Author’s response to reviews

Title: FAM3B/PANDER inhibits cell death and increases prostate tumor growth by modulating the expression of Bcl-2 and Bcl-XL cell survival genes

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Author’s response to reviews:

Dear Editor:

Spencer Gibson

BMC Cancer

Thank you for the opportunity to submit a revised version of our manuscript “FAM3B/PANDER inhibits cell death and increases prostate tumor growth by modulating the expression of Bcl-2 and Bcl-XL cell survival genes” (BCAN-D-17-00023) for your appreciation and possible publication in your journal. In order to facilitate the work of the reviewers, we provided a detailed response to each reviewer/editorial point raised, describing exactly what amendments have been made to the manuscript text and where these can be viewed. We are sending a clean as well as marked-up version of the manuscript where all changes are indicated.
We think the changes suggested by the reviewers significantly improved the presentation of the manuscript. We agree with all criticism raised by the reviewers and we have addressed the concerns the cell types, proliferation assays, inhibition assays and immunodetection, as well as, all explanations requested by the reviewers.

Therefore, we believe that our study, although with some limitations can contribute to the scientific community. Accordingly, we hope that you will now find the manuscript suitable for publication in BMC Cancer.

Thanking in advance,

Sincerely yours,

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Spencer Gibson (Reviewer 1):

1. TNFalpha induced cell death was effectively blocked by FAM3B but not etoposide induced cell death. This needs to be explained as Bcl-2 overexpression does block etoposide induced cell death in other cell types.

R: It is well known that treatment with DNA damaging anticancer drugs such as camptothecin and etoposide induces p53 activation ultimately leading to cell death by apoptosis in several cell lines. DU-145 cells do not have a functional p53 and thus require JNK-dependent Fas activation for the occurrence of etoposide-induced apoptosis. Furthermore, this pathway is not inhibited by mitochondrial anti-apoptotic proteins [1, 2]. In fact, DU145 cells die by apoptosis after treatment with docetaxel (by disruption of the microtubule network and mitosis), cisplatin, and tumor necrosis factor (TNF-α) but overexpression of Bcl-2 gene attenuates these cell death processes.
On the other hand, sensitivity to cisplatin fluctuates widely among cancer cell lines and cell lines with higher levels of Bcl-2 are desensitized. More important, caspase inhibitors did not prevent DU145 cell death induced by docetaxel or cisplatin, whereas they did inhibit cell death induced by TNF-α. Thus, the absence of p53 explains why FAM3B failed to inhibit apoptosis after treatment with etoposide and camptothecin in DU145 cells overexpressing FAM3B gene, despite high levels of Bcl-2 expression.

2. The cell viability assay in figure 2A is not a measure of cell death but of cell proliferation. The same issue in Figure 2B with TNF treatment.

R: For assays with several doses of rhFAM3B, DU145 cells were cultured under non-stimulated (serum starvation) conditions (DMEM media + 0.5% FBS). As demonstrated by Tang et al, DU145 cells under serum starvation have an initial phase of aggressive growth (0-12h) and then undergo apoptosis due to the activation of several pro-apoptotic molecules. However, cell loss by apoptosis is compensated by cell proliferation activated later on (96h), conferring DU145 cells not only a late proliferation capacity, but also resistance against apoptosis under serum-deprived conditions when cultivated for more than 5 days [4]. We confirmed these findings in the experiment shown in Fig. 2A and 3B. Therefore, since DU145 cells were treated with rhFAM3B during 48h, it is reasonable to assume that cell death is the main cellular process being inhibited by FAM3B to increase the cell population. Moreover, as measured by BrdU assays, there is an absence of a proliferative phenotype after FAM3B overexpression (see Additional file 1), confirming that FAM3B has an anti-apoptotic rather than proliferative role in DU145 prostate tumor cells. In contrast, TNF-α by itself did not significantly induce cell death in DU145 cell line [5], but cell death induced by TNF-α did occur after the addition of cycloheximide (CHX), a protein synthesis inhibitor, as previously reported [3, 6]. Therefore, the decreased cell viability of DU145 cells in response to TNF-α (Fig. 2B) represents an increased sensitivity of DU145 cells to TNF-α-mediated cell death rather than a diminished proliferative phenotype. Taken together, Fig 2B shows that cell death induced by TNF-α + CHX was inhibited by rhFAM3 (100pM), confirming the anti-apoptotic role of this protein in DU145 cells.

3. For tumors grown in nude mice, the amount of cell death should be measured by tunel assay and amount of cell proliferation needs to be determined by Ki67 staining.

R1: As shown in supplementary Figure S1, we have not found any evidence that FAM3B induces proliferation in DU145 cells. Additionally, as shown in Fig 8, increased tumor growth
correlated well with both the expression of FAM3B and anti-apoptotic protein Bcl-2. Nonetheless, we agree with the referee and have performed additional immunohistochemistry assays to measure the expression of Proliferating Cell Nuclear Antigen (PCNA), a marker for DNA synthesis, in DU145 tumor samples from nude mice xenografts. As shown in the revised Additional file S1 (See revised manuscript, Figure legends, line 770, page 33) no differences in positive staining were evidenced in DU145-control as compared to DU145-FAM3B tumors, supporting the hypothesis that FAM3B does not induce proliferation in DU145 cells.

Additional File 1. Cell proliferation assays in DU145/FAM3B cells and tumor xenografts. (C) Representative images of immunohistochemical (IHC) slide showing the expression of Proliferating Cell Nuclear Antigen (PCNA) in tumor xenografts tumors derived from DU145/FAM3B and DU145/control cells

Xiaopei Cao (Reviewer 2):

1) Except the mRNA expression, the PANDER protein expression should be checked in the cell lines and in the tumor tissues.

R: FAM3B protein in DU145-FAM3B cells was measured by western blot (Figure 3A), but, unfortunately, we could not check for the expression of FAM3B by western blot in all prostate cell lines. We agree with the referee that protein expression is very important, however, we expect that mRNA overexpression should correlate with the corresponding protein overexpression in tumor tissue. Nonetheless, we have attached one micrograph of one immunohistochemistry assay done previously, which showed the expression of FAM3B protein in tumor tissues. We have not added this IHC because of the low quality, and we would like to add it as supplementary data, if considered necessary by the reviewer.

Representative images of immunohistochemical (IHC) slide showing the expression of FAM3B in tumor xenografts tumors derived from DU145/FAM3B and DU145/control cells

2) It is necessary for the author to perform the same experiments in a PANDER knockdown LnCAP cell line (with high PANDER expression) to confirm the findings in the PANDER overexpression DU145 cells (with low PANDER expression).
R: LNCaP cells are prostate cancer hormone-responsive cells, but DU145 cells do not respond to androgen steroids. The growth signaling pathways leading to proliferation and/or cell death - two key cellular processes in tumor growth and progression – are very different in these two cell types [7]. Therefore, we have used DU145 as a model to show there is a role for FAM3B in non-hormone-responsive prostate tumors, known for their high invasiveness (prominent DU145 feature) and mortality in the clinical setting. However, we acknowledge the need for future experiments and suggest that it would be more appropriate to change the title of the manuscript to "FAM3B / PANDER inhibits cell death and Increases tumor growth in hormone unresponsive DU145 cells by modulating the expression of Bcl-2 and Bcl-XL cell survival gene".

3) The protective effect of PANDER was only observed in the TNF-alfa induced cell death. Then, what is the signal pathway of the downstream of TNF-alfa. This should be addressed and checked.

R: TNF-α can trigger both NF-κB and apoptosis signaling simultaneously, so suppression of TNF-α-induced NF-κB signaling by CHX can potentiate TNF-α-induced apoptosis through caspase-3 activation in DU145 cells, inhibited by Bcl-2 and Bcl-XL overexpression [3, 8]. In fact, our results suggest that overexpressing FAM3B promotes survival in DU145-FAM3B cells through increased Bcl-2 and Bcl-XL expression and inhibition of caspase-3 cleavage. Although the inhibition of caspase activity was observed only after TNF-α + CHX treatment, it is reasonable to think that FAM3B might directly or indirectly upregulate the anti-apoptotic caspase inhibitors, such as survivin, and significantly hinder cell death. Previous authors have shown that inhibition of Bcl-2, Bcl-XL, and survivin are key to sensitize DU145 cells to TNF-α-induced apoptosis [5, 9].

On the other hand, the inhibition of upstream caspase-8 appears not be a key step for FAM3B antiapoptotic effects in DU145 cells because this inhibition was clearly observed only at 48 h after TNF-α + CHX treatment. Considering that an alternative mechanism of caspase-8 activation is regulated by procaspase-3 cleavage, therefore, enhancing a newly caspase-3 processing by caspase-8 may accelerates apoptosis [10], we hypothesize that FAM3B may be inhibiting indirectly this positive feedback rather than directly inhibition of TNF-α-mediated caspase-8 activation. Thus FAM3B may be using alternative pathways other than inhibition of classical upstream caspases of extrinsic pathway to attenuate TNF-α-mediated apoptosis in DU145 cells. Moreover, inhibition of caspase activation-9 could be explained by the indirect inactivation of the Bid protein. Bid is cleaved by activated caspase-8 and then translocated to mitochondria, where it promotes cytochrome c release by interacting with Bax, leading to activation of apoptosis via caspase-9 [11, 12]. Therefore, in absence of early caspase-8 activation and substantial down regulation of Bax, we suppose that this pathway is not initially regulated by
FAM3B in DU145 cells after TNF-\(\alpha\) treatment. In summary, the antiapoptotic effect of FAM3B in DU145 cells could be mediated through increased expression of Bcl-2 and Bcl-xL, and it is quite likely a previous upregulation of transcription factor NF-kB and AP-1 [13, 14], which results in the inactivation of caspases and decreasing rate of DU145 cells undergoing apoptosis.

4) In figure 6, the western blot for cleaved caspase3 and caspase9 are not quite clear.

R: As caspase-3 and -9 needed to be proteolytically processed by other caspases (mainly caspase-8) for reached the full enzymatic activity, the detection of active forms are indispensable to conclude that cell death are mediated by caspase-dependent mechanisms. The active (cleaved) form of caspase-3 is identified as an approximately 17 kDa band in the western blot and the active form of caspase-9 as a 10 kDa band (marked in the WB figure). Thus, as showed in Fig. 6 we confirmed a significantly diminished activity of caspase-3 (24 hours after TNF-\(\alpha\)-treatment) and caspase-9 (48h after TNF-\(\alpha\) treatment) in DU145/FAM3B cells when compared to control cells. These data, confirmed by enzymatic assays with synthetic substrates, are suggesting that FAM3B inhibits cell death by caspase-dependent mechanisms.

5) Further experiment data is needed to confirm that whether inhibition of bcl2 and bcl-XL pathway contribute to PANDER effects on prostate tumor cell viability.

R: We agree with the reviewer and performed inhibition assays for Bcl-2 and Bcl-XL in order to verify the contribution of these proteins to the anti-apoptotic effect of FAM3B in DU145 cells.

As it was not possible to perform knockdown and silencing gene assays because these are very time-consuming assays, we used two specific inhibitors to perform the experiments:

a) Obatoclax Mesylate (GX15-070), an antagonist of Bcl-2, that also acts upon Mcl-1

b) ABT-737, a BH3-mimetic pan-inhibitor of Bcl-xL, Bcl-2, and Bcl-w, with no inhibition observed against Mcl-1

We used these inhibitors alongside TNF-\(\alpha\) (1 and 5 ng /ml) + 1 \(\mu\)M CHX to confirm the role of Bcl-2 and Bcl-X in FAM3B-mediated cell death resistance to TNF-\(\alpha\)

These procedures were added at the revised manuscript Methods section, line 225, page 12)
Following treatment with both TNF-α doses and 10 μM ABT-737, a statistically significant decrease in the resistance against TNF-α + CHX-induced cell death was observed in DU145-FAM3B cells. On the other hand, 0.1 μM GX15-070 (OBTX) only decreased resistance to cell death when 1 ng/ml TNF-α was used (Fig 6A). These data confirm the key role of Bcl-2 and Bcl-XL in FAM3B-mediated protection against cell death in DU145 cells. In a dose-dependent assay we determined that in the presence of 2 ng/ml TNF-α, GX15-070 (OBTX) does not have any significant effect (Fig. 6B).

These results were added at the revised manuscript Results section, line 369, page 18)

Since GX15-070 can also inhibit the anti-apoptotic protein Mcl-1 [15], we concluded that there are different mechanisms of cell death being mediated by Bcl-2 and Mcl-1, at low doses of TNF-α + CHX in DU145 cells. On the other hand, it is well known that different doses of TNF induce different effects on prostate tumors [16], reinforcing the hypothesis that FAM3B may inhibit different mechanisms and types of cell death in DU145 cells, including autophagy and necrosis as suggested [17]. This hypothesis should be further addressed in future studies. These commentaries were added at the revised manuscript Discussion section, line 474, page 23)

A

B

Figure 6. Inhibition of Bcl-2 anti-apoptotic family proteins decreases protective effects of FAM3B. (A) DU145 cells were treated with 1 ng/ml and 5 ng/ml TNF-α + 10 mM CHX and 10 μM ABT-737 or 0.1 μM GX15-070 (OBTX) (B) a dose-dependent assay between 0.13 and 5 ng/ml TNF and 0.1 μM GX15-070 (OBTX). The results are expressed as means of three independent experiments and as relative percent of control cells or FAM3B cells respectively. Statistical differences were determined by Two-way Annova test. (# p < 0.001; & p < 0.01).

6) As this data presented results opposite to the previous studies, the possible underlying reasons should be discussed.

FAM3B has been primarily localized to the endocrine pancreas and identified in both α- and β-cells [18, 19]Initial characterization evaluating the role of FAM3B on pancreatic islets revealed induction of pancreatic β-cell apoptosis via caspase-3 and cyclin-dependent kinase inhibitor 1A (p21) pathways, suggesting that FAM3B is a potential activator in a setting of type 1 diabetes [20]. However, recent evidence using a FAM3B knockout mouse model revealed another
biological role for this protein in the regulation of glycaemia via regulation of liver and pancreas functions[21].

In this study we show, for the first time, the role of FAM3B in tumor cell death, tumor growth, and invasiveness in prostate cancer. However, previous papers have suggested a putative mechanism for FAM3B-mediated metastasis in colorectal carcinoma through activation of SLUG [22]. SLUG is a transcription factor that promotes EMT-transition, and consequently metastasis, in prostate tumor cells by directly inhibiting the tumor repressor PTEN [23]. Moreover, in HTC8 colorectal carcinoma cell line, the knockdown of FAM3B triggers apoptosis through a p53-dependent pathway. All these studies address different effects triggered by FAM3B in tumor cells.

Some hypothesis supporting the pleiotropic role of FAM3B can be proposed:

1) As noticed above, the involvement of SLUG in cell viability and survival of DU145 cells has been demonstrated, suggesting that activation of SLUG by FAM3B could be regulating several tumor-associated cellular processes in different stages of prostate tumor progression, and probably, upon activation by several stimuli. Indeed, other molecules that are known FAM3B targets in β- pancreatic cells and hepatocytes, such as cyclin D1, Cdc42, E-cadherin and JAM [22, 24, 25] also participate in survival and metastasis pathways in DU145 cells.

2) Several molecules regulated by FAM3B to facilitate glycemic control and lipogenesis, such as MCP-1, DGAT1, and FOXO1 [25-27] play an important role in prostate tumor progression [28-31].

3) Furthermore, we attempted to establish an association of FAM3B-induced upregulation of Bcl-2 and Bcl-XL with the invasive phenotype of DU145-FAM3B cells. Interestingly, the association of increased levels of Bcl-2 and Bcl-XL expression and the progression from pathologically localized to disseminated stages with an androgen-independent phenotype of prostate cancer has been previously established [32-34]. Recent studies recognized Bcl-2 as a prognostic biomarker for prostate cancer [35] and others showed association of high Bcl-2 expression with higher Gleason scores and lower survival in patients with advanced prostate cancer [36].
4) Thus, we conclude that FAM3B-mediated upregulation of Bcl-2 anti-apoptotic family members can be contributing to both inhibition of apoptosis and metastasis induction in DU145 cells.

REFERENCES


