Reviewer 1.

PI3K/AKT/β-catenin signaling regulates Vestigial-2 like 1 which predicts poor prognosis and enhances 3 malignant phenotype in gastric cancer

Kim et al present an interesting and potentially therapeutically important discovery that Vestigial-like 1 (VGLL1) is involved in gastric cancer proliferation and metastasis. The authors posit that VGLL1 is regulated by PI3K/AKT/beta-catenin activity and that VGLL1 regulates metastasis through MMP9 expression. The paper is well written and generally includes appropriate controls to support the authors’ conclusions. There are, however, several points which should be clarified or supported with further experiments, as detailed below. If the authors can satisfy these concerns, the paper would be appropriate for publication in Cancers.

Major points:
- Page 2, Line 54: the authors say that TCGA has identified four sub-types of gastric cancer, but they only describe three in the paragraph: CIN, MSI, EBV. What is the fourth type?
  ► We appreciate your valuable comment. We somehow missed GS subtype. We inserted following sentences of GS subtype into the introduction section
  “Genomically stable (GS) subtype which is associated with diffuse gastric cancer has frequently mutations in RHOA and CDH1 gene or fusions involving Rho-family GTPase-activating proteins (GAP).”

- The title for Figure 1 is “Figure 1. VGLL1, a prognostic biomarker, is crucial for the proliferation of gastric cancer cells.” This title is not supported by the data. The data in this figure shows that VGLL1 expression is correlated with gastric cancer and PI3K.
  ► We appreciate your important comment. We changed the title of Figure 1 to “VGLL1 expression is correlated with gastric cancer and PI3K.”

- Figure 1G: are these Pearson correlations or Spearman correlations? It appears from the methods that these are Pearson correlations, but the authors should explicitly mention this in the legend.
  ► We appreciate your important comment. They are Pearson correlations. We inserted “Pearson” in the title of Figure 1g

- In Figure 1, is VGLL1 more upregulated in any of the four types of gastric cancer? For example, since the EBV sub-type is more likely to have PI3K mutations, is more VGLL1 expression observed in these cancers? The authors should include this information about VGLL1 by sub-type (assuming the sub-type for their tumors measured by microarray is known).
We appreciate your valuable suggestion that we should analyze whether VGLL1 expression status is correlated with EBV subtype GC. We agree that such additional information would help understand GC biology further. However, unfortunately, in our 556 GC cases, we have not assessed the EBER ISH or DNA/RNAseq assay so that we are unable to provide EBV status of each case. Thus, we are afraid we could not investigate the relationship between EBV subtype and VGLL1 expression in the current study.

- Page 11, “Microarray analysis” Have the authors applied an FDR correction to their significance test? P < 0.001 is likely not significant for microarray with >10k genes.

We did not apply to select only those with biological relevance. The analysis was performed according to the data processing described in Methods 4.4. Methods 4.5 (page 11) analyzed p <0.001 as significant in statistical analysis such as comparison test and Pearson correlation based on microarray results. We deleted the part because it seems to confuse the contents.

- Overexpression of VGLL1 in NUGC3 cells. From Figure 1B, NUGC3 cells already overexpress VGLL1. Why have these cells been chosen as the model cell line for overexpression of VGLL1 in Figures 2C and 3? NUGC3 seems an appropriate model cell line for the shRNA knockdown experiments, but the authors should show the effects of VGLL1 overexpression in non-VGLL1 overexpressing gastric cell lines.

We appreciate your valuable comments. In Figure 2b, we showed increased proliferation of ACS and HEK293T cells which expressing relatively low VGLL1 (refer to Figure 1b). We further examined the effect of VGLL1 overexpression on cell proliferation in SNU484, SNU638 and SNU668 cells, which express VGLL1 at low level. Cells were transfected with pcDNA3.1 and pcDNA3.1–VGLL1 for 72 h, and then stained with sulforhodamine B. Obviously, VGLL1 overexpression increased the proliferation of gastric cancer cells tested.

- Figure 2E: why are the authors using HEK293T cells with VGLL1 overexpression (and not a gastric cancer cell line)? They have VGLL1 overexpressing gastric cancer lines (notably NUGC3 cells, which are used in Fig. 2C, although as described in my previous comment these are not the ideal model for VGLL1 overexpression). The authors need to either justify the switch to HEK293T or re-do the experiment with a gastric cancer cell line.
We appreciate your valuable comment. We wanted to observe the effect of VGLL1 overexpression in normal cells expressing low VGLL1. However, we could not obtain normal gastric cancer cells. Therefore, we selected HEK293T cells because it is close to normal cells and expresses low level of VGLL1. We confirmed the increase of proliferation by VGLL1 overexpression in HEK293T cells (Figure 2b).

- Figure 4E-F: The authors show “P-AKT” Western blotting. What phosphorylation site is being tested here? The authors list the product number Cell Signaling 92271. I looked on the CST website and couldn’t find this antibody. I think the authors are referring to product 9271 (not 92271) which is pSer473-AKT, but they should clarify.

We appreciate your critical point. It was our mistake. The product number of P-AKT (Ser473) antibody is 9271. We corrected the product number in line 392.

- Figure 4F: The authors use “constitutively active AKT” but don’t describe what is this protein. Point mutation? Myristoylation? I also don’t see this vector described in the methods. The authors need to clarify.

We appreciate your critical point. We should have addressed it. Constitutively active Akt has S473D/T308D double mutation (Ref. Apoptosis 2014;19:179). We modified phrase to “constitutively active AKT containing S473D/T308D double mutation” in line 159.

- Figure 4H: The authors have overexpressed b-catenin-FLAG in a cell line (though it’s not clearly stated which cell line in the legend) that appears to express endogenous b-catenin (as shown by the siControl lane at left). Why don’t the RT-PCR and Western blot at right with b-catenin FLAG show endogenous expression of b-catenin in the pcDNA3 lanes? Is the overexpression so substantial that the endogenous mRNA / protein are not detectable at these exposures?

First of all, I'm sorry for not clarifying the experiment conditions in detail. Figure 4H experiment was performed using NUGC3 cells. Differences in endogenous expression or overexpression of b-catenin level in RT-PCR was due to program setting for RT-PCR cycles. For b-catenin knockdown, we set 30 cycle for RT-PCR. For b-catenin overexpression, we set program to 28 cycle for RT-PCR. In the western blot experiment, we confirmed overexpression of b-catenin using anti-flag antibody (Sigma-aldrich, F1804) because we constructed Flag-tagged b-catenin.

- Figure 4: the effects of b-catenin knockdown and overexpression on VGLL1 levels are rather modest (Fig. 4I, for example) and the data for b-catenin’s role is all indirect (knockdown of TCF4 or LEF1, for example). Can the authors show a ChIP demonstrating that b-catenin is localized to the VGLL1 promoter? Can the authors show that PI3K knockdown or LY294002 treatment reduces the amount of b-catenin at the VGLL1 promoter? This data is needed to convincingly show that b-catenin is what is driving VGLL1 expression in gastric cancer. It could well be that PI3K/AKT are working through another mechanism to regulate VGLL1 transcription.
We appreciate your critical point that we missed. We performed ChIP assay to examine whether b-catenin is localized to the VGLL1 promoter. We found that b-catenin bound to the region containing LEF1 and TCF4 binding sites in the VGLL1 promoter. As expected, binding of b-catenin to the VGLL1 promoter was reduced by LY294002 treatment. We inserted this result in Figure 4g.

Minor points:

- Page 2, Line 59, This sentence is repeated: “The Epstein-Barr virus (EBV) subtype has a high rate of PIK3CA mutations and 59 high PD-L1/L2 expression.”
- Page 4, Line 116: “contro” should be “control”

We removed the repeating sentence. We also corrected “control” in line 116 and 117.