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A Direct Role for NKG2D/MICA Interaction in Villous Atrophy during Celiac Disease

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Summary

MICA molecules interact with the NKG2D-activating receptor on human NK and CD8 T cells. We investigated the participation of the MICA/NKG2D pathway in the destruction of intestinal epithelium by intraepithelial T lymphocytes (IEL) in Celiac disease and its premalignant complication, refractory sprue. We show that MICA is strongly expressed at epithelial cell surface in patients with active disease and is induced by gliadin or its p31-49 derived peptide upon in vitro challenge, an effect relayed by IL-15. This triggers direct activation and costimulation of IEL through engagement of NKG2D, leading to an innate-like cytotoxicity toward epithelial targets and enhanced TCR-dependent CD8 T cell-mediated adaptive response. Villous atrophy in Celiac disease might thus be ascribed to an IEL-mediated damage to enterocytes involving NKG2D/MICA interaction after gliadin-induced expression of MICA on gut epithelium. This supports a key role for MIC/NKG2D in the activation of intraepithelial immunity in response to danger.

Introduction

Celiac disease (CD) is a T cell-mediated immune disease of the small intestine triggered by wheat gliadin, which leads to villous atrophy, cryptic hyperplasia, and malabsorption. Its only current treatment is a lifelong exclusion of gluten from the diet.

CD is characterized by the infiltration of the small intestine by a T cell "cocktail" composed mainly of activated CD4 T cells in the lamina propria and CD8 T cells in the epithelium (for review see Shan et al., 2002; Sollid, 2002). It occurs in genetically susceptible individuals expressing the HLA-DQ2 or -DQ8 molecules, which are the restriction elements for CD4 T cells recognizing gliadin peptides (Dieterich et al., 1997; Lundin et al., 1993; Molberg et al., 1998; Shan et al., 2002). The role of CD8 TCR$\alpha\beta$ or TCR$\gamma\delta$ intraepithelial lymphocytes (IEL) remains one important unsolved question. Indeed, this IEL infiltration, without any other sign of mucosal pathology, may represent the first stage of CD (Maki et al., 2003). Furthermore, disruption of intraepithelial lymphocyte homeostasis in CD can ultimately lead to the emergence of the lymphoid malignancies characteristic of CD, as recently demonstrated by the study of refractory celiac sprue (RCS). This severe enteropathy complicates CD in a small subset of patients primarily or secondarily refractory to a strict gluten-free diet. Now considered a low-grade intraepithelial lymphoma, it is characterized by a massive intraepithelial infiltration by atypical CD7+ IELs with clonal TCRγ rearrangement and intracellular CD3ϵ chain but no surface CD3 (sCD3)/TCR complexes (Cellier et al., 1998, 2000). Although IELs from CD and RCS are able to kill epithelial cells through the perforin/granzyme pathway (Di Sabatino et al., 2001; Mention et al., 2003), the effector and target cell receptors involved in this destruction have yet to be unearthed to shed light on the apparent complex pathophysiology of this disease.

The human MICA and MICB proteins (Bahram et al., 1994) are nonconventional HLA class I molecules that serve as ligands for the activating NKG2D receptor expressed at the surface of all CD8 $\alpha\beta$T cells, $\gamma\delta$T cells, and most NK cells. Under normal conditions, expression of MIC molecules is restricted to intestinal and thymic epithelium (Groh et al., 1996; Hue et al., 2003), but is stress inducible in various epithelial cells and is upregulated in tumors (Groh et al., 1999; Vetter et al., 2002) and upon exposure to intracellular pathogens (Das et al., 2001; Groh et al., 2001). MICs function as signals of cellular distress and trigger a range of immune effector mechanisms including cellular cytotoxicity, cytokine secretion, and cellular proliferation (for review see Bahram, 2000; Vivier et al., 2002). In TCR$\alpha\beta$ CD8 T cells, NKG2D/MIC engagement delivers a costimulatory signal that complements TCR-mediated antigen recognition on target cells (Groh et al., 2001). In NK cells, NKG2D acts as an activating immunoreceptor, which can transduce positive intracellular signaling (Bauer et al., 1999; Cosman et al., 2001; Pende et al., 2001). Lastly, MIC can be recognized by intraepithelial or tumor-infiltrating $\gamma\delta$T cells and is able to deliver both a TCR-dependent and an NKG2D-dependent costimulatory signal for a subset of Vδ1 $\gamma\delta$ T cells (Wu et al., 2002).

NKG2D cell surface expression requires association with the adaptor molecule DAP10 (Wu et al., 1999), which contains a consensus YxxM tyrosine-based motif in its

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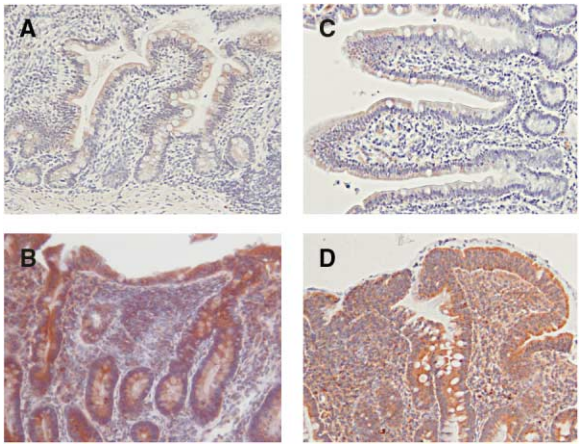

Figure 1. MICA Protein Expression Is Increased in Small Intestinal Mucosa of CD Patients

Paraffin-embedded biopsy sections from a non-CD control (A), active CD patient (B), CD patient on a gluten-free diet (C), and patient with RCS (D). Magnification 100×.

cytoplasmic domain. In human cells, engagement of NKG2D results in tyrosine phosphorylation of DAP10, and recruitment and activation of the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (Billadeau et al., 2003). In mice, however, the distinct functions of NKG2D in NK cells and CD8 T cells is due, at least in part, to the existence of two NKG2D protein species encoded by distinct mRNA splice isoforms and to their association with either DAP10 or the immunoreceptor tyrosine-based activation motif (ITAM)-containing KARAP/DAP12 adaptor protein (Diefenbach et al., 2002; Gilfillan et al., 2002).

The remarkably restricted expression of MIC in the intestinal mucosa, its induction by cellular distress, and its recognition by NKG2D at the surface of CD8 and $\gamma\delta$ T cells suggested a possible role of MIC/NKG2D interaction in the IEL-mediated destruction of gut epithelial cells in the course of CD.

Results

MIC Is Overexpressed in the Small Intestine of Patients with Active CD

Staining of duodenal sections from four normal control subjects who had undergone biopsies for diagnostic purpose showed that MIC was expressed in villous epithelial cells, as originally described (Groh et al., 1996). However, this staining was only of moderate intensity, heterogeneous within and between villi and seemed mainly intracellular (Figure 1A). By contrast, in active CD patients with villous atrophy (n = 6), epithelial expression of MICA was not only much more intense but also diffuse from the surface to the bottom of the crypts and accompanied by staining of mononuclear cells within the lamina propria (Figure 1B). Remarkably, in CD patients who had recovered normal histology under gluten-free diets (n = 5), staining pattern returned to normal (Figure 1C). However, in RCS patients under strict gluten-free diets (n = 4), MICA expression was even more intense and diffuse than in active CD (Figure 1D).

To determine whether MICA was expressed at the cell surface of villous epithelium, we next isolated gut epithelial cells from fresh intestinal biopsy samples and analyzed them by flow cytometry (Figure 2, left). In controls and treated CD patients, the percentage of epithelial cells expressing MICA at their surface was very low (mean ± SEM, 2.5% ± 0.9% in controls and 3.6% ± 2.6% in treated CD), suggesting that most immunoperoxidase staining observed in normal gut biopsies detects intracellular MICA. Indeed, staining of epithelial cells isolated from normal jejunum revealed intracellular MICA in a majority of permeabilized cells, while this antigen was not detected by surface staining (upper right quadrant). In contrast, in active CD and RCS patients, the percentage of epithelial cells expressing MICA at their surface was significantly increased (mean 29.8% ± 5% in active CD and 32% ± 18.6% in RCS, $p < 0.008$ for comparison between the four groups, nonparametric ANOVA test). This percentage even reached 87% in patient BER who presented a particularly severe RCS with 90% of circulating mononuclear cells sharing the same aberrant phenotype and clonality as IELs. These results indicate that MIC, normally expressed in the cytoplasm of gut epithelial cells, is induced at their surface during the course of CD, and this appears to correlate with the severity of the disease.

Gliadin Induces MICA Expression in the Intestine of Treated CD Patients

To assess whether gliadin, the CD-triggering factor, may induce expression of MICA in the small intestine, fresh biopsies were incubated for 4 to 48 hr with or without a peptic-tryptic digest of gliadin in an organ culture chamber prior to staining (Maiuri et al., 1998) (Figure 3 and Table 1). In biopsies from healthy individuals (n = 5), gliadin did not modify MICA expression. In untreated CD patients (n = 3), the already strong expression of MICA and the severe epithelial alterations induced by the culture with gliadin precluded any analysis. By contrast, in treated patients on gluten-free diets (n = 9), a clear increase in MICA expression was observed on both epithelium and lamina propria cells (Figure 3D). This effect was observed very early (within 4 hr of in vitro challenge) and was maximal after 24 hr of organ culture. No modification of MICA expression was induced by a peptic-tryptic digest of bovine serum albumin (BSA). These data indicate that gliadin can induce MICA protein expression in intestinal epithelial cells from treated CD patients.

To understand the mechanisms of MICA induction by gliadin, we investigated the effect of two gliadin-derived peptides known to mediate distinct effects. The 33-mer p57-89 gliadin peptide activates the CD4-mediated adaptive immune response in CD patients: it contains three concatemerized immunodominant T cell epitopes and was shown to efficiently stimulate CD4+ T cell lines derived from the intestine of CD patients (Shan et al., 2002). The p31-49 peptide, known to induce small intestinal damage in vitro and in vivo in CD patients, is not recognized by CD4 intestinal T cells but was recently reported to activate the innate immune system (Maiuri et al., 2003). The hSA peptide was used as a control. p31-49 induced a strong expression of MICA in the epi-

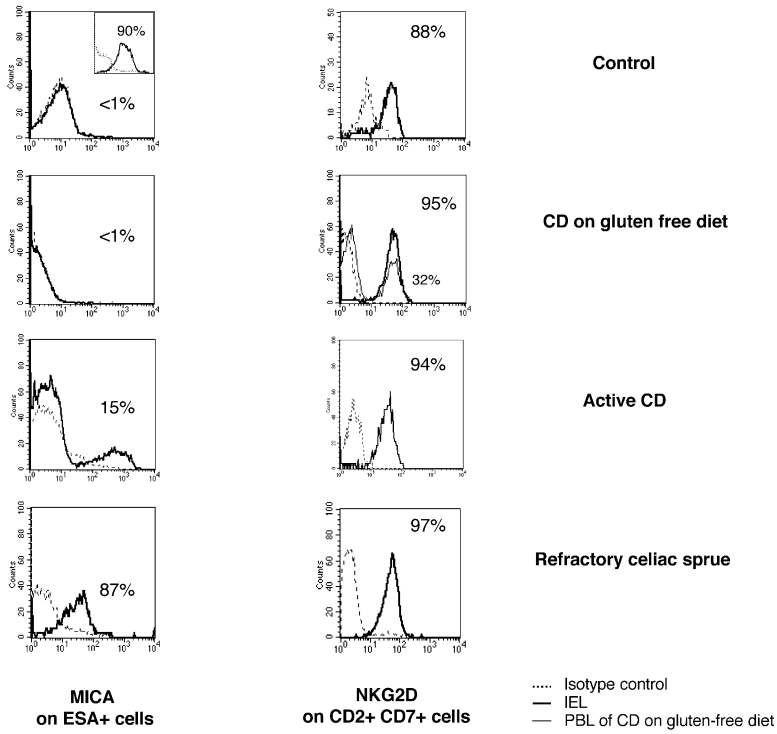

Figure 2. Induction of MIC at the Surface of Gut Epithelial Cells and Expression of NKG2D on Intraepithelial Lymphocytes in Active CD and RCS

Flow cytometry analysis of epithelial cells and IELs freshly isolated from duodenal biopsies in control subjects (n = 4), treated CD (n = 5), active CD (n = 4), and RCS (n = 4) patients. The numbers indicate the percentage of gated cells. A representative experiment from each group is shown.

Left: ESA-positive epithelial cells stained with PE-labeled anti-MICA SR116 mAb. In the upper right quadrant, intracellular staining of permeabilized cells is shown in the same control subject.

Right: CD2+ CD7+ IELs stained with FITC-labeled MICA tetramers. In parallel to IELs, staining of PBMC is shown in a treated patient.

thelium of CD patients. Induction was detectable after only 4 hr of culture but was more intense after 12 hr. The same peptide tested in three controls failed to induce MIC in two cases. In the third control, a very discreet increase of MICA expression was noticeable. The nonimmunodominant p57-89 peptide was a much less efficient inducer of MIC. Induction was detectable only in two out of six tested patients, and this peptide had no effect in the controls. The hSA control peptide had no effect on MICA expression.

To more deeply investigate the molecular mechanisms involved in MICA induction by gliadin peptides, we next studied the role of IL-15. This cytokine, overexpressed in the intestine of CD patients, not only mediates epithelial damage but also sustains a persistent activation of the adaptive immune system (Maiuri et al., 2000). Our previous data suggested that IL-15 can be upregulated upon gliadin challenge in biopsies of treated CD patients (Di Sabatino et al., 2001; Mention et al., 2003). Furthermore, it was shown that p31-49 stimulates IL-15 production in lamina propria dendritic cells and macrophages of CD patients (Maiuri et al., 2003). As shown in Figure 3, IL-15 induced a strong expression of MICA in biopsies from both treated CD patients and healthy controls. Moreover, culturing biopsies from treated CD patients in the presence of neutralizing anti-IL-15 antibody abrogated the p31-49-induced expression of MICA, while it had no effect on the p57-89-mediated MICA induction. Consistent with a direct effect of IL-15 on enterocytes, some accumulation of intracellular MICA was observed after stimulation of HT29, T84, and Caco2 epithelial cell lines cultured for 4–24 hr in the presence of IL-15. This effect remained, however, modest, perhaps due to the already high constitutive expression of MIC and IL-15 in these cell lines. In contrast, gliadin had no effect (data not shown).

Altogether (Table 1), these results demonstrate that gliadin can induce expression of MICA in the intestine of CD patients. Induction mainly depends on an early innate response triggered by the p31-49 peptide and relayed by IL-15. A contribution of adaptive mechanisms activated by the immunodominant p57-89 peptide in an IL-15-independent way can, however, be detected in a subset of patients.

IELs Can Lyse Epithelial Targets via NKG2D

Since epithelial cells of the small intestine express MICA after exposure to gliadin, they could become the targets of NKG2D-positive IELs during active CD. We first confirmed by flow cytometry analysis that NKG2D was expressed on most freshly isolated IELs, as described (Roberts et al., 2001) (Figure 2, right). The percentage of IELs able to bind MICA tetramers was not significantly different between non-CD controls, treated, or untreated CD patients (77% ± 10%, 79% ± 4%, and 82% ± 6%, respectively, mean 82.5%), while it reached 98% ± 1% in RCS patients ($p = 0.01$ compared to the three other groups, ANOVA test). The level of MICA tetramer binding to IELs, as defined by mean fluorescence intensity, was comparable in controls and CD patients whatever their status and the percentage of MICA-positive epithelial cells.

We then determined whether IELs from active CD or RCS could mediate killing of target cells through NKG2D/MICA interaction. Because the small number of IELs directly recovered from intestinal biopsies prevented extensive further experiments, we derived T cell lines from these very biopsies in nine patients (five active CD and four RCS) with IL-15 as previously described (Di Sabatino et al., 2001; Mention et al., 2003). These cell lines were then tested in a redirected lysis assay

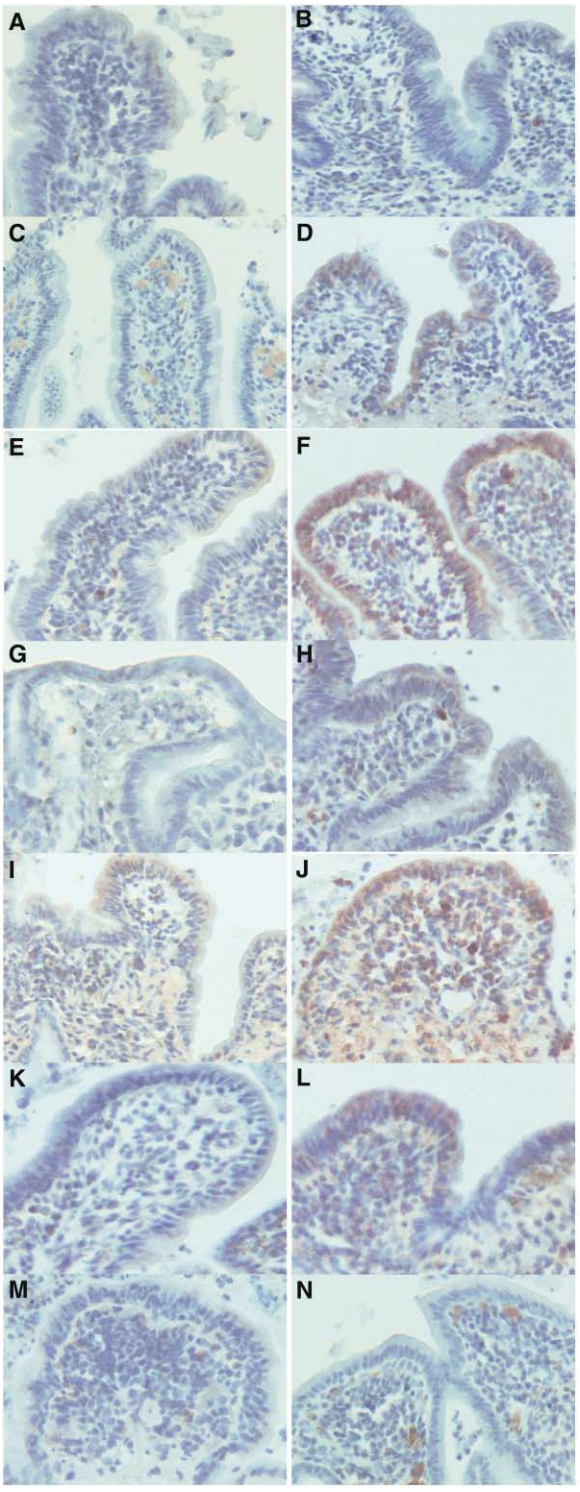

Figure 3. MICA Expression Is Induced by Gliadin or Gliadin-Derived Peptides in the Small Intestine Mucosa of CD Patients

Biopsy specimens from healthy controls (A, C, E, G, and I) and CD patients on a gluten-free diets (B, D, F, H, J, and K–N) were incubated for 12 hr in medium alone (A and B) or in presence of peptic-tryptic digest of gliadin (C and D) or p31-49 gliadin peptide (E and F), p57-89 gliadin peptide (G and H), IL-15 (I and J), p31-49 plus anti-IL-15 neutralizing mAb (K), p57-89 plus anti-IL-15 mAb (L), peptic-tryptic digest of BSA (M), and hSA control peptide (N) prior to staining with SR99 anti-MIC mAb. In (A) and (N), weak nonspecific staining of red blood cells is observed in some lamina propria blood vessels. Magnification 160×.

against FcγR-bearing P815 target cells (Bauer et al., 1999). Cell lines from active CD patients expressed a classical TCR$\alpha\beta$+ CD3+ CD8+ NKG2D+ phenotype at the time of testing. Crosslinking NKG2D alone on these IEL lines had no or only minor effect even at a 30:1 effector:target cell ratio, although TCR$\alpha\beta$ engagement by 1 ng/ml of anti-CD3 induced strong target cell lysis. However, crosslinking NKG2D significantly augmented CD3-mediated redirected lysis when using suboptimal concentration of anti-CD3 (0.01 ng/ml), although the efficiency of lysis varied from line to line (Figure 4A). These results indicate that in T cell lines from active CD patients, NKG2D functions as a costimulatory receptor in a conventional way, similar to what has been described in CD8 T cells.

Surprisingly, however, when redirected cytotoxicity assays were repeated with the same CD8 T cell lines from two different patients at various kinetic points of the cell culture, some differences appeared. Indeed, at very narrow time points, NKG2D-mediated lysis was sometimes observed at day 15 of the cell culture, while it was not detected the day before (Figure 4B). This phenomenon was transient, as CD8 T cells rapidly recovered their classical cytotoxicity profile 4 days later. It thus appears that in particular circumstances, NKG2D alone can mediate IEL cytotoxicity.

We then analyzed the four cell lines from RCS patients, which all expressed the aberrant sCD3$^-$ CD7$^+$ NKG2D+ phenotype. In all cases, NKG2D was able to mediate alone the killing of P815 target cells, even at a low effector:cell ratio (Figure 4C). Although the percentage of target cell lysis varied depending on the patient (21%–93% at 30:1 E:T ratio, mean 51.3%), this NKG2D-mediated killing was constant and maintained over time. Thus, in sCD3$^-$ CD7$^+$ IELs, NKG2D has a direct activating function triggered by engagement of its ligand MICA, similar to what is observed in NK lines (Bauer et al., 1999).

To confirm that IELs from RCS can indeed mediate killing of enterocytes via NKG2D, we performed a direct cell-mediated cytotoxicity assay on epithelial target cells. As previously shown, all cell lines from RCS patients were able to kill MICA-positive HT29 or HeLa epithelial cell lines even at a low effector:cell ratio (Di Sabatino et al., 2001; Mention et al., 2003). In the case of patient BER (Figure 4D), anti-NKG2D mAb fully inhibited the lysis by more than 60%. Since in this patient, 87% of intestinal epithelial cells expressed surface MICA, the NKG2D/MICA pathway seems to be directly involved in the massive destruction of the gut epithelium, leading to villous atrophy. Interestingly, one subclone of this patient's cell line lost surface NKG2D expression. Both redirected lysis of P815 cells and cell-mediated cytotoxicity of epithelial target cells were completely abolished (data not shown), confirming that the cytolytic effect of IELs is fully mediated by NKG2D signaling in this patient. In the other RCS patients, however, inhibition by anti-NKG2D did not exceed 35%, suggesting that killing of

Table 1. Gliadin-Induced MICA Expression in the Intestine of Healthy Controls and Treated CD

	Peptic-Tryptic Digest of Gliadin	p57-89	p31-49	IL-15	p31-49 + anti-IL-15 mAb
Controls	0/5	0/3	1*/3	3/3	n.d.
Treated CD patients	7/7	2*/6	7/7	4/4	0/3

Results are given as the number of subjects with increased expression of MICA among five healthy controls and nine treated CD patients after 12 hr of in vitro organ culture in the presence of gliadin or gliadin-derived peptides.
*Weak MICA induction.
n.d., not done.

epithelial cells involves the engagement of additional triggering receptor(s). Indeed, in agreement with this hypothesis, we observed that high concentration of IELs from the five active CD patients were also able to mediate direct epithelial cell lysis (100:1 E:T ratio) (Figure 4E). This cytotoxic activity was only partly inhibited by anti-NKG2D mAb (range 4.7%–31.5% of inhibition), arguing in favor of the participation of other activating receptors in this antigen-independent killing.

Human NK and CD8 T cells use the DAP10-PI3K pathway for NKG2D activation. To determine whether the same pathway is utilized by sCD3$^-$ IELs from RCS, we measured the lytic activity of the cell line from patient BER, which is largely dependent on NKG2D in the presence of 1 μM of the PI3K inhibitor LY 294002. In both redirected lysis assay and cell-mediated cytotoxicity experiments toward epithelial cells, lysis was abrogated by more than 80%, indicating that the PI3K pathway has a key role in the direct cytotoxicity mediated via NKG2D (Figures 4B and 4D).

Altogether, these results are evocative of a gradient in the potential of NKG2D to mediate epithelial cell destruction in the different stages of Celiac disease. In active CD, NKG2D mainly plays a costimulatory role on conventional IELs, and a TCR-mediated signal is required for complete activation. In the more severe form RCS, NKG2D mediates by itself a direct activating signal in abnormal sCD3$^-$ IELs, endowing these cells with strong epithelium killing capacity.

High Levels of Soluble MIC Molecules Are Present in the Serum of Active CD Patients

The release of a soluble form of MICA (sMICA) in the serum of some cancer patients was recently reported (Groh et al., 2002; Salih et al., 2002). We tested the presence of sMICA in the serum of CD patients with a highly sensitive sandwich ELISA (Figure 5A). Sera from 24 healthy individuals or control patients with non-CD enteropathy were all negative. Nineteen of 54 CD sera (35.2%) contained sMICA (range 0.45–370 ng/ml, mean 57 ng/ml). Among these patients, sMICA was detected in 16 of 32 (50%) untreated patients on gluten-containing diets, but in only three of 22 (13.6%, $p = 0.008$) patients on gluten-free diets. Two patients tested sMICA positive at the time of diagnosis and became negative after 6 months on gluten-free diets. In addition, among the eight RCS patients on strict gluten-free diets, three had sMICA. Therefore, the presence of sMICA is correlative

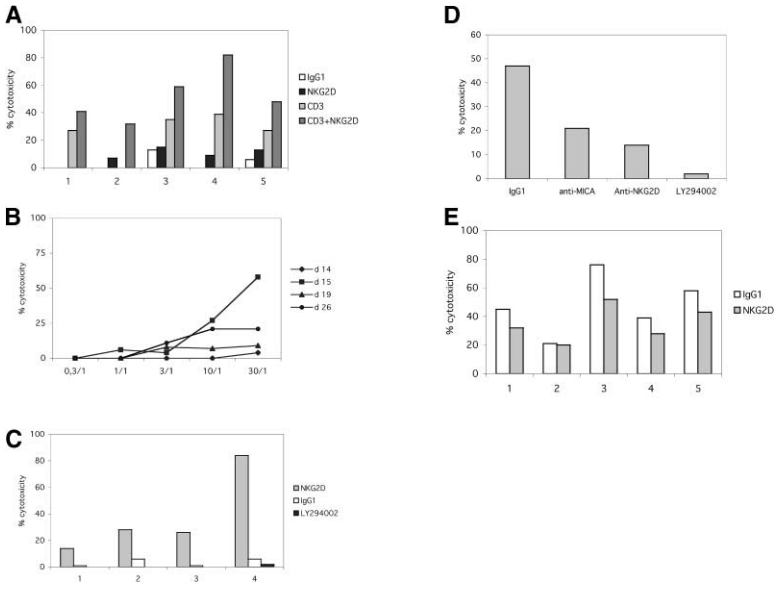

Figure 4. NKG2D-Mediated Cytotoxicity of IELs in Active CD and RCS

(A–C) Redirected cytotoxicy of FcγR+ P815 cells by IEL cell lines. Results represent triplicate of lysis for each cell line in a given experiment. (A) IEL cell lines from five different active CD patients are tested at a 30:1 effector:target cell ratio, in the presence of anti-CD3 mAb (0.01 ng/ml) and/or anti-NKG2D mAb (1 μg/ml), mAb, or isotype control IgG1. (B) IEL cell line from one representative patient with active CD, tested at different kinetic time points of the cell culture (day 14, 15, 19, and 26), shows transient capacity in the NKG2D-mediated killing of P815 cells. (C) NKG2D-mediated killing of P815 cells by IEL cell lines from four patients with RCS in the presence of control IgG1 or anti-NKG2D mAb (effector:target cell ratio 10:1). Lysis is fully abrogated by the PI3K inhibitor LY 294002 (patient BER, $n = 4$).
(D and E) Direct cytotoxicity of MICA-positive epithelial cells by IEL cell lines. (D) Cytotoxicity of HT29 epithelial cells by IEL cell line from RCS patient BER (10:1 E:T ratio) in presence or absence of isotype control IgG1, anti-MICA, or anti-NKG2D mAb or LY294002. (E) IEL cell lines from five active CD patients show variable direct cytotoxicity of HeLa cells at high effector:cell ratio (100:1). Lysis is only weakly abrogated by anti-NKG2D mAb.

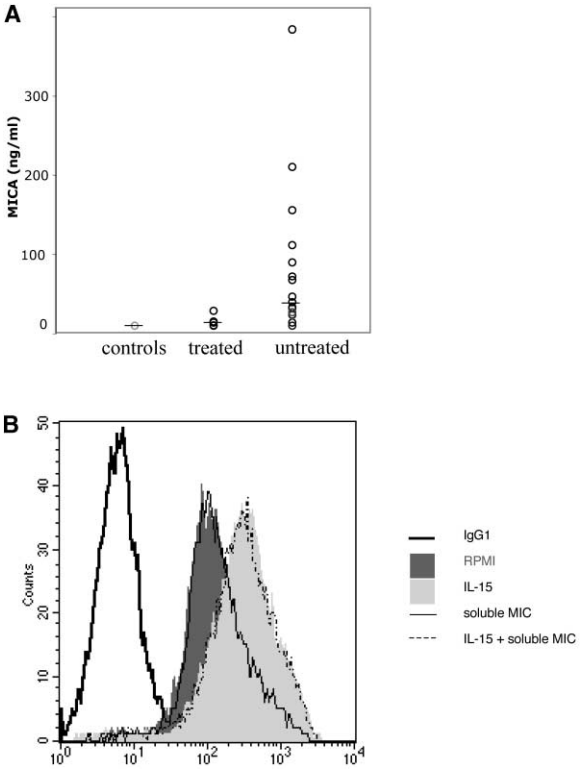

Figure 5. Soluble MICA in the Serum of CD Patients Does Not Downmodulate NKG2D Levels on IELs

(A) Soluble MICA is present in the serum of CD patients. Serum samples from 24 healthy individuals, 22 CD patients on gluten-free diets, and 32 untreated CD patients were investigated by ELISA with recombinant sMICA*008 as a standard. The data shown are means of triplicates. Horizontal lines indicate mean value of respective groups.

(B) Failure of soluble recombinant MICA to downmodulate NKG2D on IEL lines from active CD or RCS patients. Two-color staining of IEL cells incubated in the absence or presence of soluble recombinant MICA (10 μg/ml) with or without IL-15 (20 ng/ml). Data are representative of staining on three different IEL lines.

with the presence of gluten in the diet but is also detected in a fraction of RCS patients on gluten-free diets.

The binding of sMICA induces downmodulation of NKG2D on CD8 T cells and NK cells from cancer patients (Groh et al., 2002), which may serve to limit T cell activation. This raised the question of why NKG2D levels were maintained at high levels on IELs and circulating CD8 T cells of active CD and RCS patients, even in the presence of a high concentration of sMICA. Since IL-15 and TNFα are able to induce NKG2D expression (Groh et al., 2003; Roberts et al., 2001), we measured soluble IL-15 and TNFα in sMICA-containing sera. No significant amount of these cytokines was detected (below 5 pg/ml in all tested sera). Furthermore, the NKG2D level at the surface of IEL cell lines from CD patients was not downregulated after incubation with recombinant soluble MICA or whatever exogenous IL-15 was added or not to the culture medium (Figure 5B). Therefore, at variance with what is observed on $CD4^+$ $CD28^-$ T cells from patients with rheumatoid arthritis (Groh et al., 2003), sMICA does not induce NKG2D downmodulation on IELs from active CD, and this is not related to an opposing activity of soluble IL-15 or TNFα. These results suggest an intrinsic capacity of IELs from CD patients to maintain high levels of NKG2D at their surface.

Table 2. Distribution of Transmembrane MICA Alleles in CD Patients and Controls

MICA	CD Patients n = 107 (%)	Controls n = 90 (%)
4	21 (19.6)	18 (20)
5	12 (11.2)	20 (22.2)
5.1	74 (69.2)	50 (55.6)
6	55 (51.4)	40
9	16 (15)	28 (31.1)*

*The number of alleles compared multiplied by p (Pc) not significant.

The MICA5.1 allele is characterized by a frameshift mutation causing a premature termination codon within the transmembrane region. In order to rule out the presence of an aberrant allele of MICA in CD patients responsible for the presence of sMICA and given the conflicting reports on the role of MICA alleles in susceptibility to CD (Bilbao et al., 2002; Fernandez et al., 2002; Lopez-Vazquez et al., 2002), a large series of patients and controls was genotyped for MICA. We did not find any significant difference in the allelic distribution of MICA transmembrane polymorphism between 107 CD patients and 90 healthy controls (Table 2) or in the frequency of MICA5.1 homozygosity (19/107, 17.7% versus 11/90, 12.2%). MICA5.1 frequency was not significantly different among patients with serum sMICA compared to others (71.4% and 60.2%, respectively). Therefore, the presence of sMICA in the serum is not related to the presence of a particular MICA genotype.

The MICA5.1 variant molecule is aberrantly transported to the apical surface of transfected canine kidney epithelial cells, in contrast to other alleles located at the basolateral surface (Suemizu et al., 2002). This might prevent interaction of MICA5.1-expressing epithelial cells with NKG2D-positive IELs at the basolateral interface. By immunohistochemistry analysis, we did not observe any difference in the localization of MICA in biopsy samples from active CD or RCS patients expressing MICA5.1 at the homozygous or heterozygous state, or not expressing the MICA5.1 allele. These results indicate that MICA5.1 homozygosity is unlikely to protect human epithelial cells from their destruction by NKG2D+ IELs.

Discussion

Villous atrophy in active CD is associated with prominent enterocyte apoptosis indicative of active epithelial destruction (Ciccocioppo et al., 2001; Monteleone et al., 2001). However, the processes leading to mucosal damage remain unclear. Several lines of evidence point to a contribution of IELs activated via both innate and adaptive arms of the immune system. One the one hand, HLA-A2-restricted gliadin-specific CD8 T cells have been identified in the mucosa of CD patients (Gianfrani et al., 2003). On the other hand, the antigen-independent cytotoxicity of IL-15-stimulated IELs against epithelial targets points to the role of innate receptors enabling

damage of epithelial cells (Ebert, 1998; Mention et al., 2003; Roberts et al., 2001). Thus, a selective increase of IELs expressing CD94, the HLA-E-specific NK receptor, is observed in active CD (Jabri et al., 2000).

Among other immunoreceptors expressed at the surface of IELs, NKG2D is a likely candidate. It behaves as a sentinel used by CD8 T cells and NK lymphocytes to detect cells that have upregulated ligands such as MIC as a result of cellular insults (Diefenbach and Raulet, 2003). In the present study, we show that only a very small percentage of normal enterocytes express MICA at their surface, MIC expression being mostly intracellular. As NKG2D is constitutively expressed on resident IELs (Roberts et al., 2001), this low amount of surface MICA may prevent inappropriate epithelial attack by IELs in healthy individuals. We then show that MIC expression is strongly increased in active CD and becomes detectable at the surface of duodenal epithelial cells, which may therefore become targets for NKG2D-expressing IELs. NKG2D functions as a costimulatory signal in CD8 T cells, amplifying antigen-specific signals provided by the TCR. Since CD8 T IELs come into direct contact with epithelial cells, one may thus expect an enhancement of TCR-mediated destruction of epithelial target cells through NKG2D/MIC interaction in CD patients. Indeed, we show that engagement of NKG2D in T IEL lines from active CD patients strengthens TCR-mediated, redirected killing of target cells and allows efficient cytotoxicity even at limited antigen concentration. Turning our attention to RCS, a cryptic intestinal T cell lymphoma characterized by the presence of an aberrant $sCD3^-$ clonal intraepithelial T cell population, we then demonstrate that MIC/NKG2D interaction can directly induce killing of epithelial target cells by IELs, independently of any signaling via the TCR. Therefore, as the disease becomes resistant to the gluten-free diet, the contribution of NKG2D in mediating gut epithelium destruction by IELs shifts from a conventional costimulatory function in classical CD to an NK-cell receptor-like activating signal in RCS. However, the participation of other triggering receptors on IELs remains to be unraveled. First, NKG2D blockade of spontaneous cytotoxicity of $sCD3^-$ RCS cell lines against epithelial cells was only partial in three-fourths of the tested patients. Second, we observed that in extreme conditions, such as elevated levels of IL-15 and high density of effector cells, IELs from active CD patients could mediate direct epithelial cell lysis in an antigen-independent manner, a phenomenon previously described as an IL-15-induced bystander activation of memory phenotype CD8 T cells (Liu et al., 2002). This cytotoxic activity was only partly inhibited by anti-NKG2D mAb. Engagement of NKG2D, alone or in combination with other activating receptors on IELs, could therefore account for a significant part of IEL cytotoxicity against epithelial cells, depending on the complexity of the array of ligands available on target cells, as recently described for tumor-specific CD8 T cell clones (Maccalli et al., 2003).

MIC molecules, like a wide variety of transmembrane proteins, can be shed as soluble forms in the serum of patients (Groh et al., 2002; Salih et al., 2002) after proteolytic cleavage of the membrane-anchored molecules by an as yet unidentified metalloproteinase. Interestingly, levels of metalloproteinases MMP-1 and MMP-3 are increased in the mucosa of patients with active CD as a consequence of lamina propria mononuclear cell activation (Daum et al., 1999). Thus, an increased production of metalloproteinases in untreated CD patients might favor the release of sMIC, as we observed in a large percentage of our patients. However, the levels of sMICA in the serum were not correlated to the severity of histological lesions or clinical symptoms, indicating that sMICA cannot be used so far as a marker of activity or of tolerance to gluten-containing diet. The functional consequence of the shedding of MICA remains unclear. sMICA may play a role in immune escape of human tumors in vivo by inducing NKG2D downregulation, thereby decreasing the functional capacity of antitumor effector T cells or NK cells (Groh et al., 2002). We show that NKG2D levels are comparable on IELs from CD or RCS patients with high levels of sMICA and on IELs from controls or treated patients without sMICA. Since NKG2D levels on IELs are upregulated by IL-15 (Roberts et al., 2001), which is overexpressed during CD, it is conceivable that normal levels of NKG2D are maintained on IELs even in presence of sMIC. Although we did not detect soluble IL-15 in sMICA-containing sera of patients, it is known that IL-15 is massively exposed at the surface of enterocytes during CD (Mention et al., 2003). Furthermore, surface bound IL-15 can be presented in *trans* to neighboring cells (Dubois et al., 2002), and is likely to prevent ligand-induced internalization of NKG2D. However, the inability of recombinant MICA to downmodulate NKG2D on IELs from CD patients suggests that other mechanisms tending to maintain NKG2D levels on these cells are likely to be involved.

There is considerable genetic variation in the MICA gene, with several polymorphisms reported both in the transmembrane and extracellular domains (Fodil et al., 1999). Allelic variants of MICA substantially differ in their binding affinity for NKG2D (Steinle et al., 2001), which could have significant effects in the modulation of T cell responses. In addition, the MICA gene exhibits a triplet repeat microsatellite polymorphism in the transmembrane region, which might be associated with variable secretion of soluble MICA or with aberrant MICA localization at the surface of epithelial cells. We show, however, that sMICA is detected in patients' sera regardless of allelic MICA differences and that MICA is similarly distributed in gut epithelium of CD patients whatever MICA alleles expressed.

The mechanisms leading to upregulation of MIC and its translocation to the surface of epithelial cells in CD remain to be elucidated. As indicated by intracellular staining of permeabilized cells, a rapid appearance of MICA at the cell surface may be due, at least in part, to redistribution of intracellular protein. In addition, we show that gliadin rapidly induces MICA expression in gut epithelium and lamina propria of organ cultures from treated CD patients. MICA induction appears as a consequence of the inflammatory response induced by gliadin in the intestine of sensitive individuals rather than as a direct effect of gliadin per se, and one important relay in the induction of MICA is IL-15. Thus, the MICA-inducing effect of gliadin was reproduced by the p31-49 gliadin-derived peptide. This nonimmunodominant peptide was recently shown to induce IL-15 in innate immune cells in the lamina propria of CD patients and

to induce enterocyte apoptosis, the latter effect being inhibited by neutralizing IL-15 (Maiuri et al., 2003). Consistent with this observation, we observed that MICA induction by p31-49 was inhibited by neutralizing IL-15 during organ culture. Furthermore, IL-15 induced accumulation of intracellular MICA in epithelial cell lines. Together with the recent finding that IL-15 induces MICA in human dendritic cells (Jinushi et al., 2003), these data support the hypothesis of a direct inducing effect on IL-15 on MICA expression. This effect of IL-15 might explain the intense expression of MICA on the epithelium of RCS patients who have no more gluten in their diet but retain massively increased levels of IL-15 in their epithelium (Mention et al., 2003). Another gliadin-derived peptide, p57-89, which is a very efficient stimulus of the CD4-mediated adaptive immune response (Shan et al., 2002), had a much more modest effect on MICA induction at the 12 hr time point chosen, and this effect was not sensitive to IL-15 blockade. A delayed effect of this peptide via other proinflammatory relay is thus not excluded. Therefore, the p31-49 peptide could represent a danger signal in CD patients, through enhanced expression of stress proteins, such as MIC. A noticeable increased expression of hsp65 has been observed in jejunal epithelial cells of CD patients (Iltanen et al., 1999), and the heat-shock protein hsp110, produced by the intestinal epithelium, induces the expression of another nonclassical MHC class I molecule, CD1d, on intestinal epithelial cells (Colgan et al., 2003).

In conclusion, we demonstrate that in CD, gliadin, via a pathway involving IL-15, induces MICA expression at the surface of gut epithelial cells, thereby providing an epithelial target to IELs whose cytotoxic properties are simultaneously turned on by enterocyte-derived IL-15. MICA induction will trigger both direct activation and costimulation, permitting an early innate-like response and a gliadin-specific CD8 T cell-mediated adaptive response. In a broader context, interplay between NKG2D/MIC and IL-15 might be the paradigm of a normal defense mechanism allowing the immediate recruitment and activation of IELs in response to intestinal aggressions by pathogens. In CD, the control of this primordial response could help protect mucosa from the damaging effects of gliadin.

Experimental Procedures

Patients

CD was diagnosed in childhood according to EPSGAN criteria (McNeish et al., 1979) and in adulthood on the basis of villous atrophy, antiendomysium antibodies, and HLA-DQ2 or -DQ8 phenotype. Patients with RCS failed to improve clinically and histologically despite strict gluten-free diets for at least 6 months (Cellier et al., 2000). The study was performed in accordance with the Declaration of Helsinki and received approval of the local Ethical Committee.

Cells

HeLa (carcinoma of cervix), HT-29, CaCO2, T84 (colonic or colorectal adenocarcinoma), and P815 (mouse mastocytoma) cell lines (ATCC, Rockville, MD) were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), glutamine, and antibiotics.

Generation of Soluble MICA and MICB Molecules and Fluorescent Tetramers

Recombinant MICA and MICB were produced as secreted proteins in Sf9 insect cells as previously described (Hue et al., 2003). Tetramers were generated by mixing MICA or MICB monomers with fluorochrome-labeled streptavidin in a 4:1 ratio (streptavidin-PE or streptavidin-FITC, Pharmingen, San Jose, CA).

Production of Anti-MICA Monoclonal Antibodies

Anti-MICA monoclonal antibodies were obtained by immunizing mice with purified recombinant MICA*008. SR99 (IgG1), SR104 (IgG1), and SR116 (IgG2a) mAbs were selected on the basis of both reactivity against recombinant MICA by ELISA and staining of HT-29 and HeLa cells by flow cytometry (Hue et al., 2003).

Immunohistochemical Analysis

Deparaffinized sections of duodenal biopsies were treated with 40% normal human serum for 20 min at room temperature and incubated with the monomorphic anti-MICA SR99 mAb for 1 hr or isotype-matched control Ig. Antibody binding was visualized by using biotinylated goat antimouse Ig and the peroxidase EnVision System (Dako, Carpinteria, CA).

In Vitro Organ Cultures

For in vitro challenge, 8–16 fresh biopsy specimens were obtained for each of nine treated CD patients and five healthy controls, placed on iron grids, and cultured for 4, 12, 24, or 48 hr in an organ culture chamber at 37°C as described (Maiuri et al., 1998) in the presence of medium alone, with peptic-tryptic digest of gliadin or bovine serum albumin (500 μg/ml), or with peptides (200 μg/ml) before being fixed in 10% formalin. IL-15 (50 ng/ml) was also tested in four patients and three controls. In selected samples incubated in the presence of peptides, we additionally analyzed the effect of anti-IL-15 neutralizing monoclonal antibody (10 μg/ml, R&D Systems) or isotype-control antibody.

The gliadin peptides p57-89 (LQLQPFPQPQLPYPQPQLPYPQP QLPYPQPQPF) (Shan et al., 2002) and p31-49 (LGQQQPFPPQQPY PQPQPF) (Maiuri et al., 2003; Matysiak-Budnik et al., 2003) and the control human serum albumin hSA peptide (p64-76, VKLVNEVTEF AKT) were synthesized by Epytop (Nîmes, France) and were >95% pure.

All protein digests and peptides were tested for the absence of detectable levels of LPS by the Limulus Amebocyte Lysate assay (Charles River Laboratories, Charleston, SC). All reagents were found to contain <0.1 endotoxin U/ml except the control BSA digest, which contained approximately 5 U/ml.

Isolation of Cells and Flow Cytometry Analysis

PBMC from healthy controls were purified by ficoll density centrifugation. For isolation of IEL and epithelial cells, six to eight fresh biopsy specimens were pooled and incubated under constant shaking in RPMI 1640 (Gibco BRL, Rockville, MD) containing 1% FCS, 1.5 mM MgCl2, and 1 mM ethylen glycol-bis (b-aminoethyl-ether)-N,N,N′,N′-tetraacetic acid for 30 min at 37°C. The supernatant containing the IEL and contaminating epithelial cells was passed through a nylon filter, and cells were washed twice in PBS supplemented with 5% human AB serum. Intestinal epithelial cells were identified by the expression of epithelial surface antigen (FITC-ESA, Biomeda, Foster City, CA).

For cell-surface staining of MICA, epithelial cells were incubated with 10 μg/ml SR104 mAb or isotype-control antibody at 4°C for 30 min, washed, and then stained with phycoerythrin-labeled goat antimouse IgG (Caltag, Burlingame, CA). Free FcR were blocked with a 50 μg/ml dilution of mouse Ig before adding FITC-ESA mAb. In selected experiments, cells were permeabilized by 2% saponin before staining.

For cell-surface staining of NKG2D, 1–2 × 10^6 PBMC or 10^5 IEL were incubated at 22°C for 90 min with MICA tetramers (at 10 μg/ml of MICA protein), then for 20 min at 4°C with antibodies to CD3, CD8 (BD Pharmingen), αβTCR, γδTCR, or CD103 (Beckman Coulter, Miami, FL). Cells were fixed in PBS with 1% paraformaldehyde and immediately analyzed on a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, Palo Alto, CA). Data were analyzed with Cell Quest software (Becton Dickinson).

Cytotoxicity Assays

To derive cell lines from IELs, intestinal biopsy specimens from five patients with active CD and four patients with RCS were cultured in 24-well plates (1 biopsy/well) in lymphocyte culture medium containing IL-15 (20 ng/mL, R&D Systems) as described (Mention et al., 2003). Wells were fed every 3 days with IL-15-containing medium. On confluence, cells were transferred into 6-well plates and diluted at 10^6 mL every 3 days in the same medium and their phenotype monitored by flow cytometry before assessing cytotoxicity.

For redirected lysis assay, cells were washed and incubated for 4 hr with ^{51}Cr-labeled FcγR+ P815 target cells at various E:T ratios in the presence of the M585 anti-NKG2D mAb (1 μg/ml, kindly provided by Immunex/Amgen) or mouse IgG1 control and/or anti-CD3 (OKT3) mAb (0.01–100 ng/ml).

For direct cell-mediated cytotoxicity assay, cells were incubated for 4 hr with ^{51}Cr-labeled HeLa or HT29 cells at various E:T ratios in the presence or absence of recombinant soluble MICA protein (10 μg/ml), anti-MICA mAb (10 μg/ml), anti-NKG2D M585 mAb (1 μg/ml), or isotype control mAb.

PI3K inhibition experiments were done by pretreating effector cells with the PI3K inhibitor LY 294002 (1 μM, Sigma). The mean of duplicates of lysis for each cell line was expressed as a percentage of specific release.

ELISA of Soluble MICA and Modulation of NKG2D on IEL Lines

Soluble MICA was measured in the serum with a sandwich ELISA as described (Hue et al., 2003). Recombinant soluble MICA*001 was consistently detected at concentration of 0.3 ng/ml. The amount of IL-15 in patients' sera was determined by commercial ELISA with matched antibody pair in relation to standard pair (R&D Systems).

Modulation of NKG2D levels on IEL cell lines from active CD or RCS patients was examined by flow cytometry after 24 hr of incubation with soluble recombinant MICA (10 μg/ml) in the absence or presence of IL-15 (20 ng/ml).

MICA Genotyping

A total of 107 CD patients and 90 healthy controls, previously described (Djilali-Saiah et al., 1998), was MICA genotyped. The trinucleotide repeat microsatellite polymorphism in the transmembrane region of the MICA gene was amplified with PCR primers flanking the TM region as described (Mizuki et al., 1997; Sugimura et al., 2001). The PCR products were electrophoresed in an automated DNA sequencer (Applied BioSystems, Foster City, CA), and the number of microsatellite repeat was estimated automatically using the Genescan 672 software. Allelic frequencies were calculated by direct counting, and the significance of the association was determined by using the chi-square test. The level of significance was set at 0.05, and the correction of Bonferonni for multiple tests was applied by multiplying p by the number of alleles compared (Pc).

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