Lack and restoration of sensitivity of lung cancer cells to cellular attack with special reference to expression of human leukocyte antigen class I and/or major histocompatibility complex class I chain related molecules A/B

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Both cytotoxic T lymphocytes (CTL) and natural killer (NK) cells may play major roles in the host defense against cancer. However, their relationship against the same tumor remains to be elucidated. Among 26 human lung cancer cell lines established in our laboratory, 10 (38%) exhibited human leukocyte antigen (HLA)-class I haplotype loss and three (12%) lost HLA-class I expression totally by flow cytometry analysis. The two cell lines (E522L and C831L) that lost their expression of HLA-class I *in vitro* and *in vivo* were applied for further evaluations. Genetic abnormalities of β2-microglobulin gene were observed in both E522L (loss of mRNA) and C831L (point mutation). Transduction of the wild-type β2-microglobulin gene rendered them positive for HLA-class I expression. The CTL were induced from autologous peripheral blood mononuclear cells or regional lymph node lymphocytes by stimulation with wild-type β2-microglobulin transduced-E522L or -C831L, and they showed tumor-specific cytotoxicity against wild-type β2-microglobulin-transductant, but not parental cells. In NK cell cytotoxicity, E522L showed high sensitivity to NK cells; however, C831L showed resistance despite loss of HLA-class I expression. E522L expressed MHC class I chain related molecules A/B, but C831L did not. The transduction of the MHC class I chain related molecule A gene from E522L rendered C831L positive for expression and sensitive to NK cell cytotoxicity. Reconstruction of HLA-class I and MHC class I chain related molecules A expression could abrogate evasion from cellular attack by CTL and NK cells, and it may lead to a breakthrough in the development of cancer immunotherapy. (*Cancer Sci* 2007; 98: 1795–1802)

As reported previously, many kinds of lung cancer associated antigens that are able to induce specific CTL have been identified.[1–5] Tumor-specific-CTL are one of the important effectors in cellular immunity in patients with tumor expressing HLA-class I.[6–9] NK cells also play an important role in the antitumor immune response by attacking particularly cancer cells with down-regulation or loss of the HLA-class I expression.[10,11] However, in the past, the antitumor responses by CTL and NK cells have been analyzed independently[12,13] and the relationship between them against the same tumor remains to be elucidated. CTL and NK cells showed a mutually complementary or exclusive antitumor activity against cancer cells, and their relationship should be important in considering the host defense against cancer.

Cancer cells have been reported to have several escape mechanisms from the host's immunosurveillance.[14–16] It is thought that one of the main escape mechanisms is HLA-class I abnormality.[2,17–19] In non-small cell lung cancer, the rate of down-regulation of HLA-class I expression has been reported to range from 25 to 94%.[20–22] CTL can not recognize cancer cells if they have lost HLA-class I expression. In a previous report, however, we showed that the precursors of tumor-specific CTL remained *in vivo* even though cancer cells lost their haplotype of HLA-class I, and that the CTL with strong cytotoxicity against cancer cells could be induced by reconstruction of the HLA-class I.[2] Therefore, the first strategy to overcome the tumor escape mechanism is a restoration of the HLA-class I expression on such cancer cells.

In contrast, the HLA-class I molecule is well known to play an inhibitory role in NK cell cytotoxicity. NK cells usually show high cytolytic activity against cancer cells that have lost their HLA-class I expression.[11] Therefore, the loss of HLA-class I expression is one tumor escape mechanism from CTL-mediated cytotoxicity; at the same time HLA-class I deficient cancer cells might be suitable targets for NK cells. It is thought that the second strategy for anticancer immunotherapy against HLA-class I deficient cancer is to enhance the activity of NK cells.

The present study was conducted to elucidate the precise escape mechanisms from the host's immunosurveillance using HLA-class I expression-deficient lung cancer cell lines, and to elucidate the method by which such escape mechanisms are overcome.

Materials and Methods

The study protocol was approved by the human and animal ethics review committee of University of Occupational and Environmental Health, Kitakyushu, Japan, and a signed consent form was obtained from each subject before obtaining the tissue samples used in this study.

Lung cancer cell lines. Lung cancer cell lines have been established from pericardial effusion or surgically resected samples, respectively, as described previously.[23] E522L was established from pericardial effusion of patient E522 who was a 53-year-old male with

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Abbreviations: β2m, β2-microglobulin; C831L-MICA-A5, MICA (A008/5)-transduced C831L; C831L-wβ2m, wβ2m transduced-C831L; CTL, cytotoxic T lymphocytes; E522L-wβ2m, wβ2m transduced-E522L; EBV-B, Epstein–Barr virus transformed B cell line; HLA, human leukocyte antigen; IFN-γ, interferon-γ; NK, natural killer; mAb, monoclonal antibody; MHC, major histocompatibility complex; MICA/B, MHC class I chain related molecule A/B; ORF, open reading frame; PBMC, peripheral blood lymphocyte; PCR, polymerase chain reaction; PHA, phytohemagglutinin; RLNL, regional lymph node lymphocyte; RT-PCR, reverse transcription–polymerase chain reaction; TM, transmembrane domain; wβ2m, wild-type β2m.

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Table 1. Lung cancer cell lines established in our laboratory and human leukocyte antigen (HLA)-class I abnormality

Histology	Cell lines	HLA-class I		Source of primary culture
		Surface expression	Genetic analysis	
Adenocarcinoma	A110L	+	Haplotype loss	Primary tumor
	A129L	+	Haplotype loss	Primary tumor
	A925L	+	Normal	Primary tumor
	B203L	+	Normal	Primary tumor
	B901L	+	Normal	Primary tumor
	C422L	+	Haplotype loss	Primary tumor
	D611L	+	Haplotype loss	Primary tumor
	E522L	Loss	Normal	Pericardial effusion
	F1121L	+	Normal	Primary tumor
	G821L	+	Haplotype loss	Primary tumor
	H1224L	+	Normal	Primary tumor
	K420L	+	Normal	Primary tumor
	L619L	+	Normal	Primary tumor
	L804L	+	Haplotype loss	Primary tumor
Squamous cell carcinoma	B1203L	+	Normal	Primary tumor
	C1026L	Loss	Normal	Primary tumor
	H1215L	+	Haplotype loss	Primary tumor
	L1023L	+	Haplotype loss	Primary tumor
Large cell carcinoma	A904L	+	Haplotype loss	Primary tumor
	C311L	+	Normal	Primary tumor
	C831L	Loss	Normal	Primary tumor
	J206L	+	Normal	Primary tumor
Pleomorphic carcinoma	G603L	+	Normal	Primary tumor
Adenosquamous carcinoma	A529L	+	Haplotype loss	Primary tumor
Small cell carcinoma	D1008L	+	Normal	Primary tumor
Unclassified carcinoma	F1012L	+	Normal	Subcutaneous metastasis

primary lung adenocarcinoma in the right lower lobe. C831L was established from resected primary lung cancer of patient C831 who was a 54-year-old male with large cell carcinoma of the right lung. To identify their HLA genotypes, PCR was performed by Shionogi Biomedical Laboratories (Osaka, Japan). HLA genotypes were HLA-A*0207/2402, -B*4006/5901, -Cw*0102/0801 in E522L and HLA-A*0206/2601, -B*0702/3501, -Cw*0702/0801 in C831L.[23]

Other cell lines or cells. E522 PHA blasts and C831 PHA blasts were induced from autologous PBMC. EBV-B from patient C831 was produced by an infection with supernatant from Epstein–Barr virus producer line B95.8. K562 is an erythroleukemia cell line that is sensitive to NK cell cytotoxicity. PBMC derived from healthy donor were used as NK cells after removing the monocytes without any stimulation.[12,24] Functions of NK cells were assessed by cytolytic activity against K562 when used. PLAT-A cells, which produced an infectious retrovirus, were kindly donated by Dr T. Kitamura (University of Tokyo, Tokyo, Japan).[25]

Monoclonal antibody. Anti-HLA-class I mAb (W6/32) and anti-HLA-DR mAb (L243) were used for both flow cytometry and the HLA-restriction-assay of CTL. Anti-HLA-class I mAb, EMR8-5, used for immunohistochemistry, was kindly donated by Dr N. Sato (Sapporo Medical University, Sapporo, Japan).[26] MICA/B mAb (6D4), purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), was used for the detection of MICA/B expression. Anti-NKG2D mAb (1D11), purchased from Biolegend (San Diego, CA, USA), was used for a blocking assay of NK cell cytotoxicity.

Flow cytometry analysis for HLA-class I expression of tumor cells. After staining, the samples were assessed using flow cytometry (EPICS XL; Coulter International; Fullerton, CA, USA).[23] To examine the HLA-class I expression of tumor cells *in vivo*, flow cytometry was performed with cancer cells in the pericardial effusion, which were cryopreserved at the time of pericardiocentesis in patient E522.

RT-PCR for β2m and MICA of tumor cell lines. The cDNA converted from the mRNA of each cell line served as a template for PCR using a β2m-specific primer (forward primer, 5′-ATGTCTCGCTCCGTGGCC-3′, and reverse primer, 5′-TTACATGTCTCGATCCCACTT-3′), a MICA specific primer (forward primer, 5′-ACTGCTTGAGCCGCTGAGAG-3′ and reverse primer, 5′-CTGCCTGGCTGTAGAGTCTAG-3′) and a β-actin (internal control) specific primer (forward primer, 5′-GGCATCGTGATGGACTCCG-3′ and reverse primer, 5′-GCTGGAAGGTGGACAGCGA-3′). The PCR products were sequenced using the Dye terminator V3.1 cycle sequencing kit (Applied Biosystems, Tokyo, Japan).

Transduction of wβ2m or MICA into lung cancer cell lines. Wβ2m cDNA was obtained from a lung cancer cell line, B203L. MICA cDNA (MICA-A008/5) was obtained from E522L. Wβ2m- or MICA-specific PCR products were cloned and then was inserted into a pMXs retrovirus plasmid vector.[25] To obtain retrovirus vector containing the wβ2m gene or the MICA gene, retrovirus plasmid vectors were transfected into PLAT-A cells using LipofectAmine 2000 (Invitrogen, Tokyo, Japan), and then the supernatant, including the retrovirus vector, was collected as reported previously.[25] The supernatant of the retrovirus vectors, which contained the wβ2m gene or the MICA gene, was placed on plates coated with RetroNectin (Takara, Otsu, Japan) and cancer cells were placed into the RetroNectin plates. The cells infected with retrovirus were selected with puromycin (0.1 μg/mL).[25]

Induction of autologous tumor specific CTL in patient C831 and E522. Tumor specific CTL were induced from the autologous lymphocytes obtained from PBMC in patient E522 and RLNL in patient C831, as described previously.[27] Briefly, the lymphocytes were stimulated with irradiated (100 Gy) wβ2m-transduced E522L (E522L-wβ2m) or wβ2m-transduced C831L (C831L-wβ2m)

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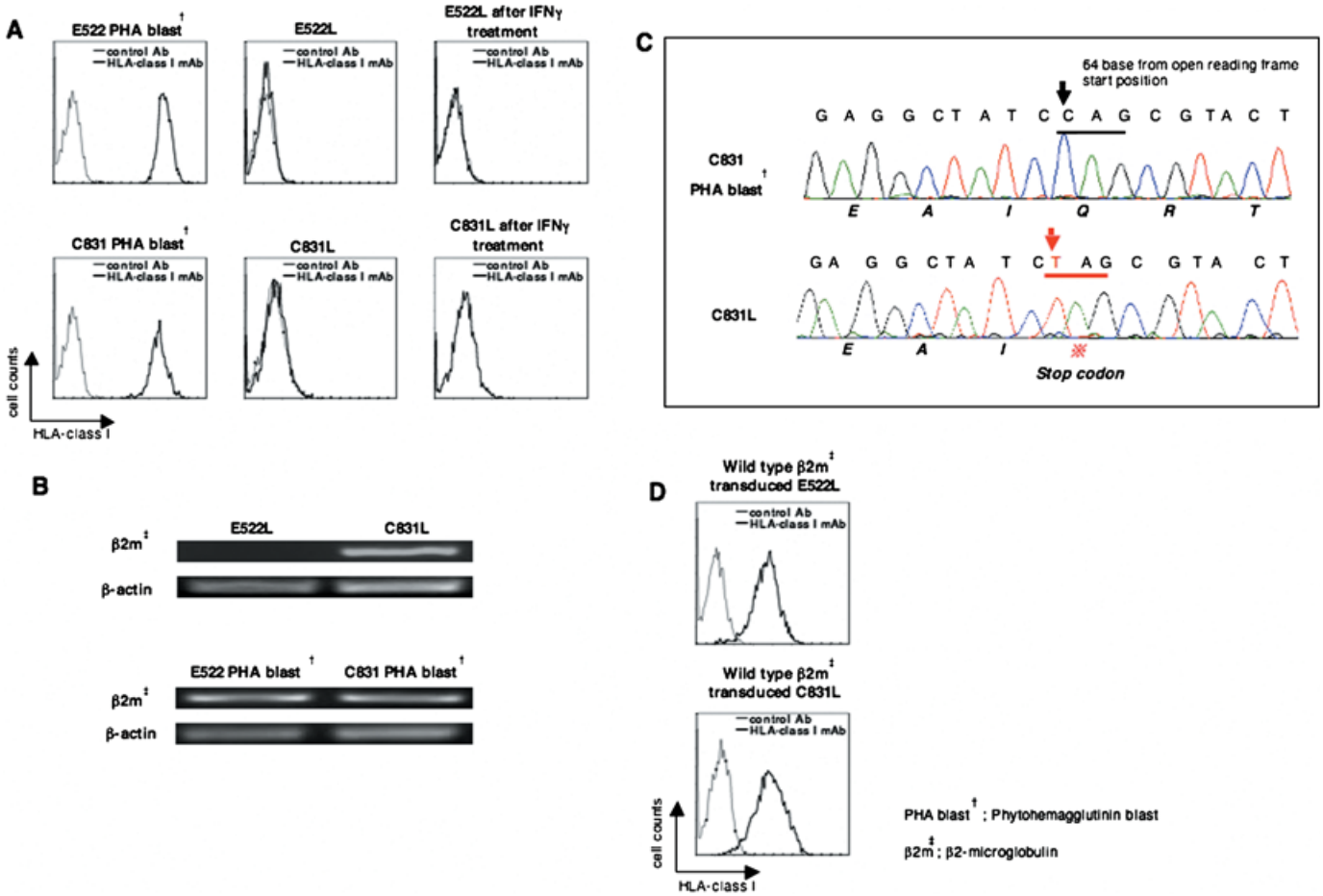

Fig. 1. The loss of human leukocyte antigen (HLA)-class I expression on cancer cells (E522L and C831L) and restoration of HLA-class I expression by the transduction of wβ2m. (A) Flow cytometry was performed to determine the cell surface expression of HLA-class I. Neither E522L nor C831L expressed HLA-class I. Phytohemagglutinin (PHA) blasts of the patients normally expressed HLA-class I. Treatment with interferon (IFN)-γ did not induce HLA-class I expression in these cancer cells. (B) The expression of the β2m gene was examined using reverse transcription–polymerase chain reaction (RT-PCR). β2m was expressed in C831L, C831 PHA blasts, and E522 PHA blasts, but not in E522L. (C) A sequence analysis of β2m was performed using PCR products. The C831 PHA blasts were identified as having wβ2m, but C831L exhibited a point mutation. Cytosine at position 64 base was substituted to thymine, thus resulting in the formation of an early stop codon. (D) After the transduction of the wβ2m gene, both E522L and C831L restored the HLA-class I expression.

weekly at a tumor-to-lymphocyte ratio of 1:10 in culture medium with 20 U/mL of rIL2 (donated by Takeda Chemical Industries, Osaka, Japan) for 3 weeks. The CTL activity was assessed at 7 days after the last stimulation. To generate the CTL clone, a limiting dilution method was performed as reported previously.[1]

Assay of CTL and NK cell activity. The cytotoxicity of CTL and NK cells was assessed using a standard ^{51}Cr release assay as described previously.[27] The CTL were co-cultured with target tumor cells at different effector : target ratios (1:1, 3:1, 10:1, 30:1). NK cell activity was evaluated at an indicated effector : target ratio (10:1, 20:1, 40:1, 80:1) for 4 h at 37°C. The CTL activity was also assessed by the cytokine production. In brief, CTL (3×10^3) was co-cultured with stimulator tumor cells (3×10^4) for 8 h, and the amount of IFN-γ in the supernatant was measured using a human IFN-γ ELISA test kit (Biosource, Camarillo, CA, USA).[2] In a blocking assay of CTL, one-quarter of the diluted culture supernatant of hybridomas (W6/32 or L243) was added to the co-culture of CTL and tumor cells. In a blocking assay of NK cell cytotoxicity, anti-NKG2D mAb (1D11) was added in the co-culture of NK cells and targets.

Measurement of soluble MICA in culture supernatant of E522L and C831L. Soluble MICA was measured using a DuoSet ELISA Development System (R&D systems, Minneapolis, MN, USA). Culture supernatants were obtained after 48 h-culture of 1×10^6 cells in a 25 cm^2 flask and applied for the ELISA kit. The difference between the two groups was statistically analyzed using Student's *t*-test.

Results

HLA-class I expression status of lung cancer cell lines. Twenty-six permanent lung cancer cell lines have been established in our laboratory since 1994, as shown in Table 1. Three cell lines (E522L, C831L, C1026L) lost their whole HLA-class I expression (12%) based on the finding of a flow cytometry analysis, as shown in Table 1. Ten cell lines had haplotype loss of HLA (38%) by comparing the HLA genes between cancer cells and normal lymphocytes. In total, 13 cell lines (50%) showed an abnormal expression of HLA-class I.

The results of flow cytometry analysis, staining with anti-HLA-class I mAb against E522L and C831L, are shown in Fig. 1A. HLA-class I expression in these cell lines could not be induced even after treatment with IFN-γ (200 U/mL) for 48 h (Fig. 1A).

Genetic analysis of β2m in E522L and C831L. Because the abnormalities of the β2m gene have been reported to cause the loss of HLA-class I expression,[13,17] we genetically analyzed β2m genes in E522L and C831L. RT-PCR showed a total loss of the β2m

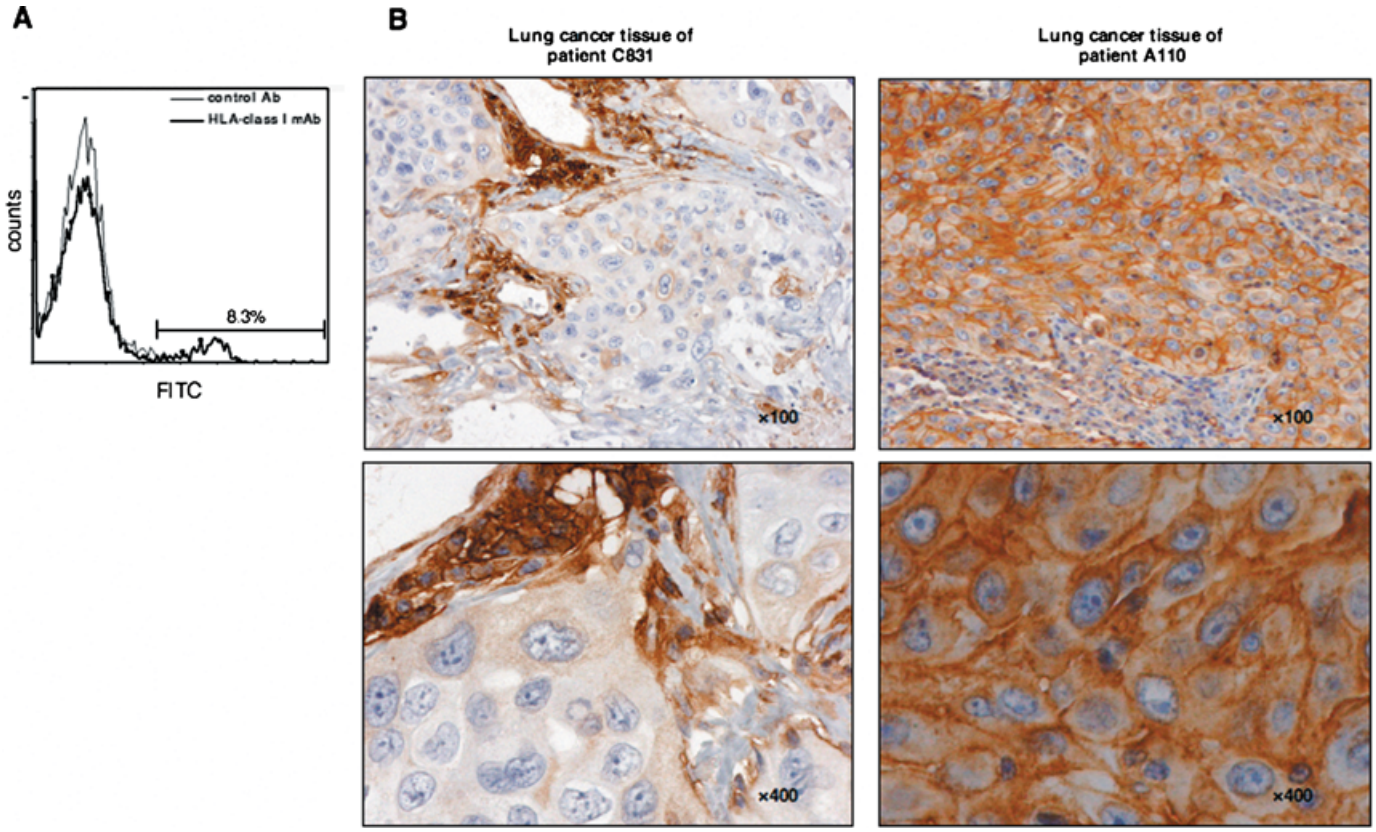

Fig. 2. The human leukocyte antigen (HLA)-class I expression in original cancer tissue or cancer cells. (A) To examine the expression of HLA-class I *in vivo*, tumor cells in pericardial effusion of patient E522 was examined using flow cytometry. After the purification of cancer cells by Ficoll–Hypaque gradient centrifuge, the cancer cells (over 90% of purity) were stained with anti-HLA-class I monoclonal antibody (mAb; W6/32). Most cells (91.7%) lost their HLA-class I expression. (B) Immunohistochemical staining of primary lung cancer tissues specimens of patient C831 and patient A110 (as positive control) with anti-HLA-class I mAb, EMR8-5, was performed. In patient C831, the cancer cells did not express HLA-class I, even though the interstitial normal cells expressed it. In patient A110, both the cancer cells and interstitial cells expressed HLA-class I.

gene in E522L (Fig. 1B). In C831L, mRNA of the β2m gene was normally expressed. However, the sequence analysis of β2m revealed a point mutation in the ORF (Fig. 1C). Cytosine at 64 base from the start position of ORF was substituted for thymine, and this change resulted in the formation of an early stop codon.

Restoration of HLA-class I expression by transduction of wβ2m. The total loss of HLA-class I expression in both the cell lines was thus ascribed to genetic abnormalities of β2m. Therefore, the wβ2m gene was transduced into E522L and C831L, used in order to restore HLA-class I expression, as described in the Materials and Methods. After selecting by the antibiotics, a flow cytometry analysis revealed that E522L-wβ2m and C831L-wβ2m restored the expression of HLA-class I on their cell surface (Fig. 1D).

HLA expression in original cancer cells or cancer tissue. To confirm the negative expression of the HLA-class I of original cancer cells *in vivo*, purified cancer cells using the Ficoll–Hypaque gradient method (approximately 90%) from pericardial effusion were examined by flow cytometry in patient E522, and showed that 92% of the cells had lost their HLA-class I (Fig. 2A). The primary lung cancer tissue of patient C831 was evaluated using immunohistochemical staining with anti-HLA-class I mAb (EMR8-5). As shown in Fig. 2B, interstitial cells strongly expressed HLA-class I on their cell surfaces, but HLA-class I was not expressed on cancer cells at all. The control cancer tissue (A110) expressed HLA-class I on the surfaces of cancer cells. These findings indicated that most cancer cells in both patient E522 and patient C831 had already lost their HLA-class I expression of tumor cells *in vivo*.

CTL response against cancer cells with or without HLA-class I expression. The CTL of patient E522 were induced from autologous PBMC by stimulations with wβ2m transduced autologous tumor cells (E522L-wβ2m). The CTL showed cytotoxicity to E522L-wβ2m, but not to parental E522L, E522 PHA blasts or K562 (Fig. 3A).

In patient C831, one CTL clone was established from autologous RLNL, as described in the Materials and Methods, and showed tumor-specific reactivity. The CTL clone killed C831L-wβ2m, but not parental C831L, C831EBV-B or K562 (Fig. 3B). Moreover, the reactivity of the CTL clone was elucidated by IFN-γ production in response to C831L-wβ2m and the response was inhibited by the addition of anti-HLA-class I mAb (Fig. 3C).

NK cell cytotoxicity against E522L or C831L. NK cell-sensitivities of E522L and C831L were examined using NK cells produced by PBMC from a normal healthy donor. E522L was as sensitive to NK cells as K562. However, the NK cell-sensitivity was completely abrogated by the restoration of the expression of HLA-class I with the transduction of wβ2m (Fig. 4A). In contrast, C831L was insensitive to NK cells despite the loss of the HLA-class I expression (Fig. 4B). These findings indicated that E522L and C831L clearly have different pathways to react with NK cells.

Analysis of the difference in sensitivity to NK cells between E522L and C831L. To explore the mechanisms regarding the difference in the sensitivity against NK cells between E522L and C831L, we examined the surface expression of NK cell-activating ligands on both cancer cells. MICA/B is a ligand for NK cell-activating receptor, NKG2D. Flow cytometry revealed that C831L did not express MICA/B, whereas E522L highly expressed it (Fig. 5A). A DNA sequence analysis of the MICA gene in these cell lines

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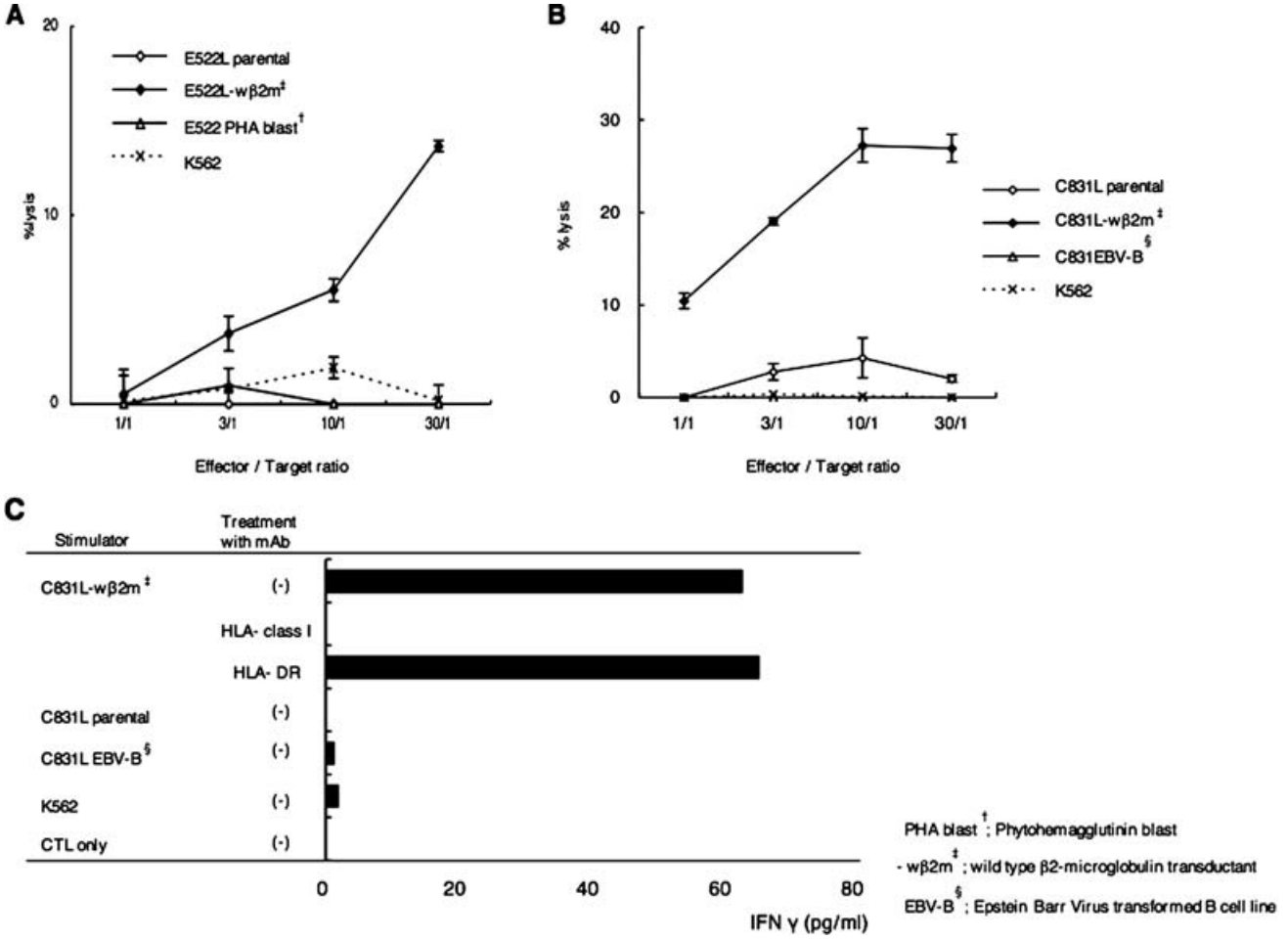

Fig. 3. The activity of cytotoxic T lymphocytes (CTL) induced from autologous lymphocytes. (A) In patient E522, CTL were induced from autologous peripheral blood mononuclear cells (PBMC) by weekly stimulation with irradiated E522L-wβ2m. The cytotoxicity of CTL was assessed by a standard ^{51}Cr release assay. The CTL killed E522L-wβ2m, but not parental E522L without either human leukocyte antigen (HLA)-class I expression, E522-PHA blasts or K562. (B) In patient C831, CTL were induced from autologous regional lymph node lymphocyte (RLNL). The CTL showed a cytolytic activity against C831L-wβ2m, but not against parental C831L, C831 EBV-B or K562. (C) Reactivity of the CTL clone induced from patient C831 was assessed by the interferon (IFN)-γ production in response to stimulators. The restriction of the CTL clone was analyzed by the addition of anti-HLA class I or anti-HLA-DR monoclonal antibody (mAb). The CTL produced IFN-γ in response to C831L-wβ2m and the response was inhibited by the addition of anti-HLA-class I mAb, but the CTL did not show any response against parental C831L, C831EBV-B or K562.

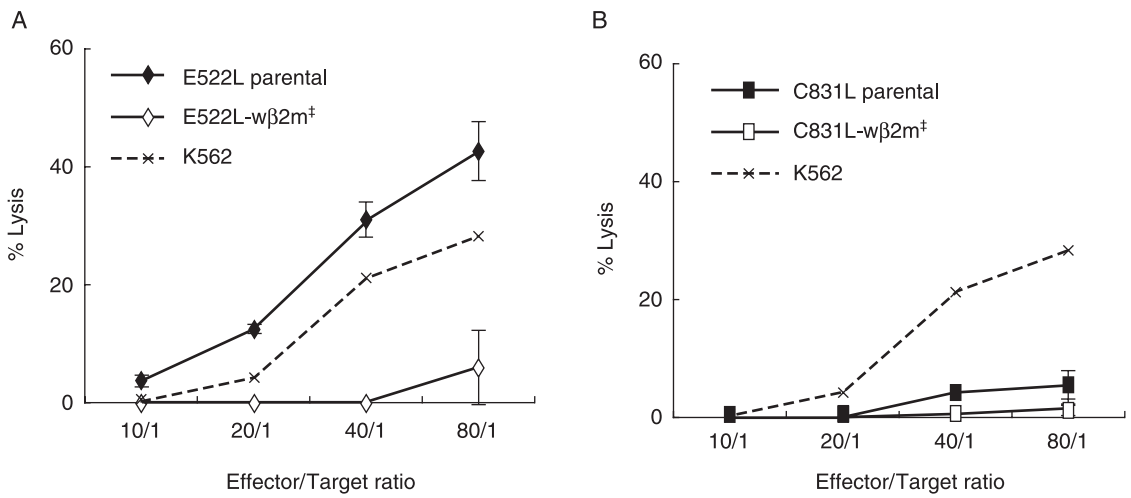

Fig. 4. The natural killer (NK) cell activity against E522L and C831L. (A) To examine the sensitivity of E522L to NK cell activity, NK cells derived from healthy donor peripheral blood mononuclear cells (PBMC), was applied for ^{51}Cr release assay. K562, sensitive to NK cell activity, was used as a positive control. E522L was as sensitive as K562 to NK cell activity. The restoration of HLA-class I expression completely abrogated the sensitivity. (B) Cytotoxic assay of NK cells against C831L. C831L exhibited resistance to the NK cell activity.

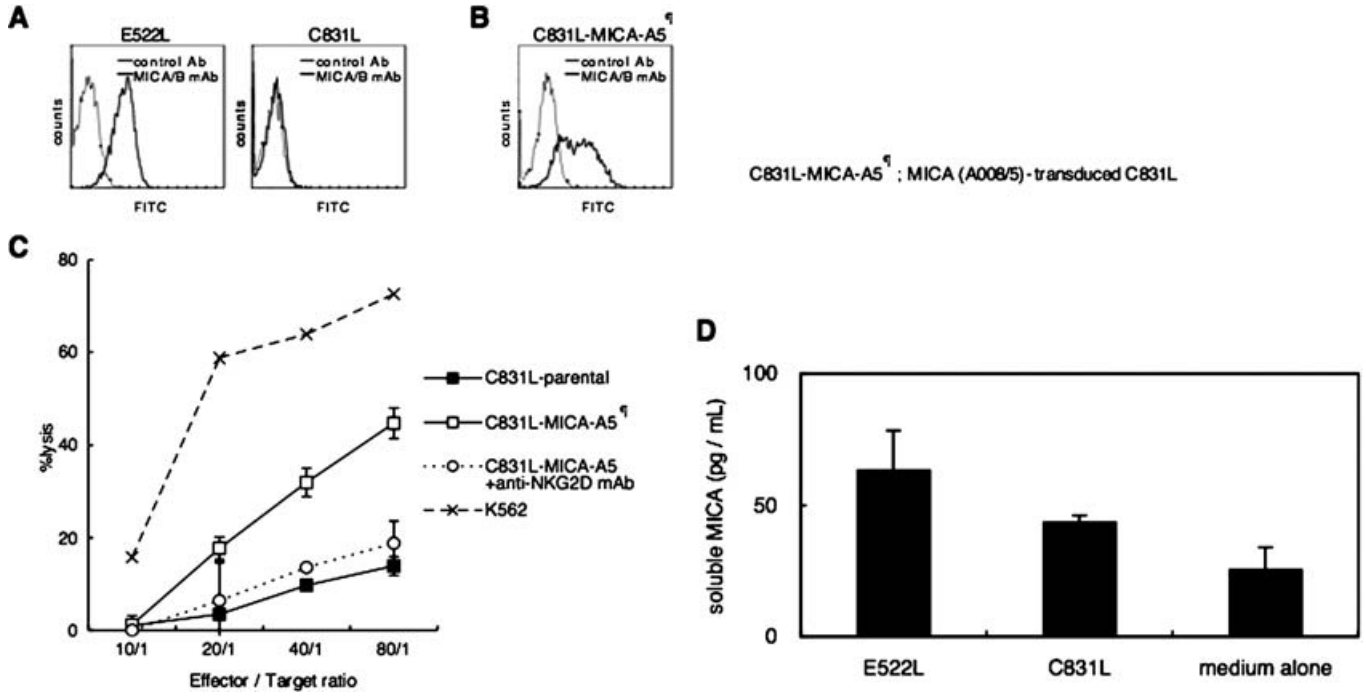

Fig. 5. Expression of major histocompatibility complex (MHC) class I chain related molecules A (MICA) and natural killer (NK) cell cytotoxic activity in E522L and C831L. (A) Flow cytometry stained with anti-MICA/B mAb revealed that E522L expressed MICA/B, while C831L did not. (B) After the transduction of the MICA gene (MICA-A008/5.1) cloned from E522L, C831L expressed MICA on the cell surface. (C) ^{51}Cr release assay was performed to examine the sensitivity of C831L-MICA-A5 to NK cell cytotoxicity. K562 was used as a positive control. C831L showed resistance to NK cell cytotoxicity, but C831L-MICA-A5 revealed sensitivity to it. The addition of anti-NKG2D mAb inhibited sensitivity of the C831L-MICA-A5. (D) Soluble MICA was measured in culture supernatant of E522L and C831L by ELISA. Soluble MICA in culture supernatant of C831L was as low as that in E522L (*$P = 0.09$, no significant difference). The data was means of triplicate of one representative experiment.

revealed that the allele of TM was different. The allele for MICA in E522L was MICA-A008/5 (extracellular domain/TM), and the allele in C831L was MICA-A008/5.1. The MICA gene (MICA-A008/5) was cloned from E522L and transduced into C831L using retrovirus vector, as described in the Materials and Methods. After selecting by the antibiotics, a flow cytometry analysis revealed that MICA(A008/5)-transduced C831L (C831L-MICA-A5) expressed MICA on the cell surface (Fig. 5B). An NK cell cytotoxic assay showed that the MICA transduction rendered C831L sensitive to NK cells (Fig. 5C). The sensitivity of C831L-MICA-A5 was thus inhibited by the addition of anti-NKG2D mAb.

Measurement of soluble MICA in culture supernatants of E522L and C831L. To elucidate whether soluble MICA was associated with the resistance of C831L against NK cells cytotoxicity, NK cells were pretreated with culture supernatant of C831L or E522L, and cytotoxic activities against E522L and K562 were evaluated. However, the activity was not affected by the presence of culture supernatant of C831L and E522L (data not shown). The amount of soluble MICA in the culture supernatant of C831L was as low as that of E522L (Fig. 5D).

Discussion

In patient E522 and C831, the total loss of the HLA-class I expression was observed not only in cancer cell lines cultured *in vitro* (Fig. 1), but also in malignant cells from the original pericardial effusion of E522 and primary cancer tissue of C831 (Fig. 2). In previous reports, 25–94% of non-small cell lung cancers had a loss or down-regulation of HLA-class I expression in immunohistochemical staining.[20,21] However, the correlation between the expression of HLA-class I of cancer cells and the prognosis of cancer patients remains unclear. An HLA-class I loss or down-regulation on lung cancer and breast cancer has been reported to be associated with favorable survival,[24,28] but a down-regulation in colorectal cancer has been suggested to be a poor prognostic factor.[29] CTL attack against normal HLA-expressing tumor cells might cause an *in vivo* selection of cancer cells showing an abnormal expression of HLA as reported previously.[2]

In E522L, mRNA of the β2m gene was not expressed at all (Fig. 3A). In melanoma cell lines, the loss of β2m RNA was reported to be ascribed to two mutational events, a loss of heterozygosity and a microdeletion of the β2m gene in exon 1.[18,30] A mutation hotspot was suggested to be located in the CT repeat region (at position from 37 base to 44 base of the ORF) in exon 1 of the β2m gene, and mutations of this region have been identified in more than 75% of cancer cells with HLA-class I loss.[18,19] However, the mutation site in C831L (Fig. 3B) was not consistent with the reported hotspot.

Previous reports have indicated that cancer cells with a deficiency of β2m molecules were able to escape from a CTL attack; however, the CTL could attack the cancer cells, which thus restored the HLA expression by wβ2m transduction.[13] In our experiment, autologous CTL were induced by E522L-wβ2m and C831L-wβ2m, and such CTL could not respond to parental cancer cells without an HLA expression (Fig. 3). These results indicated the existence of precursor CTL against normally HLA-class I expressed cancer cells *in vivo*. These findings may suggest that cancer cells can thus evade CTL attack by means of loss of HLA-class I expression due to genetic changes of β2m.

An HLA-class I expression of tumor cells is a pivotal condition for the recognition of specific CTL. However, the absence of HLA-class I on target cancer cells may cause an activation of NK cells. Such a phenomenon is well known as the 'missing-self' hypothesis.[10,31] In fact, E522L exhibited a high sensitivity to

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NK cell, and transduction of wβ2m rendered them resistant by restoration of HLA-class I as shown in Fig. 4A. However, C831L exhibited resistance to NK cell despite the loss of HLA-class I expression, as shown in Fig. 4B. According to previous reports, there are several check points of target tumor cells for NK cell sensitivity;[1] the surface expression of HLA-class I molecules as a ligand for NK cell inhibitory receptor,[2] the surface expression of ligands for activating NK cell receptors,[3] and the surface expression of non-HLA-specific inhibitory receptors.[31] Not only HLA-class I, but also HLA-E and CD1d, may protect target cells from NK cell killing.[32,33] However, neither HLA-E nor CD1d was expressed on C831L, because wβ2m is also essential for their expression on the cell surface.[22] Therefore, we examined the expression of ligands for the activating NK cell receptors.

MICA/B has recently been reported as a ligand to activate NK cells by means of activating receptor, NKG2D.[11,33–36] The down-regulation of MICA/B has been reported to be one of the immune escape mechanisms.[14,31] In the present study, E522L, which was sensitive to NK cell, expressed MICA/B, but C831L, which was resistant to NK cell, did not express it (Fig. 5A). To elucidate the different expression of MICA/B between E522L and C831L, a sequence analysis of MICA was done, and the MICA-allele of TM in E522L was MICA-A5, and that in C831L was MICA-A5.1. A previous report has indicated that the TM-allele-A5.1 encodes soluble MICA,[37] while the present data showed that transduction of MICA gene cloned from E522L (MICA-A008/5) rendered positive for MICA expression and sensitive to NK cell cytotoxicity (Fig. 5B,C). Therefore, the difference of the TM allele may cause a loss of MICA expression on the cell surface of C831L. Soluble MICA has been reported to impair NK cell cytotoxicity.[38] However, in the present study, soluble MICA was not associated with resistance of C831L against NK cells cytotoxicity because the amount of soluble MICA of C831L was as low as that of E522L (Fig. 5D), and NK cells cytotoxicity was not affected by the addition of supernatant of C831L.

In the present study, E522L showed sensitivity to NK cells-mediated cytotoxicity on *in vitro* assay. However, it is thought that lung cancer cells in patient E522 escaped from NK cells cytotoxicity and progressed to clinical cancer *in vivo*. Similarly, HLA-class I expressing lung cancer, such as half of our lung cancer cell lines, developed although CTL could attack them.[1–5] Therefore, it is necessary to consider also the influence of the immunosuppressive circumstances surrounding cancer cells as an *in vivo* escape mechanism. It is well known that cancer cells often produce immunosuppressive cytokines, including transforming growth factor-β and interleukin-10,[39] and express immunosuppressive costimulatory molecules such as PD-L1 and indoleamine 2,3-dioxygenase.[40] The loss of tumor antigen is associated with evasion from CTL cytotoxicity.[8,41] Moreover, it has been reported that an antitumor immune response is regulated by the regulatory T cells, NK T cells, and immunosuppressive dendritic cells.[42] Therefore, such immunosuppressive factors might be associated with the evasion of cancer cells in patient E522 from NK cells cytotoxicity, or the immune escape of HLA-class I expressing cancer cells from CTL-mediated cytotoxicity *in vivo*.

In the present study, we analyzed the escape mechanisms from immunosurveillance in lung cancer cells, and revealed that: (i) 50% of lung cancer cell lines had abnormalities in their HLA-class I expression; (ii) an abnormality of the β2m gene caused lack of HLA-class I expression and transduction of wβ2m induced restoration of HLA-class I expression; (iii) HLA-class I deficient cells were exempted from an attack by CTL and reconstitution of HLA-class I expression induced CTL recognition against such cancer cells; (iv) cancer cells that lost their HLA-class I expression did not always show sensitivity to NK cell cytotoxicity; and (v) by reconstitution of the MICA expression, the NK cell-resistant cancer cells restored sensitivity to NK cell cytotoxicity.

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