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Full Length Research Paper

Targeting PLCε to attenuate rat bladder tumorigenesis triggered by mnu through the H-ras signaling cascade

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The crucial role of phospholipase C epsilon (PLC ε) in cancinogensis has been described recently. However, whether or not it decreases the methyl-nitroso-urea (MNU) induced rats' bladder tumorigenesis and the probable mechanism remains poorly understood. Here, we investigated the efficacy of PLCε knockdown to MNU induced bladder tumor and its possible mechanism. First, a plasmid contains small hairpin Ribonucleic acid (RNA) against PLC ε was constructed. Then, this plasmid were intravesically installed into SD rats to silence the expression of PLC ε. 40 PLC ε silenced SD rats and 40 normal rats were intravesically installed with MNU, respectively. Another 10 normal SD rats were intravesically installed with saline. 12 weeks after installation, all rats were sacrificed. The bladders were harvested for pathological and histological examination, Reverse transcription polymerase chain reaction (RT-PCR) and Western-blotting. The pathological and histological examination results showed that 35 rats appeared tumor in normal rats group, while no rats appeared tumor in PLC ε silenced group and saline group. RT-PCR and Western-blotting showed PLC ε , PCNA were overexpressed in 35 tumor appeared rats and lowly expressed in other rats. The results reveal that the PLCε knockdown could interrupt the signal transduction in order to prevent MNU induced rats bladder tumorigenesis.

Key words: PLCε, knockdown, MNU, Ras signal pathway and mechanism.

INTRODUCTION

Methyl-nitroso-urea (MNU) is a common carcinogen in laboratory. It has been reported to induce mammary and bladder carcinogenesis (EI-sohemy et al., 1996, Hicks, l980). MNU could induce cytotoxic damage to the urothelium, which would have invoked a "wound response", driving rapid proliferation and allowing the accumulation and perpetuation of mutations (Crallan et al., 2006). In MNU induced mammary tumorigenesis model, nearly 75% exist mutations in codon 12 of Ha-Ras (Sukumar et al., 1983). The Ras genes were identified from Ki MuSV(Kirsten murine sarcoma) oncogenic virus and Harvey sarcoma virus (HSV) almost 35 years ago (Chesterman, 1966; Kirsted et al., 1970). The Ras gene is a kind of proto-oncogene. It is involved in a wide range of growth factors and regulated cell proliferation (Cox and Der, 2002; Bos, 1989). When modified by isoprenoids, the Ras translocated from cytosol to the plasmamembrance and become a kind of oncogene (Gelb, 1997). The Ras gene family encodes a group of heterotrimeric of G-proteins which are commonly over-expessed or mutated in a variety of animal and human tumors (Barbacid, 1987; Bos, 1989). Activated Ras genes are the most frequently detected oncogenes in human tumors and are present in a very high proportion of certain tumor types (Margaret and Magali, 1993). More than 90% of pancreatic tumors have K-Ras (Almoguera et al., 1988) and leukemias contain primaryly mutated N-Ras, for example. In bladder tumor, mutation of H-Ras is identified in codons 12, 13 (Margaret and Magali, 1993; Almoguera et al., 1988) and 61 (Fujita et al., 1985; Malone et al., 1985; Visvanathan et al., 1985).

PLC ε, the sixth family member of PLC isozymes, was

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initially identified in Caenorhabditis elegans as a Ras binding protein (Song et al., 2001). It contains conserved Ras-interacting domain, including a C-terminal RA domains which interact directly with Ras-family GTPases and an N-terminal CDC25 domain which impacts guanine nucleotide exchange activity to the protein (Kelly et al., 2001; Bunney and Katan, 2006). It has been reported closely related with the development of skin and colorectal tumor (Shuzo et al., 2008; Mingzhen et al., 2009). Our group had previously proved that PLC ε was closely related with the grading and staging of human bladder cancer and it played a pivotal role in regulating bladder cell proliferation and apotosis.

We, therefore, examined the effect of PLC ε knockdown in a MNU induced bladder tumorigenesis model to determine whether PLC ε is effective enough to prevent MNU induced bladder tumorigenesis, and if H-Ras mutation is a probable mechanism for MNU induced bladder tumorigenesis. Further more, we also determined the singal pathway by which PLC ε knockdown prevented MNU induced bladder tumorigenesis.

MATERIALS AND METHODS

Chemical and reagents

MNU were purchased from Applichem Corp (Germany); PLC ε, Cyclin-D1, PCNA antibody was purchased from Takara Corp (Japan); PCR, western-blotting products were purchased from ShangHai-Huashun Biotechnology Corp (China); H-Ras anti-body, immunohistochemical kit were purchased from Beijing-Zhongshan Biotechnology Corp (China).

PLC ε shRNA plasmids construction and identification

The plasmid was constructed by inserting a small hairpin RNA into PLCε RNA. According to Gene Bank, the target site of PLCε selected was at the point between 356 to 376 GCACATACTGTCAGACGAAGT, 2208-2228 AAATGTGGCAGTTCATGGACC. Recombinant plasmids were identified by restriction enzyme digestion and DNA sequencing.

PLC ε knockdown in normal S-D rats

We clamped the rat urethra with a hemostatic forceps, injected a volume of 2 ml diluted hydrochloric acid into the rat bladders to destroy the bladder mucosa, then a volume of 1.0 ml plasmids containing 30 μg PLC ε shRNA was injected into the rat bladders, once a week, totally 8 weeks. 30 min after the injection, the hemostatic forceps were undone. The efficacy of PLC ε silencing was evaluated by RT-PCR and Western-blotting.

MNU intravesical installation

Right after the PLCε knockdown, 20 PLCε silenced rats and 40 normal rats were chosen for MNU installation. We divided these rats into three groups: PLCε silenced group (S), normal MNU installation group (N), and normal control group (C). Rats in S and N group were intravesically installed with MNU, at the dose of 2 mg per time, twice a month, totally for two months. Rats in C group were intravesically installed with saline. At the end of installation, rat's bladder was observed for cancinogensis. This process lasted for 14 weeks.

Immunohistochemical analysis of H-Ras in MNU induced rat bladder cancer

Sections of 5-μm-thick paraffin-embedded tissue microarrays were deparaffinized and rehydrated with xylene and ethanol. The expression of H-Ras protein (ZhongShan, China) was determined using the streptavidin biotin complex immunohistochemical staining kit (Boster, Wuhan, China). The activation of Ras oncogene proteins must be translocated to the cell membrane. Thus a positive outcome was brown staining in the cytoplasm.

Total tissue RNA extraction and RT-PCR

Two months after the initial MNU installation, we sacrificed rats and collected the rat bladders. Total RNA was isolated from the tissue by using Trizol Reagent (*invitrogen*, USA) and RT-PCR was performed with a RNA PCR Kit Ver.3.0 (TaKaRa, Japan) according to the manufacturer's instructions. The primers used for PLCε were: the forward, 5'-CATGGAAGGATAAGCGTTGGT-3'; the reverse, 5'-CCCAAGTCCCGTG TTAAGA-3'. The primers used for β-actin were: the forward, 5'-AGGCCAAC CGCGAGAAGATG-3'; the reverse, 5'-AGTTTCGTGGATGCCACAGG-3'. The RT reaction condition was 42℃ for 30 min，99℃ for 5 min, and 5℃ for 5 min; respectively. The PCR condition was: 94°C for 3 min, 30 cycles at 94°C for 1 min, 56°C for 0.5 min, and 72°C for 1 min in 1.5 mM MgCl2-containing reaction buffer. 5 μl PCR products were used for analysis on a BIO-RAD system.

Western-blotting analysis

The protein was extracted from rat's bladder tissues. The protein concentration was determined by Bradford methods. In brief, 150 ug of total protein was loaded into each well of an SDS-gel for separation by PAGE, and then transferred onto polyvinylidene fluoride (PVDF) membranes. The blotted membrances were blocked for 120 min with TBST containing 5% skim milk, followed by probed overnight with primary antibody and secondary antibody. After three washes with TBST, blotted proteins were detected using an image acquisition and analysis system. The antibody used for western blots was as following: anti- PLCε, anti-IgG. Also, the methods of the analysis of proliferating cell nuclear antigen (PCNA) were mentioned earlier.

Statistical analysis

Statistical differences between two groups were analyzed by one-way analysis of variance (ANOVA) or Student-Newman- Keuls (SNK) test. Data were recorded as means ± SD, and values of $P<0.05$ considered significant.

RESULTS

Identification of recombinant plasmid

All plasmids were digested with *Sal* I. Plasmid pGenesil – PLCε was digested with *Sal* I, and resulted in a band of about 400 bp, as shown Figure 1A. The correct sequence was identified by DNA sequencing. The Single-enzyme digested method and DNA sequencing showed that

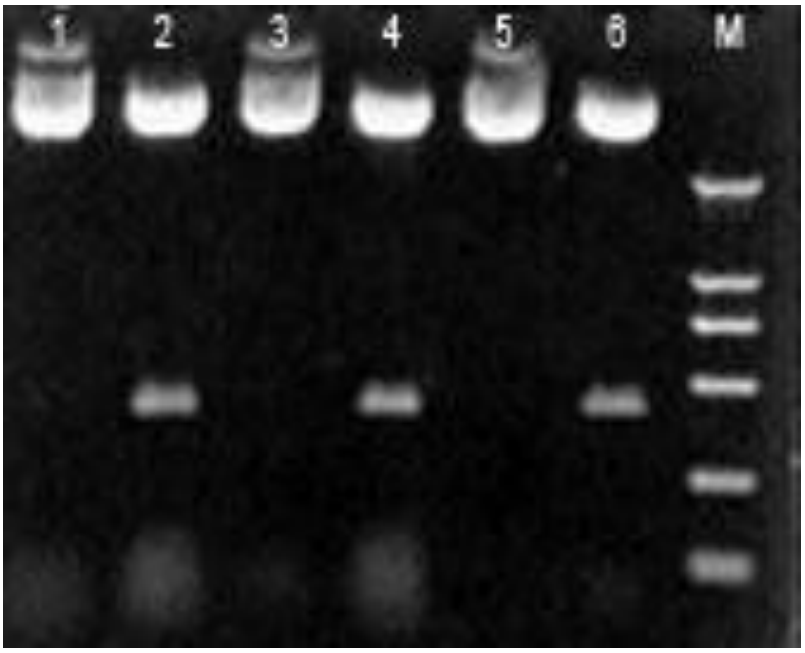

Figure 1. We identified the recombinant plasmid. The recombinant plasmids were digested with *Sal* I and resulted to a band of about 400 bp. These results strongly suggested recombinant constructed successfully. We also detected the DNA sequencing about the recombinant plasmid. The results of the DNA sequencing showed we successfully constructed the recombine plasmids.

recombinant plasmids of pGenesil –PLCε were successfully constructed.

PLCε was effectively silenced by the treatment of PLCε shRNA

The PLCε expression was detected by RT-PCR and western-blotting. We found that the PLCε expression decreased after the injection of PLCε shRNA (Figures 2A and B). We also detected the expression of PLCε at the second week, the forth week, the sixth week and 8th week after the initial injection. And we found that the expression of PLCε decreased with the time developed (Figure 2C). We also changed the injection characteristics. We evaluated the effects of varying the injection volume and dose on the efficiency of PLCε knockdown. Good PLCε silencing efficiency were seen with a volume of 1.0 ml plasmid. We also observed a dose-response relationship between the PLCε silencing efficiency and the amount of injected shRNA up to 50 ug, and saw substantial levels of PLCε silencing efficiency with only 30 ug of PLCε shRNA.

MNU induced non- PLCε-silenced tumorigenesis

At the end of 12 weeks, all rats were sacrificed and bladders were harvest for pathology and histology. 35 rats in non- PLCε-silenced (N) group appeared tumorigenesis at the end of 12 weeks; the tumorigenesis rate was 87.5%. There were 4 rats appeared tumorigenesis in S group, while there were no rats appeared tumorigenesis in C group (Figure 3A). There existed significant difference between N group and another 2 groups. We also tested the time course of the tumorigenesis in N group. We found that 13 rats appeared tumorigenesis at the end of 8 weeks after the initial installation, 16 rats appeared tumorigenesis at 9 weeks, 24 rats appeared tumorigenesis at 10 weeks, 28 rats appeared tumorigenesis at 11 weeks, 35 rats appeared tumorigenesis at 12 weeks, while no more rats appeared tumorigenesis even at a longer time.

H-Ras was positive in MNU treated group

The expression of H-Ras was determined by immunohistochemistry. We evaluated the expression of H-ras in N, S and C groups. We found that 24, 9 and 0 rats expressed H-Ras in N, S and C groups, respectively. The H-Ras positive rates were 60.00, 22.50 and 0%, respectively. The difference is statistically significant ($P < 0.05$).

PLC ε mRNA was overexpressed in N group

We measured the PLCε expression by RT-PCR at the end of 12 weeks. We found that PLCε expression were

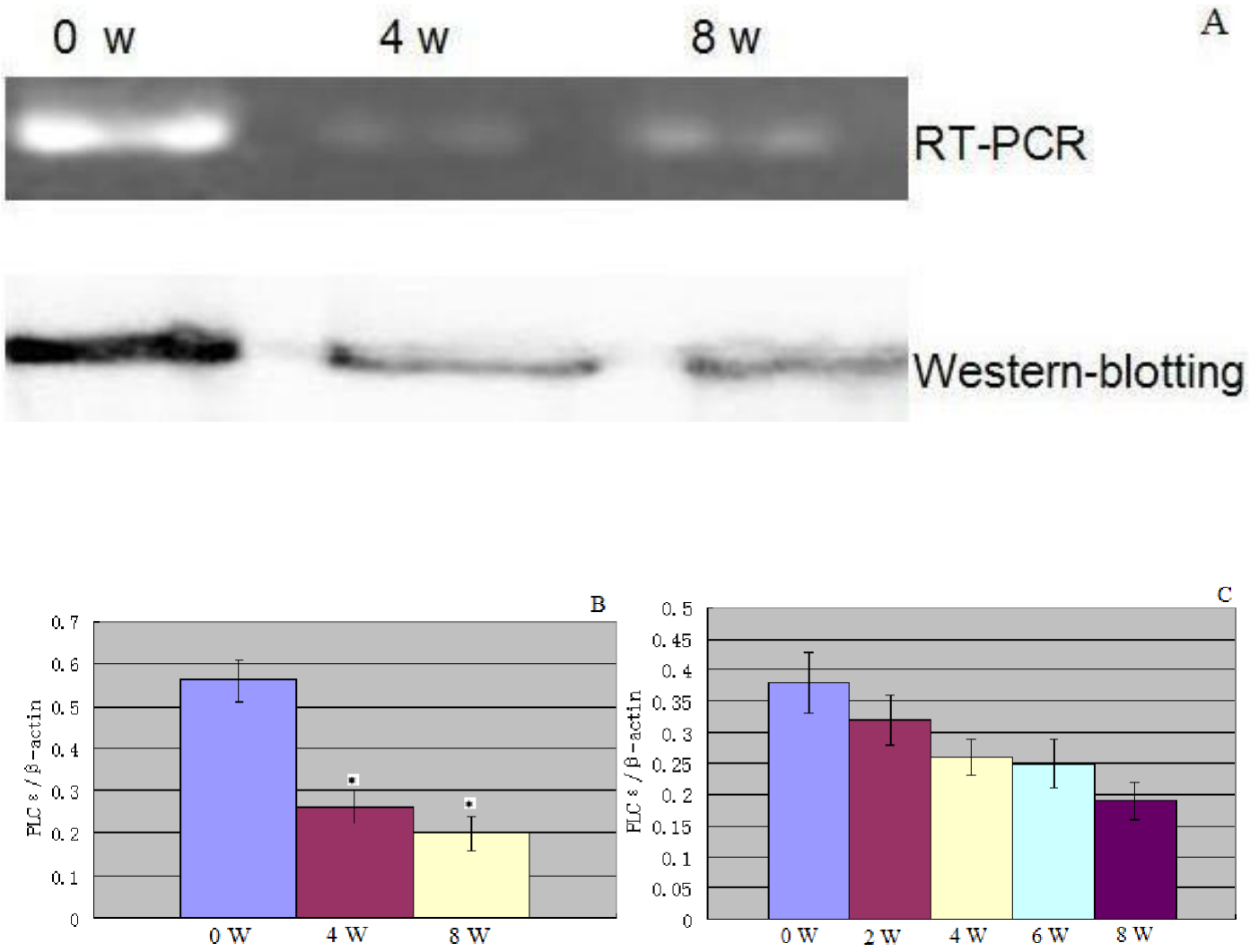

Figure 2. We silenced the PLCε by a fluid mechanics based method and measured the PLCε knockdown efficacy by RT-PCR and western-blotting. A means the RT-PCR and western-blotting results, we could be informed that PLCε expression were decreased at forth and eighth week. B means the semiquantitative analysis of PLCε expression. The PLCε expression at forth and eighth week were statistically signifacant compared with the initial PLCε expression (*P < 0.05). C means the time course of the PLCε knockdown efficacy. It showed that PLCε could be silenced by PLCε shRNA and could get a steady silencing efficacy at 8 th week.

higher in N group, and lower in the other 2 groups (Figure 4A). There existed notable difference between N group and the other 2 groups (Figure 4B). This suggested PLCε played a crucial role in chemic carcinogen induced bladder tumorigenesis.

PLCε and PCNA protein were overexpressed in N group

At the end of 12 weeks, all rats were sacrificed and the bladder was harvested for western-blotting. Proliferating cell nuclear antigen (PCNA) protein is usually taken as markers of proliferation. We investigated here the expression of PCNA protein in MNU induced bladder cancer. Western blot analysis revealed that PLCε shRNA strongly reduced the level of PLCε and PCNA protein in S group, compared with the other 2 groups (P < 0.05), as shown in Figure 5. The result demonstrated that PLCε knockdown could reduce the expression of PCNA protein in bladder cancer.

DISCUSSION

MNU-induced tumors generally have a high frequency (85%) of Ha-Ras mutations (Zarbl et al., 1985). Ras is a major focus of the signaling pathway for multiple growth factors including EGFR, Neu, FGF and VEGF, and the activation of H-Ras must be prenylated in order to be translocated to cell membrane (Cox and Der, 2002). Considered the fact that many human tumors have Ras

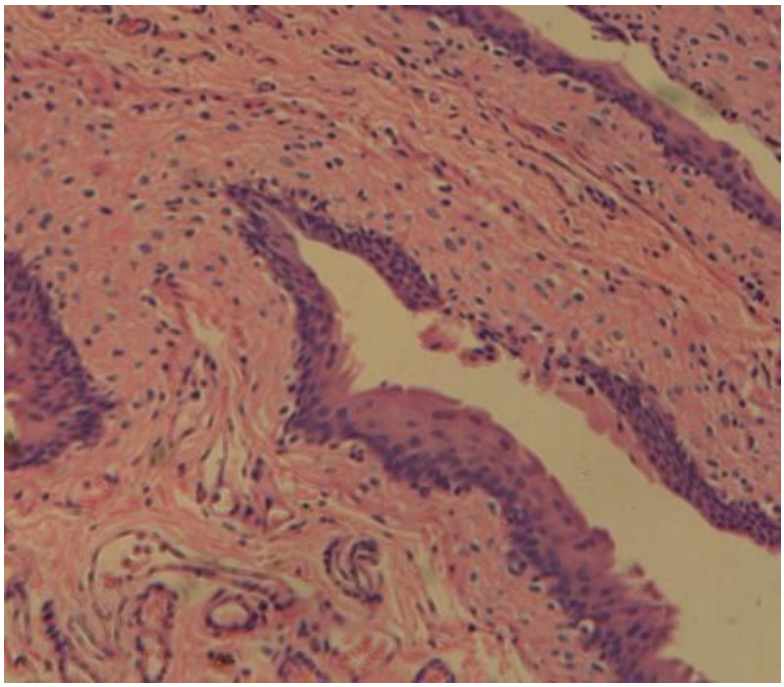

Figure 3. The histology study of rat bladder model (HE × 100). At the end of 8 weeks after the process of MNU installation, the rat bladder mucosa showed multi-center carcinogenesis. The histology study showed mucosa cell arranged disorderly.

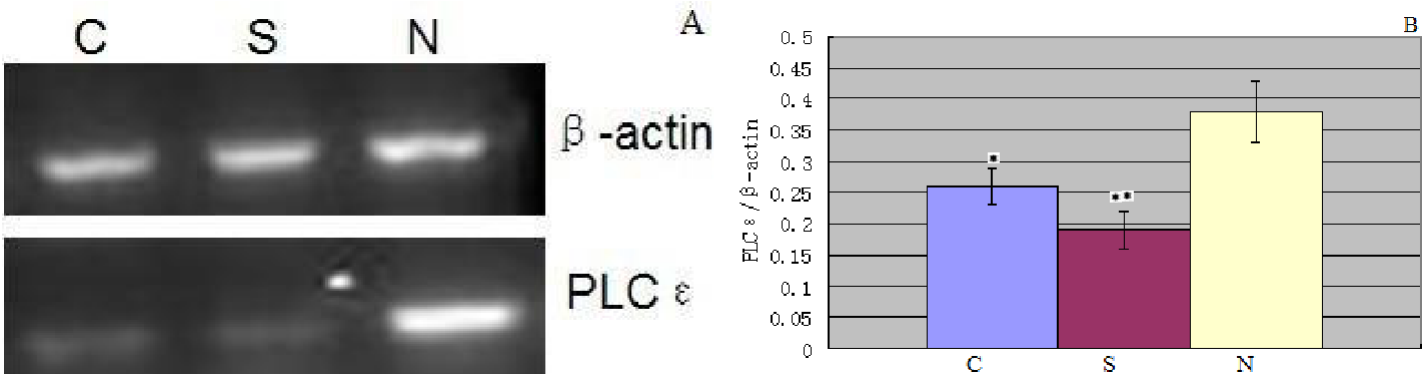

Figure 4. We measured the PLCε expression in control group (C), MNU induced normal rats bladder tumorigenesis group (N) and MNU treated PLCε silenced group (S). A means the RT-PCR of PLCε expression. It informed that PLCε were highly expressed in N group,while lowly expressed in C group and more lowly expressed in S group. B means the outcome of semiquantitative analysis of PLCε expression. The PLCε expression in C group were statistically significant compared with N group (*P < 0.05), and the PLCε expression in S group were more statistically significant compared with N group (**P < 0.01).

mutations made inhibition of Ras oncogenes function a important therapeutic target. Apparently, the tumorigenic function will be blocked by blocking the Ras signal pathway. As a known downstream signal effector (Michele et al., 2003), the PLCε shows its important role in this process. Song et al have reported that PLCε expressed ectopically in Cos-7 cells undergone translocation from the cytosol to the plasma membrane when co-expressed with a mutant form of H-ras deficient in GTPase activity (Song et al., 2001). So to prevent the downstream signal effector PLCε might be a potential approach to block the H-ras mutant effects. Interestingly, about 60% of the MNU induced rats bladder cancer has H-Ras mutation in our experiment.

We silenced the PLCε by a fluid mechanics based method according to Maruyama et al. (2002); Maruyama

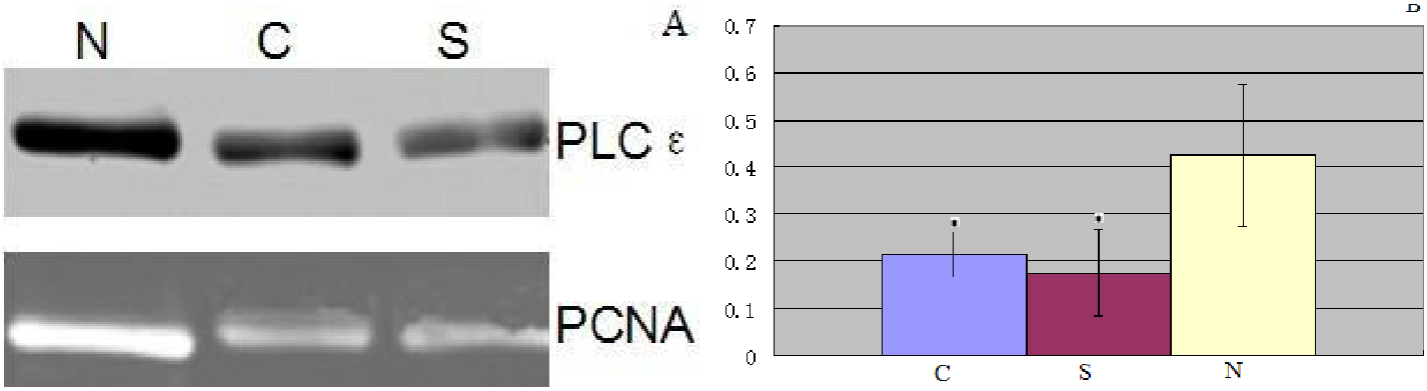

Figure 5. We measured the PLCε and PCNA protein expression in N, S, and C group with western-blotting. N, C, S in fig A means the western-blotting outcome of N, S, and C group, respectively. It informed that PLCε and PCNA protein highly expressed in N group, lowl y expressed in C group and more lowly expressed in S group. Fig B means the semiquantitative analysis of PLCε and PCNA protein expression. The PLCε and PCNA protein expression were statistically in C and S group compared with N group (*P < 0.05).

et al. (2005). We amplified the PLCε shRNA plasmids, destroyed the bladder mucosa by injection of diluted hydrochloric acid and clamping the urethra and ureters, then the amplified PLCε shRNA plasmids were injected. We changed the injection characteristics. We evaluated the effects of varying the injection volume and dose on the efficiency of PLCε knockdown. Good PLCε silencing efficiency were seen with a volume of 1.0 ml plasmid, and saw substantial levels of PLCε silencing efficiency with only 30 ug of PLCε shRNA.

In our experiment, MNU induced bladder tumorigenesis appeared in non- PLCε-silenced(N) group and PLCε-silenced(S) group .While there were no rats appeared tumorigenesis in C group. The results indicated that PLCε silencing could decrease the MNU induced rats bladder tumorigenesis efficiently (P<0.05).

In order to further clarify the probable mechanism involved in the inhibition of MNU induced bladder tumorigenesis mediated by PLCε, we investigated some proteins associated with carcinogenesis, including H-Ras and PCNA. We measured the expression of H-Ras. We found that 24, 9 rats bladder expressed H-Ras in N and S group, respectively. The positive expression rates were 60.00 and 22.50% in N and S group, respectively. PCNA functions as a cofactor for DNA polymerase is required for both DNA replication and DNA repair (Shivji et al., 1992; Xiong et al., 1992). It is usually taken as markers of cell proliferation. Our studies revealed that the protein levels of PCNA were decreased after PLCε gene silencing, indicating that cellular DNA replication and DNA repair were suppressed. These results revealed the facts that PLCε silencing could prevent the MNU induced H-Ras activation and signal transduction efficiently and decrease the PCNA expression so as to decrease the MNU induced rats bladder tumorigenesis.

In conclusion, PLCε knockdown could decrease the MNU induced rats' bladder tumorigenesis. The probable mechanism might be related to PLCε knockdown, which prevent the MNU induced H-Ras activation and signal transduction efficiently and decrease the PCNA expression. The PLC knockdown might provide us an alternative method for human bladder cancer therapy in future.

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