Author's response to reviews

Title: PKC alpha regulates netrin-1/UNC5B-mediated survival pathway in bladder cancer.

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Author's response to reviews:

Reviewer1:
Reviewer's report
Title: PKC alpha regulates netrin-1/UNC5B-mediated survival pathway in bladder cancer.
Version: 1 Date: 7 July 2013
Reviewer: Tatjana Simic
Reviewer's report:
The paper of Liu et al describes expression PKC alpha, netrin-1 and UNC5B in bladder cancer samples obtained from 120 patients and 40 specimens of adjacent non-tumor tissue as well as in bladder cancer cell lines. It also addresses the question of whether any of these parameters may have prognostic role in bladder cancer patients. Although the material presented is robust in terms of quantity of data and methodology used, there are some major concerns regarding the introduction to the problem and interpretation of the data. Besides, the paper requires extensive language editing.
- Major Compulsory Revisions
1. The research question posed by the authors can hardly be understood from the Abstract. It should be clearly pointed out why the Netrin-1 UNC5B signaling should be evaluated in bladder cancer. The sequence of presentation of the results does not correspond to that presented in the article. The conclusion refers only to the data obtained in in-vitro conditions. There are abbreviations used without an explanation.
I have changed all problems above and wrote a new “Abstract” according to the reviewer's report.
Netrin-1 and its receptor UNC5B play important roles in angiogenesis, embryonic development, cancer and inflammation. However, their expression pattern and biological roles in bladder cancer have not been well characterized. The present
study aims to investigating the clinical significance of PKC #, netrin-1 and UNC5B in bladder cancer as well as their association with malignant biological behavior of cancer cells. Netrin-1 and UNC5B expression was examined in 120 bladder cancer specimens using immunohistochemistry and in 40 fresh cancer tissues by western blot. Netrin-1 expression was positively correlated with histological grade, T stage, metastasis and poor prognosis. Immunofluorescence showed elevated netrin-1 and decreased UNC5B expression in bladder cancer cells compared with normal bladder cell line. PKC # inhibitor calphostin C, PKC # agonist PMA and PKC siRNA was employed in bladder cancer cells. CCK-8, wound healing assays and flow cytometry analysis were used to examine cell proliferation, migration and cell cycle, respectively. Cell proliferation, migration and cell cycle progression were promoted with PMA treatment while inhibited by calphostin C. In addition, PMA treatment could induce while calphostin C reduce netrin-1 expression in bladder cancer cells. The present study identified netrin-1/UNC5B, which could be regulated by PKC signaling, was important mediators of bladder cancer progression.

2. In Introduction the authors provided brief general information about Netrin-1 and UNC5 family of proteins. However, the potential significance of these proteins in bladder cancerogenesis is given in only one sentence with a bit confusing statement that “it is important to explore it’s potential mechanism on the regulation of bladder cancer apoptosis, which may unveil the functional diversity of this pathway.” Besides, the signaling pathway that relates PKC alpha with netrin1/UNC5B has not been introduced at all. PKC alpha has just been mentioned in the last sentence of the Introduction. Therefore, the hypothesis which underlies this large study is not clear at all. The Introduction should be extended and focused to the potential role of PKC alpha, Netrin-1 signaling in the regulation of bladder cancer cells survival. Overall design of the study should be provided.

All problems above have been changed as the reviewer indicates. I have written a new “Introduction” following the reviewer’s suggestions.

Bladder cancer (BC) is one of the most deadly urological malignant tumors and also the 2nd most common urologic cancer [1]. In the US, BC is the ninth most common cause of cancer-related mortality, and is the fourth most common cancer in men. Most bladder cancers are initially non-invasive and up to 15% will progress to muscle-invasive carcinoma. Although treatment of bladder cancer has been improved greatly, the mortality of this disease is still increasing [2].

As the central hub of a variety of signal transduction process, PKC involves in cell information transmission, secretion, cell differentiation and proliferation. What’s more, it participates in apoptosis and differentiation of tumor cells. PKC # is one subtype of classic protein kinase C, which is closely related to recurrence of bladder cancer [3]. PKC # can promote proliferation, migration and survival of cancer cells through the downstream signal transduction pathways ERK1/2 and NF-#B [4]. Recent research shows that activation, overexpression of PKC # as well as suppressing or depletion of PKC # can regulate the proliferation of cancer cells [5-7]. Thus it can be seen that PKC # is closely related to the biological behaviour of bladder cancer.
As a kind of proto-oncogene, Netrin-1 is the axon guidance factor that attracts the most attention in the family of dependence receptor [8]. Researches show that netrin-1 can activate PKC # after combination with its receptor, which may cause phosphorylation to promote cancer cell proliferation, and then restrain cell proliferation [9]. In recent years, netrin-1 has been found effective in inhibiting apoptosis in lung cancer, advanced neuroblastoma, breast cancer and prostate cancer [10-13]. UNC5B is one of the dependence receptors of netrin-1. Researches show that UNC5B is the downstream gene of p53, down-regulation of UNC5B using small interfering RNA Can significantly inhibit apoptosis, thus concludes that UNC5B plays a role of inducing apoptosis, and it is a kind of tumor suppressor genes[14]. According to reports in the literature, up-regulation of netrin-1transcripts can antagonize apoptosis induced by UNC5B [15]. Since PKC #, netrin-1 and UNC5B play a significant role in the process of tumor treatment. Therefore, study the mechanisms of action of PKC alpha regulates netrin-1/UNC5B-mediated survival pathway is of great significance.

In this study, we detect the expression of netrin-1/UNC5B in the bladder cancer tissues as well as in the bladder cancer cell line on both the RNA and protein levels, we found that netrin-1/UNC5B was closely related to the activation of PKC alpha state. Furthermore, netrin-1/UNC5B was closely associated with bladder cancer malignant pathological biological behavior. Therefore, we need to validate that PKC # inhibits bladder cancer cell apoptosis by regulating signaling pathway of netrin-1/UNC5B.

3. Methods section has been extensively written. Although this style is according to the journal guidelines, the description of methods still contains too many details such as frequency of rinsing and volumes of solutions added. Such information is usually the part of laboratory protocols, but rarely published except for novel procedures. It is not clear why only BIU and SV cell lines were treated with inhibitors and siRNA. Besides, drugs should be omitted in the subheading. There are typing errors such volume of “20ll” in Real time PCR, “50 lg” in Western Blotting. It should be pointed out that among so many data the overall concept of the study can hardly be followed.

Extensively written methods have been omitted. I explained why BIU and SV cell lines were treated with inhibitors and siRNA. I omitted drugs in the subheading and changed the typing errors such volume of “20ll” in Real time PCR to “20µl” and “50lg” in Western Blotting to “50µg”.

Materials and methods
Patients and specimens
One hundred and twenty bladder cancer tissues were collected by the surgical resection in the First Affiliated Hospital of China Medical University from 2008 to 2012. Bladder cancer tissues and paired adjacent normal bladder tissues were collected. None of patients underwent chemotherapy, radiotherapy or adjuvant treatment before surgery. Patients’ consent for the research use of tumor tissue was obtained, and the research protocol was approved by Ethical Committee at China Medical University. We followed up all patients for the survival time by consulting their case documents and telephoning.
Cell culture, treatment of cells with drugs and siRNA

Human BC cell lines SV, 5637, T24 and BIU-87 were purchased from Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. They were maintained in RPMI 1640, or DMEM medium supplemented with 10% fetal bovine serum (FBS). Cells were incubated at 37°C in 5% CO2.

For PMA treatment, cells were treated at the concentration of 100nmol/L for 24 hours. For calphostin C treatment, cells were treated by using 100nmol/L PMA for 4 hours first, then 50nmol/L calphostin C for 24 hours. For siRNA transfection, Lipofectamine (Invitrogen) was used. PKC siRNA sequences was as follows: forward, 5' GUG CCA UGA AUU UGU UAC UTT 3', reverse, 5' AGU AAC AAA UUC AUG GCA CTT 3'.

Real-time PCR

Total cellular RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen). First strand of cDNA was synthesized by using PrimeScript RT reagent kit (Takara). Quantitative real-time polymerase chain reaction (QPCR) was done using SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 20µl on a 7900 Real-Time PCR System (Applied Biosystems): 50°C for 2 min, 95°C for 5 min, 45 cycles of 95°C for 40 s, 60°C for 30 s. The sequences of the primer pairs are: UNC5B forward, 5' CAG GGC AAG TTC TAC GAG AT 3', reverse, 5' TGG TCC AGC AGG ATG TGA 3', netrin-1 forward, 5' GTC AAT GCG GCC TTC GG 3', reverse, 5' CTG CTC GTT CTG CTT GGT GAT 3', #-actin forward, 5' TTA GTT GCG TTA CAC CCT TTC 3', reverse, 5' ACC TTC ACC GTT CCA GTT T 3', #-actin was used as the reference gene. Relative gene expression levels were represented as CT = CT gene – CT reference; fold change of gene expression was computed by the 2^ΔΔCT method [16]. Experiments were repeated in triplicate.

Western blotting

Total protein from cells was extracted in lysis buffer (Pierce) and quantified using the Bradford method. Total protein was separated by SDS-PAGE (12%). After transferring to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA), the membranes were incubated overnight at 4°C with antibodies against UNC5B/netrin-1 (1:1000, Abcam Inc. USA), GAPDH (1:500, Santa Cruz Biotechnology). After incubation with peroxidase-coupled anti-mouse/rabbit IgG (Santa Cruz Biotechnology) at 37°C for 2h, bound proteins were visualized using ECL (Pierce) and detected using BioImaging Systems (UVP Inc., Upland, CA). The relative protein levels were calculated based on GAPDH protein as a loading control.

Immunohistochemistry and evaluation

Sections were deparaffinized in xylene, hydrated in graded alcohols. After antigen retrieval, sections were incubated in an aqueous solution of 3% hydrogen peroxide followed by incubation with 5% non-fat milk, which served as a blocking agent for nonspecific binding. Slides were incubated with UNC5B & netrin-1 rabbit polyclonal antibody with an optimal dilution of 1:100 overnight at 4°C. Biotinylated goat anti-rabbit serum IgG was used as a secondary antibody. After
washing, the sections were incubated with streptavidin–biotin conjugated with horseradish peroxidase at room temperature for 10min, and the peroxidase reaction was developed with 3, 3'-diaminobenzidine tetrahydrochloride. All the slides were evaluated by 2 pathologists. Five views were examined per slide; 100 cells were observed per view at ×400 magnification. Nucleus and/or cytoplasmic immune-staining in tumor cells were considered positively. Positive reactions were scored for both intensity of staining and percentage of positive cells. Intensity grades were 0 (no staining), 1 (weak, light yellow), 2 (moderate, yellowish brown), to 3 (intense, brown) and the percentage of positive tumor cells were scored as 0 (negative), 1 (1–50%), 2 (51–75%), 3 (≥76%). Scores of each sample were multiplied to give final scores of 0–9, and the tumors were finally determined as negative: score 0; low expression: 0<score<4; or high expression: score>4.

Cell proliferation and invasion assays

Cell Counting Kit-8 (Dojindo) was employed to determine the number of viable BIU cells. Experiments were performed according the manufacturer’s protocol. Invasion ability was examined by wound healing assay. In brief, cells were seeded at a density of 1.0×10^6 cells/well in 6-well culture plates. After they grown into confluency, scratch was performed using a pipette tip, cells were washed with PBS and cultured in the FBF-free medium for 24 hours and photographed.

Cell cycle by flow cytometry

Cells with different treatment were harvested, fixed in 1% paraformaldehyde, washed with phosphate buffered saline (PBS), and stained in 5 mg/ml propidium iodide in PBS supplemented with RNase A (Roche, Indianapolis, IN) for 30 minutes at room temperature. Data were collected using BD systems.

Immunofluorescence

Cells were washed with PBS, fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 at 37°C, and incubated in 5% BSA. Then cells were incubated with rabbit anti-human netrin-1 & UNC5B antibody (1:100) and mouse anti-human PKC # antibody (1:50) overnight at 4°C. Then fluorescently labeled goat anti-rabbit IgG (1:200) were added at 37°C for 1h. Nucleus was stained with DAPI. Cells was then observed using fluorescence microscope.

Statistical analysis

SPSS 13.0 (SPSS Inc, Chicago, IL) was used for statistical analysis. The #2 test was used to evaluate the association between the expression of netrin-1 & UNC5B and clinicopathologic variables. Kaplan-Meier method and log-rank test were used for survival analysis. The t-test was used to analyze the difference for western blot data. p values<0.05 was considered significant.

4. Results

a. Results section is divided in too many subheadings and should be organized in a way to integrate information that focuses at one topic. The text of Results should be clear without the Figures - that is not the case in this paper. For example lines 186-190: „The western blotting of 6 samples were shown in Fig. 1B & D, and the optical density of the tumor (T) & normal (N) tissues was
measured and expressed graphically (Fig.1C & E). Differences in T stage (T1, T2, T3 & T4) (Fig.2A & D) and histological grade (G1, G2 & G3) (Fig.2C & F) by RT-PCR were significant (P < 0.05). The western blot results also showed difference in tumor stage (Fig.2B & E).

Results section was unified in 3 parts that focuses at one topic. The above lines I have changed was that: The protein expression of netrin-1 and UNC5B showed the same trend as that of mRNA expression, and the optical density of all tumor (T) & normal (N) tissues were measured and expressed graphically (Fig.1). Differences of mRNA expression and protein levels in different T stage (T1, T2, T3 & T4) and histological grade (G1, G2 & G3) were significant (P < 0.05) (Fig.2).

b. How much the mean expression of Netrin-1 and UNC5B change in tumors in comparison to non-tumor tissue? Could the expression be normalized according to standard purified proteins? Regarding the correlation between netrin-1 and UNC5B the authors report the “positive” correlation of both parameters with grade, and metastasis despite the fact that there was a “negative” correlation between netrin and UNC5B. This might be the consequence of the unusual presentation of data, where, the expression of Netrin was presented as percentage of cases with low expression, while that of UNC5B as percentage of cases with low expression. This is a bit confusing. The key question is what did the authors mean by “high” and “low” expression of the parameters tested?

I defined the high” and “low” expression of Netrin-1 and UNC5B#The expression of UNC5B was finally determined T/N < 0.5 as low expression & T/N >= 0.5 as normal expression and the expression of netrin-1 was considered T/N > 2 as high expression & T/N <= 2 as normal expression.

Results

Expression of netrin-1 and UNC5B in bladder cancer tissues and association between their expressions & clinicopathologic parameters

Quantitative real-time PCR (RT-PCR) and western blot analysis were used to evaluate netrin-1 & UNC5B expression in 120 BC tissues and 40 normal bladder epithelial tissues. It showed that the increased netrin-1 expression and decreased UNC5B expression could be detected in BC samples compared with the normal bladder samples (P < 0.05). The mRNA expression of netrin-1 was found to be increased, while that of UNC5B decreased in the BC tissues as compared with the normal bladder epithelial tissues. The protein expression of netrin-1 and UNC5B showed the same trend as that of mRNA expression, and the optical density of all tumor (T) & normal (N) tissues were measured and expressed graphically (Fig.1). Differences of mRNA expression and protein levels in different T stage (T1, T2, T3 & T4) and histological grade (G1, G2 & G3) were significant (P < 0.05) (Fig.2).

The expression of netrin-1 protein in BC and normal adjacent tissues was located in both cytoplasm and nucleus, while UNC5B protein appeared to be located only in cytoplasm (Fig.3). Elevated expression of netrin-1 and down-regulated level of UNC5B was observed in T4 tumors compared with normal adjacent tissues (P < 0.01). To explore the relationship of netrin-1 over-expression and UNC5B down-regulation in a large cohort of BCs, we examined the correlation between
the immunostaining of netrin-1 & UNC5B and clinic-pathological features including age, gender, tumor size, tumor grade, etc. There was a statistically significant positive correlation between UNC5B & netrin-1 expression and high grade, aggressive stage and metastasis (Table 1 & 2), the expression of UNC5B was finally determined T/N < 0.5 as low expression & T/N >= 0.5 as normal expression and the expression of netrin-1 was considered T/N > 2 as high expression & T/N <= 2 as normal expression.

During follow-up period, 70.0% (21 of 30) of tumors with high netrin-1 expression developed metastasis compared with 5.6% (5 of 90) of tumors with low netrin-1 expression, (P < 0.01). Meanwhile, 43.2% (19 of 44) of tumors with low UNC5B expression showed metastasis, compared with only 9.2% (7 of 76) of tumors with high UNC5B expression having metastasis (P < 0.01). Therefore, high expression of netrin-1 and low expression of UNC5B were positively associated with metastasis of BC. Kaplan-Meier plots and log-rank tests showed that patients with high netrin-1 expression and low UNC5B expression in their tumor tissues had statistically significant shorter survival rate compared with those with low netrin-1 expression and high UNC5B expression (P < 0.01). However, there was no significant association between tumor recurrence and intense & feeble netrin-1 expression; recurrence curve analysis also indicated that the difference was not statistically significant with high & low UNC5B expression (P > 0.01). Moreover, we found that patients with high netrin-1 expression and low UNC5B expression had statistically significant higher metastasis rate compared with those with low netrin-1 expression and high UNC5B expression (P < 0.01; Fig.4). Log-rank analysis also showed that the expression of netrin-1 & UNC5B (P < 0.01) were significant predictors of the metastasis of BC and had statistically significant independent association with poor prognosis of the patients.

Netrin-1 & UNC5B expression and location in BC cell lines

Quantitative real-time PCR and western blot analysis were used to evaluate the expression of netrin-1 & UNC5B in human bladder cell lines SV, BIU-87, 5637 & T24, and immunofluorescence was used to detect of netrin-1 & UNC5B expression and localization. The results showed that highly invasive BC T24 cells had stronger netrin-1 expression than the superficial BC BIU-87 & 5637 cells and normal SV cells which had lowest expression. Opposite trend was observed regarding UNC5B expression (Fig.5). It was further confirmed that the expression of netrin-1 & UNC5B was positively correlated with BC grade. Quantitative real-time PCR and western blot analysis were also used to evaluate netrin-1 & UNC5B’s expression after PMA (PKC # agonist) and calphostin C (PKC # inhibitor) treatment. The results showed that netrin-1 expression were significantly inhibited by calphostin C and enhanced by PMA (treat for 24 h), while UNC5B showed the opposite trend (Fig.6). Immunofluorescence results showed that UNC5B was expressed in BC cell cytoplasm in all these four cell lines, while netrin-1 was found mainly located in cell nucleus and partly in cell cytoplasm (Fig.7).

BC cells treated with PKC # agonist & inhibitor & siRNA

PKC # inhibitor and agonist, PMA & calphostin C, were applied to treat BC BIU cells. We found that cell proliferative and invasive activities were significantly
increased after PMA treatment, but decreased by calphostin C treatment (Fig.8). Moreover, the FCS showed that cell cycle was accelerated by PMA treatment (S phase 22.33% for 24h, 36.41% for 48h; G2/M phase 23.39% for 24h, 34.42% for 48h) and blocked by calphostin C treatment at both S phase and G2/M phase (S phase 8.39% for 24h, 4.92% for 48h; G2/M phase 10.55% for 24h, 7.46% for 48h) compared with BLU cell without drugs (S phase 13.14%; G2/M phase 16.72%) (Fig.9); the cells mainly concentrated in G1/G2 phase (almost the same percentage at 57.19%) from mitotic completion to DNA replication.

Migration of bladder cancer cells by wound healing. Cells were seeded at 1.0×10^6 cells/well in 6-well plates. After grown to confluence, the cell monolayer in each well was scraped with a pipette tip to create a scratch. Cells were washed by PBS three times and cultured in the FBS-free medium. Cells were photographed after 24h and the scratch area was measured using Image software (Fig.10). PKC siRNAs were transfected into bladder cancer cells T24 & BLU-87 transiently. Real-time PCR showed that, netrin-1 expression was elevated after transfection with PKC siRNA, while UNC5B expression was decreased (Fig.11). The immunofluorescence confirmed the co-localization of PKC # and UNC5B, suggesting that the presence of their endogenous binding (Fig.12).

5. Discussion

Protein Kinase C (PKC), as the hub of a variety of signal transduction process, is not only involved in cell communication, secretion, cell differentiation & proliferation, but more importantly involved in tumor cell apoptosis and differentiation. PKC # is a classical Protein of Kinase C isoforms. Our and others’ research have shown that PKC # of high activation status is closely related to activation and apoptosis of bladder cancer recurrence [3]. UNC5B is abnormally expressed and associated with a highly malignant, chemotherapy-related and poor prognosis in colon cancer. It was reported that netrin-1 binding to its receptor can activate PKC # and lead to tumor cell proliferation, but it did not clarify PKC # and netrin-1/UNC5B’s regulatory mechanisms. To this end, we explored the mechanism of PKC # with netrin-1/UNC5B in bladder cancer. Our work shows that, PKC #, netrin-1 & UNC5B is closely related to the degree of malignancy and progress in bladder cancer and found PKC # promoted the survival of bladder cancer cell potentially through netrin-1/UNC5B signaling pathway. Thus, PKC # has an important influence on netrin-1/UNC5B signaling pathway & bladder cancer’s occurrence and development.

The expressions of netrin-1/UNC5B were detected in bladder cancer tissues & adjacent tissues and the relevance and the relationship with clinic pathological parameters was analyzed. The results showed that UNC5B had higher expression in adjacent tissues than bladder cancer tissues and it had higher
expression in the low-level cancer tissues than in high-level ones, but netrin-1 in the opposite. According to immunohistochemical results, it showed UNC5B expression in the cytoplasm and netrin-1 existing in the cytoplasm and nucleus; netrin-1’s expression gradually increased from the bladder mucosa - transitional cell carcinoma and high - grade cancer evolution, while UNC5B is gradually reduced; netrin-1/UNC5B high/low expression is closely related to bladder cancer clinical grading, staging & metastasis; and Pearson correlation analysis showed that netrin-1 and UNC5B are negatively correlated. Netrin-1/UNC5B’s expression is proved to exist in kidney cancer and prostate cancer [17, 13], and found that netrin-1 inhibits apoptosis in lung cancer, with advanced neuroblastoma, breast cancer [10-12]; UNC5B is one of the dependent receptors of netrin-1, and previous studies had demonstrated that increasing netrin-1 transcription can antagonize UNC5B induced apoptosis [15], which is consistent with the results of this study. Previously we have confirmed that PKC # is closely related to bladder cancer cell’s apoptosis & recurrence [3], and that netrin-1’s binding to its receptor UNC5B can cause PKC # phosphorylation and promote cancer cell proliferation [9], but it had not been confirmed in bladder cancer, for which we had done further research.

From the cellular level, it revealed netrin-1/UNC5B’s expression & location in bladder carcinoma. Four kinds of bladder cancer cell line T24, BIU-87, 5637 and SV malignancy has been clearly stated in previous studies: BIU-87, 5637, T24 are all bladder carcinoma cells, and their degree of malignancy increased in turn, and SV-HUC-1 is normal urothelial line [18]. We detected netrin-1/UNC5B expression in bladder cancer cell line from RNA and protein levels by Real-time PCR & Western-blot ion, UNC5B expression was the highest in normal bladder cell line (SV), and the expression was the lowest in the most malignant cells of T24, netrin-1 was the opposite. Immunofluorescence results showed that UNC5B was in bladder cancer pulp while expressions of netrin-1 existed in the cytoplasm and the nucleus. Netrin-1/UNC5B’s expression in cells and tissues shows consistent trend, and are related with the degree of malignancy of bladder cancer cell lines. PKC # has been shown to be involved in tumor cell apoptosis and differentiation. The high expression of PKC # in bladder cancer cells was found to promote cancer cell proliferation, and inhibit apoptosis and differentiation [3].

When bladder cancer cell was given PKC inhibitors and activators, and detected changes of netrin-1/UNC5B expression and bladder cell cycle, proliferation and apoptosis; it can be further confirmed that netrin-1/UNC5B are closely related with PKC # activation. When bladder cancer cell BIU-87 was given PKC inhibitors (calphoatin C) and activators (PMA), Real-time PCR & Western-blot showed that netrin-1 was inhibited after inhibitor treatment, while UNC5B was activated; netrin-1 was activated after PMA treatment, while UNC5B was suppressed. When CCK-8 and flow cytometry detection were carried out after drug treatment on bladder cancer cycle, proliferation and apoptosis. CCK-8 was found in best status by calphoatin C or PMA for 48 hours, and the inhibition rate & the activation rate increased with the increasing concentration, and at the same time it can be drawn that calphostin C of IC50 = 7.4µmol/L, PMA’s IC50 = 24nmol/L. Flow cytometry showed S and G2/M were inhibited or activated after calphoatin C or PMA treatment BIU in better condition after 48 hours. These
results could confirm that netrin-1/UNC5B was closely associated with PKC # activation, and PKC # activation or inhibition might affect the proliferation and survival of cancer cells [4, 6, 7].

After transiently transfecting PKC siRNA into the bladder cancer T24 and BIU-87 cells, it clarified PKC #’s regulatory mechanisms on netrin-1/UNC5B; Real-time PCR test results showed that netrin-1 was inhibited after PKC siRNA transfection, with its expression decreased, while UNC5B increased. Immunofluorescence results revealed the presence of co-localization of PKC # with UNC5B expression. So we speculate that there may be endogenous binding.

From the above results, we can conclude that: PKC # can promote bladder cancer cell proliferation through the regulation of netrin-1/UNC5B. On this basis, we can intervene any stage in which PKC # and netrin-1/UNC5B affect, so as to control the proliferation of bladder cancer, and provide adequate theoretical basis for bladder cancer’s diagnosis and treatment.

- Minor Essential Revisions

1. It is not clear why only BIU and SV cell lines were treated with inhibitors and siRNA. Besides, drugs should be omitted in the subheading. There are typing errors, such volume of “20ll” in Real time PCR, “50 lg” in Western Blotting...

I explained why BIU and SV cell lines were treated with inhibitors and siRNA and omitted drugs in the subheading and changed the typing errors such volume of “20ll” in Real time PCR to “20µl” and “50lg” in Western Blotting to “50µg”.

2. Table 1 and 2 it should be clarified what is meant by high or low expression

I defined the high” and “low” expression of Netrin-1 and UNC5B#The expression of UNC5B was finally determined T/N < 0.5 as low expression & T/N >= 0.5 as normal expression and the expression of netrin-1 was considered T/N > 2 as high expression & T/N <= 2 as normal expression.

3. Table 3 presents crude statistical data

I deleted table 3 as the reviewer indicates.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Not suitable for publication unless extensively edited

I have improved the quality of written English.

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests: I declare that I have no competing interests’ below

Reviewer2:

Reviewer’s report

Title: PKC alpha regulates netrin-1/UNC5B-mediated survival pathway in bladder cancer.
Reviewer's report:
Discretionary Revisions:
Tissue & cell cannot be used as key words.
Tissue & cell have been deleted as the reviewer indicates.

Reviewer: Xian-kui Liu
Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests.

Editorial Comment:
If the authors choose to revise the manuscript, special attention should be paid to the data presented in fig 1 and fig 2. All data should be used when showing correlation to stage and Grade in Fig 2, and not just 10 samples as is stated now.
Fig 1 and fig 2 were changed.
I have improved the quality of written English.