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エストロゲンレセプター陽性乳癌における microRNAの役割に関する研究

北海道大学病院乳腺・内分泌外科，名古屋市立大学病院乳腺内分泌外科*
山下　啓子　　遠山　竜也*　　吉本　信保*　　遠藤　友美*

日本内分泌・甲状腺外科学会雑誌　第29巻　第4号(2012年)　別冊

内分泌甲状腺外会誌 29 (4) : 289-292, 2012

特集2 乳癌診療の未来

エストロゲンレセプター陽性乳癌における microRNAの役割に関する研究

北海道大学病院乳腺・内分泌外科，名古屋市立大学病院乳腺内分泌外科*
山下 啓子　遠山 竜也*　吉本 信保*　遠藤 友美*

Role of microRNAs in estrogen receptor-positive breast cancer

Breast and Endocrine Surgery, Hokkaido University Hospital,
Breast and Endocrine Surgery, Nagoya City University Hospital*
Hiroko Yamashita, Tatsuya Toyama*, Nobuyasu Yoshimoto* and Yumi Endo*

乳癌組織のエストロゲンレセプター（ER；estrogen receptor）の発現量は内分泌療法や化学療法の奏効性や予後などの生物学的特性に関与する。乳癌組織のERの発現調節に関しては，ER遺伝子のメチル化やER蛋白のユビキチン化による分解などの報告があるが，臨床的に重要であるものは未だ見出されていない。最近，ERのmRNA 3'末端非翻訳領域に結合してERのmRNAと蛋白発現を抑制するマイクロRNA（microRNA）が報告された。われわれは乳癌組織におけるこれらERのmRNAに直接結合するmicroRNAの発現を定量的RT-PCR法により検討して，ERの蛋白発現や予後に関与するmicroRNAを見出した。さらに最近，ER陽性乳癌の生物学的特性に関与すると考えられるmicroRNAとその標的遺伝子を同定し，これらが治療標的あるいは薬物療法の感受性のバイオマーカーとして有用であると推測している。

Key words：乳癌（breast cancer），エストロゲンレセプター（estrogen receptor），マイクロRNA（microRNA）

はじめに

乳癌には，乳癌細胞がエストロゲンレセプター（ER；estrogen receptor）を発現してエストロゲン依存性に発生・進展するER陽性乳癌と，乳癌細胞がERを発現していなくてエストロゲン非依存性に発生・進展するER陰性乳癌が存在し，現在，両者は発生する細胞や臨床像が異なり，生物学的に異なる病気であると考えられている。エストロゲンレセプター（ER）陽性乳癌は，正常乳腺の分化の最終段階である，分化した乳腺上皮細胞から発生すると考えられている[1]。乳癌組織におけるERの発現は，現在，免疫組織化学法により評価している。ERの発現量（陽性細胞率）は症例により様々な分布を示し，0％から100％まで連続的に存在し，内分泌療法や化学療法の奏効性や予後などの生物学的特性の指標として非常に重要である。乳癌組織のERの発現調節に関しては，これまでER遺伝子のメチル化やER蛋白のユビキチン化による分解などが報告されているが，臨床的に重要であるものは未だ見出されていない。さらに，ER陽性乳癌は内分泌高感受性であるluminal Aと内分泌低感受性であるluminal Bサブタイプが存在することが明らかになっている[2]。われわれは，遺

別冊請求先：〒060-8648 北海道札幌市北区北14条西5丁目
北海道大学病院乳腺・内分泌外科 山下啓子
E-mail address：hirokoy@huhp.hokudai.ac.jp

伝子発現制御機構のひとつとして最近注目されているマイクロRNA（microRNA）について，ER遺伝子の発現やER陽性乳癌の生物学的特性への関与に関する研究を行ってきたので，本稿ではその研究の一端も含めて紹介する。

なお，本稿で用いているエストロゲンレセプターはすべて，エストロゲンレセプターアルファ（ERα；estrogen receptor α）である。

1．乳癌組織におけるエストロゲンレセプター（ER）の発現の臨床的意義

免疫組織化学法によるERの発現を正常乳腺および前癌病変で検討したAllredらの報告によると，閉経前の正常乳管上皮細胞は約30％がER陽性細胞である。一方，atypical ductal hyperplasia，atypical lobular hyperplasiaといった前癌病変ではほぼすべての上皮細胞がER強陽性となっている[3]。これに対して，low gradeの非浸潤性乳管癌（DCIS；ductal carcinoma in situ）ではほぼすべての癌細胞がER強陽性を示すが，high gradeのDCISではほぼすべての癌細胞がER陰性である。浸潤性乳癌におけるERの発現は，現在，免疫組織化学法により評価するが，ERの発現量（陽性細胞率）は症例により様々な分布を示し，0％から100％まで連続的に存在する[3]。2010年，米国臨床腫瘍学会と病理（ASCO/CAP）から，乳癌組織においてホルモンレセプター（ER/プロゲステロンレセプター：PgR）陽性と判定するカットオフを1％とすることが提唱された[4]。癌細胞の核に少なくとも1％の染色陽性細胞がある場合，ER/PgR陽性と判定することを推奨している。

乳癌組織のERの発現量と予後について，Allredらは術後内分泌療法を行った症例の予後を解析し，ERの発現量が高いほど予後良好であることを報告した[5]。最近の閉経後乳癌の術後内分泌療法（アロマターゼ阻害剤とタモキシフェンの比較）の臨床試験の報告においても，アロマターゼ阻害剤，タモキシフェンともにER/PgRの発現量が高いほど予後良好であることが示されている[6]。

われわれは内分泌療法を施行した再発乳癌における乳癌組織のERの発現量と内分泌療法の効果および予後について検討し，ERの発現量は内分泌療法有効例で有意に高く，ERの陽性細胞率1％以上の症例が有意に再発後の予後が良好であることを報告した[7]。PgRの発現量と内分泌療法の効果および予後についても同様に，PgRの発現量は内分泌療法有効例で有意に高く，陽性細胞率1％以上の症例で有意に再発後の予後が良好であった。さらにわれわれは，手術を行っていない進行乳癌において乳癌組織のERの発現量とアロマターゼ阻害剤による一次内分泌療法の奏効期間を検討し，ERの陽性細胞率2/3以上の症例は，2/3以下の症例に比べて，一次内分泌療法の奏効期間，化学療法に至るまでの期間ともに有意に長いことを報告した[8]。最近，ER陽性乳癌は内分泌高感受性であるluminal Aと内分泌低感受性であるluminal Bサブタイプが存在することが明らかになり，luminal Aサブタイプは現在の標準的化学療法の効果が期待できない可能性が指摘されている[2]。このように，乳癌組織のERの発現量は内分泌療法や化学療法の奏効性および予後に関与することが明らかとなっている。

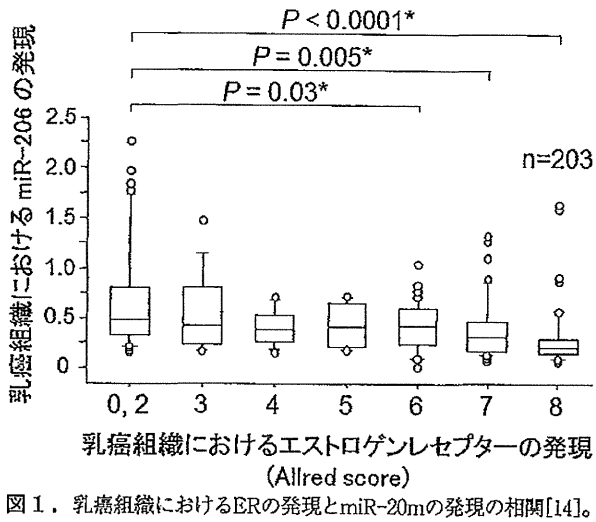

図1．乳癌組織におけるERの発現とmiR-20mの発現の相関[14]。ERαの蛋白発現量が高いほどmiR-206の発現が低い。

2．乳癌組織におけるERの発現調節

乳癌組織のERの発現調節に関してはERα遺伝子のプロモーター領域のメチル化[9]やERα遺伝子の変異[10]，ERα蛋白のユビキチン化による分解[11]などの報告があるが，臨床的に重要であるものは未だ見出されていない。2007年，マイクロRNA（microRNA）によるERαの発現調節に関する基礎研究が報告された[12]。

microRNAは20-25塩基からなる内因性の一本鎖RNAで，標的mRNAの3'末端非翻訳領域に結合して標的mRNAを分解あるいは蛋白への翻訳を抑制することにより，標的遺伝子の発現を抑制する[13]。Adamsらは，乳癌細胞を用いてmicroRNAのひとつmiR-206がERαのmRNA 3'末端非翻訳領域に結合して，ERαのmRNAと蛋白発現を抑制することを報告した[12]。われわれは乳癌組織におけるmiR-206の発現を定量的RT-PCR法により検討し，ERαのmRNAおよび蛋白発現量が高いほどmiR-206の発現が低いことを見出した（図1）[14]。この結果はmiR-206が乳癌組織におけるERαの発現を抑制していることを示唆している。miR-206をER陽性乳癌細胞MCF-7に導入すると，ERのmRNA発現は抑制され，さらに細胞増殖が抑制された。最近，miR-206以外にも幾つかのmicroRNAが乳癌細胞においてERαのmRNAに直接結合することにより，ERαの発現量を調節している可能性が報告されている[15, 16]。わ

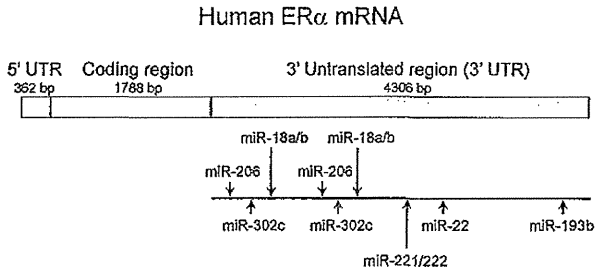

図２．ERαのmRNA 3'末端非翻訳領域（3'UTR）に結合するmicroRNA。

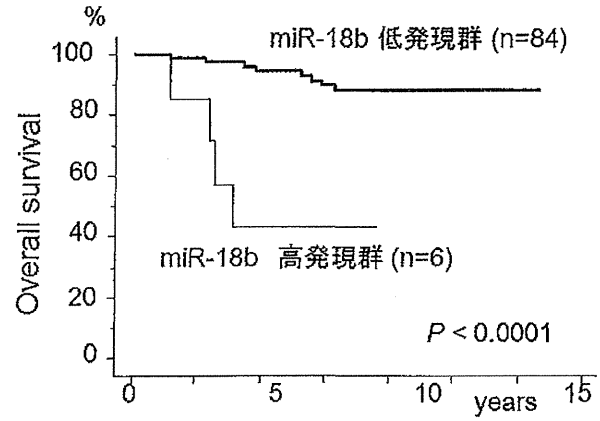

図３．ER陽性HER2陰性乳癌におけるmiR-18b発現と予後[17]。miR-18b低発現症例はmiR-18b高発現症例に比べて有意に予後（生存率）が良好である。

れわれは，ERαのmRNAに結合すると報告されている７つのmicroRNAの乳癌組織における発現を検討し，ERの蛋白発現や予後との相関を検討した(図２)[17]。その結果，miR-18aはER陰性乳癌で高発現していた。一方，miR-193bの発現は，ERの発現量が高いほど，発現量が高いという逆の結果となった。さらに，ER陽性HER2陰性乳癌において，miR-18b低発現症例はmiR-18b高発現症例に比べて有意に予後（生存率）が良好であった(図３)。

３．ER陽性乳癌におけるmicroRNAの役割

microRNAは，標的遺伝子の３'末端非翻訳領域に結合して遺伝子の発現を抑制するが，ひとつのmicroRNAの標的はひとつと限らず，通常，複数の遺伝子を標的とする[18]。また，個々のmicroRNAは複数の標的遺伝子の発現を同時に制御する。さらに，ひとつの遺伝子を複数のmicroRNAが標的とする。このように，microRNAによる遺伝子発現調節は複雑であり，microRNAは遺伝子発現を広範囲にわたって調和しつつ微調整すると考えられている。

Luらは，2005年にヒト癌におけるmicroRNA発現プロファイルを初めて報告した[19]。microRNA発現プロファイルは正常組織と腫瘍で大きく異なり，概して正常組織で発現が高いことが示されている。また，癌によりmicroRNA発現プロファイルは異なることが報告された。Iorioらは，乳癌におけるmicroRNA発現プロファイルを初めて報告した[20]。microRNA発現プロファイルは正常乳腺と乳癌で異なること，また，乳癌のなかでもプロファイルが分類されることを報告している。彼らは，ER陽性乳癌とER陰性乳癌とで異なる発現レベルを示すmicroRNA（miR-206を含む）を幾つか報告している。さらに同じサンプルを用いてmRNAとmicroRNAの発現プロファイルを検討した報告によると，mRNA発現プロファイルのサブタイプ間でmicroRNAの発現プロファイルが異なることが示された[21]。また，microRNAの発現プロファイルは，ERの発現状況やgradeによって異なることが報告されている。われわれは最近，ER陽性乳癌の内分泌高感受性乳癌／低感受性乳癌においてmicroRNAとmRNAのマイクロアレイ解析を行い，これら２群間でmicroRNAとmRNAの発現パターンが異なることを見出している。また，ER陽性乳癌の生物学的特性に関与すると考えられるmicroRNAとその標的遺伝子を同定し，これらが治療標的あるいは薬物療法の感受性のバイオマーカーとして有用であると推測している。われわれは，現在，microRNAがER陽性乳癌の生物学的特性に関与していると考えている。

おわりに

ER陽性乳癌の予後はこの30年間で明らかに改善した[22]。これは再発予防（微小転移の根絶）目的として行う内分泌療法を中心とした薬物療法の進歩によるものと考えられる。しかしながら，特に腋窩リンパ節転移陽性症例は，術後５年以降においても再発をきたす場合がある。このような症例の予後をいかに改善するかが課題のひとつとなっており，現在行っている内分泌療法や化学療法とは作用機序の異なる新たな薬物療法の開発が必要であると思われる。幾つかのmicroRNAがER陽性乳癌の発生・進展に関与していると予測され，そのメカニズムの解明が新たな治療法の確立に繋がる可能性があると考えている。

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Immunology Letters 161 (2014) 20–30

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Identification of novel helper epitope peptides of Survivin cancer-associated antigen applicable to developing helper/killer-hybrid epitope long peptide cancer vaccine

Junya Ohtake[a,1], Takayuki Ohkuri[a,1], Yuji Togashi[b], Hidemitsu Kitamura[a], Kiyotaka Okuno[c], Takashi Nishimura[a,d,*]

[a] Division of Immunoregulation, Section of Disease Control, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan
[b] Bioimmulance Co. Ltd., Sapporo, Japan
[c] Department of Surgery, Kinki University, Faculty of Medicine, Osaka-Sayama, Japan
[d] Division of ROYCE' Health Bioscience, Section of Disease Control, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan

ARTICLE INFO

Article history:
Received 12 September 2013
Received in revised form 31 March 2014
Accepted 16 April 2014
Available online 30 April 2014

Keywords:
Survivin
H/K-HELP
Cancer vaccine
Th1 and Tc1

ABSTRACT

We identified novel helper epitope peptides of Survivin cancer antigen, which are presented to both HLA-DRB1*01:01 and DQB1*06:01. The helper epitope also contained three distinct Survivin-killer epitopes presented to HLA-A*02:01 and A*24:02. This 19 amino-acids epitope peptide (SU18) induced weak responses of Survivin-specific $CD4^+$ and $CD8^+$ T cells though it contained both helper and killer epitopes. To enhance the vaccine efficacy, we synthesized a long peptide by conjugating SU18 peptide and another DR53-restricted helper epitope peptide (SU22; 12 amino-acids) using glycine-linker. We designated this artificial 40 amino-acids long peptide containing two helper and three killer epitopes as Survivin-helper/killer-hybrid epitope long peptide (Survivin-H/K-HELP). Survivin-H/K-HELP allowed superior activation of IFN-γ-producing $CD4^+$ Th1 cells and $CD8^+$ Tc1 cells compared with the mixture of its component peptides (SU18 and SU22) in the presence of OK-432-treated monocyte-derived DC (Mo-DC). Survivin-H/K-HELP-pulsed Mo-DC pretreated with OK-432 also exhibited sustained antigen-presentation capability of stimulating Survivin-specific Th1 cells compared with Mo-DC pulsed with a mixture of SU18 and SU22 short peptides. Moreover, we demonstrated that Survivin-H/K-HELP induced a complete response in a breast cancer patient with the induction of cellular and humoral immune responses. Thus, we believe that an artificially synthesized Survivin-H/K-HELP will become an innovative cancer vaccine.

1. Introduction

Survivin is aberrantly expressed in almost all cancers but poorly in differentiated adult tissues except testis, thymus, and placenta [1]. Moreover, Survivin-derived peptide is able to induce cytotoxic T lymphocytes (CTL) [2]. Therefore, Survivin has been considered as one of the attractive target molecules in cancer vaccine therapy.

It is now accepted that $CD4^+$ Th cells, especially Th1 cells play a pivotal role in antitumor immunity to induce a complete cure of tumor-bearing hosts [3–7]. However, cancer vaccine therapy using class I-binding killer epitope peptide of tumor antigen has only focused on the activation of CTL but largely ignored Th1 cells, which are essential for fully activation of CTL and memory CTL generation [3–11]. Thus, the immunological and clinical responses have been rather meager in cancer vaccine therapy using class I-binding short peptide though the therapy could induce slight increase of cancer-specific CTL and long stable diseases [8–10]. To overcome these issues, we considered that it might be a rational strategy to apply class II-binding helper peptide to cancer vaccine therapy.

Several investigators have tried to identify helper epitope peptides and applied to clinical trials. However, there have been

Abbreviations: CTL, cytotoxic T lymphocytes; EBV-B, Epstein–Barr virus-transformed B; H/K-HELP, helper/killer-hybrid epitope long peptide; HPV, human papilloma virus; MMC, mitomycin C; Mo-DC, monocyte-derived dendritic cells; PBMC, peripheral blood mononuclear cells; SLP, synthetic long peptide; Tc, cytotoxic T; Th, helper T; Treg, regulatory T.

* Corresponding author at: Division of Immunoregulation, Section of Disease Control, Institute for Genetic Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan. Tel.: +81 11 706 7546; fax: +81 11 706 7546.
E-mail address: tak24@igm.hokudai.ac.jp (T. Nishimura).
[1] These two authors are equally contributed to this work.

http://dx.doi.org/10.1016/j.imlet.2014.04.010
0165-2478/

no positive data that short helper peptide or short helper/killer hybrid epitope induced a significant antitumor immunity in cancer patients [12,13]. Recently, however, it was demonstrated that synthetic long peptides (SLP) containing helper and killer epitopes derived from naturally occurring sequence of human papilloma virus (HPV)-16 oncoproteins were superior to short peptides to induce HPV-specific Th1 and CTL responses in both animal and human systems [11]. The mixtures of SLP in cancer antigen have been also demonstrated to be beneficial for inducing anticancer immune responses in cancer patients [14]. In previous paper [15], we also developed an artificial 40 amino-acids long peptide vaccine of MAGE-A4 cancer antigen, which was designated as MAGE-A4-helper/killer-hybrid epitope long peptide (MAGE-A4-H/K-HELP). In that paper [15], we first demonstrated that cancer vaccine therapy of a colon cancer patient with artificially synthesized MAGE-A4-H/K-HELP containing both helper and killer epitopes caused the induction of superior Th1-dependent cellular and humoral immune responses in the cancer patient and inhibited the growth of colon cancer. Surprisingly, a single injection of MAGE-A4-H/K-HELP rapidly induced efficient antitumor immune responses in cancer patient likely as the case when patients were vaccinated with the mixtures (over 10 peptides) of SLP cancer vaccine.

In this paper, we identified novel helper epitope of Survivin cancer antigen and again planned to synthesize 40 amino-acids Survivin-H/K-HELP widely applicable to the therapy of cancer patients. The superior effect of Survivin-H/K-HELP on dendritic cells (DC)-dependent T cell activation *in vitro* was documented with the results of a case study in clinical trial of Survivin-H/K-HELP cancer vaccine therapy. These results indicated that artificially synthesized long peptide, Survivin-H/K-HELP would become a promising cancer vaccine peptide.

2. Material and methods

2.1. Peptides and cytokines

The synthesis of overlapping peptides covering whole amino acid sequence of Survivin-2B (SU1-SU27) and Survivin-H/K-HELP (purity > 95%) was ordered to Sigma–Aldrich Japan (Tokyo, Japan). For screening, the peptides except two supplementary SU17 and SU19 peptides were subdivided into MIX1–MIX5 5 peptide pools (MIX1; SU1–SU5, MIX2; SU6–SU10, MIX3; SU11–SU15, MIX4; SU16–SU22, and MIX5; SU23–SU27) as illustrated in Supplementary Table S1. Recombinant IL-2 was kindly donated by Shionogi Pharmaceutical Institute Co. Ltd., Osaka, Japan. Recombinant IFN-γ, IL-4, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from PeproTech Inc. (New Jersey, USA).

2.2. Cell culture

Peripheral blood mononuclear cells (PBMC) were prepared from healthy volunteers after obtaining written informed consent approved by the medical ethics committees of Hokkaido University Graduate School of Medicine and Institute for Genetic Medicine. Epstein–Barr virus-transformed B cells (EBV-B), generated from PBMC by culturing with culture supernatants from EBV-producing cells, were maintained in RPMI 1640 medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and antibiotics. Mo-DC from PBMC of healthy volunteers were cultured in AIM-V medium (Invitrogen, Carlsbad, CA, USA) without serum. T cells were cultured in AIM-V containing 5% heat-inactivated pooled human AB serum (kindly donated from Hokkaido Red Cross blood center).

2.3. Preparation of Mo-DC from PBMC as antigen presenting cells

PBMC isolated from healthy volunteers by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation were cultured in AIM-V without serum. After removing non-adherent cells, the remaining adherent monocytes were cultured in the presence of GM-CSF (50 ng/ml) and IL-4 (50 ng/ml) for 7 days. The induced CD11c-positive Mo-DC were used as antigen-presenting cells (APC) in this study.

2.4. Identification of helper epitopes using mixture of peptides derived from Survivin-2B

Non-adherent cells prepared from PBMC were suspended in the staining buffer according to the manufacture's protocol. Then, anti-CD4 microbeads (130-049-201; Miltenyi Biotech, Bergisch Gladbach, Germany) were added to the cell suspension and incubated for 15 min on ice. The cells were diluted in 10 ml PBS and washed by centrifugation at 1500 rpm for 5 min. The pelleted cells resuspended in 500 μl buffer were magnetically separated into CD4-positive or negative cells with MACS LS column (130-091-596; Miltenyi Biotech, Bergisch Gladbach, Germany) by fitting on the magnet. The purity of isolated $CD4^+$ T cells was over 90% determined by flow cytometry. Survivin 2B antigen-derived synthetic peptides (SU1-SU27; Supplementary Table S1), were dissolved in DMSO. The sequential peptides (SU1–SU5, SU6–SU10, SU11–SU15, SU16–SU22, and SU23–SU27) were mixed and used as peptide mixture (MIX1, MIX2, MIX3, MIX4, and MIX5) at 10 μg/ml, respectively. The purified $CD4^+$ T cells (2×10^6 cells) were cultured with mitomycin C (MMC)-treated PBMC (2×10^6 cells) in the presence of MIX1, MIX2, MIX3, MIX4 or MIX5 (10 μg/ml) for 7 days. The cultured $CD4^+$ T cells were restimulated with autologous Mo-DC in the presence of the cognate peptide MIX and IL-2 (10 IU/ml). After 7 days-culture, $CD4^+$ T cells were restimulated with the cognate peptide mixture to evaluate their cytokine production by ELISA. Survivin-specific Th cells were then used for further experiments to identify Survivin-2B epitope and HLA-restriction.

2.5. Induction of Survivin-specific Th1 cells and Tc1 cells from PBMC

PBMC (2×10^6 cells) were primarily stimulated with Survivin-H/K-HELP or the mixture of Survivin short peptides (SU18 plus SU22) (5 μM) in 24-well polystyrene microplate (Corning Inc., NY, USA). After 7 days-culture, the cultured T cells were restimulated with MMC-treated autologous Mo-DC (1×10^5 cells) pretreated with Th1 adjuvants, OK-432 (0.1 KE/ml) or IFN-γ (20 ng/ml) in the presence of the induction peptide and IL-2 (10 IU/ml) for 7 days. The frequency of Th1 and Th2 cell induction was determined by measuring IFN-γ and IL-5 (or IL-4) production of the expanded T cells by intracellular staining after stimulation with Mo-DC (1×10^5 cells) pulsed with the cognate peptide.

2.6. Antigen presentation capability of Mo-DC pulsed with Survivin-H/K-HELP or mixture of SU18 and SU22 short peptides

Mo-DC, which were harvested from PBMC and activated with OK-432 (0.1 KE/ml), were pulsed with Survivin-H/K-HELP or the mixture of SU18 and SU22 short peptides (5 μM) for 2 h at 37 °C. After washing with PBS twice, peptide-pulsed Mo-DC (1×10^4 cells) were cultured alone for various times (0, 24, 48, 72, 96 h) at 37 °C. The peptide-pulsed Mo-DC (1×10^4 cells) were harvested from the culture various time (0, 24, 48, 72, 96 h) after incubation and determined their antigen-presenting ability. Peptide-pulsed Mo-DC were cocultured for 24 h with Survivin-specific Th cells

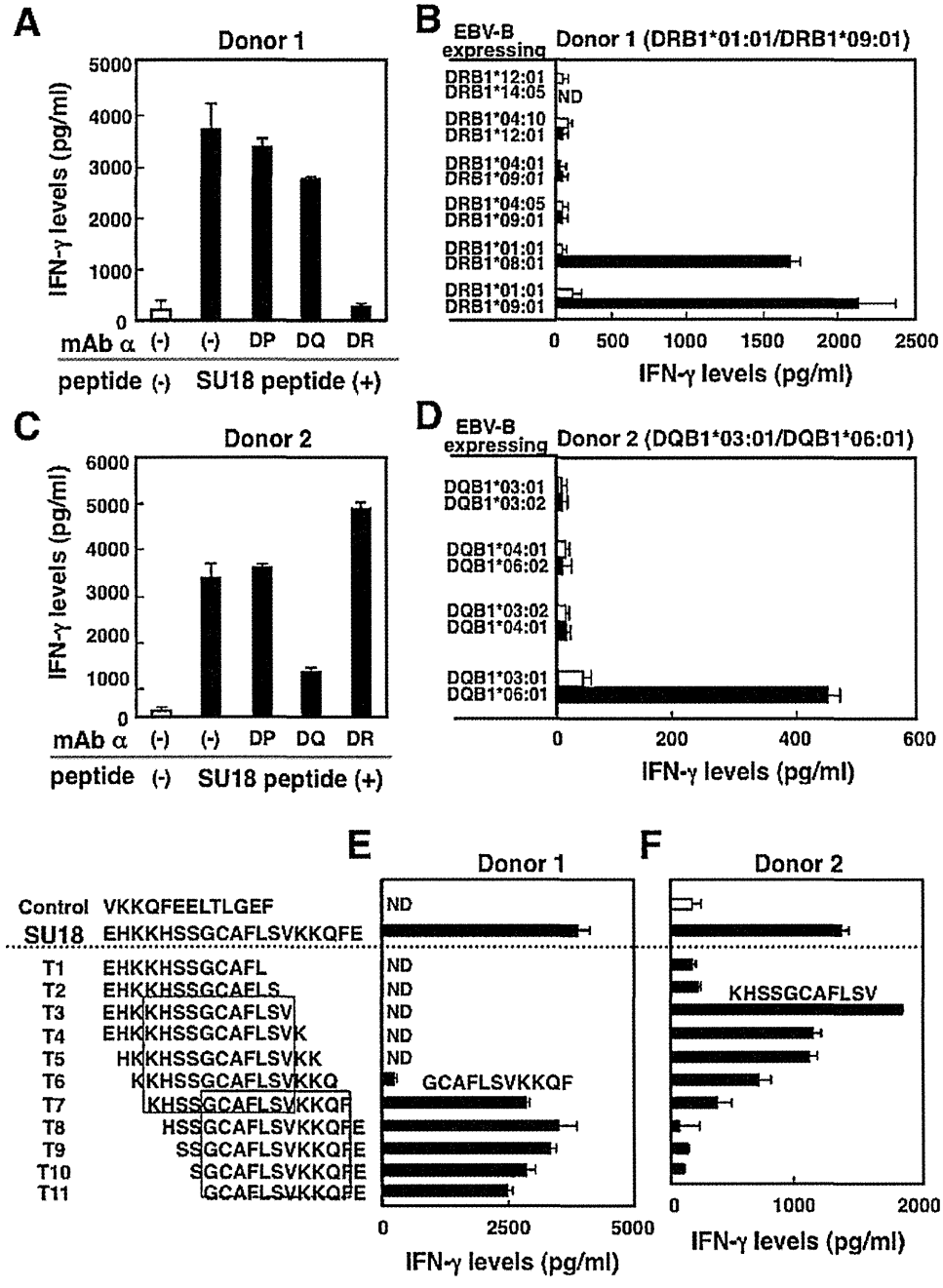

Fig. 1. Identification of novel Survivin-2B helper epitope peptide promiscuously bound to HLA-DRB1*01:01 and HLA-DQB1*06:01. Survivin-2B-specific CD4+ Th cells were induced from isolated CD4+ T cells by repetitive stimulation with MIX4 and donor 1- and donor 2-derived SU18 peptide (Survivin-$2B_{99-117}$)-reactive Th cells were used for this experiment. (A and C) Donor 1 or donor 2-derived SU18-specific CD4+ T cells were stimulated with (filled bars) or without (open bars) SU18 peptide-pulsed autologous Mo-DC in the presence of mAbs against HLA-DP (DP), HLA-DQ (DQ), or HLA-DR (DR) (5 μg/ml). (B and D) SU18-specific CD4+ T cells were stimulated with (filled bars) or without (open bars) SU18 peptide-pulsed autologous or allogenic EBV-B cells expressing indicated HLA-DR haplotypes. (E and F) SU18-specific CD4+ T cells were cultured with autologous Mo-DC in the presence of truncated 12–15-mer peptides of SU18 peptide. After culture for 20-24 h, IFN-γ levels in the culture supernatants were determined by ELISA. Means and SDs of the representative data are indicated. ND represents "not detected".

(5×10^4 cells), which were induced from autologous PBMC by stimulating with Survivin-H/K-HELP. To determine the antigen presentation capability of peptide-pulsed Mo-DC, IFN-γ levels in the culture supernatants were measured by ELISA kit (BD Biosciences, California, USA) 24 h after incubation. The change of APC function of Mo-Dc was determined by calculating the change of IFN-γ-production (%) as follows: Change of IFN-γ production (%) = (IFN-γ levels induced by co-cultured with Th1 cells and peptide-pulsed Mo-DC cultured for various hours − IFN-γ levels induced by co-cultured with Th1 cells and peptide-unpulsed Mo-DC cultured for various hours)/(IFN-γ levels induced by co-cultured with Th1 cells and peptide-pulsed Mo-DC cultured for 0 h − IFN-γ levels induced by co-culture with Th1 cells and peptide-unpulsed Mo-DC cultured for 0 h) × 100.

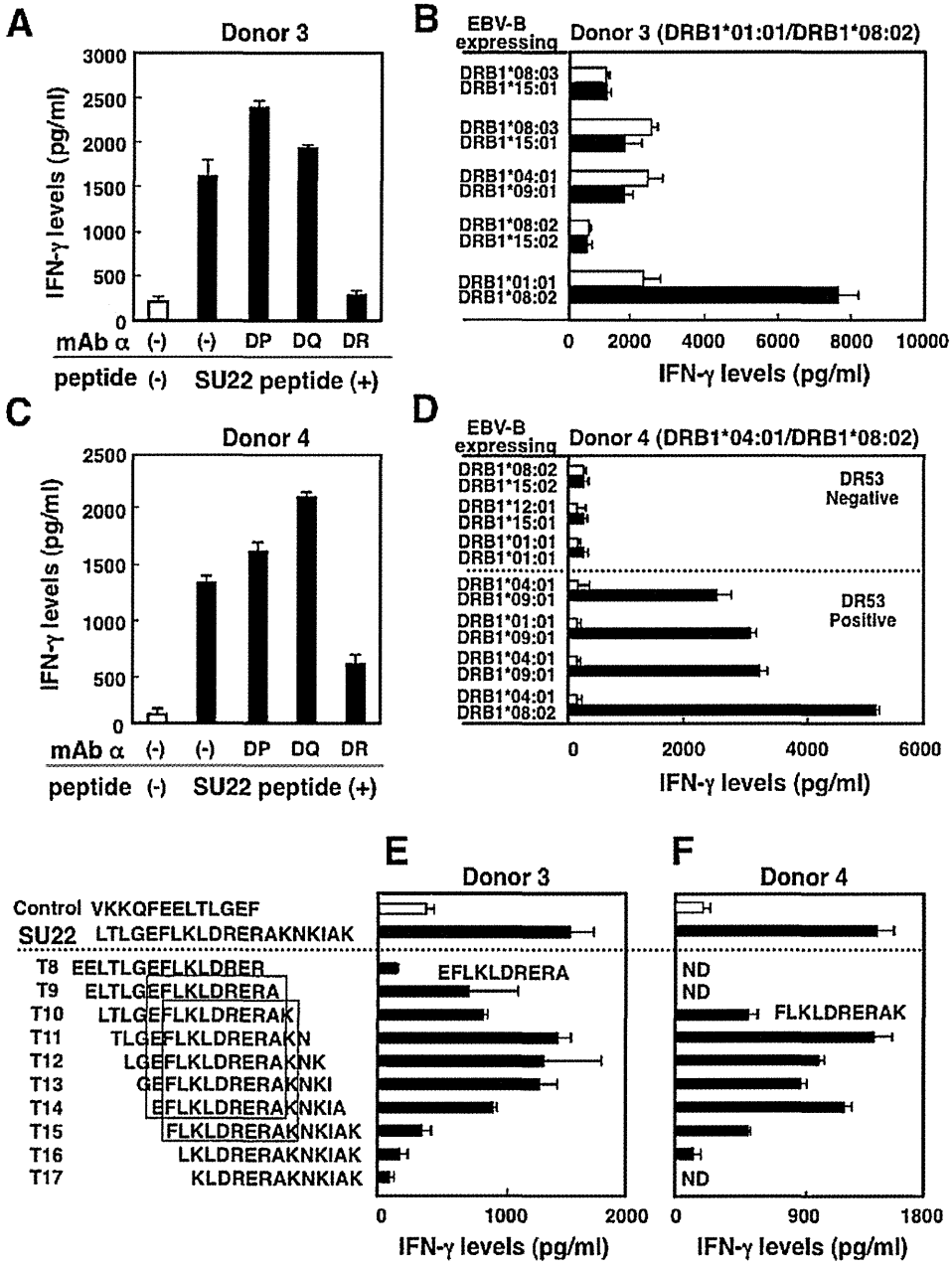

Fig. 2. Identification of HLA-DRB1*01:01- or HLA-DR53-restricted Survivin-2B helper epitope. (A and C) Donor 3 or donor 4-derived SU22-specific CD4+ T cells were stimulated with (filled bars) or without (open bars) SU22 peptide-pulsed autologous Mo-DC in the presence of mAbs against HLA-DP (DP), HLA-DQ (DQ), or HLA-DR (DR) (5 μg/ml). (B and D) SU22-specific CD4+ T cells were stimulated with (filled bars) or without (open bars) SU22 peptide-pulsed autologous or allogenic EBV-B cells expressing indicated HLA-DR haplotypes. (E and F) SU22-specific CD4+ T cells were cultured with autologous Mo-DC in the presence of truncated 12–15-mer peptides of SU22 peptide. After culture for 20–24 h, IFN-γ levels in the culture supernatants were determined by ELISA. Means and SDs of the representative data are indicated. ND represents "not detected".

2.7. Evaluation of HLA class II-restricted antigen-specific cytokine production of T cells

T cells (3×10^4 cells) expanded with Survivin-2B peptides were cultured with MMC-treated autologous PBMC (3×10^4 cells) or class II-expressing EBV-B cells (2×10^4 cells) in the presence of relevant peptide antigen for 20 h. After culture, the antigen-triggered IFN-γ levels in the culture supernatants were measured by ELISA kit (BD Biosciences, California, USA). HLA class II restriction was determined by the blocking experiment against peptide-specific T cell response (IFN-γ production) by adding 5 μg/ml anti-HLA-DR mAb (L243; BD Biosciences, California, USA), anti-HLA-DP mAb (BRAFB6; Serotech, Oxford, UK), or anti-HLA-DQ mAb (SPV-L3; Serotech, Oxford, UK). The detail HLA class II haplotype was determined by examining the APC function of EBV-B cells selected from our prepared EBV-B cell bank, which included various cells expressing various HLA class II haplotypes.

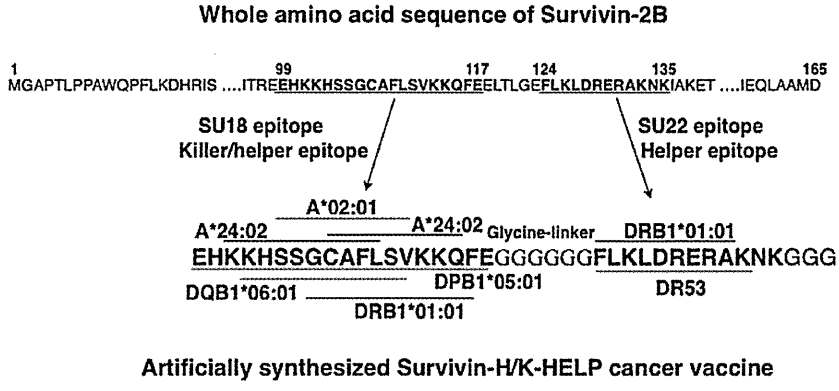

Fig. 3. Artificially synthesized cancer vaccine, Survivin-H/K-HELP containing helper and killer epitope peptides. Survivin-H/K-HELP was artificially synthesized by conjugating SU18 ($Survivin_{99-117}$) killer/helper epitope peptide (19 amino acid sequence indicated by boldface on the left) with SU22 ($Survivin_{124-135}$) helper epitope peptide (12 amino acid sequence indicated by boldface on the right) with a glycine-linker. The under lines indicated the sequence of helper and killer epitopes and HLA-restriction haplotypes. The figure illustrated Amino acid sequence and HLA-restriction portion of artificially synthesized.

2.8. Detection of antigen-specific expression of cytokine and cytotoxic molecules in T cells by intracellular staining

The induced T cells (5×10^4 cells) were stimulated with peptide-pulsed autologous Mo-DC (5×10^3 cells) for 2 h and further incubated in the presence of Brefeldin-A (Sigma–Aldrich Japan, Tokyo, Japan) for 20 h. Thereafter, the cells were first stained with anti-CD4 and anti-CD8 mAbs and then treated with Cytofix/Cytoperm (BD Biosciences, California, USA). The fixed cells were stained with anti-IFN-γ and anti-IL-5 mAbs to detect intracellular Th1 and Th2 cytokine production. Anti-Perforin, and anti-Granzyme B mAbs (BD Biosciences, California, USA) were used for detection of intracellular expression of cytotoxic molecules in $CD8^+$ T cells. The expression levels were determined by flow cytometry (FACSCanto II, BD Biosciences, California, USA).

2.9. A phase I clinical study on Survivin-H/K-HELP cancer vaccine therapy for a breast cancer patient

A phase I clinical trial of the Survivin-H/K-HELP vaccine was designed to evaluate the safety, immune response and tumor response. This clinical study was approved by all of institutional review boards in light of the Declaration of Helsinki. Written informed consent was obtained from patient before enrolling in the study. The consent for publication was also obtained from a patient by the written informed consent. The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (UMIN000003489) on April 19, 2010 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

In December 2008, a 53-year-old woman was diagnosed with locally advanced breast cancer with axillar lymph node metastasis. Four courses of an anthracycline-based regimen (THP-adriamycin, cyclophosphamide; AC) were administered as pre-operative chemotherapy. However, the tumor remained same size (no response: NR). In April 30, 2009, left mastectomy with axillary lymph node resection was performed. Microscopically, the tumor diagnosed as invasive ductal carcinoma with a negative estrogen receptor, a negative progesterone receptor, and a negative human epidermal growth factor receptor 2 score (so-called "triple negative" tumor). Adjuvant treatment of weekly taxol (80 mg/m^2) was followed 12 times during May and August 2009. In September 2009, the patient presented a clinically asymptomatic supraclavicular lymph node recurrence. As this region seemed to be resistant to the standard chemotherapy, surgical resection of supraclavicular nodes was performed in September 8, 2009 and another chemotherapy, CPT-11 (80 mg/m^2) was initiated for 12 times during October and January, 2010. After a 4-month free-of relapse period, a local recurrence was detected again in left spinal accessory node. The radiotherapy in supraclavicular and chest wall in total 50 Gy/25 times was initiated from February 1, 2010. As the CT scan showed no response after 50 Gy irradiation, 10 Gy was boosted (total 60 Gy) in the same area. As this lymph node recurrence was considered to be chemo-resistant and radio-resistant, we conducted a vaccine immunotherapy. The cancer vaccine therapy using Survivin-H/K-HELP was initiated from April 14, 2010. Survivin-H/K-HELP vaccine was subcutaneously administered biweekly 4 times. Two weeks after the last administration, the safety, immune response and tumor response were evaluated as described previously [15].

3. Results

3.1. Identification of a novel Survivin-2B helper epitope

To identify Survivin-2B helper epitopes, purified $CD4^+$ T cells from four healthy donors were stimulated with IFN-γ-treated autologous Mo-DC with Survivin-2B peptide mixture (MIX1 to MIX5), which included 5 overlapping peptides derived from Survivin-2B (Supplementary Table S1). After repetitive stimulation with the cognate mixture, we obtained MIX4-reactive Th cells from all donors. Using these Th cells, the reactive peptides in the MIX4 were determined as SU18 peptide ($Survivin\text{-}2B_{99-117}$), SU21 peptide ($Survivin\text{-}2B_{112-131}$), and SU22 peptide ($Survivin\text{-}2B_{119-138}$), respectively. In the case of SU18 peptide, two types of Th cells were generated. The IFN-γ-production of donor 1-derived SU18-reactive Th cells was greatly inhibited by anti-HLA-DR mAb (Fig. 1A) and the Th cells recognized only HLA-DRB1*01:01-expressing EBV-B cells. These evidence suggested that the SU18 peptide was presented by HLA-DRB1*01:01 (Fig. 1B). In contrast, the response of donor 2-derived SU18-reactive Th cells was inhibited by anti-HLA-DQ mAb (Fig. 1C) and it was restricted to HLA-DQB1*06:01 (Fig. 1D). Therefore, SU18 peptide is a promiscuous peptide. To identify minimal epitopes recognized by these two Th cells, a series of 15-mer overlapping truncated peptides derived from the SU18 peptide was prepared and tested for their ability to stimulate the Th cells. The HLA-DRB1*01:01-restricted Th cells recognized EBV-B cells in the presence of T7 to T11 truncated peptides, suggesting that their recognizing minimal region was $Survivin\text{-}2B_{106-116}$ (GCAFLSVKKQF) (Fig. 1E). The minimal region of HLA-DQB1*06:01-restricted Th cells was determined as $Survivin\text{-}2B_{102-112}$ (KHSSGCAFLSV) (Fig. 1F). The same analysis was also performed using SU21 and SU22 peptides and demonstrated that donor 3-derived SU21-reactive Th cells were restricted to HLA-DRB1*15:01/15:02 and

its minimal region was Survivin-2B$_{117-128}$ (EELTLGEFLKLD) (data not shown). In the case of SU22 peptide, two types of Th cells were generated from donor 3 and 4. Donor 3-derived SU22-reactive Th cells were restricted to HLA-DRB1*01:01 (Fig. 2A and B), and donor 4-derived cells were restricted to HLA-DR53 (Fig. 2C and D), suggesting that SU22 peptide was also a promiscuous peptide. Each of minimal regions was Survivin-2B$_{123-132}$ (EFLKLDRERA) and Survivin-2B$_{124-133}$ (FLKLDRERAK), respectively (Fig. 2E and F).

3.2. Development of a superior long peptide, Survivin-H/K-HELP consisting of SU18 and SU22 peptides

From the database searching, it was clarified that SU18 included a novel promiscuous helper epitope with three other killer epitopes and SU22 contained a widely applicable promiscuous helper peptide. Therefore, we decided to synthesize artificial Survivin-H/K-HELP by conjugating SU18 and SU22 Survivin-derived peptides using glycine-linker. As illustrated in Fig. 3A, Survivin-H/K-HELP consisted of two helper epitopes (presented to HLA-DRB1*01:01, DR53, DQB1*06:01, and DPB1*05:01) and three killer epitopes (presented to HLA-A*02:01 and A*24:02). The detail information concerning helper and killer epitopes presented to HLA molecules were indicated by underlines in Fig. 3A.

3.3. Survivin-H/K-HELP was superior to its component short peptides (SU18 and SU 22) for inducing Survivin-specific Th1 and Tc1 cells

CD4$^+$ T cells, isolated from 9 healthy donors were cocultured with IFN-γ-treated Mo-DC or OK-432-treated Mo-DC in the presence of Survivin-H/K-HELP. The CD4$^+$ T cells were expanded by weekly stimulation with Survivin-H/K-HELP 3 times. After 3 weeks-culture, the expanded T cells were restimulated with Survivin-peptides and their IFN-γ or IL-5 producing ability was determined by intracellular staining assay to evaluate the frequency of Th1 or Th2 cell induction. The representative FACS profile was shown in Fig. 4A. Survivin-H/K-HELP induced IFN-γ-producing Th1 cells but not IL-5-producing Th2 cells. We also demonstrated that IL-4-producing Th2 cells were not significantly induced by stimulation with short peptides (0.48%) or surviving-H/K-HELP (0%). The expanded numbers of CD4$^+$ T cells were also measured and indicated as the accumulated data of T cell growth rate of 9 donors (Fig. 4B). These results indicated that OK-432-treated Mo-DC were superior to IFN-γ-treated Mo-DC for inducing Survivin-specific IFN-γ-producing Th1 cells. Thus, we used OK-432-treated Mo-DC as antigen-presenting cells (APC) to induce Th1 cells using cancer-associated antigen peptides. Using this culture method, we assessed whether Survivin-H/K-HELP was superior to its component short peptide (SU18 and SU22) for inducing Survivin-specific Th1 and Tc1 cells. PBMC were cultured with Survivin-H/K-HELP or mixture of its component peptides (mixture of SU18 and SU22 short peptides) in the presence of OK-432-treated Mo-DC for 3 weeks. After culture, the frequency of Survivin-specific Th1 or Tc1 cells was determined by intracellular staining using flow cytometry. As shown in Fig. 5, the induction of Th1 and Tc1 cells was greatly enhanced by culture with Survivin-H/K-HELP but not with the mixture of SU18 and SU22 short peptides in 3 distinct healthy donors though SU18 (19 amino-acids) peptide contained both helper and killer epitopes as described above (Fig. 3A). The CD4$^+$ T cells and CD8$^+$ T cells, which were expanded by stimulation with Survivin-H/K-HELP recognized its component SU18 (Donor A and B) and SU22 (Donor C) short peptides on APC and produced IFN-γ (Fig. 5). The expanded CD8$^+$ T cells expressed cytotoxic molecules such as perforin and granzyme B (supplementary Fig. S1). Thus, Survivin-H/K-HELP is superior to its component short peptides

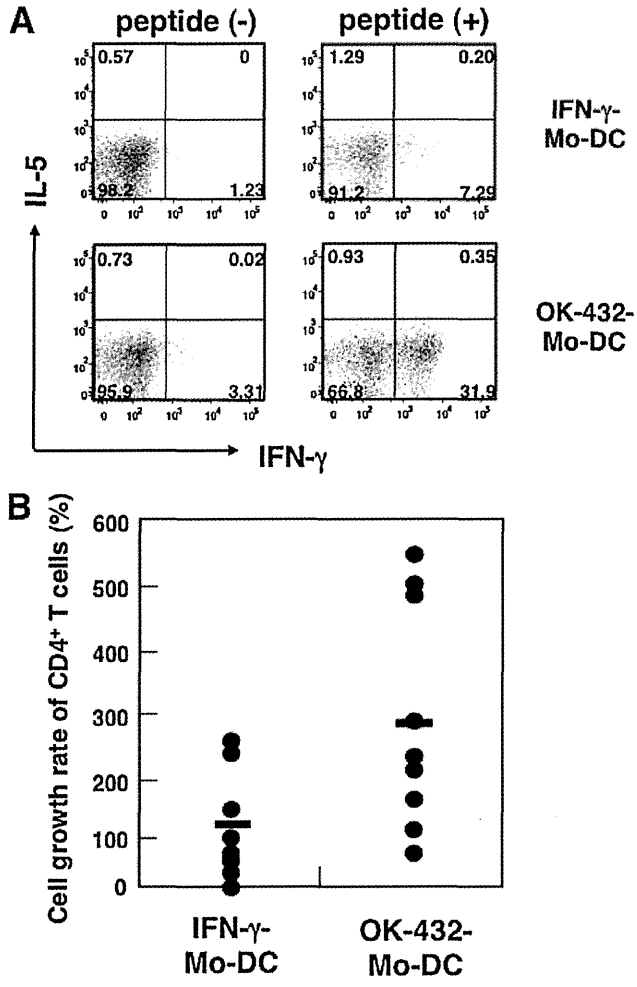

Fig. 4. OK-432-activated Mo-DC were superior to IFN-γ-treated Mo-DC for an efficient induction of Survivin-2B-specific CD4$^+$ Th1 cells by pulsing with Survivin-H/K-HELP. CD4$^+$ T cells were weekly stimulated with IFN-γ- or OK-432-treated Mo-DC pulsed with Survivin-H/K-HELP for 3 weeks. (A) IFN-γ and IL-5 production by CD4$^+$ T cells were evaluated by intracellular staining by flow cytometry after stimulation with (+) or without (−) Survivin-H/K-HELP. (B) Numbers of CD4$^+$ T cells in the culture were counted at day 21 and indicated as Cell growth rate of CD4$^+$ T cells (%), which was calculated as follows; cell growth rate of CD4$^+$ T cells (%) = numbers of activated T cells after culture/numbers of T cells before culture × 100. Means of triplicate data are presented.

(SU18 and SU22) for an efficient induction of IFN-γ-producing CD4$^+$ Th1 and CD8$^+$ Tc1 cells in the presence of OK-432-treated Mo-DC.

3.4. Superior and sustained antigen presentation of OK-432-activated Mo-DC pulsed with Survivin-H/K-HELP

We compared the antigen presentation capability of OK-432-activated Mo-DC pulsed with Survivin-H/K-HELP or its component SU18 and SU22 short peptides in the activation of the antigen-specific Th1 cells. The change of the APC function of OK432-activated Mo-DC was determined by measuring the change of Th1-dependent IFN-γ production levels in the culture. Although the antigen presentation activity of OK-432-activated Mo-DC pulsed with Survivin-H/K-HELP or its component short peptides was gradually decreased during incubation, the Mo-DC pulsed with Survivin-H/K-HELP exhibited stronger stimulatory activity to induce higher IFN-γ production from Survivin-reactive Th cells compared with those pulsed with the mixture of SU18 and SU22

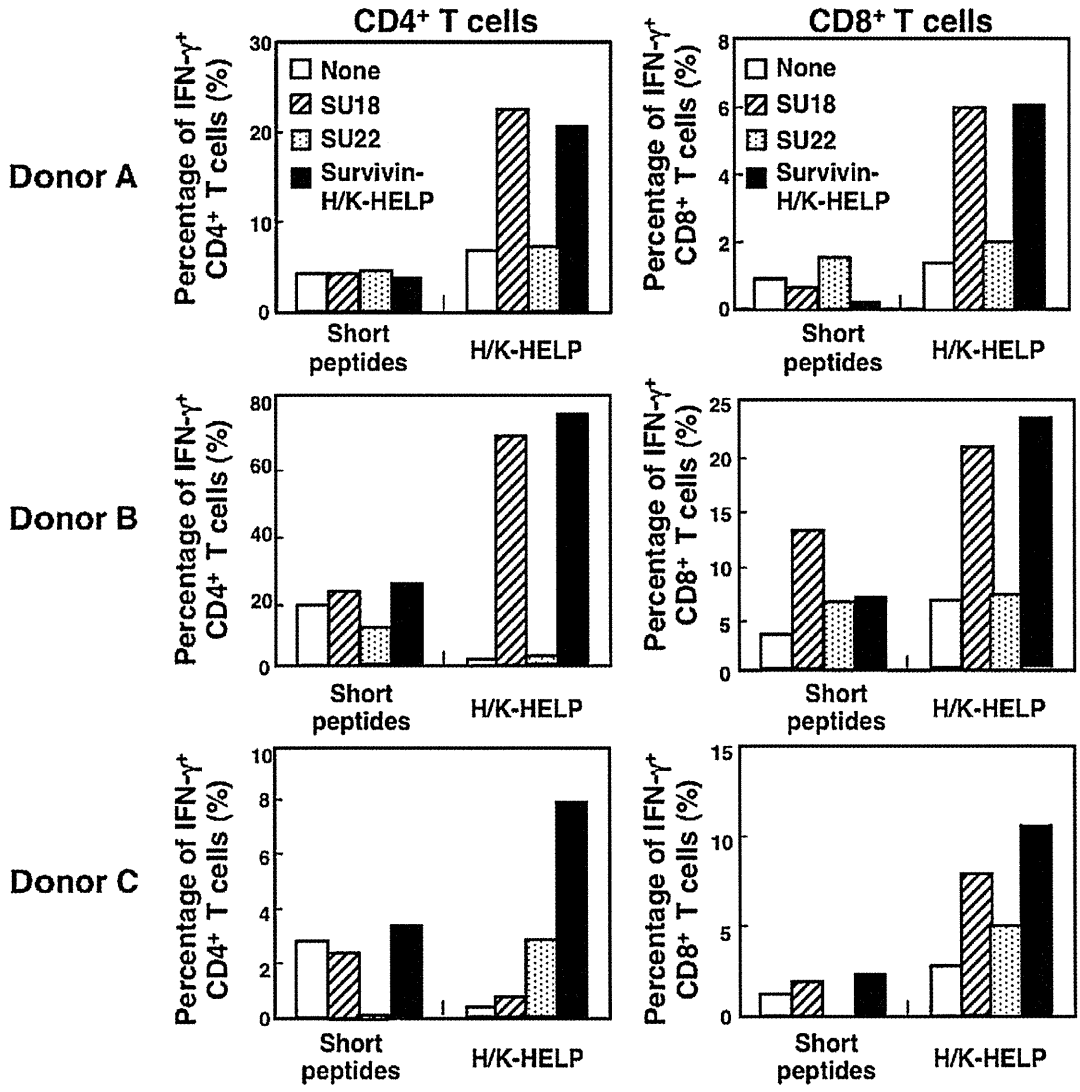

Fig. 5. Survivin-H/K-HELP induced an efficient induction of Survivin peptide-specific Th1 and Tc1 cells *in vitro*. PBMC of three donors were weekly stimulated with Mo-DC pulsed with Survivin-H/K-HELP or mixture of its component short peptides (SU18 and SU22) for 3 weeks. The induced T cells were restimulated with none (open bars), SU18 (slanted bars), SU22 (dotted bars), or Survivin-H/K-HELP (filled bars) for 20 h. IFN-γ production by stimulated $CD4^+$ T or $CD8^+$ T cells was evaluated by intracellular staining by flow cytometry. Representative data of three independent experiments are shown.

peptides at any point (24, 48, 72, 96 h) after culture (Fig. 6). Moreover, OK-432-activated Mo-DC pulsed with Survivin-H/K-HELP exhibited sustained APC function to stimulate Th1-dependent IFN-γ production. Even 96 h after the initiation of culture, OK-432-activated Mo-DC pulsed with Survivin-H/K-HELP possessed antigen-presenting capability of inducing IFN-γ from Th1 cells. While APC function of OK-432-activated Mo-DC pulsed with SU18 and SU22 short peptides almost completely disappeared 48 h after the initiation of culture.

3.5. A case study in phase I clinical trial of Survivin-H/K-HELP cancer vaccine therapy

We documented a prominent case study of breast cancer patient treated with Survivin-H/K-HEKP cancer vaccine. As shown in Section 2, a patient suffered with postoperative chemo-resistant, radio-resistant lateral deep cervical node recurrence of breast cancer was enrolled in a phase I clinical trial of Survivin-H/K-HELP cancer vaccine therapy. After confirmation of Survivin expression in cancer tissue, the patient was intradermally treated with Survivin-H/K-HELP vaccine and montanide biweekly four times (1st cycle) (Fig. 7A). Compared with the pretherapy, the size of metastatic node significantly decreased and completely disappeared 4 weeks after first cycle of Survivin-H/K-HEKP cancer vaccine therapy (71 days after initiation of vaccination) (Fig. 7B and C). In parallel with the anticancer effect of Survivin-H/K-HELP cancer vaccine, it rapidly induced Survivin-peptide-specific IFN-γ-producing Th1 cells and the elevation of Survivin-peptide specific antibodies though the generation of Tc1 cells reached peak at late time (Fig. 7D–F). It was notable that Th1-dependently complement-fixing IgG1 and IgG3 antibodies against Survivin-peptide became major subtypes at the late stage of Survivin-H/K-HELP vaccination (Fig. 7F and G).

4. Discussion

Survivin-2B is an attractive target molecule for cancer vaccine therapy because it is highly expressed in almost all cancer cells examined thus far by PCR and immunohistochemistry [16–24].

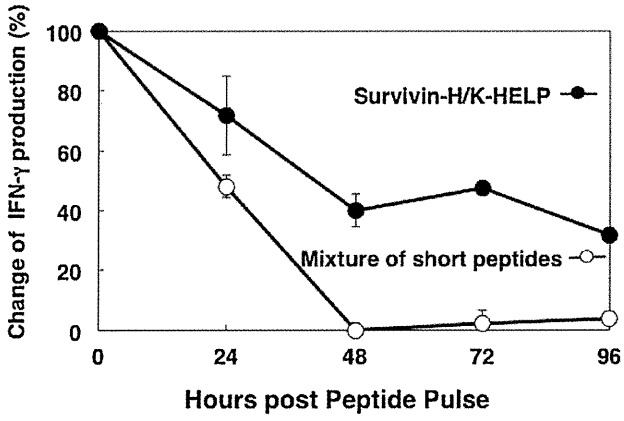

Fig. 6. Superior and sustained antigen presentation of Mo-DC pulsed with Survivin-H/K-HELP. OK-432-activated Mo-DC were pulsed with 5 μM Survivin-H/K-HELP (filled circle) or its component short peptides SU18 and SU22 (open circle) for 2 h at 37 °C. After washing twice, peptide-pulsed Mo-DC were cultured alone in AIM-V medium for various times (0, 24, 48, 72, 96 h). The peptide-pulsed-Mo-Dc were harvested from the culture 0, 24, 48, 72, or 96 h after incubation and cocultured with Survivin-peptide-reactive Th cells for 24 h to assess their antigen presentation capability. The antigen presenting ability of peptide-pulsed Mo-DC was determined by measuring Th cell-derived IFN-γ levels in the culture supernatants by ELISA. Change of IFN-γ production (%) was calculated as described in Section 2.6. Each data represents the mean of triplicate samples from representative experiment. The similar results were obtained in three separate experiments.

Here, we identify minimal peptide sequences of helper epitopes in Survivin cancer antigen. Among SU18 (Survivin-2B$_{99-117}$), SU21 (Survivin-2B$_{112-131}$), and SU22 peptides (Survivin-2B$_{119-138}$), the SU18 promiscuous helper peptide contains HLA-A*02:01- and HLA-A*24:02-restricted killer epitopes and the SU22 peptide contains a helper epitope restricted by DR53, which is a popular HLA class II phenotype in both Japanese (>60%) [25] and Caucasians (>50%) as determined by the Allele*Frequency Database. We also develop an artificially synthesized 40 amino-acid long peptide, Survivin-H/K-HELP by conjugating SU18 helper epitope and SU22 killer epitope peptides with a glycine-linker. SU18 includes a novel promiscuous helper epitope with three other killer epitopes and SU22 contains a widely applicable promiscuous helper peptide. Thus, Survivin-H/K-HELP, a long peptide including two helper and three killer epitopes, can activate both Th1 and Tc1 cells and it is applicable to the treatment of cancer patients worldwide judged from its binding HLA-haplotypes. We also demonstrate that Survivin-H/K-HELP is a superior cancer vaccine compared with its component short peptides (SU18 and SU22) to induce cancer antigen-specific Th1-dependent immunity *in vitro*.

During the past decades, many investigators have attempted to induce cancer-specific CTL in cancer patients by vaccination with class I-binding short (8–10 amino acids) peptides [26–28]. However, the overall results of cancer vaccine therapy have not been impressive [8,29–31]. This may be because of the existence of strong immunosuppression, tumor escape mechanisms and/or the lack of helper T cell activation [5,32–35].

We previously demonstrated a critical role for Th1 and Th2 immunity in tumor-bearing hosts and proposed that the introduction of Th1-dominant immunity is essential for inducing fully activated CTL and immunological memory [5,36,37]. Moreover, we demonstrated that Th1-dominant immunity could suppress the accumulation of immunosuppressive Treg cells into the tumor local site in an IFN-γ-dependent manner [38]. Therefore, the introduction of Th1 immunity in cancer patients might be a rational strategy to activate tumor-specific immunity and to inhibit immunosuppressive Treg functions. Indeed, recently, it was demonstrated that a mixture of various SLP containing both helper and killer epitopes corresponding to sequences of viral or tumor-associated antigens were superior to short peptides for inducing antitumor immunity. Melief and van der Burg et al. [11] reported that an HPV-16-derived 35 amino-acid long peptide eradicated an established HPV-16-expressing mouse tumor. SLP but not short peptides derived from the naturally occurring sequences of HPV-16 oncoproteins induced complete responses or partial responses in vulvar intraepithelial neoplasia [39]. Thus, long peptide vaccines containing both helper and killer epitopes might be a rational strategy to activate Th1-dependent antitumor immunity [5]. However, the first clinical trial using a synthetic 14 amino-acid peptide vaccine containing a naturally-occurring combination of helper and killer epitopes exhibited no significant therapeutic efficacy on the tumor [40]. We also demonstrate in this paper that a 19 amino-acid SU18 peptide, an SLP containing a naturally-occurring combination of helper and killer epitopes does not show stronger T cell stimulating activity *in vitro* compared with a 40 amino-acid Survivin-H/K-HELP containing SU18 and SU22 peptide. Therefore, long peptide with more than 23 amino acids might be suitable to induce an efficient T cell stimulation as described by Melief's group [11].

Previously [41], we demonstrated that culture of CD4$^+$ T cells with IFN-γ-treated autologous Mo-DC in the presence of helper peptides was a superior method to induce MAGE-A4-specific Th1 cells. Moreover we developed an artificially synthesized 40 amino acid long peptide, which conjugated MAGE-A4 class I-binding epitope and a defined helper epitope and demonstrated the safety and immunological effect of artificially synthesized MAGE-A4-H/K-HELP [15]. In contrast to short (14 amino-acid) hybrid peptides including helper and killer epitopes [13], administration of 40 amino acid H/K-HELP successfully induce cancer-specific Th1, Tc1, and complement-fixing antibodies (IgG1 and IgG3) [15,42]. This discrepancy may be explained as an artificially synthesized 40 amino acid long peptide, but not its component short peptides, has a beneficial structure favorable for DC presentation and subsequent activation of Th1 and Tc1. In the present paper, we develop Survivin-H/K-HELP including two helper and three killer epitopes by conjugating SU18 and SU22 short peptides. Survivin-H/K-HELP is superior to its component short peptides (SU18 and SU22) to induce Survivin peptide-specific IFN-γ-producing Th1 and Tc1 cells. Furthermore, a 19 amino acid SU18 peptide, similar to SLP containing a naturally-occurring combination of helper and killer epitopes, does not show stronger T cell stimulating activity *in vitro* compared with the 40 amino-acid Survivin-H/K-HELP containing SU18 and SU22 peptides. We do not show any cytotoxicity data of CD8$^+$ T cells induced by culture with Survivin-H/K-HELP-pulsed Mo-DC. However, we have demonstrated that Survivin-H/K-HELP can induce higher numbers of CD8$^+$ T cells expressing perforin and granzyme B compared with those stimulated with short peptides. Therefore, it is possible to speculate that IFN-γ-producing CD8$^+$ T cells induced by Survivin-H/K-HELP act as cytotoxic T cells. Moreover, in one breast cancer patient of phase I clinical study, it is demonstrated that Survivin-H/K-HELP cancer vaccine successfully induce a complete response in parallel with the induction of Th1-dependent cellular and humoral responses. It is greatly interesting that Survivin-H/K-HELP vaccination allows the elevation of complement-fixing IgG1 and IgG3 Th1-dependent antibodies in patient's serum. Thus, it is suggested that 40 amino acid long peptide, Survivin-H/K-HELP is a superior cancer vaccine *in vivo* though this is still a result of one patient. Thus, we believe that a 40 amino-acid long peptide, Survivin-H/K-HELP is a superior cancer vaccine, which may act both *in vitro* and *in vivo*.

Melief et al. [43] reported that SLP of extended class I-binding long cancer peptides were efficiently processed by professional antigen presenting cells and subsequently exhibited sustained stimulating activity of DC to induce Th-dependent tumor-specific CTL. Consistent with their results, we also demonstrate that

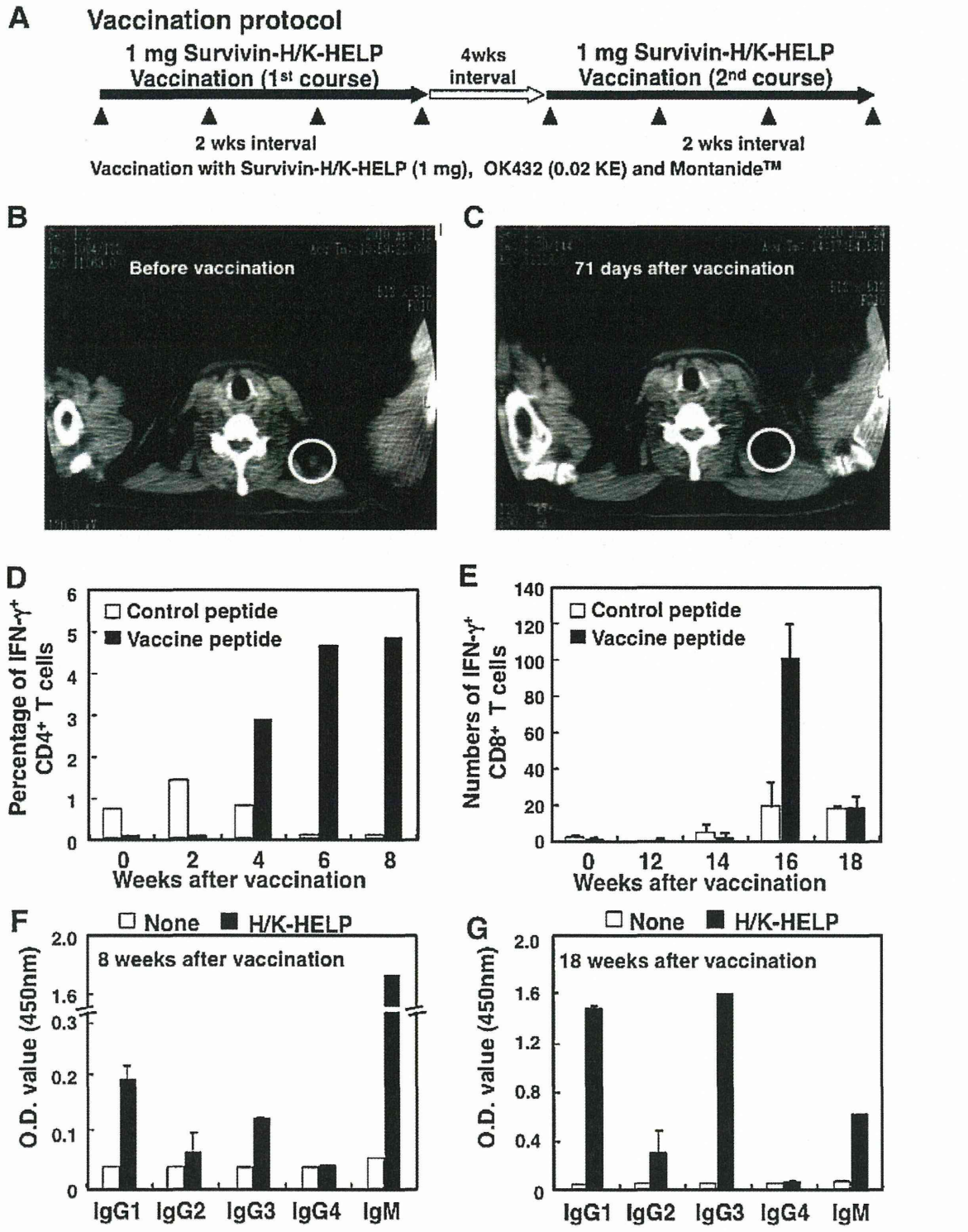

Fig. 7. Case study in phase I clinical trial of Survivin-H/K-HELP cancer vaccine therapy. A patient suffered with chemo-resistant, radio-resistant lateral deep cervical node recurrence of breast cancer was treated with Survivin-H/K-HELP vaccine therapy. (A) Vaccination protocol for the patient in phase I study. The patient was vaccinated with Survivin-H/K-HELP (1 mg) mixed with OK-432 (0.02 KE) and Montanide ISA-51 4 times (1st course) at 2 weeks interval and further 4 times (2nd course) after 4 weeks interval. (B and C) Growth inhibition of breast cancer by Survivin-H/K-HELP vaccine therapy. Tumor growth was monitored by computed tomograohy (CT) before or after the 1st cycle of Survivin-H/K-HELP vaccination. (D) Survivin-H/K-HELP-specific IFN-γ production by $CD4^+$ T cells was assessed by intracellular staining using flow cytometry various weeks after initiation of Survivin-H/K-HELP vaccination. (E) Survivin-H/K-HELP-specific IFN-γ production of $CD8^+$ T cells was determined by ELISPOT assay various weeks after initiation of Survivin-H/K-HELP vaccination. (F and G) The levels of IgG1, IgG2, IgG3, IgG4, or IgM antibody against none (□) or Survivin-H/K-HELP (■) peptide was determined using the patient's serum sample (400-fold diluted) 8 (F) or 18 (G) weeks after initiation of Survivin-H/K-HELP vaccination. Means and SDs were indicated in the figure.

OK-432-activated Mo-DC pulsed with 40 amino-acid Survivin-H/K-HELP but not its component short peptide (mixture of SU18 and SU22) allows superior antigen presentation capability to Mo-DC to stimulate IFN-γ-producing Th1 cells.

In summary, we identify novel helper epitopes and develop an innovative cancer vaccine, Survivin-H/K-HELP, which exhibits a superior capability of inducing Survivin-peptide-specific Th1 and Tc1 compared with its component short peptides (mixture of SU18 and SU22 peptides). We also demonstrate that Survivin-H/K-HELP compared with short peptides allows superior and sustained APC function to OK-432-activated Mo-DC, which is preferable for the induction of IFN-γ-producing Th1 cells. Thus, we believe that artificially synthesized Survivin-H/K-HELP of Survivin cancer-associated antigen will become an innovative cancer vaccine to induce Th1-dependent cellular and humoral immunity in cancer patients.

Acknowledgments

The authors thank the patient for consenting to publication of her clinical details. This study is a part of the 'Development of Innovative Cancer Immunotherapy by Helper T cells' sponsored by a Translational Research Promotion Project of the New Energy and Industrial Technology Development Organization (NEDO). This work was supported in part by a Grant-in-Aid for Scientific Research (B) (22300331 to T.N.) from the Ministry of Education, Culture, Sports, Science, and Technology, (grant no. 22300331 to TN) Japan. The authors thank Takuko Sawada (Shionogi Pharmaceutical Institute Co., Osaka, Japan) for kind donations of recombinant IL-2.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2014.04.010.

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Immunology Letters 158 (2014) 175–182

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Identification of a meiosis-specific protein, MEIOB, as a novel cancer/testis antigen and its augmented expression in demethylated cancer cells

Yasuo Shiohama[a,1], Junya Ohtake[a,1], Takayuki Ohkuri[a], Daisuke Noguchi[a], Yuji Togashi[b], Hidemitsu Kitamura[a], Takashi Nishimura[a,c,*]

[a] Division of Immunoregulation, Section of Disease Control, Institute for Genetic Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan
[b] BioImmulance Co., Ltd., Shin-ei-363-32, Kiyota-ku, Sapporo 004-0839, Japan
[c] Division of ROYCE' Health Bioscience, Institute for Genetic Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan

ARTICLE INFO

Article history:
Received 6 August 2013
Received in revised form
28 December 2013
Accepted 8 January 2014
Available online 16 January 2014

Keywords:
Demethylation
Cancer testis antigen
Meiosis specific with OB domains
Helper epitope
Cancer immunotherapy
5-Aza-2′-deoxycytidine

ABSTRACT

Cancer/testis (CT) antigens, which are expressed in various cancer cells but not in normal cells except germline cells of the testis, have been used as targets for cancer vaccine therapy. 5-Aza-2′-deoxycytidine (DAC), a potent inhibitor of genomic and promoter-specific DNA methylation, inhibits DNA methyltransferase activity and is reported to induce the expression of certain CT antigens by the demethylation of promoter CpG islands of the treated cells. Here, using DAC-treated cancer cells, we searched for novel attractive target molecules that would be useful for cancer immunotherapy and found a meiosis-specific protein, meiosis specific with OB domains (MEIOB), to be a novel CT antigen. Indeed, the MEIOB gene is expressed only in the testis and not in other normal tissues. The mRNA expression of MEIOB was greatly enhanced in several lung cancer cell lines after the treatment with DAC. Furthermore, we identified a variety of helper epitopes of the MEIOB antigen, which were recognized by MEIOB antigen-specific T cells in a HLA-restriction manner. Finally, we demonstrated that IFN-γ production of MEIOB peptide-specific helper T cells in response to HLA-matched cancer cells was greatly augmented by treatment with DAC and IFN-γ. Taken together, these findings show DAC to be a promising tool for finding novel CT antigens and for developing a future novel combination cancer vaccine chemotherapy.

1. Introduction

Cancer immunotherapy, including cancer vaccine, has been considered as an attractive therapy for patients with cancer because of the fewer side effects caused. Since the cancer/testis (CT) antigen was first found in a human melanoma [1], numerous CT antigens (such as MAGE and NY-ESO-1) have been identified as rational targets of various cancers. CT antigens are widely expressed in various types of human cancers, but their expression in normal cells is tightly restricted in germline cells of the testis [2–10]. It has been demonstrated that cancer vaccine therapy using the HLA class I-binding short peptide derived from the CT antigen caused an increase of tetramer-positive cytotoxic T lymphocytes (CTLs) in cancer patients and maintained the patients at long stable disease. However, we have proposed that Th1-dominant immunity in a tumor-bearing host was essential for the induction of fully activated CTLs and the subsequent generation of memory T cells [11–16].

Recently, it has been reported that the human papillomavirus (HPV)-16-derived long peptide, containing both helper and killer epitopes, remarkably eradicated established HPV-16-expressing tumors [17]. In a previous paper, we had identified the MAGE-A4-helper epitope [18] and applied it to a novel cancer vaccine immunotherapy, using the helper/killer-hybrid epitope long peptide (H/K-HELP). The H/K-HELP cancer vaccine induced superior antitumor responses, including T cell responses and antibody responses. In fact, in a phase I clinical study, we found that MAGE-A4-specific Th1 and Tc1 cells were effectively generated in patients with colon cancer after vaccination with MAGE-A4-H/K-HELP [19].

5-Aza-2′-deoxycytidine (DAC), an inhibitor of DNA methyltransferases (DNMTs), is approved by the U.S. FDA for the treatment of myelodysplastic syndromes and is currently under evaluation in phase I and II clinical trials for the treatment of solid tumors.

* Corresponding author at: Division of Immunoregulation, Section of Disease Control, Institute for Genetic Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan. Tel.: +81 11 706 7546.
E-mail address: tak24@igm.hokudai.ac.jp (T. Nishimura).
[1] These authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.imlet.2014.01.004

Interestingly, DAC has been reported to induce the gene expression of certain CT antigens by the demethylation of promoter CpG islands in the treated cancer cells [20–24]. In the present work, we searched for a novel CT antigen using DAC-treated cancer cells and attempted to determine helper epitopes that will be useful for developing an efficient cancer vaccine peptide.

2. Materials and methods

2.1. Reagents and cell culture

Cancer cell lines were maintained and treated with DAC (1 μM; Sigma–Aldrich) as described previously [18,24]. The studies using human blood and tissue specimens were approved by the medical ethics committees of the Institute for Genetic Medicine, Hokkaido University and Hokkaido University Graduate School of Medicine.

2.2. Microarray analysis

Experimental RNA samples were isolated from A549 cells, treated with or without DAC, by using an RNeasy kit (Qiagen), and the gene expression levels were evaluated by using 3D-Gene (Toray).

2.3. Polymerase chain reaction analysis

Total RNA was prepared with the ISOGEN kit (Nippon gene) and cDNAs were then prepared by reverse transcription. The MAGE-A4, XAGE, BAGE, Survivin, MEIOB, and β-actin genes were amplified with a thermal cycler system (PerkinElmer), using the corresponding primer pairs (Supplementary Table S1A). Quantitative-PCR was performed with another thermal cycler system (LightCycler, Roche). The sequences of primers used and the respective Universal ProbeLibrary are given in Supplementary Table S1B. Sample signals were normalized to the housekeeping gene GAPDH according to the ΔΔCt method: $\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$. Percentages against the control sample were then calculated for each sample.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2014.01.004.

2.4. Overlapping peptides

Overlapping peptides of MEIOB were synthesized and designated as #1–62 peptides (Sigma–Genosys; Supplementary Fig. S1). For the initial screening assay, the 62 peptides were subdivided into MIX1–MIX12 peptide pools to induce the peptide-specific T lymphocytes. Subsequently, each peptide was individually used for the secondary screening. The MEIOB gene is reported to have 2 transcripts that differ in a coding region near their C-termini. In this study, we used 2 amino-acid isoform sequences, because isoform 2 is part of the amino-acid sequence of isoform 1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2014.01.004.

2.5. Preparation of dendritic cells

Dendritic cells (DCs) were induced from peripheral blood mononuclear cells (PBMCs) as described previously [18], but with slight modification, where 50 ng/mL GM-CSF (Wako) and 50 ng/mL IL-4 (Wako) were used instead of 30 ng/mL GM-CSF (KIRIN) and 30 ng/mL IL-3 (KIRIN), respectively.

2.6. In vitro induction of antigen-specific T cells with overlapping peptides

MEIOB-specific $CD4^+$ T cells were induced from PBMCs by using MEIOB-derived overlapping peptides (5 μM) instead of protein, and restriction of human leukocyte antigen (HLA) was determined as described previously [18]. For the restimulation on day 7, mitomycin C-treated autologous DCs pulsed with OK432 were used.

2.7. Measurement of antigen-specific responses with established T cells

MEIOB-specific T cells (3×10^4), induced from PBMCs, were mixed with HLA-matched or -mismatched lung cancer cell lines (3×10^4), pretreated with or without IFN-γ (100 ng/mL; Wako) and DAC in the presence or absence of anti-HLA class I and anti-HLA class II mAbs for 72 h. The expression of HLA class I and HLA class II was evaluated by flow cytometry using HLA-A/B/C or HLA-DR/DP/DQ mAbs conjugated with fluorescein isothiocyanate (BD-Pharmingen). IFN-γ production by MEIOB-specific $CD4^+$ T cells was evaluated by using ELISA kits (BD-Biosciences) or the ELISPOT assay, performed as described previously [19].

2.8. Western blotting

Cancer cells, treated with DAC (1 μM) for 72 h, and equal numbers of cells were lysed with Cell Lysis Buffer (Cell Signaling) in the presence of protease inhibitors. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (ATTO). Membranes were blocked with EzBlock Chemi (ATTO) and probed with anti-MEIOB (ab178756; abcam) or α-tubulin (SIGMA) Abs. Membranes were washed and incubated with a Horseradish Peroxidase conjugated secondary Ab (goat anti-rabbit IgG (H&L) or goat anti-mouse IgG (H&L); American Qualex Antibodies). The protein levels were detected using a C-DiGit Blot Scanner (LI-COR) with ECL Prime Western Blotting Detection Reagent (GE Healthcare).

2.9. Statistical analysis

All experiments were independently repeated at least 3 times. The mean values and their standard deviations were calculated for *in vitro* data. Significant differences in the results were determined by the 2-tailed Student's *t*-test. A probability value of $P < 0.05$ was considered to be significant in the present study.

3. Results

3.1. Identification of a novel CT antigen, MEIOB, from DAC-treated cancer cells

To investigate the effect of DAC treatment on the gene expression of CT antigens, 3 human lung cancer cell lines, LC-OK, A549, and LC-MS, were treated with DAC. Before and after the treatment with DAC, we examined the mRNA expression levels of CT antigens (MAGE-A4, XAGE, BAGE, and Survivin) in these cancer cells. The results showed that MAGE-A4 was spontaneously expressed in LC-MS cells. XAGE was expressed in LC-OK and A549 cells, and BAGE was expressed in LC-OK cells. On the other hand, Survivin gene expression was observed in all 3 cell lines. DAC treatment of the cells caused a remarkable induction of MAGE-A4 in the LC-OK and A549 cells. Moreover, the expressions of XAGE in LC-MS cells and BAGE in A549 and LC-MS cells were augmented by DAC

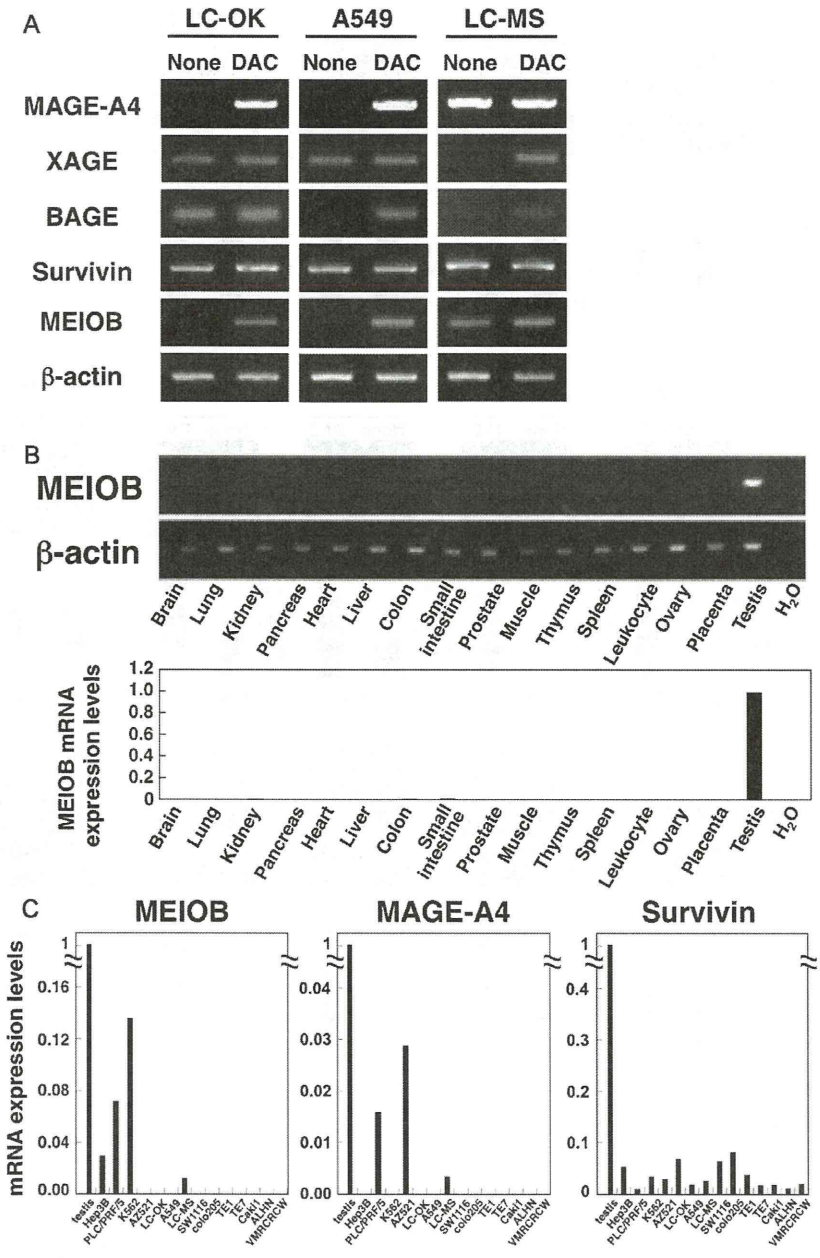

Fig. 1. Gene expression levels of the CT antigen in DAC-treated cancer cells. (A) DAC-treated cancer cell lines were analyzed by RT-PCR. (B) MEIOB gene expression of normal tissues, evaluated by RT-PCR and quantitative-PCR. (C) CT antigen mRNA expression levels in various human cancer cell lines, evaluated by quantitative-PCR. The target gene in the testis was assigned an expression value of 1, and the fold increases in the expression levels were determined by comparison. Three independent experiments were performed, and the representative results are indicated in the figure.

treatment (Fig. 1A). Next, we screened novel genes expressed in DAC-treated A549 cells by using cDNA microarrays with 25,000 human transcripts. Among the 25,000 genes, 1755 had differential expression patterns between untreated and DAC-treated A549 cells (data not shown). We further evaluated the expression levels of the candidate genes enhanced by DAC treatment in normal tissues, using multiple tissue cDNA panels. Finally, we confirmed that MEIOB was expressed only in the testis and not in other normal tissues (Fig. 1A and B). We analyzed the gene expression levels of MEIOB, MAGE-A4, and Survivin in various human cancer cell lines (liver: Hep-3B and PLC/PRF/5; leukemia: K562; stomach: AZ521; lung: LC-OK, A549, and LC-MS; colon: SW1116 and colo205; esophagus: TE1 and TE7; and renal: Caki1, ALHN, and VMRCRCW). MEIOB was detected in Hep-3B, PLC/PRF/5, K562, and LC-MS cells, whereas MAGE-A4 was expressed in PLC/PRF/5, AZ521, and LC-MS cells. Survivin, on the other hand, was detected in almost all the tested cell lines (Fig. 1C). In the present experiment, we found that the MEIOB gene expression levels were maintained in Hep-3B, PLC/PRF/5, K562, and LC-MS cells (Fig. 2A), but augmented in AZ521, LC-OK, A549, SW1116, colo205, TE1, TE7, Caki1, ALHN, and VMRCRCW cells by DAC treatment (Fig. 2B). These findings suggested that MEIOB is a novel CT antigen and might be a promising target for T-cell-mediated cancer immunotherapy.