Author's response to reviews

Title: Fibulin-1 is Epigenetically Down-regulated and Associated with Bladder Cancer Recurrence

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Author's response to reviews:

Dear editor:

The manuscript has been revised according to reviewers’ comment. The following is the point-to-point reply to reviewers’ comment. Besides, the reference style has been changed as BMC Cancer style using the Endnote software.

Thank you very much.

Sincerely,

Hua Xu

2014/8/21

Reply to reviewer 1 (Marcus Horstmann)

First of all, we really appreciate your professional comment. It is very helpful for this manuscript and also for our future study. Here is a point to point reply to your comment.

1. In the introduction: If the search for molecular markers in BC is motivated by its high recurrence rate, how do the authors think a molecular marker might help the clinicians to improve recurrence rates or progression? This links to molecular
markers and remains completely unclear and should be explained more substantially.

Reply:
We are sorry that there may be some phrase problem to make you confused. What we meant to say here is intensive researches on new molecular markers and therapeutic targets are carried out to improve current diagnosis and management of bladder cancer.

We think fibulin-1 is not only a molecular marker for recurrence, but also a potential therapeutic target. As we showed in this paper, upregulation of fibulin-1 could inhibit the tumor cell growth in both cell lines and animal model.

Limited to the length of the article, we did not give an intensive introduction in the paper, but we revised the word presentation to make it clearer.

2. The clinical data of this paper only support that fibulin is associated with tumour recurrence in NMIBC. The association between fibulin and tumour progression is not supported by clinical data but only cell cultures and the association between tumour grade and fibulin expression. The end points „recurrence” and „progression” need to be more clearly differentiated and critically discussed though out the paper.

Reply:
After much thought and discuss, we agreed that your suggestion is correct and thoughtful. Our clinical data only support that fibulin-1 is associated with tumor recurrence in NMIBC, though fibulin-1 expression associated with tumor grade in NMIBC, the experimental data in this paper is mainly from MIBC cell lines, so the original conclusion was not exact. We have revised the conclusion presentation and discussed it in the Discussion.

3. Even though the methods are well described it is a major draw back of this paper that it is only stated in the discussion that the cell lines „J82 or T24“ are from MIBC and not from NBIC. This is especially important as otherwise the paper only deals with NMIBC. This needs to be put more clear.

Reply:
Thank you for your good suggestion. The design of this study is to investigate the role of fibulin-1 in bladder cancer in general, not specifically in MIBC or NMIBC. So we used all the bladder cancer cell lines we have to perform the experiments. The reason why we used the NMIBC patient samples is only because we just have this cohort with ideal follow-up. As you know, a complete follow up in patients with BC is difficult to obtain. We did not expect that this would cause such confusion.

As you suggested, the statement of cell line origin now has been emphasized in both Method and Result part to put it clearer.

4. p3 „as the prognosis of patients’ remains poor, with a high recurrence rate, it is also one of the most expensive cancers to treat“ Please specify what you mean by „the prognosis remains poor“? The reader would probably think in this
situation that you talk about survival rates. But this is probabyl not the cas as your paper is rather about recurrence rates of NMIBC. It should be more clearly stated prognosis for what: PFS, RFS or overall survival.

Reply:
This part of introduction has been rewritten to make it exacter and clearer.

5. The question for this paper is generally well defined however it should be more clearly stated in the introduction that the aim of the study is to evaluate fibulin 1 in NMBIC and not in BC in general. Here the endpoints should also be cearly stated (see above PFS or RFS?)

Reply:
It has been explained in the above and this part of introduction has been rewritten to make it more exact and clear.

6. Beside that, telephone follow up was taken every month. We all know that a complete follow up in patients with BC is difficult to obtain. In this paper an ideal follow up seems to be obtained, even thoug in China to my knowledge patients often only come for interventions to the hospital. Please comment on this problem and explain what you asked for in the „telephone follow – up“ every month. This seems for the PFS or RFS a little bit overdone.

Reply:
As you know, patients in China usually only come for interventions to the hospital, which did make it difficult to take the follow-up. To solve this problem, staffs were specially employed for follow up and the patients’ information was managed professionally. Every month the patients was follow up by telephone and mainly focused whether there were hematuria, dysuria or other symptoms. Patients were asked to hospital when hematuria, dysuria or other specific symptoms happened. Patients were seen postoperatively at least every 3 to 4 months for the first 2 years and semiannually thereafter if there was no specific symptoms in telephone follow up.

7. The following sentences in the discussion need rewording:

1. We conjectured that in the progression progress of bladder cancer, the methylation degree of FBLN1 promoter follow increased, which resulted the progression loss of fibulin-1 expression, so that enhanced the bladder tumor growth and ability of metastasis and angiogenesis.

2. In conclusion, our study provides evidences that FBLN-1 functions as a novel candidate tumor-suppressor gene in bladder cancers and its down-regulation may (be??) due to the promoter hypermethylation.

P5 There was no case of death in the study. This contradicts to your initial stament of BC having a poor prognosis. Please change your introduction accordingly as stated before.

Reply:
These sentences have been rephrased as request.
Reply to reviewer 2 (Mathilde Borg Houlberg Thomsen)

First of all, we really appreciate your professional comment. It is very helpful for this manuscript and also for our future study. Here is a point to point reply to your comment.

1. Figure 1B: The authors conclude that the protein expression levels are significantly lower in the four selected bladder cancer cell lines compared to the normal bladder cell line – however levels are so low that this cannot be determined.

Reply:
We are sorry but expression of fibulin-1 is really low in these cell lines, we have reset the contrast (without any other change) of the figure to make it clearer.

2. Figure 2B: The authors state that hypermethylation of promoter CpG islands is detected in all four bladder cancer cell lines which isn’t the case for 5637 and HT1376. This is problematic since all downstream experiments are performed using these two cell lines. Therefore the authors need to clarify whether the promoter region of FBLN1 is indeed methylated in these two cell lines in order to draw valid conclusion on the epigenetic influence.

Reply:
We are sorry but we had really performed this detection in all four BC cell lines including 5637 and HT-1376 by Methylation-specific PCR (MSP) as shown in figure 2B. The original figure seems a little blurry so we have reset the contrast (without any other change) of the figure to make it clearer in this revision.

3. Figure 3A: decreased viability is observed with pEGFP-FBLN1 compared to Mock and pEGFP in both cell lines, however it cannot be concluded that proliferation is reduced since the assay measures cell number. Also, the authors show that the pEGFP-FBLN1 undergoes apoptosis to a higher degree compared to Mock and pEGFP, thus it can only be concluded that FBLN1 induces reduced cell viability/apoptosis, but not a slower growth rate/proliferation.

Reply:
Thank you for your suggestion. We agree that the CCK-8 assay is not an exact detection for cell proliferation though many studies are still doing so. To draw an accurate conclusion, we performed EdU (5-Ethynyl-2'-deoxyuridine) proliferation assay. (See new Figure 3B)

4. Figure 3C+D: Cell viability should also be tested using the Lenti vector system in order to rule out, that the reduced colony formation is not due to transfected cells undergoing apoptosis and not due to reduced tumorigenicity.

Reply:
We agree with that. A new CCK-8 assay using Lenti-virus expression system has been performed as you request.(see new Figure 3A)
5. Figure 1D: Normal and tumor samples should be normalized to sample size in order to compare the two groups.
Reply: This figure has been redone as you request.(see new Figure 1D)

6. Figure 2C: Tumor samples should be normalized to their respective normal sample and when presenting relative expression, normals should be set to 100.
Reply:
In this figure, we want to show not only the methylation difference between normal/tumor, but also that between the two pairs of tissue sample, combined with the MSP(Figure 2B) and Pyrosequencing(Figure 2D) results, we can see that the fibulin-1 mRNA expression correlated with the methylation status in these tissues. So the Case1 normal has not set to 100, but normalized to Case2 normal.

7. Figure 4E: The different conditions should be applied to the figure rather than letters a-d. Also, HUVEC could be indicated in E and F.
Reply: Thank you. This figure has been redone as you request, and it seems really more comprehensive.

8. Please rephrase the sentence in the discussion: “Interestingly, the expression of fibulin-1 in cancer cell lines wasn’t fit well on the notion that found in tissues”.
Reply:
This sentence has been rephrased:” Interestingly, the expression of fibulin-1 in cancer cell lines was a little different with tissue samples”.

Reply to reviewer 3 (Jiaoti Huang)
First of all, we really appreciate your professional comment. It is very helpful for this manuscript and also for our future study.
We admit that this study is mainly descriptive. We are trying to investigate the molecular mechanism that underlying the function of fibulin-1 in Bladder cancer, however, the mechanism seems complicated. So it’s really hard to address all these in just one paper and we will continue further studies.
We fully agree that it is important for the authors to use the new grading system, and we have reanalyzed our clinical data using WHO / ISUP classification 2004. (see new table 1 and 2)
The manuscript has been read carefully and revised.