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European Journal of Cancer 37 (2001) 1413–1418

European Journal of Cancer

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Overexpression of HER-2/*neu* enhances the sensitivity of human bladder cancer cells to urinary isoflavones

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Received 23 October 2000; received in revised form 24 January 2001; accepted 15 February 2001

Abstract

Soybean foods have been suggested to be practical chemopreventives for human urinary tract cancers. Recently, we demonstrated that the co-operative action of isoflavones results in an increased dose-dependent growth inhibition and apoptosis than any single isoflavone compound. This study aimed to examine the potential of HER-2/*neu* as a biological target for soy isoflavones. The sensitivity of the bladder cancer cell lines ($n=7$) to the isoflavones was inversely related to the amount of HER-2/*neu* expressed. By using HER-2/*neu* transfection experiments, all three stable transfectants showed a significant growth inhibition by the isoflavone mixture at concentrations attainable in normal adult urine. An increased inhibition of tyrosine phosphorylation of proteins immunoprecipitated by HER-2/Neu was observed in the *neu*-transfectants compared with controls. The results of this study suggest that HER-2/*neu* may be a practical biochemical target for urinary isoflavones *in vivo*. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Bladder cancer; Chemoprevention; Soy isoflavones; HER-2/*neu*; Growth inhibition; Tyrosine phosphorylation

1. Introduction

Transitional cell carcinoma (TCC) is the most important cancer of the urinary tract and generally has a protracted clinical course. Animal studies have found that a soybean diet has a protective effect on bladder carcinogenesis in Swiss albino mice [1] and on a transplantable murine tumour [2]. The inhibitory effects of soy isoflavones on human bladder cancer cells have also been confirmed *in vitro* and *in vivo* [2,3]. Given that the IC_{50} values of most cancer cell lines (3–5 μg/ml or 7.9–13.2 μM) are within reach of the urine levels of daidzein (14.7 μM) and genistein (8.4 μM) following a soy challenge [4], the physiologically relevant targets and the mechanisms by which isoflavones exert their anticancer effects need to be clarified.

Since the discovery that genistein is a potent inhibitor of tyrosine kinase activity in mammalian cells, epidermal growth factor receptor (EGFR) was suggested to be the main target [5]. However, neither EGF-stimulated EGFR tyrosine autophosphorylation nor tyrosine phosphorylation of the intracellular target proteins could be demonstrated at concentrations of genistein showing growth inhibition [6,7]. Other tyrosine kinase genes warrant investigation in the context of the anticancer effects of soy isoflavones.

The HER-2/*neu* gene, the second member of the EGFR family receptors, encodes a 185-kDa transmembrane growth factor receptor with intrinsic tyrosine activity. Experiments *in vitro* have suggested a central role for HER-2/*neu* in mediating the responses of EGFR family receptors to their cognitive ligands, and that these altered ligand-dependent responses play an important role in tumorigenesis *in vivo* [8]. Since HER-2/*neu* could be expressed in premalignant lesions of urinary bladders [9], this study was performed to examine the potential of HER-2/*neu* as a biological target for soy isoflavones. By using a HER-2/*neu* transfection experiment, the effects of the isoflavones were examined

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PII: S0959-8049(01)00110-1

2. Materials and methods

2.1. Cell lines and cell culture

The human bladder cancer cell lines Sca-BER, TCC-SUP, HT-1197, T24, HT-1376, 5637 and J82 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The BFTC-905 and TSGH8301 cell lines were established locally and propagated for use as previously described in Ref. [3]. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL, NY, USA) containing 10% fetal bovine serum (GibcoBRL, NY, USA) in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. Each cell line was seeded at 1×10^4 cells/well for each experiment.

To analyse the effectiveness of growth inhibition, appropriate concentrations of isoflavones, including genistein (GibcoBRL, NY, USA), daidzein (Calbiochem-Novabiochem, CA, USA) and/or biochanin-A (Sigma Chemical Co., MO, USA) were added into each well and incubated with the cells for 3 days. Then the cell number was counted using the crystal violet elution method [3]. Dimethyl sulphoxide (DMSO; Sigma Chemical Co., MO, USA) at a concentration of 0.5% (v/v) was used as a control. The cell cycle analysis and the measurement of apoptosis were estimated by fluorescent activated cell sorter (FACS)sort cytometer (Becton Dickinson, San Jose, CA, USA) according to the procedure described earlier in Ref. [3].

2.2. Western blot analysis

Total cell lysate was prepared by directly adding 150 μl of 2×sodium dodecyl sulphate (SDS) loading buffer (50 mM Tris–HCl; pH 6.8, 2% SDS, 10% glycerol, and 100 mM dithiothreitol) to confluent cells grown on a 10-cm dish, and detaching the cells with a rubberpoliceman. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA). 25 μg of total cell lysates were separated by SDS polyacrylamide gel electrophoresis (SDS–PAGE) on an 8% gel. The proteins were electro-transferred onto a nitrocellulose membrane at 100 mA for 2 h by using a TE70 semidry transfer unit (Amersham Pharmacia Biotech Inc. NJ, USA). Equal loading of cell lysates was confirmed by staining the membrane with amido black before antibody probing. Then the nitrocellulose membrane was blocked with 5% skimmed milk (Difco Laboratories, Inc., MI, USA) for 1 h, followed by incubation with the monoclonal antibody for HER-2/*neu* (Ab-3, Oncogene Science, NY, USA) for 2 h as previously described in Ref. [3]. The protein bands were visualised with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech Inc. NJ, USA) using horseradish peroxidase-labelled secondary antibody as suggested by the manufacturer. Finally, the membrane was stripped and reprobed for actin as a control for loading and transfer.

2.3. Transfection of the *HER-2*/neu *gene*

Plasmids for the expression of the wild-type rat HER-2/*neu* gene were generous gifts from Dr Mien-Chie Hung (University of Texas, M. D. Anderson Cancer Center, USA). TCC-SUP cells with the lowest expression of HER-2/*neu* among the bladder cancer cell lines were selected for the transfection experiments. HER-2/*neu* was introduced by a calcium phosphate transfection procedure described in Ref. [10]. Either pSV-*neu* or pMam-*neo* (Clonetech Inc., CA, USA) were cotransfected at a ratio of 10:1. Then G418-resistant transfectants were isolated from the medium containing 400 μg/ml of G418.

2.4. Soft agar assay

Cells (2×10^4) were seeded in six-well plates in semi-solid medium containing 0.33% Bacto Agar (Difco Laboratories, Inc., MI, USA) supplemented with 10% fetal calf serum layered on top of the 0.6% Bacto Agar layer. Colonies were scored after 2 weeks of incubation with or without treatment of the isoflavone mixture (3 μg/ml) at 37°C in 5% CO_2 in air. Each experiment was performed in triplicate.

2.5. Immunoprecipitation and phosphotyrosine immunoblotting

For tyrosine phosphorylation analysis, TSGH8301, TCC-SUP and TCC-SUP-*neu* cell lines cultured to 80% confluence were incubated with appropriate concentrations of isoflavones for 12 h, and then were lysed as described for the Western blotting experiments. A total of 100 μg of total cell lysates were incubated with Ab-3 antibody overnight followed by a further 60-min incubation with 40 μl of protein-A-Sepharose (Amersham Pharmacia Biotech Inc., NJ, USA). Immune complexes were washed three times in cold extraction buffer and eluted by boiling in non-reducing Laemmli buffer. Eluted proteins were then boiled with β-mercaptoethanol and resolved by SDS–PAGE on a 10% gel. The resolved proteins were transferred and immunoblotted with monoclonal anti-phosphotyrosine antibody (Sigma Chemical Co., MO, USA) at dilution of 1:1000 as previously described in detail in Ref. [11].

2.6. Statistical analysis

Results are expressed as means±standard deviation. Unpaired Student's *t*-test (two sides) was used to determine the differences in the IC_{50} values by Statworks (Cricket Software, Inc., Sale, Australia). The level of significance was set at $P<0.05$.

3. Results

3.1. The sensitivity of human bladder cancer cells to isoflavones

The effects of isoflavones on human bladder cancer cell lines ($n=7$) were tested with genistein, daidzein and biochanin-A, either individually or as an equal-proportion mixture of all three compounds at various concentrations. The IC_{50} values of each cell line are summarised in Table 1; the results for TSGH8301, Sca-BER, BFTC905, T24, HT-1376 and J82 have been previously reported [3]. Genistein was among the most potent growth inhibitor, followed by biochanin-A and daidzein, respectively. The isoflavone mixture showed a greater inhibitory effect than any single compound. The TCC-SUP, HT-1197 and HT-1376 cells had significantly higher IC_{50} values than the rest of cell lines ($P<0.05$).

3.2. The relationship between HER-2/neu expression and sensitivity to isoflavones

Expression levels of HER-2/*neu* were determined by Western blotting. Most bladder cancer cell lines were found to have a high HER-2/*neu* expression, while TCC-SUP had the lowest expression which was below the level of detection (Fig. 1). There was a trend towards higher IC_{50} values for the cancer cells expressing a low/undetectable level of HER-2/*neu* (HT-1376, HT-1197 and TCC-SUP) than those cells expressing higher levels of HER-2/*neu* (Table 1). The results imply that overexpression of HER-2/*neu* may enhance the sensitivity of bladder cancer cells to isoflavone treatment.

3.3. Establishment of TCC-SUP-neu transfectants

To confirm the causal relationship between HER-2/*neu* expression and sensitivity to isoflavones, TCC-SUP cells were selected to express the exogenous HER-2/*neu* gene. A total of three stable transfectants (TCC-SUP-N5, TCC-SUP-N10 and TCC-SUP-N14) were obtained in this experiment. Overexpression of HER-2/*neu* was confirmed in the three transfectants, but not in the control (pMam-*neo*) (Fig. 2).

Table 1
The IC_{50} values of isoflavones (μg/ml) on human bladder cancer cell lines[a]

Cell lines	HER-2/*neu*	Genistein	Biochanin-A	Daidzein	Mixture[b]
TSGH8301	High	3±0.2	12±3	40±0.1	2±1
Sca-BER	High	5±0.1	15±0.1	40±0.1	4±1
BFTC905	High	5±0.1	15±0.1	35±5	4±1
T24	High	7±2	15±0.1	45±5	5±0.1
5637	High	8±2	12±3	36±4	5±1
J82	High	10±0.2	15±0.1	40±0.2	5±0.1
HT-1376	Low	25±4	30±5	50±6	10±2
HT-1197	Low	17±3	20±3	40±0.1	10±2
TCC-SUP	Low	20±0.1	25±0.1	40±0.1	12±3

[a] Cells were treated various concentrations of isoflavones, and the viable cells were determined by the crystal violet method after exposure to the drugs for 3 days. The values obtained are from triplicate experiments and are means±standard deviations (S.D.).

[b] Genistein + biochanin-A + daidzein.

3.4. The effect of HER-2/neu expression on the sensitivity of bladder cancers to isoflavones

The *neu*-transfectants were then tested for their sensitivity to isoflavones at concentrations achievable in human urine (Table 2). All three transfectants were more sensitive to isoflavones than TCC-SUP ($P<0.05$), but there was no apparent difference between the *neu*-transfectants and the cancer cells expressing high levels of HER-2/*neu*. In addition, apoptosis was demonstrated in the *neu*-transfectants by flow cytometric analysis whereas this did not occur in the TCC-SUP cells (Fig. 3). The results support the theory that overexpression of HER-2/*neu* enhances the sensitivity of bladder cancer cells to urinary isoflavones. In addition, the IC_{50} values of the *neu*-transfectants (1–3 μg/ml) are within reach of the urine levels of isoflavones after soy challenge [4], supporting the clinical relevance of HER-2/*neu* as one of the biochemical targets for urinary isoflavones *in vivo*.

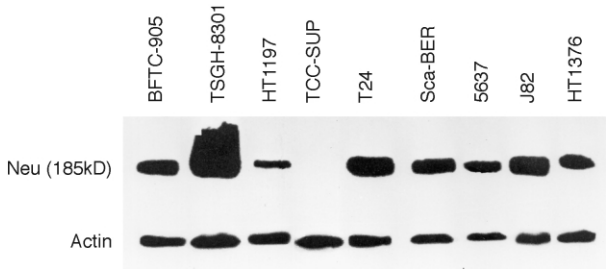

Fig. 1. Western blot analysis of HER-2/*neu* in the bladder cancer cell lines. The position of the HER-2/*neu* protein is indicated on the left and nine bladder cancer cell lines are shown. The human actin protein level was used as an internal control for loading and transfer.

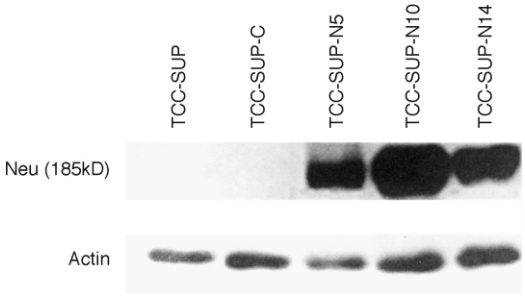

Fig. 2. Western blot analysis of HER-2/*neu* in HER-2/*neu* transfectants established from TCC-SUP cells. The position of the HER-2/*neu* is indicated on the left. TCC-SUP, normal HER-2/*neu* transfectants (N5, N10, N14) and the control transfectant (TCC-SUP-C) are shown. The human actin protein level was used as an internal control for loading and transfer.

To test our hypothesis, the anchorage-independent growth of HER-2/*neu* transfectants was determined *in vitro* by a soft agar assay. The colony number of transfectants (N5, N10 and N14) was significantly higher from 13 to 23 times, than that of the control transfectants and TCC-SUP cells (Fig. 4). The isoflavone mixture (3 μg/ml) successfully suppressed the growth of all three transfectants, supporting the theory that urinary isoflavones may inhibit the tumorigenicity of HER-2/*neu* high-expressing bladder cancer cells *in vivo*.

3.5. Downregulation of tyrosine phosphorylation by the isoflavones

To elucidate the molecular mechanisms through which the isoflavones inhibit the growth of cancer cells that express high levels of HER-2/*neu*, tyrosine phosphorylation of the *neu*-transfectants was compared with TSGH8301 cells after treatment with the isoflavones. There was a dose-dependent inhibition of tyrosine phosphorylation by the isoflavones, with the isoflavone mixture being the most potent suppressor. Representative results for TSGH8301 cells and the *neu*-transfectants (N5 and N14) are shown in Fig. 5. There was a trend towards a higher inhibition of tyrosine phosphorylation in the *neu*-transfectants than that of TSGH8301 cells. However, there seems to be variation in the inhibitory effect of the different isoflavone compounds, and in the effects on the TSGH8301 and *neu*-transfectants, implying that an additional mechanism may be involved in HER-2/*neu*-related growth inhibition. More studies are underway to verify our hypothesis.

4. Discussion

The development of bladder cancer is postulated to be a multi-step and multifocal (field effect) process, possibly involving the spread of premalignant clones along the urothelial mucosa. As a result, neoplastic lesions of the bladder are uniquely suited to the development and evaluation of chemopreventive approaches. Because carcinogens are concentrated in the urine, high concentrations of chemopreventive agents are required to exert a systemic or local effect on the mucosa of the upper urinary tract and bladder. Since isoflavone levels after soy intake are highest in the urine compared with other human body fluids [12], the molecular mechanisms of the biological effects of the physiological levels found in human urine deserve thorough investigation.

We have demonstrated that isoflavones induce a G2/M cell cycle arrest and/or apoptosis of bladder cancer cells at concentrations attainable in normal adults, and that downregulation of cdc2 kinase activity is one of the molecular mechanisms responsible for this cell cycle arrest [3]. In this study, we showed that the IC_{50} values of HER-2/*neu* transfectants were significantly lower than in the parental cells, suggesting that isoflavones

Table 2
The IC_{50} values of the HER-2/*neu* transfectants and the parental cell line[a]

Cell lines	Genistein	Biochanin-A	Daidzein	Mixture[b]
TCC-SUP	20±0.1	25±0.1	40±0.1	12±3
TCC-SUP-C	18±3	24±3	42±4	14±2
TCC-SUP-N5	3±0.1	12±3	27±3	2±1
TCC-SUP-N10	3±0.1	12±3	30±0.1	2±0.2
TCC-SUP-N14	3±0.1	12±3	25±0.1	2±0.1

[a] Cells were treated with various concentrations of isoflavones, and the viable cells were determined by the crystal violet method after exposure to the drugs for 3 days. The values obtained are from triplicate experiments.

[b] Genistein + biochanin-A + daidzein.

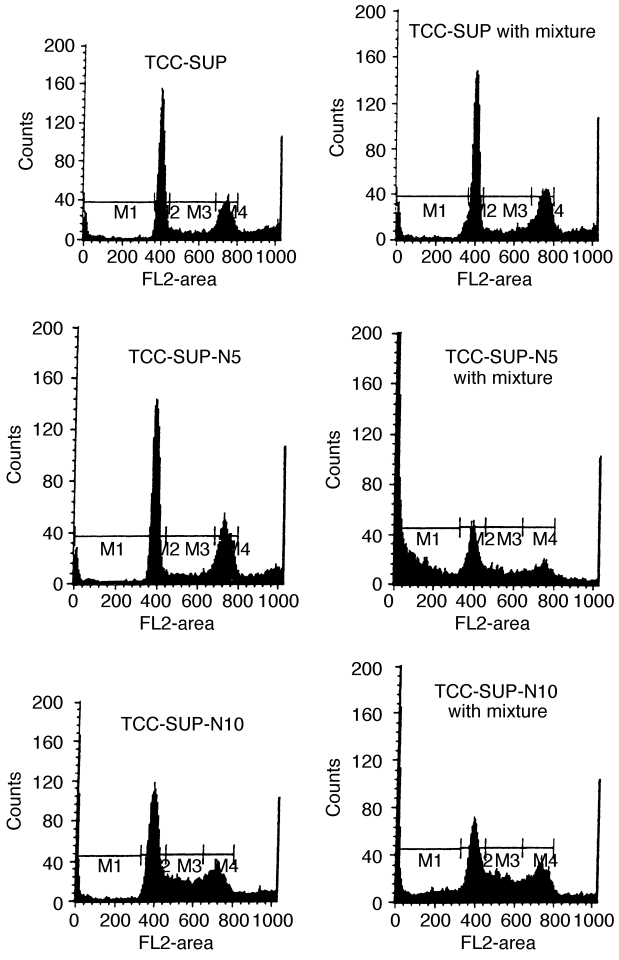

Fig. 3. Apoptosis induction in HER-2/*neu* transfectants. Cells (1×10^5) were cultured with or without treatment with isoflavones. The cell cycle status and apoptosis were estimated by flow cytometry. Representative data from TCC-SUP, TCC-SUP-N5, and TCC-SUP-N10 without treatment are shown on the left-hand side. After treatment with the isoflavone mixture (3 μg/ml) for 48 h, an apoptotic peak appeared with a G2/M cell cycle arrest occurring in the HER-2/*neu* transfectants compared with TCC-SUP (see right-hand side).

selectively inhibit the HER-2/*neu*-related signalling pathway. By using transfection experiments, overexpression of HER-2/*neu* was estimated to enhance the sensitivity to isoflavones from 5- to 7-fold. The successful inhibition of tyrosine phosphorylation in HER-2/*neu* transfectants supports the theory that the receptor is one of the biochemical targets for urinary isoflavones *in vivo*. Moreover, studies are underway to elucidate the additional mechanism(s) involved in HER-2/*neu*-mediated growth arrest induced by isoflavones.

A number of clinical studies have examined the prognostic value of individual HER-2/*neu* receptor expression in human bladder cancers; however, the results are contradictory in different series [13–17]. Despite this, experiments *in vitro* have demonstrated a stronger biological effect for HER-2/*neu*-containing heterodimers of EGFR family receptors than the respective homodimers [18]. HER-2/*neu* was also found to sensitise tumour cells to the mitogenic effects of heterologous growth factors by retarding the degradation of liganded EGFR heterodimers [19]. However, co-expression of HER-2/*neu* with ErbB3 may contribute to the progression of breast cancer through epidermal growth factor and/or betacellulin stimulation [20], suggesting that HER-2/*neu* may be the master regulator for the interactive network of EGFR family receptors *in vivo* [8].

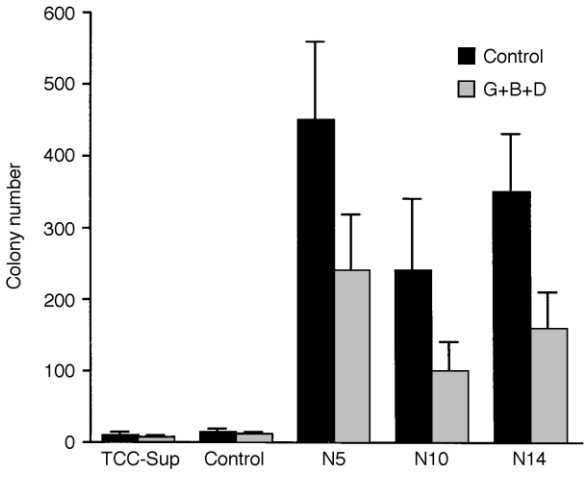

Fig. 4. The effect of isoflavones on anchorage-independent growth of HER-2/*neu*-overexpressing bladder cancer cells. Anchorage-independent growth was determined *in vitro* by a soft agar assay. G + B + D = isoflavone mixture.

Further support for the clinical relevance of HER-2/*neu* comes from a recent clinical study showing that natural immunity to EGFR family receptors, either single receptor or multiple (two or three) subfamily members, can be detected in approximately half of the sera from patients with different types of epithelial cancer [21]. In addition, antibody against HER-2/*neu* was among the most frequently detected immune response, implying a crucial role for HER-2/*neu*-associated signalling in the development of human cancer. Since HER-2/*neu* is aberrantly expressed in the field mucosa of bladder cancer patients [9], the receptor protein may serve as an excellent target for chemoprevention. Given that the IC_{50} values of all *neu*-transfectants are within reach of urine levels of isoflavones [4], our data support the notion that soybean foods are practical chemopreventives for bladder cancer, especially for those HER-2/*neu*-positive tumours [3].

Taken together, our study suggests that HER-2/*neu* may be one of the biochemical targets for urinary isoflavones *in vivo*, and thus is an excellent candidate for developing anticancer agents for bladder cancer. Additional investigations are required to examine the implication of our hypothesis for carcinomas of other cell lineages.

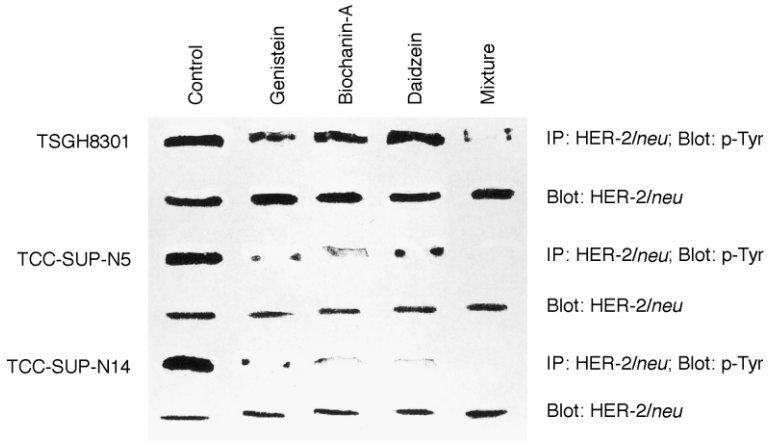

Fig. 5. Inhibition of tyrosine phosphorylation by soy isoflavones. Levels of tyrosine phosphorylation of HER-2/*neu* receptor were examined after incubation with each isoflavone compound (20 μg/ml) or their cocktail mixture for 12 h. After immunoprecipitation with Ab-3, the membranes were immunoblotted with anti-phosphotyrosine (p-Tyr) antibody (upper panels). The same amounts of total cell lysates were transferred onto another membrane and probed with anti-HER-2/*neu* antibody for comparison (lower panels).

Acknowledgements

This study was supported by NSC 89-2314-B-006-027 from the National Science Council, and by NHRI-GT-EX89 from the National Health Research Institutes, TAIWAN, Republic of China.

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