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Reviewer A

There have been a number of studies published this year using a similar approach and databases eg Huang et al Front Endocrinol. This diminishes the impact of your manuscript. You need to critically discuss your results in the context of these other studies examining KLR1 expression and immune cells in breast cancer, how does your data/approach differ from these?

Response: Thank you for your suggestion. Huang's article, also used the data of 1,109 breast cancer patients in TCGA database for research [1]. But there are some differences in the specific content of our study. First, we not only analyzed the relationship between KLRB1 and the expression and prognosis of breast cancer, but also analyzed the relationship between KLRB1 and the expression and prognosis of different subtypes of breast cancer. Secondly, we analyzed the differentially expressed genes of KLRB1 in breast cancer by GESA, found KEGG factory transmission, Reactome factory signaling pathway, NABA restricted factors, KEGG cytokine-cytokine receptor interaction, and KEGG systemic lupus erythematosus, This is different from Huang's discovery. Next, we also studied the correlation between the top 10 up and down regulated DEG and KLRB1, revealing the significant correlation between most DEG and KLRB1, which was also not studied in Huang's article. Finally, we also used clinical samples of breast cancer and two different breast cancer cell lines for experimental verification. Huang's article did not use clinical samples for verification.

To sum up, although Huang's article has been published, our research on KLRB1 in breast cancer still has certain uniqueness and significance.

[1] Huang G, Xiao S, Jiang Z, Zhou X, Chen L, Long L, Zhang S, Xu K, Chen J, Jiang B. Machine learning immune-related gene based on KLRB1 model for predicting the prognosis and immune cell infiltration of breast cancer. Front Endocrinol (Lausanne). 2023 Jun 7;14:1185799. doi: 10.3389/fendo.2023.1185799. PMID: 37351109; PMCID: PMC10282768.

The detail in the QPCR and IHC methodology needs more detail, what were the sequences of GAPDH primers, cycling conditions etc. The description of the IHC staining needs expanding, what is the nature of the cells staining in normal vs tumour? The image of the normal tissue appears to have significant levels of background staining in comparison to the tumour image. This needs rectifying, where all sections stained together for identical time periods? Was the QPCR performed using a delta delta Ct approach?

Response: Thank you for your invaluable comments. We have enriched the details of

qPCR and IHC methods. We need more details, the sequence of GAPDH primers, and circulation conditions.

RNA isolation and qPCR analysis

Tissue RNA extraction was conducted utilizing the TRIzol reagent. Subsequently, the obtained RNA was reverse-transcribed into cDNA utilizing the QuantiTect Reverse Transcription Kit. RNA was extracted from tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Quantitative PCR (qPCR) uses real-time fluorescence to measure the quantity of DNA present at each cycle during a PCR. Real-time qPCR analyses were quantified with SYBR-Green (Takara, Otsu, Shiga, Japan), and GAPDH served as an internal control. The primers employed were as follows: KLRB1 forward primer, 5′-AATTTGCCCTGAAACTTAGCTG-3′; reverse, 5′-GGATGTCACTGAAACACTCAAC-3′. GAPDH forward primer, 5′-GTCTCCTCTGACTTCAACAGCG-3′; reverse, 5′-ACCACCCTGTTGCTGTAGCCAA-3′. The qPCR value was calculated using the 2-delta delta Ct method. We set the Ct value to 15-35, and Ct values not in this range will be excluded. (lines 149 to 162)

Immunohistochemistry

Immunohistochemistry was performed by two-step method according to the instructions (PV-9000; ZSGB-BIO, Beijing, China). BRCA samples were fixed in 10% formalin, embedded in paraffin, and processed into 5-μm sequential sections. The samples were de-waxed with ethanol and blocked to inhibit the endogenous peroxidase activity. After this, samples were heated in a microwave for antigen retrieval, cooled to room temperature, and blocked using goat serum for 30 min at 37°C. The samples were incubated overnight at 4°C with rabbit anti-KLRB1 (Thermo Fisher Scientific 17-5941-82) (1:200), followed by incubation with horseradish peroxidase-coupled goat anti-rabbit secondary antibody (PV-9000; ZSGB-BIO, Beijing, China) at 37°C for 30 min. The samples were then stained with 3,3’-Diaminobenzidine (DAB). Cell nuclei were stained blue with hematoxylin. The sections were then dehydrated, cleared with xylene, and mounted. KLRB1 expressions were determined by immunohistochemistry (IHC) using the streptavidin peroxidase method, with adjacent tissues serving as the controls. The experimental procedure was performed as per the manufacturer’s instructions. Image-Pro Plus 6.0 Software (Media Cybernetics, USA) was used to analyze protein expression and perform statistical analysis of the results obtained by IHC. (lines 164 to 179)

For “what is the nature of the cells staining in normal vs tumour?”Hematoxylin stains the nucleus and intercellular substance blue. According to the expression of the target protein, antibody coupling is used in immunohistochemistry, and the color development is further amplified by the cascade of secondary antibody and DAB to make the target protein appear brown. Therefore, the tissue with weak expression of the target protein is blue, and the tissue with high expression of the target protein is brown. In this study, the protein level of normal breast tissue KLRB1 was highly expressed, and the tissue

color was brown. The protein level of tumor tissue KLRB1 is low, and the tissue is shown as blue.

For “The image of the normal tissue appears to have significant levels of background staining in comparison to the tumour image. This needs rectifying, where all sections stained together for identical time periods?” Gene Ontology (GO) annotations related to KLRB1 include transmembrane signaling receptor activity and carbohydrate binding. As KLRB1 secreted protein, KLRB1 is mainly distributed in the cytoplasm, membrane and intercellular space outside the primary nucleus. In this study, the protein level of normal breast tissue KLRB1 was highly expressed, and The image of the normal tissue appears to have significant levels of background staining.

Did you check if KLRB1 was expressed in MCF-7 transfected cells? What was the expression level, was this supra-physiological? If as you state there is a decrease in KLRB1 expression in non-tumour cells the authors should modify expression in immune cells, and determine the effect on function. If levels are changing in non-tumour cells why modify it in MCF-7 cells?

Response: Thank you for your invaluable comments. We examined the expression of KLRB1 in MCF10A cell line, MCF7 cell line and MDAMB231 cell line. The expression of KLRB1 in breast cancer cell line decreased significantly. Because KLRB1 expression is low in breast cancer cell lines, KLRB1 expression is high in breast cancer cell lines after our transfection, so this must be super rational (**Figure 8 B-C**).

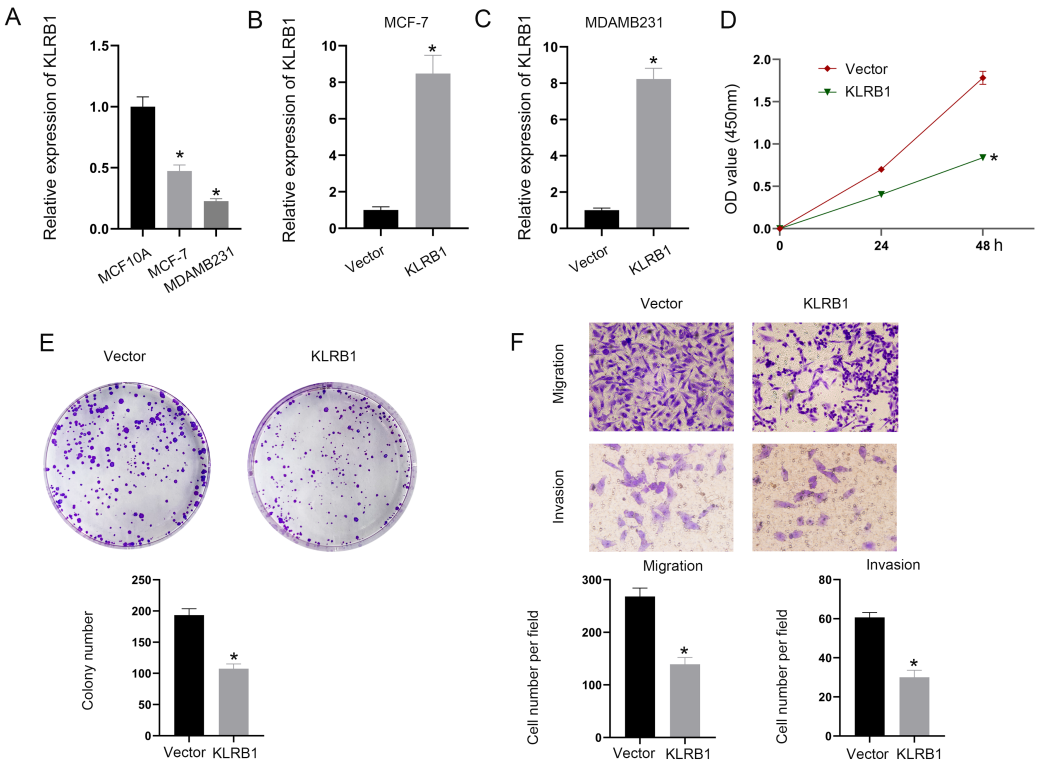

Figure 8: (**A**) The expression of *KLRB1* in MCF10A cells, MCF7 cells and

MADAMB231 cells. (**B-C**) The expression level of KLRB1 after transfection in MCF7 cells and MCF10A cells. (**D-E**) Decreased proliferation of MADAMB231 cells transfected with *KLRB1* vector. (**F**) *KLRB1* inhibits the migration and invasion ability of both MCF7 and MDAMB231 cells. *$P < 0.05$.

Reviewer B

This manuscript describes the role of KLRB1 gene in the human breast cancer by examining TCGA dataset by statistic and bioinformative methods. Furthermore, they validated their results by analyzing breast cancer specimens and MCF7 cell line.
This is very interesting. Most of the results can support their conclusions.
However, some results are still preliminary level and ambiguous to lead their conclusion.
Therefore, the authors need to clearly state some ambiguities.
The manuscript is suitable for publication, but some minor revisions should be considered.

Major concerns:
(1) The authors should examine KLRB1 gene expression level and prognosis in each breast cancer subtypes. It is well known that breast cancer is classified into Luminal A, Luminal B, Her2 type, and Triple negative type (TNBC). Therefore, it is very important to analyze the gene expression and prognosis.
Response: Thank you for your invaluable comments. We analyzed the expression and prognosis of KLRB1 in luminal a, luminal B, HER2 and triple negative breast cancer, and found that there was no significant difference in the expression and prognosis of KLRB1 in breast cancer subtypes.

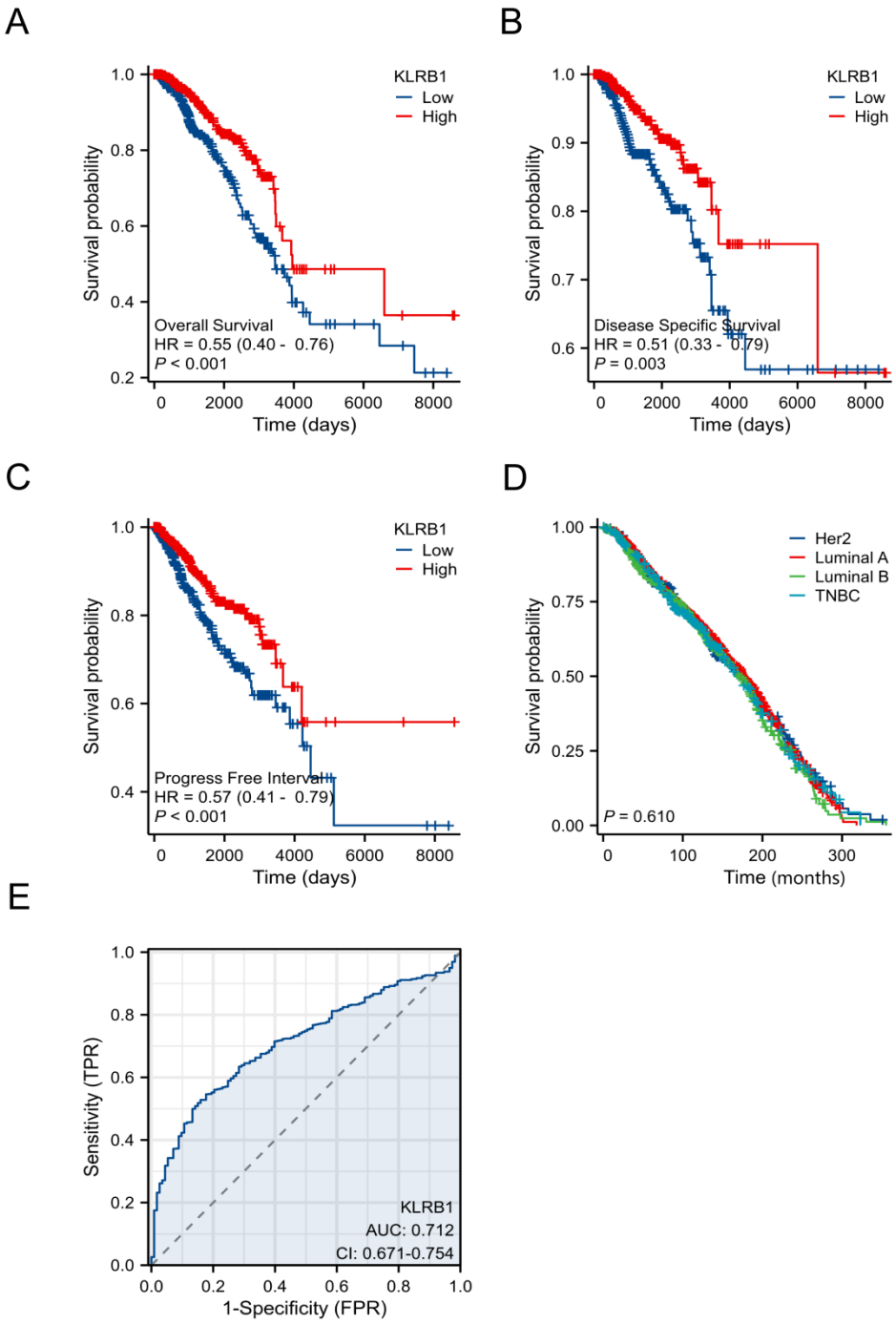

Figure 2: The survival rate of individuals with BRCA with elevated and reduced expression levels of *KLRB1*. (**A)** OS. (**B)** DSS. (**C)** PFI. (**D**) No significant difference in the prognosis of different breast cancer subtypes in breast cancer patients with low KLRB1 expression. (**F)** ROC analysis showed that *KLRB1* could accurately distinguish BRCA tumor tissues from healthy tissues.

(2) Luminal A and Luminal B types is ER positive breast cancer which is association with KLRB1 gene expression level. MCF7 cell line is Lumina A type. Therefore, the authors need to perform analysis using cell lines derived from subtypes that are highly correlated with KLRB1 gene expression and prognosis, according to the analysis results for each subtype.

Response: Thank you for your invaluable comments. First, we analyzed the prognosis of different subtypes of breast cancer, and found that there was no significant difference between the expression of KLRB1 and the prognosis of different subtypes of breast cancer. We then used the triple negative breast cancer cell line mdamb231 cell line for experimental verification, and the results were still similar to those of MCF7 cell line.

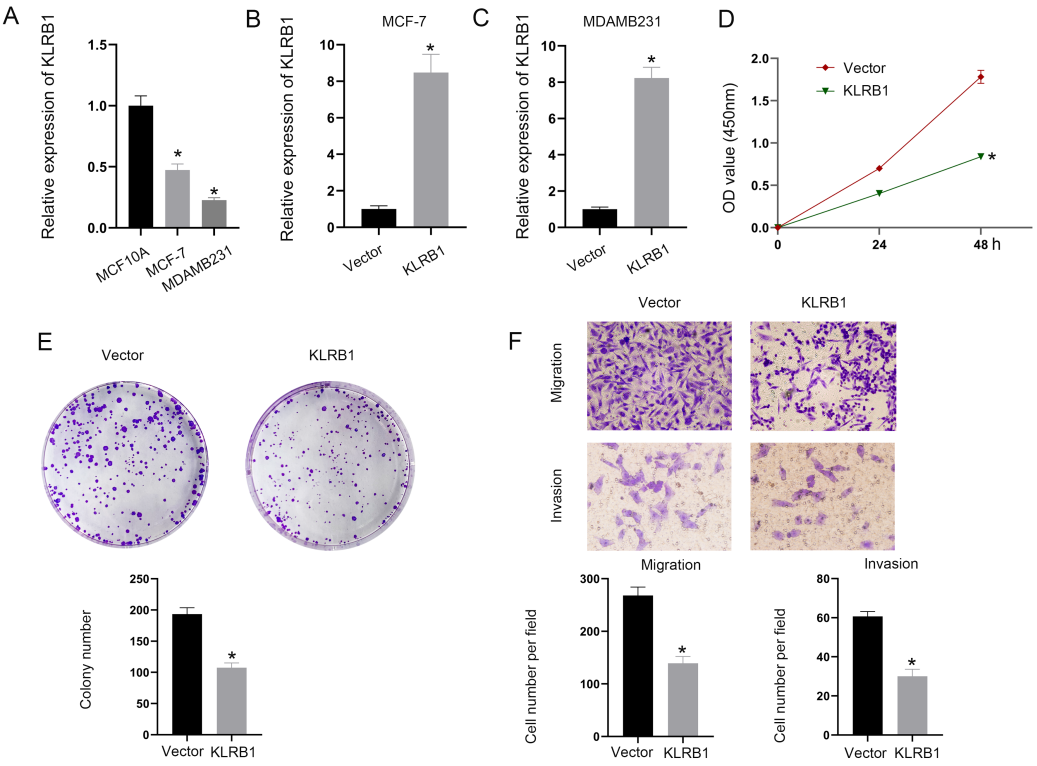

Figure 8: (**A**) The expression of *KLRB1* in MCF10A cells, MCF7 cells and MADAMB231 cells. (**B-C**) The expression level of KLRB1 after transfection in MCF7 cells and MCF10A cells. (**D-E**) Decreased proliferation of MADAMB231 cells transfected with *KLRB1* vector. (**F**) *KLRB1* inhibits the migration and invasion ability of both MCF7 and MDAMB231 cells. *$P < 0.05$.

(3) The authors should discuss the reason why KLRB1 gene expression is downregulated in several cancers including breast cancer. Example for, the authors can easily download and examine there are KLRB1 gene deletion or not in breast cancer using TCGA data set or Metabric data set. Metabric data set can be obtained from cBioPortal freely.

Response: Thank you for your invaluable comments. We downloaded the expression data of KLRB1 in the metabric data set and found that gene deletion was an important factor in KLRB1 down-regulation.

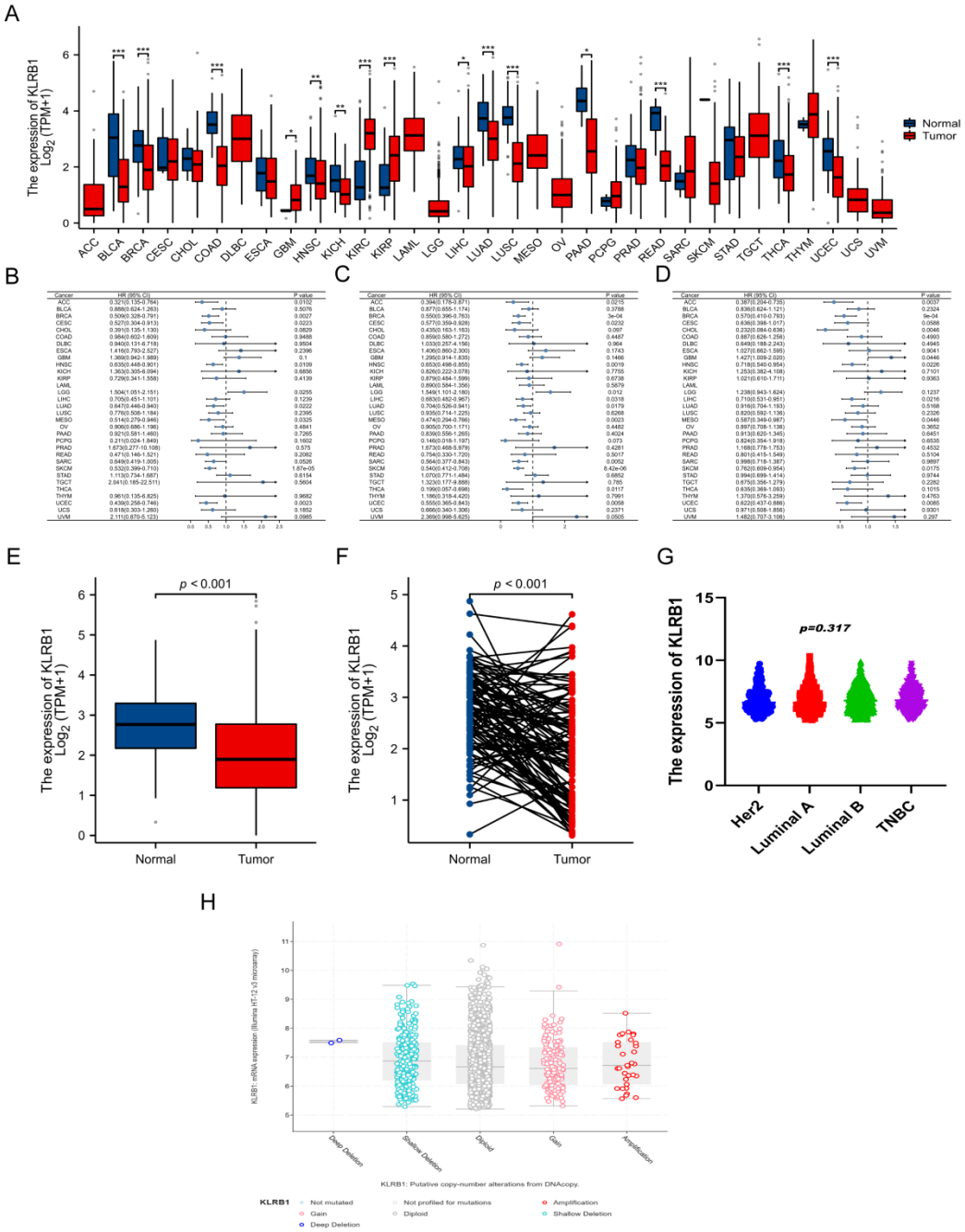

Figure 1: Association between *KLRB1* mRNA expression level and prognosis based on

TCGA database. (**A)** The mRNA expression of *KLRB1* in distinct cancer tissues and adjoining healthy tissues. (**B)** Prognostic correlation between *KLRB1* expression and distinct cancer types (OS). (**C)** Prognostic correlation between *KLRB1* expression and distinct cancer types (DSS). (**D)** Prognostic association between *KLRB1* expression and various cancer types (PFI). (**E)** *KLRB1* mRNA expression in BRCA tissues and healthy tissues. (**F)** *KLRB1* mRNA expression in BRCA tissues and paired sample tissues. (right represents HR > 1 (risky); left represents HR < 1 (protective)). (**G**) *KLRB1* mRNA expression in Her2, Luminal A, Luminal B and TNBC BRCA tissues. (**H**) Putative copy-number alterations of *KLRB1* in BRCA.“ns” represented $P > 0.05$, “*” represented $P <= 0.05$ and “***” represented $P <= 0.001$.

(4) The authors validated the results obtained from TCGA data analysis by IHC and qPCR of breast cancer specimens and MCF7 cell. This is good but the cohort is small population and no classification of breast cancer subtypes. Please validate KLRB1 gene expression level and prognosis in each breast cancer subtypes analyzing Metabric data set as larger cohort.
Response: Thank you for your invaluable comments. We downloaded the metafabric data set and analyzed the expression and prognosis of KLRB1 in luminal a, luminal B, HER2 and triple negative breast cancer. It was found that there was no significant difference in the expression and prognosis of KLRB1 in breast cancer subtypes.