

学位論文

**Phosphorylated vimentin as an immunotherapeutic  
target against metastatic colorectal cancer**

(リン酸化ビメンチンを標的とした大腸癌に対する  
腫瘍反応性ヘルパーT細胞応答に関する研究)

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# Phosphorylated vimentin as an immunotherapeutic target against metastatic colorectal cancer

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## Abstract

Colorectal cancer (CRC) patients with metastatic lesions have low 5-year survival rates. During metastasis, cancer cells often obtain unique characteristics such as epithelial–mesenchymal transition (EMT). Vimentin a biomarker contributes to EMT by changing cell shape and motility. Since abnormal phosphorylation is a hallmark of malignancy, targeting phosphorylated vimentin is a feasible approach for the treatment of metastatic tumors while sparing non-tumor cells. Recent evidence has revealed that both CD8 cytotoxic T lymphocytes (CTLs) and also CD4 helper T lymphocytes (HTLs) can distinguish post-translationally modified antigens from normal antigens. Here, we showed that the expression of phosphorylated vimentin was upregulated in metastatic sites of CRC. We also showed that a chemotherapeutic reagent augmented the expression of phosphorylated vimentin. The novel phosphorylated helper peptide epitopes from vimentin could elicit a sufficient T cell response. Notably, precursor lymphocytes that specifically reacted to these phosphorylated vimentin-derived peptides were detected in CRC patients. These results suggest that immunotherapy targeting phosphorylated vimentin could be promising for metastatic CRC patients.

**Keywords** CD4 T cell · Vimentin · Post-translational modification · Phosphorylation · Epitope · Colorectal cancer

## Abbreviations

APCs Antigen-presenting cells  
CRC Colorectal cancer

CTLs CD8 cytotoxic T lymphocytes  
DCs Dendritic cells  
EMT Epithelial–mesenchymal transition  
HTLs CD4 helper T lymphocytes  
L-cells Fibroblast cell lines  
mAb Monoclonal antibody  
PBMcs Peripheral blood mononuclear cells  
SCC Squamous cell carcinoma  
TAA Tumor-associated antigen  
TCR T cell receptors

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## Introduction

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer-related deaths worldwide. The treatment of metastatic CRC patients still remains challenging [1]. Although CRC patients having mismatch-repair deficiency can receive a clinical benefit from immune checkpoint blockade, the frequency of mismatch-repair deficiency is only around 20% in CRC, indicating that novel

therapeutic breakthroughs are necessary to cure the majority of patients [2, 3].

Since metastatic tumors possess different features such as epithelial-to-mesenchymal transition (EMT) compared to primary lesions, it is rational to target these characteristics to treat patients with metastatic CRC. Among EMT-related proteins, the expression and function of vimentin have been examined in detail [4]. Several groups have reported that the expression of vimentin is related to the poor prognosis of CRC patients [5–8]. In addition, the phosphorylation of vimentin enhances the growth of metastatic tumors [9]. Since phosphorylation is a tightly regulated post-translational cytosolic event, excessive phosphorylation of cellular proteins is a characteristic feature of malignant conversion [10, 11]. We recently focused on post-translationally modified antigens that might be more immunogenic than wild-type antigens [12, 13] and reported that human CD4 helper T lymphocytes (HTLs) can specifically recognize phosphorylated p53 [12]. Thus, phosphorylated vimentin might be a suitable target to overcome metastatic CRC.

Based on the success of immune checkpoint inhibitors, immunotherapy is considered as an effective armamentarium to treat cancer. Among immunotherapies, peptide vaccines are an active immunotherapy to stimulate antigen-specific T cells. Although the selection of peptides, immune adjuvants, and the route of administration should be improved to enhance antitumor responses [14], peptide vaccine therapy has benefits such as cost-effectiveness, simplicity of synthesis, and a general acceptance in clinical practice [15]. Tumor antigen-specific CD8 cytotoxic T lymphocytes (CTLs) tend to reduce their cytotoxic activity without the aid of CD4 helper T lymphocytes (HTLs) [16]. As we and other researchers have reported, peptide-reactive HTLs play a pivotal role not only in helping CTLs, but also in exhibiting direct toxicity toward tumor cells [13, 17]. Therefore, the identification of HTL epitopes from tumor-associated antigens (TAAs) is a key factor in developing an effective peptide vaccine against tumors.

In the present study, we identified phospho-peptide epitopes from vimentin that could elicit an effective HTL response. The treatment of tumor cells with a chemotherapeutic agent enhanced the expression of phosphorylated vimentin and augmented the ability of HTL responses against tumor cells. Moreover, we identified precursor T cells specific for phosphorylated vimentin in CRC patients, suggesting that phosphorylated epitopes from vimentin have sufficient immunogenicity. The combination of our vimentin-derived phosphorylated peptide vaccine with chemotherapy could be a novel approach to treat CRC patients.

## Materials and methods

### Cell lines

We used mouse fibroblast cell lines (L-cells) that were transfected with plasmids expressing individual human HLA-DR molecules (DR4, DR8, DR9, DR14, and DR53), CRC cell lines SW480 (DR1/13) and SW620 (DR1/13), lung squamous cell carcinoma (SCC) cell lines Calu-1 (DR7/14, -53) and Jurkat (T cell lymphoma, a cell line not expressing HLA-DR), gingival SCC cell line Sa-3 (DR9/10, -53), lung large cell carcinoma cell line Lu65 (DR4/15, -53), and renal cell carcinoma cell line SW839 (DR1/9, -53). All cell lines were maintained as recommended by the supplier.

### Immunohistochemistry

Immunohistochemistry was performed as previously described [12]. Polyclonal rabbit antihuman phospho-vimentin S39 (1:200, Cell Signaling Technology (CST), #13614, Denver, CO) and rabbit antihuman phospho-vimentin S72 (1:200, Abcam, ab52944, Cambridge, MA) served as primary antibodies. Cases with more than 5% of phospho-vimentin-stained cells were regarded as positive. Anti-HLA-DR (TAL.1B5, 1:100, DAKO, Glostrup, Denmark) mAb was used to stain HLA-DR. FFPE sections were stained with VENTANA BenchMark GX (Roche Diagnostics, Rotkreuz, Switzerland) utilizing a VENTANA ultraView Universal DAB Detection Kit (Roche Diagnostics) and treated with Cell Conditioning 1 buffer (Roche Diagnostics) for antigen retrieval. HLA-DR expression in specimens with 10% tumor cell membranous staining was considered “positive” as previously reported [18]. Representative data were obtained by a BZ-X710 microscope (Keyence, Tokyo, Japan).

### Western blot analyses

Expression of phosphorylated vimentin was evaluated by western blotting after the treatment of tumor cell lines with or without 1  $\mu$ M doxorubicin for 24 h as previously described [12]. Antibodies used to identify specific protein expression were as follows: rabbit antihuman wt vimentin mAb (1:1,000, CST, #5741), rabbit antihuman phospho-vimentin S39 mAb (1:1,000, CST, #13614), rabbit antihuman phospho-vimentin S72 mAb (1:10,000, Abcam, EP1070Y), rabbit antihuman phospho-vimentin S83 mAb (1:1,000, CST, #3878), and mouse anti- $\beta$ -actin (1: 1,000, Santa Cruz, sc-47778, C4, Santa Cruz, CA).

### Synthetic peptides

We designed peptide epitopes from the previously described sequences [19]. Synthetic peptides were

purchased from GenScript (Tokyo, Japan). Wild-type (wt) vimentin<sub>28-49</sub> (LRSYVTTSTRTYSLGSALRPSTS), wt-vimentin<sub>63-90</sub> (TRSSAVRLRSSVPGVRLQLQDSVDFSLAD), vimentin<sub>28-49/phospho-S39</sub>, vimentin<sub>63-90/phospho-S72</sub>, vimentin<sub>63-90/phospho-S83</sub>, and vimentin<sub>63-90/phospho-S72 and S83</sub> (referred to as p-Vim<sub>S39</sub>, p-Vim<sub>S72</sub>, p-Vim<sub>S83</sub>, and p-Vim<sub>S72/S83</sub>) peptides were used throughout this study. The tetanus toxoid (TT<sub>830-843</sub>; QYI-KANSKFIGITE) peptide was used as a positive control that could bind to multiple HLA class II molecules [20].

### In vitro induction of phosphorylated vimentin peptide-reactive CD4 helper T cells

The technique utilized for the induction of peptide-reactive HTL cell lines from peripheral blood mononuclear cells (PBMCs) of healthy volunteers (volunteer 1: HLA-DR4/9, DR53; volunteer 2: HLA-DR9/14, 53; and volunteer 3: HLA-DR4/8, 53) has been previously described in detail [21].

Briefly, we purified HTLs using CD4 MACS microbeads (Miltenyi Biotec, Auburn, CA). Then, we repeatedly stimulated these HTLs with peptide-pulsed autologous dendritic cells (DCs) or  $\gamma$ -irradiated autologous PBMCs and peptides (3  $\mu$ g/mL). The HTL responses or the cytotoxic activity to phosphorylated vimentin peptide-loaded autologous PBMCs was examined by measuring the levels of cytokines (IFN- $\gamma$  and GM-CSF ELISA kits, BD Pharmingen, Granzyme B ELISA, MABTECH, Stockholm, Sweden). An analysis of direct recognition of tumor cells by phosphorylated vimentin-reactive HTLs was performed as previously reported [22]. To increase phosphorylation of vimentin, the tumor cells were preincubated with or without 1  $\mu$ M doxorubicin (CST) during the last 24 h of IFN- $\gamma$  treatment.

### Measurement of peptide-specific responses in CRC patients

PBMCs were collected from fresh heparinized whole blood of CRC patients by Lymphoprep<sup>TM</sup> (Axis-Shield PoC AS, Oslo, Norway) centrifugation and co-cultured with the peptides (10  $\mu$ g/mL) as described previously [18, 23]. The PBMCs were stimulated for 2 weeks (1 stimulus/week). The production of IFN- $\gamma$  was evaluated 7 days after the second peptide stimulation.

### Statistical analysis

The data were analyzed using Student's *t* test.  $p < 0.05$  indicates statistical significance. Error bars in the data indicate the standard error of the mean (SEM). GraphPad Prism 6 was used for analyses.

## Results

### Expression of phosphorylated vimentin in primary- and metastatic-site specimens of colorectal cancer

We first examined the expression of phosphorylated vimentin in surgical specimens of primary CRC and their metastatic sites by immunohistochemistry. Twenty-two CRC patients in the Department of Surgery at Asahikawa Medical University who later developed distant metastases were included. The clinical factors of these CRC patients are described in Table 1. Phospho-Vim<sub>S39</sub> and phospho-Vim<sub>S72</sub> were expressed in primary tumor specimens of 12/22 (55%) and 15/22 (68%) patients and in metastatic tumor specimens of 15/22 (68%) and 20/22 (91%) patients, respectively. Phospho-vimentin expression was positive in the cytoplasm. Representative data are shown in Supplementary Fig. 1A–C. Phospho-vimentin was not expressed in healthy colon tissue (Supplementary Fig. 3). In addition to CRC, phospho-vimentin is also expressed in head and neck cancer, suggesting that the phosphorylation of vimentin is a common feature of malignant diseases. These results showed that multiple serine residues of vimentin were simultaneously phosphorylated in CRC, indicating the potential of phosphorylated vimentin as an immunotherapeutic target. Because MHC class II was expressed in 60% of patients whose primary tumor expressed HLA-DR (Supplementary Fig. 2), patients with metastatic CRC are candidates for CD4-based immunotherapy.

### Expression of phosphorylated vimentin in tumor cell lines

We next assessed the expression of phosphorylated vimentin in tumor cells. Western blot analysis revealed that phosphorylated vimentin S39 and S72 were expressed in several tumor cell lines including CRC cell lines (Fig. 1a–c). Sa3 (head and neck squamous cell cancer) cells expressed neither wt (wild-type, non-phosphorylated) nor phosphorylated vimentin. Since chemotherapy is a standard strategy to treat metastatic CRC, we used chemotherapeutic reagents (doxorubicin, paclitaxel, and 5-FU) to determine whether such reagents could enhance the expression of vimentin. As shown in Fig. 1b, c, doxorubicin augmented the expression of both wt and phosphorylated vimentin. Neither paclitaxel nor 5-FU affected the expression of wt or phosphorylated vimentin (data not shown). Although doxorubicin is not included in the standard chemotherapy regimen for CRC, it is applied for liver metastasis of CRC patients [24]. Moreover, doxorubicin is combined with a checkpoint inhibitor for

**Table 1** Clinical features and phospho-vimentin and HLA-DR expression of CRC patients

Case no.	Age	Sex	T	N	M	Stage	Primary tumor			Metastatic tumor		
							p-v S39	p-v S72	HLA-DR	p-v S39	p-v S72	HLA-DR
1	77	F	3	2a	1a	IVA	–	–	–	Cytoplasm	Cytoplasm	–
2	53	M	2	2a	1a	IVA	–	–	–	–	–	–
3	59	F	4a	2a	1a	IVA	–	–	–	Cytoplasm	Cytoplasm	–
4	44	F	2	2a	1a	IVA	Cytoplasm	Cytoplasm	+	–	Cytoplasm	+
5	68	M	2	0	0	I	–	–	+	–	–	–
6	70	M	3	0	1a	IVA	Cytoplasm	Cytoplasm	+	–	Cytoplasm	+
7	54	F	4a	2b	0	IIIC	Cytoplasm	–	–	–	Cytoplasm	–
8	78	F	3	2a	1a	IVA	Cytoplasm	Cytoplasm	+	–	cytoplasm	–
9	66	M	3	1b	1a	IVA	–	–	–	–	Cytoplasm	–
10	50	F	4a	1b	1a	IVA	–	–	+	–	Cytoplasm	+
11	48	F	3	0	0	IIA	–	Cytoplasm	–	Cytoplasm	Cytoplasm	–
12	71	M	3	0	1a	IVA	Cytoplasm	Cytoplasm	–	–	Cytoplasm	–
13	54	F	3	1b	1b	IVB	–	Cytoplasm	+	–	Cytoplasm	–
14	65	F	3	0	1a	IVA	Cytoplasm	Cytoplasm	–	Cytoplasm	Cytoplasm	–
15	67	M	3	1b	1a	IVA	–	Cytoplasm	+	Cytoplasm	Cytoplasm	–
16	52	M	3	1c	0	IIIB	Nucleus	Cytoplasm	–	Nucleus	Cytoplasm	–
17	60	M	3	0	1a	IVA	Cytoplasm	Cytoplasm	–	Cytoplasm	Cytoplasm	–
18	77	F	3	1a	0	IIIB	Cytoplasm	Cytoplasm	–	Cytoplasm	Cytoplasm	–
19	65	F	3	2a	1a	IVA	–	Cytoplasm	+	Cytoplasm	Cytoplasm	+
20	66	M	4a	1b	1b	IVB	Cytoplasm	Cytoplasm	+	Cytoplasm	Cytoplasm	+
21	65	F	3	2a	1a	IVA	Cytoplasm	Cytoplasm	–	Cytoplasm	Cytoplasm	–
22	60	F	3	0	1a	IVA	Cytoplasm	Cytoplasm	+	Cytoplasm	Cytoplasm	+

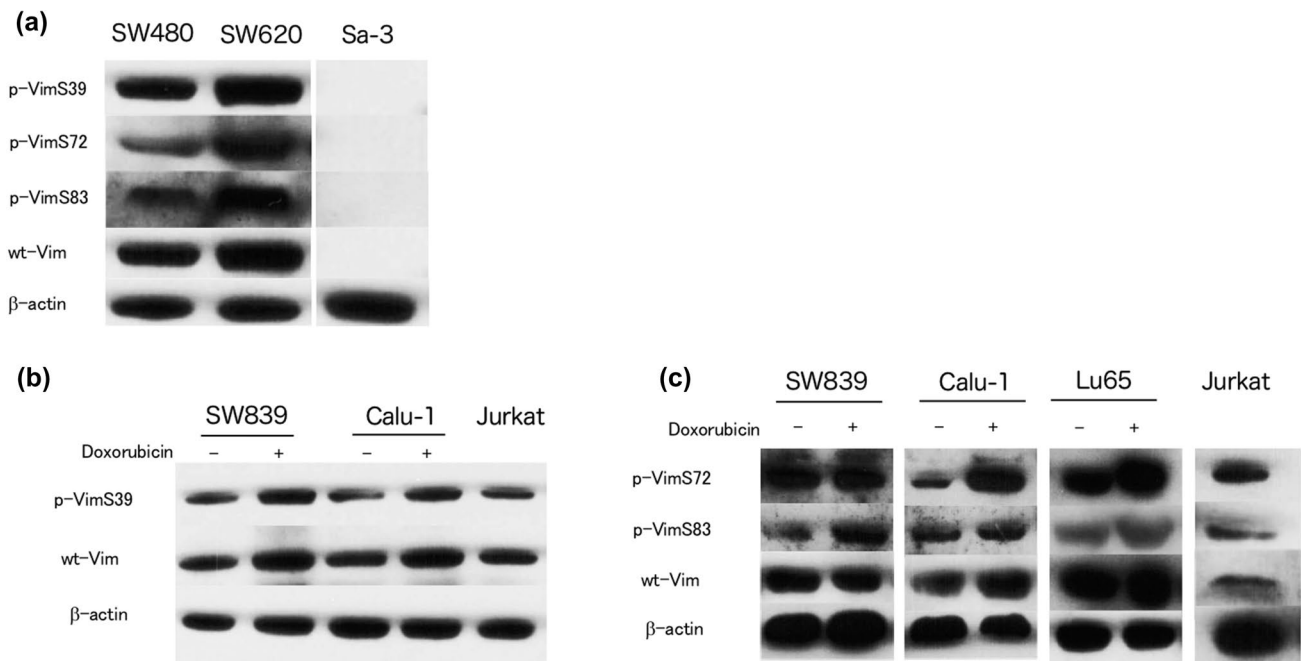
The case is regarded positive when more than 5% of cells were stained and the location information was described

CRC in the form of cancer photochemotherapy, indicating that doxorubicin might be useful to induce phosphorylated vimentin peptide epitopes in a clinical setting [25].

### In vitro induction of CD4 T cells that react to phosphorylated vimentin

To use vimentin as a peptide vaccine to treat CRC, we next sought to find the phosphorylated epitope peptide from its amino acid sequence. The citrullinated peptide epitope, cit-vimentin<sub>28-49</sub>, has been previously reported as a naturally processed epitope recognized by human HTLs [19]. Since vimentin<sub>28-49</sub> contains a phosphorylated serine 39 residue, we investigated the immunogenicity of vimentin<sub>28-49/phospho-S39</sub> peptide. In addition, we selected three other peptides, vimentin<sub>63-90/phospho-S72</sub>, vimentin<sub>63-90/phospho-S83</sub>, and vimentin<sub>63-90/phospho-S72 and S83</sub>, which possess phosphorylated serine 72 and serine 83 residues. We investigated whether the four peptides can elicit the phosphorylated peptide-specific HTL response from human PBMCs. As a result, these four epitope peptides were capable of inducing phosphorylated peptide-specific HTL cell lines (Fig. 2). The mono-phosphorylated peptide vimentin<sub>28-49/phospho-S39</sub> (referred to

as p-Vim<sub>S39</sub>) elicited phosphorylated peptide-specific HTL responses from two healthy donors (H7 and T17). These HTLs only reacted to p-Vim<sub>S39</sub> in a concentration-dependent manner, but not to wt vimentin<sub>28-49</sub> (referred to as wt-Vim<sub>28-49</sub>), indicating that the T cell receptors (TCR) of these HTLs are specific to phosphorylated Ser39. The dual phosphorylated peptide vimentin<sub>63-90/phospho-S72 and S83</sub> (referred to as p-Vim<sub>S72/S83</sub>) also elicited phosphorylated peptide-specific HTL responses (K26). This HTL clone reacted to p-Vim<sub>S72</sub> but not to p-Vim<sub>S83</sub>, demonstrating that TCR of this clone specifically recognize phosphorylated Ser72. Since these responses were inhibited by an anti-HLA-DR mAb (L243), which is specific for HLA-DR, the HTL clones were stimulated in the context of HLA-DR–peptide–TCR molecules (Fig. 2b). Subsequently, a panel of mouse fibroblasts (L-cells) expressing single HLA-DR molecules were used as antigen-presenting cells (APCs) to determine which HLA class II molecules present the phosphorylated vimentin peptides to the HTLs. As shown in Fig. 2c, HTLs H7 and K26 recognized the phosphorylated vimentin peptide in the context of HLA-DR53, while the response of T17 was restricted by HLA-DR14. These results showed that the phosphorylated vimentin peptides were able to bind to several HLA-DR



**Fig. 1** Expression of wild-type (wt) vimentin protein and phosphorylated vimentin in tumor cells. **a** The expressions of wt vimentin protein and phosphorylated vimentin (phosphorylated Ser39, Ser72, or Ser83) in tumor cell lines was examined by western blotting. **b, c** The tumor cell lines were treated with or without doxorubicin (1  $\mu$ M)

for 24 h before assessment. SW480 and SW620: colon cancer. Calu-1: lung squamous cell carcinoma. Sa-3: head and neck squamous cell carcinoma. Lu65: lung adenocarcinoma. SW839: renal cell carcinoma. Jurkat: T cell lymphoma

molecules that cover a broad population of cancer patients. In summary, these findings revealed that HTL precursors, which react to p-Vim<sub>S39</sub> and p-Vim<sub>S72</sub> and <sub>S83</sub>, exist in healthy human PBMCs.

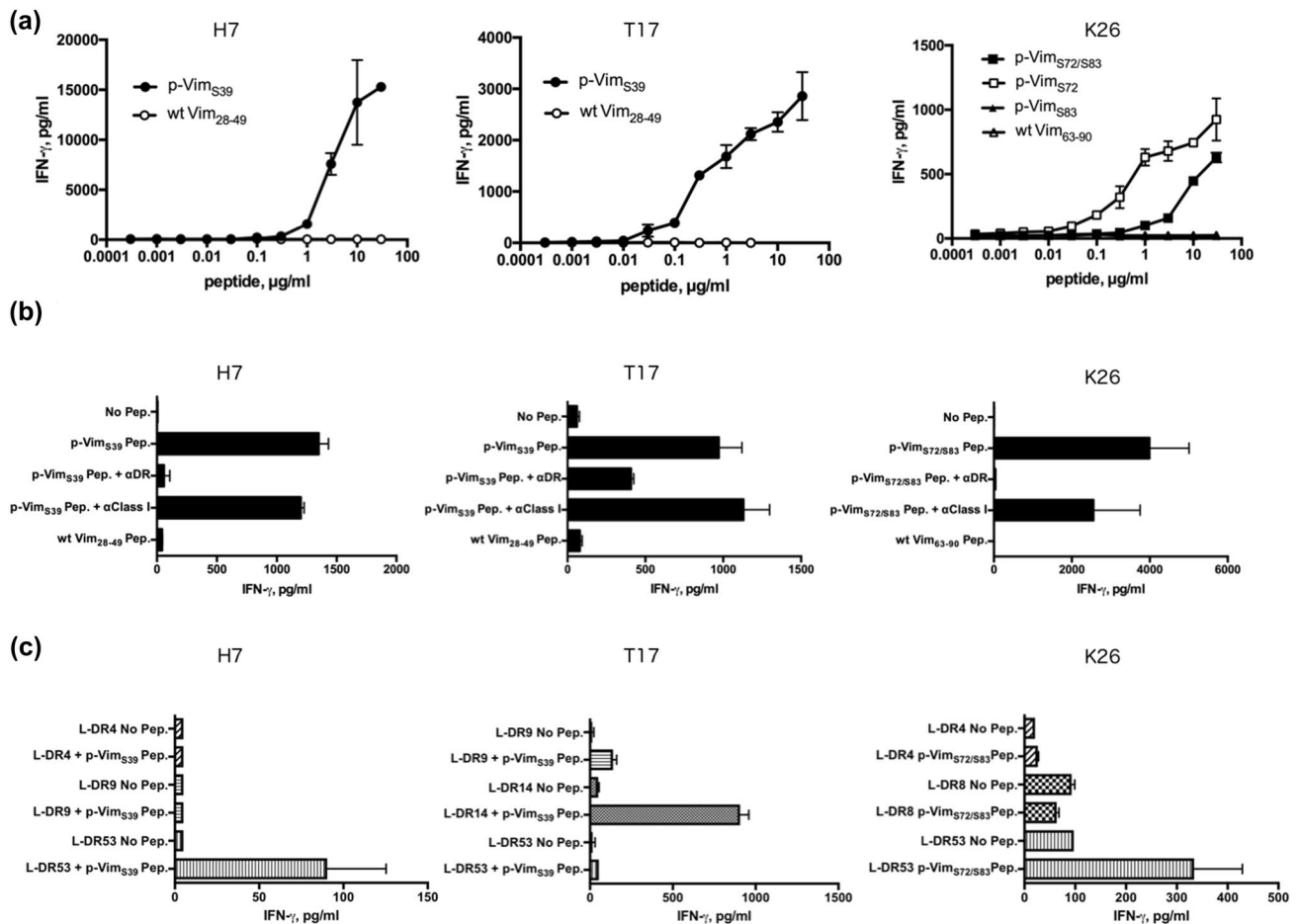
### Direct recognition of phosphorylated vimentin-positive tumor cells by peptide-reactive CD4 T cells

Next, we assessed whether phosphorylated vimentin-reactive HTLs could directly recognize phosphorylated vimentin-expressing tumor cells. Peptide-reactive HTLs and HLA-DR-matched phosphorylated vimentin-positive tumor cells were co-cultured, and IFN- $\gamma$  levels in the culture supernatant were measured. All phosphorylated vimentin-specific HTLs (H7, T17, and K26) directly recognized HLA-DR-matched phosphorylated vimentin-positive tumor cells, but did not react to HLA-DR-unmatched phosphorylated vimentin-positive tumor cell lines, HLA-DR-matched phosphorylated vimentin-negative tumor cell lines, or a HLA-DR-negative tumor cell line (Fig. 3a). In addition, p-Vim<sub>S39</sub>-reactive HTLs (H7) produced granzyme B against HLA-DR-matched phosphorylated vimentin-positive tumor cells (Fig. 3b), suggesting that these HTLs exhibited cytotoxicity against them. In accordance with the increased expression of phosphorylated vimentin

with treatment by doxorubicin (Fig. 1b, c), all phosphorylated vimentin-reactive HTL clones produced more IFN- $\gamma$  and granzyme B when tumor cells were pretreated with doxorubicin before co-culturing (Fig. 3a, b). These results showed that phosphorylated vimentin-reactive HTLs reacted to tumor cells in an HLA-DR-dependent manner, and the phosphorylation of vimentin was preserved during the antigen-processing phase. Moreover, the chemotherapeutic agent doxorubicin can act as an adjuvant to increase the expression of phosphorylated vimentin.

### Indirect recognition of phosphorylated vimentin-positive colorectal tumor cells by phosphorylated peptide-reactive CD4 T cells

We next evaluated whether phosphorylated vimentin peptide can be processed from tumor cell lysates and successfully presented through MHC class II molecules in autologous DCs. As shown in Fig. 4a, b, p-Vim<sub>S39</sub>-reactive HTLs (H7) and p-Vim<sub>S72/S83</sub>-reactive HTLs (K26) were capable of producing IFN- $\gamma$  in response to colon cancer cell lysate-loaded DCs, and these reactions were suppressed by an anti-HLA-DR mAb (L243), indicating that phosphorylated vimentin epitopes are preserved during the exogenous antigen-processing pathway.



**Fig. 2** Induction of CD4 T cell responses using phosphorylated vimentin peptides. **a** Phosphorylated vimentin-reactive CD4 T cells (H7, T17, and K26) were assessed for their ability to recognize various concentrations of phosphorylated and wild-type (wt) peptides. These cell lines were selected from peptide-reactive CD4 T cells using limiting dilution. Autologous PBMCs were used as antigen-presenting cells (APCs). **b** MHC restriction analysis of phosphorylated vimentin-reactive CD4 T cells. Peptide-induced T cell responses of

phosphorylated vimentin-reactive CD4 T cells (H7, T17, and K26) in the presence of anti-HLA-DR mAb L243 or anti-HLA class I mAb W6/32 (negative control) were evaluated. Autologous PBMCs were used as APCs. **c** CD4 T cell responses to phosphorylated p53 peptides were measured using L-cells transfected with individual HLA-DR genes as APCs to determine the restricting HLA class II molecules

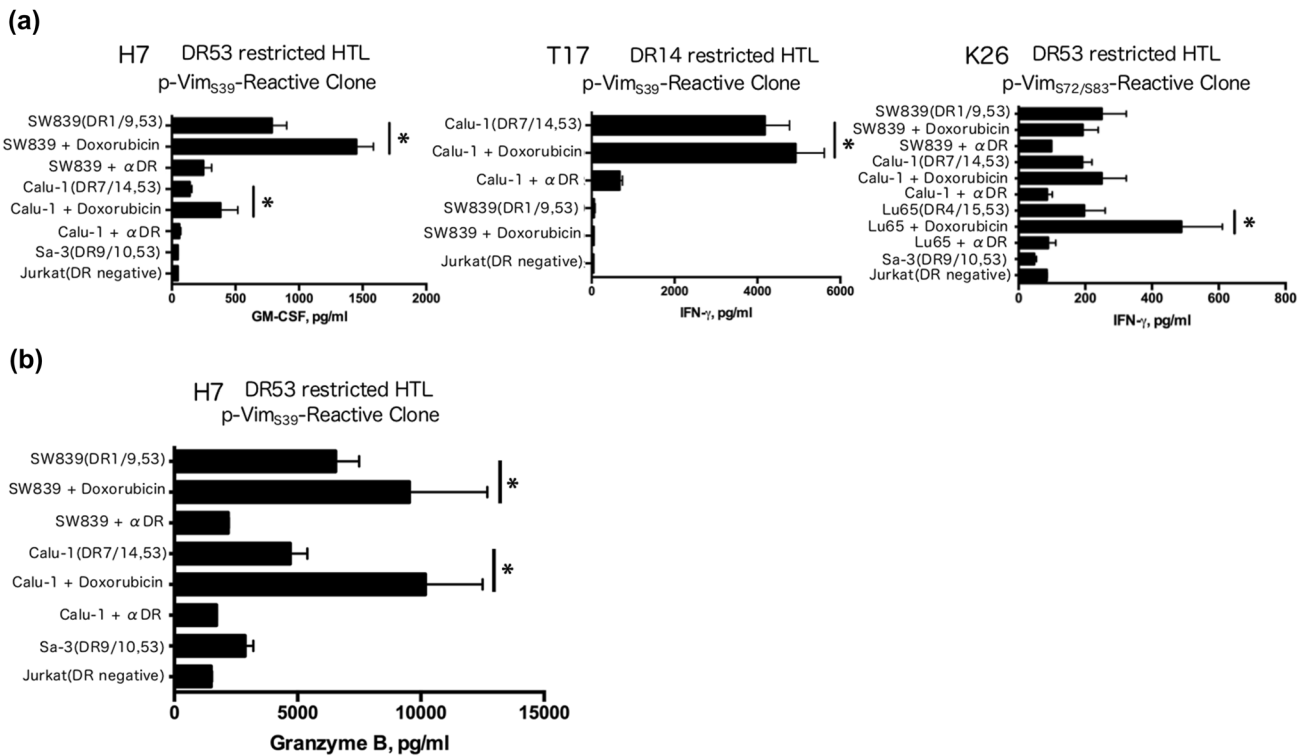
## Recognition of phosphorylated vimentin peptides by PBMCs of CRC patients

Since it is possible that tumor antigen-specific precursor T cells are diminished in cancer patients, it is important to verify the existence of precursor T cells specific for phosphorylated vimentin peptides in CRC patients to validate the utility of these peptides as a cancer vaccine. To determine the HTL responses to phosphorylated vimentin in CRC patients, we performed short-term culture experiments using peptide-stimulated PBMCs from six CRC patients. Since the volume of blood obtained from these patients was small, it was impossible to induce HTL cell lines for detailed HLA restriction analysis. Since tetanus toxoid (TT<sub>830-843</sub>) peptide is capable of eliciting strong HTL responses in the

vast majority of people regardless of their HLA-DR alleles [20], we used this peptide as a positive control. As shown in Fig. 5, considerable T cell responses to phosphorylated vimentin were observed in five CRC patients. It is intriguing that wild-type vimentin did not induce T cell responses. These responses were inhibited by an anti-HLA-DR mAb, indicating that cytokine production was induced by phosphorylated peptide-reactive HTLs.

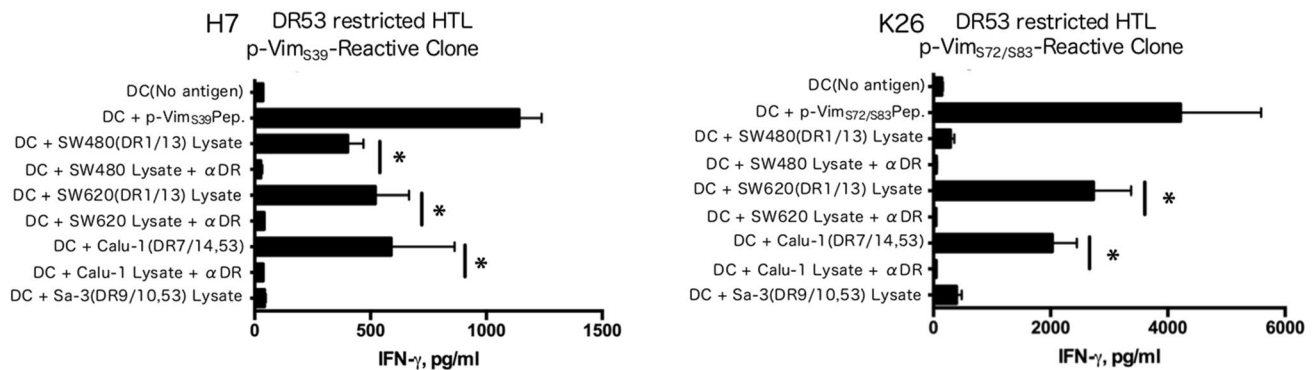
## Discussion

We showed in the present study that phosphorylated vimentin peptides are likely to be useful as a helper peptide vaccine to treat CRC. Since CTLs are considered to be a direct



**Fig. 3** Direct tumor recognition by phosphorylated peptide-specific CD4 T cells. Direct tumor recognition of naturally processed vimentin antigen expressed in tumor cells by p-Vim<sub>S39</sub>-reactive clones (H7 and T17) and the p-Vim<sub>S72/S83</sub>-reactive clone (K26) is shown. **a** Phosphorylated vimentin-reactive T cells (DR53-restricted-clone H7, K26, and DR14-restricted-clone T17) were evaluated for their capacity to recognize HLA-DR53 positive/p-Vim-positive (SW839, Calu-1, and Lu65), HLA-DR14 positive/p-Vim-positive (Calu-1), DR53-positive/

vimentin-negative (Sa-3), and DR-negative (Jurkat) tumor cells. Sa-3 and Jurkat cells were used as negative controls. Tumor cells were treated with 1  $\mu$ M doxorubicin for 24 h before co-culturing with T cells. **b** Granzyme B production by the p-Vim<sub>S39</sub>-reactive clone (H7) and the p-Vim<sub>S72/S83</sub>-reactive clone (K26) during co-culturing with tumor cells. Tumor cells were treated with 1  $\mu$ M doxorubicin for 24 h before co-culturing with T cells. \* $p < 0.05$



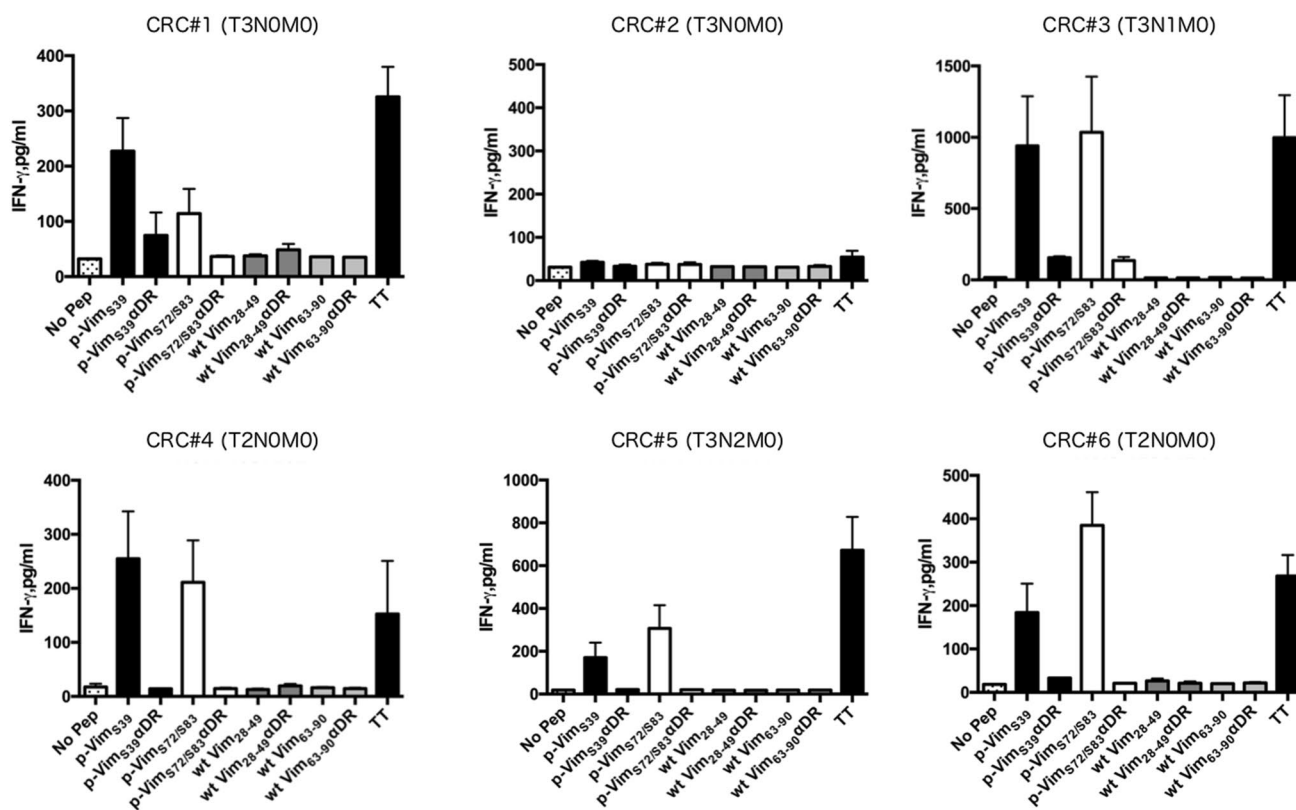
**Fig. 4** Indirect tumor recognition by phosphorylated peptide-specific CD4 T cells. The ability of p-Vim-reactive CD4 T cells to recognize naturally processed exogenous phosphorylated vimentin was assessed by measuring IFN- $\gamma$  production. Dendritic cells (DCs) were used as antigen-presenting cells (APCs), and HLA-DR-unmatched CRC

tumor cell lysates served as sources of phosphorylated vimentin protein. L243 mAb was used to verify the HLA-DR restriction of these responses. DCs pulsed with p-Vim<sub>S39</sub> and p-Vim<sub>S72/S83</sub> were used as positive controls. \* $p < 0.05$

player that kills tumors in cancer immunotherapy, clinical trials using HTLs have shown promising results [16, 26]. Previously, we showed that peptide-reactive HTLs that were

positive for granzyme B could directly kill tumor cells in a killing assay [27]. HTL cell lines that we established in this study produced cytotoxic cytokines, suggesting that HTLs





**Fig. 5** Evaluation of T cell responses to wild-type and phosphorylated vimentin in CRC patients. PBMCs from six CRC patients (CRC #1–6) were stimulated with peptides, and T cell responses were measured as described in the Materials and methods

play an important role in direct tumor cell toxicity. Vimentin, a cytoskeletal protein, is one of the clinical parameters of EMT. We showed that more than half of primary CRC and more than 70% of metastatic CRC samples were positive for phosphorylated vimentin (Table 1). Moreover, the precursor lymphocytes that recognize phosphorylated vimentin existed in CRC patients (Fig. 5). Thus, it is feasible to target phosphorylated vimentin as peptide vaccine to treat CRC.

Abnormal phosphorylation of cellular proteins is a hallmark of malignant transformation [10]. Akt, Rho-associated kinase (Rho-kinase), or Polo-like kinase 1 (Plk1) phosphorylate vimentin at Ser39, Ser72, or Ser83 enhance tumor growth, metastasis, and cytokinesis [9, 28, 29]. In oral squamous cell carcinoma, metastatic tumor cells express more vimentin than their non-metastatic counterparts [30]. This datum is consistent with the results of our IHC analysis in CRC patients showing that metastatic tumors tended to express higher levels of phosphorylated vimentin than primary tumors (Table 1). As shown in Supplementary Fig. 1, the expression of phosphorylated vimentin was heterogeneous. Because chemotherapeutic reagents induce phospho-vimentin through the TGF- $\beta$  and PI3K pathways [31], cells that suffer from cellular stress such as at an invasion front may have higher expression of phospho-vimentin compared

to non-stressed cells. Because immune checkpoint blockade with chemotherapy significantly prolongs the survival of patients with lung cancer compared to chemotherapy alone, the combination of immune-suppressive chemotherapy and immunotherapy is a clinically effective approach [32, 33]. It is plausible that chemotherapy-resistant memory T cells survive during chemotherapy [34] and are activated by released tumor antigens from destroyed tumor cells.

Since HTLs have been described to specifically identify post-translationally modified epitopes, including phosphorylated epitopes on MHC II molecules [12, 35, 36], post-translational modification can be a unique target to generate peptide vaccines. To the best of our knowledge, there are no reports focusing on phosphorylated vimentin-specific T cell responses in cancer immunotherapy. The phosphorylated vimentin epitope peptides that we found in this study bound to multiple HLA-DR alleles, including HLA-DR53 (Fig. 2c), indicating that these peptides can be applied to a large population of patients. It should be noted that phosphorylated but not wild-type vimentin induced T cell responses in PBMCs of CRC patients (Fig. 5). This result suggested that the immunogenicity of phosphorylated vimentin is higher than that of wild-type vimentin. Another possibility is that the increased antigen load in CRC patients upregulates

the number of precursor T cells against phosphorylated vimentin. Further studies are required to evaluate these possibilities.

In pathology, vimentin is usually used to distinguish mesenchymal tumors from epithelial tumors. Increased expression of vimentin is considered to represent the activation of EMT in malignant tissues. Although it was not significant because of the sample size, metastatic CRC tissues expressed higher levels of phosphorylated vimentin (S72) than primary CRC tissues (Table 1). To improve the efficacy of peptide vaccines, the selection of adjuvants and administration routes is necessary [37]. As shown in this study (Figs. 1, 3), chemotherapeutic reagents such as doxorubicin might be suitable adjuvants to increase the amount of tumor antigen (phosphorylated vimentin) in tumor cells followed by T cell stimulation. Since we proved that phosphorylated peptides were preserved during the exogenous antigen-processing pathway, chemotherapy can also increase the presentation of phosphorylated peptides from dead tumor cells on professional APCs. Because the expression of phosphorylated vimentin in healthy tissues has not been fully investigated, it is important to confirm that phospho-vimentin-reactive HTLs spare healthy tissues in future *in vivo* studies. As shown in Supplementary Fig. 2, the expression of phosphorylated vimentin was not expressed in healthy colon tissues. The existence of phospho-vimentin-reactive T cell precursors in both healthy donors and colon cancer patients suggests that these cells might not be self-reactive. It is possible that the affinity of these HTLs might be low enough to spare healthy tissues, but is sufficient to react with tumor cells that present high amounts of phosphorylated vimentin-derived peptides.

## Conclusions

We discovered novel helper epitopes from phosphorylated vimentin that can elicit antigen-specific HTL reactions. Variants in the amino acid sequences of these peptides have not been reported [38], indicating that these peptides are strongly conserved. Moreover, doxorubicin might work as an adjuvant for peptide-based cancer immunotherapy by upregulating phosphorylated vimentin. Since post-translationally modified epitopes are selectively expressed in malignant tumor cells [12, 13], a peptide vaccine using these phosphorylated peptides could be a suitable therapy for CRC with metastatic lesions, which express a higher level of phosphorylated vimentin.

**Author contributions** MO, KO, TN, and YH-N contributed to data acquisition. AK, MN, RH, SH, and YY contributed to data acquisition and analysis. TK, TO, and KO contributed to the design and concept

and drafting of the manuscript. All authors were involved in data interpretation, preparation, and review of the manuscript draft and approved the final manuscript version for submission.

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## Compliance with ethical standards

**Conflicts of interest** The authors declare that they have no conflict of interest.

**Ethical approval and ethical standards** This study followed the principles of the Helsinki Declaration, and the study protocol was approved by the Asahikawa Medical University Institutional Ethics Committee (approval number #16040-3).

**Informed consent** All blood samples from healthy human volunteers and CRC patients (Asahikawa Medical University Hospital, 2016–2018) were obtained after obtaining written informed consent (Institutional Ethics Committee approval number #16040-3). The patients and healthy donors agreed to the use of their specimens and data for research and publication.

**Cell line authentication** The CRC cell lines SW480 and SW620 were purchased from KAC Co., Ltd. (Kyoto, Japan). Lung SCC cell lines Calu-1 and Jurkat (T cell lymphoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The gingival SCC cell line Sa-3 and lung large cell carcinoma cell line Lu65 were supplied by the RIKEN Bio-Resource Center (Tsukuba, Japan). The renal cell carcinoma cell line SW839 was obtained from the Cell Resource Center for Biomedical Research Institute of Development (Aging and Cancer, Tohoku University, Sendai, Japan). Cell authentication assays were performed by each company, and the cell passaging was no more than five times.

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