Author’s response to reviews

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

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Response to Reviewers’ Comments

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LGR5 overexpression confers poor relapse-free survival in breast cancer patients

Editor Comments:

1. Please add a 'Declarations' heading on your declarations section.

Author reply:

We thank the Editorial for their comments. A 'Declarations' heading was added in the revised manuscript (page 13, line 1).

2. Under Funding (within declarations) - please add the phrase: 'The funding body had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.'

Author reply:

Author thank the Editorial’s comments. The phrase: “The funding body had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.” is added in the revised manuscript (page 13, line 11).

3. Please improve the overall resolution of your figures and particularly of the figure legends. Please remove the figure titles included in the figures (ie 'Figure 1', '(A)', '(B)' etc).

Author reply:
Author thank the Editorial’s comments. The title of every figure was removed and the corrected Figures are shown in the revised manuscript and represented as below:

Figure 1.

Figure 2.

Figure 3.

Figure 4.

4. In order to offer readers a chance to fully assess your methods and findings, your manuscript needs to be copyedited. We recommend that you ask a native English speaking colleague to help you copyedit the paper. If this is not possible, you may need to use a professional language editing service. For authors who wish to have the language in their manuscript edited by a native-English speaker with scientific expertise, BioMed Central has a new in house editing service. The new editing tool can provide both scientific and language editing: http://authorservices.springernature.com/.

Author reply:

We thank the Editorial for their comments and our manuscript has been edited by the professional language editing. The extensive changes were made for grammar, clarity, and readability of this manuscript. The inappropriate references and sentences were corrected in the revised manuscript and the detail changes were described as follow:

In the Abstract section (page 3, line 2)

“Background: Breast cancer has been viewed as a complex, heterogeneous disease, and cancer stem cells (CSCs) are believed to play a role in malignant transformation via multiple signaling pathways, including Wnt/β-catenin signaling. Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) has been identified as a Wnt-regulated target gene, but its clinical significance remains elusive. This study intends to investigate whether the LGR5-β-catenin axis is of clinical significance in breast cancer patients.” is changed to “Background: Cancer stem cells (CSCs) are believed to promote the malignant transformation of breast cancer via multiple signaling pathways, including the Wnt/β-catenin pathway. Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) has been identified as a CSC-associated Wnt-regulated target gene, but its clinical significance in the context of breast cancer remains elusive. Therefore, the purpose of this study was to investigate the clinical significance of the LGR5-β-catenin axis in breast cancer.”

In the Abstract section (page 3, line 9)
“Methods: Breast cancer tissue blocks from 126 patients were sampled for tissue microarray (TMA). Follow-up information and histopathological and clinical data including age, tumor size, estrogen receptor (ER) level, progesterone receptor (PR) level, human epidermal receptor 2 (HER2) level, tumor grade, lymph node (LN) status, clinicopathological features and survival were obtained from the cancer registry and medical charts. The breast TMA was evaluated for LGR5 and β-catenin expression using immunohistochemical staining and scores.” is changed to “Methods: Breast cancer tissue blocks from 126 patients were used to construct a tissue microarray (TMA). Histopathological and clinical data including age; tumor size; estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) level; tumor grade; lymph node (LN) status; and survival were obtained from the cancer registry database and patients’ medical records. Tissue on the breast TMA was scored for LGR5 and β-catenin expression using semi-quantitative immunohistochemical (IHC) staining. We also analyzed LGR5 expression in cellular datasets available through ONCOMINE, a web-based cancer microarray database.”

In the Abstract section (page 3, line 18)

“Results: The immunohistochemical results revealed that 58 (46%) tumors showed high LGR5 expression and 56 (47%) tumors showed high β-catenin expression. There was a statistically significant relationship between tumor size (T≧2 cm) (p = 0.002), lymph node involvement (N≧1) (p= 0.044) and triple-negative breast cancer (TNBC) and high levels of LGR5 expression (p= 0.029), which is consistent with the cancer microarray database of cell lines and integrated data-mining platform (ONCOMINE). Furthermore, β-catenin-expressing breast cancers were positively correlated with HER2 overexpression. In terms of clinical outcomes, patients with high levels of LGR5-β-catenin axis expression have the worst relapse-free survival (RFS) (p=0.027).” is changed to “Results: Immunohistochemical staining revealed that 58 tumors (46%) exhibited high LGR5 expression, whereas 56 tumors (47%) displayed high β-catenin expression. High LGR5 expression were significantly associated with tumor size (p = 0.002), LN metastasis status (p = 0.044), and triple-negative breast cancer (p = 0.029), consistent with our findings from the ONCOMINE database. In addition, we also found that β-catenin-expressing breast cancers were positively correlated with HER2 overexpression. Finally, with respect to clinical outcomes, patients with high levels of LGR5-β-catenin axis expression exhibited poorer relapse-free survival (RFS) compared to patients with low levels of LGR5-β-catenin axis expression (p = 0.027).”

In the Abstract section (page 4, line 2)

“Conclusion: LGR5 overexpression is statistically related to high T and LN metastasis values. The decrease in RFS may be due to high levels of LGR5-β-catenin axis expression in breast cancer patients, and this could represent a promising prognostic marker in breast cancer patients.” is changed to “LGR5 overexpression was significantly associated with high T stage and LN metastasis status. High LGR5 expression was also associated with reduced RFS, indicating that LGR5 may represent a promising prognostic marker for breast cancer patients.”

In the Background section (page 5, line 2)
Among women worldwide, breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death (1). Breast cancer in Asia, including Taiwan, is characterized by early tumor onset and thus a relatively younger median age at diagnosis than in Western populations (2). Specifically, breast cancer is a heterogeneous population (basal-like subtype and luminal subtype) of neoplasms originating from the epithelial cells lining the milk ducts, and some breast cancers are thought to originate in potential cancer stem cells (3). Additionally, the hormones associated with full-term pregnancy cause the permanent differentiation of vulnerable breast stem cells, which could be responsible for the susceptibility of the mammary glands to carcinogenesis (4). Clinically, the expression of estrogen receptor (ER) and progesterone receptor (PR) and the amplification of HER-2/Neu have been associated with distinct cancer subtypes, with prognostic and therapeutic implications in breast cancer (5). Interestingly, TNBC represents approximately 10–15% of all breast cancers that have poor prognoses as compared to the other molecular subtypes of breast cancer (5).“Breast cancer is the most frequently diagnosed cancer type and the second-leading cause of cancer-related deaths among women worldwide [1]. Breast cancer in Asia, including Taiwan, is characterized by early tumor onset, and thus exhibits a relatively younger median age at diagnosis than in Western populations [2]. Breast cancer is a heterogeneous disease consisting of several molecular subtypes. Indeed, estrogen receptor (ER) and progesterone receptor (PR) expression, as well as human epidermal growth factor receptor 2 (HER2/Neu) amplification, are associated with distinct subtypes, with prognostic and therapeutic implications [3]. In particular, triple-negative breast cancers (TNBCs), which account for approximately 10-15% of all breast cancer cases, are negative for ER, PR, and HER2, and exhibit poor prognoses relative to other breast cancer subtypes [3]."

The original Ref#4 and #5 were removed and the order of reference was rearranged in the revised manuscript.

In the Background section (page 5, line 13)

“LGR5, also known as GPR49, has been selected from a panel of intestinal Wnt target genes in crypt base columnar cells and is necessary for the most rapidly self-renewing tissue in the intestinal epithelium of adult mammals (6). Recently, 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). Surprisingly, a previous study has shown that in breast cancer, Wnt/β-catenin signaling is responsible for breast cancer progression and the maintenance of cancer stem-like cells (CSCs) via LGR5 overexpression (8). This evidence has revealed that 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.” is changed to “Breast cancer originates from the epithelial cells of the mammary gland, and, in some cases, is thought to arise from putative cancer stem cells (CSCs) [4]. CSCs are believed to promote the malignant transformation of many cancer types, including breast cancer, in part via activation of the Wnt/β-catenin pathway. Moreover, the CSC-associated marker leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) has been previously shown to promote breast cancer progression and CSC maintenance, in part through activation of Wnt/β-catenin
signaling [5]. However, little is known about the associations between LGR5 expression and breast cancer clinicopathological features.”

The original Ref#7 was removed and the order of reference was rearranged in the revised manuscript.

In the Background section (page 5, line 22)

“In this clinical study, we first examined LGR5 expression level by using ONCOMINE, a cancer microarray database and web-based data-mining platform aimed at facilitating research. Subsequently, we enrolled 126 specimens obtained from breast cancer patients in tissue microarray (TMA), and then used specific LGR5 and β-catenin antibodies to perform immunohistochemistry and score evaluation. We further investigated whether LGR5 and β-catenin could affect the clinical results upon 5-year follow-up in terms of RFS in breast cancer by using Kaplan-Meier analysis.” is changed to “In the present study, we first examined cellular LGR5 expression levels using datasets available through ONCOMINE, a web-based cancer microarray database. Subsequently, we constructed a tissue microarray (TMA) using specimens from 126 breast cancer patients, and performed semi-quantitative immunohistochemistry (IHC) for LGR5 and β-catenin. We further investigated the associations between LGR5 and β-catenin expression level and 5-year relapse-free survival (RFS) in breast cancer patients.”

In the Method section (page 6, line 7)

“Primary tumor tissues were obtained from 126 breast cancer patients undergoing surgical resection at Kaohsiung Medical University Hospital between 2004 and 2008. Patients’ initial characteristics and clinical outcomes were collected until death, censorship or loss to follow-up. For each patient, representative tissue cores of the breast tumors were carefully collected and subjected to tissue microarray. This study was approved by the ethics committee of the Institutional Review Board of Kaohsiung Medical University Hospital (KMUH-IRB-2013033). Informed consent was obtained from all sample donors in accordance with the Declaration of Helsinki at the time of donation. The clinical parameters and overall survival data were collected from a chart review. 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.” is changed to “This study was approved by the ethics committee of the Institutional Review Board of Kaohsiung Medical University Hospital (KMUH-IRB-2013033). Informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Primary tumor tissues were obtained from 126 breast cancer patients undergoing surgical resection at Kaohsiung Medical University Hospital between 2004 and 2008. Patient characteristics and clinical outcomes were collected until death, censorship, or loss to follow-up. Breast tumor tissue cores were collected from each patient, and used to construct a TMA. Clinical parameters and overall survival data were obtained from patients’ medical records. Patients who had not experienced disease recurrence or metastasis at the end of the study were censored at the date of the last follow up.”

In the Method section (page 6, line 19)
Immunohistochemistry (IHC) was used to detect LGR5 and β-catenin protein expression. The LGR5 antibody (orb137136) was purchased from Biorbyt (Cambridge, UK) and the β-catenin antibody (610154) was purchased from BD Transduction Laboratories™ (NJ, USA). Paraffin-embedded breast cancer tissue sections (4-μm) on poly-1-lysine-coated slides were deparaffinized and rinsed with 10mM Tris-HCl (pH 7.4) and 150mM sodium chloride. Peroxidase was quenched with methanol and 3% hydrogen peroxide. The slides were then placed in 10mM citrate buffer (pH 6.0) at 100 °C for 20 min in a pressurized heating chamber. After incubation with a 1:100 dilution of LGR5 antibody and a 1:300 dilution of β-catenin antibody for 1 h at room temperature, the slides were thoroughly washed three times with phosphate-buffered saline (PBS). Bound antibodies were detected using the EnVision Detection Systems Peroxidase/DAB, Rabbit/Mouse kit (Dako, Glostrup, Denmark). The slides were then counterstained with hematoxylin. Finally, the slides were photographed with a BX50 microscope (OLYMPUS, Japan). Colonic adenocarcinoma was used as positive control for LGR5 and β-catenin immunohistochemistry. Negative controls were obtained by performing all of the IHC steps but leaving out the primary antibody. The criteria to be evaluated were chosen according to a previous report (9), and the intensities of the signals were evaluated by two board-certified pathologists. 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.” is changed to “Immunohistochemistry was used to detect LGR5 and β-catenin expression. The anti-LGR5 antibody (orb137136) was purchased from Biorbyt (Cambridge, UK) and the anti-β-catenin antibody (610154) was purchased from BD Transduction Laboratories™ (Franklin Lakes, NJ, USA). Formalin-fixed paraffin-embedded breast cancer tissue sections (4-μm) on poly-1-lysine-coated slides were deparaffinized with xylenes and rinsed with 10 mM Tris-HCl (pH 7.4) and 150 mM sodium chloride. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol. The slides were then placed in 10 mM citrate buffer (pH 6.0) at 100°C for 20 min in a pressurized heating chamber. Slides were then incubated with LGR5 (1:100) and β-catenin (1:300) antibodies for 1 hour at room temperature, and washed three times with phosphate-buffered saline (PBS). Bound antibodies were detected using the EnVision Detection Systems Peroxidase/DAB, Rabbit/Mouse kit (Dako, Glostrup, Denmark) and counterstained with hematoxylin. Finally, the slides were photographed with a BX50 microscope (OLYMPUS, Japan). Colonic adenocarcinoma was used as positive control for LGR5 and β-catenin expression. Negative controls were obtained by performing all of the IHC steps, excluding addition of the primary antibody.

The signal intensities of the slides were evaluated by two board-certified pathologists. Immunostaining score (range: 0, 2-8) was defined as proportion score + intensity score in accordance with a previous report (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) [6]. The median IHC staining score (6) was used as the cut-off point for the dichotomization of both LGR5 and β-catenin; Scores greater or equal 6 (≥6) were defined as
indicating “high” immunostaining, while scores of less than 6 were considered to indicate “low” immunostaining.”

In the Method section (page 7, line 24)

“A P-value of less than 0.05 was considered to indicate statistical significance.” is changed to “A p-value of less than 0.05 was considered to indicate statistical significance.”

In the Results section (page 8, line 2)

“Tumor size and lymph node metastasis are correlated with high LGR5 expression

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. Next, specimens obtained from 126 breast cancer patients were enrolled in the study to investigate whether LGR5 expression had clinical importance. LGR5 expression in tumors was detected via immunohistochemistry, and representative results are shown in Figure 2.

To determine whether LGR5 expression was linked with clinico-pathological parameters, further statistical analysis was performed using 126 breast tumors. The original histopathological classification of these 126 breast tumors was invasive ductal carcinoma (IDC) in 121 patients (96.0%) and other special types in five patients (4%) (Table 1).

The clinico-pathological parameters measured included tumor grade, age, tumor size, lymph node status, ER status, PR status and HER2 status. 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 results indicated that high levels of LGR5 expression were positively correlated with tumor malignancy level.” is changed to “Tumor size and lymph node metastasis are associated with high LGR5 expression

We analyzed LGR5 expression in cellular datasets available through ONCOMINE (http://www.oncomine.org), an online collection of microarrays. Using the Zhao breast microarray dataset [7], we observed that LGR5 expression was lower in normal breast compared to breast cancer tissue (17 invasive ductal carcinomas and 20 lobular carcinomas) (Figure 1A). Additionally, mining of the Zhao and Tabchy breast microarray datasets [7, 8] revealed that TNBCs exhibited higher levels of LGR5 expression than non-TNBCs (Figure 1B and C). These results imply that LGR5 expression is associated with cancer progression. We next used specimens obtained from 126 breast cancer patients to investigate the clinical importance of LGR5 expression. Tumor LGR5 expression was detected via IHC, and representative results are
shown in Figure 2. Of the 126 tumors included in this study, 121 (96.0%) were invasive ductal carcinomas (Table 1). We then determined whether LGR5 expression was associated with clinicopathological tumor features, including age, tumor grade, tumor size, lymph node (LN) status, ER status, PR status, and HER2 status. We found that tumor size $\geq 2$ cm, LN metastasis, and TNBC were associated with high levels of LGR5 expression ($p = 0.002$, $p = 0.044$ and $p = 0.029$, respectively; Table 1). These results indicate that LGR5 expression levels were correlated with a degree of tumor malignancy.

In the Results section (page 8, line 21)

“Association between LGR5 and $\beta$-catenin in triple-negative breast cancer

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/$\beta$-catenin signaling in CSCs (8, 12). A recent study showed that the blockade of the Wnt/$\beta$-catenin signaling pathway preferentially reduced metastatic potential by altering CSC activity in a mouse model of breast cancer (13). We have further investigated $\beta$-catenin staining in the serial sections. The results revealed that $\beta$-catenin was not correlated with tumor 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 $\beta$-catenin was associated with LGR5 in breast cancer by stratifying cancers according to molecular subtype. As shown in Table 2, $\beta$-catenin was positively correlated with LGR5 in the TNBC group ($p=0.013$, Table 2).” is changed to “Association between LGR5 and $\beta$-catenin in TNBC

LGR5 in adult breast CSCs may maintain stemness by activating Wnt/$\beta$-catenin signaling [5, 9]. Accordingly, blockade of the Wnt/$\beta$-catenin signaling pathway reduced metastatic potential by altering CSC activity in a mouse model of breast cancer [10]. Therefore, we used our TMAs to investigate $\beta$-catenin staining levels in human breast tumors. Our results indicated that $\beta$-catenin expression was positively correlated with HER2 status ($p = 0.037$), but was not associated with age, tumor differentiation status, tumor size, LN metastasis, histopathological features, ER status, and PR status (Table 1). We also examined whether $\beta$-catenin was differentially associated with LGR5 as a function of breast cancer molecular subtype. As shown in Table 2, $\beta$-catenin and LGR5 expression were positively correlated among TNBCs ($p = 0.013$, Table 2).”

In the Results section (page 9, line 8)

“LGR5 expression is associated with relapse-free survival (RFS) in breast carcinoma patients

There is a positive association between LGR5 and tumor size $\geq 2$ cm, the presence of lymph node metastasis, and TNBC in breast cancer patients. Herein, we further investigated whether LGR5 and $\beta$-catenin could affect breast cancer patients’ recurrence and/or death rates. Based on the results shown in Table 3, 13 of 126 (9%) breast cancer patients suffered recurrence and/or death. A Kaplan-Meier analysis showed that patients with high levels of LGR5 expression had statistically shorter RFS periods as compared to patients with low levels of LGR5 expression in the non-TNBC group ($p=0.033$, Figure 3) and that eight of 46 high-LGR5 non-TNBC patients
suffered recurrence and/or death (13.0 %, Table 3).” is changed to “LGR5 expression is associated with shorter RFS in non-TNBC breast carcinoma patients

We next investigated associations between LGR5 expression, breast cancer recurrence, and patient mortality. As shown in Table 3, 13 patients (9%) experienced recurrence and/or death. A Kaplan-Meier analysis showed that, in the non-TNBC group, patients with high levels of tumor LGR5 expression exhibited significantly shorter RFS periods compared to patients with low levels of LGR5 expression (p=0.033, Figure 3). Additionally, 8 of 46 non-TNBC patients (13%) with high LGR5 expression experienced recurrence and/or death (Table 3).

In the Results section (page 9, line 17)

“LGR5-β-catenin signaling axis expression confers poor relapse-free survival (RFS) in breast carcinoma patients

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 high β-catenin expression levels in their tumors had the worst prognoses due to their high rates of recurrence and/or death (p=0.027, Figure 4).” is changed to “High simultaneous expression of both LGR5 and β-catenin is associated with poor RFS in breast carcinoma patients

Patients whose tumors exhibited high β-catenin levels, but not high LGR5 levels, did not display significantly shorter RFS periods compared to patients with low levels of tumor β-catenin expression (Figure 3D-F). However, patients with high tumor β-catenin expression were more likely to experience recurrence and/or death (Table 3). Moreover, Kaplan-Meier analysis of LGR5 together with β-catenin expression revealed that breast cancer patients with high simultaneous expression of tumor LGR5 and β-catenin expression had the worst prognosis (p = 0.027, Figure 4).”

In the Discussion section (page 10, line 2)

“This study has shown that high levels of LGR5 expression were significantly associated with tumor size ≥ 2 cm and lymph node metastasis in breast cancer patients (Table 1) and that patients with high levels of LGR5 expression tended toward poor differentiation (p=0.072, Table 1). Therefore, the results revealed that LGR5 could be a promising marker of poor prognosis. Based on the Kaplan-Meier analysis, we found that high levels of LGR5 expression were statistically significantly associated with shorter RFS in the non-TNBC group but not in the TNBC group, though this may be due to the limited sample size (Figure 3).” is changed to “This study showed that high levels of LGR5 expression were significantly associated with tumor size ≥ 2 cm and LN metastasis in breast cancer patients (Table 1), and that poorly differentiated tumors exhibited a trend toward higher LGR5 expression (p = 0.072, Table 1). Using a Kaplan-Meier analysis, we also found that high levels of LGR5 expression were significantly associated with shorter RFS in non-TNBC patients. This result was not observed in the TNBC group,
potentially due to the limited sample size (Figure 3). Therefore, these findings indicate that LGR5 is a promising marker of poor prognosis, particularly in non-TNBC patients.”

In the Discussion section (page 10, line 10)

The sentences “A recent study showed that distant metastasis tumors underwent the methylation of the lgr5 promoter and experienced LGR5 expression. In contrast, no normal colon tissues were positive for lgr5 methylation but all did exhibit LGR5 expression (14). The interconvertibility of LGR5-negative expression cell and LGR5-positive colon cancer cells have been seen in the tumor reconstitution for adaptation of the tumor microenvironment (15), but the underlying mechanism via which LGR5 expression acts in breast cancer is not fully understood.” were deleted in the revised manuscript.

The original Ref#14 was removed and the order of reference was rearranged in the revised manuscript.

In the Discussion section (page 10, line 10)

“On the other hand, LGR5 has gradually come to be accepted as the most reliable colorectal, breast, pancreatic and gastric cancer stem-like cell marker (8, 15-19). In fact, studies of the eyes, brain, hair follicles, mammary glands, stomach and reproductive organs have discovered that LGR5 expression is increased in rare stem cells and that these cells may become cancer stem cells in tumors (5). 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.” is changed to “LGR5 has been gradually accepted as the most reliable marker for colorectal, breast, pancreatic, and gastric CSCs [5, 11-15]. In fact, studies of the eye, brain, hair follicle, mammary gland, stomach, and reproductive organs have demonstrated that LGR5 expression is increased in rare stem cells, and that these cells may ultimately become CSCs [5]. Recent studies have explored the function of LGR5 in various cancer types. For example, in skin squamous cell carcinoma, LGR5 modulates Wnt/β-catenin signaling by interacting with and cointernalizing Wnt receptors and delaying endosome degradation [15]. In addition, interconversion of LGR5-positive CSCs to LGR5-negative cells has been shown to facilitate drug resistance in colon cancer [15]. However, the mechanism by which LGR5 may promote breast cancer is not fully understood. Therefore, given that β-catenin was positively correlated with HER2 status in the present study (Table 1), we suggest that the LGR5-β-catenin axis is responsible for breast cancer progression.”

The original Ref#20 is removed in the revised manuscript.

In the Discussion section (page 10, line 23)

“Overall, our clinical evidence showed that breast cancer patients with high levels of LGR5 expression have shorter RFS as compared to those with low levels of LGR5 expression. The
development of drugs that inhibit LGR5 expression will be an important strategy in treating breast cancer patients. On the other hand, although we used an online dataset to analyze LGR5 expression in various cell lines, the augmentation of this dataset with pharmaceutical and genomic studies is crucial.” is changed to “Overall, our clinical data showed that breast cancer patients with high levels of tumor LGR5 expression have shorter RFS compared to those with low levels of tumor LGR5 expression. However, given that we used an online dataset to analyze LGR5 expression in various cell lines, further pharmaceutical and genomic studies are crucial. Nevertheless, taken together, our study indicates that the development of drugs that inhibit LGR5 expression will be an important strategy for breast cancer treatment.”

In the Conclusion section (page 11, line 5)

“Our study revealed that breast cancer patients with high LGR5-β-catenin axis expression have poorer clinical outcomes as compared to those with less Wnt/β-catenin axis expression. Additionally, ONCOMINE, a cancer microarray database and integrated data-mining platform, was used to show that LGR5 expression was higher expressed in TNBC cases than in non-TNBC cases. Therefore, LGR5 is a likely therapeutic target in breast cancer patients.” is changed to “Our study revealed that breast cancer patients with high tumor LGR5-β-catenin axis expression have poorer clinical outcomes than those with low tumor LGR5-β-catenin axis expression. Additionally, analysis of ONCOMINE data revealed that LGR5 expression was more highly expressed in TNBC compared to non-TNBC cases. Therefore, LGR5 is a likely therapeutic target in breast cancer patients.”

In the Abbreviation section (page 12, line 4)

“…HER2: human epidermal growth factor receptor 2; CSC: cancer stem cell.” is changed to “…HER2: human epidermal growth factor receptor 2; CSC: cancer stem cell; LN: lymph node.”

In the Declarations section (page 13, line 5)

“Funding

This study was funded by grants MOST 103-2314-B-442-002-MY3 and MOST 106-2314-B-442-001-MY3 from Ministry of Science and Technology, Taiwan; MOHW106-TDU-B-212-144007 from the Health and Welfare Surcharge of Tobacco Products from Ministry of Health and Welfare, Taiwan; RB17004 from Show Chwan Memorial Hospital, Taiwan; and KMUH105-5R27 from Kaohsiung Medical University Hospital. The funding body had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.” is changed to “Funding

This study was funded by the following grants: MOST 103-2314-B-442-002-MY3 and MOST 106-2314-B-442-001-MY3 from the Ministry of Science and Technology, Taiwan; MOHW106-TDU-B-212-144007 from the Health and Welfare Surcharge of Tobacco Products from Ministry of Health and Welfare, Taiwan; RB17004 from Show Chwan Memorial Hospital, Taiwan; and KMUH105-5R27 from Kaohsiung Medical University Hospital. The funding bodies had no role
in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.”

In the page 13, line 14

“The datasets used and analysed during the current study are available from the corresponding author on reasonable request.” is changed to “The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.”

In the page 13, line 25

“The authors declare that they have no competing interests.” is changed to “The authors declare that no competing interests exist.”

In the page 14, line 6

“Written consent was provided by participants to be included in the study.” is changed to “Written informed consent was provided by study participants.”

In the reference section, the original reference, #4, 5, 7, 14 and 20 were removed and the order of reference was rearranged in the revised manuscript. In additional, author corrected the wrong format of cited reference and listed as below:


“#9. Rangel MC, Bertolette D, Castro NP, Klauzinska M, Cuttitta F, Salomon DS. Developmental signaling pathways regulating mammary stem cells and contributing to the


In the Figure legends section (page 18, line 2)

“Figure 1. LGR5 expression of breast tissue in the datasets. (A) LGR5 expression grouped by cancer and normal type in Zhao breast dataset. (B) and (C) LGR5 expression grouped by triple negative status in Zhao breast dataset and Tabchy breast dataset, respectively. TNBC: negative: triple negative breast cancer. N/A: not available.” is changed to “Figure 1. LGR5 expression in ONCOMINE breast tissue datasets. (A) LGR5 expression stratified by tissue type (cancer vs. normal) in the Zhao breast dataset. (B-C) LGR5 expression stratified by triple negative status in the Zhao (B) and Tabchy (C) breast datasets. TNBC: triple-negative breast cancer; N/A: not available.”

In the Figure legends section (page 18, line 7)

“Figure 2. Representative immunohistochemical stain results for LGR5 and β-catenin expression.” is changed to “Representative immunohistochemical staining of LGR5 and β-catenin expression.”

In the Figure legends section (page 18, line 12)

“Figure 3. Kaplan-Meier analysis of the influence of LGR5 and β-catenin expression on RFS in breast cancer patients. (A) LRG5 expression status in all patients. (B) LGR5 expression status in non-TNBC patients. (C) LGR5 expression status in TNBC patients. (D) β-catenin expression status in all patients. (E) β-catenin expression status in non-TNBC patients. (F) β-catenin expression status in TNBC patients.” is changed to “Figure 3. Kaplan-Meier analysis of the association of LGR5 and β-catenin expression with RFS in breast cancer patients. (A) LGR5 expression level in all patients. (B) LGR5 expression level in non-TNBC patients. (C) LGR5 expression level in TNBC patients. (D) β-catenin expression level in all patients. (E) β-catenin expression level in non-TNBC patients. (F) β-catenin expression level in TNBC patients. TNBC: triple-negative breast cancer.”

In the Figure legends section (page 18, line 19)

“Figure 4. Kaplan-Meier analysis of the influence of the combination of LGR5 and β-catenin expression on RFS in breast cancer patients. (A) All patients. (B) non-TNBC patients. (C) TNBC patients.” is changed to “Figure 4. Kaplan-Meier analysis of the association of combined LGR5
and β-catenin expression with RFS in breast cancer patients. (A) All patients. (B) Non-TNBC patients. (C) TNBC patients. TNBC: triple-negative breast cancer.”

Reviewer comment-1:

Page 9 line 1 and 2: "scores over 6" and "scores less than 6": can you further clarify which category included scores of 6? I mean it would be helpful if you add "≤6" or "≥6" when appropriate.

Author reply:

Author thank the reviewer’s valuable comments. The sentence was added in the page 7, line 17. “Scores greater or equal 6 (≥6) were defined as indicating “high” immunostaining, while scores of less than 6 were considered to indicate “low” immunostaining”

Reviewer comment-2:

Page 9 line 8: please change "significant" into "significance".

Author reply:

In the Method section, page 7, line 24. “A P-value of less than 0.05 was considered to indicate statistical significant.” is change to “A p-value of less than 0.05 was considered to indicate statistical significance.”

Reviewer comment-3:

In the Page 14 line 6: please change "prognoses" into "prognosis".

Author reply:

We thank the reviewers for his/her comments. In the Discussion section, page 10, line 8. “Therefore, the results revealed that LGR5 could be a promising marker of poor prognoses.” is changed to “Therefore, these findings indicate that LGR5 is a promising marker of poor prognosis…”

Reviewer comment-4:

Author reply:

Page 16 line 6: please change "expression" into "expressed".

We thank the reviewers for his/her comments. In the Conclusion section, page 11, line 8. “Additionally, ONCOMINE, a cancer microarray database and integrated data-mining platform,
was used to show that LGR5 expression was higher expression in TNBC cases than in non-TNBC cases.” is changed to “Additionally, analysis of ONCOMINE data revealed that LGR5 expression was more highly expressed in TNBC compared to non-TNBC cases.”

Reviewer comment-5:

Results section page 10 line 13-14: "These results imply that LGR5 expression is associated with cancer progression”. I do not understand this statement, since the authors state that LGR5 expression was higher in normal breast tissue than in human breast cancer tissue (line 7-8 on page 10). Can the authors clarify why they state that LGR5 expression is associated with cancer progression? Is this statement based on higher LGR5 expression levels in TNBC than non-TNBC? I known TNBC have a poorer prognosis than non-TNBC, but the higher expression of LGR5 in normal breast tissue than in breast cancer does not corroborate your statement.

Author reply:

We thank the reviewers’ valuable comments and apologize for the serious typing error. In the Results section, page 8, line 5. The original statement: “…LGR5 expression was higher in normal breast tissue than in human breast cancer tissue….” is changed to “…LGR5 expression was lower in normal breast tissue than in human breast cancer tissue ….” Based on the above correction, the higher LGR5 expression is associated with breast tumor progression and implied that LGR5 is a promising marker of poor prognosis.

Reviewer comment-6:

In addition: the quality of figure 1 is too poor to be able to read. Quality of all other figures is excellent. Could you please supply a figure 1 of better quality?

Author reply:

We thank the reviewers’ valuable comments and apologize for the poor quality of supplied figure 1. A re-paint Figure 1 has been reproduced and shown in the revised manuscript.