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**Studies on effective induction of cancer antigen-specific
T cells and its application for cancer immunotherapy**

(がん抗原特異的 T 細胞の効果的な誘導と
がん免疫治療への応用に関する研究)

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At the present day, cancer patients are treated with chemotherapy, radiotherapy, and surgical therapy as standard therapies. Progress in medicine remarkably improves our life-span, however cancer is still the number one cause of death in Japan. In late years, research and development of immunotherapy are executed as the fourth cancer treatment. Cancer vaccine therapy had started since discovery of the tumor-specific antigen in the early 1990s. Various basic and clinical studies have been performed for the cancer antigen specific immunotherapy. Recently, clinical studies using HLA class I-restricted killer peptides have been performed to activate tumor antigen specific cytotoxic T cells (CTLs). Although there are many reports about prolongation of prognosis and the limited complete regression of tumors by the cancer peptide vaccine therapy, it does not become a standard therapy for all of cancers yet.

There are several issues for this poor clinical efficacy in immunotherapy including cancer peptide vaccine. In general, suppression of antitumor immunity caused by the defectiveness of immune status in cancer patients is considered as a great factor. To overcome this problem, it is necessary that more effective cancer vaccines introducing and maintaining antitumor immunity in vivo must be established. Moreover, we need development of the standardized monitoring methods and novel biomarkers to evaluate the immune condition predicting a clinical response of an individual cancer patient before and under treatment.

In this study, I focused on Survivin, tumor antigen expressed in various kinds of cancers and identified helper epitopes inducing antigen-specific helper T (Th) cells to apply to novel cancer immunotherapy. In addition, we identified biomarkers judging immune status of cancer patients on the basis of preclinical and clinical study.

In this study, minimal peptide sequences of helper epitopes in Survivin cancer antigen were identified. Among SU18 peptide (Survivin-2B₉₉₋₁₁₇), SU21 peptide (Survivin-2B₁₁₂₋₁₃₁), and SU22 peptide (Survivin-2B₁₁₉₋₁₃₈), Su18 promiscuous helper peptide contained HLA-A*02:01- and HLA-A*24:02-restricted killer epitopes. SU22 peptide contained a helper epitope restricted by HLA-DR53, which was a popular HLA class II phenotype in both Japanese (Over 60%) and Caucasian (Over 50%). A 40 amino acid long peptide, Survivin-long peptide was synthesized by conjugating the SU18 helper and SU22 killer epitope peptides by glycine-linker. SU18 included a novel promiscuous helper epitope with other three killer epitopes and SU22 contained widely applicable promiscuous helper peptide. Thus, Survivin-long peptide including two helper peptides and three killer epitopes, can activate both Th1 and Tc1 cells and applicable to cancer patients in all over the world judging from

Survivin-peptide-binding HLA haplotypes. Survivin-long peptide was a superior cancer vaccine compared with its component's short peptides (SU18 and SU22) to induce cancer antigen-specific Th1-dependent immunity *in vitro*.

During past decades, many investigators have tried to induce cancer-specific CTLs in cancer patients by vaccination with class I-binding short (8-10 amino acids) peptides^{1,38}. However, the overall results of the cancer vaccine therapy have not been so impressive though it induced an increase of the numbers of tetramer positive-cancer-specific CTLs and long stable diseases. Thus, the cancer vaccine therapy has focused on only CTL activation appeared to be suboptimal to destruct cancer. This may be because of the existence of a strong immunosuppression, tumor escape mechanism and the lack of helper T cell activation.

Previous studies demonstrated the critical role of Th1 and Th2 immunity in tumor-bearing host and introduction of Th1-dominant immunity was essential for inducing fully activated CTLs and immunological memory. Moreover, Th1-dominant immunity could suppress the accumulation of immunosuppressive Treg cells into tumor local site in an IFN- γ -dependent manner. Therefore, it is possible to speculate that the introduction of Th1 immunity in cancer patients may be a rational strategy to activate tumor-specific immunity and to inhibit immunosuppressive Treg function. Recently, it has been demonstrated that a mixture of various synthetic long peptide (SLP) containing both helper and killer epitopes corresponding to the sequence of viral or tumor-associated antigen is superior to short peptides to induce antitumor immunity. Melief and van der Burg et al. reported that HPV-16-derived 35 amino-acid long peptide eradicated the established HPV-16-expressing mouse tumor. SLP but not short peptides derived from naturally occurring sequence of HPV-16 oncoproteins are demonstrated to induce complete responses or partial response in vulvar intraepithelial neoplasia. Thus, long peptide vaccine containing both helper and killer epitopes appears to be a rational strategy to activate Th1-dependent antitumor immunity. However, a first clinical trial using a synthetic 14 amino-acid peptide vaccine containing naturally-occurring combination of helper and killer epitopes exhibited no significant impact for therapeutic efficacy of tumor. In this study, SU18 peptide, which was likely as SLP containing naturally-occurring combination of helper and killer epitopes did not show stronger T cell stimulating activity *in vitro* compared with 40 amino acid Survivin-long peptide conjugating SU18 and SU22 peptides. Therefore, more than 23 amino acids might be suitable long peptide to induce an efficient T cell stimulation as described by Melief's groups.

Previous study demonstrated that artificially synthesized 40 amino acid long

peptide, which conjugated MAGE-A4 class I-binding epitope and our defined helper epitope showed the safety and immunological effects. In contrast to short (14 amino-acid) peptide including helper and killer epitopes, the long peptides successfully induced cancer-specific Th1, Tc1 cells, and complement-fixing Abs (IgG1 and IgG3). This discrepancy may be because artificially synthesized 40 amino acid long peptide, but not its components, short peptides has a beneficial structure for favorable DC presentation and subsequent activation of Th1 and Tc1 cells. In this study, Survivin-long peptide was prepared by conjugating two helper and three killer epitope peptides. Survivin-long peptide was superior to its component's short peptides (SU18 and SU22) to induce Survivin peptide-specific IFN- γ -producing Th1 and Tc1 cells. It was also demonstrated that 19 amino-acids SU18 peptide, which is likely as SLP containing naturally-occurring combination of helper and killer epitopes did not show stronger T cell stimulating activity in vitro compared with 40 amino-acids Survivin-long peptide conjugating SU18 and SU22 peptides. A phase I clinical study using Survivin-long peptide demonstrated that vaccination of a breast cancer patient with Survivin-long peptide caused a complete response in parallel with the induction of Th1-dependent cellular and humoral responses. Thus, it is confirmed that 40 amino acid long peptide is a superior cancer vaccine both in vitro and in vivo.

Melief group reported that SLP of extended class I-binding long cancer peptides were efficiently processed by professional antigen presenting cells (APCs) and subsequently exhibited sustained stimulating activity of DCs to induce Th-dependent tumor-specific CTLs. In consistent with their results, monocyte-derived DCs (Mo-DCs) pulsed with 40 amino-acids Survivin-long peptide but not its components (the mixture of SU18 and SU22) exhibited sustained antigen presentation capability of stimulating Th cells.

In summary, Survivin-long peptide composed of novel helper epitopes, identified in this study, revealed a superior capability of inducing Survivin-specific-Th1 and Tc1 cells. Survivin-long peptide allowed the long-sustained antigen presentation by Mo-DCs to stimulate T cells compared with short peptides. Thus, Survivin-long peptide of Survivin cancer antigen will become a promising tool to induce Th1-dependent cellular and humoral immunity during cancer vaccine therapy.

In Chapter 2, I reported about effective induction of cancer antigen-specific immune responses in phase I clinical study on cancer vaccine therapy using Survivin- or MAGE-A4-long peptide. In phase I first in man clinical study of cancer vaccine therapy using MAGE-A4- or Survivin-long peptide, patients with MAGE-A4 or Survivin-expressing cancers were treated by vaccinating the MAGE-A4- or

Survivin-peptide (1 mg or 10 mg) mixed with Picibanil OK-432 (0.02 KE) and Montanide ISA-51 (0.5 or 1.1 ml) subcutaneously or locally once every 2 weeks in four doses and evaluated the safety and immunological responses. The study subjects consisted of 16 patients, including five patients with head and neck cancer, four patients with colon cancer and one patient with extramammary Paget's disease, renal cancer, breast cancer, malignant melanoma, epitheloid sarcoma, and cervical cancer. Although several vaccine-related adverse events were observed, the treatment was well tolerated. Vaccination using MAGE-A4- or Survivin-peptide with Picibanil and Montanide resulted in an increase or induction of an antibody response in 11 of 15 patients vaccinated. An increase or induction of CD4⁺ T and/or CD8⁺ T cell responses was also observed in 10 of 15 patients. These findings confirmed the immunogenicity of the MAGE-A4- and Survivin-vaccination. Furthermore, one patient with colon cancer showed SD after six doses of the vaccination as reported previously in case report and one patient with breast cancer showed CR after eight doses of the vaccination. The summation of the size of cancer was almost unchanged in one patient with extramammary Paget's disease during the vaccine therapy.

It has been well accepted that Th cells licensed DCs for their efficient antigen cross-presentation and subsequent CD8⁺ T cell stimulation. Therefore, we hypothesized that the conjugation of a Th epitope with currently available CTL short epitope peptides facilitated the increased immunogenicity of CTL vaccines. Interestingly, Th and CTL epitopes are sometimes located in close proximity or are even overlapped, in the molecules, for example, in the case of the human papillomavirus and Her-2/neu. Zeng *et al.* reported that NY-ESO-1 157–170 (SLLMWITQCFLPVF) was recognized by both NY-ESO-1-reactive CD4⁺ T and CD8⁺ T cells. Recently, synthetic long peptides were demonstrated to be superior to short peptides for use as vaccines. Long peptides do not bind to major histocompatibility complex (MHC) class I molecules directly, and the antigen is presented after up-taking and processing by professional APCs such as DCs. Therefore, the use of long peptides prevents the antigen peptides from direct binding to MHC class I molecules on nonprofessional antigen-presenting cells such as B cells and T cells, which may cause the induction of CD8⁺ T cell tolerance because of appropriate costimulatory signals. Thus, long peptide vaccine containing both helper and killer epitopes appeared to be a rational strategy to activate Th1-dependent antitumor immunity. In contrast to viral-related cancer antigenic long peptide, p53-long peptide vaccine induced no complete or partial response in a clinical trial of human cancers, though it induced significant T cell responses. Moreover, the first clinical trial using a synthetic 15 amino acid peptide vaccine containing a naturally occurring combination

helper and killer epitopes of gp100₁₇₅₋₁₈₉ exhibited no significant impact for therapeutic efficacy of melanoma. Therefore, what kinds of long peptide is a beneficial therapeutic vaccine for human cancer remains unclear.

To overcome current problems, we tried to synthesize longer (40 amino acids) vaccine peptide containing both helper and killer epitope. Using our identified MAGE-A4-derived promiscuous helper epitope (MAGE-A4₂₈₀₋₂₉₉), we prepared an artificially synthesized long peptide, which conjugate MAGE-A4₂₇₈₋₂₉₉ helper epitope with MAGE-A4₁₄₃₋₁₅₄ killer epitope by a glycine-linker to activate antigen-specific CD8⁺ T cells in addition to CD4⁺ T cells. Similarly, using our identified Survivin-derived promiscuous two helper epitope (Survivin₉₉₋₁₁₇ and Survivin₁₂₄₋₁₃₅) as described in Chapter 1, we prepared an artificially synthesized long peptide to activate CD8⁺ T cells in addition to CD4⁺ T cells. This study showed that two long peptides, MAGE-A4- and Survivin-long peptide, spanning a peptide region MAGE-A4₂₇₈₋₂₉₉, Survivin₉₉₋₁₁₇ and Survivin₁₂₄₋₁₃₅ were immunogenic and induced the antigen-specific antibodies, CD4⁺ T and/or CD8⁺ T cell responses in cancer patients.

It is now accepted that an immune-related tumor response should be evaluated by different criteria from that for a tumor response induced by cytotoxic agents. Clinical response resulting from immunotherapy can be appreciated generally after an initial increase in tumor volume sometimes associated with the appearance of new lesions evaluated as PD by the Response Evaluation Criteria in Solid Tumors (RECIST) or WHO criteria. Thus, immune-related response criteria (irRC) were proposed recently. The MAGE-A4- and Survivin-vaccine elicited humoral, CD4⁺ T and/or CD8⁺ T cell responses in the vaccinated patients. The increase or induction of a MAGE-A4 or Survivin antibody response was observed in 4 out of 8 vaccinated patients. The sera from the vaccine-immunized patients reacted with MAGE-A4 or Survivin helper and killer peptides as well as the MAGE-A4- or Survivin-long peptide, suggesting elicitation of an antibody response by an artificially synthesized long peptide vaccine including a dominant B cell epitopes. The increase and induction of CD4⁺ T and/or CD8⁺ T cell responses were also detected after peptide vaccination in 10 of 15 patients. Although the numbers of patients was small, the responses were comparable or even stronger in terms of the frequency and characteristics of the immune responses, when compared with various preparations of other vaccines using short peptides. Moreover, Th1-dependent antibodies (IgG1 and IgG3) were preferentially induced in cancer patients after the vaccination with MAGE-A4- and Survivin-peptide. In addition, IFN- γ but not IL-4 productions by T cells were detected in the high clinical response patients, suggesting that Th1-type immune responses might be related with the clinical efficacy

of cancer vaccine therapy using long peptide. We are now conducting a phase II clinical trial for breast or colon cancer patients using Survivin-long peptide.

In conclusion, the established long peptides contain dominant regions in the MAGE-A4 and Survivin molecules that include multiple epitopes frequently recognized by antibodies, CD4⁺ T, and CD8⁺ T cells. The use of long peptide in cancer vaccine therapy will practically allow inclusion of most, if not all, patients into a study irrespective of their HLA type. The vaccine using long peptide, caused little toxicity and strong humoral and cellular immune responses, suggests the usefulness of synthesized long peptide vaccines in the clinical management of cancer patients.

In Chapter 3, I reported about novel biomarkers for predicting and checking immune status or responses of cancer patients. miRNAs, short non-coding RNAs, play a key role in the regulation of target gene expression and the subsequent cellular responses for many biological functions. miRNA is targeted to approximately 1/3 of the human mRNA⁷⁶. Recent study demonstrated that miRNAs played an important role in both innate and acquired immunity. In this study, miR-20b and miR-224 affected cytokine production in T cell immune responses. miR-20b enhanced Th2 cytokine production from healthy donor-derived PBMCs. In contrast, miR-224 Th1 cytokine production by PBMCs. These results revealed that two kinds of microRNA, miR-20b and miR-224 might participate in regulation of Th1/Th2 cytokine balance in our body. These findings suggest that miRNAs may be related with excessive activation or suppression of Type 1 and Type 2 immunity that causes immune-related diseases including autoimmune, allergy, and tumorigenesis. Furthermore, introduction of miRNA into human Mo-DCs could alter cytokine production by antigen-specific Th cells. These results that strategies using miRNA mimics or inhibitors may contribute to control T cell immune responses in various disorders in the patients to improve the excessive or suppressive Th1/Th2 immune state.

Recent clinical study on cancer immunotherapy reported that improving T cell immunity by using anti-PD-1 antibody (Nivolumab) and/or anti-CTLA-4 antibody (Ipilimumab) in cancer patients showed good therapeutic effect. This implies that immune status based on T cell responses is severely defecting in cancer patients. However, it is also reported that effective cancer immunotherapy occasionally causes adverse events according to the excessive immune responses including autoimmune. Therefore, in addition to proper regulation of T cell immune responses against cancer, identification of accurate biomarker to evaluate immune status of individual cancer patients are required for development of effective cancer immunotherapy.

Recent studies demonstrate that evaluation of serum and cellular miRNA levels can

become a promising biomarker for prediction of various disorders including malignancy or life expectancy of cancer patients. Recently, it is reported that cytokine profiles of each helper T cell subsets are closely correlated with the activity, inactivation, and the risk of tumor. Previous study demonstrated that miRNAs involved in proliferation, differentiation, and activation of Th cells⁸⁰. It was reported that the correlations of miR-20b and miR-224 were confirmed in various human cancer, although it was unclear whether these two kinds of miRNA were correlated with cytokine production in T cell immune responses.

The present findings suggest that miRNAs such as miR-20b and miR-224 will become a useful biomarker for prediction and evaluation of Th1/Th2 immune status in various diseases such as allergy, autoimmune, and cancers in addition to the immunotherapy through control of T cell immune responses.