PTD-J domain acts as an antigen carrier for cell mediated immunity

Tai-Yun Lin, Yu-Hsiu Su and Chin-kai Chuang*

*Correspondence: jkjuang@mail.atri.org.tw

Division of Animal Technology, Laboratories of Animal Technology, Agricultural Technology Research Institute No. 1, Lane 51, Dahu Rd., Xiangshan District, Hsinchu City 30093, Taiwan.

Abstract

BALB/c mice elicited specific cell-mediated immunity after transplantation with CT26 cells stably expressing human telomerase reverse transcriptase (CT26(hTERT#10)). Resting memory T cells were reactivated in vitro by CT26(hTERT#10) cells and PTD-J-hTERTepi recombinant proteins, in which the polypeptide hTERTepi comprised TH and TC epitopes, as indicated by interferon γ (IFNγ) secretion. Pre- and post-injections of PTD-J-hTERTepi with in vitro expanded and differentiated dendritic cells reduced CT26(hTERT#10) tumor size. Moreover, Hsp72 activated numerous splenocytes to perform IFNγ secretion and further reduced tumor sizes.

Keywords: PTD-J domain, hTERT, epitopes, cellular immunity

Introduction

hTERT is a ubiquitous candidate for cancer immunotherapy

Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of human telomerase and is reportedly expressed in 85% of human malignant tumors, but not in normal somatic tissues [1-3]. Hence, telomeres and telomerase are potential targets of cancer therapy [4,5], and hTERT has emerged as the first bona fide common tumor antigen [6,7] and is actively investigated as a target for cancer immunotherapy [8]. Although hTERT is rapidly degraded in cancer cells and the resulting peptides are presented on de novo synthesized major histocompatibility complex class I (MHC I) molecules, the resulting signals lack sufficient intensity to elicit cytotoxic lymphocyte (CTL) responses with assistance from specific CD4+ T helper 1 (Th1) cells. MHC I molecules load peptides in the endoplasmic reticulum (ER) and display them for T cell receptors (TCR) on CD8+ T lymphocytes (Tc). MHC class II molecules (MHCII) acquire peptides from endosomes and present these complexes to TCR on CD4+ T lymphocytes. Among adaptive immune cells involved in anti-tumor responses, Tc cells have been considered as the main protagonists because they elicit direct cytotoxic activity toward cancer cells. However, tumor reactive Th1 cells that produce IFNγ, TNFα, and IL-2 play critical roles in the orchestration of cell-mediated immunity against tumors [9].

PTD-J-X recombinant protein behaves as both intracellular and extracellular antigen

As fore mentioned, intracellular antigens are processed and primed onto MHC I in the ER, whereas extracellular antigens are processed and presented on endosomal MHC II. Recombinant proteins are usually considered as extracellular antigens; however cell penetrating peptides and their cargos can traverse the cytoplasm membrane [10,11] and can be considered as intra cellular antigens. Recently, we reported an expression system for are combinant PTD-J-cargo protein comprising a protein transduction domain (PTD), a J domain of Hsp40, and a cargo polypeptide [12]. Subsequently, we showed that the cargo polypeptide assembly displayed epitopes of FMDV VP1 protein fused with PTD-J and elicited humoral immunity against VP1 and Hsp72 which is able to associate with the J domain can enhance the humoral immunogenicity of the cargo of PTD-J-cargo [13]. Since we had proven that the cell penetrating activity of PTD-J-DsRed was stronger than that of PTD-DsRed [14]. It was interesting to test whether the PTD-J-cargopolypeptide could be used as intracellular antigen to elicit cellular immunity or not. The PTD-J-hTERTepi, where the cargo polypeptide hTERTepi is composed of MHC I and MHC II binding peptides of hTERT, was expressed and utilized to verify this idea. The ectopic Hsp72 recombinant protein was also supplemented to test whether it could enhance cellular immunity or not. Moreover, bone marrow derived syngeneic dendritic cells were used to present the antigen.

Materials and methods

Stable expression of hTERT in CT26 cells

The hTERT.Flag2-IREShyg expression cassette was separated...
from the pCX-hTERT-Flag2-IREShyg vector (Supplementary Figure S1) using Not I digestion and was transfected into CT26 colon carcinoma cells. Cell colonies resistant to 400 µg/mL hygromycin B were picked and expanded in the presence of 200µg/mL hygromycin B. Selected cells expressing recombinant hTERT-Flag2 were cultured without hygromycin B for one week and recombinant protein expression was analyzed using western blotting. Cell lines with stable expression of recombinant hTERT-Flag2 (such as CT26(hTERT #10)), and those that stopped expressing it (such as CT26(hTERT #4)) in the absence of hygromycin B were collected.

Preparation of the pET22b-PTD-J-hTERTepi expression vector
Published hTERT peptides associated with various well characterized human class I and class II MHC alleles [8,15,16] and predicted hTERT epitopes of class I and class II MHC from the mouse H-2d locus were identified using neural network [17] and stabilized matrix methods [18] (Figure 1a). According to these data, hTERT602–641 (SEAEVRQHREARPALLTSRLRFIPKPDGLRPNMDYVVG) and hTERT1088–1121 (TYVPLLGSLRTAQTQLSRKLPGTLTALEAAANP) were selected and linked by an FFRK peptide to produce hTERTepi. Corresponding cDNA with E. coli optimized codons was synthesized as shown in Supplementary Figure S2 and inserted into a pET22b-PTD1J1(Tb) vector [12] (Supplementary Figure S3) between EcoRI and XhoI sites to produce the pET22b-PTD-J-hTERTepi expression vector.

Expression and purification of the PTD-J-hTERTepi recombinant protein
E. coli Rosetta-gamiB (DE3)pLysSRARE cells carrying pET22b-PTD-J-hTERTepi were cultured overnight in LB supplemented with 30µg/mL carbenicillin and 25µg/mL chloramphenicol, and were then centrifuged at 5,000g for 10 min. Cell pellets were suspended in five volumes of 2×YT medium supplemented with 0.4% glucose and 30µg/mL carbenicillin, and were cultured for about 2 h until the OD600 value was around 1. IPTG concentrations were then adjusted to 1 mM to induce recombinant protein expression for 4h. Subsequently, PTD-J-hTERTepi recombinant protein was expressed in an insoluble inclusion body form, which was then separated from soluble lysates by centrifugation at 20,000 g for 20 min. The pellet was then dissolved in a buffer containing 50mMTris–HCl (pH 8.0), and insoluble debris was removed by centrifugation at 20,000 g for 30 min. The supernatant fraction was applied to a Ni-NTA sepharose (fast flow, GE Healthcare) column with a flow rate of 5 column volumes per hour. The column was then washed with 10 column volumes of detergent buffer containing 20mMTris–HCl(pH 8.0), and 500mMNaCl, the recombinant protein was eluted in an elution buffer containing 20mMTris–HCl(pH 7.4), 300mMNaCl, and 250mM imidazole.

Transplantation of cancer cells into BALB/c mice
CT26, CT26 (hTERT#10), and CT26 (hTERT#4) cells (1×106) were implanted subcutaneously into dorsal–lateral sides of 5 sets, three mice per set, of BALB/c mice (Figure 2). Sets one, two, and three received CT26, CT26 (hTERT #10), and CT26 (hTERT #4) cell implants into both sides, respectively (sets I–III), and sets four and five received cell implants of CT26 and CT26 (hTERT#10) (set IV), and CT26 and CT26 (hTERT#4) (set V) cells into either side, respectively. Ten days after cancer cell implantation, long (L) and short (S) diameters were measured twice a week using a caliper and tumor volumes were estimated as ½ LS².

Immunization of BALB/c mice
Bone marrow derived dendritic cells (DCs) were prepared from

---

**Figure 1. CT26(hTERT) cell lines.**
(a) Hygromycin B resistant hTERT-Flag2-IREShyg transfected CT26 (C) cell colonies were picked, and hTERT-Flag2 expression in cell lines #1, #2, #3, #4, #5, #6, #8, #10, and #12 was assessed using a monoclonal antibody M2 against the flag tag and a goat polyclonal anti-hTERT antibody (Santa Cruz,sc-7215). An anti-β-actin monoclonal antibody (abcam, ab8226) was used as a calibration control, and hTERT-Flag2, mTERT, and β-actin signals were indicated by red, black, and blue arrow heads, respectively. (b) After removal of hygromycin B for 1 week, hTERT-Flag2 expression remained stable in CT26(hTERT #10) cells and was abolished in CT26(hTERT #4) cells.
doi: 10.7243/2055-2394-2-1

Figure 2. Transplantation of CT26, CT26(hTERT#10), and CT26(hTERT#4) cell lines into BALB/c mice.
(a) Five sets of mice, three mice per set, received surgery. CT26, CT26(hTERT#10), and CT26(hTERT#4) cells were injected subcutaneously into both dorsal and lateral sides (set I, set II, and set III respectively), CT26 and CT26(hTERT#10) cells were injected into either side (set IV), and CT26 and CT26(hTERT#4) cells were injected into either side (set V). (b) Long (L) and short diameters (S) were measured twice a week from 10 days after cancer cell implantation using a caliper and tumor volumes were estimated as 1/2LS2. (c) Two months after the experiment, RBC-depleted splenocytes were collected from the second set of mice and from untreated age-matched control mice. T cells with memories of hTERT epitopes were evoked by treatment with PTD-J-hTERTepi or CT26(hTERT#10) for 24 h.

8-week-old BALB/c mice as previously described [19]. Briefly, DC progenitor cells were expanded in RPMI1640 medium containing 10% FCS, 20ng/mL recombinant murine Flt3L, 10ng/mL recombinant murine IL-3, and 10ng/mL recombinant murine SCF for 18 days. Subsequently, progenitor cells were differentiated using 20ng/mL IL-4 and 20ng/mL GM-CSF for 5 days, and 1×10⁶ differentiated isolated DCs mixed with 35µg of PTD-J-hTERTepi or with 35µg of PTD-J-hTERTepi and 137µg of recombinant murine Hsp72 [12] in 0.5 mL of PBS were injected intraperitoneally into 6-week-old BALB/c mice. At 7 days after CT26(hTERT#10) implantation, L and S diameters were measured every fifth day as mentioned.

IFNγ ELISPOT assay
PVDF membranes of ELISPOT plates’ (Millipore, Cat. No., MAIPS-4510) wells were coated with 0.25µg of rat anti-mouse interferonγ (clone R4-6A2, BioLegend, Cat. No. 505701) for more than 4 h at 4°C. After blocking, 1×10⁶ RBC-depleted splenocytes (response cells) with 0.5µg of PTD-J-hTERTepi, 5×10⁵ CT26, or 5×10⁵ CT26(hTERT#10) cells (stimulators) were added to each well and cultured for 24 h. Cells were removed by washing three times with PBS and then 3 times with PBS containing 0.05% Tween-20. Subsequently, 0.1µg of biotinylated rat anti-mouse monoclonal antibody clone XMG1.2 (BioLegend, Cat. No. 505803) in PBS containing 0.05% Tween-20 was added to each well and incubated for 2 h at room temperature. After four washes with PBS containing 0.05% Tween-20, 100µL of a 1:100 dilution of avidin-HRP (BD Biosciences, Cat No. 557630) was added to each well and incubated for 1 h at room temperature. The plate was then washed three times with PBS containing 0.05% Tween-20. Finally, 100µL of AEC substrate (BD Biosciences, Cat No. 551951) was added to each well and spots were developed. The color forming reaction was stopped by rinsing the plates with tap water, and spots were observed under a dissecting microscope after air drying the plates.

Results
BALB/c mice recognized hTERT epitopes on syngenic CT26 cells
After transfection with the hTERT-Flag₂-IREshyg gene expression cassette, CT26 derived cell lines were selected under 400µg/mL hygromycin B. Among these cell lines, CT26(hTERT#4), CT26(hTERT#10), and CT26(hTERT#12) cells expressed higher levels of recombinant hTERT-Flag₂ (Figure 1a). The hTERT-Flag₂ recombinant protein expression level would be abolished in CT26(hTERT#4) after the removal of hygromycin B selection, whereas CT26(hTERT#10) cells expressed it consistently (Figure 1b). Besides the hTERT-Flag₂ protein, endogenous mouseTERT (mTERT) was expressed in all the CT26 cell lines (Figure 1a).

Transplanted CT26 cells grow infinitely in syngenic BALB/c mice indicating that CT26 cells can escape from the immune surveillance of the hosts. It is curious that CT-26 expressing xenogenically intracellular hTERT can be recognized by the immune system or not. The CT26(hTERT#10) which consistently expresses hTERT-Flag₂, CT26(hTERT#4) which expresses hTERT-Flag₂ only under the presence of hygromycin B, and
CT26 cell lines were chosen to figure out this question. Five sets, three mice per set, of BALB/c mice were implanted subcutaneously into dorsal–lateral sides with 1×10^6 cells per site of CT26, CT26(hTERT#10), and CT26(hTERT#4) (Figure 2a). As mentioned above, the CT26 tumors proliferated most quickly. In contrast, CT26(hTERT#10) tumors grew for about 14 days after transplantation and then receded rapidly, becoming undetectable by 24 days after transplantation. These mice were observed for another two months and no tumor relapse was observed. This result indicated that certain immunity was elicited by CT26(hTERT#4) cells which escaped growth suppression from the 17th day after transplantation (Figure 2, set I, II and III). In further experiments, CT26 cells were injected into single sides of mice, and the other side was injected with either CT26(hTERT#10) or CT26(hTERT#4) cells. CT26 tumor growth rates were not influenced by the presence of CT26(hTERT#10) or CT26(hTERT#4) tumors, and relative tumor sizes reflected nutrient competition (Figure 2, set IV and V). In this situation, CT26(hTERT#10) tumors grew slowly but continuously during the experimental period (Figure 2, set V).

Preparation of the PTD-J-hTERTepi recombinant protein
Since hTERT was demonstrated to be an intracellular antigen that elicited cellular immune responses, an immunogenic composition was designed. Based on the published human MHC I and MHC II binding hTERT peptides, and those carrying predicted associations with mouse MHC I and MHC II of H-2d genotypes summarized in Figure 3a, two fragments hTERT_{602–644} and hTERT_{1088–1121} contained both human and murine candidates and were selected to assemble the multiepitopic hTERTepi polypeptide (Figure 3b). Subsequently, the recombinant PTD-J-hTERTepi protein was expressed by E. coli as an inclusion body, and was refolded and purified (Figure 3c). Very few isolated splenocytes from CT26(hTERT#10) transplanted mice (Figure 2b) were sensitive to CT26 cells at two months after the experiment. However, a certain fraction of splenocytes were invoked by PTD-J-hTERTepi protein or CT26 (hTERT#10) cells to express IFNγ (Figure 2c).

PTD-J-hTERTepi and Hsp72 proteins (DCs/PTD-J-hTERTepi/Hsp72) in 0.5 mL of PBS. At 7 days after implantation of CT26 (hTERT#10) cells, tumor volumes were measured every fifth day using a pair of calipers (Figure 4a). In these experiments, CT26(hTERT#10) tumor volumes were about 105 mm^3 at 12 days after transplantation, but were subsequently diminished by host immune activities thereafter. CT26 (hTERT#10) tumor sizes in mice that were pre-treated or post-treated with DCs, DCs/PTD-J-hTERTepi, or DCs/PTD-J-hTERTepi and Hsp72 were significantly smaller than those of untreated mice. Comparisons of tumor volumes on the 17th day (Figure 4b) indicated that injection of in vitro expanded and differentiated DCs from autologous bone marrow retards tumor growth. Moreover, injections of DCs with recombinant PTD-J-hTERTepi protein further limited CT26 (hTERT#10) tumor growth, whereas the addition of Hsp72 to DCs/PTD-J-hTERTepi had marginal effect on the reduction of tumor volumes.

Injection of PTD-J-hTERTepi and Hsp72 with DCs increases numbers IFNγ-expressing splenocytes
Both pre- and post-injections of DCs with recombinant PTD-J-hTERTepi could further reduce CT26(hTERT#10) tumor volumes than treated with DCs alone. These results indicated that cytotoxic immunity might be elicited by PTD-J-hTERTepi. This cytotoxic immunity might be augmented by Hsp72. ELISPOT experiments were performed to determine numbers of IFNγ-expressing splenocytes from RBC-depleted spleen at 7 days after two successive injections of PBS, DCs, DCs/PTD-J-hTERTepi or DCs/PTD-J-hTERTepi/Hsp72. These experiments showed very few IFNγ secreting splenocytes (9 in 10^6) from the DCs injected group in comparison with PBS treated controls. The numbers of IFNγ secreting splenocytes after injection of DCs/PTD-J-hTERTepi increased significantly (about 510 in 10^6), and out of scale numbers (more than 3000 in 10^6) were illustrated in the DCs/PTD-J-hTERTepi/Hsp72 cases (Figure 5).

Discussion
BALB/c mice specifically recognized xenogenic hTERT antigen expression in syngeneic CT26 cancer cells
Syngeneic CT26 colorectal cancer cells escaped immune surveillance in BALB/c mice, resulting in tumor growth (Figure 2, set I), whereas CT26(hTERT#10) cells were eliminated by immune activities (Figure 2, set II). However, after transplantation of CT26 and CT26(hTERT#10) cells at separate sites in the same mice, CT26 tumor formation was similar, even a little faster, to that in the mice of set I, and CT26 (hTERT#10) tumors grew very slowly (Figure 2, set IV). The CT26 (hTERT#10) tumors in set II were completely eliminated by 24 days, however, those in set IV were not. It revealed that the accompany CT26 tumors in set IV mice could suppress the immunity of the hosts. These results indicate that intracellular xenogenic hTERT expression in CT26 cells elicited immune responses in BALB/c mice. Moreover, about 230 of 10^6 splenocytes from set II mice were reactivated at two months after transplantation with CT26.
Figure 3. Amino acid sequence analyses, expression vector construction, and recombinant hTERT T-cell epitope expression.

(a) Published human MHC class I (HLA-A1, A2, A3, A24 and B7 allele) and class II (HLA-DR1, DR4, DR7, DR11 and DR15 allele and pan DR, DQ and DP) associated hTERT peptides are summarized. Sequences of hTERT peptides were predicted using ANN [17] and SMM methods [18] and associations with mouse MHC class I and II of H-2d genotype with IC50 values of <150 nM are listed. (b) The peptides hTERT[602-641] and hTERT[1088-1121] were selected and linked using an FFRK peptide to give hTERTepi. Subsequently, cDNA encoding the hTERTepi with codons optimized for E. coli was synthesized and inserted into the pET22b–PTD1J1(Tb) vector to express the recombinant PTD-J-hTERTepi protein. (c) Recombinant PTD-J-hTERTepi was expressed in E. coli (T, total lysate) but was not found in the soluble fraction (S). The insoluble pellet was suspended in 6M guanidine–HCl (GT) and the insoluble fraction (GP) was removed by centrifugation. The supernatant fraction (GS) was applied to a Ni-NTA sepharose (fast flow, GE Healthcare) column and the recombinant protein was refolded and purified as described in the Materials and methods. Most recombinant protein was trapped in the column and little was observed in the flow-through fraction (F). No protein was found in detergent (D), cyclodextrin (C), or wash buffer (W) fractions. A few contaminating proteins were detected in the early elution fraction (E1) and pure recombinant PTD-J-hTERTepi protein was found in the peak elution fraction (E2). The gel was stained by Coomassie Blue R-250.

(hTERT#10) cells as demonstrated in ELISPOT experiments (Figure 2c). The major IFNγ secreting cells were activated T,1 and CTL. Recombinant PTD-J-hTERTepi protein also activated about 35 of 10⁶ splenocytes (Figure 2c), indicating that it penetrated actively or passively into splenocytes and was subsequently presented on MHC I and II molecules, leading to activation of latent memory T cells.

PTD-J-hTERTepi suppressed CT26 (hTERT#10) tumor growth

Because the hTERTepi polypeptide contained epitopes of hTERT, we examined the efficacy of PTD-J-hTERTepi as a vaccine against CT26 (hTERT#10) cancer cells. In these experiments, syngeneic DCs were used to augment antigen presentation, and recombinant Hsp72, which associates with the J domain
was added. In addition, extra-cellular Hsp72 also acts as a danger signal [20] and activates innate immunity via Toll-like receptor (TLR) 2 and TLR4 pathways [13, 21]. Accordingly, DCs were peritoneally injected alone, with PTD-J-hTERTepi, and with PTD-J-hTERTepi and Hsp72 at 9 and 2 days before CT26(hTERT#10) cell transplantation. As shown in Figure 4a, significant CT26(hTERT#10) tumor growth was observed in untreated mice until the twelfth day after transplantation and subsequently decreased to undetectable volumes by the 27th day. Tumor sizes were significantly smaller on mice pretreated with DCs, DCs/PTD-J-hTERTepi, and DCs/PTD-J-hTERTepi/Hsp72 than on untreated mice, and receded from the seventh day after transplantation. On the 17th day, significant differences in tumor volumes between treatments demonstrated that injections of DCs suppressed CT26 (hTERT#10) tumor growth (p=0.0015), and that co-injection of PTD-J-hTERTepi and PTD-J-hTERTepi with Hsp72 further suppressed CT26 (hTERT#10) tumor growth (p=0.015 and 0.017, respectively). The effects of DCs, DCs/PTD-J-hTERTepi and DCs/PTD-J-hTERTepi/Hsp72 on CT26(hTERT#10) tumor sizes following intraperitoneal injections on days 9 and 2 after transplantation led to similar observations as those after pretreatments. Accordingly, tumor volumes on the 17th day differed significantly between post treatment groups, and indicate suppression of CT26 (hTERT#10) tumor growth by DCs (p=0.0071) and additional suppression by PTD-J-hTERTepi and PTD-J-hTERTepi with Hsp72 (p=0.076 and 0.038, respectively). However, the tumor suppressive effect of Hsp72 treatment alone was not significant (p=0.23). Besides the cross presentation of the hTERT epitopes from PTD-J-hTERTepi to the host immune system, the bone marrow derived DCs themselves could suppress CT26 (hTERT#10) tumor growth (Figure 4a). And the effect of DCs on CT26 (hTERT#10) tumor suppression was significant (Figure 4b). This phenomenon could be interpreted that the injected DCs enhanced the probability to cross present the hTERT epitopes from CT26 (hTERT#10) tumors. That is to say,
the bone marrow derived DCs of oneself could be used as a tumor suppressing agent.

DCs with PTD-J-hTERTepi stimulate splenocytes to secrete IFNγ

Suppressive effects of pre- and post-transplantation injections of DCs on CT26 (hTERT#10) tumor growth likely reflect the consequent induction of host immunity. Accordingly, only 9 of 10^6 splenocytes secreted IFNγ at seven days after the second injection of DCs, where as about 510 of 10^6 splenocytes secreted IFNγ at seven days in mice treated with DCs/PTD-J-hTERTepi (Figure 5), suggesting a predominant role of PTD-J-hTERTepi in the induction of IFNγ. Previous studies show that recombinant Hsp72 activates innate immunity via Toll-like receptor TLR2 and TLR4 pathways [13,21] and enhances humoral immunity against PTD-J-recombinant proteins [12,13]. In agreement, the present data show that more than 3,000 of 10^6 splenocytes secreted IFNγ after treatment with DCs/PTD-J-hTERTepi/Hsp72. Moreover, membrane-bound and exported Hsp70 reportedly initiated secretion of pro-inflammatory cytokines [22]. Hence, the dramatic increase in numbers of IFNγ secreting cells following treatments with Hsp72 may indicate the presence of a proinflammatory T,1 host response via TLR2 [23]. However, further experiments are needed to characterize this phenomenon.

Additional files

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

<table>
<thead>
<tr>
<th>Authors' contributions</th>
<th>TYL</th>
<th>YHS</th>
<th>CKC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Research concept and design</td>
<td>--</td>
<td>--</td>
<td>✓</td>
</tr>
<tr>
<td>Collection and/or assembly of data</td>
<td>✓</td>
<td>✓</td>
<td>--</td>
</tr>
<tr>
<td>Data analysis and interpretation</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Writing the article</td>
<td>✓</td>
<td>--</td>
<td>✓</td>
</tr>
<tr>
<td>Critical revision of the article</td>
<td>--</td>
<td>--</td>
<td>✓</td>
</tr>
<tr>
<td>Final approval of article</td>
<td>✓</td>
<td>--</td>
<td>✓</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>✓</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Acknowledgement and funding

This work was supported by the grants NSC102-2320-B-059-001-MY3 and NSC 99-2313-B-059-004-MY3 from the Ministry of Science and Technology, Taiwan to CKC.

Publication history

Editor: Shao-An Xue, University College London Medical School, UK.
Received: 15-Oct-2015 Final Revised: 20-Nov-2015
Accepted: 30-Nov-2015 Published: 09-Dec-2015

References


