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Dendritic cell based personalized immunotherapy based on cancer antigen research

Yutaka Kawakami, Tomonobu Fujita, Chie Kudo, Toshiharu Sakurai, Masaru Udagawa, Tomonori Yaguchi, Go Hasegawa, Emiko Hayashi, Yoko Ueda, Tomoko Iwata, Qinfu Wang, Starlyn Okada, Nobuo Tsukamoto, Yuriko Matsuzaki, Hidetoshi Sumimoto

Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University, School of Medicine, Tokyo, Japan

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1. ABSTRACT

Human tumor antigens were identified using various immunological and genetic methods, and immune responses to the identified antigens were evaluated in cancer patients. Autologous tumor specific unique antigens derived from genetic alterations in cancer cells were isolated from patients with favorable prognosis after immunotherapy, indicating that they are attractive targets for immunotherapy. Immunogenicity of shared antigens was found to differ among patients due to antigen expression in cancer cells and patients' immunoreactivity. These observations suggest that personalization may be applied for cancer immunotherapy. We therefore developed intratumoral DC administration protocols that are able to induce immune responses to both unique and shared tumor antigens expressed in each individual cancer. By combining cryoablative tumor pretreatment and TLR stimulated DC, the anti-tumor effect of the intratumoral DC administration was significantly augmented in a murine tumor model. This improved protocol enhanced systemic induction of anti-tumor $CD8^+$ CTL, and was able to regress relatively large remote untreated tumors. In clinical trials, systemic immune induction was observed by intraturmoral DC administration following cryoablative tumor treatment, although anti-tumor effects are relatively weak, indicating that additional interventions are required for more effective immunotherapy.

2. IDENTIFICATION OF HUMAN TUMOR ANTIGENS WITH VARIOUS IMMUNOLOGICAL AND GENETIC METHODS

Identification of human tumor antigens is essential for the understanding of anti-tumor immune responses to cancer cells and development of effective immunotherapy. With the identified tumor antigens, we are able to evaluate *in vivo* anti-tumor immune responses quantitatively and qualitatively to reveal various tumor escape mechanisms to be overcome, and to design more effective immunotherapy protocols (1, 2).

DNA expression cloning has recently been improved with various modifications and is one of the essential techniques for the isolation of tumor antigens recognized by $CD8^+$ T cells (Table 1). To isolate tumor antigens relevant to *in vivo* tumor rejection, we used cultured tumor infiltrating T cells (TIL) whose administration resulted in tumor regression for screening DNA libraries. We have isolated shared tissue specific antigens such as MART-1 and gp100, cancer-germ line antigens such as NY-ESO-1, and mutated antigens such as beta-catenin and Ski acyltransferase using stable or transient cDNA expression cloning (3,4,5). Identification of T cell epitopes revealed molecular basis of immunological recognition of human cancer cells by T cells, including

Table 1. Isolation methods for human tumor antigens

Criteria for isolation	Methods
Immunogenicity	cDNA expression cloning with tumor reactive T cells cDNA expression cloning with patient's serum (SEREX)
Specific gene expression	
• Genomic DNA	DNA sequencing (mutations, polymorphism) Comparative genomic hybridization (gene amplification)
• mRNA	cDNA subtraction (RDA, PCR differential display) cDNA profile comparison (DNA chip/microarray, SAGE, EST databases)
• Protein	Protein expression profile comparison (2D-EP, MS, protein chip, protein databases)
• HLA bound peptide	Isolation and identification of HLA bound peptides using HPLC and MS

SEREX (Serological Analysis of Autologous Tumor Antigens by Recombinant cDNA Expression Cloning), SAGE (Serial Analysis of Gene Expression), RDA (representational differential analysis), EP (electrophoresis), MS (mass spectrometry), HPLC (high performance liquid chromatography)

generation of epitopes from alternative open reading frames and introns, and through various posttranslational modifications such as protein splicing. These results have important implications for their clinical use, including their low immunogenicity and improvement by amino acid substitution, and differential processing between tumor cells and professional antigen presenting cells (APC)(1,6-,8).

cDNA expression cloning using serum IgG Ab from cancer patients, SEREX (serological analysis of recombinant cDNA expression libraries), was also used for isolation of tumor antigens in various cancers, since generation of tumor reactive T cells is relatively difficult for many human cancers (9). One of the problems of the SEREX method is difficulty to select useful tumor antigens among many isolated antigens, because some of which were not related with anti-tumor immune responses. Various modifications have been attempted for efficient isolation of useful tumor antigens. For examples, we isolated several cancer germ line antigens by screening the cDNA library made from testis, instead of cancer cells, and isolated novel tumor antigens using sera from patients who were frequently immunized with autologous tumor cell derived molecules. We have also developed SEREX using murine sera containing human IgG Ab produced by human B cells infiltrating or surrounding tumor tissues from immunodeficient mice implanted with human tumor tissues. Enrichment of human IgG Ab specific for tumor antigen p53 was confirmed in the sera from the SCID mice implanted with human colon cancer tissues, indicating efficient isolation of antigens associated with immune responses to tumor cells. With these modified SEREX methods, we have isolated various tumor antigens, including mutated CDX2, cancer-germ line antigen CAGE, tissue specific antigen KU-MEL-1, etc (10, 11, 12, 13).

Tumor antigen candidates can be identified using various systematic gene analyses along with the recently well established gene databases. Mutated antigens may be identified through mutation search by systematic DNA sequencing. Overexpressed antigens may be identified through gene amplification search using comparative genomic hubridization (CGH). A common mutation (V600E) of BRAF was identified by systematic sequencing, gene amplification of BRAF was detected by CGH, and its immunogenicity was confirmed by presence of serum IgG Ab specific for BRAF in melanoma patients (14, 15). BRAF is involved in malignant phenotypes of melanoma cells, including proliferation, invasion, and immunosuppression (16,17). Tissue specific antigens, cancer-germ line antigens, and over-expressed antigens can be isolated using various cDNA subtraction methods among various normal tissues and cancer cells, and comparison of cDNA profiles obtained by SAGE (serial analysis of gene expression), DNAChip / microarray analysis, or EST databases. With these technologies, we have identified many tumor antigen candidates in various human cancers (18-21).

Immunogenicity of the products of the candidate genes was evaluated by testing recognition by patients' T cells or IgG Ab (reverse immunology). The recombinant proteins made from candidate cDNAs can be screened with patients' serum IgG Ab. T cell recognition can be evaluated by *in vitro* induction of tumor reactive T cells from PBMC or TIL of patients by stimulation with the candidates such as synthetic peptides predicted based on the motifs for HLA allele specific binding and proteasome cleavage. We also use a newly developed HLA-A24 binding peptide prediction algorithm and HLA-A24 transgenic mice for the T cell epitope identification (21).

3. ANALYSIS OF IMMUNE RESPONSES TO THE IDENTIFIED TUMOR ANTIGENS

Autologous tumor specific peptides derived from genetic alterations in tumor cells, which are sometimes involved in tumorigenesis, are often isolated using T cells and IgG Ab from patients with good prognosis after treatment. For examples, we isolated mutated peptides of beta-catenin and Ski acyltransfrerase using melanoma reactive TIL, whose administration with high dose IL2 resulted in tumor regression (5, 22). These mutations may be involved in melanoma formation. T cell epitopes of these mutated antigens are often generated through their acquisition of HLA binding ability by anchor amino acid substitution caused by the mutations. Mutated antigens such as CDX2 and p53 were also isolated by SEREX. The tumor specific frameshift CDX2 peptide was identified as a colon cancer antigen from a hereditary non-polyposis colorectal cancer (HNPCC) patient with microsatellite instability (MSI) positive colon cancer (11). This type of colon cancer has unique clinicopathological features, including relatively good prognosis after treatment, infiltration of T cells, particularly $CD8^+$ T cells, in tumor tissues, suggesting that T cell responses to the frameshift peptides may contribute to maintenance of tumor free status. Thus, these mutated antigens, particularly involved

Table 2. Differential immunogenicity of the newly identified shared tumor antigens among patients

Antigen (positive/total)	1	2	3	4	5	6	7	8	9	10
Melanocyte Ags										
KU-MEL1 (8/25)	+	+	-	+	+	+	+	-	-	-
PAX3 (3/25)	-	-	+	-	-	+	-	+	-	-
AIM-1 (10/25)	-	-	-	-	+	+	+	+	+	-
Cancer-germ line Ags										
CAGE (2/25)	-	-	-	-	-	-	+	-	-	+
CRT2 (5/11)	nd	nd	-	+	nd	+	+	+	+	nd
Other Ags										
FABP7 (11/25)	+	-	+	+	-	+	+	-	-	-

Examples of melanoma patients, + presence of serum IgG Ab, - absence of Ab, nd; not done

in malignant phenotypes of cancer, are less likely to develop antigen loss variants, and are promising targets for immunotherapy (23). Personalized immunotherapy to induce immune responses to such antigens needs to be developed, since mutated antigens are often unique among patients, and are difficult to be identified for each patient.

In melanoma, tissue specific shared antigens such as MART-1 are frequently detected. Some of the T cell epitopes appear to be relatively cryptic epitopes those are not presented at high density on the cell surface of normal cells or professional APC in healthy individuals (3, 6). The recent observation that adoptive transfer of MART-1 specific T cells with lymphodepletive pretreatment resulted in tumor regression, indicated that these self antigens have ability to reject tumor cells (24). However, it may be necessary to modify the peptides to increase their immunogenisity to use in active immunization (cancer vaccine). We have succeeded in generation of highly immunogenic peptides by substituting an amino acid with appropriate one at the anchor site for peptide / HLA binding. With the modified peptides, immune induction and subsequent anti-tumor effects were augmented (7, 8).

We have evaluated immunogenicity of shared tumor antigens by screening with sera from many cancer patients, and found that even for shared antigens, immunogenicty of each antigen varies among the patients due to expression of antigens on tumor cells and patients' immunoreactivity, partly defined by their HLA type (Table 2). Presence of serum IgG Ab and expression of the antigen in tumor cells were positively correlated with ability to induce $CD8^+$ CTL *in vitro* against NY-ESO-I (25), and induction of IgG Ab against the immunized peptides for $CD8^+$ CTL was positively correlated with better prognosis in those patients (26). Thus, tumor antigens may be preselected by evaluation of their expression in tumor cells and immunogenicity measured using rapid serum IgG Ab screening or *in vitro* T cell induction. Immunization with the highly immunogenic, shared tumor antigens may trigger additional immune responses to unique antigens expressed on the same tumors (antigenic spreading) as observed in some clinical trials (27).

4. DEVELOPMENT OF DENDRITIC CELL BASED PERSONALIZED IMMUNOTHERAPY

The results of analyses of immune responses to the identified human tumor antigens indicated importance of development of personalized immunotherapy for maximal immune induction to both highly immunogenic unique and shared antigens. To induce immune responses to unique antigens, two strategies, including immunization with autologous tumor cell molecules and immune interventions into individual tumors, are currently attempted (Table3).

Immunization with tumor cells modified by various gene transfection or hapten conjugation, as well as immunization with various tumor derived molecules, including tumor lysates, extracted HLA bound peptides, mRNA, or heat shock proteins from autologous tumor cells, have been attempted. An immunization trial with dendritic cells pulsed with autologous tumor lysates for melanoma patients along with low dose IL2 administration was conducted, and resulted in 1 stable disease and 2 mixed responses among 10 patients (28). Interestingly, multiple metastases regressed in 2 patients with rapid central necrosis probably caused by collapse of tumor circulation. Immune responses to carbonic anhydrase-II which is highly upregulated on tumor vessel endothelial cells, were detected in these responded patients (29), suggesting possible contribution of immune responses against tumor vasculatures to tumor regression.

Intratumoral interventions include intratumoral administration of adjuvants, viruses, or immature DC. Based on the observation that dendritic cells were important for induction of anti-tumor T cells and tumor regression by intratumoral injection of modified HSV (30, 31), we developed a protocol for intratumoral DC administration (Figure 1). By combining cryoablative tumor pretreatment and short term DC culture with TLR stimulating reagents such as BCG-CWS and OK432, the anti-tumor effect of the intratumoral DC administration was significantly augmented in murine tumor models. The enhanced uptake of tumor antigens by the injected DC due to necrosis and apoptosis caused by cryoablation, and the partial activation of DC by the TLR stimulation, resulted in significant enhancement of anti-tumor $CD8^+$ CTL induction. This improved protocol was able to regress relatively large untreated tumor implanted at the remote site by systemically induced tumor antigen specific $CD8^+$ CTL. Additional treatments, including inhibition of regulatory T cells (T reg) or use of DC pulsed with highly immunogenic tumor antigens, further enhanced this anti-tumor effect (32).

Based on the mouse study, the first clinical trial, intratumoral administration of KLH pulsed DC was performed for patients with colon or esophageal cancer. We have established 2 protocols for *ex vivo* generation of monocyte-derived DC for clinical use, one is the generation from plastic adherent cells, the other is from purified monocytes using GMP grade magnetic anti-CD14 mAb and

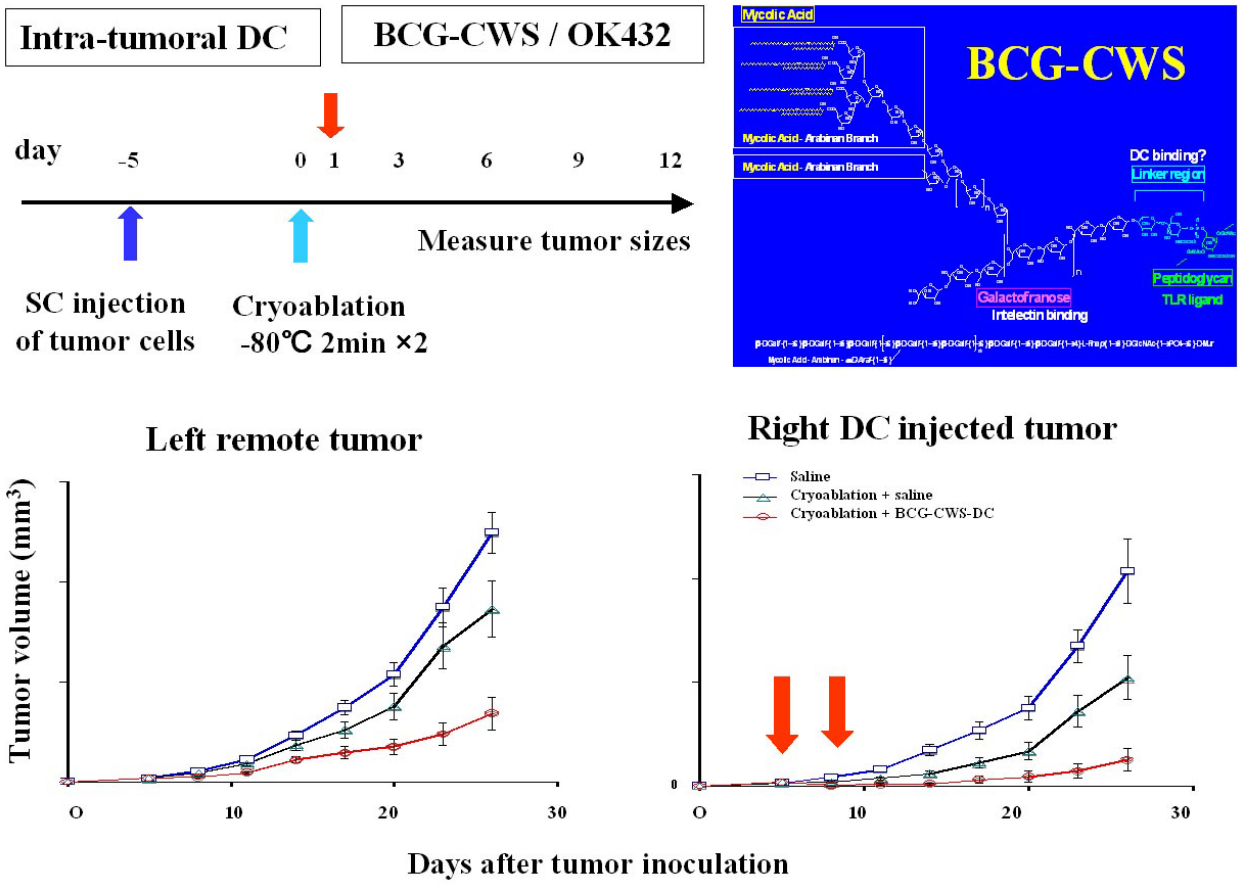

Figure 1. Intratumoral administration of BCG-CWS treated DC after cryoablative tumor treatment in murine tumor model. The treatment on only right tumors resulted in tumor regression of both the right treated tumors and left untreated tumors through the systemic induction of anti-tumor T cells. The cryoablation enhanced uptake of tumor antigens by the injected DC through necrosis and apoptosis of tumor cells.

a CliniMACS separation device. The latter technique was found to be superior to the former, regarding with purity of DC, stability of DC yield, and risk of bacterial contamination. Thus, we applied the CliniMACS method for the clinical trials. We found that yield of DC was relatively low from advanced cancer patients who received multiple chemotherapies. DC from one patient had severely decreased maturation ability upon LPS stimulation. Nevertheless, intratumoral administration of KLH pulsed DC into tumors resulted in the induction of systemic immune responses to the viral antigens and KLH which were pulsed on the injected DC, indicating that intratumoral DC administration into large tumors with immunosuppressive microenvironment, was able to induce systemic immune responses in cancer patients. However, the anti-tumor effect was observed only one patient whose liver metastases disappeared in this trial. The additional 3 clinical trials with cryoablative tumor pretreatment, intratumoral administration of BCG-CWS pulsed DC following cryoablation on subcutaneous melanoma metastases and primary lung cancer, and intratumoral administration of KLH pulsed DC following cryoablation on liver metastasis of digestive organ cancers, have been performed (Figure 2). Clinical response, SD or PR with decreased tumor markers, was obtained in some patients with induction of systemic immune responses. Additional improvements, including tumor debulking, use of highly immunogenic tumor antigen pulsed DC, and inhibition of various tumor escape mechanisms, appear to be required for more effective immunotherapy.

5. TUMOR ESCAPE MECHANISMS FROM IMMUNE SYSTEM TO BE OVERCOME FOR EFFECTIVE IMMUNOTHERAPY

Cancer cells not only produce various immunosuppressive molecules, but also produce molecules which induce immunosuppressive cells, including T reg, myeloid suppressor cells (MSC), tolerogenic DC (tDC), etc. To make the current immunotherapy more effective, it is important to further understand the mechanisms for generation of the cancer induced immunosuppressive microenvironment (Table 4). Culture supernatants from melanoma cells with enhanced MAPK signaling via mutated BRAF (V600E) or activated STAT3 signaling, inhibited LPS induced IL12 production by monocyte derived DC. Treatment of these melanoma cells with lentiviral shRNA specific for BRAF (V600E) or STAT3,

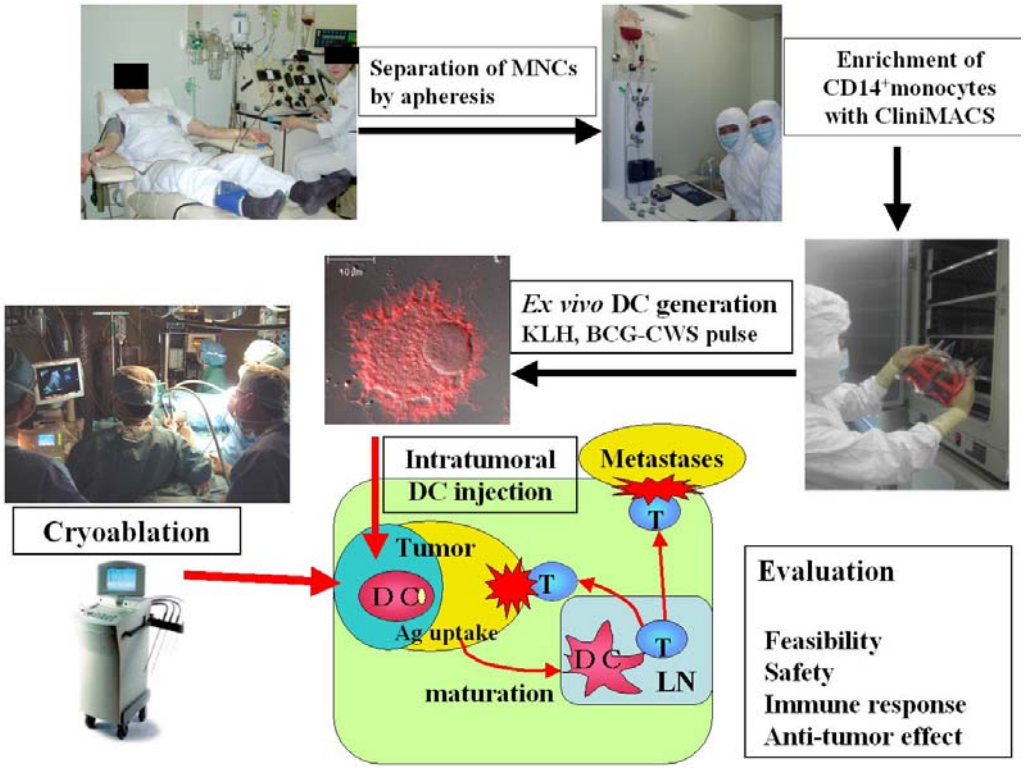

Figure 2. A scheme for the clinical trial with intratumoral administration of BCG-CWS treated DC after cryoablative tumor treatment.

Table 3. Personalized immunotherapy

A. Immunization with highly immunogenic identified shared tumor antigens
1. Selection of antigens based on
-Antigen expression in tumor cells
-Immunogenicity in patients (by serum IgG screening / *in vitro* T cell induction, HLA type)
2. Immunization with peptides, proteins, recombinant DNA, or RNA etc. (+/- dendritic cells)
3. Possible Ag spreading (induction of immune response to unknown unique antigens)
B. Immunization with autologous tumor cell molecules containing unknown unique antigens
1. Tumor derived peptides, proteins, RNA, hsp, etc. (+/- Dendritic cells)
2. Modified tumor cells by gene introduction, hapten conjugation, and viral infection
C. *In situ* immunization (intratumoral administration of DC, adjuvants, or viruses)

Table 4. Tumor escape mechanisms from T cell responses

1. Low immunogenicity of tumor antigens
low MHC binding
low processing (different processing by APC proteasomes)
2. Loss of molecules necessary for antigen processing and presentation
Antigens
MHC heavy chain, ß2-microglobulin
TAP, LMP
3. Blocking factors against anti-tumor immune responses
Tolerance induction (deletion, anergy)
Suppressive immune responses (T reg, Tr1, Th3, MSC, tDC)
Suppressive factors from tumor cells or host cells (IL10, IL6, VEGF, TGF-beta, PGE2, IDO, Arginase)
T cell counteracting molecules on tumor cells (Fas L, B7-H1, B7H4, MICA/B)
Altered T cell signaling of T cells (decreased TCR zeta)
Th1/Th2 shift
Unknown mechanisms

inhibited the suppressive activity of the supernatants through inhibiting production of IL10, IL6, and VEGF (17). Culture supernatants from some melanoma cells also induce high IL10 producing tDC and *FoxP3*$^+$ T reg. These results indicate that the altered genes in cancer cells are involved in the generation of immunosuppressive microenvironment, and that combined use of their specific inhibitors or siRNA targeting at the altered signals may augment the anti-tumor activity of the current immunotherapy through simultaneous inhibition of multiple immunosuppressive mechanisms.

6. CONCLUDING REMARKS

The identification of human tumor antigens led to further understanding of immunobiology of human cancers and development of effective immunotherapy. Our results suggest that personalized protocols should be considered for future development of immunotherapy. In addition, it is critical to develop methods to overcome various immune escaping mechanisms of cancer cells towards effective immunotherapy (1, 6).

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Abbreviations: TIL: tumor infiltrating T cells; APC: antigen presenting cells; SEREX: serological analysis of recombinant cDNA expression libraries; CGH: comparative genomic hybridization; SAGE: serial analysis of gene expression; MSI: microsatellite instability; TLR: toll like receptor; T reg: regulatory T cells; MSC: myeloid suppressor cells; tDC: tolerogenic dendritic cells; KLH: keyhole limpet hemocyanin; HNPCC: hereditary non-polyposis colorectal cancer

Key Words: Tumor antigens, T cells, Dendritic cells, Cryoablation, Immunotherapy, Review

Send correspondence to: Dr Yutaka Kawakami, Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo, 160-8582, Japan, Tel: 81-3-5363-5778, Fax: 81-3-5362-9259, E-mail: yutakawa@sc.itc.keio.ac.jp

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