RESEARCH HIGHLIGHT

Functional consequences of genetic polymorphisms in the NKG2D receptor signaling pathway and putative gene interactions

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NKG2D (NK group 2, member D) is an activating natural killer (NK) receptor, which is expressed on NK and CD8$^+$ T cells. On NK cells, NKG2D elicits cytotoxicity and release of cytokines. On CD8$^+$ T cells, it functions as a co-stimulatory molecule. The receptor recognizes several ligands including the major histocompatibility complex (MHC) class I chain-related molecules A (MICA) and B (MICB) as well as the UL16-binding proteins (ULBP). The diversity of NKG2D ligands is further increased by a high degree of genetic variability of the ligands. Recently, an amino acid exchange from valine to methionine at position 129 in MICA has been found to be associated with the outcome of allogeneic hematopoietic stem cell transplantation (HSCT), and the functional consequences of this specific genetic variation have been elucidated. The clinical associations found after HSCT were explainable by the functional differences of the MICA-129 variants. Herein, we discuss how the genetic polymorphisms of NKG2D ligands and NKG2D itself interact and may affect the outcome of HSCT and the susceptibility to other diseases, which have been associated with polymorphisms in the NKG2D signaling pathway.

Keywords: NKG2D; NKG2D ligands; MICA; hematopoietic stem cell transplantation; graft versus host disease; genetic polymorphism

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Introduction

NKG2D (NK group 2, member D) is an activating natural killer (NK) receptor, which is encoded by the *KLRK1* gene in the human NK gene complex on chromosome 12 [1]. The expression of NKG2D at the plasma membrane requires its association with the adaptor protein DNAX-activation protein (DAP)10 [2]. Notably, a truncated isoform of NKG2D, i.e. $NKG2D^{TR}$, lacking the extracellular domain can be generated by alternative splicing. $NKG2D^{TR}$ can compete with the full-length protein for DAP10 resulting in a reduced expression of the functional NKG2D receptor at the cell surface [3]. NKG2D is expressed on almost all human NK cells, resting $CD8^+$ αβ T cells, many γδ T cells and iNKT cells as well as a small subset of effector or memory $CD4^+$ T cells [2, 4, 5]. The expression of NKG2D is up-regulated by interleukin (IL)-2, IL-7, IL-12, and IL-15, but down-regulated by transforming growth factor (TGF)-β, interferon (IFN)-β1, and IL-21 [2]. On NK cells, NKG2D signaling elicits degranulation and killing of target cells [6] as well as secretion of cytokines such as IFN-γ [7]. On $CD8^+$ αβ T cells, NKG2D functions as a co-stimulatory molecule and provides, in addition to the first signal received by the T cell receptor, a second signal to elicit proliferation and differentiation into effector cytotoxic T lymphocytes (CTL) [8, 9]. NKG2D has been demonstrated to be important for the elimination of tumor cells [10] and for defense against pathogens [11, 12].

The NKG2D receptor recognizes several ligands (Figure 1A) including the major histocompatibility complex (MHC) class I chain-related molecules A (MICA) and B (MICB). The *MICA* and *MICB* genes are located within the human leukocyte antigen (HLA) complex in close proximity to *HLA-B* [13, 14]. The protein structure of MICA and MICB is similar to classical class I molecules with three extracellular domains (α1, α2, and α3), a transmembrane segment, and a carboxy-terminal cytoplasmic tail. However, in contrast to classical MHC class I molecules, MICA and MICB are not associated with β2-microglobulin and do not present peptides. NKG2D binds also to a second family of ligands, the UL16-binding proteins (ULBP) encoded by the *RAET1* gene family, which is also localized on chromosome 6 but outside the HLA complex [15]. Six loci encode functional proteins, i.e. *RAET1I* (ULBP1), *REAT1H* (ULBP2), *RAET1N* (ULPB3), *RAET1E* (ULBP4), *RAET1G* (ULBP5), and *RAET1L* (ULBP6) [16].

NKG2D ligands are constitutively expressed only on a few cell types [17]. They can be expressed during embryonic development and on pluripotent stem cells [18] but in general, the proteins are absent on healthy adult cells. However, almost all cells exposed to cellular or genotoxic stress can express NKG2D ligands [17, 19]. Therefore, NKG2D ligands are frequently found on malignant or virally infected cells and mark these cells for recognition by NK cells and other lymphocytes expressing NKG2D [20]. Several mechanisms have been described that permit tumors or virally infected cells to escape the NKG2D-mediated immune surveillance. One mechanism is that MICA and other NKG2D ligands can undergo a proteolytic shedding that results in an immune escape mediated not only by the loss of ligands on target cells but in addition by the generation of immunosuppressive soluble NKG2D ligands [16, 21, 22]. Several clinical studies showed an association between tumor-associated or soluble NKG2D ligands and disease progression or poor prognosis in different malignant diseases [23].

The diversity of NKG2D ligands is further increased by genetic polymorphisms (Figure 1B). *MICA* is the most polymorphic non-classical class I gene and currently 100 alleles are known that encode for 79 protein variants (http://www.ebi.ac.uk/imgt/hla/, release 3.17.0). *MICB* is also very polymorphic with 40 alleles encoding 26 protein variants. The *RAET1* gene cluster is less polymorphic and the variation is found mainly within the *RAET1E* (ULBP4), *REAT1L* (ULBP6), and *RAET1N* (ULBP3) genes [24, 25]. The importance particularly of *MICA* polymorphisms for cancer has been widely studied [26, 27].

A single nucleotide polymorphism (SNP) (rs1051792) at nucleotide position 454 (G/A) of *MICA* causing a valine (Val) to methionine (Met) exchange at amino acid position 129 in the α2 domain of the protein has gained specific interest because the *MICA* alleles can be separated into two groups with respect to this polymorphism. MICA variants containing a methionine at position 129 bind NKG2D with high avidity, whereas those with a valine bind NKG2D with low avidity [28]. The potential relevance of this difference is highlighted by several disease associations described for this SNP. The MICA-129 dimorphism has been associated with the risks for nasopharyngeal carcinoma [29] and hepatitis B virus-induced hepatocellular carcinoma [30]. Moreover, it has been associated with several autoimmune diseases, including ankylosing spondylitis [31], rheumatoid arthritis [32], inflammatory bowel disease [33, 34], lupus erythematosus [35], type I diabetes [36], and psoriatic disease [37]. Recently, an association with the severity of chronic Chagas heart disease has been described [38]. Notably, this SNP has also been associated with changes in the serum level of soluble MICA (sMICA). In patients with ulcerative colitis, carriers of the *MICA-129Val/Val* genotype had higher sMICA serum levels [34]. The *MICA-129Met* allele on the other hand has been associated with lower sMICA serum levels in healthy

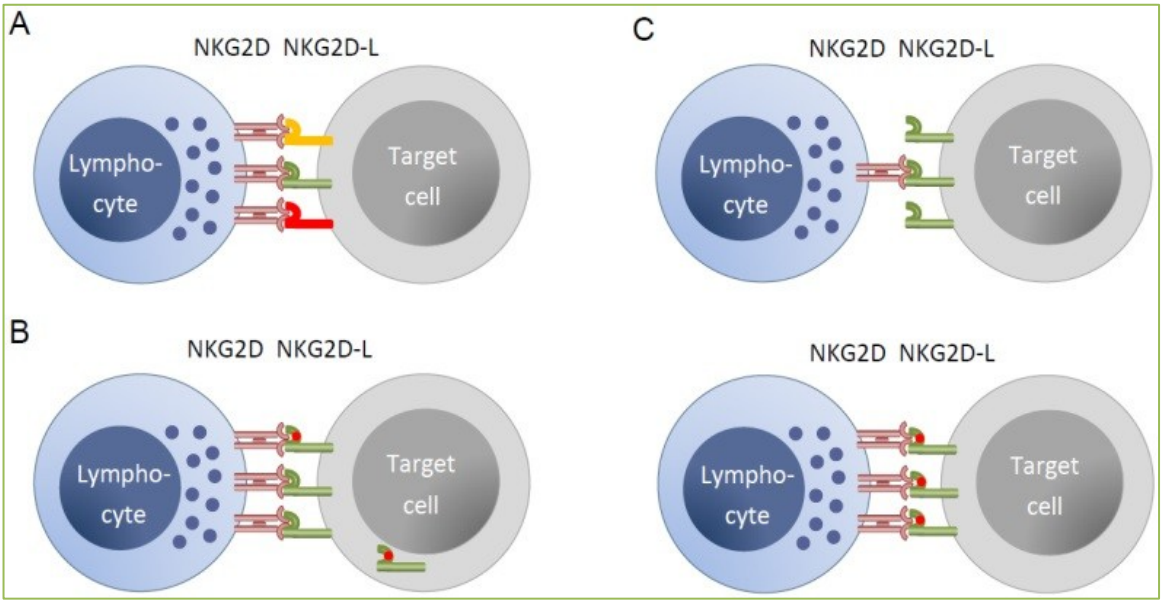

Figure 1. Summary of the effects of variation in the NKG2D receptor and NKG2D ligand pathway. (A) The NKG2D receptor on lymphocytes can interact with several NKG2D ligands on target cells, which may differ in their efficacy to elicit NKG2D signaling. (B) NKG2D ligands are polymorphic and isoforms of the same ligand, e.g. MICA, vary in their cell surface expression and their capacity to elicit NKG2D signaling. (C) NKG2D vary in the intensity of cell surface expression due to genetic polymorphisms. The functional consequences of polymorphisms in NKG2D and NKG2D ligands may be cooperative or counteracting. The interaction of the variants could be highly important for the outcome of NKG2D signaling and disease associations of the NKG2D signaling pathway.

controls and hepatitis B virus-induced hepatocellular carcinoma patients [30].

We became interested in the MICA-129 dimorphism in the context of hematopoietic stem cell transplantation (HSCT). HSCT is a potentially curative therapy for several hematological diseases. However, the success is limited by post-transplant complications including graft versus host disease (GVHD), relapse of malignancy, and infections [39]. The MICA-129 dimorphism has been associated with the incidence of chronic GVHD and relapse of malignancy after HSCT [40]. However, the finding that high avidity MICA-129Met variants were associated with an increased risk of relapse, whereas the low avidity MICA-129Val variants were associated with an increased risk of chronic GVHD [40] appeared to be counterintuitive in view of the functions of NKG2D. Thus, we analyzed another cohort of 452 patients undergoing HSCT and found that the *MICA-129Met* allele was associated with an improved survival and a reduced risk to die from acute GVHD. Nonetheless, carriers of the *MICA-129Met/Met* genotype had an increased risk to experience acute GVHD [41].

To better understand the associations of the MICA-129 dimorphism with the outcomes of HSCT, we determined functional differences of the MICA-129Met and MICA-129Val isoforms [41, 42]. Binding of the MICA-129Met isoform to NKG2D stimulated a stronger phosphorylation of SRC family kinases in NK cells than binding of the MICA-129Val isoform. Subsequently, the MICA-129Met ligand triggered more degranulation and IFN-γ production of NK cells than the MICA-129Val ligand. Notably, the extent of degranulation and IFN-γ secretion correlated clearly with the MICA expression intensity on target cells for the MICA-129Val isoform. The expression intensity of the MICA-129Met isoform, in contrast, had either none or even a negative effect on the extent of degranulation, target cell killing, and IFN-γ release. On $CD8^+$ T cells, the MICA-129Met isoform induced an earlier co-stimulatory activation than the MICA-129Val isoform. Importantly, the MICA-129Met ligand induced also a stronger down-regulation of NKG2D on both NK and $CD8^+$ T cells than the MICA-129Val ligand. This down-regulation of NKG2D impaired the capability of NK and $CD8^+$ T cells to receive signals via NKG2D. Thus, MICA-129Met ligands, which elicit strong NKG2D responses, stimulate in parallel a robust negative feedback signal by down-regulation of NKG2D and this appears to limit the initially stronger effects of MICA-129Met ligands [41]. In target cells, more of the MICA-129Met isoform was retained in intracellular compartments and when transported to the cell surface, it was more susceptible to shedding than the MICA-129Val isoform [42]. Both processes appear to limit the expression of the high avidity MICA-129Met isoform at the plasma membrane.

These results show how the MICA-129 dimorphism affects the function of NK cells and CTL. In HSCT recipients, carriers of two *MICA-129Met* alleles had an increased risk to experience acute GVHD, which could be the result of rapid and strong effects of the MICA-129Met isoform on NKG2D signaling. A faster co-stimulatory activation of $CD8^+$ T cells by the MICA-129Met isoform could be essential for this difference. On the other site, having at least one *MICA-129Met* allele conferred a lower probability of death due to acute GVHD. This finding is explainable by a rapid down-regulation of NKG2D on allo-reactive $CD8^+$ T cells mediated by engagement of the high avidity MICA-129Met isoform, which limits the NKG2D-mediated co-stimulation of allo-reactive donor $CD8^+$ T cells. In heterozygous recipients, also the risk of occurrence of acute GVHD was reduced suggesting that the effect of the MICA-129Met isoform on the cell surface expression of NKG2D was decisive for this outcome. Consequently, we found an increase in survival after HSCT for recipients carrying a *MICA-129Met* allele. Consistently, recipients having two *MICA-129Val* alleles were at hazard to develop a fatal acute GVHD and they appeared to mainly profit from treatment with anti-thymocyte globulin (ATG), which depletes T cells. This is explainable by a failure to efficiently down-regulate NKG2D on allo-reactive $CD8^+$ T cells when having only low avidity MICA-129Val variants. Therefore, this finding is of potential therapeutic relevance for recipients having two *MICA-129Val* alleles. These patients may specifically profit from ATG or other T cell depleting therapies, which are used in some treatment protocols to reduce the risk of acute GVHD.

It has previously been reported that the risk of chronic GVHD was increased for patients having the *MICA-129Val/Val* genotype, whereas the *MICA-129Met/Met* genotype was associated with an increased risk of relapse [40]. These associations, although not found in our cohort [41], are also explainable by functional effects of the MICA variants on NKG2D. Sustained NKG2D-mediated activation of allo-reactive $CD8^+$ T cells is likely if only a MICA-129Val isoform is present in a patient and this could increase the risk of chronic GVHD. On the other hand, sustained NKG2D-mediated activation of anti-leukemic $CD8^+$ T cells and NK cells would be predicted to reduce the risk of relapse.

We demonstrated that the MICA-129Met isoform triggers more NKG2D signaling at low expression intensities. The MICA-129Val isoform, in contrast, produces more NKG2D effects at high expression intensity, at which the MICA-129Met isoform already down-regulates NKG2D leading to a diminished function [41]. Since MICA expression intensities can vary for certain *MICA* alleles [43], a functional interaction of several SNPs within the *MICA* gene can be postulated. The principal relevance of NKG2D signaling for the outcome of allogeneic HSCT has been further demonstrated in mouse models, which developed less GVHD when receiving NKG2D-deficient T cells after transplantation [44]. The importance of the pathway is also emphasized by human studies demonstrating effects of the genotypes of *RAET1L* [45] encoding the NKG2D ligand ULBP6 and the NKG2D-encoding gene *KLRK1* [46] on overall survival of patients after HSCT. Therefore, also gene-gene interactions within the NKG2D signaling pathway presumably affect the outcome of HSCT and other diseases, which have been described to be associated with SNPs in NKG2D ligands or NKG2D itself.

The *KLRK1* gene has a limited degree of variation with only one SNP leading to an amino acid substitution (rs2255336, A>G, Thr72Ala). Despite this limited variation at the protein level, *KLRK1* haplotype alleles constructed from five or three SNPs have been associated with low and high NK cell cytotoxicity and increased or decreased risk of cancer development [47] and an interaction of the *KLRK1* genotype and lifestyle risk factors for cancer was observed [48]. It was the *NKG2D-HNK1* haplotype, a haplotype expected to induce greater NK cell activity, that has been associated with significantly improved overall survival after HSCT [46].

SNPs within the *KLRK1* gene have been associated also with the risk of cholangiocarcinoma in patients with primary sclerosing cholangitis [49]. The rs2255336 G genotype encoding the NKG2D-72Ala variant was found to be associated with an increased risk of cervical carcinoma and progression to advanced stages of the disease [50]. For homozygous carriers of this variant, an increased risk for systemic lupus erythematosus (SLE) was observed [51]. In accordance with these data, presence of the NKG2D-72Thr variant appeared to confer protection against SLE [52]. The G/G genotype was also associated with an increased risk of early symptomatic infection in cases with congenital cytomegalovirus infection [53]. The SNP rs2617160 T/T genotype was associated with susceptibility to chronic hepatitis B [54].

The functional consequences of the *KLRK1* polymorphisms are only partly understood. Notably, the *KLRK1* haplotypes associated with low and high NK cell cytotoxicity [47], respectively, were described to be associated with low and high expression intensity of NKG2D on NK and $CD8^+$ T cells [55] (Figure 1C). In homozygous carriers of the NKG2D-72Thr variant, a trend was observed towards a higher proliferation in response to stimulation with antibodies against CD3 and NKG2D suggesting a functional

effect also of this polymorphism [51].

In conclusion, we have shown that the MICA-129 dimorphism affects the strength and kinetics of NKG2D signaling as well as MICA cell surface expression and shedding [41, 42]. This results in differences in the NK cell cytotoxicity and cytokine secretion as well as $CD8^+$ T cell co-stimulation and it affects the NKG2D expression on NK and $CD8^+$ T cells by a counter regulatory mechanism. The MICA-129 dimorphism has a significant impact on autoimmune diseases, infections, malignancies and the outcome of HCST. In the future, it might be worthwhile to investigate the interaction of gene variants in the NKG2D signaling pathway. Specific combinations of polymorphisms in NKG2D and NKG2D ligands or downstream signaling molecules may have a higher impact on the outcome of associated diseases than single SNPs in one of the respective genes.

Conflicting interests

The authors have declared that no conflict of interests exists.

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Author contributions

RD drafted the manuscript, which AI, DM, SM, DS, PS, GM, GW, DK, and HB edited. All authors approved the final manuscript.

Abbreviations

ATG: anti-thymocyte globulin; CTL: cytotoxic T lymphocytes; DAP10: DNAX-activation protein 10; GVHD: graft versus host disease; HLA: human leukocyte antigen; HSCT: hematopoietic stem cell transplantation; IFN: interferon; IL: interleukin; MHC: major histocompatibility complex; MICA: MHC class I chain-related molecules A; NK: natural killer; NKG2D: NK group 2, member D; NKG2D-L: NKG2D ligand; Smica: soluble MICA; SNP: single nucleotide polymorphism; TGF: transforming growth factor; ULBP: UL16-binding protein.

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