Published in final edited form as:

Biochem Biophys Res Commun. 2009 September 25; 387(3): 476–481. doi:10.1016/j.bbrc.2009.07.062.

An Six-Amino Acid Motif in the $\alpha 3$ domain of MICA Is the Cancer Therapeutic Target to Inhibit Shedding

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Abstract

Expression of the MHC class I chain-related molecules A and B (MICA/B) on tumor cell surface can signal the immune receptor NKG2D for tumor immune destruction. However, MIC was found to be shed by tumors in cancer patients, which negatively regulates host immunity and promotes tumor immune evasion and progression. The mechanisms by which tumors shed MIC are not well understood although diverse groups of enzymes are suggested to be involved. The functional complexity of these enzymes makes them unfeasible therapeutic targets for inhibiting MIC shedding. Here we identified an six-amino acid (6-aa) motif in the $\alpha 3$ domain of MIC that is critical for the interaction of MIC with ERp5 to enable shedding. Mutations in this motif prevented MIC shedding but did not interfere with NKG2D-mediated recognition of MIC. Our study suggests that the 6-aa motif is a feasible target to inhibit MIC shedding for cancer therapy.

Keywords

MHC class I chain related molecule (MIC); tumor shedding; protein disulphide isomerase ERp5; NKG2D; tumor immunity; cancer therapeutic target

Introduction

The MHC class I chain related molecules A and B (MICA/B) are generally absent in normal human tissues and expressed in most transformed human tumors, including but not limited to breast, lung, ovary, prostate, kidney, and colon carcinomas [1–3]. MIC was identified as a ligand of NKG2D, a C-type lectin-like stimulatory immune receptor that is expressed by all human nature killer (NK) cells, CD8 T cells, and a subset of gamma/delta T cells [4–6]. *In vitro* experiments have shown that engagement of NKG2D by MIC expressed on the tumor cell surface triggers NK cell anti-tumor cytotoxic responses [4]. This engagement also co-stimulates antigen-specific cytotoxic T lymphocyte (CTL) mediated anti-tumor immunity and

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is necessary for activation of V δ 1 γ δ T cells [6,7]. Thus, MIC is proposed as a specific target on tumor cells to mark nascent tumors for NKG2D-mediated immune destruction [8,9].

Ligand-induced activation of NKG2D on NK cells and CTLs has been demonstrated to be very effective in tumor destruction in experimental animal models [10,11]. In humans, NKG2D-mediated immune destruction of tumors is subverted. Mounting evidence suggests that tumor shedding of MIC is at least one of the mechanisms by which human tumors evade NKG2D-mediated immune destruction and progress [12–17]. High levels of soluble MIC (sMIC) molecules in sera correlate strongly with poor clinical outcomes in patients with various types of cancer, including colon [13], prostate [14], pancreatic carcinomas [16], and multiple myeloma [15]. sMIC(A) induces host immune suppression by down regulation of surface NKG2D expression on NK cells and CTLs [12–17] and indirectly propagating the expansion of tumor infiltrated immune suppressive CD4⁺NKG2D⁺ T cells [18]. *In vivo* studies have shown that preventing MIC shedding inhibits tumor initiation in xenograft animal models [19].

Because of the apparent suppressive effects of MIC shedding on host immunity, inhibition of tumor shedding of MIC may have therapeutic potentials. The mechanisms that regulate tumor shedding of MIC are not fully understood, although studies have suggested that it is a proteolytic process [20] and that multiple enzymes are involved [21–23]. The thiol isomerase ERp5 has been shown to be required to initiate MICA, and presumably MICB, shedding by reducing the disulphide bond of MIC to allow access of proteolytic enzymes [21]. Metalloproteinase family members ADAM-10 and ADAM-17 have been indicated to be involved in MICA shedding [22] and ADAM-17 has also been suggested to be involved in MICB shedding [23]. Both ERp5 and ADAMS have been proposed as targets for therapeutic intervention to inhibit MIC shedding. However, given the knowledge that these enzymes are also involved in many aspects of normal cellular and physiological function [24,25], application of specific inhibitors to these enzymes to inhibit MIC shedding may not be clinically feasible.

We hypothesize that the most feasible strategy to inhibit MIC shedding for therapy would be to block the interaction of ERp5 with MIC. In this report, we identified an six-amino acid (6-aa) motif in the α 3 domain of MICA that is critical for interaction of MICA with ERp5. Mutations of this motif inhibited MICA to interact with ERp5 and prevented MICA shedding, but did not interfere with the recognition of MICA by NKG2D. Our studies suggest that the 6-aa motif is a feasible target to inhibit MIC shedding for cancer therapy.

Materials and methods

Cell lines

The mouse prostate tumor cell line TRAMP-C2 was grown in DMEM medium supplemented with 10% FCS, 5 μ g/ml insulin, 5 μ g/ml transferrin, and 5 ng/ml selenium (ITS). The human B cell lymphoma cell line C1R was cultured in RPMI-1460 supplemented with 10% FCS. The Eco-Phoenix and Ampho-Phoenix retrovirus package cell lines were cultured in DMEM medium supplemented with 10% FCS and 25 mM sodium pyruvate. NK-92 cells were maintained in MEM- α media supplemented with 12.5% FCS, 12.5% horse serum, and 1000 U/ml of IL-2.

Overexpressing human MICA and MICA mutants in TRAMP-C2 and C1R cells

The cDNA of wild-type MICA*01 (wtMICA) was kindly provided by Dr. A. Steinle (University of Tübingen, Tübingen, Germany). cDNAs of MICA mutants were generated by recombinant PCR techniques as described [19]. cDNAs were subcloned into the retroviral

vector pBMN-GFP (Orbigen Inc). To overexpress the wtMICA and MICA mutants in TRAMP-C2 and C1R cells, the Eco-Phoenix and Amphi-Phoenix package cell lines were transfected with the recombinant plasmids respectively. The recombinant retrovirus from the respective package cells were used to infect TRAMP-C2 and C1R cells.

Flow cytometry

For detection of cell surface expression of wtMICA or MICA mutants, single cell suspension was incubated with the mAb 6D4.6 (Biolegend) followed by a PE-conjugated secondary reagent. Cells were analyzed with a BD FACScan. Data were analyzed with the CellQuest software (BD Bioscience).

Immunoprecipitation and Western-blotting

Cells were lysed in Baserga lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100) with Complete Protease Inhibitors (Roche Applied Science). Cell culture supernatant or clear cell lysate was incubated with the mAb 6D4.6 and ultralink immobilized protein A/G plus (Pierce). Immune complexes were treated with PNGase F to remove oligosaccharide chains, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. The membrane was blotted with the rabbit anti-human MICA polyclonal Ab H-300 (Santa Cruz Biotechnology) and donkey anti-rabbit-IgG-HRP (Santa Cruz Biotechnology). Proteins were detected with ECL reagents (GE Healthcare).

Co-immunoprecipitation

To assess the interaction of MICA mutants with ERp5, cells were surface biotinylated with EZ-Link Sulfo-NHS-SS-Biotin (Pierce) and fixed with 10% (w/v) TCA (trichloro acetic acid) for 30 min before lysed in 1% NP-40 lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 5mM EDTA, 40 mM N-ethylmaleimide and Complete Protease Inhibitors (Roche Applied Science). Clear cell lysates were neutralized to pH 7.0 with 1 M Tris buffer (pH 9.5) and incubated with the anti-MIC mAb 6D4.6 and ultralink immobilized protein A/G plus (Pierce). Immune complexes were resolved in SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blotted with HRP-Streptavidin (KPL), or the rabbit anti-human MICA polyclonal Ab H-300 (Santa Cruz Biotechnology), or the rabbit anti-ERp5 (also called P5) antibody ab11432 (Abcam). In order to distinguish ERp5 and MICA by molecular mass, MICA was not deglycosylated with PNGase F in this assay.

MICA shedding assay

Cells were seeded at the density of 4×10^5 cells / well in a 6-well plate in complete media overnight and replaced with 1 ml / well serum-free media for 24 h. Supernatant was collected and filtered through an 0.45 μ m membrane. Cells were lysed with 1 ml Baserga lysis buffer. The amount of soluble MICA in the supernatant and the amount of MICA in the cell lysates were measured using human MICA DuoSet sandwich ELISA kit (R&D Systems). The degree of shedding is expressed as the ratio of sMICA in the culture supernatant to MICA in cell lysates.

Cytotoxicity assay

NK-92 cells were used as effector cells. Cytotoxicity assay was performed in triplicates using the standard 4 h ⁵¹Cr release assay as previously described [19]. For blocking the activity of NKG2D receptor, NK cell was incubated with 30 ng/ml of rsMICA (GenWay Biotechnology Inc.) for 1 h prior to being used for cytotoxicity assay.

Purification of tumor shedding-derived sMICA from cell culture supernatant

The mAb 6D4.6 was conjugated to the NHS-activated Sepharose Fast Flow (GE Health care) by following the manufacture's instructions. Cell culture supernatant containing sMICA was filtered through 0.2 μ m membrane before loaded onto the antibody conjugated Sepharose Fast Flow column. The column was washed with 50 mM NaOAc (pH 4.5) buffer and sMICA was eluted by 0.1 M citrate buffer (pH 2.5). The elution was neutralized with 1.5 M Tris (pH 8.3) immediately and concentrated by the Biomax PES-5 column (Centricon Plus-20, Millipore).

Tandem mass-spectrometry analysis

Purified sMICA was treated with PNGase F before loading on a 10% SDS-PAGE. After Commassie Blue staining, the sMICA bands were excised, washed with 50 mM NH_4HCO_3 , dehydrated in acetonitrile (ACN), and vacuum dried. The protein bands were incubated with 6.25 ng/ μ L of trypsin overnight at room temperature. The peptide digests were analyzed by electrospray ionization in the positive ion mode using the LTQ-Orbitrap (Thermo Fisher). Database search was performed with Phenyx software (GeneBio SA, Geneva, Switzerland) against the human International Protein Index (IPI) database.

Statistical Analysis

Data were analyzed using JMP software. Significance between two comparison groups was determined by student's *t*-test. $P < 0.05$ was considered significant.

Results and discussion

Mutation of an 6-aa motif in the $\alpha 3$ domain of MIC prevents shedding

Our previous study has suggested that partial mutation of the $\alpha 3$ domain (aa 215 to 274) of MIC prevented MIC shedding [19] (Fig. 1a). To further define residues or peptide motif(s) that may be critical for shedding of MICA as potential therapeutic targets, we constructed a panel of MICA mutants within this region by replacing interested residues or motifs with corresponding sequences of HLA-A2 (Fig. 1b). These mutants were stably expressed in the MIC-negative mouse prostate tumor TRAMP-C2 cell lines using a retroviral system. All these mutants were expressed on the cell surface as shown by flow cytometry analyses (Fig. 1c). To assess the shedding nature of these MICA mutants, cell culture supernatant and cell lysates were collected and immune precipitated with the anti-MIC mAb 6D4.6. Immune complexes were analyzed by Western blotting with the anti-MIC polyclonal antibody H-300.

We first constructed three mutants that cover different regions of aa 218–274: M1 (residues Q²¹⁸ to D²³⁶), M2 (residues C²⁵⁰ to F²⁵⁷), and M3 (residues N²³⁸ to R²⁴⁸). Only mutant M3 was shown to be shedding-resistant, specifically, no sMICA was seen in the culture supernatant while full-length MIC was detected in the cell lysates (Fig. 2a). This suggests that residues in the region covered by mutant M3 (N²³⁸ to R²⁴⁸) are critical for shedding. We further constructed several mutants covering variable lengths of the region N²³⁸ to R²⁴⁸ (M4 to M6, Fig. 1b). Mutant M4 (N²³⁸ to V²⁴⁵) and M5 (N²³⁸ to T²⁴³) were shedding-resistant whereas mutant M6 (N²³⁸ to Q²⁴²) was not, suggesting that the 6-aa motif covered by M5 (N²³⁸ to T²⁴³) is critical for MICA shedding. Mutants M5 and M6 differ in one amino acid T²⁴³ which was shown not critical for MICA shedding as the mutation of T²⁴³ to Ala (M7) did not prevent MICA shedding. Together, these results suggest that the 6-aa motif (N²³⁸ to T²⁴³) is critical for MICA shedding, possibly by maintaining a biological conformation of MICA. We also observed the same results with similar mutations in the MICB molecule (Supplement material S1).

We evaluated shedding of MICA using an ELISA. In a given number of cells, the amount of sMICA released to the culture supernatant and MICA in the cell lysates was measured. The

degree of shedding was indicated as ratio of sMICA in the culture supernatant to MICA in the cell lysates. Consistent with western-blot analyses, no shedding was seen in mutants M3, M4, and M5 (Fig. 2b). Together, we identified an 6-aa shedding motif in the $\alpha 3$ domain of MIC that is critical for shedding.

The 6-aa shedding motif is not host-specific and is conserved among all MIC alleles

We investigated whether the MICA mutants M4 and M5 are also shedding-resistant in human tumor cell lines. wtMICA and mutants M4, M5, and M6 were stably expressed in C1R cell lines using retroviral expression vector. Culture supernatant was collected for immunoprecipitation and Western-blot analyses. The degree of shedding was also assayed by ELISA as described above. As shown in Figs 2a and 2b, no shedding was detected with mutant M4 or M5 whereas wtMICA and mutant M6 was shed by C1R cells. These results suggest that our identified 6-aa shedding motif is not host-specific.

MIC is highly polymorphic. There are now 51 recognized human MICA alleles and at least 22 MICB alleles [26]. Multiple alignment comparison showed that the sequence of 6-aa shedding-motif, residues N²³⁸ to T²⁴³, is identical among all recognized MICA and MICB alleles (Supplement material S2 and S3). These results suggest that the 6-aa shedding motif of MIC is evolutionarily selected to be conserved for a specific function.

The 6-aa shedding motif does not contain MICA cleavage site(s)

To address whether the 6-aa shedding motif contains potential MICA shedding site(s), we performed in-gel trypsin digestion and tandem mass spectrometric analyses of sMICA purified from the culture supernatant of TRAMP-C2-MICA cells. In agree with similar analyses of MICA by other studies [22], in three independent experiments, staggered non-tryptic C-terminus of sMICA were identified in the near transmembrane region (Fig 3a); no non-tryptic C-termini was identified within the 6-aa shedding motif. These results suggest that the 6-aa shedding motif does not contain potential proteolytic MICA cleavage site.

The 6-aa shedding motif is critical for MICA to interact with ERp5

We further pursued the mechanisms by which the 6-aa shedding motif is critical for MIC shedding. Since the 6-aa motif does not contain MIC cleavage site(s), it may play a regulatory role in MIC shedding. Studies have shown that the protein disulphide isomerase ERp5 is required for enabling MICA shedding through disulphide-bond interaction with the $\alpha 3$ domain of MICA, and presumably with MICB as well [21]. Since our identified 6-aa shedding motif is located between the two Cysteine (C) residues that form the disulphide bond in the $\alpha 3$ domain of MIC (Fig. 1a), we thus hypothesize that the 6-aa motif is critical for the physical interaction of MIC with ERp5. To test this hypothesis, we performed co-immunoprecipitation of MICA with the anti-MIC mAb 6D4.6 from the cell lysates of TRAMP-C2 cells overexpressing wtMICA and the shedding-resistant MICA-M5. To detect cell surface proteins interacting with MICA, cells were surface biotinylated before being lysed for co-immunoprecipitation. The immunocomplexes were resolved in SDS-PAGE and blotted with HRP-streptavidin, the anti-MICA polyclonal antibody H-300, or the anti-ERp5 polyclonal antibody ab11432. As shown in Figs. 3b–3d, wtMICA forms a complex with ERp5; on the contrary, the shedding-resistant MICA mutant M5 does not form a complex with ERp5. These results suggest that the 6-aa shedding motif covered by the mutant M5 is critical for MICA, and presumably MICB, to interact with ERp5 to enable shedding.

Mutation of the 6-aa motif does not affect the recognition of MICA by NKG2D

Since NKG2D only interacts with the $\alpha 1\alpha 2$ ectodomain of MICA [27], mutations in the 6-aa shedding motif (M5) are not likely to impair the recognition of MICA by NKG2D. To confirm

this, we performed standard 4 h NK cell cytotoxicity assays. Pure populations of TRAMP-C2 and C1R cells expressing comparable levels of wtMICA and the MICA mutant M5 were isolated by repeated flow cytometry sorting (Fig 4a) and used as target cells for NK-92 cells. Both wtMICA and MICA-M5-expressing TRAMP-C2 and C1R cells are sensitive to the cytotoxicity of NK-92 cells and the sensitivity was inhibited by pre-incubating NK-92 cells with 30 ng/ml of rsMICA. This confirms that disruption of the 6-aa shedding motif does not impair NKG2D-mediated recognition of MICA by NK cells. These results suggest that potential antibodies or small molecules binding to the 6-aa shedding motif to block the interaction of MICA with ERp5 would not interfere with the sensitivity of MICA-expressing cells to NK cells.

Of note, although wtMICA was shed by TRAMP-C2 and C1R cells, there was no significant difference ($p = 0.12$ at LD_{50}) in sensitivity to NK cells between cells expressing wtMICA and mutant M5 in the 4 h *in vitro* cytotoxicity assay. This observation is consistent with our previous report [19], showing that in the 4 h *in vitro* cytotoxicity assay, the killing ability of NK cells was not significantly affected by soluble MIC resulting from target cells shedding. Indeed, in a dynamic shedding study, we show that there is little accumulation of sMICA in the culture supernatant within 6 h of culture (Supplement material S4).

In conclusion, although MIC was cleaved at multiple sites and potentially by multiple enzymes, we have identified an 6-aa motif that can be an effective target to block the interaction of MIC with ERp5 and thus to inhibit MIC shedding. The fact that the 6-aa motif is conserved among all recognized MIC alleles and that MIC is generally absent in normal tissues makes it feasible to target the 6-aa motif for therapeutic intervention. Our current study has laid an essential ground work for designing small molecules or antibodies to inhibit MIC shedding for cancer therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by DOD-USMRC New Investigators Award W81XWH-04-1-0577, DOD-USMRC IDEA Development Award W81XWH-06-1-0014, NW Prostate SPOR Program, and NIH Temin Award 1K01CA116002.

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a.

151 MKTKTHYHAMHADCLQELRRYLKSGVLLRRTVPPMVNVTRSEASEGNITV
 201 TCRASGFYPWNITLSWRQDGVSLSHDTQQWGDVLPDGN^{*}NGTYQTWVATRIC
 251 QGEEQRF^{*}TCYMEHSGNHSTHPVPSGKVLVLQSHWQTFHVSTMAVAAAAIFVI

α3

b.

Sequence	designation	shedding
211 EDQTQDTELV-ETRPAGDGT ^{FQK} WAAVVVPSGQE ^{QRY}	HLA-A2	
211 VLSHDTQQWGDVLPDGN ^{NGTYQTWVATRIC} QGEEQRF	wtMICA	+
EDOTQDTELV-ETRPAG ^{NGTYQTWVATRIC} QGEEQRF	MICA-M1	+
VLSHDTQQWGDVLPDGN ^{NGTYQTWVATR} VPSGQE ^{QRY}	MICA-M2	+
VLSHDTQQWGDVLPDGD ^{DGT} FQK ^{WAAVV} ICQGEEQRF	MICA-M3	-
VLSHDTQQWGDVLPDGD ^{DGT} FQK ^{WAA} ATRICQGEEQRF	MICA-M4	-
VLSHDTQQWGDVLPDGD ^{DGT} FQK ^W VATRICQGEEQRF	MICA-M5	-
VLSHDTQQWGDVLPDGD ^{DGT} FQ ^Q TWVATRICQGEEQRF	MICA-M6	+
VLSHDTQQWGDVLPDGN ^{NGTYQ} AVVATRICQGEEQRF	MICA-A7	+

c. **Fig. 1.**

a. Partial of the MICA sequence in the α3 domain. Residues in gray area were selected for mutation analyses. Arrows indicate beginning and end of the α3 domain. Residues in the boxed region were identified to be critical for MICA shedding. Asterisks indicate the two Cysteine residues that form a disulphide bond. **b.** Sequence and designation of MICA mutants. The underlined sequences were mutated. The shedding assay results (from Fig. 2) are also indicated. +, shedding. -, no shedding. wtMICA, wild type MICA. **c.** Flow cytometry histograms showing surface expression of MICA mutants in TRAMP-C2 cells (filled profiles). Open profiles, negative control TRAMP-C2 cells stained with the mAb 6D4.6. Note that these were unsorted cells and that the histograms showed heterogeneity in the expression levels of the mutants.

a. **b.** **Fig. 2.**

Mutation of an 6-aa motif prevents MICA shedding. **a.** Western blots showing the shedding resulting sMICA in the culture supernatant (SN) and full-length MICA in the cell lysates (LY). PNGase F was used for deglycosylation. **b.** ELISA quantitatively assayed the degree of shedding of MICA mutants in TRAMP-C2 and C1R cells. (LY). Results represent three independent experiments. *, $P < 0.001$ compared to wtMICA

a. **b.** **Fig. 4.**

Mutation of the 6-aa motif does not interfere with recognition of MICA by NKG2D. **a.** Flow cytometry showing surface wtMICA and MICA mutant M5 expression in isolated pure populations of TRAMP-C2 (TC2) and C1R cells (open profiles). Filled profiles, negative control TC2 or C1R cells stained with the anti-MIC mAb 6D4.6. **b.** Cytotoxicity assay showing susceptibility of cells expressing shedding-resistant MICA to NK cell killing. E : T, effector : Target. Data also show that specific lysis was blocked by pre-incubating effector NK-92 cells with 30 ng/ml of rsMICA. Data represents three independent experiments.