RESEARCH ARTICLE

Orphan Nuclear Receptor Nurr1 as a Potential Novel Marker for Progression in Human Prostate Cancer

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Abstract

A number of studies have indicated that Nurr1, which belongs to a novel class of orphan nuclear receptors (the NR4A family), is important for carcinogenesis. Here we investigated expression of Nurr1 protein in benign and malignant human prostate tissues and association with clinicopathologic features using immunohistochemical techniques. Moreover, we also investigated the ability of Nurr1 to influence proliferation, migration, invasion and apoptosis of human prostate cancer cells using small interfering RNA silencing. Immunohistochemical analysis revealed that the expression of Nurr1 protein was higher in prostate cancer tissues than in benign prostate tissue ($P < 0.001$), levels being positively correlated with tumor T classification ($P = 0.003$), N classification ($P = 0.017$), M classification ($P = 0.011$) and the Gleason score ($P = 0.020$) of prostate cancer patients. *In vitro*, silencing of endogenous Nurr1 attenuated cell proliferation, migration and invasion, and induced apoptosis of prostate cancer cells. These results suggest that Nurr1 may be used as an indicator for prostate cancer progression and be useful for novel potential therapeutic strategies.

Keywords: Clinical progression - Nurr1 - prostate cancer - PC-3 cells

Asian Pacific J Cancer Prev, **14 (3)**, 2023-2028

Introduction

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer-associated death among men in the USA (Siegel et al., 2012). The incidence of prostate cancer in Asian countries, while still lower than in Western nations, is increasing rapidly due to a more westernized lifestyle (Namiki et al., 2010). Important issues in the care of patients with prostate cancer are how to screen and diagnose prostate cancer earlier, and how to determine a therapeutic strategy based on outcome prediction. Biomarkers could play a pivotal role in accomplishing these tasks. Despite extensive research efforts, very few biomarkers of prostate cancer, such as prostate specific antigen (PSA), have been successfully implemented into clinical practice (Bjartell et al., 2011; Shariat et al., 2011; Zhou et al., 2012). Thus, it is essential to seek new valuable biomarkers for diagnosis, disease monitoring, prognosis and development of new targeted therapies.

Nurr1 (NOT/NR4A2) belongs to a NR4A family of nuclear receptors that includes two other members, Nor-1 (MINOR/NR4A3) and Nur77 (NGIF-B/NR4A1). It exerts an important function in a number of biological processes, including regulation of proliferation (Bonta et al., 2010; Sirin et al., 2010), apoptosis (Ke et al., 2004), migration (Maijenburg et al., 2012) and differentiation (Lee et al., 2010) in a cell type-specific manner. Recently, the oncogenic activities of Nurr1 are emerging. Nurr1 may stimulate progression of colorectal cancer by protecting cell survival (Holla et al., 2006), and induce suppression of apoptosis in cervical cancer cells (Ke et al., 2004), and is an independent prognostic marker for tumor progression and survival in patients with bladder cancer (Inamoto et al., 2010). To date, there have been no studies regarding the significance of Nurr1 expression in human prostate cancer. It is noteworthy that increased levels of thromboxane A2 receptor (TPR) in the prostate cancer were all significantly associated with higher Gleason scores and pathologic stages (Dassesse et al., 2006), moreover, activation of thromboxane A2 receptors may induce Nurr1 expression (Li and Tai, 2009). Therefore, we hypothesized that prostate cancer progression is associated with the level of human Nurr1 protein expression.

On the basis of these observations, we examined Nurr1' association with clinicopathologic features of prostate cancer, and identified the effects of Nurr1 on cell growth, migration and invasion, and investigated the role of Nurr1 in the pathogenesis and progression of prostate cancer.

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Materials and Methods

Clinical specimens

One hundred and eighteen PCa specimens and 22 benign prostate tissue specimens were collected from the patients registered at People's Hospital of Jinzhou City (Jinzhou, China) between 2006 and 2011 with patients' consent and ethical committee approval. The PCa specimens were derived from patients undergoing radical prostatectomy, and the benign prostate tissue samples were derived from benign prostatic hyperplasia (BPH) patients. All clinical and clinicopathologic data, including age, Gleason score and tumor node metastasis (TNM) staging, were obtained from medical records. The median age of the 118 patients with PCa was 70 years (mean 71 years, range 42–82 years), with 45 cases aged less than 70 years and 73 cases exceeding 70 years. In all PCa specimens, 42 cases had a Gleason score of 4–6 and 76 cases had a Gleason score of 7–10. The distribution of TNM stage in all PCa specimens was as follows: T_1-T_2 in 56 cases and T_3-T_4 in 62 cases; N_0 in 90 cases and N_1 in 28 cases; and M_0 in 94 cases and M_1 in 24 cases.

Immunohistochemistry

The expression of Nurr1 in human prostate tissues was assayed by immunohistochemical method based on routine protocols. In brief, antigens in 5-μm sections prepared from formalin-fixed and paraffin-embedded specimens were exposed by boiling in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. After blocking with Bovine Serum Albumin (BSA) (Santa Cruz, CA, USA) for 30 min, tissue sections were incubated with primary rabbit anti-Nurr1 polyclonal antibodies (diluted in the ratio 1: 100, Santa Cruz, CA, USA) at 4 ºC overnight. The sections were then incubated with biotinlabeled goat anti-rabbit secondary antibody (Earthox LLC, San Francisco, USA) and streptavidin-peroxidase for 30 min each. The samples were developed with 3, 3'-diaminobenzidine tetrahydrochloride substrate (Sigma, Steinheim, Germany) and counterstained with hematoxylin (Sigma, Steinheim, Germany). As a negative control, primary antibody was omitted and replaced with phosphatebuffered saline.

The immunohistochemically stained tissue sections were scored independently by two pathologists blinded to the clinical parameters. The final score for Nurr1 was the average of the scores obtained by the two observers. The intensity of staining was evaluated subjectively on a scale of 0-3, where 0 = no staining, 1 = weak equivocal staining, 2 = unequivocal moderate staining and 3 = strong staining. The extent of the staining, defined as the percentage of positive staining cells in relation to the whole field, was scored on a scale of 0 to 4 as 0 (0%), 1 (1 to 25%), 2 (26 to 50%), 3 (51 to 75%), or 4 (76 to 100%). The sum of the staining intensity and staining-extent scores (0 to 7) was used as the final staining score for Nurr1. For statistical analysis, a final staining score of < 3 and ≥ 3 were respectively considered to be low and high expression levels (Soumaoro et al., 2004; Cao et al., 2010).

Cell Culture

Prostate cancer cell line PC-3 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). PC-3 cells were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 100 IU mL−1 penicillin G sodium, 100 mg mL−1 streptomycin sulfate and 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA). They were incubated at 37 ºC with 5% CO_2 in a humidified incubator.

Silencing of Nurr1

Small interfering RNA-oligo targeting Nurr1 (siRNA-Nurr1) was obtained from Ambion (Austin, Tex, USA); sense: 5'-GGCUUGUAAAUUUACCCAATT - 3', antisense: 5'-UUGGGUAAAUUUACAAGCC TT - 3'. As nontargeting control, Silencer Negative Control small interfering RNA (Ambion) was used (Inamoto et al., 2010). Cells were incubated to 60% - 80% confluency in 6-well plates and transfected with specific or nonspecific small interfering RNA using siRNA Transfection Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's instructions. Cells were assayed within 24 - 72 h after the addition of fresh normal growth medium.

Western blotting

Forty-eight hours after silencing of Nurr1,total cellular proteins were extracted. Equal amounts of each protein sample (50 μg) were separated by electrophoresis on 10% SDS-PAGE gel, and transferred to nitrocellulose membranes Invitrogen) using a wet transfer. After being blocked with blocking buffer (phosphate buffered saline [PBS] containing 0.1% Tween 20 and 5% nonfat dry milk) for 2 h, the membranes were incubated with primary rabbit anti-Nurr1 polyclonal antibodies (diluted in the ratio 1: 500, Santa Cruz, CA, USA) and primary mouse anti -β-actin monoclonal antibodies (diluted in the ratio 1: 1000, Santa Cruz, CA, USA) for 2 h at room temperature. After primary antibody incubations, membranes were incubated with DyLight 680-conjugated secondary antibodes (diluted in the ratio 1: 15000, KPL, USA) for 1.5 h at room temperature in the dark. Membranes were scanned using an Odyssey scanner (LI-COR 9120-00, LI-COR, USA) and analyzed using Odyssey imaging software 3.0. This experiment was repeated five times.

MTT assay for cell proliferation

After silencing of Nurr1, cells were seeded in 96-well plates at a density of 4000 cells per well and cultured for 24 h, 48 h, 72 h and 96 h in 200 μL of complete medium. 3-(4,5–dimethylthiazol–2-yl)-2.5-diphenyltetrazolium-bromide (MTT) (Sigma, Steinheim, Germany) was used to assess cell proliferation reflected by metabolic activity of the cells. After the indicated times, MTT was added to a final concentration of 1 mmol L−1 and incubated for 4 h. The formazan product was dissolved in dimethyl sulfoxide and optical density was measured on a Dynatech MR 5000 plate reader at 490 nm. These experiments were performed in quintuplicate.

Table 1. Protein Expression of Nurr1 in PCa and BPH and Correlation of Clinicopathologic Features of the Patients with PCa and Expression of Nurr1 Protein

	No.	Nurr1 protein expression (%)		*P*
		High	Low	
Group				
BPH	22	4 (18.2)	18 (81.8)	<.001
PCa	118	83 (70.3)	35 (29.7)	
Age (year)				
<70	45	34 (75.6)	11 (24.4)	0.330
≥70	73	49 (67.1)	24 (32.9)	
Gleason score				
4-6	42	24 (57.1)	18 (42.9)	0.020
7-10	76	59 (77.6)	17 (22.4)	
CS (TNM)				
T stage				
T_1-T_2	56	32 (57.1)	24 (42.9)	0.003
T_3-T_4	62	51 (82.3)	11 (17.7)	
N stage				
N_0	90	59 (65.6)	31 (34.4)	0.017
N_1	28	25 (89.3)	3 (10.7)	
M stage				
M_0	94	61 (64.9)	33 (35.1)	0.011
M_1	24	22 (91.7)	2 (8.3)	

PCa, prostate cancer; BPH, benign prostatic hyperplasia

Cell invasion and migration assay

The invasive and migratory potential of cells was evaluated using 24-well cell culture with 8 μm pores (Coring, NY, USA). For invasion assay, after silencing of Nurr1, 4.0×10^4 cells in 5% FBS medium were seeded into insert chamber pre-coated with matrigel matrix (BD, NJ, USA), and a volume of 0.5 mL 10% FBS medium was added to the matched lower chamber. Chambers were incubated at 37 ºC under 5% CO_2 atm for 24 h. At the end of the incubation period, non-invaded cells were removed from the upper surface of the transwell membrane with a cotton swab, and invaded cells on the lower membrane surface were fixed in methanol, and then stained with Hexamethylpararosaniline chloride. For migration assay, the procedures were similar, except that 2.0×10^4 cells were seeded into the inserts without matrix gel pre-coated and incubated for 12 h. Five random fields at 100 × magnification for each insert were counted. Inserts were conducted in quintuplicate in five separate experiments.

Cell apoptosis assay

Forty-eight hours after silencing of Nurr1, transfected or no-transfected cells were washed in PBS and resuspended in binding buffer at a concentration of 1×10^6 cells mL-1. After incubation, 195 μL of the solution was transferred into a 5 ml culture tube with 5 μL annexin V-FITC (Keygen, Nanjing, China). The tube was then incubated for 30 min at room temperature in the dark. The cells were washed with binding buffer and resuspended in 190 μL binding buffer, with 10 μL PI added. Finally, the tube was gently vortexed and incubated for another 30 min in the dark. Cells were analyzed by FACSCalibur (Becton Dickinson, San Jose, CA, USA)with WinMDI2.9 software. Five independent series of experiments were performed.

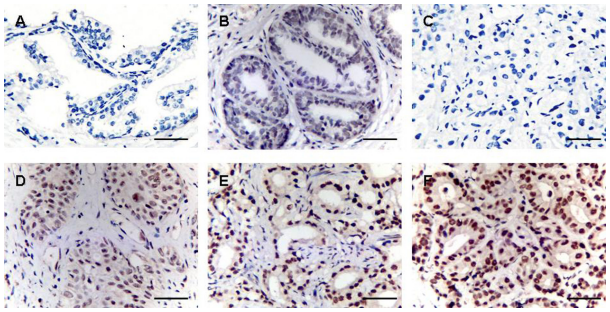

Figure 1. Expression of Nurr1 in BPH and PCa Samples (magnification 400×; Scale bars = 50 μm)(A-F). (A): Control group of BPH showing no staining when the primary antibody was omitted; (B): Weak expression of Nurr1 in BPH sample; (C): Control group of PCa showing no staining when the primary antibody was omitted; (D): Immunohistochemistry staining of Nurr1 in well-differentiated PCa sample; (E): Immunohistochemistry staining of Nurr1 in moderately differentiated PCa sample; (F): Immunohistochemistry staining of Nurr1 in poorly differentiated PCa sample

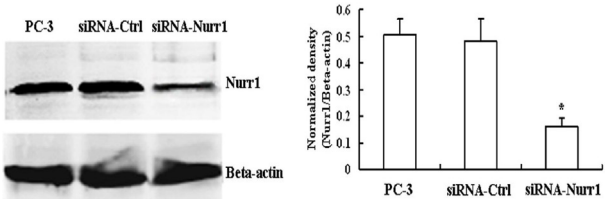

Figure 2. Expression of Nurr1 in Different Groups. Western blotting assay shows significantly decreased protein expression of Nurr1 in siRNA-Nurr1 cells comparing to siRNA-Ctrl and the parental PC-3 cells. β-actin was used as the internal control. Data were presented as mean ± SD for five independent experiments. **P* < 0.05, as compared to PC-3 and siRNA-Ctrl cells

Statistical analysis

All data were analyzed for statistical significance using SPSS 13.0 software. The χ^2 test was applied to the analysis of relationship between Nurr1 expression levels and clinicopathologic characteristics. Statistical values are presented as mean ± SD of the mean. One-way ANOVA was used to determine the differences between groups for all analyses. Statistical significance was defined as $P < 0.05$.

Results

Relationship between clinicopathological characteristics and Nurr1 expression in PCa patients

The relationship between clinicopathological characteristics and Nurr1 expression in patients with PCa is summarized in Table 1. We observed that the level of Nurr1 protein was significantly greater in formalin-fixed paraffinembedded 118 PCa tissue samples than the 22 BPH tissue samples ($P < 0.001$).We did not find significant association of Nurr1 expression with age in 118 patients with PCa. However, we found that the level of Nurr1 expression was closely correlated with the Gleason score (G4 - G6 vs. G7 - G10, $P = 0.020$), T classification (T_1 - T_2 vs. T_3 - T_4, $P = 0.003$), N classification (N_0 vs. N_1, $P = 0.017$) and M classification (M0 vs. M1, $P = 0.011$) in PCa patients (Table 1). Images of the immunohistochemical staining can be seen in Figure 1.

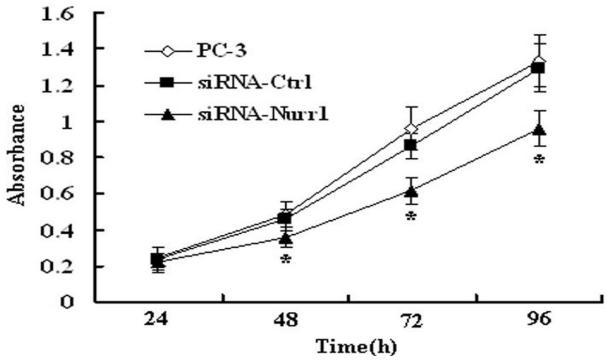

Figure 3. Reduced Expression of Nurr1 Inhibited Cell Proliferation. The cell growth of parental PC-3 cells and their stable derivatives, siRNA-Ctrl and siRNA-Nurr1, were examined by MTT assay over a four-day period. Data were presented as mean ± SD for five independent experiments. *$P < 0.05$, as compared to PC-3 and siRNA-Ctrl cells

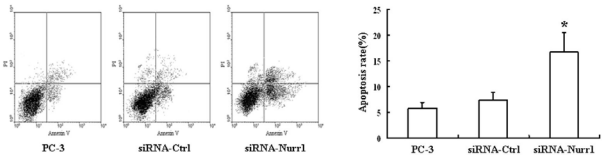

Figure 4. Flow Cytometric Analysis for Quantification of Apoptotic Cells. Cells were stained with FITC-annexin V and propidium iodide. Cell apoptosis percentages were evaluated by flow cytometry. Flow cytometric assay shows significantly increased decreased apoptotic rate in siRNA-Nurr1 cells comparing to siRNA-Ctrl and the parental PC-3 cells. Data were presented as mean ± SD for five independent experiments. *$P < 0.05$, as compared to PC-3 and siRNA-Ctrl cells

Suppression of Nurr1 expression by RNAi

To study the biological function of Nurr1, we used a small interfering RNA-oligo targeting Nurr1 to knock down the endogenous expression of Nurr1 in PC-3 cells. As shown in Figure 2, comparing to the controls (blank control and scrambled siRNA control), cells transfected with siRNA-Nurr1 had significantly decreased levels of Nurr1 protein: the normalized density value of siRNA-Nurr1 group (0.16 ± 0.03) was lower than the values of PC-3 (0.50 ± 0.06) or siRNA-Ctrl groups (0.48 ± 0.09) ($P < 0.05$).

Knock-down of Nurr1 inhibited proliferation of PC-3 cells in vitro

In order to determine whether RNAi-Nurr1 had inhibitory effects on the growth of PC-3 cells, MTT assay was conducted to determine the cell proliferation. Results showed that the absorbance value of the siRNA-Nurr1 transfected group was significantly weaker than control groups with increasing time of cells culture. The statistical analysis showed that the value of absorbance from the second day difference between siRNA-Nurr1 and control groups was statistically significant (siRNA-Nurr1 group vs. PC-3 group, $P < 0.05$; siRNA-Nurr1 group vs. siRNA-Ctrl group, $P < 0.05$) (Figure 3).

Knock-down of Nurr1 induced apoptosis of PC-3 cells in vitro

The induction of apoptosis by siRNA-Nurr1 was quantified by flow cytometry using annexin V and propidium iodide staining. The results showed that the knock-down of endogenous Nurr1 expression could significantly induce apoptosis as compared to the no-transfected or transfected with scrambled siRNA cells: the apoptosis ratio of siRNA-Nurr1 group (16.66% ±3.78%) was higher than the ratios of PC-3 (5.81% ± 1.2%) or siRNA-Ctrl groups (7.44% ± 1.49%) ($P < 0.05$) (Figure 4).

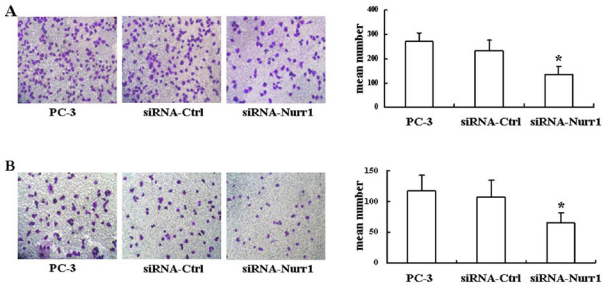

Figure 5. Reduced Expression of Nurr1 Inhibited Cell Migration and Invasion. The cell migration (A) and invasion (B) capabilities of parental PC-3 cells and their stable derivatives, siRNA-Ctrl and siRNA-Nurr1, were examined using transwell assay. Data were presented as mean ± SD for five independent experiments. *$P < 0.05$, as compared to siRNA-Ctrl and PC-3 cells

Knock-down of Nurr1 inhibited the invasion and migration of PC-3 cells in vitro

The cell migration and invasion are integral steps for the process of tumor development and metastasis. We used transwell chambers with or without pre-coated matrigel to determine whether the RNAi- Nurr1 could weaken the invasion and migration of PC-3 cells. The results showed that the knock-down of endogenous Nurr1 expression could significantly reduce cell migration and invasion as compared to the no-transfected or transfected with scrambled siRNA cells (siRNA-Nurr1 group vs. PC-3 group, $P < 0.05$; siRNA-Nurr1 group vs. siRNA-Ctrl group, $P < 0.05$) (Figure 5).

Discussion

It is known that the majority of prostate cancer deaths result from tumor metastases rather than from primary tumors. However, the molecular mechanisms regulating the cell invasion and metastasis of prostate cancer remain incompletely understood. Some studies show oncogenic activities of Nurr1 in a cell type-specific manner (Ke et al., 2004; Holla et al., 2006; Li and Tai, 2009). There is also one study showing that the cytoplasmic mislocalization of Nurr1 protein is associated with cancer progression (Inamoto et al., 2010). No earlier studies have compared the actual expression of Nurr1 protein with prostate cancer stages and grades. Through our systematic study of the Nurr1 protein in benign and malignant prostate tissues, we found that Nurr1 was expressed in BPH and prostate cancer, and was associated with the cancer's TNM classification and Gleason score. Our results showed that the expression of Nurr1 in many of the benign prostate tissue specimens was at low levels. In many of our prostate cancer tissue specimens, in contrast,an overexpression of Nurr1 was frequently detected, and the frequency of

Nurr1 overexpression increased with ascending of the TNM classification and Gleason score in prostate cancer. These findings suggest the possibility that upregulated expression of Nurr1 may provide a selective advantage in the occurrence and progression of prostate cancer.

Malignant tumor cell proliferation, migration and invasion were important characteristics of cancer development, and apoptosis has an important role in the inhibition of oncogenesis, tumor development and growth. To investigate the biological functions of Nurr1 in prostate cancer, we chose to use PC-3 cells exhibiting the higher expression level of endogenous Nurr1, and employed the loss-of-function approach that is by knocking down the expression level of endogenous Nurr1. Using this system, we identified the roles of Nurr1 in promoting cell proliferation, migration and invasion. Our study showed that Knock-down of Nurr1 inhibited the proliferation, migration and invasion, and induced apoptosis of PC-3 cells. These results strongly suggest an oncogenic role of Nurr1 in the prostate cancer development.

NR4A family members consist of an N-terminal domain mediating transactivation and a C-terminal ligand-binding domain (Hsu et al., 2004). These members are thought to act as constitutively active transcription factors that bind the promoter of target genes on consensus NBRE (AAAGGTCA) sites. Only a few direct target genes, such as vascular endothelial growth factor (VEGF)-A, have been identified to date (Pols et al., 2007; Zhao and Bruemmer, 2010; Zeng et al., 2006). Specific ligands for the NR4A family of transcription factors have not been identified, classifying them as orphan nuclear receptors (Bonta et al., 2006). Furthermore, gene expression of NR4A members is induced by various growth factors and cytokines in a variety of cell types (Pei et al., 2005; Nomiyama et al., 2006).

Recently, it was reported that Nurr1 has antiapoptotic effects. Nurr1 has an ability to down-regulate the expression of proapoptotic protein Bax, which is directly transactivated by tumor suppressor p53 (Zhang et al., 2009). By microarray analyses, overexpression of Nurr1 downregulates caspase-3 and other apoptotic factors in neural stem cells (Sousa et al., 2007). Nurr1 is also a key target gene of thromboxane A2 receptor-signaling pathway and is critical for the proliferation of lung carcinoma cells by regulating cyclin D1 expression (Li and Tai, 2009). There is also study showing that Nurr1 is upregulated in migratory mesenchymal stromal cells (MSC), and overexpression of Nurr1 in nonmigratory fetal bone marrow MSC (FBMSC) significantly increases migration compared with mock-transduced cells (Maijenburg et al., 2012). The above studies further support oncogenic role for Nurr1 in promoting the cancer development.

In conclusion, our study provided the first evidence that Nurr1 may be involved in the development of prostate cancer. In addition, we also demonstrated that Nurr1 may be used as a useful molecular marker for prostate cancer and an indicator for tumor progression. In combination with other biomarkers of prostate cancer, Nurr1 would be useful for novel therapeutic strategies. Further works are needed to investigate the mechanisms and pathways of prostate cancer pathogenesis mediated by Nurr1.

Acknowledgements

This work was supported by Guangdong Natural Science Foundation (No.10452402301006027).

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