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Mitogen-activated protein kinase inhibition augments the T cell response against *HOXB7*-expressing tumor through human leukocyte antigen upregulation

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Abstract

Homeobox B7 (*HOXB7*) is a master regulatory gene that regulates cell proliferation and activates oncogenic pathways. Overexpression of *HOXB7* correlates with aggressive behavior and poor prognosis in patients with cancer. However, the expression and role of *HOXB7* in head and neck squamous cell carcinoma (HNSCC) remain unclear. In this study, we observed that most samples from patients with oropharyngeal cancer and HNSCC expressed *HOXB7*. As no direct inhibitor has been reported, we identified a potent peptide epitope to target *HOXB7*-expressing tumors through immune cells. A novel *HOXB7*-derived peptide epitope ($HOXB7_{8-25}$) elicited antigen-specific and tumor-reactive promiscuous $CD4^+$ T cell responses. These $CD4^+$ T cells produced γ-interferon (IFN-γ) and had the direct ability to kill tumors through granzyme B. Notably, downregulation of *HOXB7* using siRNA enhanced human leukocyte antigen class II expression on tumor cells by decreasing the phosphorylation of MAPK. Mitogen-activated protein kinase inhibition augmented IFN-γ production by *HOXB7*-reactive $CD4^+$ T cell responses without decreasing the expression of *HOXB7*. These results suggest that combining *HOXB7* peptide-based vaccine with MAPK inhibitors could be an effective immunological strategy for cancer treatment.

KEYWORDS
cancer immunotherapy, HLA, HOXB7, MAPK, peptide vaccine

Abbreviations: APC, antigen-presenting cell; bFGF, basic fibroblast growth factor; CFSE, carboxyfluorescein succinimidyl ester; CIITA, MHC class II transactivator; CTL, $CD8^+$ CTL; DC, dendritic cell; E: T, effector : target; EMT, epithelial mesenchymal transition; HNSCC, head and neck squamous cell carcinoma; HOXB7, homeobox B7; HTL, $CD4^+$ helper T lymphocyte; ICI, immune checkpoint inhibitor; IFN-γ, γ-interferon; IHC, immunohistochemistry; SCC, squamous cell carcinoma; TAA, tumor-associated antigen.

Hiroki Komatsuda and Risa Wakisaka contributed equally to this study.

1 | INTRODUCTION

More than 600,000 new cases are diagnosed as HNSCC each year.[1,2] Despite advances in comprehensive treatment with surgery, radiation therapy, and chemotherapy, the survival rate for patients with HNSCC remains less than 50%, and more than 65% of patients suffer from recurrent and/or metastatic disease.[3–5] Recently, ICIs have been clinically applied as an innovative treatment; however, the response rate is below expectations (approximately 20%).[6] The limitation of ICIs is that their antitumor effect depends on the patient's immune cells, which may be few or exhausted. Accordingly, there is an urgent need to develop a novel strategy to increase the number of tumor-reactive immune cells in patients with HNSCC.

Tumor vaccines are ideal immunotherapy for selectively expanding tumor-reactive T cells. Tumor-specific antigens, such as mutation-derived neoantigens, have become a topic of interest as tumor vaccine candidates.[7] However, neoantigens are a challenging therapy considering their instability in mutation-prone tumors, and identifying a personalized mutation-derived epitope is expensive.[8] When applied to nonselected patients with common HLA-DRs, tumor vaccines targeting TAAs are an attractive method for stimulating tumor-reactive T cells.[9,10] Although TAAs are limited by their low immunogenicity based on past clinical trials,[11,12] recent reports have shown that modification of peptide structure, administration routes, and use of effective adjuvants could provide sufficient antitumor effects with TAAs.[9,11] We have previously identified potent epitopes from TAAs, confirmed their antitumor effect in vitro, and discovered an appropriate adjuvant and administration route to achieve in vivo antitumor responses with peptide vaccines, indicating that TAA-targeted peptide vaccines are a feasible method to induce tumor-reactive T cells.[10,12,13]

HOXB7, a homeobox gene family member, encodes a homeodomain protein that regulates embryonic cell development and differentiation.[14–16] *HOXB7* is highly expressed in breast, pancreatic, lung, prostate, and oral squamous cell carcinomas and is associated with tumor cell proliferation, differentiation, invasion, and angiogenesis through PI3K/Akt and MAPK pathways.[16–21] Although *HOXB7* expression is correlated with clinical progression and poor prognosis in patients with cancer,[22] an inhibitor of *HOXB7* has not been identified. As ovarian cancer patients showed significant serologic reactivity to recombinant HOXB7 protein,[23] the immunogenicity of this protein regarding T-cell epitope has not been elucidated. Thus, immunotherapy targeting *HOXB7* could be a novel and potent method for treating cancer patients with poor prognoses.

In this study, we report that *HOXB7* is highly expressed in HNSCC tissue. A novel epitope derived from *HOXB7* could induce antigen-specific $CD4^+$ HTLs from the PBMCs of healthy individuals and patients with HNSCC. These *HOXB7*-specific HTLs showed direct cytotoxicity in HNSCC cells. Furthermore, *HOXB7* downregulated HLA class II expression through the MAPK pathway. Upregulation of tumoral HLA class II expression by MAPK inhibitors increases the antitumor effect of *HOXB7*-specific HTLs. These results suggest that *HOXB7* is a potential target for cancer immunotherapy, and the combination of a peptide vaccine and MAPK inhibitors could be a novel therapeutic strategy to treat *HOXB7*-expressing tumors.

2 | MATERIALS AND METHODS

2.1 | Patients and IHC

We obtained pretreatment biopsy tissues from the primary sites of 66 patients with HNSCC treated at Asahikawa Medical University. Informed consent was obtained using the opt-out method on the Asahikawa Medical University website. The collection and analysis of clinical data were approved by the Asahikawa Medical University Ethical Committee and Review Board (#16217). Table 1 shows their clinical characteristics. The TNM staging was based on the 8th edition of the UICC classification.

The expression of *HOXB7* was analyzed in formalin-fixed and paraffin-embedded tissues from HNSCC patients. Rabbit polyclonal Ab against *HOXB7* (NBP2-14098, 1:100 dilution; Novus Biological) served as the primary Ab. Ventana Benchmark GX, Cell Conditioning 1 buffer, and Ventana ultraView Universal DAB Detection Kit (all Roche Diagnostics) were used for IHC. Staining intensity scores for *HOXB7* in tumor cells were graded as follows: 0, no staining; 1, weak; and 2, strong. Quantity scores for *HOXB7* were assessed from the percentage of positively stained tumor cells and graded as follows: 0, <5%; 1, 5%–25%; 2, 26%–50%; 3, >50%. The IHC score was calculated as the sum of the staining intensity and quantity scores, with four or more scores defined as the high expression and three or fewer scores defined as low expression. The expression of *HOXB7* was analyzed using the Human Protein Atlas database (https://www.proteinatlas.org/).[24]

2.2 | Cell lines

We used HNSCC cell lines HSC3 (tongue SCC, HLA-DR 15), HSC4 (tongue SCC, HLA-DR 9/10, 53), HPC92Y (hypopharyngeal SCC, HLA-DR4/9, 53), and SAS (tongue SCC, HLA-DR 9/15. 53). HSC3 and HSC4 were supplied by RIKEN BioResource Center. HPC-92Y and L cells (mouse fibroblast cell lines) expressing individual human HLA-DR molecules (HLA-DR4, 9, 15, and 53) were provided by Dr. S. Yanoma (Yokohama Tsurugamine Hospital), Dr. R. Karr (Karr Pharma), and Dr. T. Sasazuki (Kyushu University). SAS cells were purchased from ATCC. All cell lines were maintained in tissue culture, as recommended by the supplier.

2.3 | Western blot analysis

The total protein extraction kit for cultured animal cells and tissues (Invent Biotechnologies, Inc.) was used for protein extraction from HNSCC cell lines. Protein extracts were electrophoresed using a 4%–12% NuPAGE Bis-Tris SDS-PAGE gel (Invitrogen, Thermo Fisher

TABLE 1 Relationship between clinicopathologic features of patients with oropharyngeal cancer and *HOXB7* expression

Clinical feature	*HOXB7* IHC score		*p* value
	Low	High	
Gender			
Female	5	3	0.2561
Male	22	36	
Age (years)			
<70	16	23	>0.9999
≥70	11	16	
Tobacco			
Never smoker	7	5	0.2063
Current or former smoker	20	34	
Alcohol			
Never drinker	8	2	0.0117
Current or former drinker	19	37	
HPV status			
Negative	11	24	0.1333
Positive	16	15	
T classification			
T1,2 (early)	16	15	0.1333
T3,4 (advanced)	11	24	
N classification			
N0 (negative)	16	7	0.0007
N1,2,3 (positive)	11	32	
M classification			
M0	16	49	0.4091
M1	0	1	
Stage			
I, II	13	20	>0.9999
III, IV	14	19	

Abbreviations: IHC, immunohistochemistry; HPV, human papillomavirus.

Scientific Inc.) and transferred onto an Immobilon-P membrane (Merck Millipore).

The membrane was then incubated with mouse anti-human *HOXB7* Ab (40–2000, 1:100 dilution, Invitrogen), rabbit anti-phospho-p44/42 MAPK (pERK1/2) Ab (Thr202/Tyr204; Cell Signaling Technology), rabbit anti-p44/42 MAPK (ERK1/2) Ab (137F5; Cell Signaling Technology), mouse anti-CIITA Ab (7-1H; Santa Cruz Biotechnology), rabbit anti-phospho-Stat1 Ab (D4A7; Cell Signaling Technology), rabbit anti-Stat1 Ab (42H3; Cell Signaling Technology), and mouse anti-human β-actin Ab (C4; Santa Cruz Biotechnology). The Amersham ECL Prime Western Blotting Detection System (GE Healthcare Life Sciences) and Invitrogen iBright Imaging Systems 1500 (Invitrogen) were used for detection by chemiluminescence. ImageJ was used to analyze the expression values of the target bands.[25]

2.4 | Synthetic peptide

We used the Immune Epitope Database Analysis Resource (IEDB, RRID: SCR_013182, https://www.iedb.org/) and SYFPEITHI (http://www.syfpeithi.de/) to investigate the common HLA-DR-binding amino acid sequence of *HOXB7*. We selected a $HOXB7_{8–25}$ peptide that has the potential to bind to multiple HLA-DRs (DR1, 4, 7, 11, and 15). $HOXB7_{8–25}$ peptide was synthesized by Hokkaido System Science.

As a positive control, we used the PADRE peptide (aK-Cha-VAAWTLKAAa, where "a" denotes D-alanine and "Cha" denotes L-cyclohexylalanine) that can bind with multiple HLA-DR molecules.

2.5 | In vitro induction of *HOXB7*-specific $CD4^+$ T cells

The methods utilized for the generation of peptide-specific HTLs from the PBMCs of healthy individuals have been previously described in detail.[26] Briefly, HTLs and monocytes were purified from PBMCs using MACS microbeads for CD4 and CD14, respectively (Miltenyi Biotec). Monocytes were differentiated into DCs using granulocyte-macrophage colony-stimulating factor (50 ng/ml) and interleukin-4 (1000 IU/ml) for 7 days. Helper T lymphocytes were stimulated with $HOXB7_{8–25}$ peptide-pulsed autologous DCs for one cycle and γ-irradiated autologous PBMCs for two cycles. Helper T lymphocytes were assessed for IFN-γ production with $HOXB7_{8–25}$ peptide stimulation using ELISA kits (BD Pharmingen) according to the manufacturer's instructions. The absorbance of the supernatants was measured at 450 nm using a Glomax Discover Microplate Reader (Promega). Positive microcultures showing at least a threefold increase in IFN-γ production compared to that in the unstimulated control were subsequently expanded. Finally, $HOXB7_{8–25}$-specific HTLs were isolated using limiting dilution. AIM-V medium (Invitrogen) supplemented with 3% human male AB serum (Innovative Research) was used as a complete culture medium for all experiments.

2.6 | Antigen-specific responses by *HOXB7*-specific HTLs

The method to assess HTL responses to target peptides has been described previously.[26] Established HTLs ($1–1.5\times10^5$) were co-cultured with APCs. Irradiated autologous PBMCs ($1–1.5\times10^5$), HLA-DR-expressing L cells (3×10^4), or *HOXB7*-expressing HNSCC cell lines (3×10^4) were used as APCs. The anti-HLA-DR Ab L243 (HB-55; ATCC) and anti-HLA class I Ab W6/32 (HB-95; ATCC) were used to evaluate HLA restriction. To upregulate HLA-DR expression (Figure S1), HNSCC cell lines were treated with IFN-γ (500 U/ml; PeproTech) or MEK1/2 inhibitor (U0126, 3 μM; Promega) for 48 h before the assay.

2.7 | *HOXB7* peptide-specific responses in HNSCC patients

Peripheral blood mononuclear cells from six HNSCC patients were cocultured with $HOXB7_{8-25}$ peptides or PADRE (a peptide that can bind to pan HLA-DR molecules) in 96-well plates, as described previously.[10] Seven days after the second stimulation, the supernatants were collected and evaluated using an IFN-γ ELISA kit. All experiments were approved by the Institutional Ethics Committee of Asahikawa Medical University (#16217), and written informed consent was obtained from all participants.

2.8 | Cytotoxicity assay

The cytotoxic activity of *HOXB7*-specific HTLs was evaluated by granzyme B ELISA and flow cytometry. Tumor cells were treated with IFN-γ (500 IU/ml) for 48 h before coculturing with HTLs to enhance HLA-DR expression. After coculturing HTLs with target HNSCC cells, supernatants were collected and evaluated using the Granzyme B ELISA kit (Mabtech) according to the manufacturer's instructions. In the killing assay, the target HNSCC cells were labeled using the CellTrace CFSE Cell Proliferation Kit (Invitrogen). After 6 h of coculturing with various E : T ratios, dead cells were labeled with 7-AAD viability staining solution (BioLegend). Dead tumor cells were detected as CFSE and 7-AAD double-positive cells.

2.9 | Cell proliferation assay

Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8; DOJINDO Laboratories, Kumamoto, Japan). The tumor cells (1×10^4/well) were cultured in a 96-well plate. After removing the supernatant, the CCK-8 solution (10 μl) and culture medium (90 μl) were mixed and incubated for 1 h. The absorbance of each well was measured at 450 nm wavelength using a Glomax Discover Microplate Reader (Promega).

2.10 | Flow cytometry

After pretreatment, HLA-DR expression in HNSCC cell lines with a MEK1/2 inhibitor (U0126, 3 μM; Promega) for 48 h was evaluated by flow cytometry. Anti-HLA-DR Abs conjugated with FITC (BD Pharmingen) and anti-MHC class I Abs conjugated with FITC (BD Pharmingen) were used. Immunoglobulin G1 (MOPC-21; BioLegend) and IgG2a (MOPC-173; BioLegend) were used as isotype controls. Samples were analyzed using a CytoFLEX LX flow cytometer and CytExpert (Beckman Coulter).

2.11 | Small interfering RNA

Lipofectamine RNAiMax Reagent (Invitrogen) and OptiMEM I Reduced Serum Medium (Invitrogen) were used to downregulate *HOXB7* according to the manufacturer's instructions. *HOXB7* siRNA (ID#s6807; Thermo Fisher Scientific) and control siRNA (Silencer Select negative control sequence #2; Thermo Fisher Scientific) were used in this study. After 72 h of incubation with the tumor cells, the expression of *HOXB7* was assessed using western blotting.

2.12 | Statistical analysis

All data were assessed using Student's *t*-test or Fisher's exact test. Statistical significance was set at $p<0.05$. GraphPad Prism 9 (GraphPad Software) was used for all the analyses.

3 | RESULTS

3.1 | *HOXB7* expression in HNSCC patients and cell lines

HOXB7 is highly expressed in HNSCC and other types of cancer.[27] In the database analysis, we observed that more than 30% of patients with HNSCC had high *HOXB7* expression, and their prognosis was inferior to that of patients with low *HOXB7* expression (Figure 1A). To verify this finding, we undertook IHC staining of tissues from 66 patients with HNSCC and evaluated the correlation between *HOXB7* expression and clinical characteristics. *HOXB7* was expressed in 50/66 (76%) HNSCC tissue samples (Figure 1B). Although the relationship between the survival rate and the *HOXB7* positivity was not apparent in our samples, lymph node metastasis was significantly increased in the patient with high *HOXB7* expression (Table 1). It should be noted that *HOXB7* expression was also associated with lymph node metastasis in hepatocellular cancer.[28] In addition, current drinkers highly expressed *HOXB7* compared to nondrinkers.

Next, we examined the expression of *HOXB7* in HNSCC cell lines (HSC3, HSC4, HPC92-Y, and SAS). As shown in Figure 1C, *HOXB7* was expressed in all the examined cell lines. The proliferation of HNSCC cells was inhibited by *HOXB7* knockdown, indicating that *HOXB7* is a vital factor in HNSCC progression and could be a favorable target for treating HNSCC (Figures 1D and S2).

3.2 | Generation of $HOXB7_{8-25}$-reactive $CD4^+$ T cell lines

Due to the lack of *HOXB7* inhibitors and difficulty targeting nuclear proteins such as *HOXB7*, peptide-based immunotherapy could be a feasible approach to target *HOXB7*. Tumor-reactive HTLs, but not $CD8^+$ CTLs, initiate antitumor immunity by supporting surrounding natural killer cells[29] in addition to direct cytotoxicity to tumor cells.[30–34] Thus, we focused on HLA class II-restricted epitopes that could elicit HTL responses in this study. Based on computer-based algorithms, $HOXB7_{8-25}$ (PLLLKLLKSVGAQKD) was selected as a potential candidate for eliciting antigen-specific HTL responses. Purified HTLs from PBMCs of healthy donors

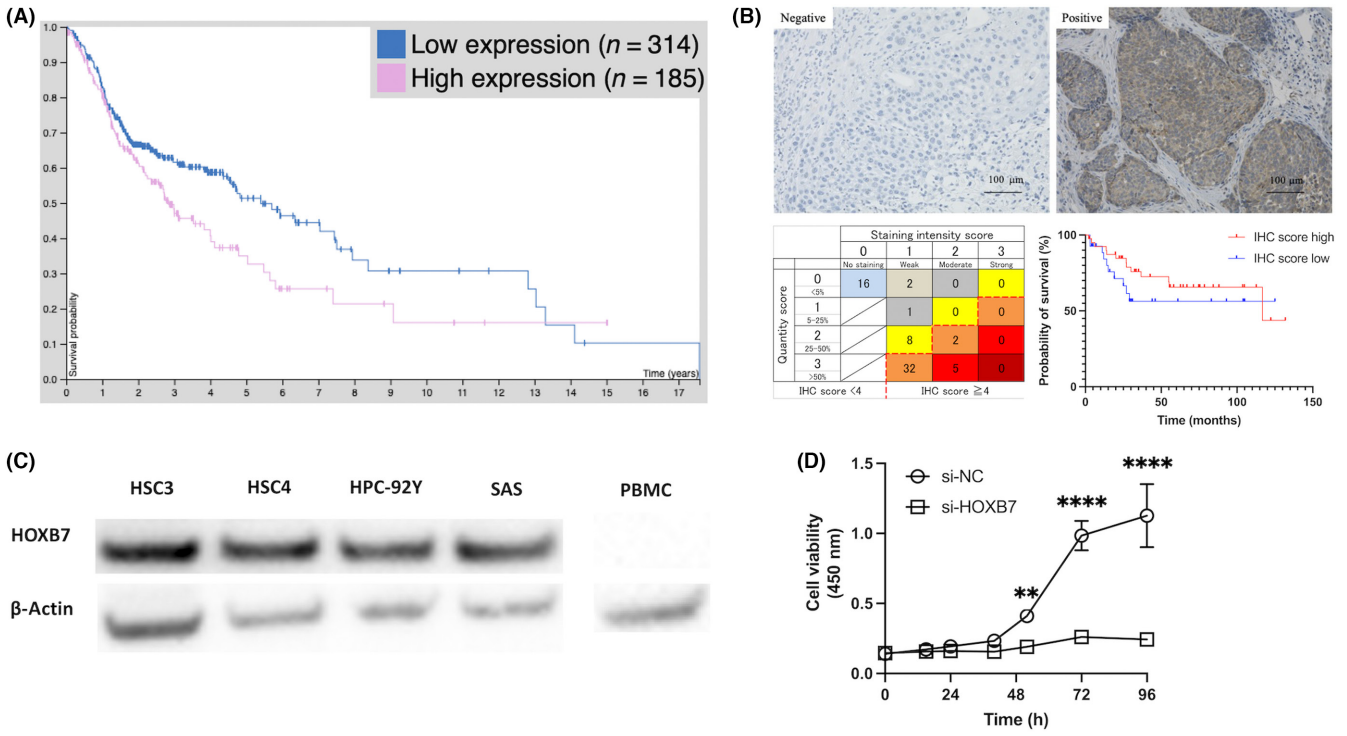

FIGURE 1 Expression of *HOXB7* in head and neck squamous cell carcinoma (HNSCC) patients and cell lines. (A) Expression of *HOXB7* was analyzed using the Human Protein Atlas database (https://www.proteinatlas.org/ENSG00000260027-HOXB7/pathology/head+and+neck+cancer). The 5-year survival was low in HNSCC patients with high *HOXB7* expression (cut-off value, 7.47; *p* score, 0.014). (B) Representative immunohistochemical (IHC) images of *HOXB7* expression. The expression of *HOXB7* was classified based on the quantity and intensity score. The Kaplan–Meier curve of high (IHC score ≥4) or low (IHC score <4) *HOXB7* expression in patients with oropharyngeal cancer is shown. (C) Western blot analysis of *HOXB7* expression in HNSCC cell lines. β-Actin was used as a control. (D) Proliferation of SAS cells with siRNA-mediated gene silencing (control or *HOXB7*) was assessed with the CCK-8 assay. Experiments were performed in triplicate. $^{**}p < 0.01$, $^{****}p < 0.0001$.

were repeatedly stimulated with the $HOXB7_{8-25}$ peptide, and $HOXB7_{8-25}$ peptide-reactive HTLs (S1, S2, and T3) were induced. These HTLs reacted with the $HOXB7_{8-25}$ peptide and released IFN-γ in a dose-dependent manner (Figure 2A). T cell responses were blocked by anti-HLA-DR Ab but not by anti-MHC class I Ab, indicating that these responses were restricted to MHC class II (HLA-DR) (Figure 2B). To determine which HLA-DR is involved in the interaction with each HTL, we used L cells expressing a single HLA-DR as APCs. S1 and S2 reacted with L cells expressing HLA-DR4, whereas T3 reacted with L cells expressing HLA-DR53 (Figure 2C). An HTL cell line was obtained from a donor with HLA-DR11. However, DR11-restriction could not be examined because of the availability of HLA-DR11-expressing APCs. These results indicate that the $HOXB7_{8-25}$ peptide can be presented through several HLA-DR molecules.

3.3 | Direct tumor recognition and cytotoxic activity by $HOXB7_{8-25}$-reactive $CD4^+$ T cell lines

To evaluate whether $HOXB7_{8-25}$ peptide-reactive HTLs can directly recognize tumor cells, HTLs were cocultured with *HOXB7*-expressing HNSCC cell lines. Each $HOXB7_{8-25}$ peptide-reactive HTLs reacted with HLA-DR-matched tumor cell lines and produced IFN-γ (Figure 3A). HLA-DR-unmatched tumors (S1 and S2: SAS and HSC3; T3: HSC3) did not induce $HOXB7_{8-25}$ peptide-reactive HTL responses. Inhibition of HLA-DR canceled the T cell reaction, demonstrating that T cell responses were mediated in the context of the HLA-DR/peptide/T cell receptor complex. In addition to IFN-γ, HTLs produced granzyme B, a tumoricidal cytokine (Figure 3B). To confirm whether $HOXB7_{8-25}$ peptide-reactive HTLs show direct tumor cytotoxicity, CFSE-labeled tumors were cocultured with these T cells. As shown in Figure 3C,D, $HOXB7_{8-25}$ peptide-reactive HTLs directly killed HLA-DR-matched tumor, suggesting that the $HOXB7_{8-25}$ peptide is a potent target for an antitumor peptide vaccine that can induce tumor-reactive cytotoxic HTLs.

3.4 | $HOXB7_{8-25}$-reactive T cells in peripheral blood from HNSCC patients

The presence of peptide-reactive T cells in patients is required to achieve T cell responses with peptide vaccines. However, these T cells might lose their ability to react with peptides in HNSCC patients with suppressed antitumor immunity. To prove the rationality of the $HOXB7_{8-25}$ peptide vaccine in clinical settings,

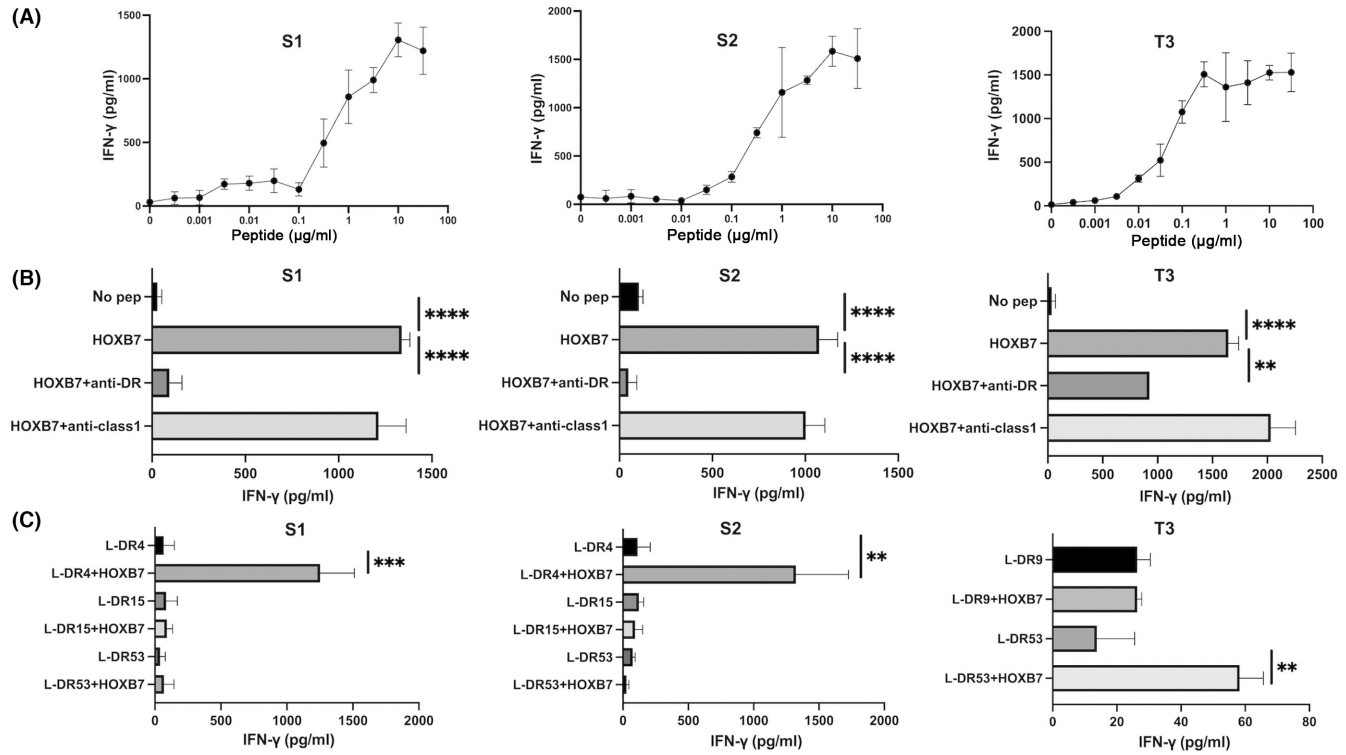

FIGURE 2 Characteristics of $HOXB7_{8-25}$-reactive helper T lymphocytes (HTLs). (A) Evaluation of the peptide dose–responses of $HOXB7_{8-25}$-reactive HTLs (S1, S2, and T3). These T cells were cocultured with autologous PBMCs as antigen-presenting cells (APCs) with various $HOXB7_{8-25}$ peptide concentrations. (B) HLA restriction of $HOXB7_{8-25}$-reactive HTLs. The γ-interferon (IFN-γ) production from T cells cocultured with autologous PBMCs pulsed with $HOXB7_{8-25}$ peptide (3 μg/ml) was evaluated in the presence of an anti-HLA-DR Ab or anti-HLA class I Ab (negative control). (C) HLA-DR allele restriction of the $HOXB7_{8-25}$-reactive HTLs. The response of HTLs to $HOXB7_{8-25}$ peptide was evaluated using peptide-pulsed (3 μg/ml) L cells transfected with individual HLA-DR alleles. IFN-γ production was measured by ELISA after coculturing with APCs for 48 h. Symbols and error bars indicate mean and SD, respectively. Experiments were performed in triplicate. $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

we evaluated T cell reactivity to the $HOXB7_{8-25}$ peptide using PBMCs from HNSCC patients. Peripheral blood mononuclear cells isolated from five HNSCC patients were stimulated twice with the $HOXB7_{8-25}$ peptide, and IFN-γ production was measured. The clinical characteristics of patients are shown in Table 2 and Figure S3. *HOXB7* expression was observed in tumor tissues from all the patients. Notably, all patients reacted with the $HOXB7_{8-25}$ peptide and produced IFN-γ, which was abolished by adding the anti-HLA-DR Ab (Figure 4). We undertook a short-term peptide reactivity assay using healthy donors without HOXB7-positive tumor. As a result, no responses were observed in three PBMCs from healthy donors (Figure S4). These results demonstrate the presence of precursor T cells that can respond to the $HOXB7_{8-25}$ peptide in patients with HOXB7-positive HNSCC.

3.5 | Inhibition of MAPK augments *HOXB7*-targeted immunotherapy through HLA upregulation

HOXB7 is an upstream molecule of MAPK and PI3K, which are both essential for cell proliferation. *HOXB7* downregulation inhibited the expression of phosphorylated MAPK but not total MAPK (Figures 5A and S5). Because inhibition of MAPK also increased HLA-DR expression in tumors (Figure 5B), it is plausible that *HOXB7* suppresses tumoral HLA-DR through MAPK. As we previously reported, inhibition of MAPK increases CIITA expression in tumor.[35] However, only HPC92Y cells showed an increase in CIITA by HOXB7 inhibition alone, while IFN-γ was required for the other cell lines (Figure S6). As STAT1 phosphorylation was also elevated in accordance with CIITA expression, HOXB7 may suppress MHC class II expression through the MAPK and/or IFN-γ pathway (Figure 5C). Because genetic modification is difficult to translate in clinical settings, MAPK inhibitors are a feasible approach as immune adjuvants to upregulate HLA-DR expression. Coculture of MAPK inhibitor-treated HNSCC cell lines with $HOXB7_{8-25}$ peptide-reactive HTLs showed that the MAPK inhibitor upregulated T cell responses against tumors (Figure 5D). Because *HOXB7* expression was unaffected by MAPK inhibition (Figure S7), MAPK inhibitors might be a potent adjuvant to combine with *HOXB7*-targeted peptide vaccines by inducing MHC class II expression without losing tumor antigenicity.

4 | DISCUSSION

We identified a novel T cell epitope peptide from *HOXB7* that generated antitumor HTL responses against tumors expressing *HOXB7*. In

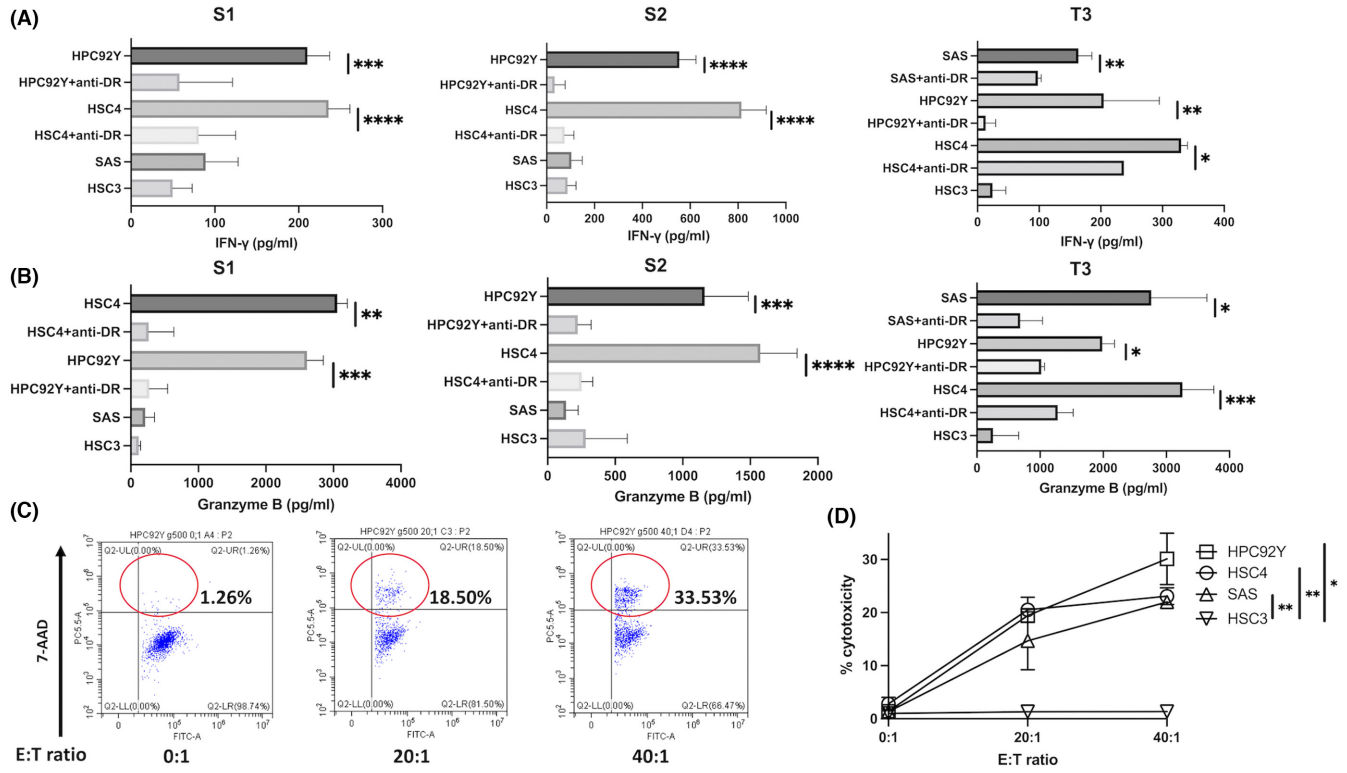

FIGURE 3 Direct cytotoxicity to *HOXB7*-expressing head and neck squamous cell carcinoma (HNSCC) by $HOXB7_{8-25}$-reactive helper T lymphocytes (HTLs). (A) Direct recognition of tumor cells expressing *HOXB7* by $HOXB7_{8-25}$-reactive HTLs was evaluated by coculturing HLA-DR matched or unmatched HNSCC cell lines with or without anti-HLA-DR Ab. The production of IFN-γ in supernatants was measured by ELISA after coculturing for 48 h. S1 and S2 were restricted to HLA-DR4, whereas T3 was restricted to HLA-DR53. HSC3: HLA-DR15; HSC4: HLA-DR1/4, 53; SAS: HLA-DR9/15, 53: HPC-92Y: HLA-DR4/9, 53. (B) Production of granzyme B from $HOXB7_{8-25}$-reactive HTLs cocultured with HLA-DR matched or unmatched tumor cell lines was examined. (C, D) Killing activity of $HOXB7_{8-25}$-reactive HTLs in HLA-DR matched and unmatched tumor cell lines. HLA-DR53 restricted T3 cell lines were cocultured with tumor cell lines with several effector to target (E:T) ratios. HSC3 (HLA-DR15) was used as a negative control. $HOXB7_{8-25}$-reactive HTLs were cocultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled tumor cells for 6 h. Dead cells were labeled with 7-AAD, and the percentage of dead tumor cells ($CFSE^+$ $7\text{-}AAD^+$ cells) was determined using flow cytometry. Representative flow cytometry (HPC-92Y) is shown. Symbols and error bars indicate mean and SD, respectively. Experiments were performed in triplicate. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

TABLE 2 Clinical characteristics of head and neck squamous cell carcinoma patients responding to $HOXB7_{8-25}$ peptide

								HOXB7 IHC score	
No.	Gender	Age (years)	Primary site	TNM	Stage	Tobacco	Alcohol	Intensity	Quantity
1	F	50	Nasopharynx	T4N1M0	IVa	Current	Never	1	3
2	M	78	Oropharynx	T2N1M0	I	Current	Current	1	3
3	M	83	Larynx	T3N1M0	III	Current	Current	1	3
4	M	67	Oropharynx	T0N1M0	I	Never	Current	N/A	
5	F	68	Larynx	T4aN2cM0	IVa	Current	Never	2	3

Abbreviations: F, female; IHC, immunohistochemistry; M, male; N/A: not available.

the past, most reports on the development of antitumor vaccines focused primarily on CTLs. However, CTLs alone are insufficient to generate the T cell memory required to sustain the antitumor effect of the vaccine.[9] The benefit of HTLs induced by the TAA peptide vaccine is that they provide cytokines and costimulatory molecules such as CD40 to APCs to assist the antitumor effects of CTLs. In addition, HTLs can kill tumor cells.[11] Our results indicated that *HOXB7* peptide-specific HTLs recognized tumors and showed cytotoxic activity against HNSCC cell lines as well as IFN-γ production. These results suggest that the novel *HOXB7* peptide is a promising candidate for a tumor vaccine to induce tumor-reactive HTLs. As $HOXB7_{18-26}$ (SSVFAPGAF) might bind to HLA-A*26:01 in in silico analysis, the elongation of $HOXB7_{8-25}$ to $HOXB7_{8-26}$ would improve the antigenicity of the peptide by inducing both HTLs and CTLs.

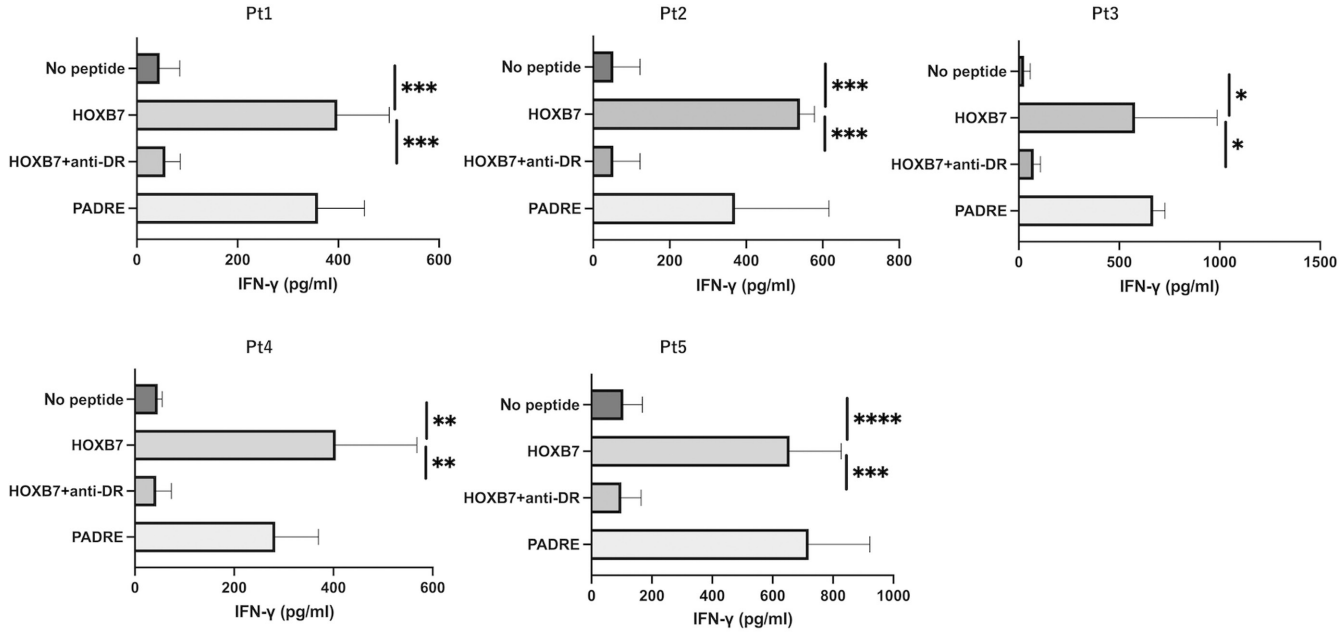

FIGURE 4 Reactivity to $HOXB7_{8-25}$ peptide in PBMCs from head and neck squamous cell carcinoma (HNSCC) patients. PBMCs derived from HNSCC patients were cultured in the presence of $HOXB7_{8-25}$ or PADRE peptide (10 μg/ml) for the first 7 days and restimulated with peptide-pulsed (10 μg/ml) irradiated autologous PBMCs for the following 7 days. The production of γ-interferon (IFN-γ) in the supernatant was measured using ELISA. The PADRE peptide was used as a positive control, and an anti-HLA-DR Ab was used to inhibit the peptide present via HLA-DR. Symbols and error bars indicate mean and SD, respectively. Experiments were performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

In this study, *HOXB7* was selected as the TAA. The relationship between *HOXB7* overexpression and poor prognosis has been reported in various types of cancer.[16,21,36–38] As in previous reports, the present study also found an association between HOXB7 and alcohol consumption.[39] The overexpression of *HOXB7* increases the proliferation, invasion, and migration of tumor cell lines,[27,36,38] and *HOXB7* knockdown inhibits the proliferation of HNSCC cells. Liao et al. reported that *HOXB7* might be a useful prognostic biomarker for colorectal cancer.[21] Likewise, we confirmed that HNSCC patients with high *HOXB7* expression had relatively lower survival rates than negative patients ($p = 0.014$) using the Human Protein Atlas database.[40] Although no significant correlation between *HOXB7* expression and prognosis was observed in our samples, the prevalence of *HOXB7* in the tissues of HNSCC patients was more than 75%, suggesting that *HOXB7* is a rational target in the treatment of HNSCC.

The drawback of targeting TAAs, such as *HOXB7*, is their potential to induce autoimmune diseases. T cells with high-affinity T-cell receptors to self-antigens are depleted in the thymus, a phenomenon known as negative selection. The TAA-reactive T cells with low-affinity T-cell receptors can survive and only react to tissues with high antigen expression. Immunohistochemical staining revealed that healthy tissues surrounding the tumor did not express *HOXB7*. Moreover, we identified precursor cells of *HOXB7*-reactive T cells from both healthy donors and all examined HNSCC patients without any autoimmune diseases. These T cells can directly react with and kill tumor cells. These results suggest that *HOXB7*-reactive T cells that pass through negative selection in the thymus would have low-affinity T-cell receptors and recognize *HOXB7*-high tumors but ignore *HOXB7*-low normal tissues. Notably, the precursor of *HOXB7*-reactive T cells existed in patients with HNSCC, rationalizing *HOXB7* as a target of peptide vaccines. Further research, including an in vivo model, is required to confirm the safety and feasibility of *HOXB7*-targeting immunotherapy.

As shown in this study, we confirmed that inhibition of HOXB7 upregulates the expression of CIITA and phosphorylation of STAT1, which potentiated the effect of IFN-γ. Our results suggest that HOXB7 inhibits the IFN-γ/STAT1/CIITA pathway. In addition, the MAPK pathway is downstream of *HOXB7*. Phosphorylation of MAPK decreased following *HOXB7* knockdown with siRNA. Wang et al. reported that *HOXB7* activates the MAPK pathway through the induction of bFGF secretion following *FGFR3* stimulation, and suppression of bFGF inhibits *HOXB7*-induced MAPK activation.[41] As we previously identified that *FGFR1* was expressed in HNSCC cells,[35] *FGFR3* was not expressed in HNSCC cells (data not shown). Thus, *FGFR3* could be dispensable in the *HOXB7*/MAPK pathway. The MAPK pathway is considered a vital pathway for downregulating tumoral MHC expression to evade antitumor immune responses.[42] As MAPK is reported to negatively regulate CIITA, the master regulator of MHC class II,[43] and CIITA induces MHC class II mRNA,[44] HOXB7 knockdown might upregulate HLA-DR expression at the mRNA level. Our results indicated that *HOXB7* inhibits MHC class II expression not only through the IFN-γ/STAT1/CIITA pathway but also through the MAPK pathway (Figure S8). Inhibition of MAPK augmented IFN-γ production from HTLs through MHC class II upregulation. Because IFN-γ might be insufficient to induce HLA-DR expression in the naïve tumor microenvironment, the introduction of MAPK

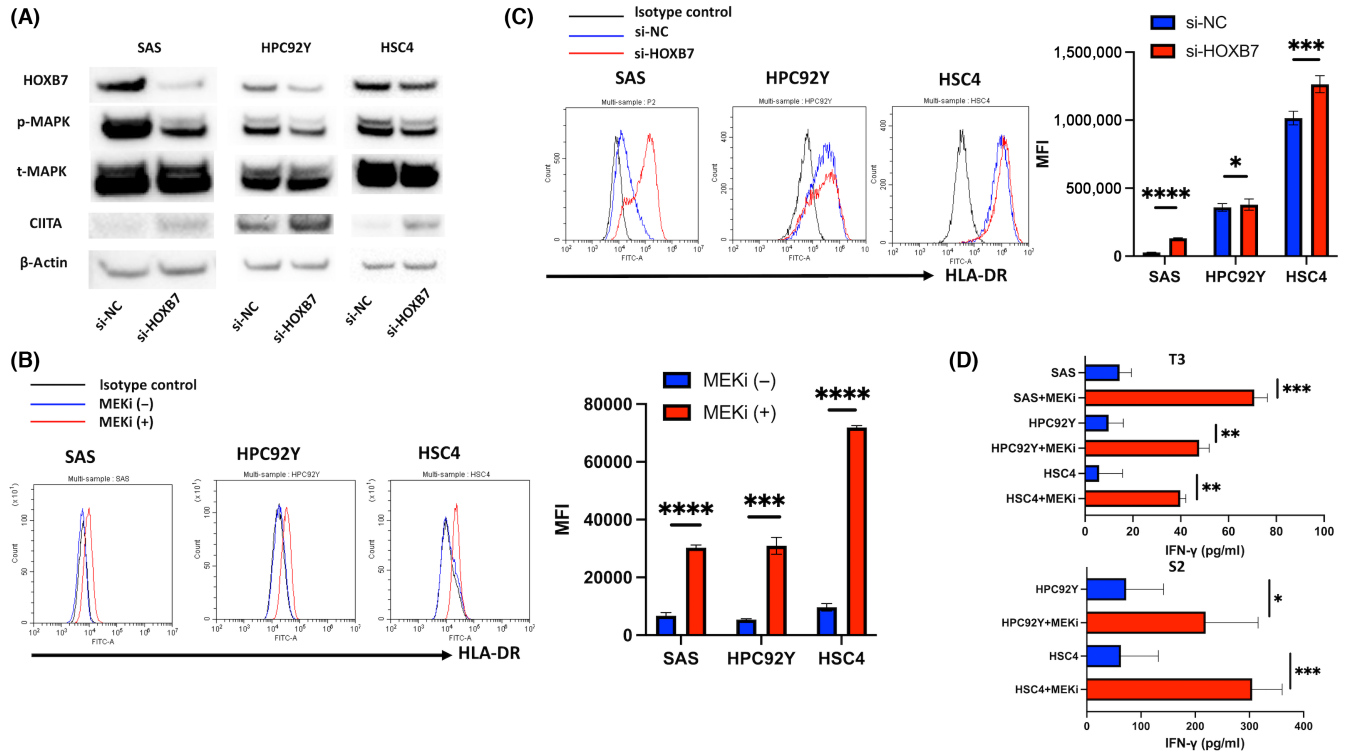

FIGURE 5 Inhibition of MAPK augments the tumor recognition of *HOXB7*$_{8-25}$-reactive HTLs through HLA-DR upregulation. (A) The expression of total MAPK (t-MAPK), phosphorylated MAPK (p-MAPK), and MHC class II transactivator (CIITA) in siRNA-mediated *HOXB7* knockdown head and neck squamous cell carcinoma (HNSCC) cell lines was evaluated using western blot analysis. All tumor cells were treated with γ-interferon (IFN-γ; 50 IU/ml) for 48 h before the assay. si-*HOXB7*, HNSCC cells transfected with *HOXB7* siRNA; si-NC, HNSCC cells transfected with negative control siRNA. (B) Expression levels of HLA-DR on HNSCC cell lines incubated with or without MAPK inhibitors were evaluated by flow cytometry. Black, isotype control; blue, HLA-DR expression on untreated tumor cells; red, HLA-DR expression on tumor cells treated with MAPK inhibitors (3 μM) for 48 h. (C) Expression levels of HLA-DR on HNSCC cell lines were evaluated by flow cytometry. Black, isotype control; blue, HLA-DR expression on tumor cells transfected with negative control siRNA; red, HLA-DR expression on tumor cells transfected with *HOXB7* siRNA. All tumor cells were treated with IFN-γ (50 IU/ml) for 48 h before the assay. (D) Responses of *HOXB7* $_{8-25}$-reactive HTLs to tumor cell lines pretreated with MAPK inhibitors (3 μM) for 48 h were evaluated by measuring IFN-γ production. After coculturing for 48 h, the production of IFN-γ was measured by ELISA. Blue, IFN-γ production levels of T cells cocultured with untreated tumor cells; red, IFN-γ production levels of T cells cocultured with MAPK inhibitor-pretreated tumor cells. Symbols and error bars indicate mean and SD, respectively. Experiments were performed in triplicate. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

inhibitors would be a promising immune adjuvant to trigger massive T cell responses with peptide vaccines. Mitogen-activated protein kinase inhibitors have been clinically applied to several carcinomas, such as melanoma. Clinical trials of MAPK inhibitors combined with ICIs or molecular-targeted therapy are currently underway for many types of carcinoma.[45,46] Our results provide preliminary evidence to confirm the rationality of ongoing clinical trials.

In conclusion, *HOXB7* is highly expressed in HNSCC patients. We identified a novel helper epitope from *HOXB7* that triggered an antigen-specific HTL response. *HOXB7*-specific HTLs recognized tumor cell lines and directly killed the tumor, and the response was HLA-restricted. The precursors of *HOXB7*-specific HTLs were identified in patients with HNSCC and healthy donors. In addition, the antitumor response of *HOXB7*-specific HTLs was augmented by MAPK inhibitors through upregulation of MHC class II. We believe that *HOXB7* is a potential target for antigen-specific immunotherapy and that combining a peptide vaccine and MAPK inhibitors could be an effective strategy for cancer treatment.

AUTHOR CONTRIBUTIONS

HKom, RW, MK, TK, RH, HY, RS, and TN took part in acquisition, analysis, and interpretation of data. KO, MT, and AKa were involved in statistical analysis of data. TO, AKo, and HKob participated in material support. HKom, TK, TN, and HKob were involved in development of methodology. TK took part in conception, design, and supervision of the study. Hkom and TK were involved in writing the paper. TK, TN, and HKob participated in review of the paper. All authors agree to the content of the manuscript.

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DISCLOSURE

The authors declare no conflicts of interest.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: This study was approved by the Research Ethics Committee of Asahikawa Medical University and was performed in accordance with the Declaration of Helsinki.

Informed consent: Written informed consent was obtained from all donors who provided samples.

Registry and registration no. of the study/trial: N/A.

Animal studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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