RESEARCH COMMUNICATION

β3GnT8 Regulates Laryngeal Carcinoma Cell Proliferation Via Targeting MMPs/TIMPs and TGF-β1

Dong Hua1&, Fang Qin2&, Li Shen2,3, Zhi Jiang2, Shi-Tao Zou2, Lan Xu2, Zhi-Hong Cheng1*, Shi-Liang Wu2*

Abstract

Previous evidence showed β1, 3-N-acetylglucosaminyltransferase 8 (β3GnT8), which can extend polylactosamine on N-glycans, to be highly expressed in some cancer cell lines and tissues, indicating roles in tumorigenesis. However, so far, the function of β3GnT8 in laryngeal carcinoma has not been characterized. To test any contribution, Hep-2 cells were stably transfected with sense or interference vectors to establish cell lines that overexpressed or were deficient in β3GnT8. Here we showed that cell proliferation was increased in β3GnT8 overexpressed cells but decreased in β3GnT8 knockdown cells using MTT. Furthermore, we demonstrated that change in β3GnT8 expression had significant effects on tumor growth in nude mice. We further provided data suggesting that overexpression of β3GnT8 enhanced the expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) at both the mRNA and protein levels, associated with shedding of tissue inhibitors of metalloproteinase TIMP-2. In addition, it caused increased production of transforming growth factor beta 1 (TGF-β1), whereas β3GnT8 gene knockdown caused the reverse effect. The results may indicate a novel mechanism by which effects of β3GnT8 in regulating cellular proliferation are mediated, at least in part via targeting MMPs/TIMPs and TGF-β1 in laryngeal carcinoma Hep-2 cells. The finding may lay a foundation for further investigations into the β3GnT8 as a potential target for therapy of laryngeal carcinoma.

Keywords: β3GnT8 - proliferation - laryngeal carcinoma - MMP - TGF-β1 - therapy

Introduction

Laryngeal carcinoma is a common head and neck malignancy with high incidence as it accounts for approximately 2.4% of new malignancies worldwide every year (Marioni et al., 2006; Papadas et al., 2010). Use of tobacco products and drinking too much alcohol can affect the risk of developing this cancer (Van Belle, 2008). The functional treatment of this condition poses a considerable challenge because it is resistant to chemotherapy and radiotherapy during advance stage. In addition, the formation and progression of laryngeal carcinoma is a chronic and multistep process resulting from mutagenic damage to growth-regulating genes and their products that ultimately leads to the development of invasive or metastasis cancers (Yoshida et al., 1999). Thus, it is essential to investigate the mechanism involved in the development and progression of laryngeal carcinoma.

The basement membrane and transmembrane proteins contain signals for cell survival and growth, but loss of these signal results in cell death or suppression of proliferation in both normal and cancerous cells. Matrix metalloproteinases (MMP) belong to a family of proteinases, which play an important role in the degradation of basement membrane or transmembrane proteins. Recent studies have indicated that MMP-2 silencing by recombinant lentivirus mediated RNA interference can inhibit invasion and growth of human laryngeal carcinoma Hep-2 cells (Sun et al., 2008a). Statistical data showed that 54 of 102 patients (52.9%) who underwent laryngectomy for their laryngeal cancer had positive cytoplasmic staining for MMP-2 and MMP-2 expression was related with worse overall and disease-free survival (Mallis et al., 2012). It also has been reported that MMP-9 gene silence can inhibit invasion and growth of laryngeal cancer cells (Sun et al., 2008b). The expression of both MMP-2 and MMP-9 are up-regulated early in development of laryngeal carcinoma (Uloza et al., 2011). Moreover, the activation of MMP-2 and MMP-9 causes a loss of growth regulation, which is characterized by proliferation and subsequent invasion. However, there is paucity of data with regard to regulators of MMP-2 and MMP-9 and activity in laryngeal carcinoma.

The complex sugar chains of glycoprotein (O-glycans
and N-glycans), which are formed through the reactions of different glycosyltransferases, are thought to play a critical role in mediation cell–cell and cell–ECM (extracellular matrix) interactions that are clearly critical as cells undergo oncogenesis (Guo et al., 2003). Meanwhile, there is substantial empirical evidence indicating that MMPs, particularly MMP-2 and MMP-9, seem to be essential in tumor cell-mediated degradation of ECM (Decock et al., 2011; Gialeleli et al., 2011). That means the expression of MMP-2 and MMP-9 may be correlated with aberrant glycosylation of cell surface glycoprotein. Furthermore, the altered expression of glycosyltransferases was also found to in laryngeal carcinoma. For example, the study of the various chondroitin/dermatan synthesizing enzymes revealed that they were differentially expressed in human laryngeal carcinoma, leading to specific chondroitin/dermatan structures which contributed to proteoglycan formation with specific features (Kalathas et al., 2010). The expression of N-acetylgalactosaminyltransferasemyltransferases were demonstrated in laryngeal cancer but were deficient in relation to the normal adjacent counterpart (Kuhns and Schoentag, 1981). The saccharides of the MUC 1 mucin-type glycoprotein produced by Hep-2 cells in the presence of aryl-N-acetyl-α-galactosaminides was also observed. Although under the current research, MMP-2 and MMP-9 can be up-regulated by TGF-β1 and has been shown to interact with TIMP-2, it is now appreciated that the expression of MMP-2 and MMP-9 in laryngeal cancer can also be induced by glycosyltransferases (Wiercinska et al., 2011; Gao et al., 2012).

β-1, 3-N-acetylgalactosaminyltransferase-8 (β3GnT8), which has been cloned and characterized by our and another groups, catalyzes the transfer of GlcNAc to the non-reducing terminus of the Galβ1-4GlcNAc of tetraantennary N-glycan in vitro (Huang et al., 2004; Ishida et al., 2005). It was reported that the transcripts of β3GnT8 were abundant in most colorectal cancer cell lines and tissues (Seko and Yamashita, 2005). Additionally, subcellular localization and tumor distribution of β3GnT8 by antiserum showed the enzyme was expressed significantly higher in some tumor tissues than in normal tissues, indicating its biofunction in tumorigenesis (Jiang et al., 2010). Previously, we have also reported that β3GnT8 was highly expressed in larynx tissue (Huang et al., 2004). However, so far, accumulating evidence has shown that β3GnT8 plays a central role in carcinogenesis, but its potential role in regulating the development of laryngeal carcinoma, has not been characterized. Therefore, we investigated the role of β3GnT8 on cell proliferation behavior of laryngeal carcinoma cell line Hep-2 in vitro and in vivo. Further, we also explored the effect of β3GnT8 on MMP-2, MMP-9, TIMP-2 and TGF-β1 regulation. To our knowledge, this is the first study on the relationship between β3GnT8 and the development and progression of laryngeal cancer.

Materials and Methods

Cell culture

Human laryngeal carcinoma cell line Hep-2 was purchased from Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 supplemented with 10% Fetal bovine serum (FBS) in a humidified atmosphere with 5% CO₂ at 37 °C (Heraeus, Germany).

Construction and identification of β3GnT8 sense or interference vector

For overexpression of β3GnT8 the full coding sequence of β3GnT8 was cloned into the mammalian expression vector pEGFP-C1 (Clontech, Germany) and verified by sequencing. For gene knock-down, siRNA expression vectors was constructed in which 21-nt sense and antisense sequences against the β3GnT8 gene were placed using SilenCircleRNAi Kit (Allele Biotechnology, CA, USA). We have also demonstrated the most effective sequence of β3GnT8 siRNA (Liu et al., 2011). The following sequences were used: sense: 5’-CAUUCGGCCUCUGGAACATT-3’; antisense 5’-UGUUUUCCAGGCG- AAUGCT-3’. The ligated plasmids were transformed into Escherichia coli (DH5α) and the transformed clones were then further confirmed by automated DNA sequencing. The pEGFP-c1-β3GnT8 plasmids were identified by digestion with restriction enzymes XhoIand EcoRI, while the pSilenCircle-β3GnT8 plasmids was digested by Snu (MBI, Fermentas, Lithuania). Digestion products was confirmed again on a 1% w/v agarose gel.

Transfection and generation of stable cell lines

One day before transfection, the cells were seeded in 6-well cell culture plates to provide a final density of 60-70% confluence. Transfections of pEGFP-C1-β3GnT8 or pSilenCircle-β3GnT8 plasmids into Hep-2 cells were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Transfected cells were selected in the presence of 500 mg/L G418. Stable clones were selected for at least 4 weeks before single colonies were picked and analyzed for β3GnT8 expression by RT-PCR and Western blot. The transfected cells had a stable deficiency of β3GnT8 expression (herein termed as β3GnT8-si), and β3GnT8overexpression (β3GnT8-s). In parallel, pEGFP-C1 empty plasmids were used to generate vector control stable cell lines.

MTT assay

Cells were plated at 5 × 10⁴ cells per well in 96-well plates with six replicate wells. After transfection as described previously, 20 μl of MTT (5 g/L, Sigma, USA) was added into each well at each day of consecutive 4 days after treatment and the cells were incubated for additional 4 h, the supernatant was then discarded. 200 μl of DMSO was added to each well to dissolve the precipitate. Optical density (OD) was measured at wave length of 570 nm. The data was presented as the mean ± SD, which was derived from triplicate samples of at least three independent experiments.

Tumor growth in nude mice

Four-week-old female nude mice (SPF BalB/c,
cells/0.2 ml). Total of ten mice per group were used. The mice were sacrificed 28 d after inoculation with tumor cells. This time point was chosen because it gave a reproducible number of overt metastases without significant morbidity. A similar model has been used by other investigators (Dhawan et al., 2005; Lu et al., 2008). Then, the tumor diameter and weight was measured. The volume was calculated according to the formula $V=0.4 \times \text{largest diameter} \times \text{smallest diameter}$. The growth curve of each tumor was plotted and incidence of tumor formation was calculated.

**Western blot analysis**

Total proteins from above cells were extracted and quantified after normalization with the density of β-actin.

**Statistical analysis**

Values are expressed as means ± SD of three independent experiments and a two-sample Student’s t-test was performed to compare the treated groups with untreated and control group. Values of P<0.05 were regarded as statistically significant.

**Results**

*Identification of β3GnT8 sense or interference expression vector*

The pEGFP-c1-β3GnT8 and pSilenCircle β3GnT8 vectors were identified via enzyme digesting method to be the expected size (Figure 1 A, B). The results of DNA sequencing were completely identical to the β3GnT8 sequences on GenBank. Restriction enzyme digestion analysis showed the effectiveness of the plasmids and confirmed that β3GnT8 overexpressing and knockdown vectors had been successfully constructed.

**Establishment of β3GnT8 overexpressing or knock down cell lines**

To investigate the potential role of β3GnT8 in laryngeal carcinoma, stable cell lines overexpressing β3GnT8 and β3GnT8 knockdown cells were established using the Hep-2 cells as parental cell line. β3GnT8 mRNA and proteins expression levels were determined respectively by RT-PCR and Western blot. The results revealed that β3GnT8 expression at mRNA and protein level were significantly higher (P<0.05) in the cells transfected with pEGFP-c1-β3GnT8 than in the cells transfected with pSilenCircle-β3GnT8, but there was no difference (P>0.05) in the expression level of β3GnT8 between the cells untreated with β3GnT8 Regulates Laryngeal Carcinoma Cell Proliferation Via Targeting MMPs/TIMPs and TGF-β1 ScXK2007-0005) were obtained from the laboratory animal center of Soochow University (Su Zhou, China). All studies on mice were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Soochow University Experimental Animal Care Committee. After being grown to subconfluency, transfected (pEGFP-c1-β3GnT8, pSilenCircle-β3GnT8 or pEGFP-c1 empty vector) and non-transfected cells were trypsinized and harvested, washed twice with PBS and resuspended in 0.2ml PBS (7.5 ×10⁶ cells/0.2 ml). Total of ten mice per group were used. The mice were sacrificed 28 d after inoculation with tumor cells. This time point was chosen because it gave a reproducible number of overt metastases without significant morbidity. A similar model has been used by other investigators (Dhawan et al., 2005; Lu et al., 2008). Then, the tumor diameter and weight was measured. The volume was calculated according to the formula $V=0.4 \times \text{largest diameter} \times \text{smallest diameter}$. The growth curve of each tumor was plotted and incidence of tumor formation was calculated.

**Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)**

Total RNA from vector-treated or untreated Hep-2 cells was extracted by TRIZol (Gibco-BRL), according to the manufacturer’s protocol. Complementary DNA (cDNA) was generated from total RNA, using M-MLV RT (MBI, Fermentas). Amplification reaction protocol was performed for 30 cycles consisting 94 °C for 1 min, 52 °C for 15 sec, 72 °C for 1 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel and transferred to PVDF membrane. The membrane was blocked in 5% skim milk for 1 h and then incubated with a specific antibody for 2 h. Rabbit anti-human β3GnT8 affinity pAb was purification by our Lab (Jiang et al., 2010). Anti-β-actin rabbit mAb, anti-MMP-2 rabbit mAb, anti- TIMP-2 rabbit mAb, anti-MMP-9 rabbit mAb and anti-TGF-β1 rabbit mAb as well as the anti-rabbit second antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For each treatment, an equal amount of proteins from the parallel samples was mixed and used for the blots. The band density of specific proteins was quantified after normalization with the density of β-actin.

**Results**

To investigate the potential role of β3GnT8 in laryngeal carcinoma, stable cell lines overexpressing β3GnT8 and β3GnT8 knockdown cells were established using the Hep-2 cells as parental cell line. β3GnT8 mRNA and proteins expression levels were determined respectively by RT-PCR and Western blot. The results revealed that β3GnT8 expression at mRNA and protein level were significantly higher (P<0.05) in the cells transfected with pEGFP-c1-β3GnT8 than in the cells transfected with pSilenCircle-β3GnT8, but there was no difference (P>0.05) in the expression level of β3GnT8 between the cells untreated

![Figure 1. Identification of β3GnT8 Sense or Interference Expression Vector](image-url)
and transfected with pEGFP-c1 empty vector (Figure 2A-D). These results imply the successful construction of the β3GnT8-overexpressing or -knockdown cell lines. Thus, these stable cell lines can be effectively used for further study.

**Effect of β3GnT8 on the growth of Hep-2 cells in vitro**

The effect of β3GnT8 on proliferation of Hep-2 cells was investigated using MTT assay. The results showed that the cell proliferation was increased in β3GnT8 overexpressed cells but decreased in β3GnT8 knockdown cells. As shown in Figure 3, there was no difference in cellular proliferation rate between the untreated and cells transfected with empty vector (P>0.05). The cell viability was found to be significantly enhanced after treatment with pEGFP-c1-β3GnT8 (P<0.05). However, after transfection with pSilenCircle-β3GnT8, proliferation of the Hep-2 cells at these time points was inhibited significantly (P<0.05) compared to that of the untreated and control group cells.

**Effect of β3GnT8 on tumor growth in a mouse model.**

Based on our cell culture data, we postulated that β3GnT8 expression would affect tumor formation in vivo. Therefore, to examine whether these in vitro data were correlated with the in vivo effects, Hep-2 cells transfected with pEGFP-c1-β3GnT8, pSilenCircle-β3GnT8 or pEGFP-c1 empty vector were grown in nude mice to form tumors which were measured at regular time points, as described in the Materials and methods. In mice injected with the β3GnT8 sense vectors transfected cells, measurable tumors appeared within 1 week and grew to an average volume of 1240 mm$^3$, respectively, by 4 weeks after injection. In contrast, tumors from mice injected with β3GnT8 interference vectors transfected cells became detectable only after 2 weeks of growth, and the average tumor volume was only 293 mm$^3$ at 4 weeks after injection. Untreated and control group started developing tumors at 1-2 weeks after injection, and the average tumor volume was 719 and 692 mm$^3$ after 4 weeks of growth, respectively.

The tumor weight in β3GnT8-si group was smaller than that of the untreated and control group (P < 0.05), while the incidence of tumor formation following subcutaneous injections was only 60% in mice injected with β3GnT8 knockdown cells, compared with 100% in mice receiving β3GnT8 overexpressing cells.

**Change of β3GnT8 expression regulates MMP-2 and MMP-9 levels**

The expression of MMP-2 and MMP-9 appear to play...
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Effect of modulation of β3GnT8 expression on tumor formation in a mouse model

Table 1. Effect of Modulation of β3GnT8 Expression on Tumor Formation in a Mouse Model

| Groups       | Average days | Average tumor volume (mm³) | Average tumor weight (mg) | Average tumor incidence of tumor formation |
|--------------|--------------|----------------------------|---------------------------|------------------------------------------|
| Untreated    | 9            | 719±28                     | 617±14                    | 90%                                      |
| β3GnT8-s     | 7            | 1240±35*                   | 1048±22*                  | 100%                                     |
| Control      | 10           | 692±27                     | 602±15                    | 90%                                      |
| β3GnT8-si    | 14           | 293±16*                    | 355±12*                   | 60%                                      |

Effect of modulation of β3GnT8 expression on tumor formation in nude mice was determined as described in the text. The mice were sacrificed 28 days after cell injection, and average tumor volume and weight were measured. The results indicate the mean ± SD from three independent preparations (*P<0.05 compared to the untreated and control group)

Discussion

All cells of every species studied to date are covered by a dense coating of glycans. Specific changes in the glycosylation pattern of cell surface glycoproteins have been shown to correlate with the enhancement of the metastatic efficiency of tumor cells (Calle et al., 2000; Hossler et al., 2009). The complex sugar chains of glycoprotein, which are formed through the reactions of different glycosyltransferases, are also important for the regulation of cell survival and proliferation (Calle et al., 2000). As we all know, proliferative phenotype is fundamental components of malignant disease and functional inactivation of tumor suppressor genes causes a loss of growth regulation (Gao et al., 2005). Substantial alterations of the cellular glycoprotein pattern are expected to occur during cell proliferation. The change of glycosyltransferases directly modify carbohydrates on cell surface receptors and cell adhesion molecules, which then promotes or inhibits tumor cell growth. For example, the biosynthesis of glycoproteins in endothelial and smooth muscle cells is subject to growth-dependent regulation (Vischer and Buddecke, 1985). The deficiency of LH3 glycosyltransferase activities, especially in the extracellular space, causes growth arrest revealing the importance of LH3 for cell growth and viability (Wang et al., 2009). Loss of the glycosyltransferase Egghead affects membrane signaling and activation of PI3K signaling in glia of the peripheral nervous system, and suggest that Egghead may suppress proliferation (Dahlgaard et al., 2012). Therefore, this report mainly concerned the relationship between β3GnT8 and cell proliferation behavior.

Recently, we and others previously showed that β3GnT8 was highly expressed in human lung adenocarcinoma, gastric cancer, chronic marrow leukemia cells, and colon cancer (Ishida et al., 2005; Jiang et al., 2010). However, whether β3GnT8 is associated with cellular proliferation and its possible mechanisms of action in laryngeal carcinoma remain unclear. In this regard, we modulated the expression level of β3GnT8 in Hep-2 cells by generation stable cell lines overexpressing β3GnT8 and β3GnT8 knockdown cells. Here, we found laryngeal carcinoma Hep-2 cells could express β3GnT8 gene. Down-regulation of β3GnT8 markedly inhibited the growth in vitro of Hep-2 cells. We observed similar inhibitory effects of β3GnT8 on tumors growing in vivo in immunodeficient nude mice. Additionally, increased β3GnT8 completely exhibited the contrary effect. The cell viability in vitro and tumor formation in vivo was
found to be significantly enhanced after treatment with pEGFP-c1-β3GnT8. In this study, we first show that tumor growth was controlled by altered β3GnT8 expression in laryngeal carcinoma. That means the expression of specific N-glycans as constituents of cell surface glycoproteins, which caused by β3GnT8 in particular, was directly linked to regulation laryngeal carcinoma proliferation.

Matrix metalloproteinases (MMPs) are believed to play a pivotal role in malignant behavior of cancer cells such as rapid growth, invasion, and metastasis by degrading ECM. MMP-2, is essential in tumor cell-mediated degradation of ECM. MMP-9 belongs to gelatinases and most investigations supported that there was a positive correlation between MMP-9 and tumor progression. Numerous reports have demonstrated that the expression of MMP-2 and MMP-9 is closely correlated with cell proliferation and process of tumor progression. It has been reported that MMP-2 can regulate growth in stellate cells directly (Dahlgaard et al., 2012). Others have showed that protein kinase D1 inhibits cell proliferation through MMP-2 and MMP-9 in prostate cancer (Biswas et al., 2010). Differential expression of MMP-2 and MMP-9 could reflect biologic features, such as proliferation and differentiation, of retinoblastoma cells (Kim et al., 2010). Moreover, increased expression of MMP2 and MMP-9 has also been found in laryngeal carcinoma. Therefore, it is important to understand the molecular mechanisms associated with β3GnT8 regulation of MMP-2 and MMP-9. We observed that both knockdown and overexpression of β3GnT8 revealed a direct correlation with MMP-2 and MMP-9. Up-regulation of β3GnT8 enhanced the expressions of MMP-2 and MMP-9 at both the mRNA and protein levels, whereas down-regulation of β3GnT8 caused a reverse effect. The change of MMP-2 and MMP-9 at the level of mRNA and protein expression displayed the similar trend with that of β3GnT8. The findings provide evidence of a positive feedback loop in which induction of β3GnT8 leads to enhanced cell proliferation and MMP-2 and MMP-9 expression.

We also explored the role of β3GnT8 in regulating TIMP-2. MMP-2 and MMP-9 expression was affected by many factors, such as membrane activator, matrix components and TIMPs. TIMP-2 is a secreted protein that is complex with MMP-2 and involved in the inhibition of MMP-2 activity. Recent experimental data suggest that TIMP-2 not only have MMP-2 inhibitory functions, but also are multifunctional molecules, with apparent paradoxical effects on tumor progression (Giannelli et al., 2002). In addition, there was correlation between the presence of TIMP-2 and the different from MMP-9. DNA ploidy analysis confirmed that MMP-9 activity increased during tumor progression a corresponding increase in TIMP-2 level also occurred. Relatively high level of TIMP-2 could be induced by MMP-9 (Zhang et al., 2005). In this study, we found that β3GnT8 gene knockdown increased, and its overexpression decreased, the expression of TIMP-2 in laryngeal carcinoma cells. This proved that TIMP-2 displayed a negative feedback effect in the regulation of β3GnT8 and β3GnT8 can interact with TIMP-2.

Although under the current research, TGF-β1 is a multifunctional cytokine, which modulates a wide variety of biological processes, including cell growth, differentiation, apoptosis, immunity, extracellular matrix production, angiogenesis, migration and invasion. However, MMPs might negatively regulate cancer cell growth by TGF-β1. For example, MMP-2 expression is suppressed by a threshold level of active TGF-β1, which in turn promotes a contractile vascular smooth muscle cells phenotype that prevents the up-regulation of MMP-2 (Risinger et al., 2010). TGF-β1 modulates the mRNA and protein levels of MMPs (MMP-2 and MMP-9) as much as their inhibitors (TIMP-2 and RECK) (Gomes et al., 2012). Consistent with this concept, we showed that β3GnT8 suppression decreased the expression of TGF-β1 while the overexpression of the β3GnT8 gene increased TGF-β1 expression. The change of TGF-β1 expression exhibited the related trend with that of MMP-2 and MMP-9. This data provide further molecular evidence for the antiproliferation of β3GnT8.

In summary, we describe β3GnT8 as a novel regulator of laryngeal carcinoma cell proliferation. This indicates that aberrant N-glycosylation of cell surface glycoproteins is important for cell survival and proliferation. Down regulation of β3GnT8 is sufficient to affect tumorigenesis and cancer progression. The proposed anti-proliferation mechanisms might be mediated through the inhibition of MMP-2, MMP-9 and TGF-β1, as well as enhancement of TIMP-2. What is more, the examination of the regulatory mechanisms of β3GnT8 will guide to the exact understanding of the various pathways associated with cancer and thus will contribute to more targeted and effective treatment. In the future, further work should be addressed to establish whether anticancer therapies combining specific drugs that inhibit the expression of N-glycan structures and β3GnT8-depleting agents are effective treatments in vivo.

Acknowledgements

This work was supported by National Natural Science Foundation of China to Q.C. (nos. 30670462 and 31170772).

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DOI:http://dx.doi.org/10.7314/APJCP.2012.13.5.2087

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Asian Pacific Journal of Cancer Prevention, Vol 13, 2012  2093