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Expression and shedding of MHC class I-related chain (MIC) A and B molecules in human carcinoma cell lines

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A Dissertation

By

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1. Abbreviations

3'UTR Three prime untranslated region

ADCC Antibody-dependent cell-mediated cytotoxicity

ADAM A disintegrin and metalloproteinases

ADAMTS A disintegrin and metalloproteinase with thrombospondin motifs

APP Amyloid precursor protein

ATM/ATR Ataxia-telangiectasia mutated and ATM- and Rad3-related kinases

BACE Beta-secretase 1

CHO Chinese hamster ovary

CIK Cytokine induced killers

CMV Cytomegalovirus

DAP10 DNAX-activating protein

DLL1 Delta-like protein 1

ELISA Enzyme-linked immunosorbent assay

ERK Extracellular-signal-regulated kinases

FC Flow cytometry

Grb2 Growth factor receptor-bound protein 2

HDAC Histone deacetylase

HCMV Human Cytomegalovirus

IE1/IE2 Immediate early gene products 1 and 2

IC Intracellular

INF Interferon

INF-γ Interferon gamma

IP Immunoprecipitation

MCA Methylcholanthrene

MEK Mitogen-activated protein kinase kinase

MICA MHC class I chain-related molecule A

MICB MHC class I chain-related molecule B

MMP Matrix metalloproteinase

MULT1 Murine ULBP-Like transcript 1

NCR Natural cytotoxic receptor

NF-Y Nuclear transcription factor Y

NKG2D Natural killer group 2D

PI3K Phosphatidylinositol 3-kinases

PMA Phorbol 12-myristate 13-acetate

RAET Retinoic acid early transcript protein

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SiRNA Small interfering RNA

Sp1, 3 Specificity Protein 1 and 3

SVMPs Snake venom metalloproteinases

TGF-α Transforming growth factor alpha

TLR Toll-like receptor

TNF-α Tumor necrosis factor alpha

TRAMP Transgenic adenocarcinoma of the mouse prostate model

tPA Tissue plasminogen activator

TSA Tumor specific antigen

ULBP UL16-binding protein

uPA Urokinase-type plasminogen activator

WB Western blotting

2. Introduction

2.1 Cancer and the immune System

Scientific evidence has proven the important role of the immune system in the prevention of malignant transformation. In the process referred to as tumor immunosurveillance the immune system can specifically recognize and destroy transformed cells based on the expression of tumor specific antigens (TSA) or stress-induced molecules. The adaptive immune system with its effector $\alpha\beta$-T cells can recognize tumor antigens in the context of major histocompability (MHC) molecules and effectively eliminate transformed cells. However, the innate immune system with its effector cells (NK, NKT, $\gamma\delta$-T cells) can recognize "stress-induced" danger signals without MHC presentation and eliminate transformed cells before the acquired, adaptive immune system starts to function (Murphy et al, 2008). Many types of cancer cells selectively downregulate MHC class I molecules on the cell surface (Khanna, 1998) and in these cases, an anti-stress response of the innate immune system might be of particular importance in tumor immunosurveillance.

2.2 NK cells

A prototypical example of leukocytes sensing different forms of cellular stress are natural killer (NK) cells. Initially, NK cells were described as large granular lymphocytes with natural cytotoxicity against tumor cells (Kiessling et al., 1975). NK cells are major effectors of the innate immune system combining both cytotoxic and cytokine-producing effector functions. They develop mainly in the bone marrow and circulate in the peripheral blood (2-18% of lymphocytes) (Cooper et al., 2001). The cytotoxic potential of NK cells is mainly based on the release of granules containing perforin and granzymes (Trinchieri, 1989). They are activated upon type I interferons (INF) as well as IL-2, IL-12, IL-15 and IL-18. Following activation, NK cells produce INF-γ and TNF-α (Vivier et al., 2008).

The main targets of NK cells are "altered self" cells that experienced various forms of cellular stress (DNA damage-related stress, malignant transformation, microbial infection) (Luci and Tomasello, 2008). The wide range of inhibitory and activating receptors expressed on NK cells enables the discrimination between potentially dangerous target cells from healthy cells. The recognition of self MHC class I molecules by inhibitory NK cell receptors (killer cell immunoglobulin like receptors (KIR) and lectin-like CD94-NKG2A heterodimers) provides a negative signal leading to tolerance, whereas the ligation of killer activating receptors such as natural killer (NK) group 2D (NKG2D) and natural cytotoxic receptors (NCR) transmits a positive signal that elicits the activation of NK cells (Vivier et al., 2008). The balance between these negative and positive signals defines the activation status of NK cells (Fig.1).

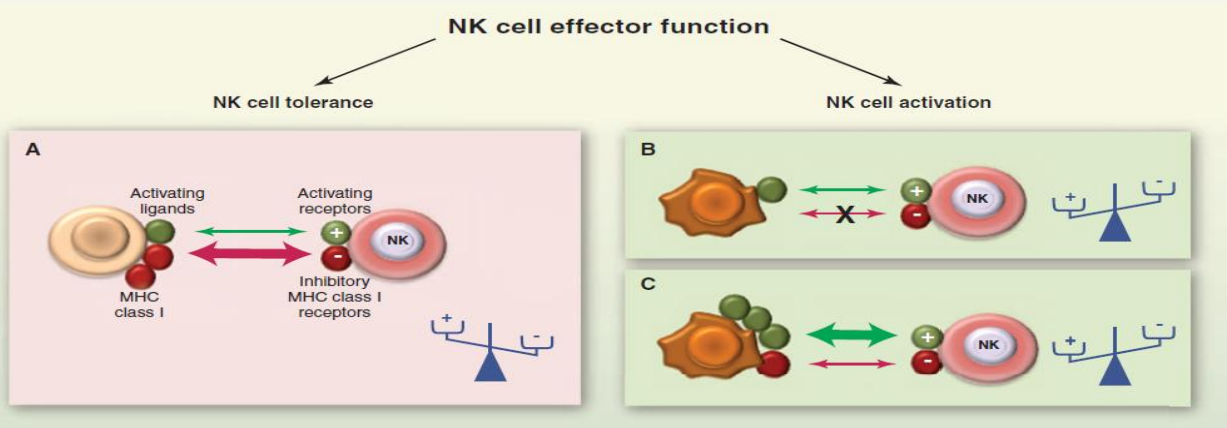

Fig.1 (A) Cells expressing self MHC class I molecules and low amounts of stress-induced molecules are tolerated by NK cells. (B) NK cells selectively target cells in "distress" that downregulated inhibitory MHC class I molecules or upregulated stress-induced self molecules on the cell surface (Vivier et al., 2011).

2.3 NKG2D receptor

NKG2D is the best studied activating receptor expressed on NK cells, NKT cells, γδ T cells, $CD8^+$ T cells and on some $CD4^+$ T cells. Depending on the cell type NKG2D serves either costimulatory or direct activatory function. In TCR-activated $CD8^+$ T cells NKG2D has a costimulatory function (Raulet, 2003) while in γδ T cells NKG2D might trigger both costimulatory or primary activatory pathways (Rincon-Orozco et al., 2005). In NK cells the ligation of NKG2D directly elicits activating signals inducing cytotoxic effector function (Raulet, 2003).

The NKG2D receptor gene is located in the NK gene complex on chromosome 12 in humans and on chromosome 6 in mice. NKG2D is a homodimeric type II transmembrane protein with a short cytoplasmic tail. For signal transduction each homodimer of NKG2D associates with 2 adaptor molecules of DNAX-activating protein of 10kD (DAP10) in human and with DAP10 or DAP12 in mice. DAP10 contains a YINM signaling motif and the phosphorylation of the YINM motif by Src family kinases leads to the recruitment of the p85 subunit of PI3-K and Grb2 to DAP10. This results in the activation of a PI3K-Vav1-Rac-PAK1-MEK-ERK signaling cascade essential for the release of cytotoxic granules and for NK cell effector functions in humans. However, the molecular details of this signal transduction pathway remain to be elucidated (Coudert and Held, 2006).

2.4 The ligands for the NKG2D receptor

A hallmark of the NKG2D receptor is the multitude of ligands identified so far (Gonzalez et al., 2008). These include MHC class I chain-related molecules A and B (MICA/B) and the UL 16-binding protein

(ULBP) family with its members ULBP1-6 in humans (Champsaur and Lanier, 2010). In the murine system no MIC molecules but 7 member of the RAET family (5 retinoic acid early transcripts 1 (RAE1) proteins, H60 and MULT1) have been identified as the ligands for the NKG2D receptor (Cerwenka et al., 2000). Since this study concerns human MICA and MICB molecules, these molecules will be described in more detail.

MIC genes are localized within the human HLA locus. Like classical MHC class I molecules (homology 28-35%) MIC proteins contain 3 extracellular Ig-like domains ($\alpha1-\alpha3$), a transmembrane domain and a short cytoplasmic tail (Fig.2). They are highly polymorphic and the different allelic variants are expressed co-dominantly (Stephens, 2001). To date 84 alleles of MICA and 35 alleles of MICB have been identified, which results in encoding 67 and 23 variants of MICA and MICB proteins, respectively (http://www.ebi.ac.uk/imgt/hla/stats.html). Different alleles of MICA and MICB have been reported to be associated with various diseases including Celiac disease, Behçet's disease, psoriasis (Gonzalez et al., 1999, Stephens, 2001, Fernandez et al., 2002), Kawasaki disease and dengue fever (Garcia et al., 2011, Collins, 2004, Huang et al., 2000). In contrast to MHC molecules, MICs do not associate with $\beta2$ microglobulin nor do they present peptides (Eleme et al., 2004).

The function of MIC proteins is to alert the immune system to various forms of cellular stress. MICA/B are upregulated on the cell surface upon different types of stress and their NKG2D-dependent recognition leads to the activation of cytotoxic lymphocytes and the subsequent elimination of dangerous cells (Waldhauer and Steinle, 2008). Thus, the NKG2D/NKG2DL system enables the sensing of potentially dangerous conditions and the subsequent activation of immune cells. In this context, the expression of these molecules has to be tightly regulated. Indeed, in healthy adults the expression of MICA and MICB is mostly restricted to the thymic epithelium and to the gastrointestinal mucosa (Eagle et al., 2009). In contrast, MICA/B are up-regulated upon certain danger conditions including heat shock, viral infection, genotoxic stress, oxidative stress, ionizing radiation, histone deacetylase (HDAC) inhibitors (Kato et al., 2007), malignant transformation and apoptosis (Stern-Ginossar and Mandelboim, 2009).

The molecular pathways leading to the upregulation of NKG2DL remain poorly understood. In general, any condition activating the major DNA damage checkpoint pathway initiated by ataxia teleangectasia mutated (ATM) and ATM and Rad3 related (ATR) protein kinases induces the transcription of NKG2D ligands (Gasser et al., 2005). Chromatin remodeling might be another mechanism responsible for MIC upregulation. The inhibition of histone deacetylases which switch DNA to its 'open' conformation and in this way enable the access of transcription factors to the MICA/B promotors results in gene activation (Zhang et al., 2009). The exact mechanisms of MICA/B

upregulation upon viral infection are also elusive, although chromatin remodeling has been functionally associated with this process (Kato et al., 2007). The transcription factors involved in MICA/B upregulation include ubiquitous Sp1, Sp3, NF-Y and the heat shock-induced heat shock transcription factor HSP-1 (Gonzalez et al., 2008, Venkataraman et al., 2007) (Fig.2).

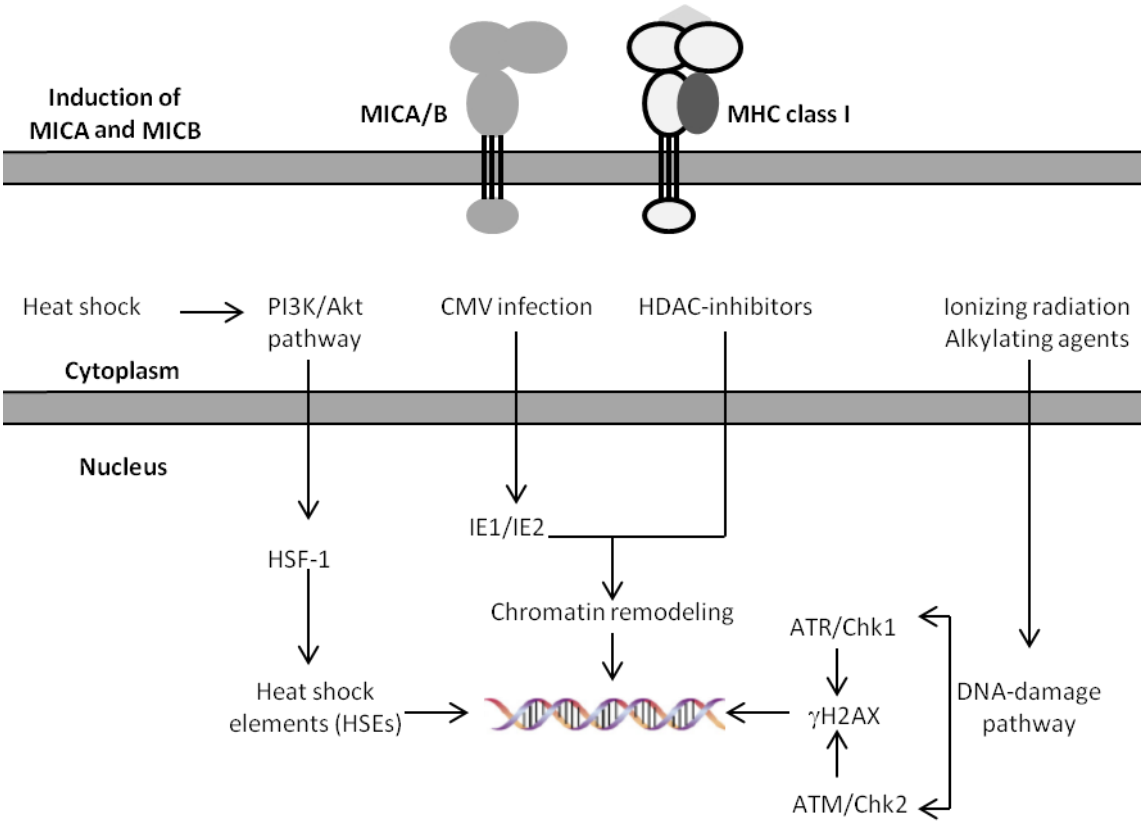

Fig.2 The regulation of NKG2D ligand transcription. NKG2D ligands expression is regulated by diverse mechanisms. Heat shock elements (HSE) in the promotor region of MIC respond to heat shock factor 1 (HSF-1). Viral immediate early proteins (IE1/IE2) from Cytomegalovirus (CMV) or histone deacetylase Inhibitors enhance NKG2D ligand expression by inducing the remodeling of chromatin. The DNA damage checkpoint pathway, initiated by ATM and ATR protein kinases also induces the transcription of NKG2D ligands (Modified from (Lopez-Larrea et al., 2008)).

Since genetic instability is the main characteristic of transformed cells, many types of cancer over-express at least one of these ligands on the cell surface. However, it still remains elusive, whether malignant transformation itself is sufficient for MICA/B transcription (Boissel et al., 2006, Spies, 2008, Okita et al., 2012). MICA/B expression has been proven on many progressing tumors including melanomas, neuroblastomas (Pende et al., 2002) breast, lung, prostate, ovarian, renal, prostate and colon carcinomas (Groh et al., 1999) and also in different leukemia cell lines (Weiss-Steider et al., 2011, Salih et al., 2003).

Additionally, it has been shown that toll-like receptor (TLR) signaling pathways and different cytokines could also affect the expression of NKG2D ligands. For example, TLR signaling triggers the NKG2DL expression in dendritic cells (Ebihara et al., 2007), macrophages (Hamerman et al., 2004) and activated T cells (Rabinovich et al., 2003); this might be a regulatory mechanism of NK cells limiting the function of overactivated immune cells (Eagle and Trowsdale, 2007). Induced MIC expression has been also documented in human cytomegalovirus (HCMV)-infected fibroblasts and endothelial cells (Groh et al., 2001). In response to INF-α MICA was expressed on dendritic cells (Jinushi et al., 2003) whereas INF-γ and TGF-β downregulated MICA expression in melanomas (Schwinn et al., 2009) and in gliomas, respectively (Eisele et al., 2006).

In order to avoid unwanted damage, the expression of MICA/B has to be tightly regulated. Interestingly, it has been shown that different cells and tissues express NKG2DL mRNA but lack protein expression, suggesting regulatory mechanisms on the posttranscriptional/posttranslational level. Endogenous miRNAs repress their translation by binding to three prime untranslated region (3'UTR) of MIC and thus regulate the MICA/B expression on the posttranscriptional level (Stern-Ginossar and Mandelboim, 2009, Nice et al., 2009). Ubiquitination might display a posttranslational regulatory mechanism of MICA expression. Ubiquitin ligase K5 down-regulates cell-surface expression of MICA by ubiquitination of the MICA cytoplasmic tail lysine residues and results in redistribution of MICA from the plasma membrane to an intracellular compartment, but does not result in an increased rate of degradation (Thomas et al., 2008).

2.5 The role of the NKG2D system in tumor-immune interaction

Research over the past two decades suggests a pivotal role of NKG2D system in cancer immuno-surveillance. The interaction of the NKG2D receptor with MIC molecules expressed on different tumor cells elicits cytotoxic effector functions initiating the elimination of malignant cells (Fig. 3).

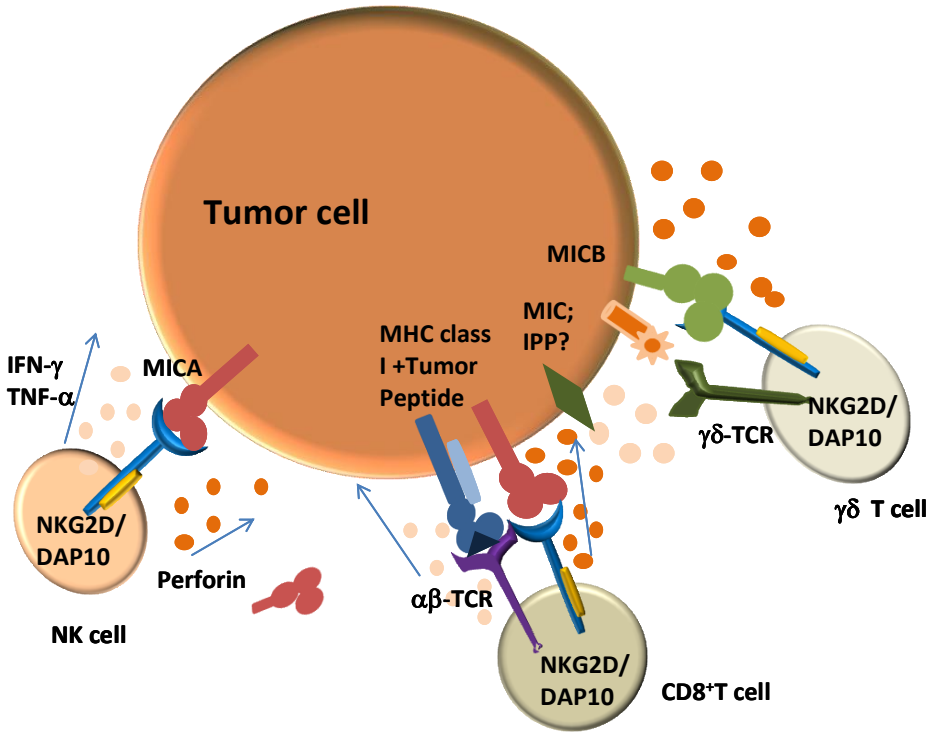

Fig.3 The role of the NKG2D system in tumor-immune interaction. Expression of NKG2D ligands on transformed cells facilitates recognition by NKG2D receptor-expressing NK cells, $CD8^+$ αβ-TCR^+ T cells and γδ-TCR^+ T cells and triggers cytotoxic effector functions. (Modified from (Lanier, 2001)).

The critical role of the NKG2D system has been shown in different model systems. *In vitro*, in melanomas and leukemia cell lines, the cytotoxic effect mediated by the NKG2DR-L interaction was correlated with the expression and surface density of MICA and other NKG2D ligands as well (Pende et al., 2002). Enhanced expression of MICA/B by sodium butyrate, a potent histone deacetylase inhibitor resulted in an increased susceptibility of cervical carcinoma and hepatoma cells to NK cell mediated lysis in an NKG2D-dependent manner (Zhang et al., 2009). In leukemia cells treatment with trichostatin A (histone deacetylase inhibitor) increased the cytotoxic potential of NKG2D expressing NK and $CD8^+$ T cells in a MICA/B-dependent manner (Kato et al., 2007). Furthermore, a MICA-containing bispecific antibody (MICA-CD20 fusion protein) increased the cytotoxic activity of NK cells against lymphoma cell lines and enhanced antibody-dependent cell-mediated cytotoxic (ADCC) activity of daratumumab (antineoplastic monoclonal antibody) in freshly isolated leukemias (Kellner et al., 2012).

Apart from the *in vitro* studies described above, *in vivo* mice models have confirmed the critical role of the NKG2D system in cancer immunosurveillance as well. NKG2D-deficient transgenic adenocarcinoma of mouse prostate models (TRAMP) appear to be defective in the control of spontaneous malignancies compared to wild type mice. These mice displayed early-arising, malignant

prostate tumors, while NKG2D-positive mice mostly developed late-arising benign tumors (Guerra et al., 2008). In another model, mice treated with neutralizing anti-NKG2D antibodies were more susceptible to methylcholantrene-induced (MCA) sarcomas than the mice treated with the isotype control, pointing out the important role of the NKG2D system in the control of newly arising tumors (Smyth et al., 2005). The importance of the NKG2D system was further confirmed in the clinic. The tumoricidal effect of cytokine-induced killer cells (CIK) cells (*ex vivo* expanded polyclonal T effector cells cultured under cytokine stimulation) is mostly dependent on NKG2D-mediated recognition of its cognate ligands, MICA and MICB on the target cells (Verneris et al., 2004). CIK immunotherapy has shown promising outcome in several early clinical trials in the United States (Leemhuis et al., 2005) and are routinely used in practice in China (Linn and Hui, 2010). So far CIK cells are the most effective example of an adoptive immunotherapy.

2.6 Escape mechanisms from NKG2D system-mediated immune surveillance

In a process referred to as "immunoediting", tumor cells have developed several mechanisms to actively bypass immune surveillance mediated by the NKG2D system. Tumor cells reduce their immunogenicity by modulating the cell surface expression of MICA and MICB via several mechanisms as well as affect the expression of NKG2D by releasing suppressive cytokines (Vesely et al., 2011).

Downregulation and degradation of NKG2D ligands and the receptor by tumor-derived cytokines: TGF-β as the main immunosuppressive cytokine released by transformed cells downregulates MICA expression, simultaneously induces NKG2D receptor internalization (Castriconi et al., 2003) and thus interferes with NK cell-mediated cytotoxicity (Lee et al., 2004). Consequently, the depletion of TGF-β increases the immunogenicity of tumors and susceptibility to NK cell-mediated killing (Eisele et al., 2006).

Altered epigenetic profile: histone deacetylases are frequently overexpressed in cancer cells (Glozak and Seto, 2007). Since inhibitors of HDAC increase the expression of MIC molecules (Kato et al., 2007, Huang et al., 2011) alterations in epigenetic gene regulation could limit the cell surface expression of MICA/B and decrease the immunogenicity of transformed cells.

Intracellular retention of NKG2D ligands: In some melanomas, MIC molecules are not expressed on the cell surface, but accumulate as immature forms in the endoplasmic reticulum (ER). The lack of surface MIC expression has been correlated with lower NK cell-mediated killing (Fuertes et al., 2008).

Ectodomain shedding of MICA/B: The proteolytic cleavage of NKG2DL at an extracellular site reduces the surface expression and releases the soluble extracellular domain and is regarded as the main immune escape mechanism. The amount of soluble MICA/B in the sera of cancer patients is significantly higher compared to healthy individuals (Salih et al., 2002) and is associated with poor prognosis (Holdenrieder et al., 2006). Shedding of MICA/B promotes tumor progression in various ways: (i) It decreases the cell surface expression of these molecules and reduces the immunogenicity of tumors. (ii) Soluble forms of MICA/B molecules impair the cytotoxic potential of NK cells by interfering with NKG2D binding sites and inducing the internalization of the NKG2D receptor on effector cells (Duan et al., 2011, Groh et al., 2002). (iii) Soluble MIC molecules promote the expansion of an immunosuppressive $CD4^{+}CD25^{+}NKG2D^{+}$ T cell population (Groh et al., 2006). Various members of the metalloprotease family have been identified as inducers of MICA/B cleavage. However, most of the studies have been performed in MIC over-expressing systems (Boutet et al., 2009, Waldhauer et al., 2008, Salih et al., 2002) and the exact mechanism of the shedding of endogenous MICA and MICB molecules requires further investigation.

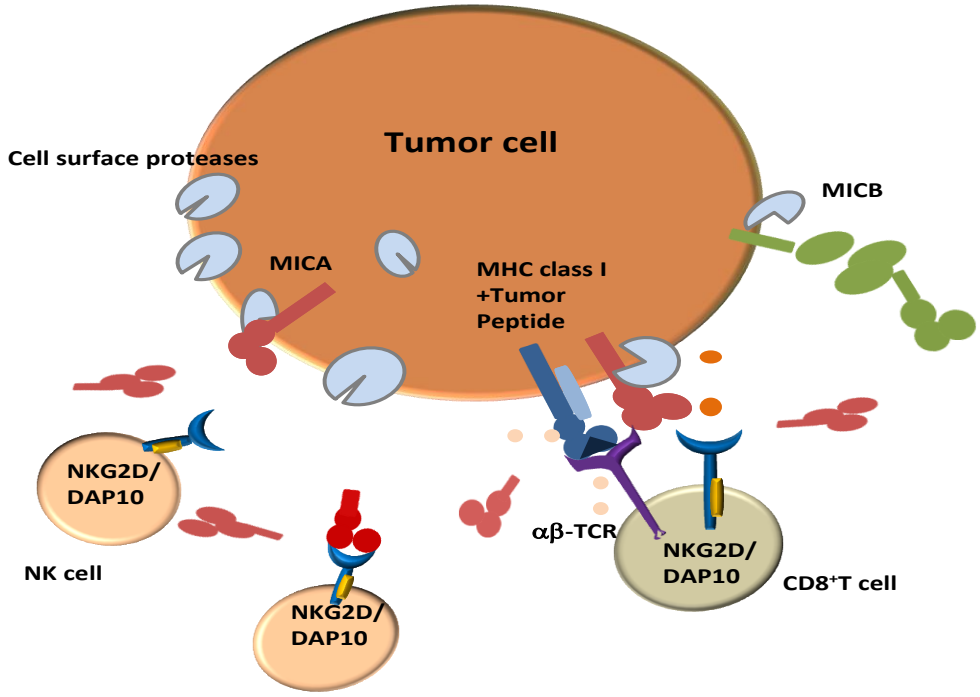

Fig. 4 Tumor immune evasion by cleavage of MICA/B. The ectodomain shedding of MICA/B from the surface of tumor cells decreases the immunogenicity of cancer cells. The soluble form of MICA/B interferes with the binding of NKG2D to its ligands.

2.7 Suspected proteases in the proteolytic cleavage of NKG2D ligands

2.7.1 Ectodomain shedding

Ectodomain shedding is a posttranslational mechanism that regulates the expression of cell surface proteins by cleavage. This cleavage usually occurs in the stalk region near the plasma membrane and leads to the release of the extracellular part of the protein (Arribas and Borroto, 2002). This results in the downregulation of cell surface expression and the generation of the soluble form of the protein, in many cases in a fully active form (Hooper et al., 1997).

Targets of ectodomain shedding include transmembrane growth factors, adhesion molecules, membrane receptors and ligands. Hence, ectodomain shedding regulates many (patho)physiological processes including cell development, migration, inflammation and tumorigenesis (Garton et al., 2006, Higashiyama et al., 2011).

Ectodomain shedding is regulated by Erk/MAPK and p38/MAPK signaling pathways. The activation of these pathways by different stimuli drastically increases the basal ectodomain shedding observed in non-stimulated cells (Fan and Derynck, 1999).

The major executors of ectodomain cleavage are members of the "a disintegrin and metalloproteases (ADAM)" (Huovila et al., 2005) and the matrix metalloproteases (MMP) family (Gialeli et al., 2011). However, extracellular proteolysis can also be mediated by extracellular serine, cysteine or aspartic proteases. Type II transmembrane serine proteases (matriptases) or tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) serine proteases have been involved in extracellular proteolysis during metastasis and invasion (Dano et al., 2005, List et al., 2006). Cysteine proteases belonging to the cathepsins are localized on the cell surface during malignant transformation and play a role in extracellular matrix degradation (Sloane et al., 2005). The membrane-anchored aspartic protease, BACE, releases the β-site of Amyloid Precursor Protein APP and is involved in the pathogenesis of Alzheimer's disease (Yan et al., 1999).

2.7.2 ADAM metalloproteases

ADAM proteases belong to the metzincins- family of zinc proteases. The metzincins include serralysins, astacins, matrixins, and adamalysins. ADAM family members belong to the adamylasins together with snake venom metalloproteinases (SVMPs) and ADAMTS (ADAMs containing thrombospondin motifs). Twelve ADAMs (ADAM8, 9, 10, 12, 15, 17, 19, 20, 21, 28, 30, 33) out of 40 described ADAM family members are catalytically active. Structurally ADAMs consist of an N-terminal signal sequence followed by a prodomain, a metalloprotease domain, a disintegrin domain with a

cysteine-rich region, an EGF domain, a transmembrane domain and a cytoplasmic tail (Seals and Courtneidge, 2003). The interaction of a cysteine residue in the prodomain with the zinc ion in the catalytic site maintains the ADAMs in the inactive state and the removal of the prodomain is required for the activation of ADAM proteases (Anders et al., 2001). ADAMs have been implicated in diverse (patho)physiological processes such as cell development, cell migration, adhesion, inflammation, autoimmune disorders and most importantly in carcinogenesis (Reiss and Saftig, 2009).

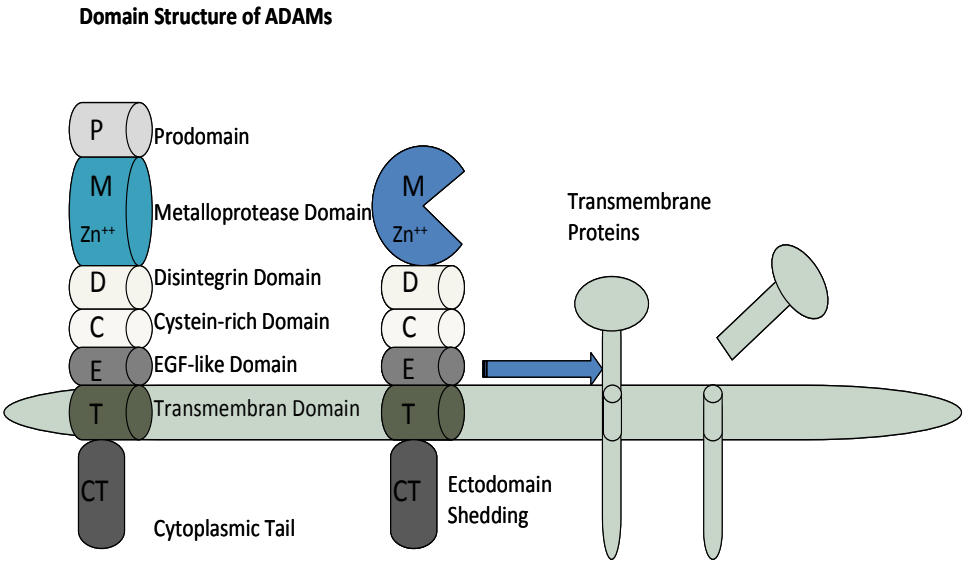

Fig. 5 The structure and activating mechanism of ADAMs. Removal of the prodomain switches ADAM proteases to their active form. Activated ADAM cleaves the substrate from the cell surface and releases the extracellular domain of the protein.

Recent evidence shows that the increased expression of individual ADAM proteases is associated with various types of cancer (Lu et al., 2008). In gastric cancers the upregulation of ADAM9/12/15 has been observed (Carl-McGrath et al., 2005) whereas in breast cancers the particular role of ADAM9/12/17 has been documented (Lendeckel et al., 2005). ADAMs may promote cancer progression by the release of stimulating growth factors such as members of the EGFR/HER family of ligands. By the cleavage of adhesion molecules, ADAMs increase the invasiveness and metastatic potential of tumors (Duffy et al., 2011).

2.7.3 ADAM10 and ADAM17

ADAM10 and ADAM17 are the best characterized members of the ADAM family so far. The substrates of ADAM17/10 include growth factors and their receptors, chemokines, adhesion molecules and many other transmembrane proteins involved in different regulatory processes.

The particular role of ADAM10 has been documented in several (patho)physiological conditions. By the cleavage of the Notch receptor and Delta Like Ligand-1 (DLL1), a receptor-ligand system controlling the cell fate in a broad range of tissues, ADAM10 plays an important role in cellular development (Pan and Rubin, 1997, Six et al., 2003). Moreover, ADAM10 is involved in pathogenesis of Alzheimer´s disease by the cleavage of APP (Allinson et al., 2004). By cleaving Fas ligand (the ligand of death receptor Fas) ADAM10 is an important regulator of FasL induced cell death (Schulte et al., 2007).

So far documented substrates of ADAM17 are TNF-α (Black et al., 1997), TGF-α, L-selectin (Peschon et al., 1998), TNF receptors I and II (Reddy et al., 2000, Tokumaru et al., 2000), IL-6 Receptor (Chalaris et al., 2007) and several other molecules involved in cell growth, inflammation, cell migration, carcinogenesis and adhesion (Reiss and Saftig, 2009).

ADAM10 and 17 are ubiquitously expressed and upregulated in cancers (Duffy et al., 2011, Duffy et al., 2009). By activation of different growth-stimulating factors (Schafer et al., 2004, Duffy et al., 2011, Sahin et al., 2004), by shedding of adhesion proteins and thus affecting the adhesion properties of tumor cells (Janes et al., 2005), ADAM10/17 promote tumor progression and increase both the metastasic potential and the invasiveness of cancers. Moreover, recent findings indicate that ADAM10/17 contributes to carcinogenesis by cleaving the stress antigens MICA/MICB from the cell surface of tumors (Waldhauer et al., 2008, Boutet et al., 2009). Considering this ADAM10/17 might be strategic targets for cancer therapy.

3. Aim of the study

The ectodomain shedding of MICA and MICB molecules from tumor cells decreases the killing capability of NKG2D receptor-expressing immune effectors and promotes tumor immune escape. However, the shedding of endogenous MICA/B molecules is not precisely defined and the better understanding of this cleavage machinery might be beneficial for the development of effective cancer therapeutic strategies.

In this study we aimed to investigate:

1. Expression of endogenous MICA/B molecules in different carcinoma cell lines. We aimed to analyze different carcinoma cell lines for the baseline and PMA-induced expression of endogenous MICA/B molecules.

2. Ectodomain shedding of endogenous MICA/B molecules. With inhibitory studies and siRNA-mediated targeting of individual proteases, we aimed to define the proteases responsible for the shedding of endogenously expressed MICA and MICB molecules.

4. Materials

4.1 Cell biology

4.1.1 Cell lines

Panc89	Pancreas ductal adenocarcinoma	Institute for Experimental Cancer Research, Kiel, GER
PancTu-I	Pancreas ductal adenocarcinoma	Institute for Experimental Cancer Research, Kiel, GER
PC-3	Prostate adenocarcinoma	ATCC, USA
MDA-MB-231	Breast adenocarcinoma	ATCC, USA

4.1.2 Reagents, buffers and media used for cell culture

RPMI-1640 medium (25 mM HEPES, L-glutamine)	Invitrogen, Carlsbad, USA
FCS (heat-inactivated and sterilized by filtration)	Invitrogen, Carlsbad, USA
Penicillin/streptomycin (1 x 10^4 U/1 x 10^4 µg/ml)	Biochrom AG, Berlin, GER
Trypsin-EDTA (0,05%/0,02% (w/v))	Biochrom AG, Berlin, GER
PBS w/o Ca^{2+}/Mg^{2+}	Cell Concept, Freiburg, GER

Inhibitor	Specificity	Used Concentration	Source
GM6001 (in DMSO)	Metalloproteases	5 µM	Calbiochem, GER
TAPI-I (in DMSO)	Metalloproteases	5 µM	Calbiochem, GER
AEBSF Hydrochloride (in H_2O)	Serine proteases	25-100 µM	Calbiochem, GER
Leupeptin (in H_2O)	Cystein/serine Proteases	100 µM	Calbiochem, GER
Pepstatin A(in DMSO)	Aspartate Proteases	10 µM	Calbiochem, GER
Cycloheximide	Inhibitor of protein syntesis	0.033-0.15 µg/ml	Calbiochem, GER

Stimulator		
Phorbol 12-Myristate-13-Acetate (PMA)	50-100 ng/ml	Sigma, GER

4.1.3 Materials used in flow cytometry

Buffers and reagents

FACS washing buffer	
1x PBS	Biochrom AG, Berlin, GER
1% (w/v) BSA	Carl Roth, Karlsruhe, GER
0.1% (w/v) NaN3	Merck KGaA, Darmstadt, GER

Fixation buffer	
1% (w/v) PFA (in PBS)	Merck KGaA, Darmstadt, GER

BD Cytofix/Cytoperm Fixation/Permeabilization Kit	
Kit Components	(BD Biosciences, USA)
Fixation/Permeabilization solution (125 ml)	
BD Perm/Wash buffer 10x (diluted 1:10 prior to use in distilled water)	

FcR blocking reagent	Miltenyi Biotec, USA
diluted 1:10 prior to use in FACS buffer	

Antibodies used for cell surface staining

Specificity	Clone #	Used Concentration	Species	Isotype	Source
Isotype control-PE	133303	25 µg/ml	mouse	IgG2b	R&D Systems, USA
Anti-human MICA-PE	159227	25 µg/ml	mouse	IgG2b	R&D Systems, USA
Anti-human MICB-PE	236511	25 µg/ml	mouse	IgG2b	R&D Systems, USA
Isotype control-PE	x40	25 µg/ml	mouse	IgG1	BD Biosciences, USA
Anti-human NKG2D-PE	149810	25 µg/ml	mouse	IgG1	R&D Systems, USA
Anti-human ADAM17-PE	111633	25 µg/ml	mouse	IgG1	R&D Systems, USA

Antibodies used for intracellular (IC) staining

Specificity	Clone #	Used Concentration	Species	Isotype	Source
Isotype control-PE (IC)	G155178	0,25 µg/ml	mouse	IgG2a	BD Pharmingen
Anti-human ADAM10-PE (IC)	163003	0,25 µg/ml	mouse	IgG2b	R&D Systems, USA

4.1.4 Materials used in enzyme-linked immunosorbent assay (ELISA)

Buffers and reagents

Reagent Diluent (RD)	
1x PBS	Biochrom AG, Berlin, GER
1% (w/v) BSA	Carl Roth, Karlsruhe, GER
ELISA washing buffer	
1x PBS	Biochrom AG, Berlin, GER
0,05% (v/v) Tween-20	Merck KGaA, Darmstadt, GER
Substrate Reagent Pack	R&D Systems, USA (#DY999)
Color Reagent A	Stabilized hydrogen peroxide
Color Reagent B	Stabilized tetramethylbenzidine

Materials provided in human MICA ELISA duo set kit

R&D Systems, USA (#DY1300)	Dilution
Capture Antibody 360 µg/ml in PBS	1:180 in PBS
Detection Antibody 72 µg/ml in RD	1:180 in RD
Standard MICA 180 µg/ml in RD	1:45 in RD
Streptavidin-HRP 1ml	1:200 in RD

Materials provided in human MICB ELISA duo set kit

R&D Systems, USA (#1599)	Dilution
Capture Antibody 720 µg/ml in PBS	1:180 in PBS
Detection Antibody 72 µg/ml in RD	1:180 in RD
Standard MICA 370 µg/ml in RD	1:37 in RD
Streptavidin-HRP 1ml	1:200 in RD

4.2 Molecular biology

4.2.1 Small interfering RNAs used for gene silencing

Stealth RNA		Invitrogen, Carlsbad, USA	Final Concentration
ADAM10 stealth RNA	Sense	GGUGUUGCUGAGAGUGUUAAUUCU	25 nM
	Antisense	AGAAUUAACACUCUCAGCAACACCA	
ADAM17 stealth RNA	Sense	CCAGGGAGGGAAAUAUGUCAUGUAU	25 nM
	Antisense	AUACAUGACAUAUUUCCCUCCCUGG	
Stealth control RNAi	Negative Control Med GC Duplex		25 nM

Lipofectamine RNAiMAX	Invitrogen, Carlsbad, USA	1,67 µL/ml
Opti-MEM Reduced Serum Medium	Invitrogen, Carlsbad, USA	

4.2.2 Materials used for the isolation of genomic DNA

Materials provided in genomic DNA purification kit (MACHEREY-NAGEL, GER)

Lysis Buffer BQ1
Washing Buffer BQ2
Elution Buffer BE
Lyophilized Proteinase K, dissolved in PB buffer
NucleoSpin®Blood Columns
Collection Tubes

4.3 Protein biochemistry

4.3.1 Cell lysis buffers and reagents

TNE lysis buffer	
1% (v/v) NP-40	Fluka Riedel-de Häen/Sigma, GER
50 mMTris	Merck KGaA, Darmstadt, GER
150 mM NaCl	Merck KGaA, Darmstadt, GER
2 mM EDTA	Merck KGaA, Darmstadt, GER

NP-40 lysis buffer	
1% (v/v) NP-40	Fluka Riedel-de Häen/Sigma, GER
20 mM Tris	Merck KGaA, Darmstadt, GER
150 mM NaCl	Merck KGaA, Darmstadt, GER

RIPA buffer:	
20 mM Tris-HCl (pH 7.5)	Merck KGaA, Darmstadt, GER
150 mM NaCl, 1 mM Na_2EDTA	Merck KGaA, Darmstadt, GER
1 mM EGTA	Merck KGaA, Darmstadt, GER
1% NP-40	Fluka Riedel-de Häen/Sigma, GER
1% sodium deoxycholate	
2.5 mM sodium pyrophosphate	
1 mM β-glycerophosphate	

Inhibitors cocktail: (1:100)	Sigma-Aldrich, St. Luis, USA
104 mM AEBSF	1.4 mM E-64
80 µM Aprotinin	2 mM Leupeptin
4 mM Bestatin	1.5 mM pepstatin A
Bradford Reagent	Thermo Scientific, Karlsruhe, GER

4.3.2 Materials used for immunoprecipitation

Materials used in immunoprecipitation and deglycosylation

Protein G sepharose™ 4B beads	Zytomed, Munich, GER
G7 reaction buffer 10x	New England BioLabs, UK
PNGaseF 500.000 U/ml	New England BioLabs, UK
10% NP-40	New England BioLabs, UK
Denaturing buffer 10x	New England BioLabs, UK

4.3.3 Materials used for SDS-page

4.3.3.1 NuPAGE system mini gels (Invitrogen)

Buffers and reagents used in mini gels

2x SDS sample buffer	
125 mM Tris	Merck KGaA, Darmstadt, GER
4% (w/v) SDS	Merck KGaA, Darmstadt, GER
20% (v/v) glycerol	Merck KGaA, Darmstadt, GER
0.005% (w/v) bromophenol blue	Merck KGaA, Darmstadt, GER
10% (v/v) β-2-mercaptoethanol	Merck KGaA, Darmstadt, GER

10x Running buffer	
30 g/l Tris	Merck KGaA, Darmstadt, GER
144 g/l glycine	Carl Roth, Karlsruhe, GER
10 g/l SDS	Merck KGaA, Darmstadt, GER

Prestained high-range Standard	Bio-Rad, Hercules, USA
Prestained low-range Standard	Bio-Rad, Hercules, USA

4.3.3.2 Protean II system gels (Bio Rad)

Materials for Protean II system gels

Running gel (Protean II system, 10%, 30 ml)	
10.0 ml 30% acrylamide/0.8% bisacrylamide	Carl Roth, Karlsruhe, GER
11.2 ml 1 M Tris, pH 8.8	Merck KGaA, Darmstadt, GER
0.3 ml 10% (w/v) SDS	Merck KGaA, Darmstadt, GER
8.7 ml aqua ad iniectabilia	Braun, Melsungen, GER
0.1 ml 20% APS	Merck KGaA, Darmstadt, GER
0.02 ml TEMED	Carl Roth, Karlsruhe, GER

Stacking gel (Protean II system, 10 ml)	
1.67 ml 30% acrylamide/0.8% bisacrylamide	Carl Roth, Karlsruhe, GER
1.25 ml 1 M Tris, pH 6.8	Merck KGaA, Darmstadt, GER
0.1 ml 10% (w/v) SDS	Merck KGaA, Darmstadt, GER
7.0 ml aqua ad iniectabilia	Braun, Melsungen, GER
0.05 ml 20% APS	Merck KGaA, Darmstadt, GER
0.01 ml TEMED	Carl Roth, Karlsruhe, GER

3x reducing sample buffer (Protean II system)	Bio-Rad, Hercules, USA
Precision Plus Protein All Blue Standard	Bio-Rad, Hercules, USA

4.3.4 Materials used for western blotting and IP

Antibodies in WB and IP	Clone#	Concentration	Dilution	Species	Source
Anti-human ADAM10 (WB)	Polyclonal	1 mg/ml	1/1000	Rabbit	Millipore, GER
Anti-human ADAM17 (WB)	Polyclonal	1 mg/ml	1/1000	Rabbit	Millipore, GER
Anti-human MICA/B (WB)	BAMO1	1 mg/ml	1/1000	mouse IgG1	BAMOMAB, GER
Anti-human MICA (IP)	159227	360 µg/ml	1ug/ml	mouse IgG2b	R&D Systems, USA
Anti-β actin(WB)	AC-15	2,9 mg/ml	1/10000	mouse IgG1	Sigma, GER

HRP-conjugated antibodies	Dilution	Species	Source
Anti-rabbit IgG-HRP	1/10000	donkey	GE Healthcare,UK
Anti-rabbit IgG-HRP	1/10000	donkey	GE Healthcare,UK

Buffers and reagents used in western blotting

Transfer buffer

25 mM Tris	Merck KGaA, Darmstadt, GER
192 mM glycine	Carl Roth, Karlsruhe, GER
20% (v/v) methanol	Sigma-Aldrich, St. Louis, USA
0.015% (w/v) SDS	Merck KGaA, Darmstadt, GER

Washing buffer (TBS-T)

Tris 10 mM	Merck KGaA, Darmstadt, GER
NaCl 150 mM	Merck KGaA, Darmstadt, GER
Tween 20 0,05% (v/v)	Merck KGaA, Darmstadt, GER

Blocking buffer

5% (w/v) BSA in TBS-T	Serva Electrophoresis GmbH, GER
5% (w/v) Dry Milk in TBS-T	Granovita, GER
Ponceau S staining solution	Sigma-Aldrich, St. Luis, USA
ECL™ detection reagents	GE Healthcare, UK

4.4 Consumables

Laboratory consumables	
Centrifuge tubes (15ml, 50ml)	Greiner, Kremsmünster, AUT
Reaction tubes (1.5 ml, 2.0 ml)	Sarstedt AG, Nümbrecht, GER
Pipette (filter) tips	Sarstedt AG, Nümbrecht, GER
	Greiner, Kremsmünster, AUT
	Biozym, Hessisch Oldendorf, GER
Cryotubes	Nunc, Roskilde, DEN
Cell culture flasks	Greiner, Kremsmünster, AUT
FACS tubes	Dunn, Asbach GER
96-well plates	Nerbe, Winsen, GER
	Nunc, Roskilde, DEN
24-, 6-well plates	Greiner, Kremsmünster, AUT
NuPAGE™ 4-12% Bis-Tris gels (1.5 mm)	Invitrogen, Carlsbad, USA
Nitrocellulose membrane Hybond™ C (0.4 µm)	GE Healthcare, UK
Amersham Hyperfilm ECL™	GE Healthcare, UK

4.5 Laboratory equipment

CO_2-humified, water jacketed incubator	Forma Scientific, Marietta, USA
Flow cytometer FACSCalibur	BD, East Rutherford, USA
Microplates-Photometer reader 'infiniteTM M200'	Tecan, Crailsheim, GER
Development machine Curix60	Agfa, Mortsel, Belgium
NanoDrop™ ND-1000	Thermo Scientific, USA
SmartSpec™3000 spectrophotometer	Bio-Rad, Hercules, USA
Protean® II electrophoresis chamber	Bio-Rad, Hercules, USA
Transfer-Tank (Mini) Trans-Blot®	Bio-Rad, Hercules, USA
Film cassettes	Amersham/GE Healthcare, UK
Inverse light microscopes	Carl Zeiss, Jena, GER
Neubauer hemocytometer	Fischer, Frankfurt, GER
Megafuge 10	Heraeus, Osterrode, GER
Biofuge 15R	Heraeus, Osterrode, GER
Shaking water bath	GFL, Burgwedel, GER
Rocking device	Fröbel, Lindau, GER
Vortex-Genie 2	Scientific Industries, USA
Calibrated densitometric gel scanner GS-800	Bio-Rad, Hercules, USA
Pipetting aid accu-jet pro	Brand, Wertheim, GER
Pipettes	Eppendorf, Hamburg, GER
	Gilson, Middleton, USA

5. Methods

5.1 Cell biology

5.1.1 Cell culture

The pancreas ductal adenocarcinomas Panc89 and PancTu-I and the breast cancer cell line MDA-MB-231 were cultivated in RPMI-1640 growth medium. The prostate cancer cell line PC-3 was cultured in F-12K medium. The growth media were supplemented with 10% heat inactivated Fetal Calf Serum (FCS), 100 U/ml Penicillin and 100 µg/ml Streptomycin. All cell lines were cultivated at 37°C in a humidified atmosphere with 5% CO_2.

For passaging the adherent cells (Panc89 and PancTu-I, MDA-MB-231 and PC-3) cells were detached by sterile Trypsin/EDTA (0,05%/0,02%) and centrifugated at 300 g for 5 minutes. Cells were seeded in fresh medium at a density to reach 80-90% confluence within 48-72 hours.

In inhibitor studies, fixed numbers of cells were treated with non-toxic concentrations of inhibitors and with PMA where indicated. After 24 hours of cultivation, the conditioned medium and cellular lysates were subjected to ELISA.

5.1.2 Flow cytometry

Flow cytometry is a method for the identification and quantification of cellular antigens. The main principle of flow cytometry is to measure the light,emitted by the cell as it passes through a focused laser beam. Cells coated with specific, fluorochrome-conjugated antibodies are excited by the laser and the specific emission light correlates with amount of antigen. The data provides information about the granularity and size of the cell and about the expression of a given protein (Murphy et al, 2008).

To determine the cell surface expression of MICA, MICB and ADAM17 1 x 10^5 cells were transferred to 96-well plates and washed twice (centrifugation at 300g, 4°C, 4 min) with 180 µL FACS washing buffer. Samples were blocked with 20 µL Fc receptor blocking reagent for 15 minutes at 4°C in the dark. Following another washing step, cells were incubated for 30 minutes with either 10 µL of phycoerythrin (PE)-conjugated specific antibodies or with the appropriate isotype control at 4°C. Afterwards, cells were washed twice and fixed in 1% PFA.

For intracellular staining of ADAM10, the commercially available Cytofix/Cytoperm Fiaxtion/Permeabilization Kit was used (BD Biosciences, USA). Following the FcR blocking step, cells were resuspended in 100 µL Fixation/Permeabilization solution for 20 minutes at 4°C and washed with Perm/wash buffer two times. Fixed and permeabilezed cells were stained (30 min, 4°C, in the dark) with 100 µL of PE-conjugated antibody detecting the intracellular part of ADAM10 and with the

corresponding isotype control. Following two washing steps with Perm/wash buffer, cells were resuspended in 1% PFA.
5000 living cells of each sample were measured with the FACS Calibur Flow Cytometer and analysed using CellQuest Pro software (BD Biosciences, USA).

5.1.3 Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) is a sensitive immunological technique specifically detecting an antigen and determining its concentration in liquid samples. The main principle of this approach is to detect the target molecule via specific enzyme-coupled antibodies. The antigen-antibody interaction causes the conversion of a substrate into a colored, detectable product. Light absorbance is detected by an ELISA Reader. The intensity of the signal is directly proportional to the amount of antigen captured in the plate by binding to the detection reagent (Engvall and Perlmann, 1971).
The amount of soluble and cellular MICA/B was determined by commercially available MICA and MICB Elisa kits (Human Duo set MICA and MICB, R&D Systems, USA) following manufacturer's instructions. Briefly, 96-well ELISA plates were coated with 100 µL of capture antibody overnight. After three washes with 400 µl ELISA washing buffer the wells were blocked with 300 µL of diluent reagent and incubated for 1 hour at room temperature. Following three further washing steps 100 µL conditioned medium and cellular lysates (prepared as described in 5.3.1.1 and previously diluted in diluent reagent) were added in duplicates and incubated for two hours. Serial dilutions of recombinant MICA/B served as a standard. Bound MICA/B was detected using 100 µl of a biotinylated detection antibody (2 h) followed by incubation with 100uL of HRP-conjugated streptavidin for 30 minutes. Afterwards 100 µL of a substrate solution (equal mixture of color reagents A and B) were added to each well and the color reaction was stopped after 8-10 minutes by adding 50 µL of 2N sulfuric acid. The absorbance was measured at 450 nm against 570 nm reference wavelength with the Infinite@200PRO Elisa Reader (Tecan, USA).

5.2 Molecular biology

5.2.1 Gene silencing by RNA interference

SiRNA interference is a widely used approach for the specific silencing of a given protein of interest. Small double stranded RNAs 21-23 base pairs in length; integrates in the RNA-induced silencing complex (RISC) and induce the degradation of specific mRNAs with complementary sequence. For the effective transfection, commercially available transfection reagents (Lipofectamine RNAiMAX, Invitrogen) are commonly used.

To induce ADAM10 and ADAM17 gene silencing, specific and control synthetic siRNA duplexes (Invitrogen, USA) were mixed with lipofectamine RNAiMAX (diluted in 50 µl Opti-MEM serum-reduced medium prior to use). Following incubation for 20 minutes at RT in the dark, the mixture was added to 70.000 cells suspended in 500 µl of medium and was seeded in 24-well plates and incubated for 72 hours at 37°C. Medium was replenished and the protein knockdown efficiency was confirmed by Western Blot and flow cytometry. Cells were incubated for further 24 h and the the conditioned media was subjected to ELISA.

5.2.2 Isolation of genomic DNA

For the genotyping of MICA, genomic DNA was isolated with the Genomic DNA Purification kit (MACHEREY-NAGEL, GER). 2 x 10^5 cells were resuspended in 200 µl PBS and mixed with 25 µl proteinase K and 200 µl BQ1 lysis buffer. Following incubation at 70°C for ten minutes, 200 µl pure ethanol was added and vigorously vortexed. Afterwards the samples were loaded to Nucleospin®Blood Quickpure Columns. After centrifugation at 11.000 g for one minute, Nucleospin® Blood Columns were washed twice with Washing Buffer BQ2 (350 and 200 µl, respectively). Genomic DNA was eluted by the addition of 50 µl prewarmed elution buffer BE followed by incubation at room temperature for one minute and centrifugation at 11.000 g for one minute. Samples were analyzed at the Institute of Clinical Transfusion Medicine and Immunogenetics, in Ulm, Germany (Furst et al., 2011).

5.3 Protein biochemistry

5.3.1 Preparation of cellular lysates

Cells were harvested and washed with PBS at 300 g, for 5 min. Lysis buffer supplemented with inhibitor cocktail was added to the pellet and the sample was incubated on ice for 40 min. Lysates were subjected to centrifugation at 14.000 rpm for 10 min to remove cellular debris and subjected to Bradford assay for protein content determination.

5.3.2 Bradford-assay

Protein content in cellular lysates was determined by a colorimetric Bradford assay which is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 once bound to protein. 5 µl of the sample were mixed with 995 µL of Bradford reagent and incubated at RT for 10 Min. The absorbance was measured at 595 nm with the SmartSpec 3000 (BioRad, USA).

5.3.3 Immunoprecipitation (IP)

Immunoprecipitation (IP) is a widely used approach for the isolation of specific proteins from protein mixtures. A specific antibody binds to a target protein and the antibody-bound protein is immunoprecipitated by Protein G or Protein A beads.
Cellular lysates were mixed with 1 µg of the precipitating antibody and 60 µl of protein G beads (1/1 in lysis buffer NP-40). This mixture was incubated on a rotating device at 4°C overnight. Beads were sedimented by quick spin and washed three times with NP-40 lysis buffer. Beads were resuspended in 15 µl of sterile water and incubated with 5 µl of 10x glycoprotein denaturing buffer at 100°C for 10 minute. Following denaturation, samples were subjected to deglycosylation by the addition of 5 µl sterile water, 7 µl G7 10x reaction buffer, 7 µl of 10% NP-40 buffer and 1 µl of PNGase F. Samples were incubated at 37°C for 1 hour. Following that samples were incubated at 100°C for 5 minutes in 30 µl of reducing sample buffer and afterwards subjected to SDS-PAGE.

5.3.4 SDS-PAGE

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a widely used technique for the separation of proteins based on their size. Proteins are denaturated by the anionic detergent

SDS and separated by gel electrophoresis. Subsequent Western blotting enables the detection of target proteins by specific antibodies.

In this study proteins were separated either by discontinuous gel electrophoresis according to Laemmli (Laemmli, 1970) using the Protean II system (Bio-Rad Laboratories, Hercules, USA) or by continuous electrophoresis with pre-cast Bis-Tris NuPAGE® gels (Invitrogen, Carlsbad, USA).

5.3.4.1 NuPAGE system mini gels (Invitrogen)

4x SDS sample buffer was added to 20 µg of total protein. Following a denaturation step at 70°C for 10 min, samples and prestained standards marker were loaded on 4-12% acrylamide gradient Bis-Tris gels and electrophoresis was performed in running buffer at 200 V for 45-60 minutes.

5.3.4.2 Protean II system gels (Bio Rad)

30 µl of reducing sample buffer were added to the precipitates and incubated at 100°C for 5 minutes. Samples and precision plus protein marker were loaded on protean II system gels, composed of running and stacking gel prepared according to the method of Laemmli (Laemmli, 1970). Electrophoresis was performed for 2 hours at 180 V and afterwards at 55 V overnight.

5.3.5 Western blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes 0.4 µm by tank blotting. For Protean II system gels, this was performed in Trans-Blot® tanks at 4°C and 0.8 A for two hours. In the case of NuPAGE mini-gels, Mini Trans-Blot® tanks were used and the transfer was performed for 2h at 110 V. Transfer efficiency was documented by reversible Ponceau S staining. Blots were washed with TBS for 5 minutes to remove Ponceau S and afterwards blocked at room temperature for 1 h with 5% (w/v) BSA in TBS-T or with 5% milk in TBS-T. Blots were washed tree times with TBS-T and incubated with the primary antibody diluted in TBS-T or in 2% BSA (w/v) in TBS-T for 1 h at RT or at 4°C overnight. After three to four washing steps, HRP-conjugated secondary antibodies diluted in TBS-T were added and incubated at room temperature for 1 h. Blots were washed again extensively with TBS-T and ECL solution (equal volume of the detection reagent 1 and

the detection reagent 2) was added for 1 min. ECL films were then exposed for an appropriate time in the dark and developed with the imaging device, CP1000-AGFA, GER.

5.4 Statistical analysis

For statistical analysis the paired, two-tailed student's t-Test was performed. Statistical significance was set at $p < 0.05$ and displayed as ***$p<0.001$ **$p<0.01$ *$p<0.05$.

6. Results

6.1 Differential expression and shedding profiles of endogenous MICA and MICB molecules in various epithelial cancer cell lines

Initially the expression levels and shedding profiles of endogenous MICA and MICB were determined in different human carcinoma cell lines. This analysis encompassed several epithelial cancer cell lines including the pancreatic carcinoma cell lines Panc89 and PancTu-I, the mammary carcinoma cell line MDA-MB-231 and the prostate carcinoma cell line PC-3. Flow cytometric analysis revealed that both MICA and MICB molecules are expressed on the cell surface of all analyzed cell lines, although the expression level of these molecules notably varied (Fig.1, A). In general, MICA expression was more pronounced than the expression of MICB. Only in the prostate cancer cell line PC-3 MICB was more abundant than MICA. In the mammary cancer cell line MDA-MB-231 and in the pancreas cancer cell line PancTu-I MICB expression was very low.

In parallel, conditioned medium and cellular lysates were subjected to ELISA and examined for the level of MICA/B (Fig.1, B). The overall expression of MICA/B was also determined by ELISA of cellular lysates to allow for the assessment of the relative shedding. Consistent with the flow cytometry data, expression levels of MICA as detected from cellular lysates were higher than of MICB ranging between 2845 pg/ml and 843 pg/ml for MICA and 1034 pg/ml and 509 pg/ml for MICB, respectively. The pancreatic cancer cell line Panc89 contained the highest amount of MICA in the cellular lysates (2845 pg/ml), whereas the mammary cancer cell line MDA-MB-231 released the uppermost amount of MICA (752 pg/ml) into the supernatant. Interestingly, the prostate cancer cell line PC-3 failed to secrete MICA, despite the fact that it expresses both NKG2D ligands on the cell surface. However, the release of the highest amount of MICB (1977 pg/ml) was observed from this particular PC-3 cell line. The lowest release of MICB has been observed from PancTu-1 cell line. Additionally, MICA protein was immunoprecipitated from cellular lysates. Following deglycosylation by PNGaseF, MICA was detected by western blotting (Fig.1, C) as a 42 kD protein as described previously (Bahram et al., 1994). However, in PC-3 cells MICA migrated at 33 kD.

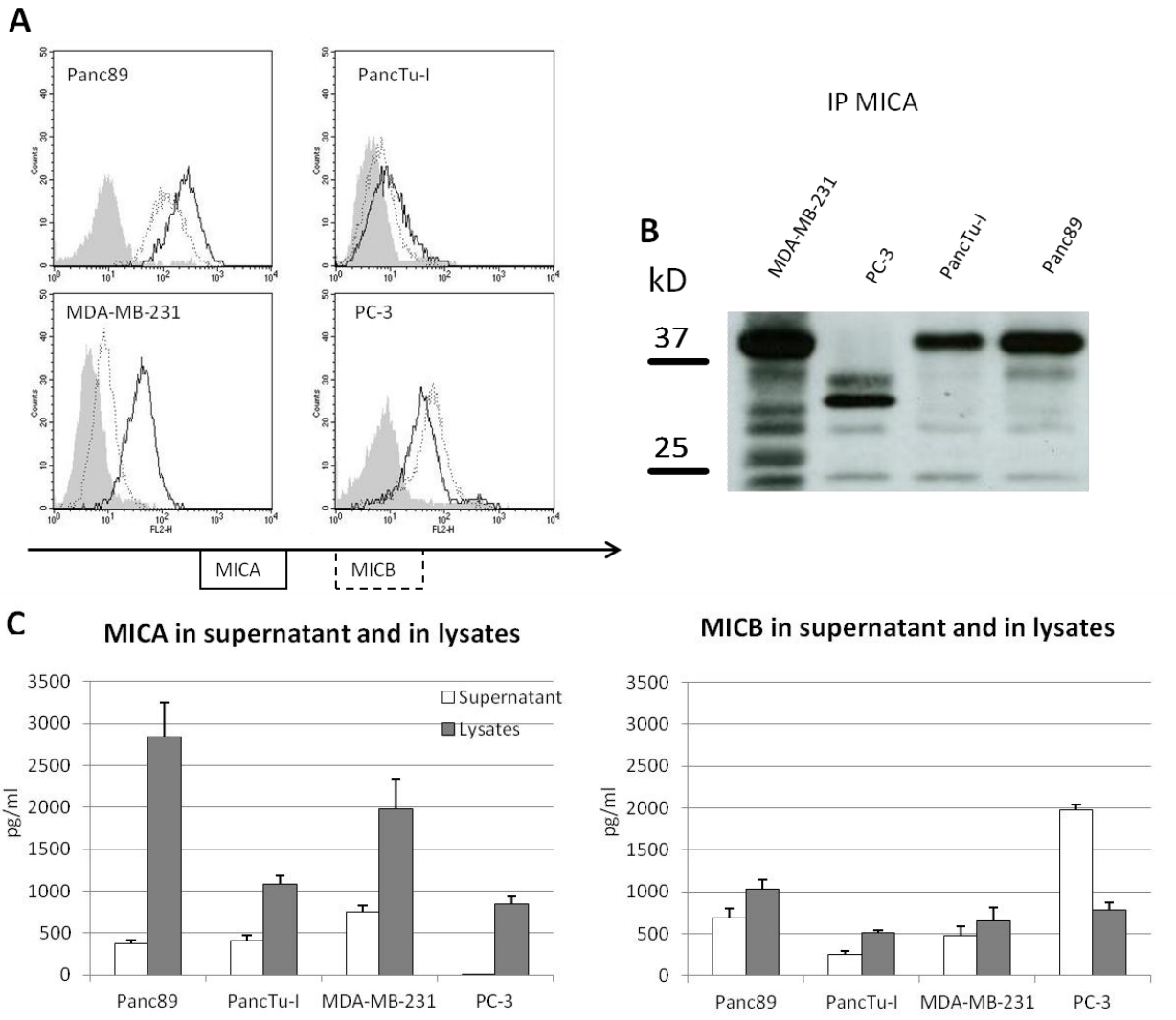

Fig.1 Epithelial cancer cell lines express and release soluble MICA and MICB into the supernatant. Pancreatic cancer cell lines Panc89 and PancTu-I, the mammary cancer cell line MDA-MB-231 and the prostate cancer cell line PC-3 were cultivated for 24 hours. (A) Cell surface expression of MICA (solid lines) and MICB (dotted lines) was determined by flow cytometry. Shaded peaks represent the Isotype control. (B) Harvested supernatants (white) and cell lysates (grey) were analyzed for the amount of MICA (left) and MICB (right) by ELISA. The data shown are mean of three different experiments with +/-SEM. (C) From cellular lysates immunoprecipitated and deglycosylated MICA was separated in SDS-PAGE and detected in western blot.

In summary all examined cancer cell lines express MICA and MICB and display it on the cellular surface. Moreover, soluble MICA and MICB are released into the supernatant. However, despite the expression of MICA, PC-3 cells do not secrete this protein.

6.2 Aspartate, cysteine and serine proteases do not affect the proteolytic cleavage of MICA and MICB

The observation that all analyzed cell lines express and release NKG2D ligands into the conditioned media, indicated that proteolytic cleavage within an extracellular site might release a soluble ectodomain of MICA/B. In order to define the proteases responsible for this process referred to as shedding, selected broad-band inhibitors for individual protease families were used. Panc89, PancTu-1, PC-3 and MDA-MB-231 cells were incubated with or without inhibitors targeting aspartate (Pepstatin), cysteine (Leupeptin) and serine (AEBSF) proteases at non-toxic concentrations. After 24 hours of incubation, the levels of released soluble MIC molecules in the conditioned medium were determined by ELISA.

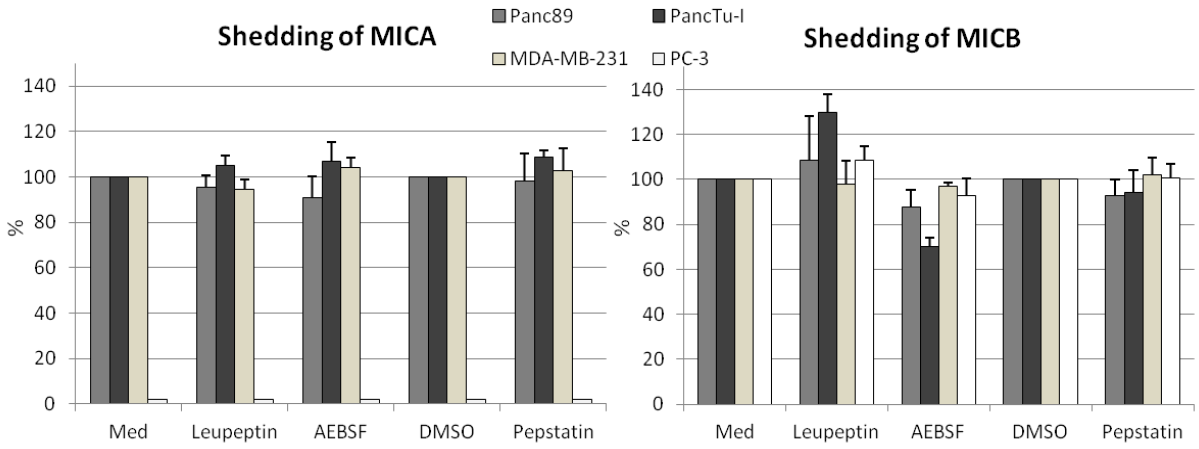

Fig.2 Aspartate, cysteine and serine proteases do not cause the release of MICA and MICB. Cells were treated for 24 hours with Leupeptin (100 µM), inhibitor of cysteine proteases, with Pepstatin (10 µM in DMSO) for inhibition of aspartate proteases and with AEBSF (25-50 µM) for serine proteases. Medium and DMSO (0.02%) served as a control. (A) Harvested supernatants were analyzed for the amount of MICA (left) and MICB (right) by ELISA. The value of control sample was set to 100%. The data shown are the mean of 3-4 different experiments with +/-SEM.

The results showed that none of these inhibitors affected the proteolytic cleavage of endogenous MICA and MICB molecules (Fig.2). Only the serine protease inhibitor AEBSF slightly reduced the amount of MICB detected in the conditioned medium of PancTu-I cells. Of note, PancTu-I cells released the lowest amount of MICB in the supernatant.

Thus, aspartate, cysteine and serine proteases are not responsible for the shedding of endogenous MICA and MICB molecules from the examined cell lines.

6.3 Metalloproteases are responsible for the basal and relative shedding of MICA and MICB

Since members of the aspartate, cysteine and serine protease families do not account for the release of MICA/B, we investigated the role of metalloproteases employing a combination of the broad range inhibitors GM6001 and TAPI-I. Cells were incubated in the presence or absence of inhibitors at non-toxic concentrations for 24 hours. The absolute shedding of MICA/B was assessed by the analysis of the harvested supernatant by ELISA. For the determination of relative shedding, the amount of the protein released into conditioned medium was divided by the amount detected in cellular lysates. The MICA/B concentration determined in untreated control samples was defined as 100%. In parallel, the modulation of cell surface expression was analyzed by flow cytometry. The results demonstrate that the treatment with GM6001/TAPI-1 dramatically decreased the shedding of MICA/B molecules and accordingly increased the cell surface (Fig.3) expression.

Inhibition of metalloproteases significantly decreased the shedding of MICA and MICB. In line with these results, the cell surface expression of MICA was upregulated on the cell surface of Panc89, PancTu-I and MDA-MB-231 cells. Again, PC-3 cells expressed MICA, but in accordance with the observation that they do not release MICA, the cell surface expression of this molecule was not affected by inhibitors of metalloproteases. Despite the fact that inhibition of metalloproteases significantly inhibited the release of MICB, surface expression was hardly modulated in PancTu-I and MDA-MB-231 cells. However, in Panc89 and PC-3 cells MICB surface expression was increased following the inhibition of metalloproteases.

These results imply that metalloproteases mediate the shedding of endogenous MICA/B molecules from human tumor cells.

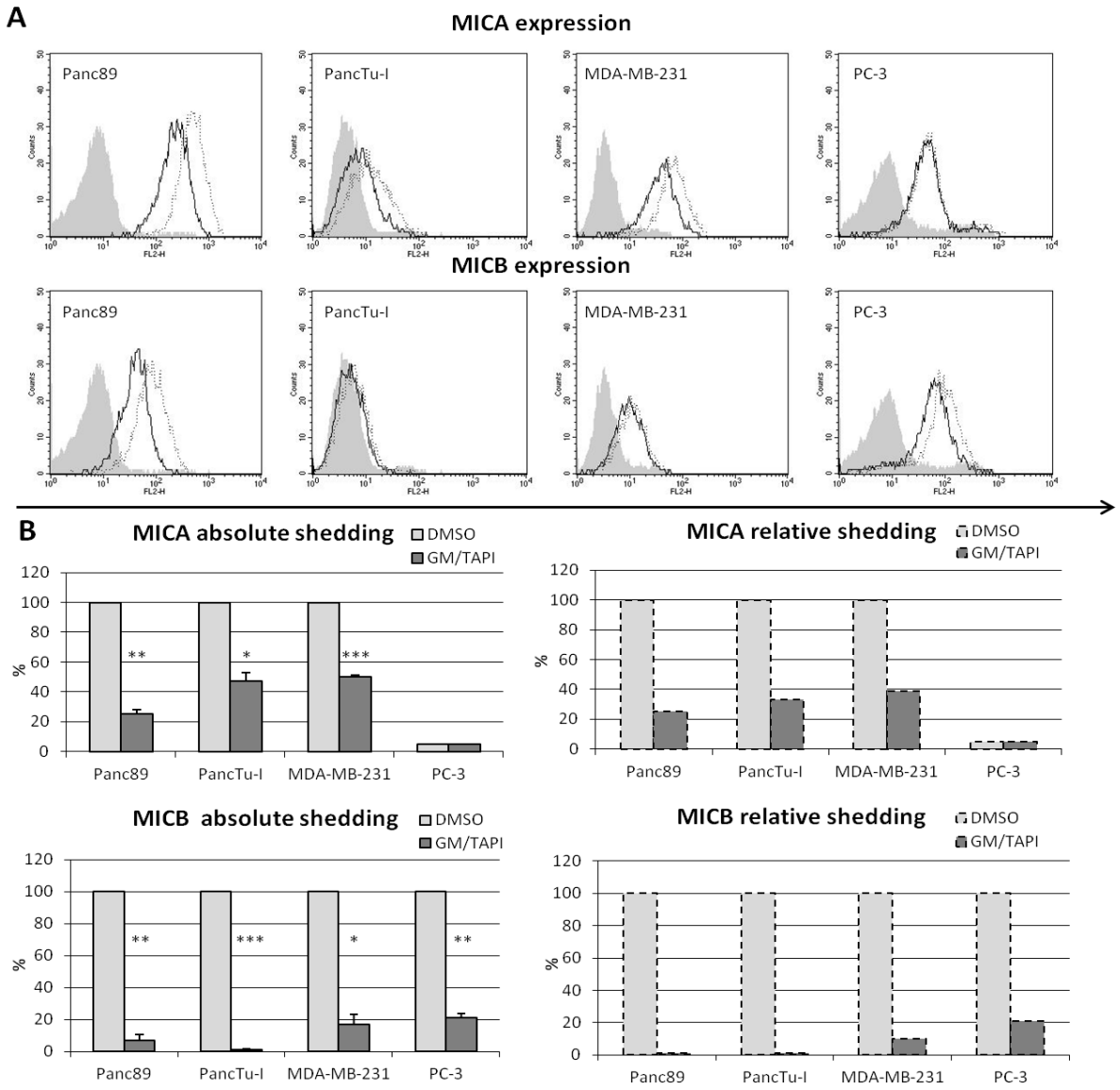

Fig.3 The inhibition of metalloproteases reduces the MICA and MICB release into supernatant and modulates their cell surface expression. Cells were treated with broad range inhibitors for metalloproteases GM6001 (5 µM) and TAPI-1 (5 µM) and with DMSO as a control (>0,1%) for 24 hours. (A) Cell surface expression of MICB is determined by flow cytometry after GM6001/TAPI-1 (dotted lines) and DMSO (solid lines) treatment. Shaded peaks represent the Isotype control. (B) The supernatants and cell lysates of DMSO treated samples (grey) and GM6001/TAPI-1 treated samples (dark grey) are analyzed for the amount of MICA/B by ELISA. To determine the absolute shedding activity (left), the amount of soluble protein in supernatant was measured by ELISA. For the calculation of the "Relative shedding" activity (right), the amount of MICA/B (pg/ml) detected in conditioned medium was divided by the amount of MICA/B (pg/ml) detected in cell lysates. The value of DMSO control is set to 100%; the data shown are the mean of three to four different experiments with +/-SEM. For statistical analysis 2-tail *t*-test is performed. ***p<0.001 **p<0.01 *p<0.05

6.4 Metalloproteases are the cause of phorbolester-induced shedding of MICA and MICB

In a next step, we aimed to define the role of metalloproteases in the induced shedding of MICA and MICB. For the induction of shedding we used Phorbol 12-myristate 13-acetate (PMA), a well-known stimulator of metalloprotease-mediated shedding (Arribas et al., 1996).

To analyze the PMA-induced release of MICA/B, cells were treated with PMA in the presence or absence of the metalloproteases inhibitors GM6001/TAPI-1. Conditioned media were analyzed by ELISA for the released amount of MICA/B and in parallel cell surface expression were determined by flow cytometry. PMA significantly stimulated the shedding of MICA/B (Fig.4 B). In the case of MDA-MB-231 and PancTu-I cells shedding of MICB was not significantly increased (Fig.4 B) even when using higher concentrations of PMA (100 ng/ml instead of 50 ng/ml). Besides the induction of shedding, PMA differentially modulated the cell surface expression of the MICA/B molecules. In Panc89 and PancTu-I cells the surface expression of MICA as well MICB (Fig.4 A) was increased (solid black line), whereas it was reduced in MDA-MB-231 and PC-3 cells following PMA stimulation. As PMA might modulate MICA/B biosynthesis, cellular lysates were subjected to ELISA as well (Fig.5). In line with the results from the cell surface staining, analysis of cellular lysates by ELISA revealed that in the pancreas carcinoma cell lines PMA stimulation increased the overall expression of MIC molecules. In MDA-MB-231 and in PC-3 cells, the level of MIC molecules was reduced as a result of the induction of proteolytic cleavage. To further investigate the effect of PMA on MICA/B expression, cycloheximide, an inhibitor of protein synthesis, was used (Baliga et al., 1969). Cycloheximide was able to abolish this effect of PMA on the pancreas cancer (Panc89) cell line (Fig.6), indicating that PMA in Panc89 cells induces the synthesis of MICA/B.

Taken together, the inhibition of metalloproteases also reduced the PMA-induced shedding of endogenous MIC molecules and accordingly increased the level of MICA/B surface expression. Again, the induced expression of MICB was hardly modulated by the metalloproteases inhibition in the case of MDA-MB-231 and PancTu-I cells. These results indicate that metalloproteases are responsible for the stimulated shedding of MICA/B.

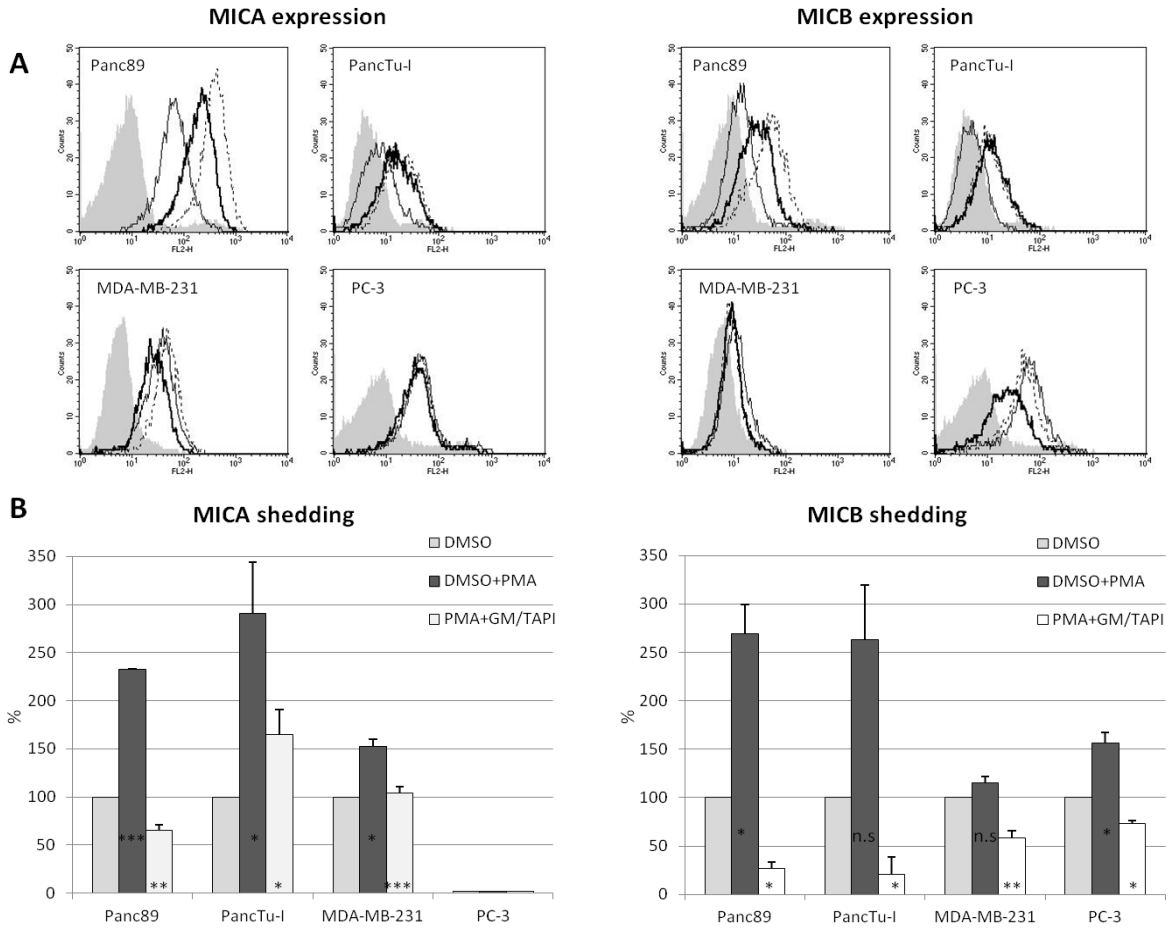

Fig.4 PMA induced shedding is inhibited by the broad range inhibitors of metalloproteases. In order to determine the role of metalloproteases in the stimulated shedding of MICA/B, carcinoma cell lines were treated with 50 ng/ml (Panc89 and PC-3) and 100 ng/ml (MDA-MB-231 and PancTu-I) PMA for 24 hours in the presence or absence of the metalloprotease inhibitors GM6001/TAPI-1. (A) Modulation of cell surface expression of MICA/B in control (thin solid line), in PMA- (thick solid line) and PMA/GM6001/TAPI-1-treated (dotted line) samples was analyzed by flow cytometry. Closed peaks represent the Isotype control. (B) Released amount of MICA/B was detected in the conditioned medium by ELISA. Data shown are the mean of 3 different experiments +/-SEM. For statistical analysis, 2-tail *t*-test is performed. n.s not significant; ***p<0.001 **p<0.01 *p<0.05

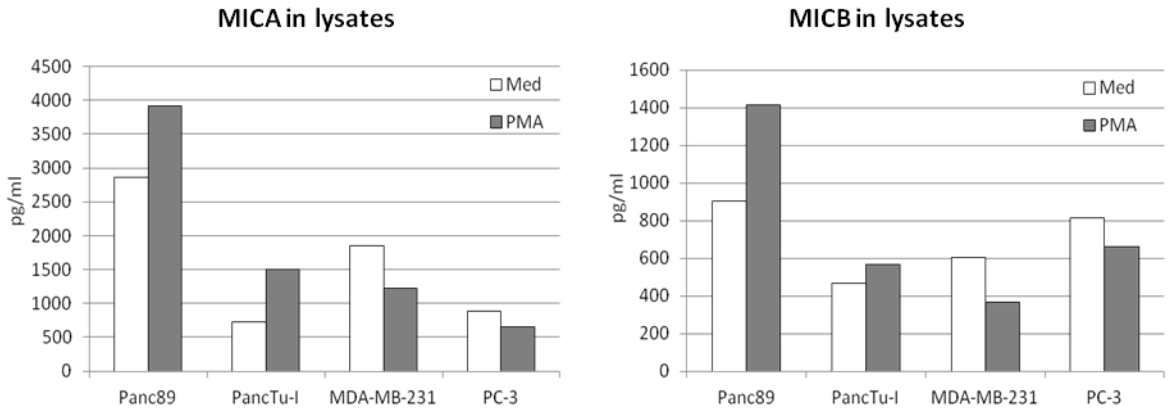

Fig.5 PMA stimulation differently modulated the endogenous MICA/B expression in epithelial cancer cell lines. Cells were cultivated in vitro for 24 hours with (grey columns) or without (white columns) PMA (50-100 ng/ml). Cellular lysates were analyzed for the amount of MICA (left) and MICB (right) by ELISA. One experiment out of three is shown.

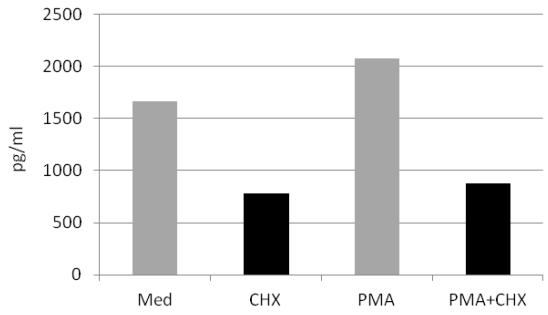

Fig.6 PMA-induced expression of MICA is abolished by Cycloheximide (CHX) in Panc89 cells. Panc89 cells were cultivated for 24 hours with non-toxic concentrations of PMA (50 ng/ml), CHX (0.15 µg/ml) and a combination of both. One representative experiment is shown.

6.5 The particular role of ADAMs in MICA/B shedding

The identification of metalloproteases as MICA/B sheddases posed the question which individual members of this heterogeneous group of proteases mediate the proteolytic cleavage of endogenous MICA/B molecules. The members of A disintegrin And Metalloprotease (ADAM) family are involved in the ectodomain shedding of a wide variety of cell surface molecules (Huovila et al., 2005) and ADAM10 and ADAM17 have already been implicated in the release of sMICA/B in overexpression systems (Waldhauer et al., 2008, Boutet et al., 2009). MDA-MB-231, the mammary cancer cell line with the highest shedding rate of MICA and PancTu-I, the pancreatic cancer cell line with the lowest shedding rate of MICA were transfected with siRNAs targeting ADAM10 and ADAM17 and appropriate control siRNAs. After 72 hours, the medium was changed and the transfected cells were further incubated in the presence or absence of PMA for additional 24 hours to address induced shedding as well. The efficacy of downregulation was confirmed by western blotting and flow

cytometry (Fig.7,8 B). In MDA-MB-231 cells (Fig.7 A) the downregulation of ADAM17 significantly decreased the amount of soluble MICA/B, whereas shedding was hardly affected by the downregulation of ADAM10. In contrast, in PancTu-I cells (Fig.8 A) both proteases were responsible for the shedding of MICB. In case of MICA, downregulation of ADAM17, and not ADAM10 inhibited the shedding in PancTu-I cells. Exactly the same picture was observed in the case of PMA-stimulated shedding.

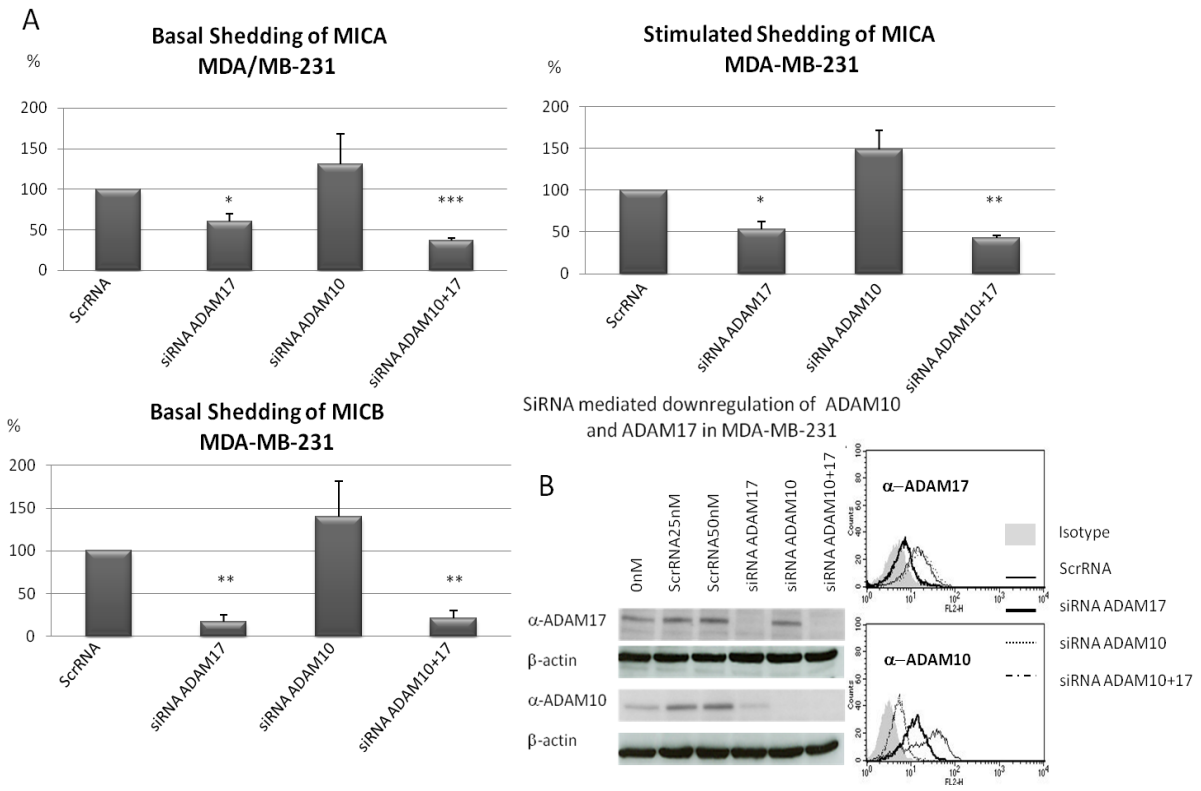

Fig.7 ADAM17 is major cause of MICA/B shedding from MDA-MB-231. (A) To define the role of ADAM10 and ADAM17 in the shedding of MICA/B MDA-MB-231 cells were transfected with siRNAs targeting A Disintegrin And Metalloproteases family members ADAM10 and ADAM17 or with scrambled RNA as a control. 72 h after transfection the medium was changed and cells were incubated for another 24 hour with or without PMA. The amount of soluble MICA/B released into the supernatant was assessed by ELISA and the amount of protein released from control RNA transfected cells was set to 100%. (B) The effective silencing of ADAM10/17 was confirmed by Western blotting and flow cytometry. Data shown are the mean of three to four independent experiments +/- SEM. For statistical analysis, a 2-tail *t-test* was performed. ***p<0.001 **p<0.01 *p<0.05

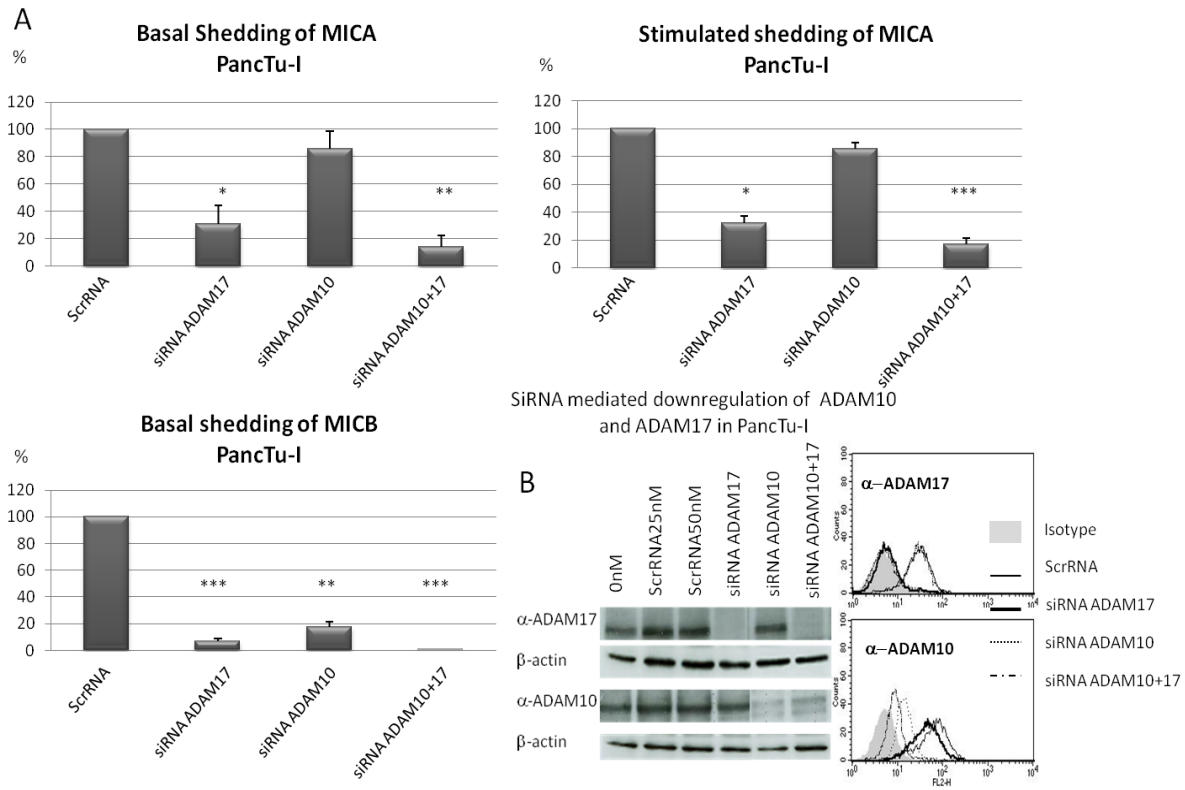

Fig.8 ADAM10 and 17 revealed to be the major cause of MICA/B shedding from PancTu-I cells. (A) To define the role of ADAM10 and ADAM17 in the shedding of MICA/B PancTu-I cells were transfected with siRNAs targeting A Disintegrin And Metalloproteases family members ADAM10 and ADAM17 or with scrambled RNA as a control. 72 h after transfection the medium was changed and cells were incubated for another 24 hour with or without PMA. The amount of soluble MICA/B released into the supernatant was assessed by ELISA and the amount of protein released from control RNA transfected cells was set to 100%. (B) The effective silencing of ADAM10/17 was confirmed by Western blotting and flow cytometry. Data shown are the mean of three to four independent experiments +/- SEM. For statistical analysis, a 2-tail *t-test* is performed. ***p<0.001 **p<0.01 *p<0.05

In summary, the specific inhibition of ADAM10 and ADAM17 by small interfering RNAs revealed the particular role of ADAM17 and/or ADAM10 in the cleavage of endogenous MICA/B molecules.

6.6 Sequence based typing of MICA

In order to determine which MICA alleles were expressed in the tested carcinoma cell lines, the samples of genomic DNA were sent to the Institute of Transplantation Immunology and Clinical Transfusions Medicine in Ulm, Germany (Furst et al., 2011). Sequence-based typing of exon2-5 of MICA alleles revealed that all the cells, used in this study were heterozygous for MICA expression. Panc89, PancTu-I and MDA-MB-231 cells harbored MICA alleles with the full cytoplasmic part, whereas PC-3 cells expressed the truncated form of MICA, (MICA*008:01:01) that lacks the cytoplasmic domain (Table 1). (http://www.ebi.ac.uk/imgt/hla/stats.html).

Table 1 **Allelic variations of MICA in the tested carcinomas.** All but PC-3 cells express MICA full cytoplasmic anchor. PC-3 cells harbor one MICA allele *008:01:01 lacking the cytoplasmic tail.

Panc89	PancTu-I	MDA-MB-231	PC-3	
*009:01	*002:01	*004	*008:01:01	MICA alleles
*027	*009:01	*027	*012:01	MICA alleles

7. Discussion

The antitumor capacities of cell-bound NKG2D ligands and in contrast, tumor promoting properties of cleaved, soluble NKG2D ligands have been described many times and the perspective of targeting the proteases responsible for the cleavage of NKG2D ligands has been under intensive investigation. Different research groups showed the prominent role of various metalloproteases in this process. However, most of the studies have been performed in artificial experimental systems using NKG2D ligand over expressing cells, whereas the proteases responsible for the shedding of endogenously expressed NKG2D ligands have not been precisely defined.

In this in vitro study we have observed that different carcinoma cells, including mammary, pancreatic and prostate carcinomas are constitutively expressing and releasing MICA/B molecules into the culture medium. However expression and shedding of MICA/B varies from cell line to cell line. All tested carcinomas differentially express MIC molecules on the cell surface and their cell surface density is associated to respective shedding activity. In PancTu-I cells, especially MICB expression is extremely low and this is accompanied by the release of very low amounts of soluble MICB into the medium. In contrast to MICB, MICA expression is overall higher in all but prostate cancer PC-3 cells. In this particular cell line, we could not detect soluble MICA in the supernatant, despite cell surface expression of this molecule. We hypothesized that the reason for the lack of MICA shedding from PC-3 cells might be the expression of a truncated allelic variant of MICA as MICA*008 for example, which represents the most common MICA allele in Caucasian populations (Zhang et al., 2001). MICA*008 lacks the cytoplasmic tail resulting in a truncated form of this protein. It is released as a full length molecule in exosomes and not shed by the cell surface proteases releasing a soluble ectodomain form (Ashiru et al., 2010). The hypothesis that PC-3 cells express the truncated variant of MICA is further confirmed by sequence based typing of MICA. Genotyping of the tested carcinomas reveals that in contrast to other cells, only PC-3 cells harbor the MICA allele *008:01 and apparently we have been unable to detect the MICA-containing exosomes in ELISA. Altogether, this clearly indicates the allele-dependent regulation of MICA release. Of note, MICA*008 released in exosomes suppresses the NK cell cytotoxicity (Clayton and Tabi, 2005) and promotes tumor immune escape to higher extent than the soluble ectodomain part of MICA molecules (Ashiru et al., 2010).

In order to avoid NKG2D-mediated immune surveillance, each genetically heterogeneous tumor cell has its own cell-dependent regulating mechanism for controlling MIC expression. However, the exact contribution of allelic polymorphism in this process is poorly understood. Recently, Shafi et al have

shown that intragenic MICA polymorphism affect MICA mRNA expression levels in Chinese hamster ovary (CHO) cells (Shafi et al., 2011). However, tumors with lower MIC expression would be more resistant to NKG2D-mediated immunosurveillance while tumors expressing truncated alleles of MICA might impair the NKG2D-mediated response to a higher extent than tumors expressing MICA with a full cytoplasmic part. On the other hand, the fact that the NKG2D receptor binds individual allelic variants of MICA with different affinities shows that the expression of alleles with high binding affinities (MICA*001 and *007) might be more beneficial for cancer immunosurveillance than the expression of alleles with low binding affinities (MICA*004, *008, and *016) (Steinle et al., 2001).

Different *in vitro* and *in vivo* model studies have been performed to characterize the NKG2D Ligand shedding responsible proteases. In line with previous studies (Waldhauer et al., 2008, Boutet et al., 2009) using over-expressing cells we were able to show that the cleavage of endogenous MICA/B is dependent on metalloprotease family members, in particular on A Disintegrin and Metalloproteases (ADAM) ADAM10/17. Aspartate, cysteine or serine proteases do not contribute to the cleavage of endogenous MICA and MICB molecules. Moreover, the broad range inhibition of metalloproteases and specific targeting of ADAM10/17 by siRNA-mediated downregulation increases the cell surface expression of MICA/B and therefore the immunogenicity of tumors. However, despite the complete abrogation of MICB release from MDA-MB-231 and PancTu-I cells lines by broad range inhibition of metalloproteases, it is almost impossible to observe the increase in MICB cell surface expression. Of note, PMA, which is a well-known stimulator of ectodomain shedding, is incapable of further stimulating MICB release in the same cells, indicating another unknown, cell- or allele-specific regulatory mechanism of MICB expression. Once again, inhibition of metalloproteases fails to increase the cell surface density of MICA in PC-3 cells expressing MICA*008, which confirms the metalloprotease-independent release of this particular allelic variant of MICA.

SiRNA-mediated inhibition of ADAM10/17 in PancTu-I and MDA-MB-231 cells has shown a remarkable degree of heterogeneity in MICA/B shedding. MICA shedding is mainly caused by ADAM17, whereas both ADAM10 and ADAM17 are responsible for the cleavage of MICB in PancTu-I cells indicating a high degree of variability in terms of protease-substrate specificity. Apart from ADAM10/17 one cannot exclude that other metalloproteases might play a role in the shedding of MICA/B. For instance MMP-9 (Sun et al., 2011), MMP-14 (Liu et al., 2010) and ADAM-9 (Kohga et al., 2010) have been reported as mediators of MICA shedding in various types of cancer cells. The diversity of MIC shedding responsible proteases points out the need of further investigation of this complex shedding machinery for the specific targeting of MIC cleavage and for the development of more effective therapeutic strategies based on the blocking of NKG2D ligand shedding.

Due to the tumor promoting properties of metalloproteases (Duffy et al., 2011), the therapeutic potential of their inhibitors in cancer treatment has been broadly investigated. In contrast to successful preclinical testing of these agents (Wilson et al., 1997, Stetler-Stevenson, 1999, Huang et al., 2011) the significant evidence of efficacy has not been observed in clinical trials due to their high toxicity and the lack of both specificity and selectivity (Fingleton, 2007). Furthermore, none of the ADAM17 inhibitors have passed a Phase II clinical trial for similar reasons (DasGupta et al., 2009). Nevertheless, a new generation of synthetic, low molecular weight inhibitors targeting ADAM10/17 specifically, for example INCB7839 (Fridman et al., 2007), are well tolerated and have been shown to inhibit tumor cell growth in several preclinical models (Zhou et al., 2006). Currently these agents are undergoing clinical investigations in breast cancer patients. Preliminary reports of these studies show a promising clinical activity of INCB7839 as a single therapeutic agent as well as in combination with EGFR-targeting immunotherapies (Infante et al. 2007). Antitumor activity of INCB7839 might partially depend on the inhibition of NKG2D ligand shedding. These specific inhibitors of ADAM10/17 represent the perfect tool for targeting MICA/B shedding and clinical use of these compounds might improve NKG2D-based cancer immunosurveillance and immunotherapy.

Different therapeutic strategies have been proposed to boost the NKG2D system-mediated cancer immunosurveillance including the removal of soluble MICA by neutralizing antibodies (Jinushi et al., 2006) or usage of *ex vivo* expanded cytokine-induced killer (CIK) cells targeting tumor cells based on their NKG2D receptor ligation (Leemhuis et al., 2005, Linn and Hui, 2010). However, this type of treatment would not be effective in patients with low or no MICA/B expression. Therefore, we assume that the selection of patient populations with lower expression of NKG2D ligands and pretreatment with selective ADAM10/17 inhibitors might improve the clinical outcome of NKG2D-system based immunotherapies. On the other hand, patients harboring truncated allelic variants of MICA would not benefit from this type of treatment. Nevertheless, the contribution of other NKG2D ligands in cancer immunosurveillance must be considered as well.

8. Summary

The interaction of the MHC class 1-related chain molecules (MIC) A and B with the corresponding Natural Killer Group 2D receptor elicits cytotoxicity of Natural Killer cells and T cell subsets. Albeit absent in normal tissue, these molecules are constitutively expressed on transformed cells and play an important role in tumor immunosurveillance. Consequently, the ectodomain shedding of MICA and MICB is regarded as an important mechanism of the immune escape of cancer cells. However, the proteolytic machinery responsible for the shedding of endogenous MICA/MICB from tumors has not been well defined.

In this study, we analyzed different human tumor entities including mammary, pancreatic and prostate carcinomas for the expression and shedding of endogenous MICA and MICB molecules. Flow cytometry and ELISA revealed that all the tested cells constitutively expressed MICA and MICB on the cell surface and also released NKG2D ligands into the supernatant. Inhibitor studies showed that metalloproteases are responsible for both the constitutive and phorbolester-induced generation of soluble MICA/B, whereas aspartate, cysteine and serine proteases are not involved in this process. Consequently, the inhibition of metalloproteases reduced the level of released MICA/B and increased cell surface expression. In the prostate carcinoma cell line PC-3, MICA was not shed at all, despite expression of these molecules on the cell surface. Genotyping of this cell line showed that the reason for this discrepancy was the expression of the truncated allelic variant, MICA*008:01, indicating an allele-specific regulation of this process. Studies employing RNA interference not only revealed a prominent role of a disintegrin and metalloprotease (ADAM) 10 and 17 in the shedding of NKG2D ligands but also a differential susceptibility of MICA to the proteolytic activity of ADAM10/17.

Altogether, inhibition of shedding responsible proteases lowers the release of tumor-promoting soluble MICA/B and increases the cell surface density of these molecules and in that way presumably also the immunogenic potential of tumors. The detailed analysis of the proteolytic machinery responsible for the shedding of NKG2D ligands such as MICA/B from tumor cells might open the field for new strategies in tumor therapy.

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Education

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In frame of the Collaborative Research Center (CRC) 877, Project A7, Expression and shedding of MICA and MICB molecules in different human tumor entities (Supervisor Prof. Dr. med. D. Kabelitz)

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· Tbilisi State Medical University (Faculty of Medicine)

Scientific Trainings and Courses

· Autumn School of Immunology, Bad Schandau, Germany 2011

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