activity as compared with KIR2DL1 Pro-I activity. It will therefore be of interest to compare Pro-I transcripts and Pro-I activity of these genes to see if a correlation exists between divergent Pro-I structures and distinct expression patterns.

In conclusion, the discovery of a novel KIR promoter associated with protein expression by mature NK cells mandates a re-examination of previous data correlating proximal promoter polymorphisms with differences in the timing, tissue specificity or level of transcription of individual KIR genes or alleles. This paradigm shift promises to provide insight into gene/allele-specific differences that have not been adequately explained by study of the proximal KIR promoter.

**MATERIALS AND METHODS**

**Cell lines**

YT cells were cultured in RPMI 1640 media containing 10% fetal bovine serum, 100 U/ml streptomycin, 100 U/ml penicillin, sodium pyruvate and L-glutamine. HEK293T cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin (P/S), sodium pyruvate and L-glutamine.

Donors and NK cell isolation

Collection of DNA and blood samples from 162 donor/recipient pairs from unrelated donor hematopoietic cell transplants performed to treat AML was facilitated by the NMDP (Minneapolis, MN, USA) Research Sample Repository. Samples were collected after informed consent, and approval from the NMDP and University of Minnesota Institutional Review Boards were obtained in accordance with the Declaration of Helsinki. Healthy volunteers were recruited through the NCI-Frederick Research Donor Program (http://ncifrederick.cancer.gov/programs/science/rdp/default.aspx). The KIR genotype of each donor was determined as previously described.

**Flow cytometric analysis of KIR expression on peripheral blood NK cells**

The proportion of NK cells expressing KIR2DL1 was assessed in whole blood by three-color flow cytometry. NK cells were identified using anti-human CD56-PE (Beckman Coulter, Pasadena, CA, USA). T cells were labeled with anti-human CD3-Pacific Blue (e Bioscience, San Diego, CA, USA). KIR2DL1-expressing cells were identified with the clone 143211-FITC. KIR2DL1-specific antibody (R&D Systems, Minneapolis, MN, USA). Briefly, 200 μl of EDTA-treated blood was incubated with a cocktail of the three mAbs for 30 min on ice. Erythrocytes were lysed by the addition of 3 ml of RBC lysis buffer (150 mM NH4Cl, 10 mM NaHCO3, 1 mM EDTA, pH 7.4). The remaining cells were analyzed with a FACSort flow cytometer (Becton & Dickinson, San Jose, CA, USA). Events (25,000) were collected in the lymphocyte gate and analyzed. The percentage of CD3-CD56+ NK cells expressing KIR2DL1 was determined.

PCR screening and sequencing of KIR2DL1 genes

PCR screening of donors for the ZEB1 SNP was performed with a KIR2DL1-specific forward primer (5'-GGGTTCACAGTCTTATTGAGCG-3') together with a reverse primer recognizing the KIR3DP1/KIR2DL5B sequence found in the low-expresser variant (5'-CTCAATATAATTTAACTCCTTTAC-3'). To obtain the complete sequence of the KIR2DL1 alleles present in each donor, 50 ng of leukocyte DNA obtained from donor blood was subjected to PCR with a series of KIR2DL1/51-specific primers to produce overlapping ~1 kb amplicons spanning the complete gene from the polyA site of the preceding KIR2DP1 gene to the polyA site of KIR2DL1. PCR fragments were cloned into the pCR2.1-topo vector (Invitrogen, Carlsbad, CA, USA). A minimum of six clones of each fragment was sequenced for each KIR2DL1/51 allele present.

RT-PCR of KIR transcripts

Total RNA was purified from 1 x 10^7 donor NK cells with the RNeasy kit (Qiagen, Valencia, CA, USA), and cDNA synthesis was carried out using
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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