Author’s response to reviews

Title: Btbd7 Contributes to reduced E-cadherin expression and Predicts Poor Prognosis in Non-small Cell Lung Cancer

Authors:

Chuifeng Fan (fanchuifeng2013@163.com)
Yuan Miao (cmumiaoyuan@yahoo.com.cn)
Xiupeng Zhang (zhang_xiupeng@126.com)
Di Liu (liudi060@163.com)
Guiyang Jiang (jggy0617@163.com)
Xuyong Lin (linxuyong@gmail.com)
Lan Luan (luanlan33@163.com)
Zhonghai Xu (jh302010@hotmail.com)
Enhua Wang (wangeh@hotmail.com)

Version: 4 Date: 5 July 2014

Author’s response to reviews: see over
List of responses to the comments:

To referee 1:

1. The authors should include a table in supplementary info with all the relevant clinical data from all the patients included in the study.

   Supplementary info was given as Professor suggested.

2. Although the manuscript is written in standard english and is readily understandable, there are numerous typos all over the text (such as "progression", "tumors", "function", "dance", "therapy" or "metastasis" that should be corrected.

   we have carefully checked the manuscript and corrected the typos as follow:
   progression---- progression
   therapy---- therapy
   tumors----tumors
   corresponding---- corresponding
   metastasis---- metastasis
   expression---- expression
   function---- function
   cancer---- cancer

To referee 2:

1. Btbd7 and prognosis

   Btbd7 expression is higher in patients with L/N metastasis. OS of patients with positive immunoreactivity of Btbd7 is shorter. Could authors show the relationship between Btbd7 expression and distant metastasis such as other organs? Btbd7 expression is found in 27 out of 61 patients with stage I and II. It means that Btbd7 expression occur in the early stage of NSCLC. I do not agree that Btbd7 expression is associated with the prognosis of NSCLC in this clinical data.

   The samples used in this study include 49 cases of III+ IV, but there are only 4 cases of IV which have distant metastasis in other organs. These cases are all with Btbd7 positive expression. Because patients with distant metastasisi commonly don’t receive surgical therapy, unfortunately it is difficult for us to get the cancer samples with distant metastasis in other organs at the moment. It is true as Professor referred that Btbd7 expression was seen in samples of I+II. In the samples, I+II were 61 cases, and III+IV were 49 cases. Positive rate of Btbd7 expression in I+II was 44.3% and was lower than that in III+IV which was 61.2%. The stastical analysis shows no significant association between Btbd7 expression and TNM stages but the p value was very close (p=0.077). However, Btbd7 expression was significantly associated with LN metastasis. It may be because the number of samples is still not enough to get significant result for association of Btbd7 expression and TNM stage. So we have done
IHC study in additional more cases with complete survival data including 130 cases. The result shows that Btbd7 expression in NSCLC was significantly associated with lymph node metastasis and advanced TNM stages (III + IV) (p<0.05). However 43.1% of the cases of I-II stages were Btbd7 positive though the positive rate was lower than that of III+IV. We can not exclude the possibility of a role of Btbd7 in early stage of NSCLC. Actually there is a report about Btbd7 mRNA expression in hepatocarcinoma indicating a role of Btbd7 to promote cancer cell proliferation though mechanism involved was not mentioned and not clear. However, here we can conclude that Btbd7 may contribute to cancer development from the result that it was associated with LN metastasis, advanced TNM stages and patients’ poor clinical outcome.

2. Btbd7 and EMT
Btbd7 expression is associated with reduced E-cadherin expression and increased N-cadherin. Downregulation of Btbd7 inhibits the invasion of lung cancer cells in vitro. I suggest that other biomarkers of EMT such as MMPs, fibronectin, #-catenin or snail should be investigated. If available, mouse models of lung cancer metastasis could prove authors’ results.

We have investigated MMP2, MMP7, snail and snail2 (slug) expression using Western blot after downregulation of Btbd7 expression in NCI-H1299 cells. (To see Result and figure. 7 and as follow):

Result
Downregulation of Btbd7 restores membrane E-cadherin expression and inhibits lung cancer cell invasion in vitro
……Western blotting study shows that downregulation of Btbd7 in NCI-H1299 cells significantly upregulated E-cadherin expression and downregulated snail 2 (slug), MMP2 and MMP7 expression in cancer cells (Fig. 7, A).……

Fig. 7 Downregulation of Btbd7 expression in NCI-H1299 cells using Btbd7 siRNA significantly upregulates E-cadherin expression. (A) Western blotting study shows effective downregulation of Btbd7 using Btbd7 siRNA significantly upregulates E-cadherin expression and downregulates slug, MMP2 and MMP7 expression (p<0.05)……

3. Btbd7 and survival
Btbd7 expression is associated with shorter survival in the survival curve of all 110 patients with NSCLC. They include the heterogenous characteristics in terms of pathology, stage and treatment modalities. I suggest that the univariate and multivariate analysis should be shown to confirm the role of Btbd expression as a prognostic predictor.

As Professor suggested, we performed the multivariate analysis and the results show that Btbd7 is an independent prognostic marker for NSCLC. (To see Result and Table.3)

Results
Evaluation of Btbd7 as a potential prognostic marker for NSCLC
……lymph node metastasis, TNM stage and Btbd7 expression are independent prognostic
To referee 3:

1. First of all, it is unclear how the authors investigate Btbd7 in NSCLC, although the authors address the possible role of Btbd7 in epithelial dynamics (or remodeling) in the background. Without lack of lead to study Btbd7 in this aspect, the value of their finding cannot be fully supported. More introduction of Btbd7 is required in the background. It is less clear what BTB/POZ domains are and why they are important. Why the protein level of Btbd7 is firstly examined in the lung cancer and why Btbd7 is assumed to play role in NSCLC metastasis.

Our team focus on the research work associated to function of P120-catenin, E-cadherin, β-catenin and Wnt signaling in non-small cell lung cancer. Btbd7 is a newly reported protein to have possible roles in regulating E-cadherin and epithelial dynamics in lung tissue formation. We have carried out experiments about those proteins associated to E-cadherin and catenins including not only Btbd7. Actually, there is very few data in pubmed about function of Btbd7 especially in cancer. The work about it is just at beginning. A search study was carried out by Yamada et al for finding a regulatory gene that might be involved in branching morphogenesis during lung tissue formation and Btbd7 was indentified by them. Another team’s report shows Btbd7 mRNA was elevated in hepatocarcinoma and may contribute to cancer cell malignancy but not any mechanism was referred. The BTB domain was first identified as a sequence motif in genes of DNA virus. It derives its name from the subsequent observation by Laski et al that the Drosophila transcription factors Bric-a-brac, Tramtrack, and Broad Complex display a region of sequence similarity at their N terminus, that they named the BTB domain. The BTB domain is a protein–protein interaction motif that is found throughout eukaryotes. It determines a unique tri-dimensional fold with a large interaction surface. The exposed residues are highly variable and can permit dimerization and oligomerization, as well as interaction with a number of other proteins. The functions of BTB containing proteins well known now are mainly transcriptional regulation and protein degradation. Yamada et al’s study shows that Btbd7 can regulate E-cadherin expression through regulating snail2 at transcriptional level. However whether this function is associated to the BTB domain of Btbd7 is yet unknown. We firstly examined Btbd7 in the lung cancer because we focus on the research works associated to fuction of P120-catenin, E-cadherin, β-catenin and Wnt signaling in non-small cell lung cancer and was inspired by Yamada et al’s study. We assum that Btbd7 may not only play roles in regulating E-cadherin and epithelial dynamics in lung tissue formation but also may contribute to invasion and metastasis in NSCLC through regulating E-cadherin. Here we added more introduction about Btbd7 and BTB domains according to Professor’s suggestion in the Background and Discussion as follow:

Background

Recently Btbd7 (BTB (POZ) domain containing 7), a BTB (POZ) domain containing protein, was found to play important roles in the development of salivary glands and lungs through regulating E-cadherin [15]. Many organs form by branching of epithelia through the formation of clefts and buds during embryonic development. The authors identified Btbd7 as a dynamic regulator of branching morphogenesis through its highly focal
expression leading to local regulation of E-cadherin and epithelial cell motility [15]. Btbd7 protein contains 1130 amino acids with two putative BTB/POZ domains. The protein family containing BTB domains are evolutionarily conserved from Drosophila to mammals [15]. The BTB domain is a protein-protein interaction motif that was first identified as a sequence motif in genes of DNA virus [16]. The functions of BTB containing proteins well known now are mainly transcriptional regulation and protein degradation [16].

Discussion

……Btbd7 protein contains two putative BTB/POZ domains. The BTB domain is a protein–protein interaction motif that determines a unique tri-dimensional fold with a large interaction surface [16]. Some BTB-containing proteins are known to control cellular processes including actin dynamics and cell-cycle regulation [16]. However, expression and function of Btbd7 in malignant tumors including lung cancer are largely unknown so far.……

2. If Btbd7 is closely associated to the level of N and E-cadherin, the level of N-cadherin and E-cadherin, shown in Figure 4 can be determined by Immunoblotting from the cancer tissues as similar as that of Figure 2A. The authors may have at least 12 cancer tissues (Fig. 2A), which show higher Btbd7 expression in 6 cancer tissues (T1, T2, T6, T7, T10, and T11). N or E-cadherin expression should be examined in this set of cancer tissue to address more convincing correlation.

We have investigated E-cadherin expression in the 12 paires of lung and corresponding cancer tissues using Western blotting study according to Professor’s suggestion. T Test analysis shows that E-cadherin expression was negatively associated to Btbd7 expression in these tissues (r=-0.445, p<0.05, see Result. 1 (Quantification of Btbd7 expression in NSCLC and corresponding non-tumor lung tissues) and Figure. 2).

3. Results in Figure 3 are less important to be present as a separate figure.

According to this suggestion, we deleted the previous “figure. 3” . To show the pattern and the comparision of the pattern of E-cadherin, N-cadherin and Btbd7 expression in normal lung tissues and cancer tissues using IHC, a new picture was made (to see the new Figure. 3): Fig. 3 Investigation of E-cadherin and N-cadherin expression in non-small cell lung cancer (T) and lung tissuees (N) using immunohistochemistry. Strong and entire

4. IHC data shown in figure 4 as a representative figure should be compared in the same set of sample with appropriate controls.

We have also carried out immunostaining in the normal lung tissues of the same set of samples which could be compared with that in cancer tissues and could be taken as inside controls (see the newly made Figure. 3):
membrane expression of E-cadherin was seen in normal bronchial epithelium. Membrane expression of E-cadherin was decreased in cancer cells. N-cadherin expression was commonly absent in normal bronchial epithelium. Diffuse cytoplasmic expression of N-cadherin was detected in cancer cells. Strong cytoplasm Btbd7 expression was accompanied by reduced membrane E-cadherin expression and accumulation of N-cadherin in cytoplasm in cancer cells (×400).

5. According to the data in Figure 6, H1299 cells, which show higher Btbd7 expression should express higher N-cadherin than the other cancer cell lines as well as higher invasive properties. Such positive correlation of Btbd7 to invasive and EMT properties in cancer cell line model would be able to convince their notion. Among lung cancer cell line model, a number of NSCLC cell lines (such as H1650) showed the distinct mesenchymal properties, such correlation should be examined in those cancer cell line model (see PMID 22272264).

According to this suggestion, we have investigated Btbd7 and N-cadherin expression in NSCLC cell lines using Western blot (see Result and Figure 5). It shows a positive correlation between Btbd7 and N-cadherin expression in these cells \( r=0.095, \ p<0.001 \). The highest level of N-cadherin expression was found in NCI-H1299 cells which has a relative higher Btbd7 expression level than the other cell lines used in this study. We also carried out Transwell study to investigate the invasion ability of NCI-H1299 with relative higher Btbd7 expression level and NCI-H157 cell with relative lower Btbd7 expression level. It shows that NCI-H1299 cell has higher invasive properties than NCI-H157 cell. The data indicate that Btbd7 expression in lung cancer cells is associated with invasive and EMT properties. We don’t have the H1650 cell in our laboratory at the time. Accoding to our data, we think NCI-H1299 cells could be used in our study to investigate the fuction of Btbd7 in lung cancer. Changes are made as follow:

Result

**Downregulation of Btbd7 restores membrane E-cadherin expression and inhibits lung cancer cell invasion in vitro**

We examined Btbd7 and N-cadherin expression in bronchial epithelial HBE cell and lung cancer cell lines including A549, BE1, LK2, NCI-H157, NCI-H460, NCI-H1299, LH7, LTE and SPC using Western blotting (Fig. 5, A, B). Expression of Btbd7 was detected in these cells with different levels. Relative higher Btbd7 expression was detected in NCI-H1299 cell with strong expression of N-cadherin (Fig. 5, A, B). Btbd7 and N-cadherin expression was positively related in these cell lines \( r=0.095, \ p<0.001 \) (Fig. 5, A, C). Transwell study shows higher invasive properties in NCI-H1299 cells than that in NCI-H157 cells with relative lower Btbd7 expression (Fig. 5, A, B, D).……

**Fig. 5** Detection of Btbd7 expression in lung cancer and bronchial epithelial cell lines. Higher expression level was detected in NCI-H1299 cells compared to other cancer cell lines and bronchial epithelial cell HBE (A, B); N-cadherin expression was positively associated with Btbd7 expression (A, C). Relative higher invasive ability (Transwell study, D) was detected in NCI-H1299 cells compared to NCI-H157 cells with relative lower Btbd7 expression.
6. Migration experiment shown in Figure 7 should not be interpreted as a ‘invasive properties’.

   We have changed it into “migration”.

   ……The scratch wounding assay shows that downregulation of Btbd7 using Btbd7 siRNA significantly inhibits the migration ability of the cells (Fig. 7). ……

   ……We found that downregulation of Btbd7 using Btbd7 siRNA significantly inhibits the migration ability of the cells examined by scratch wounding assay. ……

   Fig. 6 Scratch wounding assay shows downregulation of Btbd7 expression in NCI-H1299 cells using Btbd7 siRNA significantly inhibits cancer cell migration (* p<0.05)

7. Based on figure 7 (left panels), cells are still proliferating. To determine migration capacity solely, the cell growth should be arrested as growth retardation by knockdown of a gene of interest may also contribute the migration capacity. Due to the lack of sufficient description of the experiments in the method section and figure legend, any conclusion in this experiment is less valid.

   We have reperformed the Scratch wounding assay according to Professor’s suggestion. Mitomycin C was added to inhibit cell proliferation. The concentration was determined by MTT assay and was 1ug/ml in this study. More description of this experiment was added in the method section as follow:

   ……After monolayer of adherent cells is formed draw two vertical lines using sterile 100ul pipette tips then cells were washed 3 times with PBS to remove float cells from the mark. Mitomycin C (1ug/ml) was added to the cells to inhibit cell proliferation before drawing the lines. Then the plates are moved to an incubator (5% CO2, 37 °C). ……

   The result of this experiment can be seen in Figure 6:

   Fig. 6 Scratch wounding assay shows downregulation of Btbd7 expression in NCI-H1299 cells using Btbd7 siRNA significantly inhibits cancer cell migration (* p<0.05)

8. DAPI staining was not positive to all cells in figure 8B.

   Immunofluorescent staining was reperformed and the result can be seen in Figure 7:

   Fig. 7 …… (B) Immunofluorescent staining shows that downregulation of Btbd7 in NCI-H1299 cells effectively restores E-cadherin expression in cell membrane (×400)