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OSTEOPONTIN REGULATION BY PROTEIN KINASE B (AKT) IN HepG2 CELLS

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Aim: **The mechanism responsible for osteopontin regulation is not understood in HepG2 cells. The aim of this study was to investigate the relationship between protein kinase B (Akt), a key gene in PI3K signal transduction pathway, and osteopontin expression.** ***Methods:*** **HepG2 cells were transfected with constitutively active *Akt* and dominant negative *Akt* using lipofectin. The *Akt* transfection was confirmed by Western blot analysis. Osteopontin expression was detected by both Northern blot and Western blot.** ***Results:*** **Overexpression of exogenous Akt was detected in HepG2 cells by Western blot, indicating that HepG2 cells were successfully transfected with the *Akt* genes. In serum-free condition, the expression of osteopontin was either low or undetectable in HepG2 cells transfected with vector only, however, the expression increased after transfection of cells with constitutively active *Akt*. Osteopontin expression decreased when HepG2 cells were transfected with dominant negative *Akt*.** ***Conclusion:*** **Protein kinase B (*Akt*) gene regulated osteopontin expression in RNA level and protein level, suggesting that osteopontin synthesis can be blocked by inactivation of the *Akt* gene. This leads to a potential means of intervention for the inhibition of metastases in liver cancer.**
Key Words: **protein kinase B (Akt), osteopontin, hepatocellular carcinoma, gene transfection.**

Hepatocellular carcinoma (HCC) has been reported as the fourth most common malignant tumor in the world [1, 2]. Its incidence has increased remarkably in many countries such as the United States of America, the United Kingdom, Japan and so on. In China, hepatocellular carcinoma ranks the second in mortality rates among malignant tumors. Although there are various treatments for hepatocellular carcinoma, the efficacy is rather limited mainly because of its high rate of recurrence and strong tendency of metastasis. Therefore, the key to enhance the efficacy of treatment is to control recurrence and metastasis of HCC. Research has demonstrated that metastasis genes initiate migration and dissemination of cancer through certain signaling pathway [3, 4], including hepatocarcinoma cells [5, 6]. Thus, controlling these genes may be an effective means for controlling recurrence and metastasis of HCC.

Osteopontin (OPN), a phosphorylated glycoprotein, has been widely recognized as a product of metastasis gene or a metastasis-associated gene [3], and its close correlation with tumor metastasis has received much concern. Transfections of the OPN gene into benign tumors [4] or slightly malignant tumor cell strains [7] will lead to increased invasion of tumor cells. OPN has been shown to be involved in metastasis of breast cancer in patients with high expression levels of OPN, giving these patients a poor prognosis [8]. Studies have found that OPN expression in rat renal epithelial cells [9] and HL60 cells [10] depends on activation of PI3K [11]. This activation leads to the transmission of signals that leads to cell proliferation, differentiation, transformation and apoptosis, all characteristics that are associated with occurrence and development of tumors.

It has been shown that osteopontin is up-regulated in many kinds of cancer, including breast cancer [7, 12, 13], prostate cancer [14, 15], ovarian cancer [16, 17], brain cancer [18, 19] and lung cancer [20]. OPN is highly expressed in HCC [5, 6] and much more highly expressed in those with metastasis. We have shown that OPN expression in hepatocarcinoma is dependent on PI3K signaling pathway [21], and OPN expression in breast cancer is regulated by Akt, a key gene in PI3K signaling pathway [22], but so far no reports are available on whether OPN expression in HCC is regulated by Akt. Through gene transfection, we found that Akt could regulate OPN expression in hepatocarcinoma HepG2 cells at both the transcriptional and translational levels. This could potentially become a new strategy for gene therapy of HCC.

MATERIAL AND METHODS

Reagents. DMEM medium and probe labeling kit were purchase from Gibco BRL; RNA isolation kit and RNAeasy Mini Kit from QIAGEN; anti-OPN polyclonal antibody from Calbiochem; anti-tubulin monoclonal antibody from Sigma; BCA protein quantity kit, DNA ligase, *E. coli* DH5α, BSA, tryptone and yeast extract from Sagon (Shanghai, China); PVDF membrane from Roche; restriction endonucleases from MBI; and HepG2 cell line from The Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Plasmid pcDNA3.1 — CA-Akt (containing active Akt fragment), pcDNA3.1 — DN-Akt (containing defect Akt fragment) and vacant vector pcDNA3.1 — were kindly provided by Prof. Frank (Columbia University, USA) and plasmid pBlueScript-OPN-OPN fragment 1493bp — were from Dr. Chambers (Canada).

Cell culture. HepG2 cells were cultured in DMEM medium containing 10% FBS (Hyclone), 100 IU/ml

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Abbreviations used: CA-Akt – constitutively active Akt; DN-Akt – dominant negative Akt; HCC – hepatocellular carcinoma; OPN – osteopontin; PI3K – phosphatidylinositol- 3- kinase.

penicillin and 100 mg/L gentamycin at 37 °C — under an atmosphere of 5% CO_2.

Transfection of Akt gene. Cells in exponential growth were seeded into 6-well plates at a concentration of 1×10^5/ml. After 24 h, liposomes mixed with 3 μg of active *Akt (CA-Akt)* or deficient *Akt-DN-Akt* were used to transfect HepG2 cells. Culture medium was replaced after 6 h of incubation, and medium containing 500 μg/mL G418 was used for screening 48 h later. About 3 weeks later, ten G418-resistant clones were selected with a cloning ring for amplification in culture.

Construction of plasmid pGEM-OPN. Plasmid pGEM-OPN was constructed by PCR-cloning methods using plasmid pBlueScript-OPN containing OPN fragment as a template. The primers for OPN were: forward, 5′-ATGGATCCGATGACACTGATGATTCTCAC-3′, reverse, 5′-GCGAATTCGAATTCACGGCTGACAAA-3′ with BamHI and EcoRI digestion sites in the forward primer and reverse primer, respectively. The amplified fragment, which was 486 bp in length, was purified, digested and purified again. The resulting fragment was connected with vector pGEM by T4 DNA ligase, and the product was tranfected into DH5α strain, amplified, digested with BamHI and EcoRI to get positive clones containing the OPN fragment, and finally identified by DNA sequencing (Sagon, Shanghai).

Probe labeling and Northern blot. OPN fragment was obtained by digesting the plasmid pGEM-OPN with BamHI and EcoRI. The probe was labeled with isotope ^{32}P according to instructions provided in the kit. Northern blot was performed as follows: total RNA was extracted according to instruction of RNAeasy Mini Kit, and a 752 ultraviolet spectrophotometer was used to detect the purity. 10 μg of template RNA was subjected to formaldehyde degenerated gel electrophoresis, transferred to a nylon membrane, fixed with ultraviolet crosslinker, and incubated with OPN probe labeled with ^{32}P overnight. After washing and fixation, the membranes were stored at −80 °C, until they were visualized by autoradiography. The washed membrane was incubated with GAPDH cDNA probe to act as an inner control for correcting the sample amount of RNA.

Western blot. After discarding the medium, the cells were washed twice with PBS and lysed in RIPA buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate). Cell lysates were denatured at 100 °C for 5 min, quantitied by BCA kit. Equal amounts of protein were separated by SDS-polyacrylamide gel, and transferred to PVDF membranes. The membranes were blocked with BSA, and incubated with antibodies to osteopontin (Calbiochem). The membranes were washed with TBST buffer, incubated in a 2nd antibody, and visualized by ECL. Membranes were stripped and re-probed with antibodies to tubulin as an internal control.

RESULTS

Identification of HepG2 cells transfected with the Akt gene. We asked whether expression of osteopontin is regulated by Akt in HepG2 cells, we stably transfected HepG2 cells with the constitutively active (CA) *Akt* gene, dominant negative (DN) *Akt* gene and vector alone. After transfection of *Akt* genes into HepG2 cells, 20 colonies were selected for amplification in culture and then identified by Western blot analysis. Five clones were obtained for the active *Akt* gene and three clones for the DN form. Compared with those clones transfected with vector, the Akt protein expression was significantly increased in those transfected with *CA-Akt* or *DN-Akt* (Fig. 1, lane 3 and 4). This suggested that we have stably established a HCC HepG2 cell line that expresses exogenous *Akt* gene.

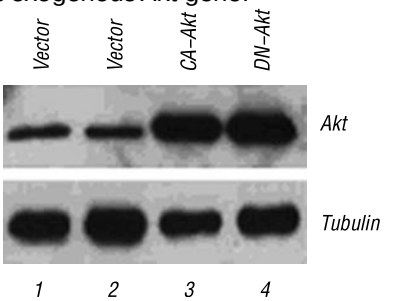

Fig. 1. Western blot analysis of expression of Akt and tubulin in HepG2 cells transfected with *CA-Akt* and *DN-Akt* genes. Although there was a difference in tubulin expression it did not influence the interpretation of the results

Constitutively active Akt (CA-Akt) induced OPN gene expression in HepG2 cells. The expression levels of the transfected genes were measured on the RNA and protein levels. After transfection of the vector into HepG2 cells, in serum-free conditions, constitutive expression of OPN was fairly low or undetectable (Fig. 2, lane 2); in contrast, OPN expression in stable transfectant of the *CA-Akt* gene was significantly increased as seen by Northern blot (see Fig. 2, *a*) and Western blot (see Fig. 2, *b*) analysis when cells were in serum-free conditions. There was no significant change in GAPDH (Northern blot) and tubulin (Western blot), which were the inner controls, suggesting that the sample amounts were equivalent.

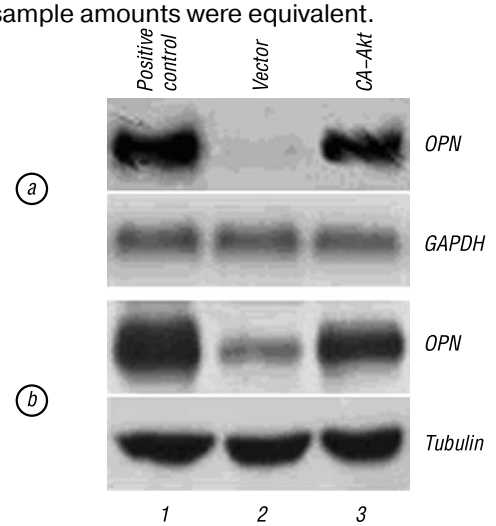

Fig. 2. Northern blot analysis (*a)* and Western blot analysis (*b)* of OPN expression in HepG2 cells transfected with vector and *CA-Akt*

Dominant negative Akt (DN-Akt) suppressed OPN expression in HepG2 cells. Compared with those clones transfected with vector only, OPN expression in HepG2 cells was significantly reduced after *DN-Akt* gene transfection, as seen in Northern (Fig. 3, *a*) and Western blot (Fig. 3, *b*) analysis. There was no obvious change in GAPDH (Northern blot) and tubulin (Western blot) again, suggesting that the sample amounts are equivalent.

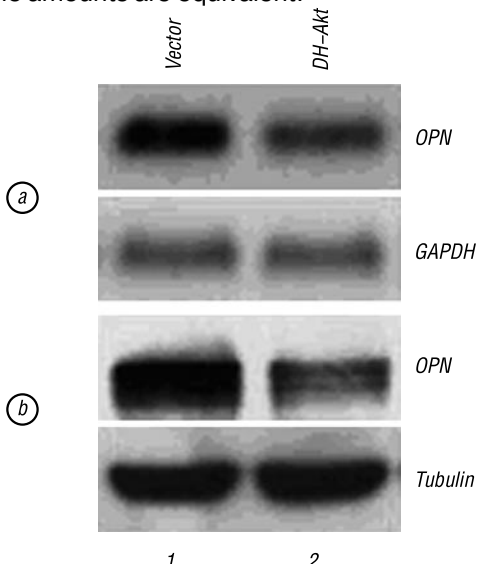

Fig. 3. Northern blot analysis *(a)* and Western blot analysis *(b)* of HepG2 cells transfected with vector and *DN-Akt*

DISCUSSION

Osteopontin, a secreted phosphorylated glycoprotein containing a RGD sequence, can promote cell chemotaxis, adhesion and migration via its receptors such as integrin and CD44 [23, 24]. Transfection of oncogene *H-ras* into NIH3T3 cells leads to cell transformation and oncogenesis. It has been shown that the expression and secretion of OPN mRNA are related with Ras expression level and cell metastasis ability [25], whereas transfection of OPN antisense RNA results in reduced metastasis ability and oncogenesis [26]. OPN is overexpressed in colorectal cancer, cells especially upan metastasis. Therefore, the relationship between OPN and tumor metastasis has prompted much concern.

Protein kinase B (PKB), also known as Akt [27], is a key enzyme in the phosphatidylinositol-3- kinase (PI3K) signaling pathway [28]. It regulates metabolism, transportation of blood sugar, synthesis of glycogen and proteins in cells, as well as regulates cell growth and inhibits cell apoptosis [29]. Deregulation of these pathways plays an important role in the occurrence and development of tumors.

In the present study, constitutively active *Akt* and dominant negative *Akt* were successfully transfected into HepG2 cells. Stable transfectants expressing exogenous *Akt* gene were obtained after screening, making them ideal for research on the characteristics and functions of the *Akt* gene. We found that OPN expression was induced in HepG2 cell transfected with *CA-Akt*, while it was reduced in HepG2 cells transfected with *DN-Akt.* These results suggest for the first tame that *Akt* regulated OPN synthesis at the transcriptional level and the protein level in HCC cells. We have also demonstrated that epidermal growth factor induced OPN expression through the PI3K signaling pathway in HepG2 cells [21]. Taken together, it could be concluded that EGF/PI3K/Akt signaling is the critical pathway for regulation of OPN expression in HepG2 cells.

We have previously reported that Akt kinase activity can induce the transactivation of the osteopontin promoter in breast cells [23]. Here, our data showed that Akt regulated the expression of OPN in HepG2 cells. An insight into the molecular mechanism can provide a theoretical basis for therapy targeting the *Akt* gene. Such processes would block the production of the metastasis gene OPN, by intervening the *Akt* gene, thereby inhibiting metastasis of hepatocarcinoma.

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РЕГУЛЯЦИЯ ЭКСПРЕССИИ ОСТЕОПОНТИНА ПРИ УЧАСТИИ ПРОТЕИНКИНАЗЫ В В КЛЕТКАХ ЛИНИИ HepG2

Цель: **механизм регуляции экспресии остеопонтина еще детально не изучен. Целью данного исследования было изучение взаимосвязи между протеинкиназой В (Akt), ключевым геном PI3K пути сигнальной трансдукции, и экспрессией остеопонтина.** ***Методы:*** **при помощи липофектина клетки HepG2 трансфецировали конститутивно экспрессирующимся геном *Akt* и доминантно негативным геном Akt. Akt-трансфектанты отбирали при помощи Вестерн-блоттинга. Детекцию экспрессии остеопонтина осуществляли методами Нозерн- и Вестерн-блоттинга.** ***Результаты:*** **эффективность трансфекции была подтверждена выявленной гиперэкспрессией экзогенного гена Akt в клетках линии HepG2. В условиях культивирования в бессывороточной среде экспрессия была низкой или даже ниже уровня детекции в клетках линии HepG2, трансфицированных только вектором; тем не менее, уровень экспрессии этого белка возрастал после трансфекции клеток конститутивно экспрессирующимся геном *Akt*. Экспрессия остеопонтина снижалась после трансфекции клеток линии HepG2 доминантно негативным геном *Akt*.** ***Выводы:*** **ген протеинкиназы В регулирует экспрессию остеопонтина на уровне РНК и белка. Предполагается, что его синтез может быть блокирован путем инактивации гена *Akt*.**

***Ключевые слова*: протеинкиназа В, Akt, остеопонтин, гепатоцеллюлярная карцинома, генная трансфекция.**