Author’s response to reviews

Title: LGR5 Overexpression Confers Poor Relapse-free Survival in Breast Cancer

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Mousumi Majumder (Reviewer 1):

Q1. TNBC is the most aggressive breast cancer but why LRG5 expression is not high in this group, explain?

Answer: The results of this study showed that tumour size $\geq$ 2 cm tumor, lymph node metastasis and TNBC were associated with high levels of LGR5 expression ($p=0.002$, $p=0.044$ and $p=0.029$, respectively; Table 1).

The description has been added in the result part (line 4, page 11).

Q2. Authors explained the importance of LRG5 in cancer stem cell. As LRG5 can regulate cancer stem cell via Wnt/beta catenin pathway; at least one marker of stem cell pathway along with LRG5 in both NTNB and TNB groups in serial sections need to be done. Co-staining would be best but it could be done in serial sections or may be if they can extract mRNA, multiple markers can be done from tissue extracted RNA/Protein. That could be new and novel findings.

Answer: Thank you very much for your kind suggestion. The results have been written in the manuscript (line 7, page 6; line 10 page 11; line 1, page 15).

LGR5 not only participates in tumorigenesis but also maintains stemness by activating Wnt/β-catenin signaling in a breast cancer cell model. However, studies of LGR5 in breast cancer, particularly those of clinical significance, have been relatively few.

Regarding the mechanism via which CSCs act in breast cancer, in adult stem cells, LGR5 may serve as a functional factor that maintains stemness by activating Wnt/β-catenin signaling in
CSCs (8, 12). A recent study showed that the blockade of the Wnt/β-catenin signaling pathway preferentially reduced metastatic potential by altering CSC activity in a mouse model of breast cancer (13). We have further investigated β-catenin staining in the serial sections. The results revealed that β-catenin was not correlated with differentiation, age, tumor size, LN metastasis, histopathological feature, ER status, and PR status, but it was positively correlated with HER2 status (p=0.037, Table 1). We also examined whether β-catenin was associated with LGR5 in breast cancer by stratifying cancers according to molecular subtype. As shown in Table 2, β-catenin was positively correlated with LGR5 in the TNBC group (p=0.013, Table 2).

Although patients with elevated β-catenin levels alone did not have statistically shorter RFS periods as compared to patients with low levels of β-catenin expression (Figure 3D-F), those with increased β-catenin expression were likely to be more likely to experience recurrence and/or death (Table 3). When LGR5 and β-catenin were combined for Kaplan-Meier analysis, the results revealed that breast cancer patients with high LRG5 and β-catenin expression levels in their tumors had the worst prognoses due to their high rates of recurrence and/or death (Figure 4).

Q3. Except in theory authors did not show any evidence of link between stem cell pathway and LRG5.

Answer: Thank you very much for your kind suggestion. The description has been written in the manuscript (line 7, page 6; line 10 page 11; line 1, page 15).

LGR5 interacts with and cointernalises Wnt receptors to modulate Wnt/β-catenin signaling and delay endosome degradation (19). Our data revealed that β-catenin was positively correlated with HER2 status (Table 1). A previous study indicated that HER2-inducible Wnt/β-catenin signaling was required for the early onset of breast neoplasia (20). Therefore, we suggest that the LGR5-β-catenin axis is responsible for breast cancer progression.

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Q4. Figure 1: Can you make it two? Put breast cancer in one chart and rest of the cancers in other. In breast cancer plot, if you could separate out triple negative breast cancer and NTNB cancer data separated would be best representation.

Answer: Data mining of the Zhao and Tabchy breast microarray dataset, obtained via the Oncomine platform, regarding LGR5 expression in human breast cancer revealed that triple-negative breast cancer (TNBC) was associated with higher levels of LGR5 expression than non-triple-negative breast cancer (non-TNBC) (Figure 1B and C) (10, 11). These results imply that LGR5 expression is associated with cancer progression. Above description has been written in the result section (line 8, page 10).

Mieke Van Bockstal (Reviewer 2):

Q1. The manuscript needs extensive language editing, as the text contains many grammatical errors. I advise the authors to contact a native speaker or a language editing service.

Answer: We agree the comment of the reviewer. The language editing has been done by a language editing service.

Q2. Please include page numbers as this would facilitate the review process.

Answer: The numbers has been added at the bottom of every pages in this manuscript.

Q3. Introduction line 58: please clarify the abbreviation "PCNA".
Answer: We have clarified the abbreviation "PCNA" by adding its full name (proliferating cell nuclear antigen). LGR5’s expression has been positively correlated with proliferating cell nuclear antigen (PCNA) and Ki-67 and inversely associated with colorectal cancer survival rate in a meta-analysis (7).

Above description has been added in the introduction section (line 19, page 5).

Q4. Introduction line 23: "... then used a specific LGR5 antibody for performing immunohistochemistry..." Please explain in the materials & methods section which tests you have performed to assess the specificity of the antibody. Did you use positive controls? If yes: were these controls batch controls or on-slide controls?

Answer: Colonic adenocarcinoma was used as batch positive control.

Above description has been written in the methods section (line 10 page 8).

Q5. Patients line 10: please mention clearly in which circumstances patients were censored.

Patients who were still alive without recurrence or metastasis at the end of the study were censored at the date of the last follow up.

Above description has been written in the methods section (line 12, page 7).

Q6. Patients: please specify whether your study population contains invasive ductal carcinoma only (=invasive breast cancer, no special type). Were there lobular cancers included? Were there any special types?

Answer: The original histopathological classification of these 126 breast tumors was invasive ductal carcinoma (IDC) in 121 patients (96.0%) and a special type in five patients (4%) (Table 1).

Above description has been written in the result section (line 18, page 10).

Q7. Immunohistochemistry and scoring, line 42: please clarify why you used a cut-off at score 6. Was this cut-off arbitrarily chosen? If not: how did you determine this cut-off?

Answer: The criteria were as follows: immunostaining score: proportion score + intensity score (range 0, 2-8) [Proportion score (0 = 0/100; 1 = 1/100~1/10; 2 = 1/10~1/3; 3 = 1/3~2/3; 4 = 2/3~1; and 5 = 100/100); Intensity score (0 = negative; 1 = weak; 2 = intermediate; and 3 = strong)]. The median score was used as the cut-off point for the dichotomization of LGR5 and β-catenin levels. A median staining score of 6 was selected for LGR5 and β-catenin immunohistochemical scoring. Scores over 6 were defined as indicating “high” immunostaining, while scores of less than 6 were considered to indicate “low” immunostaining.

Above description has been written in the methods section (line 16, page 8).
Q8. Line 14 "Tumor size and lymph node tumor are corelated with high LGR5 expression". You initially compared LGR5 expression between invasive ductal breast carcinoma and breast adenocarcinoma, but these invasive ductal carcinoma is an adenocarcinoma of the breast. Is this a typo? Did you use any normal breast tissue in your comparison? This part of the text is not clear.

Answer: Thank you very much for your kind suggestion. The typo has been deleted. We analysed the cell dataset regarding LGR5 expression obtained from the Oncomine platform (http://www.oncomine.org), an online collection of microarrays. First, based on the results of the data mining of the Zhao breast microarray dataset on the Oncomine platform, LGR5 expression was higher in normal breast tissue than in human breast cancer tissue (17 invasive ductal carcinomas and 20 lobular carcinomas) (Figure 1A) (10). Additionally, data mining of the Zhao and Tabchy breast microarray dataset, obtained via the Oncomine platform, regarding LGR5 expression in human breast cancer revealed that triple-negative breast cancer (TNBC) was associated with higher levels of LGR5 expression than non-triple-negative breast cancer (non-TNBC) (Figure 1B and C) (10, 11). These results imply that LGR5 expression is associated with cancer progression.

Above description has been written in the result section (line 3, page 10).