Downregulation of Hepatoma-Derived Growth Factor Contributes to Retarded Lung Metastasis via Inhibition of Epithelial–Mesenchymal Transition by Systemic POMC Gene Delivery in Melanoma

Han-En Tsai, Guei-Sheung Liu, Mei-Lang Kung, Li-Feng Liu, Jian-Ching Wu, Chia-Hua Huang, San-Cher Chen, Hing-Chung Lam, Chieh-Shan Wu, and Ming-Hong Tai

Abstract

The prognosis of malignant melanoma is poor due to high incidence of metastasis, underscoring the demand for development of novel therapeutic strategies. Stress hormone pro-opiomelanocortin (POMC) is the precursor for several anti-inflammatory peptides that hold promise for management of cancer-related diseases. The present study evaluated the antimetastatic potential and mechanism of POMC therapy for metastatic melanoma. Adenovirus-mediated POMC gene delivery potently inhibited the invasiveness of human and mouse melanoma cells. Moreover, after induction of lung metastasis, systemic POMC expression significantly reduced the foci formation and neovascularization in lungs. Mechanistic studies revealed that POMC therapy inhibited the epithelial–mesenchymal transition (EMT) of melanoma cells by upregulation of E-cadherin and downregulation of vimentin and α-smooth muscle actin (α-SMA). In addition, microarray analysis unveiled POMC gene transfer reduced the mRNA level of multiple prometastatic factors, including hepatoma-derived growth factor (HDGF). Cell culture and immunohistochemical studies further confirmed that POMC gene delivery significantly decreased the expression of HDGF in melanoma cells and tissues. Despite stimulating the invasion and EMT, exogenous HDGF supply only partially attenuated the POMC-mediated invasion inhibition and EMT change in melanoma cells. Finally, we delineated the contribution of melanocortins to POMC-induced inhibition of invasion, HDGF downregulation, and E-cadherin upregulation. Together, these results indicate that HDGF downregulation participates in POMC-induced suppression of metastasis and EMT in melanoma.

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Introduction

Cutaneous melanoma, which begins with benign nevi and progresses to radial and vertical growth, is one of the fastest increasing malignancies worldwide (1). Metastasis or the spread of cancer cells accounts for the major cause of cancer mortality including melanoma (2, 3). Melanoma is notorious for resistance to conventional chemotherapy and radiotherapy (4). Moreover, melanoma metastases are extremely aggressive and the average survival for patients with metastatic melanoma is only between 6 and 9 months (5). Therefore, novel modalities are demanded for control of metastatic melanoma.

Hepatoma-derived growth factor (HDGF) is an acidic heparin-binding protein originally isolated from the conditional medium of human hepatoma cell line, HuH-7 (6, 7), and could stimulate with the proliferation of fibroblast (8), vascular smooth muscle cells (9), endothelial cells (8), and several tumor cells including hepatoma and melanoma (10–12). HDGF has also been recognized as an important prognostic marker that HDGF overexpression was correlated with advanced stages and poor survival outcome in several types of cancers including liver cancer (13) and esophageal carcinoma (14). Furthermore, we have recently shown that HDGF overexpression promoted epithelial-to-mesenchymal transition (EMT) by E-cadherin downregulation and vimentin upregulation, thereby promoting the invasion and metastasis in breast cancer cell (15). However, the upstream molecules or signaling mechanisms that modulate HDGF expression...
and therefore affect cancer progression remain largely unknown.

The initiation of metastasis is characterized by the increased motility and invasiveness of cancer cells. To acquire such invasive abilities, tumor cells undergo physiologic changes such as EMT, a process in which cells lose generally immotile epithelial characteristics and gain motile mesenchymal properties (16, 17). EMT is mediated through several transcription repressors, such as Snail, Slug, Twist and ZEB1, mesenchymal markers Vimentin and N-cadherin, and these EMT inducers typically suppress the transcription of the E-cadherin gene, an epithelial cell marker and a potent suppressor of tumor cell invasion and metastasis (18, 19). Therefore, identifying the mechanisms that block the loss of E-cadherin expression is critical for preventing malignant progression via suppression of the EMT.

Stress hormone pro-opiomelanocortin (POMC) is the precursor of several anti-inflammatory neuropeptides including adrenocorticotropic (ACTH), melanocortins (α-, β-, and γ-MSH), and β-endorphin (β-EP). Recent studies indicate that the anti-inflammatory POMC therapy effectively suppresses the growth of murine primary tumors, including B16-F10 (20, 21) and Lewis lung carcinoma (22). Furthermore, POMC-derived peptide, α-MSH, has been reported to trigger melanoma/melanocyte differentiation (23, 24) and potently inhibits both the in vitro and in vivo invasion of highly invasive B16-BL6 melanoma cells (25, 26). A recent study has indicated that prophylactic α-MSH treatment decreased the metastatic potential of melanoma cells in vitro and in vivo (27). These findings suggested that peripheral POMC expression may inhibit invasive and metastatic growth. However, the underlying mechanism by which POMC-derived peptides inhibit melanoma metastasis has not been fully understood. Therefore, it will be valuable to examine whether POMC-derived peptides could be a novel regulator of EMT.

In this study, we first evaluated the therapeutic efficacy of POMC gene therapy for lung metastasis after administration of melanoma cells into circulation by bio-imaging and histologic analysis. Subsequently, the therapeutic mechanism underlying POMC therapy was explored by examining tumor angiogenesis and EMT profiles. Finally, microarray analysis was used to identify the involvement of HDGF downregulation in POMC-induced metastasis suppression.

Materials and Methods

Reagents

Generation of recombinant HDGF protein and anti-HDGF were previously described (10). TGF-β was purchased from Sigma. Rhodamine B-dextran (10,000 Mw) was purchased from Invitrogen. Anti-mouse E-cadherin, Vimentin antibodies were from Santa Cruz. Anti-mouse α-SMA antibody was from Sigma. Anti-mouse β-actin was from Millipore. ACTH, α-MSH, β-MSH, and γ-MSH were purchased from Bachem.

Cell culture

Mouse B16-F0, B16-F10, and human A2058 melanoma cells were purchased from American Type Culture Collection and cultured in Dulbecco’s Modified Eagles Medium (DMEM; Invitrogen) medium containing 10% fetal calf serum, 2 mmol/L glucose, 100 mg/mL streptomycin, and 100 U/mL penicillin at 37°C in 5% CO2 incubator. Cells were initially grown and multiple aliquots were cryopreserved. All the cell lines were used within 6 months after resuscitation, and no further cell line authentication was conducted. The GFP- luciferase-expressing B16-F10 melanoma cells were generated as previously described (20).

Preparation of adenovirus vectors

The recombinant adenovirus vectors containing GFP (Ad-GFP) and POMC (Ad-POMC) were generated as previously described (20, 28).

Metastatic melanoma model and gene delivery

Male C57BL/6J/Narl mice (4–6 weeks old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and housed under specific-pathogen-free conditions. All animal experiments were carried out under protocols approved by Animal Care and Use Committee of National Sun Yat-Sen University (Kaohsiung, Taiwan; approval ID, NSC 98-2320-B-110-004-MY3/97014).

In the metastasis model, 5 × 105 B16-F10 cells were resuspended in 0.1 mL PBS and injected into the tail vein of C57BL/6 mice at day 0 to induce the pulmonary metastasis. For liver-based gene delivery, C57BL/6 mice are injected with adenovirus vectors: Ad-POMC [1 × 109 plaque-forming units (pfu)] or Ad-GFP (1 × 109 pfu) via tail vein at day 1. Metastatic progression was monitored and quantified by using noninvasive bioluminescence as previously described (29). The mice were sacrificed on day 14 and the lungs were harvested for consequence analysis.

Immunohistochemistry

Immunohistochemistry was conducted as described previously (20). The primary antibodies are CD31 (1:100; Novoceastra), α-SMA (1:200), E-cadherin (1:250), and vimentin (1:200).

Western blot analysis

Whole-cell protein extracts were prepared as described previously (20). Proteins were separated on 8% to 16% Amersham ECL gels and western blotted with anti-α-SMA (1:1,000), E-cadherin, vimentin (1:500), and β-actin (1:5,000).

Flow cytometry

A total of 1 × 106 infected B16-F10 cells were harvested and washed twice with PBS and incubated with blocking buffer (5% bovine serum albumin in PBS) for 1 hour. For surface molecules content analysis, cells were incubated...
with primary antibodies for 1 hour (E-cadherin, 1:500) followed by incubation with Alexa 488–conjugated secondary antibodies for 30 minutes (Molecular Probes). The data from Flow cytometry were analyzed by Cell Lab Quanta SC (Beckman Coulter Inc.).

**Microarray analysis**

The total RNA was isolated from B16-F10 cells infected with Ad-GFP or Ad-POMC at multiplicity of infection of 1,000 for 24 hours using RNAzol (TEL-TEST Inc.). The integrity and quality of RNA samples were confirmed by using agarose electrophoresis and the Lab-on-chip system. Afterward, 0.5 µg RNA was amplified and labeled with Cy3 (the FC sample) and Cy5 (the MC sample), respectively, as previously described (30). A Qiagen RNeasy Mini Kit (Qiagen Inc.) was used to purify fluorescent cRNA probes. An equal amount (2 µg) of Cy3- and Cy5-labeled probes was mixed and used for hybridization on one Agilent Mouse V2 Oligo Microarray (array kit serial number US0030099; Agilent Technologies Inc.) following the protocol provided by the manufacturer. The hybridization signals were acquired by using Agilent 2100 Bioanalyzer (G2938B) and analyzed using Agilent G2567AA Feature Extraction Software (v7.5).

**Real-time PCR**

RNA was isolated from B16-F10 cells using RNAzol (TEL-TEST, Inc.). For reverse transcription, 5 µg of total RNA was used for reverse transcription with Superscript III (Invitrogen Co.) using oligo(dT) and random primers. One twentieth of reverse transcription products were used as template for real-time PCR in Lightcycler (Roche) using Taqman green I assay. PCR reaction was carried out in 20 µl SYBR Green PCR Master Mix (Roche) containing 100 µmol/L forward primers and reverse primers and approximately 30 ng cDNA. Amplification and detection were conducted by: 1 cycle of 95°C for 10 minutes, 40 cycles of 95°C for 5 seconds, 62°C for 5 seconds, and 72°C for 10 seconds. The primer sequences for mouse HDGF were: forward primer 5′-CCGGATGAGATGATGCC-TGA-3′, reverse primer 5′-TCATGGCTGCTTCCAAACT-3′, which amplified a 150-bp HDGF cDNA fragment. The β-actin mRNA level was determined using: forward primer 5′-TCACCCACACTTGCT ATATCAGA-3′ and reverse primer 5′-CAGCGGAACCGCTATGGCAAT- GG-3′, which amplified a 295-bp β-actin cDNA fragment.

** Invasion assay**

Invasion assay was assessed using a Boyden chamber as previously described (31).

**Transfection and luciferase measurement**

For transient transfection, adenovirus infected B16-F10 cells (in a 6-well plate) at 80% confluence were transfected with plasmid DNA by using the Lipofectamine plus method (Invitrogen) according to the manufacturer’s instructions. For promoter activity assay, cells were cotransfected with 1 µg HDGF-driven luciferase vector (32) or E-cadherin–driven luciferase vector (generous gift from Dr. Yu-Sun Chang, Chang Gen University, Taipei, Taiwan; ref. 33) and the 0.2 µg Renilla reniformis luciferase reporter vector (Promega). The luciferase activities in cells were determined using a Dual-Light kit (Promega) in a luminometer (Microplate Plus LB96V; Berthold Technologies) and normalized with that of R. reniformis luciferase according to manufacturer’s instructions.

**Statistical analysis**

Differences between the groups were statistically evaluated using the one-way ANOVA. The results are presented as mean ± SEM. All P values were 2-tailed, and a P < 0.05 was considered to be statistically significant.

**Results**

**POMC gene delivery inhibited the invasiveness of melanoma cells in vitro and lung metastasis of B16-F10 melanoma in vivo**

To evaluate the antimetastatic potential of POMC gene delivery, we investigated the invasiveness of murine (B16-F0) and human (A2058) melanoma cells. It was found that POMC overexpression significantly retarded the matrix-penetrating capability in these melanoma cells (Fig. 1A). Subsequently, we investigated the antimetastatic efficacy of POMC therapy on lung metastasis of B16-F10 melanoma cells in mice. For quantification analysis of metastatic events, mice were administrated with B16-F10 cells that expressed firefly luciferase (Luc-B16-F10) at day 0 and were treated with adenovirus vectors on day 1 and then subjected to bioluminescence analysis at various time intervals (Supplementary Fig. S1A). It was shown that the bioluminescence intensities in lungs of Ad-POMC–treated mice were significantly weaker than those of control groups on day 14 (Fig. 1B). Microscopic analysis revealed that lung tissues in Ad-POMC–treated mice were relatively intact with few visible foci, whereas it was grossly damaged with numerous colonies in control groups (Fig. 1C). Histologic analysis further validated that the metastatic colonies were prominently reduced in the lung tissue of Ad-POMC–treated mice (Fig. 1C).

To further investigate the time required for systemic POMC therapy to elicit metastatic suppression, bioluminescence analysis was conducted on days 0, 7, and 14 after induction of lung metastasis. It was found that POMC therapy significantly reduced the lung bioluminescence intensities on days 7 and 14 (Supplementary Fig. S2), suggesting that POMC therapy effectively perturbed the lung metastasis within 7 days of treatment.

**POMC gene delivery suppressed the neovascularization and colonization of metastatic melanoma in lungs**

As angiogenesis plays a pivotal role in tumor metastasis and colonization, we then investigated the influence of POMC therapy on lung neovascularization and colonization of metastatic melanoma. After received the
intravenous injection of GFP-B16-F10 cells for 14 days, mice were sacrificed after injection of the fluorescent dye (rhodamine label-dextran) for vessels tracking and histologic analysis (Supplementary Fig. S1B). Simultaneous analysis of rhodamine label-dextran and GFP fluorescence revealed that both metastatic foci and neovascularization in lungs were prominently reduced in Ad-POMC–treated compared with Ad-GFP–treated mice (Supplementary Fig. S3A). Moreover, CD31 immunostaining also unveiled a significant reduction in the number and size of CD31-positive blood vessels in Ad-POMC–treated foci compared with control (Supplementary Fig. S3B). Thus, these findings indicate that POMC gene delivery attenuates the lung metastasis of B16-F10 melanoma cells through inhibition of neovascularization, thereby perturbing the colonization of metastatic melanoma in lungs.

**POMC gene delivery reversed the EMT of melanoma through upregulation of E-cadherin and downregulation of vimentin and α-SMA**

As the EMT is involved in tumor metastasis, we evaluated whether POMC therapy affected the EMT of melanoma cells by examining the expression of EMT marker molecules. By using Western blot and flow cytometry analysis, it was shown that POMC gene delivery significantly elevated the protein level of E-cadherin, an epithelial marker, while reducing the protein levels of mesenchymal markers, including vimentin and α-SMA, in melanoma cells (Fig. 2A). Flow cytometric analysis also confirmed that POMC gene delivery enhanced the cell surface expression of E-cadherin in B16-F10 melanoma cells (Fig. 2B). Histologic analysis showed that E-cadherin immunostaining was increased whereas both vimentin and α-SMA immunostaining intensities were decreased in Ad-POMC–treated melanoma tissues (Fig. 2C). These observations strongly suggest POMC therapy attenuates melanoma metastasis through EMT modulation in vitro and in vivo.

**POMC gene delivery elicited HDGF downregulation in B16-F10 cells and metastatic melanoma**

In addition to regulating EMT genes expression, we searched for the potential genes that contributed to the antimetastatic function of POMC therapy by microarray analysis. As showed in Table 1, POMC gene delivery significantly decreased the gene expression of fibronectin, thymosin β4 (Tβ4), connective tissue growth factor (CTGF), and HDGF. As the role of fibronectin, Tβ4 and CTGF during melanoma metastasis has been reported (34), we delineated whether HDGF downregulation was involved in POMC-induced metastatic inhibition. By using real-time (RT)-PCR and promoter assay, we found that POMC gene delivery significantly decreased the gene expression of HDGF in melanoma cells (Fig. 3A and B). Similarly, we also showed that POMC gene delivery decreased the protein levels of HDGF by Western blot analysis (Fig. 3C). Moreover, immunohistochemical study revealed the prominent reduction in HDGF immunostaining in Ad-POMC–treated metastatic melanoma in vivo but not in Ad-GFP–treated groups (Fig. 3D).
Exogenous HDGF supply failed to reverse the POMC-induced suppression of invasiveness and EMT change in melanoma cells

Although elevated HDGF is associated with the tumor progression in melanoma (11), the function of HDGF in invasiveness and EMT in melanoma cells has not been validated. As POMC gene delivery reduced HDGF expression in melanoma cells, we investigated whether exogenous HDGF supply affected the invasiveness and EMT of B16-F10 melanoma cells. It was found that exogenous HDGF significantly stimulated the invasion (Fig. 4A) and EMT in melanoma cells (Fig. 4B). However, even

Table 1. Genes downregulated in Ad-POMC–infected B16-F10 melanoma cells by microarray analysis

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Normalized ratio (vs. Ad-GFP)</th>
<th>P</th>
<th>Quantitative RT-PCR ratio (vs. Ad-GFP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatoma-derived growth factor (Hdgf)</td>
<td>NM_008231</td>
<td>0.749</td>
<td>0.012</td>
<td>0.57 ± 0.048a</td>
</tr>
<tr>
<td>Thymosin, beta 4, X chromosome (Tmsb4x)</td>
<td>NM_021278</td>
<td>0.547</td>
<td>0.001</td>
<td>0.51 ± 0.026a</td>
</tr>
<tr>
<td>Fibronectin 1 (Fn1)</td>
<td>NM_010233</td>
<td>0.556</td>
<td>0.021</td>
<td>0.65 ± 0.028a</td>
</tr>
<tr>
<td>Connective tissue growth factor (Ctgf)</td>
<td>NM_010217</td>
<td>0.688</td>
<td>0.003</td>
<td>0.57 ± 0.075a</td>
</tr>
</tbody>
</table>

*aStatistically significant (P < 0.05).*
in the presence of excessive HDGF, POMC gene delivery still potently suppressed the invasion of melanoma cells (Fig. 4A). Moreover, HDGF treatment failed to abrogate the POMC-mediated downregulation of vimentin and α-SMA as well as E-cadherin upregulation (Fig. 4B). Therefore, exogenous HDGF supply was not sufficient to abolish the antimetastatic function of POMC therapy in melanoma cells.

Mimicking POMC gene delivery by POMC-derived peptides perturbed invasiveness and EMT in melanoma cells

To identify the neuropeptide(s) that contributed to the inhibition of invasion and reduction of EMT change by POMC gene delivery, B16-F10 were treated with various POMC peptides (ACTH and α-, β-, γ-MSH) to recapitulate the effect of POMC gene delivery. For invasion capacity, it was shown that application of ACTH, α-MSH, and β-MSH significantly retarded the matrix-penetrating capability in these melanoma cells (Fig. 5A). In contrast, γ-MSH treatment had no effect. Similarly, treatment with ACTH, α-, and β-MSH significantly decreased the HDGF expression in B16-F10 cells whereas γ-MSH had no such effect (Fig. 5B). Moreover, by evaluating the effect of POMC-derived peptides on EMT change of melanoma, it was found that application of ACTH, α-, or β-MSH significantly increased the E-cadherin expression whereas γ-MSH had no such effect (Fig. 5C). Therefore, the POMC-derived melanocortins, including ACTH, α-, and β-MSH but not γ-MSH, participated in POMC-mediated inhibition of tumor invasion and EMT change in melanoma cells.

Discussion

The novel findings of the present study are: (i) that systemic POMC gene delivery by posttreatment shows that significant retards lung metastasis via reduced tumor invasion, colonization, and angiogenesis in established melanoma. The systemic POMC expression seems tolerable given no obvious changes in body weight and feeding behavior was observed in mice receiving POMC therapy. (ii) Our findings also show that POMC gene delivery potentially inhibits EMT change by E-cadherin upregulation and vimentin, α-SMA downregulation, which contributes to tumor cell invasion and metastasis in melanoma in vitro and in vivo. (iii) Through the microarray
analysis, our data suggest that POMC gene delivery decreased the expression of HDGF, which contribute to inhibition of tumor invasion via reducing EMT change. Moreover, exogenously supplied HDGF studies confirm that HDGF expression directly regulates the invasion and EMT of melanoma cells. Together with our early studies showing that POMC therapy suppresses melanoma through MC1R/NFκB signaling and angiogenesis.
inhibition (20, 21), we herewith provide a hypothetical model for POMC-induced metastasis suppression through HDGF depletion, which leads to EMT perturbation, and angiogenesis inhibition (Fig. 5D).

Our studies showed that POMC gene delivery inhibited the motility and invasiveness in human and mice melanoma cells that suggests attenuation of invasion is involved in the POMC-mediated suppression of metastasis. In lung metastasis model, B16-F10 melanoma cells are directly injected into the bloodstream and metastatic colonies form more rapidly than that in the primary melanoma model, thereby allowing the rapid screening of antimitastatic efficacy of therapeutic agents. Current study, POMC therapy was initiated 24 hours after melanoma cells entered the circulation. On the basis of our previous studies (21), an additional 12 to 24 hours was required for the production of anti-inflammatory peptides in circulation after POMC transgene expression and processing in the liver. Despite the delayed therapy, a significant reduction in lung by bioluminescence image was observed in Ad-POMC–treated mice at as early as day 7 after implantation. Thus, systemic POMC therapy was robust even when the tumor cells took a 24-hour head start entering the circulation before the treatment began.

Tumor neovascularization plays critical roles for the development, progression, and metastasis of cancers (35), and novel therapeutic approaches have been developed for treatment of malignancies by controlling angiogenic activities (36). Our previous studies showed that POMC gene transfer disrupted the angiogenic processes of cultured endothelial cells (31, 37) and primary tumor neovascularization through α-SMA signaling pathway (21). In the present study, we further showed that systemic POMC therapy elicited the neovascularization blockade in metastatic melanoma by using fluorescent dye tracking and CD31 immunostaining. The striking correlation between neovascularized vessels and metastatic melanoma nodules strongly supports the dependence of newly colonized melanoma cells on neovascularization. Because HDGF is an angiogenic factor (12, 38), the decreased HDGF expression would likely contribute to the POMC-mediated angiogenesis inhibition and metastasis suppression. Together, these findings support that neovascularization blockade is involved in the antimitastatic mechanism of POMC therapy.

EMT was originally described by embryologists and occurs in many developmental processes; however, it is also a key step in cancer progression when cells acquire invasive behavior and disseminate (39, 40). Downregulation or loss of E-cadherin results in dedifferentiation, gain of invasiveness, and promotion of EMT in carcinoma cells including malignant melanoma (41). Our finding reveals that E-cadherin protein level is upregulated, whereas vimentin and α-SMA are downregulated in Ad-POMC–treated melanoma cells and lung colonies. Microarray analysis unveiled that POMC is a potent antimitastatic suppressor by simultaneous repression of multiple prometastatic genes such as fibronectin, CTGF (42, 43), TGFβ (27, 44), and HDGF (45). Our recent study showed that HDGF upregulation is correlated with recurrence, lymph node metastasis, and EMT in patients with breast cancer (15). Moreover, HDGF protein or gene delivery also regulates the EMT in breast cancer cells through modulation of E-cadherin and vimentin expression. Here, our findings show that inhibition of HDGF might contribute in POMC-mediated inhibition of metastatic and tumor angiogenesis in melanoma progression. However, a recent study has shown that melanocyte-specific HDGF overexpression did not lead to oncogenic transformation of melanocytes (46). The discrepancy between these studies remains to be resolved.

In summary, our study provides the proof-of-principle evidence supporting POMC therapy for control of metastatic melanoma. Moreover, POMC therapy elicited downregulation of HDGF expression, which may subsequently perturb the EMT process in melanoma. Future studies are warranted to systematically evaluate the POMC-based therapy for management of metastatic melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: H.-E. Tsai, G.-S. Liu, L.-F. Liu, J.-C. Wu, C.-H. Huang, H.-C. Lam, C.-S. Wu, M.-H. Tai

Development of methodology: H.-E. Tsai, G.-S. Liu, J.-C. Wu, S.-C. Chen, M.-H. Tai

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.-E. Tsai, G.-S. Liu, M.-L. Kung, C.-H. Tang, M.-H. Tai

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.-E. Tsai, G.-S. Liu, M.-H. Tai

Writing, review, and/or revision of the manuscript: H.-E. Tsai, G.-S. Liu, L.-F. Liu, S.-C. Chen, M.-H. Tai

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-E. Tsai, M.-L. Kung, L.-F. Liu, C.-H. Huang, S.-C. Chen, M.-H. Tai


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