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ORIGINAL ARTICLE

MHC class I chain-related molecule A and B expression is upregulated by cisplatin and associated with good prognosis in patients with non-small cell lung cancer

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Abstract MHC class I chain-related molecule A and B (MICA/B) are NK group 2 member D (NKG2D) ligands, which are broadly expressed in transformed cells. Both DNA damage-induced ataxia-telangiectasia-mutated (ATM)- and ATM and Rad3-related protein kinases (ATM–ATR) signaling and oncogene-induced PI3K–AKT signaling regulate the expression of NKG2D ligands, which promote NK cell-mediated cytotoxicity via NKG2D–NKG2D ligand interactions. NKG2D ligand overexpression was recently reported to be correlated with good prognosis in several types of cancer. However, the prognostic significance of NKG2D ligands in non-small cell lung cancer (NSCLC) remains unclear. Here, MICA/B expression was evaluated based on immunohistochemistry of 91 NSCLC samples from patients following radical surgery. In addition, expression of MICA/B was assessed in NSCLC cell lines treated with cisplatin in order to evaluate the regulatory mechanisms of MICA/B expression. Overall, 28 out of 91 (30.8 %) specimens showed high expression level of MICA/B, which was associated with low ^{18}F-fluorodeoxyglucose uptake and manifestation of adenocarcinoma. After a median follow-up of 48.2 months, high MICA/B expression was associated with good recurrence-free survival ($p = 0.037$). In vitro assays using cell lines revealed that MICA/B expression was upregulated by cisplatin via ATM–ATR signaling, resulting in enhanced NK cell-mediated cytotoxicity. Upregulated MICA/B expressions in patients with radically resected NSCLC are predictive of good disease prognosis. Cisplatin-induced MICA/B upregulation is possibly an indirect mechanism by which the innate immune system eliminates tumor cells. NKG2D–NKG2D ligand-targeting therapy is a promising avenue for future immune-chemotherapy development.

Keywords Non-small cell lung cancer (NSCLC) · Positron emission tomography (PET) · Prognostic factor · MICA/B (MHC class I chain-related molecule A and B) · NK cell · Cisplatin

Abbreviations

Ad	Adenocarcinoma
AIS	Adenocarcinoma in situ
ATM	Ataxia-telangiectasia-mutated
ATR	Ataxia-telangiectasia-mutated- and Rad3-related protein kinases
CEA	Carcinoembryonic antigen
CI	Confidence interval
CSC	Cancer stem cell
FDG-PET/CT	^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography
HR	Hazard ratio
ΔMFI	Increase in mean fluorescence intensity
rMFI	Relative mean fluorescence intensity
MICA/B	MHC class I chain-related molecule A and B
NKG2D	NK group 2 member D
NSCLC	Non-small cell lung cancer

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OS	Overall survival
RFS	Recurrence-free survival
SIS	Squamous cell carcinoma in situ
Sq	Squamous cell carcinoma
SUVmax	Maximum standard uptake value
ULBP	UL16-binding protein

Introduction

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer-related death worldwide [1]. It is known that up to 10 % of patients with stage IA non-small cell lung cancer (NSCLC) and approximately half of the patients with stage IIB disease show relapse even after the complete surgical resection of the tumor [2]. TNM classification has generally been used as the major guide for prognostic evaluation of NSCLC [2]. However, many researchers have reported other predictive factors such as ^{18}F-fluorodeoxyglucose (FDG) uptake on positron emission tomography/computed tomography (PET/CT) [3], vessel invasion [4, 5], serum carcinoembryonic antigen (CEA) [6], and pleural lavage cytology [7].

An essential step during tumor progression is the tumor's ability to escape from the host immune system. NK cells play an important role in immunosurveillance [8]. MICA and MICB (MICA/B) are NK group 2 member D (NKG2D) ligands, which are expressed in transformed or infected cells [9], and promote NK cell-mediated cytotoxicity via NKG2D–NKG2D ligand interactions [10]. Overexpression of MICA/B or other NKG2D ligands was recently reported to be correlated with good disease prognosis in breast cancer [11], cervical cancer [12], and hepatocellular carcinoma [13, 14]. In NSCLC, high concentration of serum-soluble UL16-binding protein 2 (ULBP-2) was reported to be a poor prognostic factor [15]. However, the correlation between NKG2D expression in tumor tissues and clinical outcome has not been investigated previously. In this study, we evaluated the expression of MICA/B using immunohistochemistry in resected stage I–IIIA NSCLC and correlated its expression with patient survival.

To improve clinical outcome of resected NSCLC, cisplatin-based adjuvant chemotherapy has been established [16]. Here, we demonstrated that cisplatin upregulated MICA/B expression in NSCLC cells via the ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related protein kinases (ATR) pathways, leading to enhanced NK cell-mediated cytotoxicity. Our findings suggest that MICA/B-overexpressing tumors can be used as a predictive factor for good clinical outcome; moreover, tumors expressing low MICA/B levels represent a good target for cisplatin-containing adjuvant chemotherapy to eliminate tumor cells via the upregulation of MICA/B in NSCLC.

Materials and methods

Patients and specimens

Our research was approved by the Kawasaki Medical School ethics committee (No. 1227-2) and written informed consent was obtained from all patients for the use of specimens. This retrospective study included patients with primary NSCLC, who were evaluated using ^{18}F-FDG-PET/CT (Discovery ST Elite; GE Healthcare, Fairfield, CT) before undergoing a lobectomy with lymph node dissection in the Department of General Thoracic Surgery, Kawasaki Medical School, between January 2007 and January 2011. We excluded patients with adenocarcinoma in situ (AIS) or squamous cell carcinoma in situ (SIS), as well as those with neuroendocrine tumors, those who had other malignancies, and those who received induction radio- or chemotherapy. Histological diagnosis was based on H&E staining according to the WHO 2004 criteria [17] and the IASLC/ATS/ERS classification of lung adenocarcinoma [18]. Pathological stages were defined according to the 7th edition of the TNM classification [2]. A routine postoperative checkup including physical examination, blood cell count, serum chemistry, serum tumor markers (CEA and/or cytokeratin fragment 19), and chest radiography was performed 4 times a year for the first 2 years, 3 times a year for the third year, and twice annually thereafter. CT or ^{18}F-FDG-PET/CT was performed twice a year for the first 5 years, and annually thereafter. Brain magnetic resonance imaging was not performed routinely.

Immunohistochemical staining

MICA/B expression was determined using immunohistochemistry with a mouse monoclonal anti-MICA/B antibody (clone D-8; Santa Cruz, Dallas, TX). Specimens from surgically resected tumors were fixed in 10 % formalin for 1 or 2 days and paraffin-embedded; 4-μm sections were then cut from tissue blocks and placed on glass slides. Tissue slides were processed using a manual protocol. Briefly, tissue sections were de-paraffinized and rehydrated. Epitope retrieval was performed by heating the slides 3 times for 5 min in 0.01 M citrate buffer (pH 6) at 100 °C. Endogenous peroxidase activity was inhibited using peroxidase block solution (Dako, Santa Clara, CA) for 10 min. Slides were incubated with anti-MICA/B primary antibody (1:50 dilution) overnight at 4 °C, followed by 60-min incubation with poly-HRP-conjugated goat anti-mouse/rabbit secondary antibody. Finally, 3,3′-diaminobenzidine (DAB) substrate-chromogen system was used to visualize protein expression of MICA/B (Dako). Positive control tissue comprised sections of malignant pleural mesothelioma. The primary antibody was omitted from the negative control. The

slides were examined by 2 investigators (R. Okita and T. Yukawa) who had no prior knowledge of the corresponding clinicopathological data. Cytosolic intensity of immunoreactivity was independently scored by the investigators. The intensity scoring for cytosolic staining was defined as follows: "0", no staining; "1", weak staining; "2", moderate staining; and "3", strong staining. The MICA/B score was considered "negative" if the cytosolic staining score was low (0–1) and "positive" if the cytosolic staining score was high (2–3).

Cell culture and reagents

Human NSCLC cell lines A549, RERF-LC-KJ, and LC2/Ad were obtained from Riken BRC through the National Bio-Resource Project of the MEXT (Tsukuba, Japan). PC-9 cells were obtained from the IBL cell bank (Gunma, Japan). All cell lines were authenticated by genotyping with the PowerPlex 16 STR system (Promega, Madison, WI). Cell lines were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 in RPMI 1640 medium with 2 mM L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10 % FBS (Sigma–Aldrich, St. Louis, MO) (A549, RERF-LC-KJ, and PC-9) or 15 % FBS (LC2/ad), and 50 U/mL penicillin streptomycin (Sigma–Aldrich). For cell culture work, cisplatin (Wako, Osaka, Japan) was dissolved in DMSO (Sigma–Aldrich).

Flow cytometric analysis of MICA/B expression

Tumor cells were stained with fluorochrome-conjugated antibodies, as previously described [19, 20]. PE-labeled MICA (clone 159227) and allophycocyanin-labeled MICB (clone 236511) were obtained from R&D Systems (Minneapolis, MN). PE- and allophycocyanin-labeled anti-mouse IgG1κ (clone MOPC-21) or IgG2bκ (clone MOPC-173) were obtained from BioLegend (San Diego, CA) and used as isotype controls. Data were acquired on a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA) and analyzed with the FlowJo software 6.4.7 (Treestar, Ashland, OR). The increase in MFI (ΔMFI) was calculated as follows: (MFI with specific mAb—MFI with isotype control)/MFI with isotype control. Relative MFI (rMFI) values were calculated to compare the differences between ΔMFI values of a specific treatment and control as follows: 100 × (ΔMFI of specific treatment/ΔMFI of control) [21].

siRNA assay

Tumor cells were transfected with either ATM-targeting siRNA (Santa Cruz, #sc-29761) or control siRNA (Santa Cruz, #sc-37007) using Lipofectamine 2000 (Invitrogen), and Opti-MEM I cell culture medium (Invitrogen) as previously described [20]. After 48 h, transfected cells were harvested for further experiments.

Western blot analysis

Cell extracts were prepared using CelLytic (Sigma–Aldrich) containing protease inhibitor cocktail (Sigma–Aldrich), and the protein concentrations were determined using the BCA protein assay (Takara Bio, Kusatsu, Japan), as previously described [19, 20]. Western blot was performed to assess the expression of ATM as previously described [20]. Briefly, equal amounts of protein were separated using electrophoresis on 3–8 % NuPAGE Tris–Acetate gel (Life Technologies, Carlsbad, CA); the separated proteins were then transferred to polyvinylidene difluoride membranes using the iBlot2 dry blotting system (Life Technologies). After blocking, the blots were probed with primary antibody for 2 days at 4 °C. The following antibodies were used: ATM (Santa Cruz) or β-actin (Sigma-Aldrich). The blots were then washed, incubated with HRP-linked goat anti-mouse IgG antibody (Cell Signaling Technology, Beverly, MA) at 4 °C overnight, and visualized using an ECL prime system (GE Healthcare, Fairfield, CT) according to the manufacturer's protocol. Images were digitally captured using a LAS-4000 camera system (Fujifilm, Tokyo, Japan).

NK cell isolation

The blood samples were collected only from researchers who engaged in this study; hence, written informed consent was not required. The ethics committee at Kawasaki Medical School approved the study (No. 1217-3). NK cells were isolated as previously described [20]. Briefly, NK cells were negatively isolated using an NK cell isolation kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's protocol. This protocol typically yielded >95 % CD3-CD56 + cells. Isolated NK cells were incubated overnight with 100 IU/mL of human recombinant IL-2 (Shionogi, Osaka, Japan; provided by Professor Yoshiyuki Yamaguchi, Kawasaki Medical School, Japan).

NK cell-mediated cytotoxicity assay

NK cell-mediated cytotoxicity was assessed using the LDH release assay as previously described [20]. Briefly, untreated and CDDP-treated target cells were tested for sensitivity to NK cell-mediated lysis using the CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega), according to the manufacturer's protocol. LDH release in the supernatants was determined using a Varioskan Flash spectral scanning multimode reader (Thermo Scientific, Rockford, IL). The percentage of specific lysis was calculated according

Table 1 Clinicopathological characteristics by MICA/B expression

Characteristic		Number ($n = 91$)	MICA/B expressions		
			Low	High	p
Age	67.8 (37–83)		67.5 ± 7.8	68.4 ± 9.8	0.645
Gender	Male	59 (65 %)	44	15	0.158
	Female	32 (35 %)	19	13	
Smoking[a]	Never	33 (37 %)	21	12	0.347
	Current of former	57 (63 %)	42	15	
CEA (ng/ml)			10.8 ± 31.8	5.1 ± 4.8	0.348
SUVmax			8.3 ± 5.4	5.2 ± 4.5	**0.008**
Tumor size (mm)			30.5 ± 12.7	30.1 ± 14.4	0.890
Histology	Adenocarcinoma	71 (78 %)	44	27	**0.005**
	Squamous cell carcinoma	20 (22 %)	19	1	
Histological grade	G1	38 (42 %)	23	15	0.071
	G2	29 (32 %)	19	10	
	G3	24 (26 %)	21	3	
	G4	0 (0 %)	0	0	
Pleural invasion	Negative	59 (65 %)	39	20	0.478
	Positive	32 (35 %)	24	8	
Lymphatic invasion	Negative	66 (73 %)	43	23	0.210
	Positive	25 (27 %)	20	5	
Vascular invasion	Negative	54 (59 %)	33	21	0.064
	Positive	37 (41 %)	30	7	
Lymph node metastasis	N0	72 (79 %)	47	25	0.163
	N1-2	19 (21 %)	16	3	
Pathological stage	IA	35 (38 %)	20	15	0.063
	IB-IIIA	56 (62 %)	43	13	

[a] Data not available for one patient

to the following formula: % specific lysis = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). To determine the involvement of NKG2D in the cytotoxicity of NK cells, effector cells were co-incubated with 20 μg/mL of anti-NKG2D blocking antibody (clone 1D11; BioLegend) or an isotype-matched control antibody (clone 11711; R&D Systems).

Statistical analysis

Chi-square tests or Fischer exact tests were performed to compare the expression level of MICA/B between patients. Kaplan–Meier survival analysis was performed to determine the association between MICA/B expression and recurrence-free survival (RFS) or overall survival (OS); the significance of the differences in RFS or OS between groups was estimated using a log-rank test with GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA). Univariate and multivariate analyses were performed using the Cox proportional hazards model to identify the independent prognostic factors. Statistical analyses were performed using the SPSS statistical package 17.0 (SPSS, Chicago, IL). In all cases, $p < 0.05$ was considered significant.

Results

Clinical characteristics

Between January 2007 and January 2011, 269 patients with primary NSCLC underwent surgical resection in our department, and 91 of these patients were selected for this study. Patients with limited resection (segmentectomy or wedge resection), non-curative resection (R1-2), carcinoma in situ (AIS or SIS), neuroendocrine tumor, or adenosquamous carcinoma, as well as those that received radiotherapy or chemotherapy before the surgery, or those who were not evaluated using ^{18}F-FDG-PET/CT were excluded from the study. The median follow-up time was 41.4 months (range 1–80 months) for RFS, and 48.2 months (range 1–89 months) for OS. The clinicopathological characteristics are summarized in Table 1. Patient age ranged from 37 to 83 years of age (mean, 67.8 years). The study included

59 male and 32 female patients. Following histological assessments, 71 tumors were diagnosed as adenocarcinoma and 20 were diagnosed as squamous cell carcinoma. Based on pathological staging, 65 cases were classified as stage I, 17 cases were classified as stage II, and 9 cases were classified as stage IIIA.

MICA/B overexpression as an indicator of good prognosis in resected NSCLC

Of the 91 tumors, 28 (30.8 %) showed MICA/B overexpression in the cytosol. Representative MICA/B immunohistochemical staining patterns are shown in Fig. 1a–d. MICA/B overexpression was correlated with maximum standardized uptake value (SUVmax) on ^{18}F-FDG-PET/CT ($p = 0.008$) and with histology ($p = 0.005$) (Table 1).

Survival analysis was performed in 91 patients who underwent curative resection for clinical Stage I–IIIA NSCLC. The median follow-up time was 48.2 months (range 1–89 months). The results indicated that MICA/B overexpression was associated with good RFS ($p = 0.037$), but had only a marginal effect on OS ($p = 0.095$) (Fig. 1e, f). Cox regression analyses were performed to determine the predictive value of clinical variables for RFS. Univariate analysis showed that lymphatic invasion, vascular invasion, lymph node metastasis, and MICA/B overexpression were potential predictors of RFS. In line with the TNM staging system for NSCLC, multivariate analysis showed lymph node metastasis to be a poor prognostic factor [hazard ratio (HR) 5.683, $p = 0.005$] for RFS, but identified MICA/B overexpression as an independent good prognostic factor for RFS (HR 0.303, $p = 0.046$) (Table 2). The association between clinical variables and OS was further investigated using Cox regression analyses. Univariate analysis showed that pleural invasion, vascular invasion, and lymph node metastasis were potential predictors of OS. In addition, multivariate analysis showed lymph node metastasis to be a poor prognostic factor for OS (HR 3.910, $p = 0.022$) (Table 3).

Regulation of cisplatin-induced NKG2D ligand expression by DNA stress-induced ATM–ATR signaling

We examined the effect of the cytotoxic reagent cisplatin on proliferation of NSCLC cells using the WST assay. In comparison with the other investigated cell lines, the RERF-LC-KJ cell line tended to be more sensitive to cisplatin (Fig. 2a). To analyze the ability of cisplatin to influence MICA and MICB expression, the 4 NSCLC cell lines were cultured in the presence or absence of 1 or 10 μM cisplatin for 24 h. Cisplatin upregulated MICA expression in the A549 cell line, but had only a marginal effect on the other 3 cell lines. In addition, cisplatin upregulated MICB expression in A549, PC-9, and LC2/ad cells but did not influence MICB expression in RERF-LC-KJ cells (Fig. 2b and Supplementary Fig. 1).

It was previously demonstrated that the DNA stress sensing ATM–ATR pathway can regulate the expression of cytotoxic reagent-induced NKG2D ligands [22]. To assess and confirm the influence of ATM–ATR signaling on MICA/B expression, ATM expression was silenced in A549 and PC-9 cells using RNAi. Western blot analysis showed that the addition of ATM-targeting siRNA led to lower ATM expression levels in both cell lines (Fig. 2c). To determine whether the ATM–ATR pathway regulates cisplatin-induced MICA/B expression in NSCLC, the expression of MICA/B was analyzed in A549 and PC-9 cells pretreated with ATM-targeting siRNA. ATM-targeting siRNA did not decrease the basal expression of MICA in A549 and PC-9 cells, which was in line with our recent report [20]. However, in both cell lines, ATM knockdown attenuated basal expression of MICB (Fig. 2d) and clearly blocked cisplatin-induced MICB (Fig. 2e).

Dependence of cisplatin-induced NK cell-mediated cytotoxicity on NKG2D–NKG2D ligand interaction

The NKG2D ligands MICA/B are engaged by the NKG2D receptor expressed in NK cells and CD8$^+$ T cells [9, 10]. Cisplatin clearly enhanced MICA and MICB expression in A549 cells and weakly upregulated MICB expression in PC-9 cells; hence, we investigated the effect of cisplatin on the sensitivity of these 2 cell lines to NK cell-mediated cytotoxicity. To verify whether this receptor is involved in the cisplatin-induced sensitivity to NK cell killing, purified NK cells were pre-treated with anti-NKG2D blocking antibody and were then subjected to the LDH release assay. In comparison with the untreated control cells, NK cell-mediated cytotoxicity was strongly enhanced in the cisplatin-treated A549 cells and less markedly enhanced in the cisplatin-treated PC-9 cells. The anti-NKG2D blocking antibody inhibited cisplatin-induced NK cell-mediated lysis of tumor cells, but treatment with an isotype control antibody had no effect (Fig. 3). Our findings suggest that cisplatin-induced NK cell-mediated cytotoxicity is dependent on NKG2D–NKG2D ligand interaction, but that enhanced cytolysis is also derived from other cisplatin effects. This implies that a direct causal relationship exists between upregulation of NKG2D ligand MICA/B expression by cisplatin and increased NK cell activity. Moreover, it is interesting to speculate why NK cell-mediated cytotoxicity toward PC-9 cells is strongly dependent on NKG2D, despite low MICA/B expression levels. One possible reason is that other NKG2D ligands such as ULBP family members or hitherto unknown ligands are strongly expressed and upregulated by cisplatin in PC-9 cells.

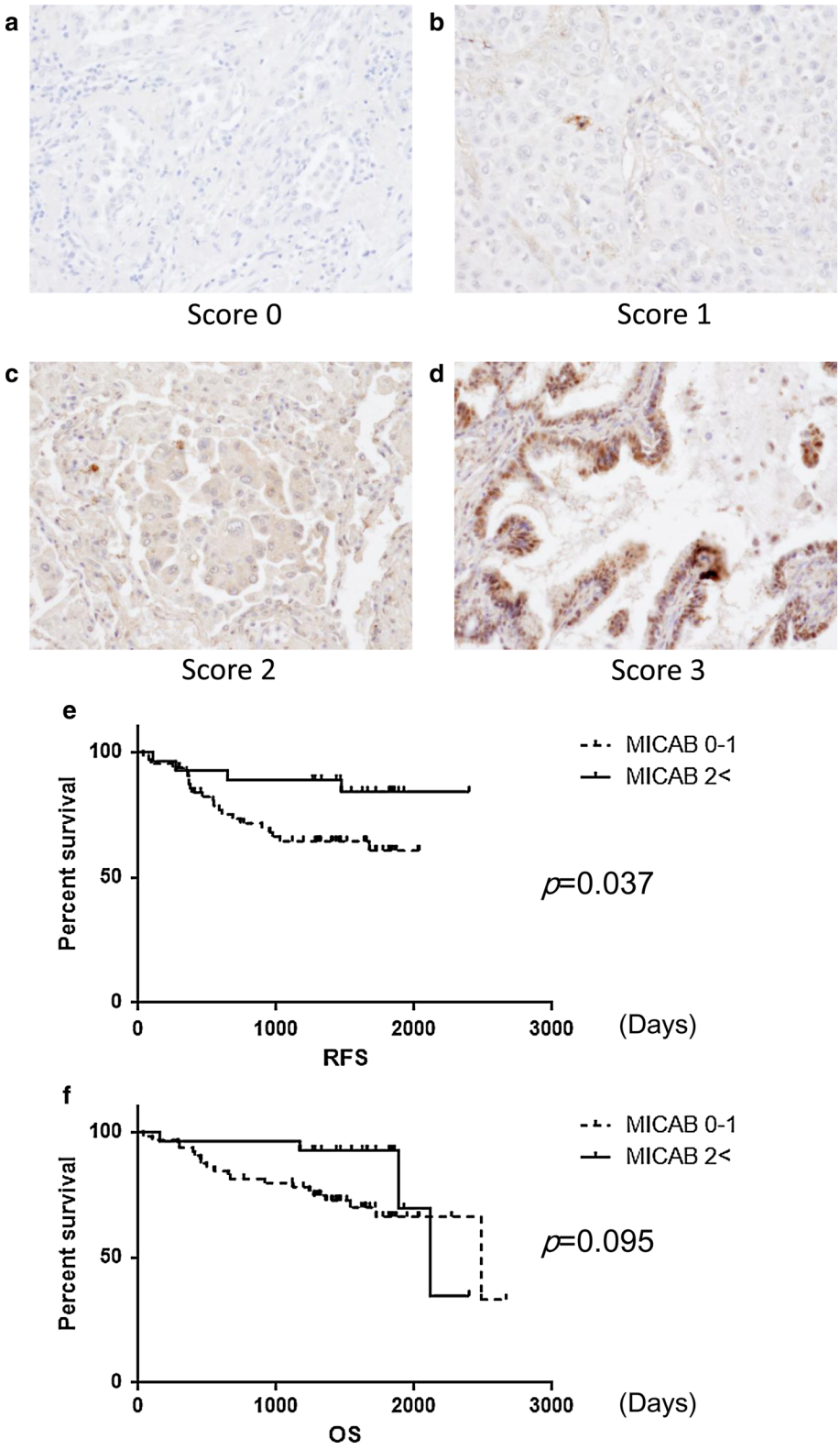

Fig. 1 MICA/B overexpression is an indicator of good prognosis in resected NSCLC. Immunohistochemical staining of MICA/B in non-small cell lung cancer (NSCLC) tissues. Representative staining for MICA/B in cancer cells (×100 magnification). The *four panels* show images corresponding to different intensity scores of MICA/B expression. **a** score 0, **b** score 1, **c** score 2 and **d** score 3. Recurrence-free survival (RFS) and overall survival (OS) in NSCLC patients. Kaplan–Meier plots showing **e** RFS or **f** OS in patients with lower (score 0–1) or higher (score 2–3) MICA/B expression

Table 2 Cox proportional hazard model for RFS (n = 91)

	Univariate		Multivariate	
	Hazard ratio (95 % CI)	*p* value	Hazard ratio (95 % CI)	*p* value
Gender (male vs. female)	0.818 (0.364–1.837)	0.624		
Smoking[a] (non-smoker vs. smoker)	1.124 (0.717–1.762)	0.612		
Histology (Ad vs. Sq)	1.070 (0.403–2.842)	0.891		
Pleural invasion (neg vs. pos)	1.347 (0.913–11.498)	0.120		
Lymphatic invasion (neg vs. pos)	5.244 (2.391–11.498)	**<0.001**	1.737 (0.508–5.942)	0.379
Vascular invasion (neg vs. pos)	2.637 (1.577–4.410)	**<0.001**	1.168 (0.559–2.441)	0.679
Lymph node metastasis (neg vs. pos)	8.087 (3.673–17.806)	**<0.001**	5.683 (1.713–18.856)	**0.005**
MICA/B expression (low vs. high)	0.331 (0.114–0.962)	**0.042**	0.303 (0.094–0.980)	**0.046**

[a] Data not available for one patient

Table 3 Cox proportional hazard model for OS (*n* = 91)

	Univariate		Multivariate	
	Hazard ratio (95 % CI)	*p* value	Hazard ratio (95 % CI)	*p* value
Gender (male vs. female)	0.554 (0.218–1.405)	0.213		
Smoking[a] (non-smoker vs. smoker)	1.264 (0.777–2.058)	0.345		
Histology (Ad vs. Sq)	0.823 (0.302–2.238)	0.702		
Pleural invasion (neg vs. pos)	1.499 (1.023–2.196)	**0.038**	1.397 (0.898–2.175)	0.139
Lymphatic invasion (neg vs. pos)	2.061 (0.900–4.720)	0.087	0.162 (0.187–2.004)	0.417
Vascular invasion (neg vs. pos)	2.247 (1.302–3.877)	**0.004**	1.542 (0.760–3.130)	0.230
Lymph node metastasis (neg vs. pos)	3.727 (1.611–8.623)	**0.002**	3.910 (1.216–12.569)	**0.022**
MICA/B expression (low vs. high)	0.533 (0.197–1.440)	0.215	0.591 (0.200–1.749)	0.342

[a] Data not available for one patient

Discussion

In this study, we have shown that overexpression of MICA/B in NSCLC cells is independently associated with good prognosis in terms of RFS. This was in line with previous studies by several groups, who reported that expression of MICA/B and other NKG2D ligands were good prognostic factors for breast cancer [11], cervical cancer [12], and hepatocellular carcinoma [13, 14]. These findings suggest that upregulation of NKG2D ligands promotes tumor susceptibility to NK cell-mediated immunosurveillance.

Interestingly, we also found that MICA/B overexpression was significantly associated with lower SUVmax and reduced adenocarcinoma histology. In addition, it increased cell differentiation in NSCLC. To the best of our knowledge, there are no previous literature regarding the relationship between MICA/B expression and SUVmax. However, Kamimura et al. [13] reported that ULBP1 overexpression was associated with enhanced cell differentiation in hepatocellular carcinoma. The SUVmax value obtained from ^{18}F-FDG-PET/CT imaging is a semiquantitative value that indicates the degree of glucose uptake at the lesion site. Tumors with high SUVmax values are considered to have higher cell proliferative potential [23], resulting in more aggressive behaviors than tumors with low SUVmax values [24]. In addition, several groups including ours have reported that expression levels of Glut-1, VEGF, p53, Ki-67, or COX2 are correlated with ^{18}F-FDG uptake in lung cancer [23, 25–29]. MICA/B expression is regulated by the DNA damage-induced ATM–ATR pathway [22] and the oncogene-induced PI3K–AKT signaling pathway [19, 21]. It is possible that MICA/B-overexpressing tumors have higher DNA damage or oncogene activation such as EGFR driver mutation [30], which has also been reported to be a good prognostic factor for cancer [31]. It is interesting that tumors with high SUVmax values had low MICA/B levels despite their aggressive potential. One possible reason is that de-differentiated tumors with high proliferative potential and high SUVmax values have been immunoselected based on lower MICA/B expression, because tumors expressing low MICA/B levels tend to escape NK cell-mediated immunosurveillance. Recently, Wang et al. [14] reported that breast cancer stem cells (CSCs) had lower MICA/B expression, contributing to resistance against NK cell-mediated cytotoxicity and resulting in metastasis. Our present results suggest that loss of MICA/B expression is associated with de-differentiation of cancer cells, and that tumors expressing low MICA/B levels are enriched in CSCs. Considering the recent advances in the field of CSC [32], we believe

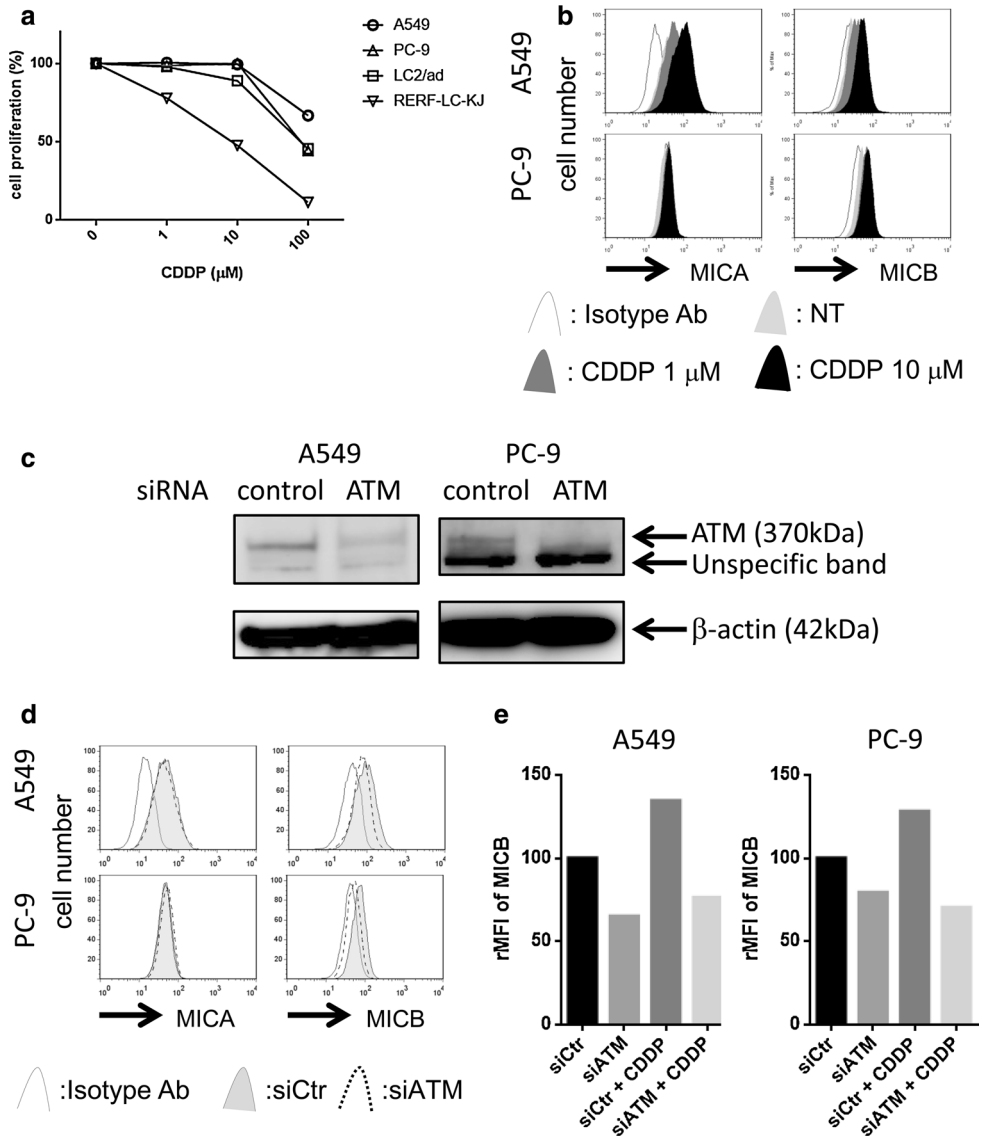

Fig. 2 Cisplatin-induced upregulation of MICB expression is regulated by the ATM–ATR pathway in A549 and PC-9 cells. **a** Four non-small cell lung cancer (NSCLC) cell lines were treated with the indicated concentrations of cisplatin for 48 h. At the end of the incubation period, WST cell proliferation assays were performed. Representative data from 3 independent experiments are shown. **b** A549 cells and PC-9 cells were cultured in the presence or absence of 1 or 10 μM cisplatin for 24 h, and the expression levels of MICA and MICB were individually assessed using flow cytometry. Representative histograms from 3 independent experiments are shown. **c** A549 and PC-9 cells were transfected with ATM-targeting siRNA or control siRNA for 48 h. The expression levels of ATM and β-actin were assessed using western blot analyses. Representative data from 3 independent experiments are shown. **d** MICA and MICB expression in A549 cells and PC-9 cells treated with ATM-targeting siRNA (siATM) or control siRNA (siCtr) for 48 h. Representative histograms from 3 independent experiments are shown. **e** MICB expression in A549 cells and PC-9 cells treated with siATM for 48 h and then with 100 μM cisplatin for 24 h. The relative MFI (rMFI) value of MICB was calculated. Representative data from at least three independent experiments are shown

that CSCs represent a promising target for NSCLC. However, further research is required before our findings can be clinically applied because considerably controversy exists regarding biological markers of NSCLC stem cells.

We also found that high MICA/B expression was associated with adenocarcinoma histology. It is well known that adenocarcinomas frequently show oncogene driver mutations such as EGFR or EML4-ALK, but that squamous cell carcinoma does not [33]. MICA/B expression can be regulated by the oncogene-activated PI3K/AKT pathway, and therefore, we hypothesized that the discrepancies in MICA/B expression between these 2 disease phenotypes are caused by different frequencies of oncogene driver mutation.

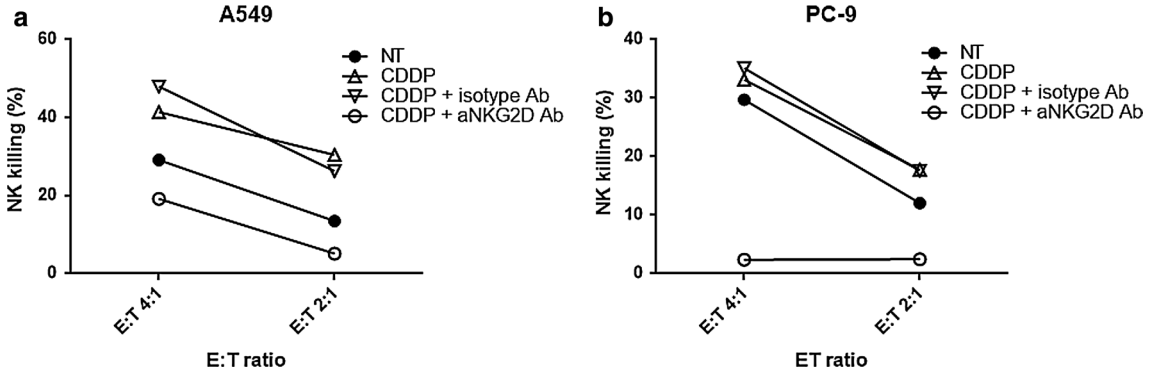

Fig. 3 Effects of cisplatin on NK cell-mediated cytotoxicity. **a** A549 cells or **b** PC-9 cells were cultured in the presence or absence of 10 μM cisplatin for 24 h and were then subjected to the LDH release assay for 4 h using IL-2 activated NK cells as effector cells. IL-2 activated NK cells were pre-treated with blocking antibodies for NKG2D or isotype control 30 min prior to the start of the cytotoxicity assay. Data are presented as the mean of triplicate samples and are representative of three independent experiments

On the other hand, our in vitro data showed that cisplatin, a key drug for NSCLC treatment as adjuvant chemotherapy, enhanced NK cell-mediated cytotoxicity via upregulation of MICA/B expression. Our findings suggest a new anti-tumor mechanism whereby cisplatin eliminates tumor cell from NSCLC patients; tumor cells are eradicated by NK cells via cisplatin-induced MICA/B expression. Many immunotherapeutic approaches including immune-checkpoint inhibitors and cancer vaccines have been developed. Recently, an NKG2D-Fc fusion protein binding multiple NKG2D ligands was shown to potently enhance the anti-tumor effect of NK cell-mediated antibody-dependent cytotoxicity against leukemia cells [34]. This suggests that MICA/B overexpression in NSCLC cells is not only a good prognostic factor for cancer, but also serves as a promising target for immune-chemotherapy using NKG2D ligand-targeting drugs in combination with cisplatin.

Taken together, MICA/B overexpression in tumor cells was correlated with good prognosis in NSCLC patients, and was associated with lower ^{18}F-FDG uptake and adenocarcinoma histology. Our findings suggest that tumors express low MICA/B levels are more aggressiveness and that administration of adjuvant chemotherapy should be considered for patients with these tumors. On the other hand, MICA/B-overexpressing tumors may be defined as a subset of tumors with lower risk of relapse, and therefore, adjuvant chemotherapy may not be required. Moreover, cisplatin-based chemotherapy may destroy tumor cells through cytotoxic effects and NK cell-mediated cytotoxicity via upregulation of MICA/B expression. Therefore, therapies targeting NKG2D–NKG2D ligand interactions may enhance the effect of cisplatin-based chemotherapy. The mechanisms of NKG2D ligand regulation in tumor cells should be further investigated to advance the field of immunotherapy and immune-chemotherapy for NSCLC.

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Author Contributions R Okita conceived, designed, and performed the experiments. R Okita and T Yukawa analyzed the data. R Okita, T Yukawa, Y Nojima, A Maeda, S Saisho, K Shimizu, and M Nakata collected clinical data and samples. R Okita and M Nakata contributed reagents/materials/analysis tools. R Okita and M Nakata wrote the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest Dr. Masao Nakata received research funding from Kyowa Kirin for this study. The sponsor had no control over the interpretation, writing, or publication of this work. All other authors declare no conflicts of interest.

Ethical approval Our research was approved by the Kawasaki Medical School ethics committee (No. 1217-3 and 1227-2).

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