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The effect of Pokemon on bladder cancer epithelial–mesenchymal transition

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ABSTRACT

Objective: This study aimed at detecting Pokemon expression in bladder cancer cell and investigating the relationship between Pokemon and epithelial–mesenchymal transition. Furthermore, we investigated the functions of Pokemon in the carcinogenesis and development of bladder cancer. This study was also designed to observe the inhibitory effects of siRNA expression vector on Pokemon in bladder cancer cell.
Methods: The siRNA expression vectors which were constructed to express a short hairpin RNA against Pokemon were transfected to the bladder cancer cells T24 with a liposome. Levels of Pokemon, E-cadherin and β-catenin mRNA and protein were examined by real-time quantitative-fluorescent PCR and Western blot analysis, respectively. The effects of Pokemon silencing on epithelial–mesenchymal transition of T24 cells were evaluated with wound-healing assay.
Results: Pokemon was strongly inhibited by siRNA treatment, especially siRNA3 treatment group, as it was reflected by Western blot and real-time PCR. The gene and protein of E-cadherin expression level showed increased markedly after Pokemon was inhibited by RNA interference. While there were no differences in the levels of gene and protein of β-catenin among five groups. The bladder cancer cell after Pokemon siRNA interference showed a significantly reduced wound-closing efficiency at 6, 12 and 24 h.
Conclusions: Our findings suggest Pokemon may inhibit the expression of E-cadherin. The low expression of E-cadherin lead to increasing the phenotype and apical-base polarity of epithelial cells. These changes of cells may result in the recurrence and progression of bladder cancer at last.

1. Introduction

Bladder cancer (BC) is the fifth most common cancer in Western countries and the first leading cause of death among urinary malignancies in China [1]. However, patients with non-muscle-invasive BC are at high risk of recurrence and progression into muscle-invasive BC, and the prognosis of patients with muscle-invasive BC is limited due to the high rate of metastasis [2]. Accumulating evidence suggests that epithelial–mesenchymal transition (EMT) plays an important role in cancer invasion and metastasis by endowing cells with a more motile and invasive phenotype [3–5]. EMT is a multistep process in which epithelial cells lose their epithelial characteristics and gain mesenchymal characteristics, such as motility and invasive properties [2]. EMT is typically characterized by an upregulation of mesenchymal markers such as vimentin and a downregulation of epithelial markers such as E-cadherin or cytokeratins [6]. E-cadherin which is expressed in epithelial cells is decreased during EMT in embryonic development, tissue fibrosis, and cancer [7]. E-cadherin repression is frequently accompanied by activation of the β-catenin/Wnt signaling cascade [8]. β-Catenin, a member of the protein complex connecting cadherins to the actin cytoskeleton at adherens junctions, plays a crucial role in the onset and progression of EMT [9,10]. However, until now, a change in expression of E-cadherin and β-catenin have been used to monitor the progress of EMT during embryonic development and cancer progression [6].

Pokemon (POK erythroid myeloid ontogenic factor), also known as FBI-1, LRF and OCZF, is a member of the POK (POZ and krüppel) family of transcriptional repressors which plays a critical role in cell transformation and malignancy. Pokemon consists of an NH2-terminal POZ/BTB domain and 4 COOH-terminal krüppel-type zinc fingers [11]. The POZ/BTB domain is involved in homodimerization or heterodimerization and recruits some corepressors such as BcoR, NcoR, and SMRT, and the krüppel-type zinc fingers domain mediates specific DNA recognition and binding [12,13]. Recent studies indicated that Pokemon is implicated in the pathogenesis of several cancers including prostate, breast, non-small cell

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lung, and ovarian carcinomas, gliomas, T-cell and B-cell lymphomas [14–19]. An aberrant overexpression of Pokemon in human tumors indicates that this protein can undergo oncogenic transformation and tumorigenesis. However, there is little known about its effect on the development and progression of bladder cancer so far.

Recent research indicates that Pokemon has numerous functions including initiating tumor formation by inhibiting the negative regulation of the ARF-p53 pathway in malignant cells and influencing signal transduction pathways, such as nuclear factor kappa-B (NF-κB), PI3K/Akt, and cell apoptosis [11,16,20]. However, little is known about the precise mechanism of Pokemon in these tumors and its downstream regulator pathways. The study [21] indicates Pokemon is in close contact with β-catenin, whether correlations may be direct or indirect. In addition, Pokemon and kaiso are members of the POK family of transcription repressor, and kaiso is the upstream of β-catenin. On the other hand β-catenin and E-cadherin are the biomarker of EMT. So we speculate that Pokemon may affect the EMT and result in the recurrences and progression of bladder cancer. In this study, we detected Pokemon expression in bladder cancer cell and investigated the relationship between Pokemon and epithelial–mesenchymal transition in bladder cancer cells.

2. Materials and methods

2.1. Cell culture

The human bladder cancer cells T24 was obtained from Chinese Academy of Science (Chinese Academy of Science, china). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 incubator and were routinely passaged at 3- or 4-day intervals.

2.2. SiRNA plasmids and transfection

A total of 3 kinds of SiRNA for Pokemon including SiRNA1 5′ CAGCAGAACGTGTACGAGA 3′, SiRNA2 5′ GCTGGACCTTGTAGATCAA 3′, SiRNA3 5′ GCACAGACACCTCAAGAAA 3′ and scrambled control 5′ ACTACCGTTGTTATAGGTG 3′ were designed and their expression vectors were constructed by Sangon Biotech company (Sangon Biotech, China). Interference plasmid and the negative control plasmid were transfected to bladder cancer cells with Lipofectamine 2000 transfection reagent (Invitrogen). 48 h after transfection, total RNA and protein were extracted from the cells, and the expression levels of Pokemon, E-cadherin and β-catenin mRNA and protein were examined by RT-PCR and Western blot, respectively.

2.3. RNA isolation and real time PCR

Acid guanidine thiocyanatephenol-chloroform extraction was used to isolate total RNA from T24 cells. Total RNA of T24 cells was extracted by Trizol reagent (Life Technologies) according to the manufacturer's instructions. The concentration and quality of the extracted total RNA were determined by measuring OD260 and the OD260/OD280 ratio. The reverse transcription of 1 μg RNA into cDNA was carried out using Superscript II reverse transcriptase (TakaRa, Japan) and stored at −80 °C until use.

PCR: Primers for human Pokemon, E-cadherin, β-catenin and β-actin genes were designed with Primer Express 2.0 software (Applied Biosystems) and synthesized by Sangon. The basic information on primers, including gene name, forward primer, reverse primer and product size (bp) was presented in Table 1. Real-time PCR was done in triplicate for each sample in a 20 μl reaction mixture, which consisted of template DNA (2 μl), primers (1 μl), SYRB premix (10 μl), ddH_2O (7 μl) (ExScript real-time PCRKit, TaKaRa). PCR was done in a 7900HT Fast real-time PCR instrument using the following thermal cycles: The cycling variables were 95 °C (30 s), 40 cycles of 95 °C (5 s), 60 °C (30 s). According to the method tested by Pfaffl, the relative expression ratio of a targeted gene was calculated based on efficiency and the Ct compared with a reference gene (β-actin).

2.4. Western blot analysis

Nuclear and cytoplasmic proteins of T24 cells were extracted by using NE-PER® unclear and cytoplasmic extraction kit (PIERCE) according to the protocol. For the whole cell extracts, cells were lysed in RIPA extraction buffer (150 mM sodium chloride, 50 mM Tris–HCl, pH = 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM PMSF, 1% Triton× −100, 1% sodium deoxycholate, 0.1% SDS, 5 mg/ml leupeptin) and were then centrifuged. The supernatant was used as the whole cell protein extract.

2.5. Wound-healing assay

The human bladder cancer cells (4×10^6) were plated in 6-well plates for 24–48 h (until they reached confluence). A diametric scratch was done using a pipette tip followed by two culture medium changes. Cells were photographed in several pre-marked spots as time 0. Multiple photographs were then taken at 6, 12 and 24 h in the same spots for comparison. The percent of wound closure was determined by the following equation: wound closure (%) = (initial wound areas − remaining wound areas)/initial wound areas. The image analysis for measurements of wound areas was done using Image-Pro Plus software (Media Cybernetics, MD). The cell scrape wound healing assay was repeated three times.

2.6. Statistical analysis

All the experiments were done in triplicate, and the results are given as the mean ± standard error. Statistical comparisons were done using the *t* test and ANOVA. All procedures were done using SPSS, version 13.0 with $P < 0.05$ considered statistically significant.

Table 1
Nucleotide sequence of primers used in real time PCR.

Gene	Primers	Nucleotide sequence 5′-3′	Length (bp)	Temperature (°C)
Pokemon	Forward	CAGCCAAGTCTGTGACTTGCACGTAC	134	60
	Reverse	CTATGTCGAAAAGTGTTTCTGTCATC		
E-cadherin	Forward	AACATCCTAGCCAAGATCC	116	57
	Reverse	GCACCTGACCCTTGTACGTG		
β-catenin	Forward	AACGGCTTTCGGTTGAGCTG	147	60
	Reverse	TGGCGATATCCAAGGGCTTC		
β-actin	Forward	TGCCTTTGTGCACTGGTATG	124	60
	Reverse	CTGGAGCAGTTTGACGACAC		

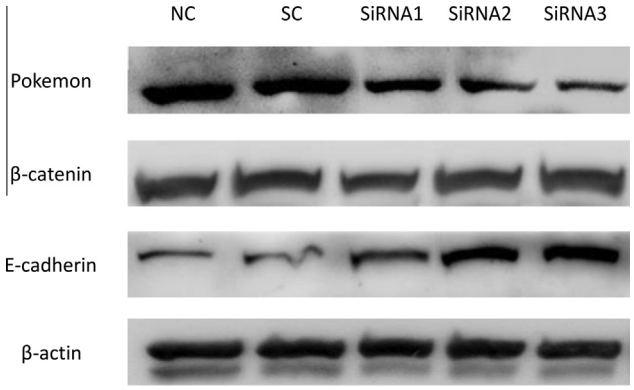

Fig. 1. Western blot analysis of β-catenin, E-cadherin and Pokemon in bladder cancer cell in five groups. Pokemon protein was strongly inhibited in siRNA treatment group, especially in siRNA3 group. No significant difference was found in β-catenin protein. While E-cadherin protein was strongly expressed in siRNA treatment group, especially in siRNA2 and siRNA3 group. (NC: negative control; SC: scrambled control; SiRNA1: SiRNA1 interference; SiRNA2: SiRNA2 interference; SiRNA3: SiRNA3 interference).

3. Result

3.1. Effect of Pokemon siRNA on expression of Pokemon in bladder cancer cells

To examine the specific effect of Pokemon siRNA treatment on Pokemon expression in bladder cancer cells, the Pokemon mRNA and protein expression levels were determined quantitatively using real time PCR and Western blot analyses, respectively. The level of protein was demonstrated in Fig. 1, Pokemon protein was strongly inhibited by siRNA treatment groups, especially siRNA3 group, as it was reflected by Western blot. The inhibition rates of Pokemon protein after infection with specific Pokemon siRNA were 34% for siRNA1, 46% for siRNA2 and 68% for siRNA3 (Table 2). The inhibition rates of Pokemon gene after infection with specific Pokemon siRNA were 59% for siRNA1, 78% for siRNA2 and 89% for siRNA3 (Table 2). The results demonstrated that Pokemon expression was decreased significantly at 48 h after infection with specific Pokemon siRNA ($P < 0.05$ compared with control groups). The level of Pokemon mRNA was demonstrated in Fig. 2A, Pokemon mRNA was strongly inhibited by siRNA treatment groups which was in accordance with the level of Pokemon protein.

3.2. Effect of Pokemon siRNA on expression of biomarkers of EMT in bladder cancer cells

To examine the specific effect of Pokemon siRNA treatment on EMT in bladder cancer cells, the E-cadherin and β-catenin mRNA and protein expression levels were determined quantitatively using real time PCR and Western blot analyses, respectively. The level of E-cadherin and β-catenin protein were demonstrated in Fig. 1, E-cadherin protein was strongly expressed in siRNA treatment groups, especially in siRNA3 group, as it was reflected by Western blot. The β-catenin protein had no significant difference among the five groups. The levels of E-cadherin and β-catenin mRNA were demonstrated in Fig. 3A and B, respectively. They had the same trends in accordance with the levels of protein.

3.3. The relationship between Pokemon and E-cadherin and β-catenin

The relationship between Pokemon and E-cadherin and β-catenin was demonstrated in Fig. 2B. We could see that the expression of Pokemon was inversely correlated with the E-cadherin expression, which suggested that Pokemon could inhibit the expression of E-cadherin. The expression of β-catenin was not associated with the expression of Pokemon and E-cadherin, which suggested that there was no relationship between Pokemon and β-catenin.

3.4. Pokemon siRNA on bladder cancer reduce cell wound healing in vitro

The bladder cancer cells after Pokemon siRNA showed a significantly reduced wound-closing efficiency at 6, 12 and 24 h. After 6 h, the percentage of wound closure in NC group was significantly

Table 2
The inhibition rate of Pokemon.

Group		NC	SC	siRNA1	siRNA2	siRNA3
Protein level	Grey ratio	1	0.91 ± 0.02	0.41 ± 0.04	0.22 ± 0.03	0.11 ± 0.02
	Inhibition rate	–	–	0.59	0.78	0.89
Gene level	Grey ratio	1.03 ± 0.04	0.96 ± 0.02	0.66 ± 0.03	0.54 ± 0.07	0.33 ± 0.03
	Inhibition rate	–	–	0.34	0.46	0.68

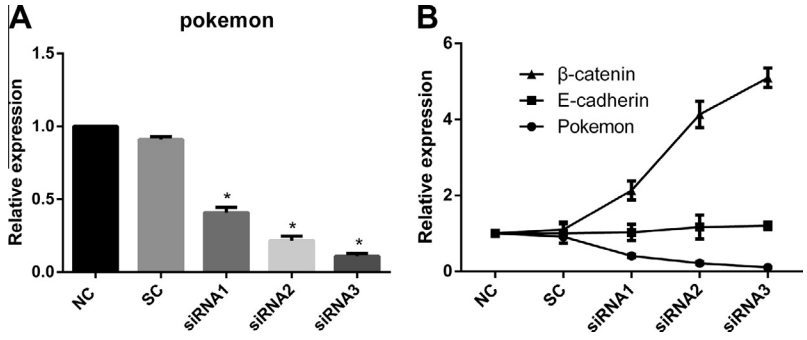

Fig. 2. The relative expression of Pokemon in bladder cancer cell in five groups (A). Pokemon mRNA was strongly inhibited in siRNA treatment groups, especially in siRNA3 group. The relationship between Pokemon, E-cadherin and β-catenin (B). The expression of Pokemon was inversely correlated with the E-cadherin expression while the expression of β-catenin was not associated with the expression of Pokemon. (NC: negative control; SC: scrambled control; SiRNA1: SiRNA1 interference; SiRNA2: SiRNA2 interference; SiRNA3: SiRNA3 interference) (*$P < 0.05$ vs NC).

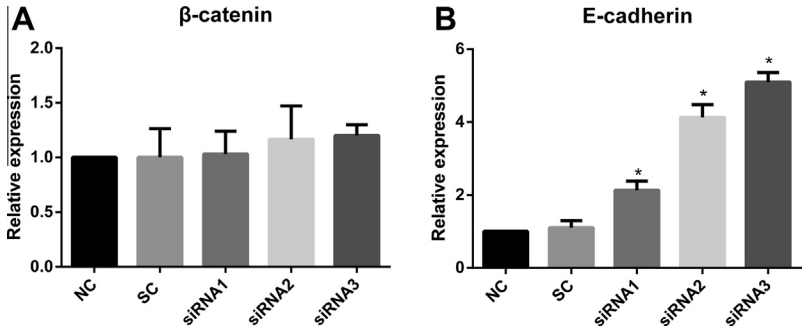

Fig. 3. The relative expression of β-catenin in bladder cancer cell in five groups (A). No significant difference was found in β-catenin gene among the five groups. The relative expression of E-cadherin in bladder cancer cell in five groups (B). The gene level of E-cadherin was strongly high in siRNA treatment groups, especially in siRNA2 and siRNA3 groups. (NC: negative control; SC: scrambled control; SiRNA1: SiRNA1 interference; SiRNA2: SiRNA2 interference; SiRNA3: SiRNA3 interference) (*$P < 0.05$ vs NC).

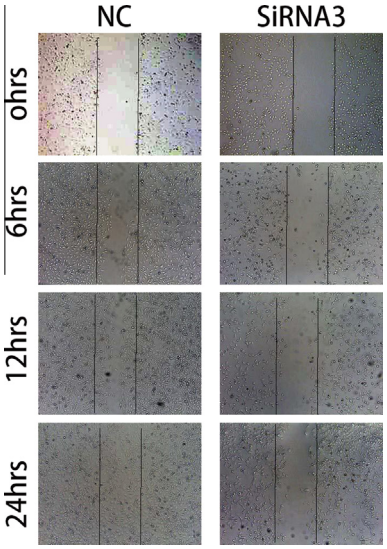

Fig. 4. The in vitro wound-healing assays of bladder cancer cell in NC and SiRNA3 groups: After 6 h, the percentage of wound closure in NC group was significantly higher (40.1% ± 3.4%) than that in siRNA3 group (16.7% ± 3.2%) ($P < 0.05$). After 12 and 24 h, the percentage of wound closure in NC group was also significantly higher (75.5% ± 2.8%, 91.8% ± 2.7%) than that in siRNA3 group (33.1% ± 3.3% ($P < 0.05$), 45.3% ± 3.5% ($P < 0.05$)) (NC: negative control; SiRNA3: SiRNA3 interference).

higher (40.1% ± 3.4%) than that in siRNA3 group (16.7% ± 3.2%) ($P < 0.05$). After 12 h and 24 h, the percentage of wound closure in NC group was also significantly higher (75.5% ± 2.8%, 91.8% ± 2.7%) than that in siRNA3 group (33.1% ± 3.3% ($P < 0.05$), 45.3% ± 3.5% ($P < 0.05$)) (Fig. 4). This result indicated that Pokemon was associated with proliferation and migration of bladder cancer cell. Based on that EMT played an important role in cancer invasion and metastasis by endowing cells with a more motile and invasive phenotype, we speculated that Pokemon may inhibit the expression of E-cadherin, then effect EMT and at last promote the proliferation of tumor cells.

4. Discussion

Bladder cancer is responsible for the deaths of 150,000 people annually and is the seventh most prevalent type of cancer worldwide [2]. At the time of the first diagnosis, about 70–80% of BCs are non-muscle-invasive BCs(NMIBCs) and the remaining 20–30% are muscle-invasive BCs (MIBCs). For NMIBCs 30–50% of these patients have recurrences after transurethral resection of the primary tumor, and 10–20% progress to MIBC. So it is fact that patients with non-muscle-invasive BC are at a high risk of recurrence and progression into muscle-invasive BC.

Epithelial cells have important barrier functions that are facilitated by their tight cell-to-cell interactions [22]. Loss of these cell-to-cell interactions can induce morphological changes in epithelial cells and increase their cellular motility which is closely associated with the invasion and metastasis of several cancers. BC is a kind of multifocality and high rates of relapse, progression, and metastasis cancer, so the EMT is likely to participate in BC as well. The most important mediator of cell-to-cell adhesion in epithelial tissues is cadherin, which is a family of cell-surface adherence junctional proteins. The first cadherins to be identified were E-cadherin, P-cadherin, and N-cadherin [23]. E-cadherin plays an essential role in epithelial cell-to-cell interactions because it mediates the connections between adjacent epithelial cells and maintains the phenotype and apical-base polarity of epithelial cells [24]. Due to these functions of E-cadherin, it plays a key tumor suppressor role in suppressing the invasiveness of cancer cells [25]. Cadherin switch is a key change during EMT, during which the normal expression of E-cadherin is replaced by the abnormal expression of N- or P-cadherin [26,27]. This down-regulation of E-cadherin is associated with the release of β-catenin, which then migrates to the nucleus and activates WNT signaling resulting in the EMT and metastasis [28]. For this reason, E-cadherin and β-catenin have often been used to monitor the progress of EMT during embryonic development and cancer progression.

Pokemon is overexpressed in multiple human cancers, and cells lacking Pokemon are refractory to oncogenic transformation. It was reported that the Pokemon protein can bind to the promoter region of the Rb gene via its POZ domain, and cause the histones H3 and H4 in the promoter sequence nucleosome to initiate deacetylation by activating histone deacetylase, resulting in inhibition of Rb transcription, uncontrollable cell cycle, and cell malignant mutation [29]. Other researchers reported that Pokemon can also influence signal transduction pathways, such as nuclear factor kappa-B (NF-κB), PI3K/Akt, and cell apoptosis [13,30,31]. Although the precise function of Pokemon in oncogenesis is unknown, previous studies showed that Pokemon may regulate a set of genes in cell growth and proliferation, which may be important in cancer development and cancer cell proliferation [20]. In our study, we found that bladder cancer cell had a significantly reduced wound-closing efficiency after Pokemon was inhibited by RNA interference. This means that Pokemon play an important role in recurrence and progression of bladder cancer. Furthermore we found that the level of E-cadherin was markedly increased after Pokemon was inhibited by RNA interference. These results imply that Pokemon may inhibit the expression of E-cadherin, and the low expression of E-cadherin lead to increase the phenotype and apical-base polarity of epithelial cells. These changes of cells might result in the recurrence and

progression of bladder cancer at last [32]. To our surprise, in our experiment β-catenin was showed the same level of gene and protein after Pokemon was inhibited by RNA interference. The β-catenin, which can migrate to the nucleus and activates WNT signaling resulting in the EMT and metastasis is associated with the level of E-cadherin [28]. But in our study, β-catenin seems no associated with the level of E-cadherin and Pokemon in bladder cancer cells. The reason of this may be that Pokemon affect the expression of E-cadherin is a complex process during which the precise signal pathway needs to be further studied. This process may be not associated with the change of β-catenin. However, E-cadherin molecule contains an ectodomain composed of 5 extracellular cadherin (EC1-5) repeats (550 aa in total), in which EC1 composes the HAV peptide sequence responsible for homophilic interactions, a transmembrane region, and a cytoplasmic tail (150 aa) [33]. The latter has a β-catenin binding domain, whose core sequence motif DEEGGGEED is important for p120-catenin binding, resulting in the stabilization of the E-cadherin/catenin complex at the cell surface [34,35]. This E-cadherin/catenin complex plays an important role in maintaining the phenotype and apical-base polarity of epithelial cells. So how the β-catenin and E-cadherin affect the bladder cancer cell after Pokemon was inhibited by RNA interference needs to be studied in the near future.

To the best of our knowledge, this is the first research to study the effect of Pokemon on bladder cancer epithelial–mesenchymal transition. We found that Pokemon may inhibit the expression of E-cadherin. The low expression of E-cadherin lead to increase the phenotype and apical-base polarity of epithelial cells. However, we also acknowledge some inherent limitations in our research. There is no enough data to reveal the mechanism of how Pokemon inhibit the expression of E-cadherin and how Pokemon affect the phenotype and apical-base polarity of epithelial cells. Given the limitations in our study, Pokemon in bladder cancer cell requires further study.

5. Conclusion

Pokemon may inhibit the expression of E-cadherin. The low expression of E-cadherin lead to increase the phenotype and apical-base polarity of epithelial cells. These changes of cells may result in the recurrences and progression of bladder cancer at last.

Conflicts of interest/disclosures

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