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**Supplementary Material**

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The MAPK Pathway Is a Predominant Regulator of HLA-A Expression in Esophageal and Gastric Cancer

Kousaku Mimura,*† Kensuke Shiraishi,*† Anja Mueller,‡ Shinichiro Izawa,‡ Ley-Fang Kua,§ Jimmy So,* Wei-Peng Yong,§ Hideki Fujii,† Barbara Seliger,‡ Rolf Kiessling,* and Koji Kono*†,‡

Downregulation of HLA class I expression may contribute to a poor prognosis in cancer patients. There is limited information about epigenetic and oncogenic regulation of HLA class I, and multiple mechanisms may be involved. In the current study, we examined the relationship between the HER2-signaling pathway (MAPK and PI3K-Akt) and the expression of HLA class I and Ag-processing machinery (APM) components. A panel of gastric and esophageal cancer cell lines was treated with wortmannin as an Akt-signal inhibitor; the MAPK signal inhibitor PD98059; lapatinib, which inhibits both the epidermal growth factor receptor and HER2 tyrosine kinase; or siRNA for MAPK. The levels of HER2-signaling molecules, APM components, and HLA class I were evaluated by Western blot, quantitative PCR, and flow cytometry. Resected gastric tumor tissues (n = 102) were analyzed for p-Erk and HLA class I expression by immunohistochemistry. As a result, inhibition of the MAPK pathway induced upregulation of HLA-A02 and HLA-A24 expression in parallel with an increase in APM components and enhanced target sensitivity to tumor Ag–specific CTL lysis. HLA-A expression was predominantly regulated by the MAPK pathway, but it was also influenced, in part, by the Akt pathway. There was a strong inverse correlation between p-Erk expression and HLA class I expression in clinical tumor samples. In conclusion, HLA-A expression is predominantly regulated by the MAPK pathway in gastric and esophageal cancer. The Journal of Immunology, 2013, 191: 6261–6272.

Reduced expression of HLA class I on tumors is often associated with disease progression and poor prognosis in diverse human tumors, including ovarian, colorectal, and breast cancer (1–3). We demonstrated previously that downregulation of HLA class I molecules occurred in 43% of the primary tumors in patients with esophageal squamous cell carcinoma (ESCC) and was associated with a poor prognosis (4). There is a general consensus that downregulation of HLA class I expression and Ag-processing machinery (APM) components is often associated with reduced sensitivity to lysis by CTLs (5, 6). Decreased sensitivity to CTLs could be a contributing factor to the poor prognosis in cancer patients.

Factors like genomic alteration, transcriptional regulation, protein transportation, and oncogene regulation may all be involved in the inactivation of HLA class I molecules (7, 8). HLA class I loci, including the HLA-A gene, are located on chromosome 6p21, and four phenotypes in the genomic alteration of HLA class I molecules are known. These include total HLA loss, HLA haplotype loss, HLA locus loss, and HLA allelic loss. Genomic alterations are known to be infrequent. For example, chromosomal loss in the short arm of chromosome 6 was observed in tumors of the colon (13.8%) and larynx (17.6%), as well as in melanoma (15.3%) and ESCC (17.2%) (7, 9, 10), indicating that the other factors regulated by epigenetic and oncogenic mechanisms could play an important role in this process. However, there is limited information about oncogenic and epigenetic regulation of HLA class I expression.

With regard to oncogene regulation of HLA class I, we (11–13) reported that there is an inverse correlation between HLA class I expression and HER2 signaling, and overexpression of functional HER2 severely impairs HLA-A02 expression and CTL-mediated recognition of several HLA-A02–restricted tumor Ags. In addition, we reported that the downregulation of HER2 with small interfering RNA (siRNA) technology on ESCC resulted in increased expression of HLA class I (11, 14). These observations strongly indicated that HER2 signaling is closely related to the regulation of HLA class I expression. Because HER family signaling is involved in two main signaling pathways, ras/MAPK and PI3K-Akt (15), it was vital to evaluate further the relationship between MAPK or PI3K-Akt and HLA class I expression.

In the current study, we examined HLA class I expression and HER2 signaling using a panel of gastric and esophageal cancer cell lines and resected tumor tissues. We evaluated whether MAPK or PI3K signaling is related to the expression of HLA class I and APM components, the downstream consequences of modulating HLA class I expression, and whether HER2-signaling...
pathway components correlate with HLA class I expression in tumor tissues.

Materials and Methods

Reagents
Penicillin streptomycin, DMSO, Tween-20, and human serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Trypan blue stain, RPMI 1640, and FCS were purchased from Invitrogen (Carlsbad, CA). Primary Abs against HER2/Erbb2, p-HER2/Erbb2 (Tyr1221/1222), Akt, p-Akt (Ser473), p44/p42 MAPK, p-p44/p42 MAPK (Thr202/Tyr204), and β-actin, as well as secondary, HRP-linked anti-rabbit IgG (Cell Signaling Technology, Danvers, MA) were used for Western blot. PE-conjugated mouse anti-HER2/neu Ab, PE-conjugated IgG1k isotype-control Ig, PE-conjugated mouse anti-human HLA-A202, PE-conjugated mouse anti-human epidermal growth factor receptor (EGFR), PE-conjugated mouse IgG2b isotype-control Ig, FITC-conjugated mouse anti-human HLA-ABC (clone G46-2.6), FITC-conjugated mouse IgG1k isotype-control Ig, FITC-conjugated Annexin-V, and 7-aminoactinomycin D (7-AAD) were purchased from Becton Dickinson Biosciences (San Jose, CA). PE-conjugated anti-human HLA-ABC (clone W6/32; eBioscience, San Diego, CA), PE-conjugated mouse IgG2a isotype-control Ig (eBioscience), PE-conjugated mouse monoclonal anti-human ErbB3 (R&D Systems, Minneapolis, MN), PE-conjugated mouse IgG1 isotype-control Ig (R&D Systems), PE labeled anti-HLA-A24 (MBL, Nagoya, JAPAN), and PE-labeled mouse IgG2b isotype-control Ig (MBL) were also used for flow cytometry.

Cell lines and HLA typing
ESC cells lines TE1, TE2, TE3, TE4, and TE5 were obtained from the University of Tohoku cell bank (Institute of Development, Aging and Cancer, University of Tohoku, Sendai, Japan). ESCC cell lines KYSE30, KYSE50, and KYSE110 and gastric cancer cell lines MKN7, NUGC2, and NUGC-3 were obtained from Health Science Research Resources Bank (Osaka, Japan). Gastric cancer cell lines NCI-N87 and KATOIII were obtained from the American Type Culture Collection (Rockville, MD), and OE19 was obtained from the European Collection of Animal Cell Cultures (Sigma-Aldrich). PC-9 (lung cancer cell line) was purchased from the IBL cell bank (Gunma, Japan), and MKN45 was purchased from the RIKEN BioResource Center (Ibaraki, Japan). The TISI cell line is a TAP mutant cell line derived from human B-lymphoblastoid cells expressing HLA-A24. All cell lines were kept in RPMI 1640 with 5% FCS (Invitrogen), 50 U/ml penicillin, and 2 mM l-glutamine. Cell lines were regularly authenticated and matched the short tandem repeat DNA profiles of the original cell lines in each cell bank.

HLA genotyping of all tumor cell lines was performed using the PCR-Luminex method in the HLA laboratory (Kyoto, Japan).

Treatment with inhibitors, epidermal growth factor, and HER3 ligand
Tumor cells were cultured in six-well plates and exposed to various concentrations of the Akt signal inhibitor, wortmannin (Cell Signaling Technology); the MAPK signal inhibitors, PD98059 (Cell Signaling Technology) and PD0325901 (Cayman Chemical, Ann Arbor, MI); the combined EGFR and HER2 tyrosine kinase inhibitor, lapatinib (GlucoSmithKline, Brentford, U.K.); or DMSO as negative control. After 1 h of incubation with each inhibitor, tumor cells were used for Western blot and PCR. After a 12–72 h incubation with each inhibitor, tumor cells were used for flow cytometry and ELISPOT assay.

Epidermal growth factor (EGF; R&D Systems), EGFR ligand, and NRG-1-β1 (R&D Systems), HER2 ligand were used to activate Akt signaling and MAPK signaling. Tumor cells were treated with DMSO or PD98059 for 1.5 h prior to stimulation with the indicated doses of EGF or NRG-1-β1. After 1 h of incubation with EGF or NRG-1-β1, tumor cells were used for Western blot. After 48 h of incubation with EGF or NRG-1-β1, tumor cells were used for flow cytometry.

Gene silencing using siRNA
Tumor cells were grown at ~60% confluence in 12-well plates and transfected with 100 nm p44/42 MAPK siRNA (Cell Signaling Technology) in Opti-MEM I Reduced Serum Medium (Invitrogen), including Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. For control transfectants, 100 nmol control siRNA (Cell Signaling Technology) was used for transfection in parallel. After 48 h, cells were analyzed for p44/42 MAPK and p-p44/42 MAPK (Thr202/Tyr204) by Western blotting and for HLA class I and HLA-A expression by flow cytometry.

MAPK- and control-siRNA transfectants were always cultured in parallel for the same period of time.

For some experiments, after the siRNA transfection, cells were treated with MAPK inhibitors or DMSO for 48 h and subjected to Western blot and flow cytometry.

RT-PCR and real-time PCR
Total cellular RNA was extracted from cells using the RNeasy Mini Kit (Machery-Nagel, Dueren, Germany), followed by digestion with DNase I (Invitrogen). CDNA were synthesized from total RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Primers used for RT-PCR of APM components were described previously (13, 16). Comparative quantification of gene expression was performed by real-time PCR on a Rotor Gene 2000 (Corbett Research, Sydney, Australia) using a quantitative SYBR Green kit (QIAGEN, Hilden, Germany) and target-specific primers (13, 16). Results were normalized to β-actin, GAPDH, PPIA, and HPRT transcription levels.

Western blot
Cell pellets were solubilized in electrophoresis sample buffer, sonicated for 10 s, and boiled for 10 min. The protein concentration of cell lysates was measured, and 3 μg protein was separated by SDS-PAGE, followed by transfer to a polyvinylidine fluoride microporous membrane (Millipore, Billerica, MA). Membranes were blocked in PBS with 5% milk powder and probed with primary Ab. After washing, the membrane was incubated with HRP-linked goat anti-rabbit Ab (Cell Signaling Technology). Blots were visualized by ECL (ECL Plus; Amersham Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer’s protocol. Results were captured digitally using a LAS1000 Lumino Image Analyzer (Fuji Photo Film, Tokyo, Japan).

Flow cytometry
Tumor cells were immunofluorescently labeled in a V-bottom 96-well plate according to a previously established immunofluorescent staining protocol (17). Tumor cells were analyzed using a four-color FACS machine (FACSCalibur) and CellQuest software (both from BD). Dead and/or apoptotic cells were excluded using Annexin-V and 7-AAD. Relative mean fluorescence intensity (MFI) was calculated as: [(MFI with specific mAb − MFI with isotype mAb)/MFI with isotype mAb]. Relative mean fluorescence intensity was calculated as: [(MFI with specific mAb − MFI with isotype mAb)/MFI with specific mAb of control treatment − MFI with isotype mAb of control treatment] (18).

Immunohistochemistry analysis
The use of clinical tumor samples from patients was approved by the Institutional Ethical Committee, University of Yamanashi, and written informed consent was obtained from all participants. Deparaffinized and rehydrated sections of tumor tissue were incubated with epitope retrieval solution (Dako) for 40 min at 95–99°C in a water bath or for 20 min at 121°C in an autoclave. For HLA class I staining, the sections were treated with ChemMate Peroxidase Blocking Solution (Dako). Sections were incubated with the Ab (Ab MR6-5 (Cosmo Bio) or the isotype control (Dako) at 4°C overnight. Tumor cells were immunofluorescently labeled in a V-bottom 96-well plate according to a previously established immunofluorescent staining protocol (17). Tumor cells were analyzed using a four-color FACS machine (FACSCalibur) and CellQuest software (both from BD). Dead and/or apoptotic cells were excluded using Annexin-V and 7-AAD. Relative mean fluorescence intensity (MFI) was calculated as: [(MFI with specific mAb − MFI with isotype mAb)/MFI with isotype mAb]. Relative mean fluorescence intensity was calculated as: [(MFI with specific mAb − MFI with isotype mAb)/MFI with specific mAb of control treatment − MFI with isotype mAb of control treatment] (18).

Immunohistochemistry analysis was performed by two independent observers (K.S. and S.I.) without prior knowledge of clinicopathological data.

Generation of HLA-A24–restricted, cancer testis Ag–specific CTL line and CTL clone
HLA-A24+ PBMCs were collected using Ficol Paque (GE Health Care, Uppsala, Sweden) gradient centrifugation. Monocytes were enriched by adherence to a plastic tissue culture flask for 1 h at 37°C and cultured in X-VIVO (Life Technologies, Gaithersburg, MD), supplemented with 1000 U/ml GM-CSF and 1000 U/ml IL-4 (both from PeproTech, London, U.K.). Five days later, 20 μg/ml OK-432 (Chugai Pharmaceutical, Tokyo, Japan)
was added for maturation of dendritic cells (DCs). After a 48-h incubation, mature DCs were pulsed with 20 \( \mu \)g/ml HLA-A24–restricted LY6K peptide (RYCNLEGPPI), which is the immunodominant peptide derived from cancer testis Ag, LY6K specific for ESCC, for 3 h at 37˚C and subsequently coincubated with autologous CD8 T cells at a 1:10 ratio in a 24-well plate. T cells were cultured in X-Vivo and supplemented with 20 IU/ml IL-2 (Shionogi & Co., Osaka, Japan) after 2 d of coincubation. Cultured T cells were restimulated with LY6K peptide–loaded, irradiated (25 Gy) autologous mature DCs every 7 d. A total of 40–80 IU/ml IL-2 (Shionogi & Co.) was replenished 2 d after each restimulation. One week after the third stimulation, the CTL lines were tested for their Ag specificity for LY6K peptide using ELISPOT assay in combination with irrelevant HLA-A24–binding peptides TTK (SYRNEIAYL), IMP3 (KTVNELQNL), CDCA1 (VYGIRLEHF), and TOMM34 (KLRQEVKQNL).

The CTL clone was obtained from the HLA-A24–restricted, LY6K peptide–specific CTL line by limiting dilution. Log-phase T cells were

Table I. HLA-A and HER family expression on ESCC and gastric cancer cell lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>HLA-A Allele</th>
<th>Flow Cytometry (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLA-A02</td>
<td>HLA-A24</td>
</tr>
<tr>
<td>ESCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TE1</td>
<td>2402, 2601</td>
<td>(−)</td>
</tr>
<tr>
<td>TE2</td>
<td>0206</td>
<td>(−)</td>
</tr>
<tr>
<td>TE3</td>
<td>0206</td>
<td>(−)</td>
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<tr>
<td>TE4</td>
<td>0207, 1101</td>
<td>(−)</td>
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<tr>
<td>TE5</td>
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<tr>
<td>KYSE110</td>
<td>0206</td>
<td>(−)</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MKN7</td>
<td>2402</td>
<td>(−)</td>
</tr>
<tr>
<td>NCI-N87</td>
<td>2301</td>
<td>(−)</td>
</tr>
<tr>
<td>KATOIII</td>
<td>0201, 0207</td>
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</tr>
<tr>
<td>OE19</td>
<td>0201</td>
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<tr>
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<tr>
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<tr>
<td>BT474</td>
<td>0101, 2902</td>
<td>(−)</td>
</tr>
<tr>
<td>Epidermoid carcinoma</td>
<td>0301</td>
<td>(−)</td>
</tr>
</tbody>
</table>

*Anti–HLA-A24 mAb cross-reacts with the HLA-A23 molecule.
(−), Negative expression; ND, not determined.

FIGURE 1. HLA class I expression of ESCC cell lines is upregulated by the MAPK inhibitor. (A) TE1, TE4, and KYSE30 cells were treated with wortmannin (Akt signal inhibitor), PD98059 (MAPK signal inhibitor), or lapatinib (EGFR and HER2 dual inhibitor) at the indicated doses or with DMSO as a negative control for 48 h. HER2-related signaling molecules were assessed by Western blot. (B) KYSE30 cells were treated with varying doses and exposures of wortmannin, PD98059, or lapatinib or DMSO as a negative control. HLA class I was assessed by flow cytometry. Dead and/or apoptotic cells were excluded based on Annexin-V and 7-AAD staining. Relative MFI (rMFI) was calculated. Representative graphs are shown. **p < 0.01, PD98059 versus DMSO.
incubated with mitomycin-treated Jiyoye and EB-3 cells (1 × 10^4 cells/well for each cell line) as feeders in a 96-well U-bottom plate in X-VIVO in the presence of 30 ng/ml anti-human CD3 (Becton Dickinson) and 125 IU/ml IL-2 (Shionogi & Co.). Specificity of the CTL clone was tested for the Ag-specific response using ELISA. EB-3 and Jiyoye cells were the kind gifts of Dr. Tsunoda (OncoTherapy Science, Kanagawa, Japan).

**ELISPOT assay**

The Ag-specific response was determined by IFN-γ ELISPOT assay. ELISPOT assay was performed, according to the manufacturer’s protocol, with a commercial kit (Mabtech, Stockholm, Sweden). Briefly, 96-well plates with nitrocellulose membrane (Millipore) were coated with a primary anti-human IFN-γ capture mAb (1-D1K) for 16 h. Subsequently, the plates were treated with X-VIVO containing 1% human serum albumin for 90 min. Target cells (2 × 10^3/well), TISI cells pulsed with antigenic peptide or tumor cells treated with inhibitors, and CTLs (5 × 10^3/well) were cocultured in the well with 200 μl X-VIVO for 24 h. A biotinylated secondary anti-human IFN-γ mAb (7-B6-1) was added for 2 h and streptavidin-alkaline phosphatase reagent was added for 1 h, followed by staining with NBT and BCIP (Invitrogen). The number of spots was quantified using an ELISPOT reader (KS ELISPOT Compact; Zeiss, Göttingen, Germany).

**Cytotoxicity assay**

The cytotoxic activity of the LY6K peptide–specific, HLA-A24–restricted CTL clone was measured using a calcine-release assay. Briefly, target cells were incubated with 5 μM calcine-AM (Becton Dickinson Biosciences) for 30 min at 37°C and 5% CO2, and targets (5 × 10^3/well) were cocultured at various ratios with the LY6K peptide–specific, HLA-A24–restricted CTL clone. Cells were cocultured in 200 μl X-VIVO for 4 h. The assays were performed in triplicate in a 96-well U-bottom plate. After incubation, 100 μl of each supernatant was transferred from each well to a 96-well flat-bottom plate. The fluorescence of each supernatant was measured at 485 nm excitation and 528 nm emission wavelength using an FLx800 fluorescence microplate reader (BioTek, Winooski, VT). Spontaneous release was obtained from target cells incubated without effector cells, and maximum release was obtained from detergent-released target cells. The percentage of specific lysis was calculated according to the following formula: percentage of specific lysis = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

**Statistics**

The Student unpaired t test was performed to determine statistical significance, and analyses were performed at a significance level of 5% (p < 0.05) using Statistical Package for Social Science Statistics 20.0 (SPSS).

**Results**

**HLA-A status on ESCC and gastric cancer cell lines**

The genotype of the HLA-A allele and the HLA-A02/HLA-A24 expression quantified by flow cytometry were examined in ESCC (n = 8) and gastric cancer (n = 6) cell lines, as were EGFR (HER1), HER2, and HER3 expression (Table I) (20, 21). The expression levels of EGFR+ (KYSE30) and HER2+ (TE4, NCI-N87, OE19, and MKN7) cell lines were comparable to A431, a well-characterized EGFR-overexpressing epidermoid carcinoma cell line, and BT474, a breast cancer cell line that overexpresses HER2 (Table I).

**Inhibition of the MAPK pathway upregulates HLA-A expression in ESCC**

Because MAPK and PI3K-Akt are the two main pathways involved in HER2 signaling (15), the ESCC cell lines TE4, TE1, and KYSE30 were treated with wortmannin as the Akt signal inhibitor, the MAPK signal inhibitor PD98059, or lapatinib, which inhibits both EGFR and HER2 tyrosine kinase. Western blot analysis (Fig. 1A) demonstrated that PD98059 markedly inhibited p-Erk and

**FIGURE 2.** Inhibition of MAPK signaling upregulates HLA-A expression in ESCC. Eight ESCC cell lines were treated with PD98059, wortmannin, lapatinib, or DMSO as a negative control for 48 h. (A and B) The expression of total HLA class I (W6/32 or G46-2.6 mAbs) and HLA-A02 or HLA-A24 was assessed by flow cytometry. Representative graphs are shown. (C) TE4, TE1, and KYSE30 cells were treated with wortmannin, PD98059, lapatinib, or DMSO as a negative control for 48 h. The expression of HLA-A02 or HLA-A24 was assessed by flow cytometry. *p < 0.05, **p < 0.01, PD98059 versus DMSO.
wortmannin downregulated p-Akt in all three cell lines in a dose-dependent manner. Lapatinib inhibited HER2 phosphorylation (p-HER2) in HER2-overexpressing TE4 cells (Fig. 1A), whereas HER2 expression was not detected on HER2− TE1 or KYSE30 cells. Predictably, lapatinib almost completely inhibited both p-Akt and p-Erk in HER2-overexpressing TE4 cells but not in HER2− TE1 or KYSE30 cells. These observations validated that PD98059, wortmannin, and lapatinib acted efficiently on the MAPK and PI3K-Akt pathways in the ESCC cell lines.

We subsequently evaluated the effect of PD98059, wortmannin, and lapatinib at different doses and exposure times on the expression of HLA class I in TE4, TE1, and KYSE30 cells by flow cytometry after dead and/or apoptotic cells were excluded using Annexin-V and 7-AAD. As shown in Fig. 1B, PD98059 markedly upregulated the expression of HLA class I after 48 h, whereas lapatinib and wortmannin did not significantly affect its expression. The same pattern was also noted for TE4 and TE1 cells (data not shown). Therefore, cells were exposed to the inhibitors for 48 h in the subsequent experiments.

Eight ESCC cell lines were treated with the HER2-signaling pathway inhibitors described above and examined by flow cytometry for HLA class I expression using two mAbs (W6/32 and G46-2.6). As shown in Fig. 2A, the expression of HLA class I was upregulated by PD98059 in five of eight ESCC cell lines, whereas lapatinib and wortmannin did not induce significant changes. Compared with overall HLA class I expression, modulation of the alleles HLA-A02 and HLA-A24 was more pronounced. HLA-A02 expression was upregulated by PD98059 treatment in all ESCC cell lines, in contrast to treatment with lapatinib and wortmannin (Fig. 2B). Similarly, treatment with PD98059 markedly increased HLA-A24 expression in both TE1 and KYSE30 cells, whereas wortmannin and lapatinib did not significantly alter its expression (Fig. 2B). PD98059-induced upregulation of HLA-A02 and/or HLA-A24 on TE4, TE1, and KYSE30 cells occurred in a dose-dependent manner (Fig. 2C).

Taken together, these findings clearly indicated that the inhibition of MAPK with PD98059, but not the inhibition of PI3K-Akt, upregulates the expression of HLA-A on ESCC cell lines.
MAPK inhibition upregulates HLA-A expression in gastric cancer cell lines

Six gastric cancer cell lines were also treated with PD98059, as described above for ESCC cells. As shown in Fig. 3, treatment with PD98059 resulted in increased expression of HLA-A02 or HLA-A24 molecules, as well as upregulation of total HLA class I in all gastric cancer cells. There was no significant change in the expression of HLA-A02/HLA-A24 or total HLA class I on any gastric cancer cell line when treated with wortmannin or lapatinib (data not shown).

Analogous to the ESCC cell lines, inhibition of p-Erk with PD98059 was confirmed by Western blot (Fig. 4A, 4B) and resulted in a dose-dependent upregulation of HLA-A02 and HLA-A24, as well as total HLA class I, on MKN7 and OE19 cells (Fig. 4C, 4D). In contrast, lapatinib downregulated both p-Erk and p-Akt in MKN7 and OE19 cells (Fig. 4A, 4B) but did not alter the expression of total HLA class I or HLA-A when used at 0.5 or 1.0 μM (Fig. 4C, 4D).

Thus, the upregulation of HLA-A with inhibition of MAPK signaling were confirmed in both ESCC and gastric cancer cell lines.

Upregulation of APM components upon treatment with MAPK inhibitor

To further analyze the effect of the MAPK inhibitor on HLA-A expression, we evaluated a panel of APM components by Western blot (8 molecules) and quantitative PCR (15 molecules).

The levels of mRNA by quantitative PCR were normalized to the levels of housekeeping genes and expressed as fold increases in comparison with DMSO controls. As shown in Fig. 5A, mRNA levels for most of the APM components in PD98059-treated MKN7 cells were increased. In particular, mRNA levels of LMP2 and LMP10 were upregulated >2-fold compared with DMSO controls. Virtually identical trends were seen with KYSE30 cells (Fig. 5A).

Western blot revealed that the protein levels of TAP-2, LMP10, and HC10 were clearly upregulated upon treatment with MAPK inhibitor in both KYSE30 and MKN7 cells in comparison with DMSO controls (Fig. 5B).

Taken together, inhibition of the MAPK pathway resulted in upregulation of APM components at both the mRNA and protein levels, as well as increased HLA-A expression. These observations further confirm that the MAPK pathway plays an important role in the regulation of HLA-A and its related APM components.

Gene silencing using siRNA for Erk1/2

To further demonstrate the involvement of MAPK signaling in the regulation of HLA-A expression, we treated KYSE30 cells with Erk1/2 siRNA, to silence Erk1/2 expression, in combination with two MAPK inhibitors (PD98059 and PD0325901). Transfection of siRNA Erk1/2 reduced the basal levels of total Erk1/2 and p-Erk1/2 in comparison with those in control siRNA (Fig. 6A), although the level of p-Erk1/2 was not completely silenced. As expected, HLA-A24, HLA-A02, and total HLA class I (W6/32 and G46-2.6 mAbs) were upregulated ∼1.5-fold in cells transfected with...
Erk1/2 siRNA (siErk1/2 + DMSO) in comparison with control-treated KYSE30 cells (siCTR + DMSO) (Fig. 6B).

Moreover, when treated with the combination of Erk1/2 siRNA and PD98059, the level of p-Erk1/2 was completely silenced (Fig. 6A), and HLA class I-related molecules were upregulated ∼2.7-fold compared with control-treated KYSE30 cells (siCTR + DMSO) (Fig. 6B). To further confirm the above phenomenon, another MAPK inhibitor (PD0325901) was used, although its ability to inhibit p-Erk1/2, as well as to upregulate the HLA-A molecules, was greater than that of PD98059 (Fig. 6A, Supplemental Fig. 1). Again, the combination of Erk1/2 siRNA and PD0325901 completely silenced the level of p-Erk1/2 and upregulated the expression of HLA class I–related molecules ∼3.0-fold in comparison with control-treated KYSE30 cells (siCTR + DMSO) (Fig. 6B).

Thus, these results further indicate that upregulation of HLA-A was induced by inhibition of p-Erk1/2, and the level of p-Erk1/2 correlated inversely with the level of HLA-related molecules.

Inhibition of MAPK signaling results in enhancement of tumor-specific CTL activity

To investigate the functional consequence of upregulated HLA-A and APM components on immune recognition, we examined Ag-specific CTL recognition of tumor targets treated with PD98059. Cytotoxic T cell lines and clones against LY6K peptide were generated by repeated stimulation with mature DCs pulsed with LY6K peptide (22). As shown in Supplemental Fig. 2, ELISPOT assays revealed that the CTL lines specifically reacted with TISI targets pulsed with the LY6K peptide in comparison with the TISI targets pulsed with several HLA-A24–binding irrelevant peptides (TTK, CDCA1, IMP3, and TOMM34). Furthermore, the CTL lines recognized the HLA-A24+, LY6K-expressing ESCC cell lines TE1 and KYSE30 but not the panel of control tumor cell lines, including HLA-A24+ adenocarcinoma cell lines and HLA-A24– ESCC cell lines (Supplemental Fig. 2).

Pretreatment of TE1 and KYSE30 with PD98059 resulted in a >2-fold increase in peptide-specific IFN-γ production, as measured by ELISPOT, in comparison with the DMSO-treated controls (Fig. 7A).

HLA-A24–restricted, LY6K peptide–specific CTL clones were generated from the cell lines described above, by the limiting-dilution method. When KYSE30 cells were pretreated with PD98059, the reactivity of the CTL clone to pretreated targets increased >2-fold in comparison with the DMSO-treated control in an ELISPOT assay (Fig. 7B). Similar results were also noted with pretreated TE1 and MKN7 cells in cytotoxic assays (Fig. 7C). Furthermore,
the increased CTL reactivity was only seen with PD98059 treatment and not with lapatinib (Fig. 7D).

Taken together, the upregulation of HLA-A and APM components induced by PD98059 resulted in enhancement of CTL reactivity.

**HLA-A expression in EGFR-overexpressing tumor cells treated with MAPK inhibitor and EGF**

Next, we examined whether ligand-induced EGFR signaling could affect HLA-A expression in EGFR-overexpressing tumor cells. When the EGFR-overexpressing ESCC cell line KYSE30 was treated with EGF, p-Akt expression was greatly enhanced by EGF (Fig. 8A, left panel), whereas p-Erk expression was marginally increased in response to EGF. When KYSE30 cells were treated with the combination of EGF and PD98059, Western blotting showed strong inhibition of p-Erk expression and enhanced p-AKT expression (Fig. 8A, right panel). In the presence of PD98059, the upregulation of HLA-A02 and HLA-A24 was further enhanced on KYSE30 cells by EGF treatment in a dose-dependent manner (Fig. 8B); cumulatively, it appears that the concurrent inhibition of p-Erk and upregulation of p-Akt resulted in the most prominent increase in HLA-A expression in KYSE30 cells.

These findings confirm that HLA-A expression is regulated predominantly by the level of p-Erk, although p-Akt levels are also involved in the regulation of HLA-A.

**HLA-A expression in HER3-expressing ESCC cell line treated with MAPK inhibitor and HER3 ligand**

Next, we investigated whether ligand-induced HER3 signaling could affect HLA-A expression on HER3-expressing KYSE30 cells.

**Inverse correlation between p-Erk expression and HLA class I expression in clinical tumor samples**

We further confirmed the relationship between p-Erk and HLA class I expression in gastric cancer tissues by immunohistochemistry ($n = 102$, Supplemental Table I). Representative immunostaining with anti–p-Erk and anti–HLA class I (EMR-5) mAbs in serial sections of gastric cancer are shown in Fig. 9A. The expression of HLA class I was empirically classified into three categories: preserved, partial loss, and loss (Fig. 9B). Similarly, the expression of p-Erk was classified into strong, weak, and negative, as shown in Fig. 9C. With this classification system, there appeared to be a strong inverse correlation between p-Erk expression and HLA class I expression in gastric cancer tissue ($p < 0.0001$, Fig. 9D). These observations further support that the MAPK pathway predominantly regulates HLA class I expression.
Discussion

It was shown that oncogenes, such as ras, myc, and HER2, can induce the downregulation of MHC class I surface expression, resulting in an escape from immunosurveillance (12, 24–27). However, there is limited information about epigenetic and oncogenic factors for downregulation of HLA-class I. To our knowledge, only one previous study (24) reported that joint action of DNA methylation and MAPK inhibition could exert a regulatory effect on HLA-A expression in colon cancer cells. In the current study, we expanded this anecdotal observation to a more general phenomenon, showing that the MAPK pathway could regulate HLA-A expression in gastric and esophageal cancer. In addition, we showed that HLA-A expression is predominantly regulated by the MAPK pathway but is influenced, in part, by the Akt pathway, as shown by the HER1 and HER3 experiments (Fig. 8), and the lapatinib treatment (Figs. 1, 4).

Although p-Akt and p-Erk were both almost completely inhibited by lapatinib in HER2-overexpressing cells (Figs. 1A, 4A, 4B), there was no upregulation of HLA-A by lapatinib treatment (Figs. 2B, 2C, 4C, 4D), in contrast with the fact that p-Erk inhibition alone by MAPK inhibitor showed the upregulation of HLA-A (Figs. 1A, 4A, 4B). The principal difference in the effect of lapatinib and MAPK inhibitor was the presence or absence of p-Akt signaling in the PI3K-Akt pathway. These observations clearly indicated that inhibition of p-Erk in the presence of PI3K-Akt activation is a limiting factor for upregulation of HLA-A expression. Furthermore, when EGFR-overexpressing KYSE30 cells were treated with the combination of EGF and PD98059, the inhibition of p-Erk and upregulation of p-Akt resulted in a greater increase in HLA-A expression in comparison with inhibition of p-Erk alone (Fig. 8A, 8B). Thus, results from both lapatinib and EGF experiments support the premise that HLA-A expression is predominantly regulated by the MAPK pathway but is influenced, in part, by the PI3K-Akt pathway. Of note, the results from the HER3 experiment (Fig. 8C, 8D) showed the opposite trends in terms of HLA-A expression compared with the EGF experiment. One of the explanations is that PD98059 combined with neuregulin could not inhibit p-Erk expression significantly, and the p-Erk levels were enhanced by the increased doses of neuregulin, despite the presence of PD98059. Then, the p-Erk levels correlated inversely with HLA-A levels, regardless of p-Akt levels (Fig. 8C). This observation further suggests that inhibition of MAPK is a more important limiting factor in the regulation of HLA-A expression. Taken together, HLA-A expression is regulated predominantly by the MAPK pathway but partially by the PI3K-Akt pathway.

The results of the in vitro experiments support the observed inverse correlation between p-Erk levels and HLA class I expression in clinical tumor samples. With regard to the potential mechanisms underlying the downregulation of HLA class I in tumors, genomic alterations are not frequent, but other mechanisms affect HLA expression (7, 9, 10). Our present observations suggest
that upregulated p-Erk activation may be one of the important factors responsible for decreased HLA class I expression in tumors, although the positive rate of upregulated p-Erk was relatively low. In fact, the activation of p-Erk was reported in a variety of tumor types through growth factor receptors, such as the HER family, IGF, FGF, and HGF (28–30).

In our previous studies, we showed that HER2 expression correlated inversely with HLA class I expression in breast cancer, ESCC, and melanoma (13, 14), and silencing of HER2 resulted in upregulation of HLA class I in vitro (11, 14). However, the up-regulation of HLA class I by HER2 silencing was relatively marginal in comparison with that induced by MAPK inhibition in the current study. We speculate that one explanation for this discrepancy is that silencing of HER2 impacts both p-Erk and p-Akt, similar to lapatinib treatment, whereas the MAPK inhibitor blocks only p-Erk. As described above, inhibition of p-Erk alone leads to remarkable upregulation of HLA-A, but inhibition of both p-Erk and p-Akt induces no or only marginal upregulation of HLA-A. Thus, silencing of HER2 and lapatinib treatment marginally affect HLA-A expression in comparison with the MAPK inhibitor.

However, it is not always true that HER2-overexpressing tumors have the reduced MHC class I expression. It may be that the balance between p-Erk and p-Akt is important for the regulation of HLA class I, even when HER2 is overexpressed (i.e., although most HER2-overexpressing tumors exhibit varying degrees of both p-Erk and p-AKT activation, the balance between p-Erk and p-Akt may be a key factor governing downregulation of HLA class I).

Although the molecular mechanisms underlying MAPK-dependent HLA-A regulation have to be elucidated in detail, it is possible that treatment with the MAPK inhibitor has novel therapeutic implications. Our findings suggest that the MAPK inhibitor may work toward enhancement of T cell–mediated antitumor immunity through upregulation of HLA-A and APM components in addition to its original antiproliferative functions in upper gastrointestinal cancer. Moreover, Sers et al. (24) reported that interference with MAPK activation restores ULBP2 expression on colon cancer cells, which is one of the important ligands for the NK activating receptor NKG2D. Recently, we showed that the PI3K-Akt pathway regulates MICA/B expression, which is another one of the important ligands for the NK activating receptor NKG2D (18). Taken together, manipulation of the HER family–related signaling pathway may influence both T and NK cell–mediated immunity, in addition to having a direct antiproliferative effect on tumor cells.

In conclusion, HLA-A expression is regulated predominantly by the MAPK pathway but is influenced, in part, by the PI3K-Akt pathway in gastric and esophageal cancer. Activation of p-Erk may be one of the important mechanisms behind downregulation of HLA class I that occurs in malignant tumors.

**FIGURE 8.** HLA-A expression in EGFR- and HER3-expressing tumors treated with MAPK inhibitor and ligands. (A and B) The EGFR-overexpressing ESCC cell line KYSE30 was treated with a combination of EGF and PD98059 at the indicated doses. After incubation with EGF, treated cells were exposed to 20 μM (PD-20) or 50 μM (PD-50) of PD98059 or DMSO (PD-0). (A) The status of HER2-signaling molecules was assessed by Western blot. (B) The relative MFI (rMFI) of HLA-A02 and HLA-A24 was assessed by flow cytometry. (C and D) The HER3-expressing ESCC cell line KYSE30 was treated with a combination of NRG-1-β1 and PD98059 at the indicated doses. After incubation with NRG-1-β1, treated cells were exposed to 20 μM (PD-20) or 50 μM (PD-50) of PD98059 or DMSO control. (C) The status of phosphorylation of p44/42 Erk and Akt was assessed in treated KYSE30 cells by Western blot. (D) The rMFI of HLA-A02 and HLA-A24 in treated KYSE30 cells was assessed by flow cytometry. Data from Western blot show one of three independent experiments with comparable results. *p < 0.01, **p < 0.05, PD98059 versus DMSO.
Disclosures
The authors have no financial conflicts of interest.

References

FIGURE 9. Immunohistochemistry for p-Erk and HLA class I expression in gastric cancer tissues. The expression of p-Erk and HLA class I in gastric cancer tissues was evaluated by immunohistochemistry (n = 102). (A) Representative immunostaining with anti–p-Erk and anti-HLA class I (EMR-5) mAbs in serial sections of gastric cancer. (B) The expression of HLA class I was classified semiquantitatively into three categories: preserved, partial loss, and loss. (C) The expression of p-Erk was classified as strong, weak, or negative. (A–C) Original magnification ×200. (D) There was a strong inverse correlation between p-Erk expression and HLA class I expression in gastric cancer tissue (p < 0.0001).


