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Immunological and Symptomatic Effects of Oral Intake of Transgenic Rice Containing 7 Linked Major T-Cell Epitopes from Japanese Cedar Pollen Allergens

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Keywords
Allergic rhinitis · Japanese cedar · Peptide immune therapy · Pollinosis · T cell epitopes

Abstract
Background: A rice-based peptide vaccine containing 7 linked human predominant T-cell epitopes (7Crp) derived from Japanese cedar (JC) pollen allergens, Cry j 1 and Cry j 2, was developed. Here, we examined the efficacy and safety of this transgenic rice in JC pollinosis patients. ***Methods:*** Transgenic rice (5, 20, and 80 g) was administered orally. We measured the T-cell proliferative activity against 7Crp, Cry j 1, and Cry j 2; the cytokine expression levels; and specific IgE and IgG4 production levels. In addition, the symptom and medication scores were monitored during the pollen season, and quality of life (QOL) was evaluated. ***Results:*** T-cell proliferative activities to Cry j 1, Cry j 2, and 7Crp were significantly depressed in a dose-dependent manner. Oral intake of 80 g transgenic rice for 20 weeks resulted in significant suppression of allergen-specific T-cell proliferation with downregulation of IL-13 and upregulation of IL-10 levels but no changes to specific IgE and IgG4 levels. The QOL symptom scores for allergic rhinitis were not significantly improved. ***Conclusions:*** Allergen-specific T-cell responses were significantly reduced by oral intake of transgenic rice in a dose-dependent manner. However, neither medication score nor QOL symptom scores could be improved during the JC pollen season with oral intake of transgenic rice for 20 weeks.

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Introduction

Japanese cedar (JC) pollinosis was first reported in 1964 [1], and the number of patients with this seasonal allergy has remarkably increased in the last 2 decades [2]. Currently, more than 30% of the Japanese population suffers from JC pollinosis between February and April each year, which is a kind of a national affliction [3]. In our recent epidemiological study, we demonstrated that the prevalence of JC pollinosis in the Tokyo metropolitan area reaches 45.6% [4], resulting in a strong social demand for an effective treatment.

The main treatment for JC pollinosis is usually pharmacotherapy, but this therapy only reduces clinical symptoms and is not curative. Allergen-specific immunotherapy based on the administration of disease-causing allergens is the only curative treatment with long-lasting clinical efficacy [5]. Treatment is typically administered through the subcutaneous or sublingual route for several years (e.g., 3–5 years) [6–8]. However, when natural intact allergens (crude allergen extract) are used as tolerogens for such immunotherapies, side effects causing severe anaphylactic shock sometimes occur [9]. Furthermore, repeated injections are laborious and inconvenient. Thus, establishing a more convenient, more effective, and safer protocol is highly desired. One promising strategy for overcoming these challenges is peptide immunotherapy [10, 11]. Dominant T-cell epitopes derived from allergens with lengths <20 amino acids cannot cross-link IgE and possess minimal inflammatory potential, indicating that high-dose administration is feasible. Thus, peptide immunotherapy may provide a safe and effective alternative to conventional subcutaneous and/or sublingual approaches.

Dominant T-cell epitopes have been characterized in mice and human for cedar pollen allergens, Cry j 1 and Cry j 2 [12]. Oral administration of a peptide containing major T-cell epitopes from Cry j 2 has been shown to induce immunological tolerance in Cry j 2-sensitized mice [13]. In addition, a synthesized peptide containing 3 dominant T-cell epitopes (3Crp) from these JC pollen allergens induced tolerance to Cry j 1 and Cry j 2 in a mouse model [12, 13]. Three peptide sequences from Cry j 1 and four from Cry j 2 have been identified as major human T-cell epitopes [14, 15], leading to the development of a peptide sequence (96 amino acids) containing these 7 linked dominant T cell epitopes (7Crp) [15]. 7Crp exhibits similar immunogenicity (T cell activity) toward Cry j 1 and Cry j 2 but lacks allergenicity (IgE-binding activities) [15]. Taken together, these results indicate that peptide immunotherapy using T-cell epitopes is safe and effective due to the lack of cross-linking to specific IgE because of short epitope lengths.

Rice seed has been used as an ideal platform for the production of recombinant proteins because of its robust production, presence of ample deposition space, high stability at ambient temperature for several years, and scalability [16]. Moreover, extensive purification is not required for administration because rice seed is generally regarded as safe. Recombinant proteins expressed in seeds are deposited in protein bodies, a protein storage organelle that is resistant to the harsh conditions found in the gastrointestinal environment when produced as a secretory protein [17]. We previously generated transgenic rice containing human 7Crp or mouse T-cell epitopes fused to soybean glycinin seed protein in endosperm tissue [18, 19]. When rice seeds containing these tolerogens are administered orally, they are expected to be effectively delivered to gut-associated lymphoid tissues (GALTs) without severe degradation by digestive enzymes due to bioencapsulation through double barriers comprising the cell wall and protein body, providing the means for innovative therapy as an oral tolerogen against allergy diseases [17]. In an experimental murine model, transgenic rice seeds containing mouse T-cell epitopes from Cry j 1 and Cry j 2 were orally administered and then challenged with JC pollen allergen extract. Treated mice had significantly reduced production of Th2-type cytokines and allergen-specific IgE and decreased histamine release and nasal sneezing, indicating that oral mucosal immune tolerance against Cry j 1 and Cry j 2 was induced [18]. Specific suppression of T-cell proliferation and IgE was also observed in mice by oral administration of transgenic rice containing 7Crp [19]. Human 7Crp was highly expressed in rice endosperm tissue at 60 μg/grain [19]. Given that oral administration of transgenic rice seeds containing 7Crp can induce immunological tolerance against JC pollen allergens, it may be possible to control JC pollinosis through diet.

In the present randomized, placebo-controlled trial, 3 different concentrations of rice seed-based peptide vaccine were orally administered as tolerogens in a formulation of pre-packed cooked rice to patients with JC pollinosis for 20 weeks from December to April overlapping pollen season (February to April). T-cell proliferation in peripheral blood mononuclear cells (PBMCs), the levels of several cytokines and specific IgE and IgG4 antibodies, clinical symptoms, and quality of life (QOL) were evaluated. We demonstrated that T-cell proliferation was significantly suppressed but clinical symptoms were not clearly improved in the transgenic rice groups compared to the placebo group.

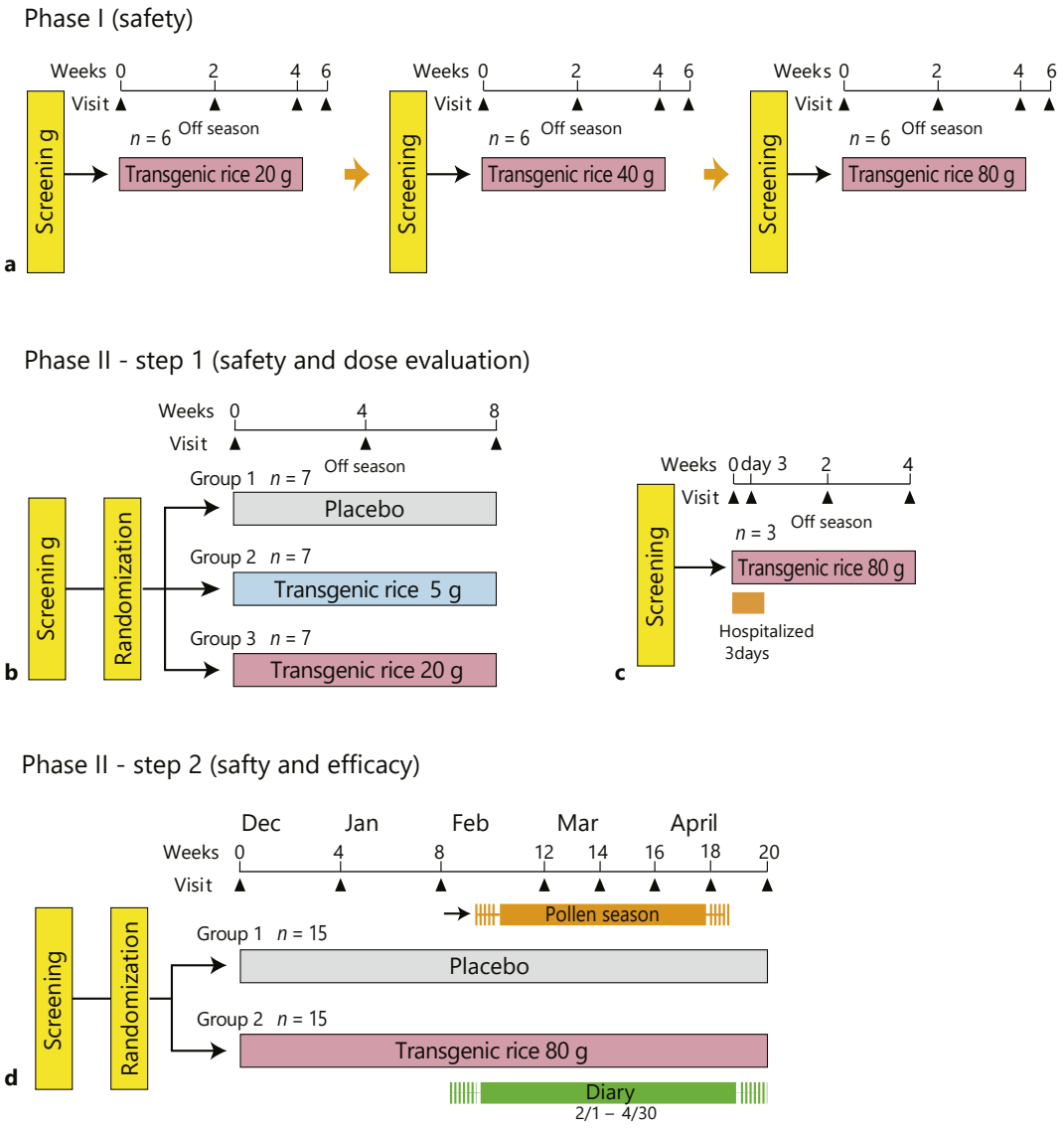

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Fig. 1. Study design of safety and efficacy of transgenic rice. **a** Safety analysis: 20, 40, and 80 g transgenic rice were orally administered step by step to a healthy person without pollinosis. **b** Dose evaluation analysis: 5 and 20 g of transgenic rice or non-transgenic rice were orally administered to Japanese cedar (JC) pollinosis patients for 8 weeks during off-pollen season. **c** 80 g of transgenic rice was orally administered to JC pollinosis patients for 4 weeks during off-pollen season. **d** Efficacy analysis: 80 g transgenic or non-transgenic rice were orally administered for 20 weeks overlapping pollen season.

Methods

Subjects

The subjects were patients with JC pollinosis for at least 2 years during JC pollen seasons who underwent medical examination. Patients who had a RAST score ≥2 and a positive response to JC pollens by provocation test and intradermal reaction were enrolled in the study.

Patients with the following diseases that prevent accurate judgment were excluded: if they were afflicted with rice allergy or nasal diseases such as acute/chronic rhinitis, nasal polyps, hypertrophic rhinitis, nasal septum deviation, and rhinosinusitis, or if they had undergone nasal surgery in the past year. Patients were also excluded if they had a current diagnosis of diabetes mellitus, liver disease, heart disease, uncontrolled asthma, immunodeficiency, malignant tumor, or severe anemia; if they were taking β-blocker

for heart disease or taking α-blocker for prostatic hypertrophy; or if they were pregnant or breastfeeding. Patients who did not have T-cell proliferation with stimulation index (SI) ≥2 were also excluded.

Study Design

The present study was conducted at Jikei University Hospital as a single-blind, randomized placebo-controlled trial (Fig. 1a, c, d) from May 2013 to April 2014 (registered clinical trial 25–057 [7,162]; UMIN000011086) and a double-blind placebo-controlled trial (Fig. 1b) from June 2014 to January 2015 (registered clinical trial 26–055 [7,560]; UMIN000016078). The protocols were approved by the Ethics Committee of Jikei University School of Medicine, and this study was carried out in accordance with the guidelines of the Declaration of Helsinki and the ethical guidance regarding clinical study of the Ministry of Health, Labor and Welfare in Japan. Written informed consent was obtained from each patient prior to participation in these studies.

In Phase I, safety was checked with healthy volunteers (Fig. 1a). The dose of transgenic rice administered was increased in a step-by-step manner (20, 40, and 80 g). In each step, prepacked cooked rice was orally administered to 6 healthy volunteers every day for 4 weeks. After ascertaining the safety, the dose was stepped up.

In Phase II – step 1 (Fig. 1b, c), to evaluate the dose of transgenic rice required for suppression of allergen-specific T-cell proliferation, 21 participants were randomly allotted to the placebo group, 5 g transgenic rice group, and 20 g transgenic rice group in a 1:1:1 ratio (Fig. 1b). Participants were asked to consume 1 prepacked cooked rice per day for 8 weeks from October. Follow-up visits were conducted on the first day of rice intake and at 4 and 8 weeks after beginning of the rice intake. Furthermore, 3 patients were administered 80 g of prepacked cooked rice every day for 4 weeks in October (off-season). They were hospitalized for the first 3 days and follow-up visits conducted on the first day of rice intake (day 0) and 3, 14, and 28 days after beginning of the rice intake (Fig. 1c).

In the Phase II – step 2 (Fig. 1d), 30 patients were blindly randomized to either the 80 g transgenic rice group or the placebo group at a 1:1 ratio. Participants were asked to consume 1 prepacked cooked rice, which contains 80 g of transgenic or non-transgenic rice, per day for 20 weeks from December to April overlapping with the JC pollen season (February to April).

Transgenic Rice

The transgenic rice line, which accumulates a hybrid peptide composed of 7 dominant human T-cell epitopes (7Crp; molecular weight 10.6 kDa) in its seed [18], was cultivated in a confined field at the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan) during 2012 and 2013. The harvested seeds were husked at NIAS and transported to the authorized rice milling company, where they were polished and processed into the prepacked cooked rice (total 80 g) with 3 different doses of transgenic rice (5, 20, or 80 g) by mixing with non-transgenic rice.

Lymphocyte Proliferation Assays

Blood was collected from patients at every follow-up visit. PBMCs were separated from blood as described previously [20]. Allergen-specific lymphocyte proliferative responses to 7Crp, Cry j 1, and Cry j 2 were measured using an in vitro radioactively labeled thymidine incorporation assay [20]. Results are expressed as the SI, which was calculated as follows: mean counts/min in the presence of the antigen divided by mean counts/min in the absence of the antigen. Intergroup comparisons were based on the mean ratio of allergen-specific responses in the group at the indicated times during treatment, which was calculated as the SI at the indicated date divided by the SI at day 0.

Analysis of Cytokines and Allergen-Specific Serum Antibodies

The concentrations of IL-4, IL-5, IL-10, IL-13, and IFN-γ secreted from PBMCs following stimulation with 7Crp, Cry j 1, and Cry j 2 were measured using standard sandwich ELISA protocols. Antigen-specific IgE antibody titers were measured by IgE capture ELISA as described previously [21]. The antigen-specific IgG4 antibody titers were measured by Nittobo Medical Co., Ltd. (Fukushima, Japan).

Clinical Symptoms

Clinical symptoms were evaluated by symptom scores (sneezing, running nose, blocked nose and itchy eyes) and medication scores, which were self-recorded by participants in a daily allergy diary, during pollen season from February 1 to April 30. Each symptom was scored on a scale of 0–4 according to severity. Total nasal symptom and medication scores (TNSMS) were comprehensively evaluated by summing individual scores for sneezing, runny nose, blocked nose, and medication score. QOL was evaluated using the JRQLQ according to the Practical Guideline for the Management of Allergic Rhinitis in Japan [6, 22]. Questionnaire responses were reported on the visit date and baseline clinical characteristics were evaluated on the registration date. The JC pollen dispersion stage was defined as >20 pollen grains/cm^2. When daily life was severely affected by JC pollinosis, 20 mg of epinastine hydrochloride was selected as the first-choice treatment. If this treatment was insufficient, cromoglycate nasal spray or cromoglycate eye drops were added to alleviate nasal or eye symptoms, respectively. Use of one of the drugs described above was defined as a medication score of 1 and the use of all 3 drugs was defined as a medication score of 3.

Statistical Analysis

Statistical analyses were carried out with IBM SPSS ver. 23 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). Symptom scores were compared using the Mann-Whitney U test for data. T-cell proliferation and cytokine production were analyzed by repeated measures ANOVA. Differences in categorical variables were evaluated using the χ^2 test. Significance was defined as $p < 0.05$.

Results

Immune Responses Induced by Oral Intake of Transgenic Rice

Since the safety of oral intake of transgenic rice was confirmed in the Phase I trial, we measured how the lymphocyte proliferation activity of PBMCs from JC pollinosis was affected by oral intake of transgenic rice.

Patients were orally administered prepacked cooked rice containing 5 or 20 g of transgenic rice for 8 weeks

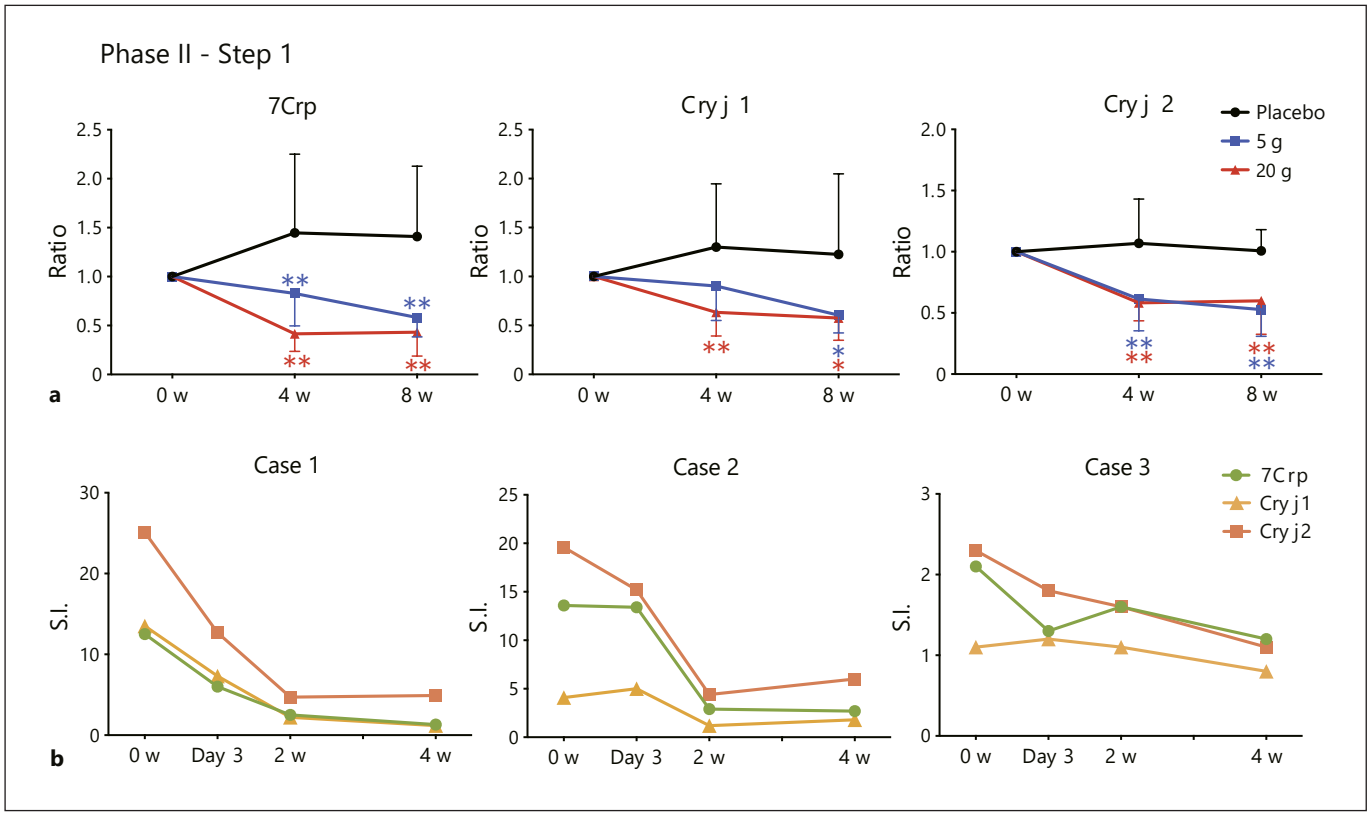

Fig. 2. Dose dependency of T-cell proliferation responses induced by oral intake of transgenic rice. **a** Changes in T-cell proliferation responses to 7Crp, Cry j 1, and Cry j 2 were examined by oral administration of 5 or 20 g of transgenic or non-transgenic rice for 8 weeks during off-pollen season. Results are expressed as means ± 95% CI. $^{*}p < 0.05$, $^{**}p < 0.01$. **b** Stimulation Index (S.I.) of T-cell proliferation responses to 7Crp, Cry j 1, and Cry j 2 were examined by oral administration of 80 g of transgenic rice for 4 weeks during off-pollen season.

during off-season, followed by stimulation with 7Crp, Cry j 1, and Cry j 2 (Fig. 1b). As shown in Figure 2a, when 20 g of transgenic rice was administered, specific T-cell proliferation against 7Crp, Cry j 1, and Cry j 2 was remarkably reduced at 4 weeks after the intake of transgenic rice compared to the non-transgenic rice group (placebo control group). On the other hand, in the case of 5 g intake, the suppression of specific T-cell proliferation against 7Crp and Cry j 1 was lower than that in the 20 g intake group at 4 weeks, although the level finally decreased to a similar level as the 20 g group at 8 weeks.

When 80 g of transgenic rice was orally administered for 4 weeks (Fig. 1c), T-cell proliferation against 7Crp, Cry j 1, and Cry j 2 was depressed even for 3 days (Fig. 2b). Furthermore, oral intake of transgenic rice for 2 weeks resulted in a reduction in T-cell proliferation to the lowest level (case 1 and case 2, Fig. 2b). These results indicate that the immune response to cedar pollen allergens (Cry j 1 and Cry j 2) can be modulated by oral intake of transgenic rice containing 7Crp. Furthermore, it was also shown that suppression of T-cell proliferative response is regulated in a dose-dependent manner. Thus, immune tolerance against Cry j 1- and Cry j 2-specific T cells is expected to be induced by oral administration of transgenic rice containing their major T-cell epitopes.

Downregulation of T-Cell Proliferation Responses Induced by Oral Intake of Transgenic Rice during Pollen Seasons

The effects of transgenic rice intake were examined in regard to the T-cell proliferation responses of JC pollinosis patients during the pollen seasons. In Phase II – step 2 (Fig. 1d), 80 g of transgenic rice or non-transgenic rice was administered daily to 2 groups for 20 weeks from December to April, overlapping with pollen season. T-cell proliferation in response to 7Crp, Cry j 1, and Cry j 2 was significantly suppressed by the intake of transgenic rice compared to placebo (Fig. 3a). Specific T-cell prolifera-

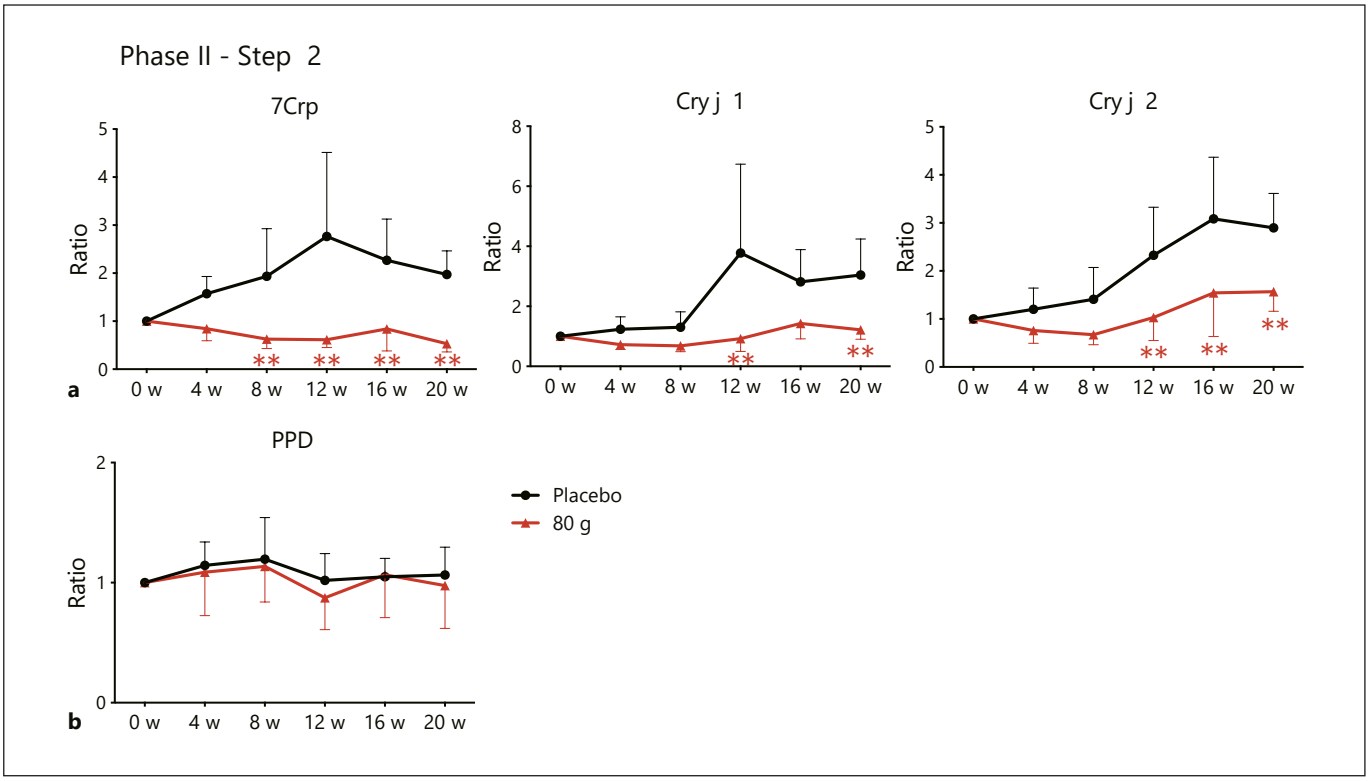

Fig. 3. Changes in T-cell proliferation responses to 7Crp, Cry j 1, and Cry j 2 (**a**), and those to purified protein derivatives of tuberculin (PPD) after oral intake of 80 g of transgenic rice for 20 weeks (**b**). Results are expressed as mean ± 95% CI. *$p < 0.05$, **$p < 0.01$.

tion against 7Crp, Cry j 1, and Cry j 2 gradually decreased until 8 or 12 weeks according to the duration of intake and then slightly increased during pollen season. In contrast, the proliferation response of the placebo group gradually increased and plateaued at 12 or 16 weeks and then dropped off, which is very similar to the annual pattern of T-cell proliferations against Cry j 1 and Cry j 2 reported previously in JC pollinosis patients [3]. These results indicate that the T-cell proliferation responses to Cry j 1 and Cry j 2 can be significantly suppressed by oral administration of transgenic rice containing 7Crp even during the pollen season.

To investigate whether such suppression of T-cell responses was specific to JC pollen allergens, T-cell proliferation responses between transgenic and placebo groups were compared following stimulation with purified protein derivatives of tuberculin. We found no difference in T-cell proliferation between the 2 groups (Fig. 3b), indicating that the oral intake of transgenic rice containing 7Crp caused the specific changes to T-cell proliferation in response to 7Crp, Cry j 1, and Cry j 2. Interestingly, oral intake of transgenic rice containing 7Crp also led to a partial reduction in T-cell proliferation in response to Japanese cypress pollen allergen Cha o 1 (see online suppl. Fig. 1; see www.karger.com/doi/10.1159/000509996 for all online suppl. material). This may be attributed to the presence of common or highly homologous T-cell epitopes between Cry j 1 and Cha o 1 [23].

Effect of Transgenic Rice Intake on Cytokine Secretion by PBMCs

In Phase II – step 2 (80 g rice intake), the levels of IL-4, IL-5, IL-13, IL-10, and IFN-γ secreted by PBMCs were measured following stimulation with 7Crp, Cry j 1, and Cry j 2 (Fig. 4). In the control (placebo) group, IL-13 secretion by Th2 cells gradually increased during the pollen season (12, 16, and 20 weeks). However, in the transgenic rice group, no such increase in IL-13 was observed following stimulation with 7Crp and Cry j 2, which was shown that the oral intake of transgenic rice for 12, 16 and 20 weeks and for 20 weeks significantly suppressed the production of IL-13 by stimulation with 7Crp and Cry j 2, respectively (Fig. 4). On the other hand, IL-10 secretion from regulatory T cells, such as FOXP3$^+$ Treg, Tr1, or Th3

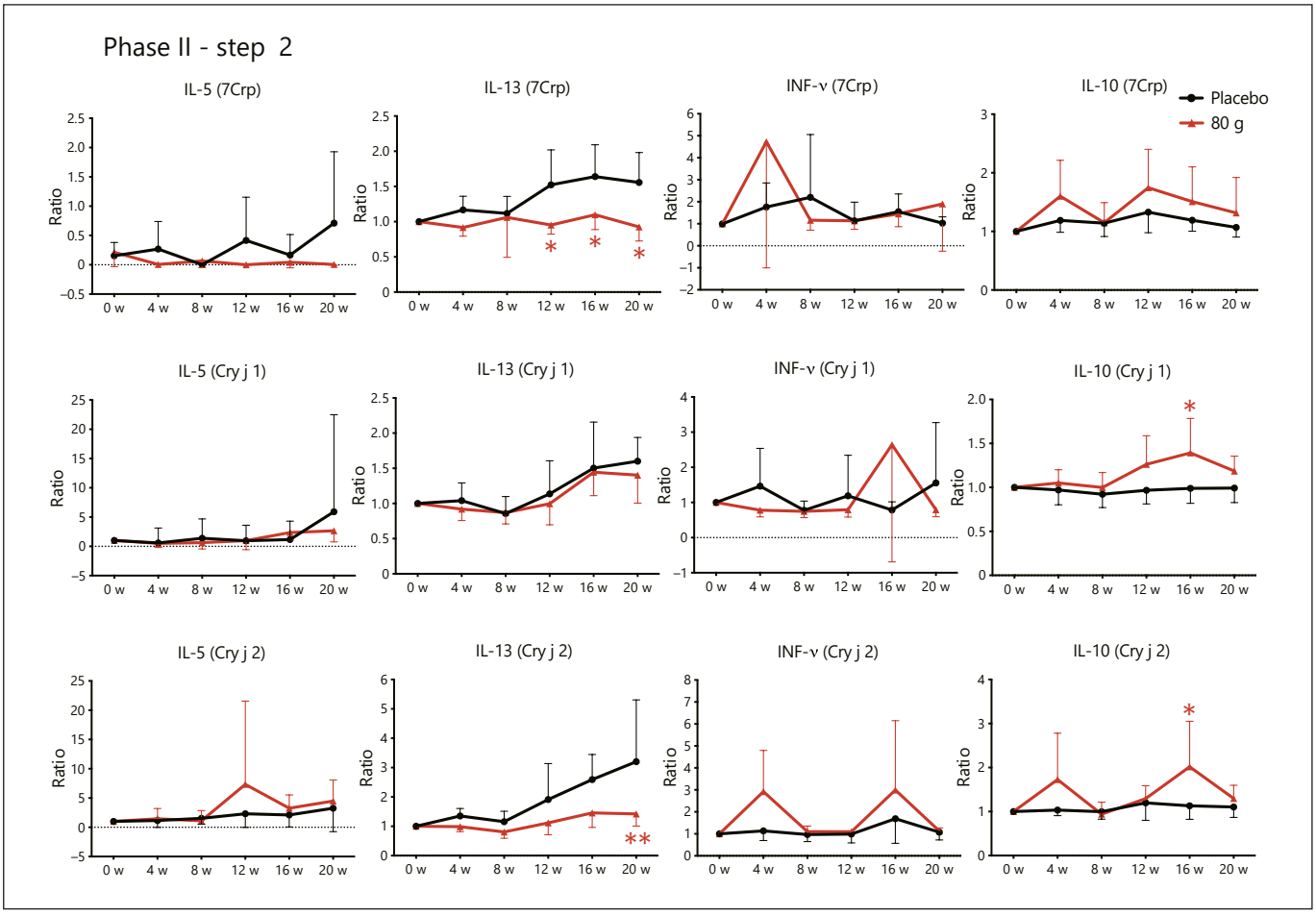

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Fig. 4. IL-5, IL-13, IL-10, and IFN-γ cytokine levels in peripheral blood mononuclear cell (PBMC) supernatants from patients with Japanese cedar pollinosis who ingested 80 g of transgenic or non-transgenic rice for 20 weeks. PBMCs were stimulated with 7Crp, Cry j 1, and Cry j 2. Results are expressed as mean ± 95% CI. *$p < 0.05$, **$p < 0.01$.

cells, was significantly increased 16 weeks after intake of transgenic rice following stimulation with Cry j 1 and Cry j 2. Increased IL-10 was also observed for 7Crp, though no significant differences were found. IFN-γ and IL-5 levels secreted from Th1 and Th2 cells were not significantly affected. IL-4 levels produced by Th2 cells were below the detectable level (data not shown).

Induction of Specific Antibodies to Cry j 1 and Cry j 2 with Oral Intake of Transgenic Rice

The levels of specific IgE and IgG4 antibodies against Cry j 1 and Cry j 2 were examined in the sera of JC pollinosis patients. When the levels were compared between the transgenic rice (80 g) and placebo groups, we found no differences in their allergen-specific IgE and IgG4 production with administration for 20 weeks (online suppl. Fig. 2).

Effects of Transgenic Rice Intake on Symptoms Related to JC Pollinosis

To evaluate the clinical effects of transgenic rice intake, symptom scores during the peak of pollen seasons were compared between the transgenic rice intake and placebo groups. Oral administration of 80 g of transgenic rice in Phase II – step 2 tended to alleviate some clinical symptoms of rhinitis, such as sneezing, runny nose, blocked nose, and itchy eyes during pollen season compared to the placebo control group, but no significant differences were detected. Furthermore, we found no difference in the medication score between the 2 groups (Fig. 5a).

When the QOL of patients at the peak of pollen season was assessed by the JRQLQ score, the symptom itchy nose was significantly improved and other symptoms, such as

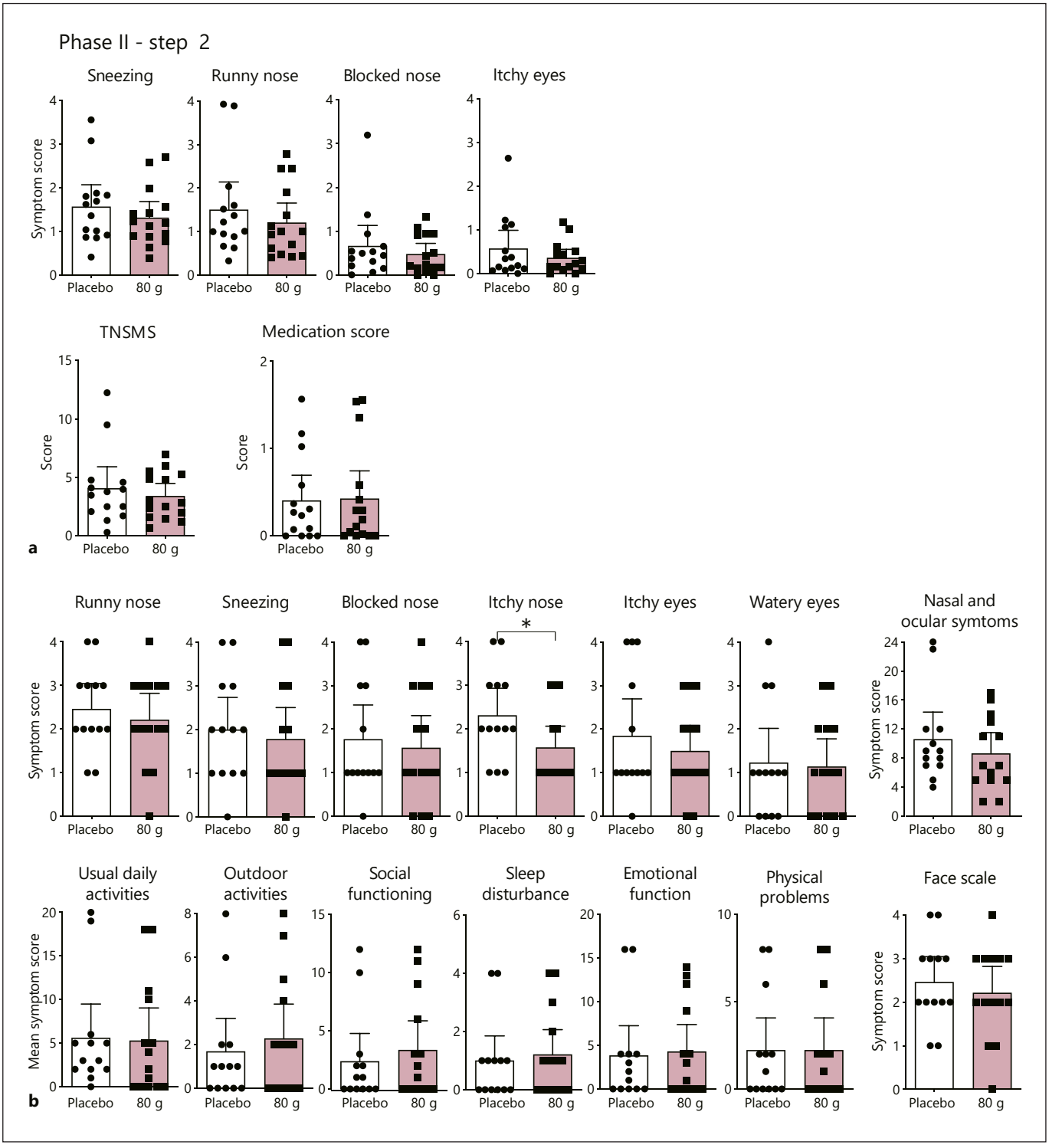

Fig. 5. Measurements of clinical symptoms after oral administration of 80 g of transgenic or non-transgenic rice for 20 weeks. **a** Symptom scores, TNSMS, and medication scores. **b** JRQLQ scores. Results are expressed as means ± 95% CI. *$p < 0.05$.

runny nose, sneezing, blocked nose, and itchy and watery eyes, were slightly relieved. However, the QOL scores and overall face scale were not improved (Fig. 5b).

Safety

In the 3 steps of Phase I (safety research), we found that no adverse events were caused by transgenic rice containing 7Crp. Furthermore, side effects associated with transgenic rice were not observed even in the case of oral administration to JC pollinosis patients, although non-severe adverse events not related to transgenic rice were reported for Phase II – step 2 in several subjects (infection with influenza, $n = 2$; diarrhea, $n = 1$; anemia, $n = 1$; abnormal liver function in hematological examination, $n = 1$). These results indicate that transgenic rice containing 7Crp serves as an oral tolerogen without severe side effects.

Discussion

In this study, the safety and efficacy of a rice-based oral peptide vaccine against JC pollinosis were evaluated by oral administration of transgenic rice to healthy subjects and patients. Allergen-specific T-cell proliferation against JC pollen allergens, Cry j 1 and Cry j 2, was significantly downregulated by oral intake of transgenic rice containing 7Crp. The suppressive effect of transgenic rice on allergen-specific T-cell proliferation was dependent on the intake dose as shown in Figure 2a. It is important to note that Cry j 1- and Cry j 2-specific T-cell proliferation was decreased within only 2 weeks after intake of transgenic rice (Fig. 2b) and their suppressive levels (immune tolerance state) were retained upon exposure to dispersed JC pollens during pollen season (Fig. 3a). Furthermore, suppression of allergen-specific T-cell proliferation was observed by oral intake of even a much lower dose (5 g) of transgenic rice (Fig. 2a).

When potential efficacy of rice-based peptide vaccine for JC pollinosis was assessed by oral administration of 80 g transgenic rice for 20 weeks in JC pollinosis patients, the daily TNSMS were slightly improved, but not significantly (Fig. 5a; online suppl. Fig. 3). The JRQLQ at peak-pollen season also indicated that the symptom itchy nose was significantly alleviated, and other nasal symptoms were slightly mitigated (Fig. 5b). These results indicate that oral administration of 80 g transgenic rice leads to a slight improvement in nasal symptoms, but the medication score and face score were not alleviated. In conclusion, transgenic rice seeds containing 7Crp did not show clinical improvement by oral administration for 1 season (20 weeks), even though T-cell proliferative activities to Cry j 1 and Cry j 2 were highly suppressed by this treatment.

Transgenic rice provided to patients in a formulation of prepacked cooked rice containing remarkably high amount of tolerogen (128 mg of 7Crp peptide/80 g rice). It is known as the oral tolerance mechanism that clonal deletion and anergy can be elicited by oral administration of large doses of allergen (10–500 mg), leading to the absence of allergen-specific T-cell proliferation [24]. In addition, 7Crp peptide deposited in PBs in transgenic rice seed is expected to be delivered to tolerogenic GALT rich in immune cells without degradation by digestive enzymes and harsh conditions in gastrointestinal tract after oral intake. Thus, the administration of high dose of tolerogen (80 g transgenic rice) and its efficient delivery to GALT are expected to give rise to significant reduction of Cry j 1- and Cry j 2-specific T cells as a result of oral tolerance. However, clinical symptoms of JC pollinosis were not improved by oral intake of this transgenic rice for 20 weeks. One possible explanation is that 7Crp peptide does not cover the full repertoire of T-cell epitopes localized in Cry j 1 and Cry j 2 molecules due to the diversity of MHC (HLA) class II alleles in patients. Although 92% of JC pollinosis patients have been reported to be covered by 7Crp [15], Cry j 1- and Cry j 2-specific T cells that cannot be suppressed by 7Crp peptide may proliferate in some patients during pollen season. Another possibility is that it takes longer duration of the intake (more than 2 seasons) to improve clinical symptoms.

The mechanisms of T-cell epitope-based peptide therapies are different from those of conventional or current SCIT or SLIT using native allergen or recombinant hypoallergenic derivative covering the whole sequence as tolerogen, which are mainly based on the induction of allergen-specific blocking IgG antibodies [25, 26]. This is attributed to T-cell epitopes <20 amino acids in length, being too short to induce allergen-specific IgG4 or IgE because of the inability of the peptides to cross-link B cell surface antigen receptors. The main mechanism of peptide immunotherapy leading to the allergen-specific immune suppression (T-cell tolerance) has been suggested to be based on (1) the induction of anergy of allergen-specific naive $CD4^+$ T cells and Th2 cells, (2) the deletion of allergen-specific Th2 cells, and/or (3) the induction of Treg cells with IL-10 production, resulting in inhibition of Th1, Th2, and inflammatory cell functions [25]. Furthermore, immune mechanisms of inactivation such as bystander suppression [27] or linked epitope suppression [28] may be involved in T-cell epitope-mediated tolerance to the allergen.

As shown in Figure 4, significant reduction of Th2 type IL-13 and increased IL-10 were observed with the administration of 80 g transgenic rice for 1 season However, this treatment neither decreased cedar pollen-specific IgE antibodies nor increased IgG-blocking antibodies. One possible reason is that oral administration of transgenic rice containing 7Crp induced immune tolerance to Cry j 1- and Cry j 2-specific T cells in mucosal immune tissues of GALT but was not sufficient to completely depress the allergy symptoms. Another reason is that JC pollen-specific IgE antibodies remaining over the season may provoke IgE cross-linking on mast cells upon cedar pollen exposure, leading to nasal symptoms. Therefore, inclusion of additional T-cell epitopes to address MHC diversity [29] and/or a more prolonged pre-seasonal treatment and longer-term treatment may be required for improvement of clinical symptoms.

Peptide immunotherapy is considered safe because few adverse effects occur due to a lack of IgE binding and minimal inflammatory potential. When different doses of transgenic rice containing 7Crp peptide were orally administered to human subjects with or without JC pollinosis, adverse effects (immediate and late-phase allergic reactions) associated with 7Crp peptide were not observed. These observations are a marked contrast to previous reports, in which T-cell-mediated late-phase side effects were reported for birch pollen and cat peptide vaccines after subcutaneous injection of T-cell epitope peptide [30, 31]. Therefore, such T-cell-mediated late-phase side effects may be avoided by rice-based oral administration.

This study was designed as a preliminary to evaluate the safety and efficacy of rice-based peptide vaccine for JC pollinosis in human patients. Because this study was the first clinical trial to administer the transgenic rice to human subjects, we gave first priority to the safety. The sample size of Phase II – step 2 might be small, but the subjects were carefully chosen according to the strict criteria and their intake compliance was good. Therefore, irrespective of minimum number of subjects, statistically significant data were obtained in allergen-specific T-cell proliferation without adverse events. In the near future, a detailed analysis with a larger sample size and multi-year intervention periods will be designed to evaluate the clinical efficacy. Follow-up studies will also be necessary to investigate how long immunological tolerance is sustained after treatment cessation. Last, the efficacy of transgenic rice must be investigated under constant conditions using an environmental exposure chamber, as symptoms change depending on the exposed pollen count and the scattered JC pollen count changes each year [2].

Acknowledgements

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Statement of Ethics

The protocols were approved by the Ethics Committee of Jikei University School of Medicine, and this study was carried out in accordance with the guidelines of the Declaration of Helsinki and the ethical guidance regarding clinical study of the Ministry of Health, Labor and Welfare in Japan. Written informed consent was obtained from each patient prior to participation in these studies.

Conflicts of Interest Statement

All authors have no conflicts of interest to declare.

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Author Contributions

T.E., D.A., and Sa.S. were involved in the conception and design of the study. H.T., Y.W., K.O., M. T., and F.T. provided the transgenic rice and technical support. T.E., D.A., Sy. S., H.K., R.M., S.T., N.S., S.O., H.K., and Sa.S. were involved in the acquisition of data. T.E., F.T., D.A., T.N., and Sa.S. analyzed and interpreted the data. T.E., F.T., M.T., T.N., and Sa.S. drafted the article. All the other authors critically revised the article for important intellectual content. All authors read and approved the final manuscript.

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