A phase I/IIa study of adjuvant immunotherapy with tumour antigen-pulsed dendritic cells in patients with hepatocellular carcinoma

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Background: To date, no adjuvant treatment has been shown to have a clear benefit in patients with hepatocellular carcinoma (HCC). In this prospective phase I/IIa study, we evaluated the safety and efficacy of adjuvant dendritic cell (DC) therapy in HCC patients who received primary treatment for HCC.

Methods: Twelve HCC patients who had no viable tumour after primary treatments were included. Dendritic cell vaccines pulsed with cytoplasmic transduction peptide-attached alpha-fetoprotein, glypican-3 and melanoma-associated antigen 1 recombinant fusion proteins were injected subcutaneously near to inguinal lymph nodes. Adverse effects, time to progression (TTP), and associated immune responses were evaluated after DC vaccination.

Results: Nine of 12 patients had no tumour recurrence up to 24 weeks after DC vaccination. Among a total of 144 adverse events, 129 events (89.6%) were regarded as adverse drug reactions, all of which were grade 1 or 2. The majority of patients showed enhanced anti-tumour immune responses after DC vaccination. Recurrence-free patients exhibited relatively stronger anti-tumour immune responses than patients who developed recurrence after DC vaccination, as evidenced by lymphocyte proliferation and IFN-γ ELISPOT assays. The median time of TTP was 36.6 months in the DC-vaccination group and 11.8 months in the control group (hazard ratio, 0.41; 95% confidence interval, 0.18–0.95; P=0.0031 by log-rank test).

Conclusions: Adjuvant DC vaccine for HCC was safe and well tolerated in phase I/IIa study, and preliminary efficacy data are encouraging to warrant further clinical study in patients with HCC after primary treatments.

Globally, hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third leading cause of cancer-related death. Surveillance programs for early detection of HCC in high-risk populations and improvement of therapeutic modalities have increased the likelihood of potentially curative treatment (Yuen et al, 2000; Bolondi et al, 2001). However, the long-term prognosis is still poor even after curative treatment due to the high frequency of recurrence in the remnant liver, which ranges up to 25% per
year (Lai et al, 1995). This high recurrence rate has led to efforts to develop adjuvant therapies to reduce recurrence. A number of studies have explored adjuvant strategies; however, the benefit of any form of adjuvant therapy remains unclear (Schwarte et al, 2002; Samuel et al, 2009), and current scientific guidelines do not recommend adjuvant therapy in patients treated with resection or local ablative therapy (Bruix et al, 2011; European Association for The Study of The Liver, European Organisation for Research Treatment of Cancer, 2012; Verslype et al, 2012).

Dendritic cells (DCs) are professional antigen-presenting cells that have a critical role in the cell-mediated immune response by stimulating proliferation and activation of antigen-specific cytotoxic T cells (Banchereau and Steinman, 1998). Autologous DCs offer a practical basis for a tumour vaccine (den Brok et al, 2005), and adoptive immunotherapy using DC vaccine has been tested in clinical trials in various malignancies including prostate cancer, melanoma, renal cell carcinoma, and HCC (Nestle et al, 1998; Höltl et al, 1999; Small et al, 2000; Lee et al, 2005; Butterfield et al, 2006; Palmer et al, 2009; Tada et al, 2012). Regarding HCC, a previous phase II study using intravenous vaccination with DCs ex vivo pulsed with HCC cell line (HepG2) lysate showed evidence of anti-tumour efficacy in some patients with advanced HCC (Palmer et al, 2009). A phase I/II study reported that strong T-cell responses against alpha-fetoprotein (AFP) were generated by immunisation with DCs pulsed with four AFP peptides as the immunogenic tumour-associated antigen (TAA) instead of tumour cell lysates in HCC patients. However, no clinical responses were observed in the treated patients (Butterfield et al, 2006).

In the present study, we used DC vaccines pulsed with multiple TAAs (i.e., AFP, glypican-3 (GPC-3) and melanoma-associated antigen 1 (MAGE-1)) to enhance the DC vaccine efficacy. Because no single antigen is ubiquitously expressed in HCC, we selected AFP, GPC-3, and MAGE-1 as target antigens for DC vaccine, which were most frequently detected in the tissue array of tumour tissues obtained from 412 Korean HCC patients at Seoul National University Hospital (H-0811-009-261). All methods and procedures associated with this study were conducted in accordance with the principles of the Declaration of Helsinki and local law. The study was designed by the sponsor (JW CreaGene, Inc., Seongnam-si, Gyeonggi-do, Korea) in conjunction with the principal academic investigators. Clinical Research Information is available on the website of the Korean Centers for Disease Control and Prevention, Ministry of Health and Welfare (http://apps.who.int/trialsearch/Trial2.aspx?TrialID=KCT0000427).

## Preparation of recombinant HCC antigens.

As described previously (Tada et al, 2012), cDNAs encoding AFP, MAGE-1, and GPC-3 were cloned into the pCTP plasmid. These three antigens were expressed in E. coli BL21 and purified with a column of nickel–nitrilotriacetic acid (Ni2+-NTA) chromatography (Qiagen, Hilden, Germany). Quality control of the antigens was performed to assure >95% purity by SDS–PAGE and <1.0 EU μg−1 endotoxin by Limulus amoebocyte lysate test.

### Autologous DC vaccine generation.

Peripheral blood mononuclear cells (PBMCs) were obtained from the patients with HCC through leukapheresis at Seoul National University Hospital. Dendritic cells were generated from blood monocytes, as described previously (Kim et al, 2007), with minor modifications. DCs were prepared in a Good Manufacturing Practice-compliant facility at JW CreaGene, Inc. Peripheral blood mononuclear cells isolated by Ficol–Paque PLUS (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation were resuspended in RPMI1640 medium (Lonza, Basel, Switzerland) supplemented with autologous heat-inactivated plasma, and then incubated in CellSTACK Culture Chambers (Corning, Corning, NY, USA). After 0.5–1 h incubation at 37°C in a 5% CO2 incubator, non-adherent cells were removed by gentle washes. The adherent monocytes were cultured in X-VIVO15 (Lonza, East Rutherford, NJ, USA) supplemented with 100 ng ml−1 of granulocyte macrophage-colony-stimulating factor (JWCreaGene Inc.) and 300 ng ml−1 of interleukin (IL)-4 (JWCreaGene Inc.) for 5 days. On day 5, nonattached immature DCs were collected and pulsed with CTP-fused human AFP, MAGE-1, and GPC-3 recombinant proteins at a final concentration of 5 μg ml−1 each. Antigen-pulsed DCs were matured in the presence of cytokine cocktail, IL-6 (Peprotech, Rocky Hill, NJ, USA), IL-1β (Peprotech), tumour necrosis factor (TNF)-α (Peprotech), prostaglandin E2 (PGE2; Sigma-Chemical Co., St Louis, MO, USA), interferon (IFN)-γ (LG Life Science; Seoul, Korea), OK432 (Chugai Pharmaceutical Co., Tokyo, Japan), and poly I:C (Sigma) for 2 days. On day 7, the DCs were collected, washed, and resuspended in 2.0 ml of cryopreserving solution containing 5% dimethyl sulfoxide (Bioniche Pharma USA LLC, Rockford, IL, USA). Finally, fully equipped DCs were packed into a sterile glass vial (5 x 107 cells per vial), sealed with a snap-cap, and then stored at an ultralow freezer until administration.

### Immunogenicity of DC vaccine in preclinical study.

T-cell proliferation and cytotoxic T lymphocyte (CTL) induced in vitro by co-culture experiments with TAA-pulsed mDCs prepared from the PBMCs of healthy donors were assessed as described in Supplementary Methods.

### Quality control of DC vaccine.

Sterility, cell size and granularity, surface phenotype, cell viability, T-cell stimulation capacity, and cytokine production profiles of DC vaccine were evaluated according to the JW CreaGene Standard and Test Guidelines approved by the Korea Ministry of Food and Drug Safety. The detailed procedures are described in Supplementary Methods.

### Treatment protocol.

Patients who provided informed consent were screened within 4 weeks before the start of immunotherapy.

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**PATIENTS AND METHODS**

**Patient selection.** Patients who had undergone treatment including surgical resection, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), or transarterial chemoembolization (TACE) as a treatment for HCC of clinical stage between I and IIIc according to the American Joint Committee on Cancer (AJCC) staging system (sixth edition) were eligible for this study (Greene, 2002). The diagnosis of HCC was made by histological examination or radiological imaging tests, mainly based on the guidelines of the American Association for the Study of Liver Diseases (Bruix et al, 2011). Detailed criteria for patient recruitment are described in Supplementary Methods.

**Study oversight.** This phase I/IIa clinical study was a prospective, open-labelled trial. The study was conducted at Seoul National University Hospital. All patients provided written informed consent before enrolment in this study. The study protocol and procedures were approved by the institutional review board at Seoul National University Hospital (H-0811-009-261). All methods and procedures associated with this study were conducted in accordance with the principles of the Declaration of Helsinki and local law. The study was designed by the sponsor (JW CreaGene, Inc., Seongnam-si, Gyeonggi-do, Korea) in conjunction with the principal academic investigators. Clinical Research Information is available on the website of the Korean Centers for Disease Control and Prevention, Ministry of Health and Welfare (http://apps.who.int/trialsearch/Trial2.aspx?TrialID=KCT0000427).
Peripheral blood mononuclear cells were collected with leukapheresis 2 weeks before the first planned vaccination. TAA-pulsed DC vaccine was injected subcutaneously (s.c.) into the thigh near the inguinal lymph nodes. Toll-like receptor-7 (TLR-7) agonist (imiquimod: Mochida Pharmaceutical Co.; Tokyo, Japan) was applied topically around the injection site for 2 consecutive days before injection. Patients received six DC vaccines over 14 weeks (four treatments every 2 weeks and then two treatments every 4 weeks). Response was evaluated 4 weeks after the fourth vaccination (10 weeks after the first vaccination) and 4 weeks after the sixth vaccination (18 weeks after the first vaccination) (Figure 1A). Detailed flow of the study evaluating the tolerated dose (TD) is provided in Figure 1B. Dose-limiting toxicity (DLT) was defined as severe adverse drug reaction of grade 3 or 4 according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. TAA-pulsed DC vaccine was designed to be administered at 5 × 10^7 cells per injection for the first three patients. If none of these three patients experienced DLT, then the next nine patients were planned to be treated at that dose, which was designated as TD. If one of the first three patients experienced DLT, three more patients were enroled at that dose level. If no more than one of six patients experienced DLT, the dose was defined as TD and the next nine patients were treated at the dose. If two of six patients experienced DLT, additional three patients were enroled and if none of the three patients experienced DLT, the dose was designated as TD. However, if ≥2 patients of the first three patients, ≥3 patients of the first six, or ≥3 patients of the first nine patients experienced DLT, then the dose was reduced by 50% and TD finding trial was repeated. If TD was defined, toxicity evaluation was repeated until sample size (12–18 patients) was reached.

Outcomes and assessment. The primary outcomes were safety of DC vaccine and the induction of TAA-specific cellular immune response in patients with HCC. Adverse events classified and graded according to the CTCAE version 3.0 and World Health Organization-Adversity Reaction Terminology (WHO-ART) version 092 were assessed from the time the patient provided written informed consent until the end of the study or dropout/withdrawal. TAA-specific cell-mediated immunity induced by DC vaccine was evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay and lymphocyte proliferation assay with blood samples of weeks 0, 4, 10, 18, and 24. The secondary outcomes including tumour recurrence and other status were evaluated as described in Supplementary Methods. In addition, 4-year follow-up study was performed, and time-to-progression (TTP) and recurrence-free survival (RFS) of the vaccinated patients were assessed in comparison with those of the historical controls during the same period.

Immune monitoring after vaccination. For immune monitoring after DC vaccination, antigen-specific IFN-γ ELISPOT assay, lymphocyte proliferation assay in vitro and immunosuppressive cytokine analysis were performed with the patients’ blood according to the Minimal Information about T-cell Assays (MIATA) guidelines (Britten et al, 2012) with some modifications as described in Supplementary Methods.

Statistics. The efficacy and outcomes were assessed according to the intention-to-treat principle. Missing values were imputed by last observation carried forward method. Changes in laboratory test results from baseline were compared by Wilcoxon signed-rank test. Repeated measures ANOVA was used to evaluate the changes in the results of IFN-γ ELISPOT and lymphocyte proliferation assays. TTP and RFS were compared using a log-rank test. Statistical significance was set at P<0.05. The statistical analysis was performed using PASW Statistics version 19.0 (IBM, Chicago, IL, USA).

RESULTS

Patient characteristics. Between 16 March 2009 and 5 January 2010, 17 patients were screened. Among them, 12 met the eligibility criteria and were enroled in this study. Table 1 illustrates the baseline demographic and clinical characteristics. Ten patients (83.3%) were male and the median age was 57 years (range, 45–71). All patients showed pre-immunisation positivity for delayed-type hypersensitivity response. Chronic hepatitis B was the predominant cause of underlying liver disease (83.3%). Four patients underwent surgical resection, four RFA, two PEI, and two TACE, for HCC within 8 weeks before study enrolment. Six patients had a history of previous treatment for HCC. According to the AJCC staging system, eight patients had stage I, two had stage II, and two had stage III, HCC. When enroled, all 12 patients did not show any residual tumour on CT or MRI according to RECIST criteria a month after primary treatments. The first three patients were enroled between 16 March 2009 and 30 March 2009, and the next nine patients between 23 November 2009 and 5 January 2010. The date of the study end was 24 November 2010. Additional follow-up study for recurrence was assessed until the end of 2014.

Characteristics of DC vaccine. In the preclinical study, we evaluated the immunogenicity TAA-pulsed mature DCs (mDCs) in vitro which were prepared from the PBMCs of three healthy donors. TAA-pulsed mDCs showed typical mDC phenotypes (Figure 2A). T-cell proliferation (Figure 2B) and CTL assay (Figure 2C) revealed that the TAA-pulsed mDCs were active enough to stimulate antigen-specific CD8+ T cells and CTLs. Given this background, DC vaccine was generated from the 12 patients with HCC. The average yield of DC vaccine was about 3.9 ± 2.0% of the initial amounts of PBMCs. Dendritic cell vaccines showed the typical features of mDC morphology under the microscope. All manufactured DC vaccines passed safety tests including sterility test, mycoplasma test, and endotoxin test (Table 2). The cell viability ranged from 75.4 to 89.8%. According to flow cytometric analysis of the cell size and granularity, the population of DCs was >80% of the cells, with a median value of 92.9% (range, 84.4–97.8). Flow cytometry results confirmed that the DCs expressed high levels of major histocompatibility complex (MHC) class I (HLA-ABC), MHC class II (HLA-DR), and co-stimulatory molecules (CD86, CD80, CD40, and CD83). The analysis of lineage markers demonstrated that the contamination of B cells (CD19) and monocytes (CD14) was mostly <2%, except in one case (CD14, 8.4%). All of these quality controls are summarised in Table 2.

Dose and administration of DC vaccines. For the first three patients, 5 × 10^7 antigen-pulsed DCs were administered per injection, and there was no DLT. Thus, 5 × 10^7 cells per injection was defined as the TD. At the same dose, there was no DLT in the next nine patients. Although two patients (no. 07 and 09) experienced grade 3 haematologic adverse events (i.e., persistent leukopenia, neutropenia, and lymphopenia from the screening test in patient no. 07 and thrombocytopenia probably related to HCC progression in patient no. 09), the cases were assessed as no adverse drug reactions and thus no DLT. All patients received six injections of DC vaccine. DC vaccinations were administered as scheduled except for two patients (no. 07 and 09). Dose administration for the sixth dose was delayed by 19 days for no. 07, and by 41 days for no.09 due to treatment for recurrence of HCC.
Safety evaluation. Table 3 summarises all adverse events. During the treatment period, a total of 144 adverse events occurred among the 12 patients, and there were no grade 3 or 4 adverse events except for the aforementioned grade 3 haematologic adverse events in two patients. Approximately 90% of adverse events (129 of 144) were assessed as adverse drug reactions. The most common adverse drug reactions were injection site pain (12 patients), fever (8 patients), myalgia (7 patients), headache (5 patients), and fatigue (4 patients) (Table 3). However, most adverse drug reactions were self-limited and resolved within 1 or 2 days. Four patients experienced a total of serious adverse events including HCC progression (five events in three patients (no. 07, 09, and 12)) and...
menorrhagia (one event in one patient (no. 11)), none of which were assessed as drug-related adverse reactions and thus did not stop the DC vaccine treatment.

DC vaccination augmented TAA-reactive T-cell response. Eleven of 12 patients (91.6%; all patients except for no. 11) showed an increase in TAA-reactive lymphocyte population after DC

Table 2. Quality control of 12 DC vaccines

| Patient no. | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 | 09 | 10 | 11 | 12 |
|-------------|----|----|----|----|----|----|----|----|----|----|----|----|
| Sterility   | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass |
| Mycoplasma  | I (PCR) | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass |
|             | II (Direct culture) | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass |
|             | Endotoxin (<10 EU ml⁻¹) | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass |
| Viability (%) | 83.1 | 85.7 | 75.4 | 77.2 | 86.8 | 80.8 | 88.2 | 88.2 | 86.1 | 76.5 | 89.8 | 78.6 |

Phenotype identification

| Size and granularity (%) | CD14 | CD19 |
|--------------------------|------|------|
| 85.1 | 90.9 | 93.6 |
| 93.8 | 96.4 | 93.7 |
| 97.8 | 96.6 | 84.4 |
| 89.1 | 91.5 | 92.2 |

Viability (%)<sup>a</sup>

| CD14 | CD19 |
|------|------|
| 1.5  | 1.6  |
| 2.0  | 0.6  |
| 8.4  | 0.9  |
| 0.3  | 0.1  |
| 0.5  | 0.3  |
| 0.6  | 1.3  |
| 1.0  | 1.9  |
| 1.4  | 0.4  |
| 1.1  | 0.5  |
| 1.2  | 0.8  |
| 1.3  | 0.2  |

*The viability of the DC vaccine was assessed by flow cytometry after propidium iodide (PI) staining, and is represented as 100 – (PI<sup>+</sup> of sample) / (PI<sup>+</sup> of control) (%).

**The % represents marker-positive cell populations based on the isotype control in flow cytometry.
Each TAA showed comparable capacity to induce lymphocyte proliferation, and the TAA-reactive T-cell populations significantly increased in proportion to the number of repeated vaccinations except at week 24 (Figure 3A, upper). Particularly, it is worth noting that nine recurrence-free patients showed greater TAA-specific T-cell proliferation when compared with the three patients with recurrence after TAA-pulsed DC vaccinations (Figure 3A, lower). To examine the proportion of TAA-responsive effecter T cells in the proliferated T-cell populations, we performed IFN-\(\gamma\) ELISPOT assay, and the results were summarised in Supplementary Table S1. As shown in Figure 3B, number of IFN-\(\gamma\) ELISPOT increased with repeated DC vaccinations (Figure 3B, upper). Among the TAAs, AFP showed the highest reactivity, while GPC-3 antigen was moderate in its capacity to induce effecter T-cell responses. The ELISPOT assay after DC vaccinations also showed that the average number of effecter T cells was larger in nine recurrent-free patients than in three recurrent patients (Figure 3B, lower). The changes in serum levels of IL-10, TGF-\(\beta\), and VEGF are provided in Table 4. There were not any clear tendencies for changes in serum cytokine levels for any patient.
β, and VEGF between baseline and week 24 were not significantly different (all \( P > 0.05 \) by Wilcoxon signed-rank test). We could not see any discernible correlation between positivity for lymphocyte proliferation tests and changes in immunosuppressive cytokine levels (all \( P > 0.05 \) by Wilcoxon signed-rank test).

**Clinical efficacy evaluation.** Clinical outcomes are also summarised in Table 4. Tumour assessments were performed using dynamic CT or MRI according to RECIST criteria. Nine of 12 patients were free of recurrence up to 24 weeks after DC vaccination. Representative CT and MRI results of two recurrence-free patients are shown in Figure 4. Three patients experienced tumour recurrence; cumulative recurrence rates at weeks 18 and 24 were 16.7% (2 of 12) and 25% (3 of 12), respectively. Tumour recurrences occurred at lymph nodes (one patient) or liver (two patients). All three patients with tumour recurrence had prior history of HCC treatment including TACE and PEI. Two patients died during the study period, and the mortalities were related to progression of the occurred tumour. The median levels of serum AFP were comparable between baseline and week 24 (6.6 (range, 2.6–5470) vs 5.6 (range, 1.8–36 600) ng ml\(^{-1}\); \( P = 0.91 \) by Wilcoxon signed-rank test). The median level of PIVKA-II increased during the study period (18.5 (range, 9–2040) vs 21.5 (range, 13–35080) nAU ml\(^{-1}\); \( P = 0.02 \) by Wilcoxon signed-rank test). There was no significant change in patient performance status according to the Kornofsky scale during the treatment period (data not shown).

In the 4-year follow-up study, an additional 4 (total 7, 58.3%) of 12 patients showed recurrence and 2 more patients (total 4, 33.3%) died of recurrent cancer, while 27 (87.1%) of 31 control patients who were matched by treatment modality from the historical control group showed recurrence during the same period. The patient composition and baseline characteristics of the control patients were summarised in Table 5. The median time of TTP was 21.5 (range, 13–1080) (starting from the primary treatment/from the first DC vaccination to the event). LN denotes lymph node.

### Table 4. The results of immunological response and clinical outcomes

| Patient no. | Lymphocyte proliferation\(^{a}\) | IL-10 change (%)\(^{b}\) | TGF-β change (%)\(^{b}\) | VEGF change (%)\(^{b}\) | AFP change (%)\(^{b}\) | PIVKA-II change (%)\(^{b}\) | SP | FU | SP | FU |
|-------------|----------------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|-----|-----|-----|-----|
| 01 Positive | 14.7                              | 4.1                    | 4.0                    | –23.0                  | 7.7                    | No                     | No  | No  | No  |
| 02 Positive | –16.8                             | –16.6                  | –6.8                   | –21.1                  | 31.6                   | No                     | No  | No  | No  |
| 03 Positive | 26.2                              | –15.8                  | –3.2                   | –47.5                  | 52.6                   | No                     | Yes (294/217)          | No  | Yes (878/801) |
| 04 Positive | –20.9                             | 35.2                   | 6.9                    | 8.6                    | 0.0                    | No                     | Yes (1075/963)         | No  | No  |
| 05 Positive | 98.1                              | 3.5                    | –2.1                   | 25.9                   | 12.5                   | No                     | Yes (917/805)          | No  | No  |
| 06 Positive | –13.3                             | 59.2                   | 36.8                   | –21.5                  | 0.0                    | No                     | No  | No  | No  |
| 07 Positive | –22.4                             | –32.3                  | 42.3                   | 63.6                   | 37.0                   | Yes (134/61)           | —   | No  | Yes (340/267) |
| 08\(^{d}\) Positive | –23.7                             | 49.5                   | –17.6                  | –10.3                  | –28.6                  | No                     | No  | No  | No  |
| 09\(^{d}\) Positive | 70.8                              | –66.8                  | –5.6                   | 569.1                  | 1619.6                 | Yes (205/91)           | —   | No  | Yes (312/198) |
| 10 Positive | 8.3                               | 8.6                    | –36.8                  | –30.8                  | 13.3                   | No                     | Yes (1226/1037)        | No  | No  |
| 11 Negative | –12.4                             | –7.5                   | 226.0                  | –16.7                  | 44.4                   | No                     | No  | No  | No  |
| 12 Positive | 26.0                              | 20.7                   | –24.0                  | 25.0                   | 122.2                  | Yes (259/139)          | —   | No  | Yes (783/663) |

\(^{a}\)Positive means that the lymphocyte proliferation (assessed by \(^{3}\)H-thymidine incorporation) showed over a 2-fold increase at both 18 and 24 weeks after DC vaccination as compared with the mean value in prevaccination.

\(^{b}\)Median = (the value at post-vaccination – the value at pre-vaccination)/the value at pre-vaccination × 100.

\(^{c}\)Recurrence and death were monitored during the study period (SP) (24 weeks from the first DC vaccination) and 4-year follow-up (FU) study. Numbers in the parenthesis represent the TTP (starting from the primary treatment/from the first DC vaccination to the event).

\(^{d}\)In these two patients, since data at week 24 were missing, data at week 18 were used according to the last observation carried forward method. LN denotes lymph node.

**DISCUSSION**

HCC development and progression are related to chronic inflammation (Grivennikov et al, 2010). Once tumours are established, mutual interactions between tumours and immune cells present during inflammation may provide conditions favourable for tumour cell survival (Ungefroren et al, 2011). Immune suppressor cells (e.g., regulatory T cells, myeloid-derived suppressive cells, or tumour-associated macrophages) facilitate tumour immune evasion (Zamarron and Chen, 2011). Natural tumour-infiltrating lymphocytes are incompletely activated, proliferate little, and fail to eradicate tumours (Korangy et al, 2010). DCs have an essential role in anti-tumour immunogenicity, particularly in the proliferation of tumour-specific CTL. However, the number and function of DCs in tumour patients are suppressed or dysfunctional. Thus, \( \text{ex vivo} \)-generated DC vaccine therapy is expected to produce an appropriate anti-tumour immune response.

In the present study, a number of strategies were applied to enhance the efficacy of DC vaccine. First, autologous DC vaccines were prepared by pulsing monocyte-derived DCs with three common HCC TAAAs (AFP, MAGE-1, and GPC-3) in order to account for the heterogeneity of HCC based on the tissue array results from 412 HCC patients in Korea (unpublished data). Second, CTP-fused TAAs were used to facilitate CTP-mediated antigen delivery into the cytoplasm of DCs (Kim et al, 2006), leading to better induction of TAA-specific CTLs. Third, topical TLR-7 agonist (imiquimod), which is known to facilitate DC migration to the regional lymph node (Prins et al, 2006), was
applied around the injection site of DC vaccines to induce a synergistic effect with DC vaccination. Fourth, instead of epitope peptides, recombinant proteins of TAAs were used to overcome the HLA restriction. Fifth, patients who had no evidence of residual HCC after primary treatment were included in this study in order to minimise tumour-mediated immune suppression (Beyer and Schultze, 2006; Diaz-Montero et al., 2009). Sixth, DC vaccines were injected s.c. into the thigh near the inguinal lymph nodes rather than intravenously (i.v.) to increase the number of DCs reaching regional lymph nodes (Lappin et al., 1999; Okada et al., 2001).

Dendritic cell vaccines are injected either intradermally (i.d.), s.c., i.v., or sometimes intratumourally depending on the tumour types and/or the study protocols designed on the basis of the preclinical study. The cases using i.d. injection were about twice as many as those using s.c. route among the DC vaccine trials registered on the NIH clinical trials site (https://clinicaltrials.gov). In our preclinical study, however, we found that there was no significant difference in efficacy between the i.d. and s.c. injections. Thus, in the present clinical study, we delivered DC vaccines s.c.

In this phase I/IIa trial, we investigated the safety and efficacy of DC vaccines using autologous TAA-pulsed DCs in 12 patients with HCC after surgical resection or loco-regional therapy. Adverse drug reactions including injection site pain, fever, and myalgia occurred in all patients. However, there was no serious adverse drug reaction limiting DC vaccination, and all 12 patients tolerated the scheduled six injections well. Nine of 12 patients remained tumour free up to 24 weeks after DC vaccination, while three patients experienced tumour recurrence. The majority of patients showed enhanced TAA-specific cellular immune response after DC vaccination, presumably leading to effective inhibition of tumour recurrence after primary treatment of HCC. Recurrence-free patients exhibited relatively stronger anti-tumour immune responses than the patients who developed recurrent tumour after DC vaccination, as evidenced by lymphocyte proliferation and IFN-γ ELISPOT assays (Figure 3). The degree of immunologic response appears to have clinical significance as it seems to correlate with the clinical efficacy results (i.e., tumour recurrence in Table 4). The 4-year follow-up study clearly showed that RFS rates

Figure 3. Immunological analysis after DC vaccination. (A) Antigen-specific lymphocyte proliferation assay was performed during and after DC vaccinations using the autologous PBMCs obtained from 12 patients (upper), and the results were further analysed in recurrence-free patients (lower left) and recurrent patients (lower right). The proliferation was determined by ³H-thymidine incorporation (dpm) using a liquid scintillation counter. Data are presented as mean ± s.e., *P<0.05. (B) ELISPOT assay was performed with the PBMCs obtained from each patient at the indicated time points after the start of DC vaccination. The results from all (12) patients (upper), the nine recurrence-free patients (lower left) and the three recurrent patients (lower right) are presented as mean ± s.e.
Figure 4. CT and MRI scan data of two representative patients (pt4 and pt6) before and after one cycle of antigen-pulsed DC vaccination. Treatment records of each patient are summarised.

Table 5. Patient compositions and baseline characteristics of historical control group

| Baseline characteristics | DC vaccinated (n = 12) | Histological control (n = 31)* |
|--------------------------|------------------------|-----------------------------|
| Age, years (median, range) | 57                    | 45–71                       |
| Gender (N, %), male, female | M10 (83.3%)           | F2 (12.7%)                  |
| HCC history, N (%), new/recurrent | New 6 (50%)           | Recurrent 6 (50%)           |
| Primary treatment         |                        |                             |
| Surgery (N, %)             | 4                      | 11                          |
| RFA (N, %)                 | 4                      | 10                          |
| TACE (N, %)                | 2                      | 4                            |
| PEIT (N, %)                | 2                      | 100.0%                      |
| Total (N, %)               | 12                     | 31                          |
| Recurrence* (N, %)         | 7/12                   | 58.3%                       |
| Baseline characteristics   | DC vaccinated (n = 12) | Historical control (n = 31) |
| DM patient, N (%)          | 10 (83.3)              | 27 (87.1)                   |
| AST, IU/l                  | 43.5 ± 10.4            | 47.4 ± 40.3                 |
| ALT, IU/l                  | 46.2 ± 21.0            | 44.4 ± 32.0                 |
| Albumin, g dl⁻¹            | 3.88 ± 0.39            | 4.03 ± 0.43                 |
| Total bilirubin, mg dl⁻¹   | 0.93 ± 0.43            | 0.97 ± 0.59                 |
| PT INR                     | 1.15 ± 0.07            | 1.19 ± 0.29                 |
| Platelet, x 10⁹/μl⁻¹       | 133.3 ± 35.8           | 132.2 ± 43.3                |
| Creatinine, mg dl⁻¹        | 0.92 ± 0.16            | 0.96 ± 0.24                 |
| AFP, ng ml⁻¹               | 7.3 (4.6, 11.4)        | 7.4 (3.8, 24.0)             |
| Tumour size, cm            | 1.8 (1.0, 2.2)         | 2.2 (1.3, 3.5)              |
| Tumour number              | 1.67 ± 0.78            | 1.52 ± 1.26                 |
| Tumour stage               |                        |                             |
| Stage I                    | 8 (66.7)               | 24 (77.4)                   |
| Stage II                   | 2 (16.7)               | 5 (16.1)                    |
| Stage IIIA                 | 2 (16.7)               | 2 (6.5)                     |

Abbreviations: AFP = alpha-fetoprotein; ALT = alanine aminotransferase; AST = aspartate aminotransferase; DM = diabetes mellitus; F = female; M = male; PEI = percutaneous ethanol injection; PT INR = prothrombin time international normalised ratio; RFA = radiofrequency ablation; TACE = transarterial chemoembolisation.

*Patients who received more than one treatment of TACE, RFA, PEIT, or Surgery for primary or recurrent HCC during the period (August 2005–October 2013) at Seoul National University Hospital (SNUH). They should be in the window of the enrolment criteria for the clinical study of DC vaccine. Treatment modality ratio (TACE/RFA/PEIT/Surgery) should be matched to that of DC vaccine group. The ratio between the number of newly diagnosed patients and recurrent patients in the historical control group should be comparable with that of DC vaccine group.

*Patients newly diagnosed or with recurrent tumour when they received primary treatment at SNUH.

*Monitored for 5.5 years starting from the 1st DC vaccination for DC vaccine group, and for the same period from the 1st primary treatment for the historical control group.

P value indicates that the two groups were comparable.
II and III studies may be warranted to evaluate the clinical efficacy of DC vaccines in HCC patients.

ACKNOWLEDGEMENTS

This study was supported by a Bio New Drug grant (A085033) of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea.

CONFLICT OF INTEREST

YL, MKHeo, and J-SS are CreaGene Inc. employees. Y-SB has been a consultant for JW CreaGene Inc. All remaining authors have declared no conflicts of interest.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)