Vaccine therapy with dendritic cells transfected with Il13ra2 mRNA for glioma in mice

Laboratory investigation

MAKOTO SAKA, M.D.,1 TAKAYUKI AMANO, M.D.,1 KOJI KAJIWARA, M.D.,1 KOICHI YOSHIKAWA, M.D.,1 MAKOTO IDEGUCHI, M.D.,1 SADAHIRO NOMURA, M.D.,1 HIROSUKE FUJISAWA, M.D.,1 SHOICHI KATO, M.D.,1 MASAMI FUJII, M.D.,1 KOJI UENO, PH.D.,2 YUJI HINODA, M.D.,2 AND MICHIAKU SUZUKI, M.D.1

Departments of 1Neurosurgery and 2Oncology and Laboratory Medicine, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi, Japan

Object. The Il13ra2 gene is often overexpressed in brain tumors, making Il13ra2 one of the vaccine targets for immunotherapy of glioma. In this study, using a mouse glioma model, the authors tested the hypothesis that vaccination using dendritic cells transfected with Il13ra2 mRNA induces strong immunological antitumor effects.

Methods. A plasmid was constructed for transduction of the mRNAs transcribed in vitro into dendritic cells. This was done to transport the intracellular protein efficiently into major histocompatibility complex class II compartments by adding a late endosomal/lysosomal sorting signal to the Il13ra2 gene. The dendritic cells transfected with this Il13ra2 mRNA were injected intraperitoneally into the mouse glioma model at 3 and 10 days after tumor cell implantation. The antitumor effects were estimated based on the survival rate, results of histological analysis, and immunohistochemical findings for immune cells.

Results. The group treated by vaccination therapy with dendritic cells transfected with Il13ra2 mRNA survived significantly longer than did the control groups. Immunohistochemical analysis revealed that greater numbers of T lymphocytes containing CD4+ and CD8+ T cells were found in the group vaccinated with dendritic cells transfected with Il13ra2 mRNA.

Conclusions. These results demonstrate the therapeutic potential of vaccination with dendritic cells transfected with Il13ra2 mRNA for the treatment of malignant glioma. (DOI: 10.3171/2009.9.JNS09708)

KEY WORDS • Il13ra2 • mRNA • malignant glioma • mouse • vaccination therapy • dendritic cell

MALIGNANT gliomas are unusual, but are considered to be one of the most aggressive tumors among brain cancers. A malignant glioma of astrocytic origin can differentiate into a WHO Grade III anaplastic astrocytoma or a Grade IV GBM. Over many years, no significant prolongation of survival has been achieved. The prognosis for patients with malignant glioma remains poor, in spite of gross resection by microsurgery with adjuvant radiotherapy and chemotherapy. The median survival of patients with anaplastic astrocytoma is about 2–3 years, and that of patients with GBM is only 9–12 months;10 patients with GBM usually die within 2 years. The combination therapy of temozolomide with radiotherapy improved the survival time of patients with GBM by 2.5 months, but a complete cure has not been achieved.29,49,50 Therefore, the development of new therapeutic strategies for malignant glioma is essential. Proposed investigations and ongoing clinical trials of immunotherapy have shown the effectiveness and possibility of clinical application of one of these new therapies, a dendritic cell–based vaccine, and many authors have reported the successful induction of tumor immunity by antitumor immune therapy in which dendritic cell–based vaccine therapies were used.4,12,39,51 A positive aspect of immunotherapy is that it selectively destroys tumor cells, and no apparent side effects are usu-
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ally observed. Therefore, immunotherapy for malignant glioma could be an attractive supplemental and alternative therapeutic option.

The CNS is an immunologically privileged site hidden behind the blood-brain barrier. Nevertheless, immune effector cells induced peripherally can be recruited into the CNS. Malignant glioma cells are considered to be poor antigen-presenting cells to the immune system, because the B7 costimulatory molecules required for direct activation of T cells with tumor cells are downregulated and because the tumor itself secretes immunosuppressive cytokines and factors such as vascular endothelial growth factor. To induce an antitumor response against malignant glioma, professional antigen-presenting cells are needed to internalize and present malignant glioma antigens efficiently to immune cells. In reported immunotherapies, dendritic cells have been used as antigen-presenting cells for the induction of efficient immune reaction. Various forms of tumor antigens have been applied to improve the induction of specific antitumor immune responses; for example, apoptotic tumor cells, tumor cell lysates, proteins, peptides, and nucleic acids. In our series, we used mRNA as a tumor antigen. The mRNA can be synthesized in vitro, transfected into dendritic cells, and subjected to molecular engineering. Furthermore, the mRNA is superior to cDNA in its transfection efficiency and safety, and is also superior to synthetic peptides, because there is no need to determine whether the peptides have been presented to the immune system.

Interleukin-13 is an immunoregulatory cytokine secreted predominantly by activated Th2 cells and is one of the mediators in the pathogenesis of allergic inflammation. The IL-13 receptor consists of IL4Ra, IL13Ra, and IL13Ra2. Over the past years, several studies revealed that IL13ra2 is highly and specifically overexpressed in high-grade gliomas, including GBM, whereas it is not expressed or is expressed at a very low level in the normal brain or low-grade gliomas. In recent years, study results have proved that IL13ra2 is an effective antitumor vaccine target when used with antigen-presenting cells. The cytotoxic T cells have a major role in immune reaction to tumor cells, and the activation of CD4+ helper T cells is essential for the induction of cytotoxic T cells in dendritic cell therapy. To induce a strong immune response, it is necessary for antigen peptides to be presented not only on MHC class I but also on MHC class II molecules. Dendritic cells capture exogenous antigens, process them in the endosomal pathway, and present on MHC class II molecules, leading to the induction of the CD4+ helper T-cell response. On the other hand, nuclear proteins are processed by degradation in proteasomes and then transported into the endoplasmic reticulum and then combined to form MHC class I molecules, which induce the CD8+ helper T-cell response. Thus, effective transport of the IL13ra2 to both the MHC class I and class II molecules (cross-presentation) is very important to induce a strong immune response against malignant gliomas.

The purpose of the present study was to demonstrate the antitumor effects of vaccine therapy by using dendritic cells transfected with IL13ra2 mRNA. An SS and late endosomal/lysosomal sorting signal were attached to the IL13ra2 mRNA for efficient presentation of the antigen, not only on MHC class I molecules, but also on MHC class II molecules of dendritic cells, for cross-presentation.

Methods

Experimental Animals

Two hundred forty adult male C57BL/6 mice (6–8 weeks old) were purchased from Japan SLC Co., Ltd. Mice were maintained in a specific pathogen-free environment and provided with sterilized food and water. Mice weighing between 25 and 35 g were used in this study. This experiment was reviewed by the Committee on the Ethics of Animal Experiments at the Yamaguchi University School of Medicine and conducted under the Guidelines for Animal Experiments at the Yamaguchi University School of Medicine and the Law (no. 105) and Notification (no. 6) of the Japanese government.

Cell Lines

The KR158B cell is a subclone of the murine glioma cell line KR158, involving the mutation of 2 tumor suppressor genes, Nf1 and Trp53. The KR158B glioma cells were cultured and maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (complete media).

Polymerase Chain Reaction

The total RNA of the KR158B glioma cells was generated by RT-PCR. The cDNA was obtained from total RNA by using TR1zol (Invitrogen) according to the manufacturer’s protocol.

Plasmid Cassette for RNA Transcription

Plasmid constructs were described previously. Briefly, for in vitro transcription, the plasmids were cloned with pSP64 vector (Promega). A TRP-2 SS fragment and TM-Cyto were amplified from TRP-2 cDNA by PCR (Ex Taq polymerase, Takara). The PCR products were cloned as a Hind III-Pst I SS fragment and BamH I-Sma I TM-Cyto fragment into pSP64 to allow in vitro transcription under the control of an SP6 promoter to transport the IL13ra2 protein efficiently to MHC class II compartments for eventual cross-presentation by both classes I and II on dendritic cells in a cognate manner.

In Vitro Transcription of mRNA

The full-length mouse IL13ra2 cDNA was generated by RT-PCR using the following forward and reverse primers: mouse IL13ra2 forward primer, 5'-AAAAGTCGACATGGCCTTTTTGCAATATCG-3' and IL13ra2 reverse primer, 5'-AACCTTAGACAGAGGTATCTTCCATCCA-3'. The products were cloned as a Sal I-IL13ra2-Xba I fragment. The fragment was inserted into a pSP64 cassette (Fig. 1). In vitro transcription was performed in a...
60-µl reaction mix at 37°C for 2 hours by using an mMESSAGE mMACHINE T7 ultra kit (Ambion).

Dendritic Cell Generation From Mouse Bone Marrow

The C57BL/6 mice (144 animals) were painlessly killed, and femurs and tibias were removed. The bone marrow was flushed with PBS by using a syringe with a 26-gauge needle, and then it was filtrated through a 70-µm cell strainer. The bone marrow cells were adjusted to 2 × 10⁵ cells/ml in complete media and plated on 100-mm dishes. They were cultured for up to 7–10 days in the presence of 1000 U/ml of granulocyte-macrophage colony-stimulating factor and 500 U/ml of IL-4 at 37°C, in 5% CO₂. On Day 4, the same amount of cytokines was added to the dishes. Seven to 10 days later, we obtained several immune cells, including dendritic cells. To isolate the dendritic cell population, we collected the cells in the dishes and suspended them in 5 ml of complete media. The same volume of 14.5% (weight/volume) metrizamide in complete media was underlain and centrifuged at 1200 G for 20 minutes at room temperature. After centrifugation, the cells in the interface (the dendritic cell population) were collected and washed with PBS. These dendritic cells were used for subsequent RNA transfection. Before and after RNA transfection, the expression of the surface molecules on the dendritic cells was analyzed with the aid of flow cytometry (Epics XL, Beckman Coulter Co.).

Transfection of mRNA to Dendritic Cells

The dendritic cells were resuspended and adjusted to 10⁶ cells/5 ml in RPMI 1640. The RNA transfection was accomplished by mixing 10⁶ dendritic cells and 10 µg of mRNA, using a TransMessenger Transfection Reagent (Qiagen), and incubated for 3 hours at 37°C. After transfection, the cells were washed with PBS and used for dendritic cell therapy.

Chloroquine Inhibition Assay

To assess the intracellular mechanisms of antigen processing to MHC class II compartments for eventual cross-presentation by both classes I and II on the dendritic cells in a cognate manner, we linked the leader sequence and the cytoplasmic domain containing a late endosomal/lysosomal sorting signal derived from the mouse TRP-2 gene to the II13ra2 gene at its N₁ and COOH terminus, respectively. We investigated whether proteins translated from such mRNA constructs with a late endosomal/lysosomal sorting signal were actually localized and degraded in the human leukocyte antigen class II processing compartments. For this purpose, EGFP and TRP2-EGFP mRNAs were used. The TRP2-EGFP is a fusion mRNA in which EGFP was linked to the N₂ terminus region of TRP-2 at nucleotide position 169, as previously described. After transfection of EGFP or TRP2-EGFP mRNA into dendritic cells, 50 µM of the lysosomal degradation inhibitor chloroquine was added to the dendritic cells immediately after mRNA transfection. The expression of EGFP was analyzed by flow cytometry. Dendritic cells were harvested at different time points after transfection and treated with DAKO Intrastain (DAKO). The EGFP was detected by anti-EGFP monoclonal antibody by using anti–mouse IgG-FITC, as described previously.

Implantation of Tumor Cells Into the Mouse Brain

The mice were anesthetized with an intraperitoneal injection of pentobarbital sodium. The head skin was incised almost 20 mm, and a 1.5-mm bur hole was made on cranial bone 2 mm to the right of the bregma. The injection device consisted of a stereotactic frame composed of a 27-gauge needle that was connected via a catheter to a 10-µl Hamilton syringe. A total of 2 × 10⁵ KR158B cells suspended in 2 µl PBS was injected into the brain over
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10 minutes. The needle was held in the same place for 10 minutes and then retracted slowly over 5 minutes. Wound closure was performed with bone wax and sutures.

The tumor-implanted mice were divided into 2 groups. One group (80 animals) was allowed to live without a time limit to evaluate the survival time. The other group (16 animals) was scheduled to be killed on Day 20 after implantation for cell toxicity assays and histological and immunohistochemical examination.

Statistical Analyses

Survival data were calculated using the Kaplan-Meier method. Statistical significance of survival data between the groups was compared using a log-rank analysis. Differences at p < 0.05 were considered statistically significant.

Vaccination With Dendritic Cells

On Days 3 and 10 after tumor cell implantation, mice received immunization. Mice implanted with tumor cells were classified into 4 groups, as follows: the first group was injected intraperitoneally with 3 × 10^5 II13ra2 mRNA-transfected dendritic cells; the second group was injected intraperitoneally with 3 × 10^5 chloroquine-treated dendritic cells after the II13ra2 mRNA transfection; the third group was injected intraperitoneally with 3 × 10^5 PBS-pulsed dendritic cells without II13ra2 mRNA; and the last group received no treatments after tumor cell implantation.

Immunocytochemical Examination for II13ra2 and Immune Cells

On Day 20 after tumor cell implantation, some mice from all groups were killed for histological and immunohistochemical examination. Their brains were fixed in cold ethanol, treated with hydrogen peroxide, and blocked with 1.5% rabbit serum before being incubated overnight at room temperature with the rat monoclonal antibodies TIB122 (anti-CD45), KT3 (anti-CD3), YTA3.1 (anti-CD4), YTS169.4 (anti-CD8), FA/11 (macrosialin), and TIB222 (anti-CD25). After being washed by PBS, the sections were incubated with horseradish peroxidase–conjugated rabbit antibody (anti-CD8), FA/11 (macrosialin), and TIB222 (anti-CD25). After being washed by PBS, the sections were incubated with biotinylated horseradish peroxidase H reagent, which were especially prepared to form ideal complexes for immunoperoxidase staining. Horseradish peroxidase was detected with dianaminobenzidine. The sections were coun-
with II13ra2 mRNA survived significantly longer (mean survival 38.5 days, 20 animals) than did the mice immunized with nontransfected dendritic cells (mean survival 26.8 days, 20 animals; p = 0.0024, log-rank analysis of survival curves) and the animals without immunization (mean survival 23.5 days, 20 mice; p = 0.0003, log-rank analysis of survival curves) (Fig. 3). In addition, the mice immunized with dendritic cells transfected with II13ra2 mRNA survived significantly longer than did the animals immunized with chloroquine-treated dendritic cells transfected with II13ra2 mRNA (mean survival 31.1 days, 20 mice; p = 0.0435, log-rank analysis of survival curves). The mice immunized with chloroquine-treated dendritic cells transfected with II13ra2 mRNA survived significantly longer than did the animals without immunization (p = 0.0047). However, there was no significant difference in survival between the mice immunized with chloroquine-treated dendritic cells transfected with II13ra2 mRNA and those with nontransfected dendritic cells (p = 0.0749), or between the mice with nontransfected dendritic cells and those without immunization (p = 0.1385).

All mice from the groups that were not immunized or vaccinated with nontransfected dendritic cells died by Day 40, and all mice from the group vaccinated with chloroquine-treated dendritic cells transfected with II13ra2 mRNA died by Day 49. However, 50% of the mice vaccinated with dendritic cells transfected with II13ra2 mRNA were still alive on Day 35. Even at Day 30, 50% of the mice in the group vaccinated with dendritic cells transfected with II13ra2 mRNA were healthy and neurologically normal.

**Immunocytochemical Analysis**

Using H & E staining, we compared the intracranial...
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tumor volumes among all 4 groups. We found that the tumor volumes in the mice vaccinated with dendritic cells transfected with Il13ra2 mRNA were smaller than those in the other groups, especially the group of nontreated mice (Fig. 4A and B).

Brain-infiltrating inflammatory cells in the intracranial implanted tumor revealed by immunocytochemical staining are shown in Fig. 5. The upper panels (Fig. 5A–D) are photomicrographs from the group vaccinated with dendritic cells transfected with Il13ra2 mRNA, and the lower panels (Fig. 5E–G) are photomicrographs from the group with no treatment. In the group vaccinated with dendritic cells transfected with Il13ra2 mRNA, CD45+ leukocytes were detected within and around the tumors (Fig. 5A). A greater number of CD3+ lymphocytes had infiltrated in and around the tumor (Fig. 5B), and these lymphocytes contained not only CD8+ (Fig. 5C) but also CD4+ (Fig. 5D) T cells. In contrast, also in the brains implanted with KR158B glioma cells from the group that received no treatment, CD45+ leukocytes were detected within and around the tumors (Fig. 5E). However, the infiltrating cells were mainly macrophages (Fig. 5F), and few CD3+ lymphocytes were present in the tumor or surrounding brain tissue (Fig. 5G). In the group vaccinated with chloroquine-treated dendritic cells, only a few T lymphocytes were detected.

The degree of T-cell infiltration in the tumor is summarized in Table 1. In the group treated with dendritic cells transfected with Il13ra2 mRNA, the degree of CD4+ and CD8+ T cells was +++, or at least ++. By contrast, in the untreated group, no CD4+ and CD8+ cells were detected.

Discussion

It has been reported that Il13ra2 protein could be an attractive vaccine target and that effective antitumor immune responses could be induced in preclinical models by using dendritic cell–based vaccines.11,24 Furthermore, it has been well documented that Il13ra2 antigen in HLA-A0201 CD8+ T cells could induce specific T-cell responses.24 Therefore, the Il13ra2 could be an HLA-A0201–restricted cytotoxic T-cell antigen in immunotherapy of patients with malignant glioma. In this study, we used the full-length mouse Il13ra2 cDNA for the preparation of mRNA for Il13ra2. We had previously reported that mRNA induced cytotoxic T-cell activity in vitro by using the same vector,15 and mRNA for Il13ra2 used in dendritic cell–based vaccines is thought to be immunogenic.

In recent studies it has been reported that the Il13ra2 chain is specifically overexpressed by glioblastomas, whereas normal brain cells do not express this protein, or express it at a very low level.7,8,10,11,21,25,26,33 The Il13ra2 chain was highly expressed in high-grade gliomas, but no or low-level expression was found in normal or low-grade glioma tissues.6,6 In our mouse glioma model implanted with KR158B glioma cells, we confirmed Il13ra2 expression by immunohistochemical investigation, whereas Il13ra2 was not detected in the normal mouse brain. Moreover, we also examined the normal mouse brain tissues that had been immunized with Il13ra2 mRNA–transfected dendritic cells by using histological studies, and could not detect any toxicity. From these results, Il13ra2 seemed to be a suitable target as an antitumor antigen for vaccine therapy of glioma. However, it was reported that Il13ra2 is, in fact, expressed in only a subset of gliomas; probably a minority.23 The previously reported data indicate that prescreening of subsets may be of benefit in future trials of Il13ra2-targeting immune therapies. In addition, the implication of cell-to-cell heterogeneity of the expression of this antigen is an important problem. Due to the antigenic heterogeneity among glioblastoma tumor cells, several peptides should be used to target a large number of tumor cells. With regard to the expression of Il13ra2 in other organs, it has been reported in the kidney, spleen, liver, lung, thymus, respiratory epithelium, and monocytes in humans.19,42 Therefore, significant toxicity might ensue in human models with specific targeting of this antigen. Although we did not find any toxicity based on our observations of the clinical features of the mouse models used in this study, there might be adverse effects that would

![Fig. 4. Photomicrographs of histological coronal sections of brain obtained at 20 days after implantation of tumor cells and stained with H & E. The tumor volume of the sections vaccinated with dendritic cells transfected with Il13ra2 mRNA was smaller than that of the other groups. (A, brain tumor vaccinated with dendritic cells transfected with Il13ra2 mRNA; B, no treatment.) Original magnification x 25.](image-url)
be revealed by detailed microscopic observation of these organs. We should pay serious attention to the possibility of unexpected adverse effects caused by IL13ra2-targeting immune therapies in human clinical trials.

To induce a strong immune response, it is necessary for antigen peptides to be presented not only on MHC class I molecules, but also on MHC class II molecules. To present antigen peptides on MHC class II molecules, CD4+ T cells that release interferon-γ. In this study, we designed modified mRNA containing SS domain– and TM-Cyto domain–sorting signals of TRP-2 to the N2 and COOH terminus of IL13ra2, and transfected it to dendritic cells. This is for effective transportation of the IL13ra2 to both the MHC class I and class II molecules, making it possible for the antigen to be cross-presented. We previously demonstrated that this unique strategy was useful for the efficient induction of specific immune responses.

With regard to the timing of a vaccine therapy in this study, we followed the model of our studies published previously. We did not test the preventive effect of a vaccination, because it would not be practical when considering clinical application. According to a recent review by Nencioni et al., most previous studies have used weekly, biweekly, or monthly injections with at least 2 vaccine administrations. It seems likely that several injections will

### Table 1: Degree of T-cell infiltration in brain tumor

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<th>Mouse No.*</th>
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* Mice 1–4 were treated with dendritic cells transfected with IL13ra2 mRNA; mice 5–8 received no treatments. – = no T cells evident; + = rare cells (1–20 cells); ++ = scattered cells (21–40 cells); +++ = larger number of cells (> 40 cells).
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be required to expand antigen-specific lymphocytes in vivo. It would have been interesting if we could have continued our weekly vaccination for a longer period, but the in vivo growth of KR158B was too fast for us to try it.

Mice in the group vaccinated with dendritic cells transfected with II13ra2 mRNA survived longer compared with mice in the untreated groups (Fig. 3). This might show that the II13ra2 peptides translated from mRNA would be effectively immunogenic and could be effective antitumor antigens. Moreover, in vaccination therapy for tumors, the immune response induced by dendritic cells transfected with mRNA has the potential to reduce tumor cell proliferation. Survival time was longer in the group treated with dendritic cells transfected with II13ra2 mRNA than in the group of chloroquine-treated dendritic cells with the mRNA, as well as other control groups. Chloroquine is an inhibitor for lysosomal degradation and raises the pH in the endosomal and lysosomal compartments, inhibiting protein hydrolysis by cathepsins. 6,30,38,46 Thus, it inhibits lysosomal degradation of antigen peptides and then suppresses their MHC class II presentation, giving rise to the decreased antitumor effects of dendritic cells. Our present results suggest that the efficient cross-presentation of antigen peptides plays an important role in the induction of a significant antitumor effect.

The tumor volume of the mice vaccinated with dendritic cells transfected with II13ra2 mRNA tended to be smaller than that of the mice in other groups. This result indicates that vaccine therapy using II13ra2 mRNA–transfected dendritic cells may suppress tumor growth. Immunohistochemical analysis revealed that greater numbers of CD3+ lymphocytes infiltrated the tumor tissues in the mice vaccinated with dendritic cells transfected with II13ra2 mRNA compared with the mice in other groups, and that these lymphocytes contained not only CD8+ T cells but also CD4+ T cells. In contrast, in the mice vaccinated with chloroquine-treated dendritic cells and in the nontreated group, we detected mainly macrophages, with a few or no T cells. From these results we deduced that our vaccine therapy could activate T cells, and then induce specific immune responses to the tumor and antitumor effects.

Even in the group treated with dendritic cells transfected with II13ra2, the survival time was still modest, and almost 90% of mice in this group finally died by 80 days after tumor implantation. Although II13ra2 could be an effective immune target, the immunogenicity might not be strong enough to cure the mice receiving intracranial transplantation of KR158B cells. One possible explanation is the lower expression level of MHC class I and class II molecules on the surface of KR158B cells. 44 Additional strategies will be necessary to improve the therapeutic efficacy of mRNA-loaded dendritic cells in this tumor model.

Conclusions

Our study showed the immunotherapeutic potential of vaccine therapy with dendritic cells transfected with II13ra2 mRNA in a mouse malignant glioma model. The results show that II13ra2 mRNA may be one of the targets of vaccine therapy for malignant gliomas. This treatment could be a supplemental therapeutic arm for brain malignant glioma protocols. Additionally, with our presented method, it is possible that we could use other mRNAs together with II13ra2 mRNA as target antigens, which might strengthen this therapeutic strategy.

Disclaimer

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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