Peer Review File

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Reviewer Comments

The authors present a well-structured work, with appropriate methodologies and expected conclusion. However, I suggest major changes for consideration.

Introduction

Comment 1: *Page 3, Lines 51 - 53. "Although the recent…" This phrase could be better worded. The second sentence in relation to breast cancer cases in China is lost if the country's incidence number is not provided.*

Reply 1: We have added the incidence rate of breast cancer in China in the text, and modified "Although" into "However". And we have modified our text as advised. (see Page 5, line 57-61)

Changes in the text: Breast cancer (BC) is one of the most common types of female cancer worldwide, threatening women's health and lives. There were 248,620 new BC cases in females, corresponding to an age-standardized incidence rate of 29 per 100,000 Chinese women, which compares with approximately 120 per 100,000 in westernized population(1).

Methods and Results

Mutations and expression of the GRP94 gene has been studied in 23 cancers, including

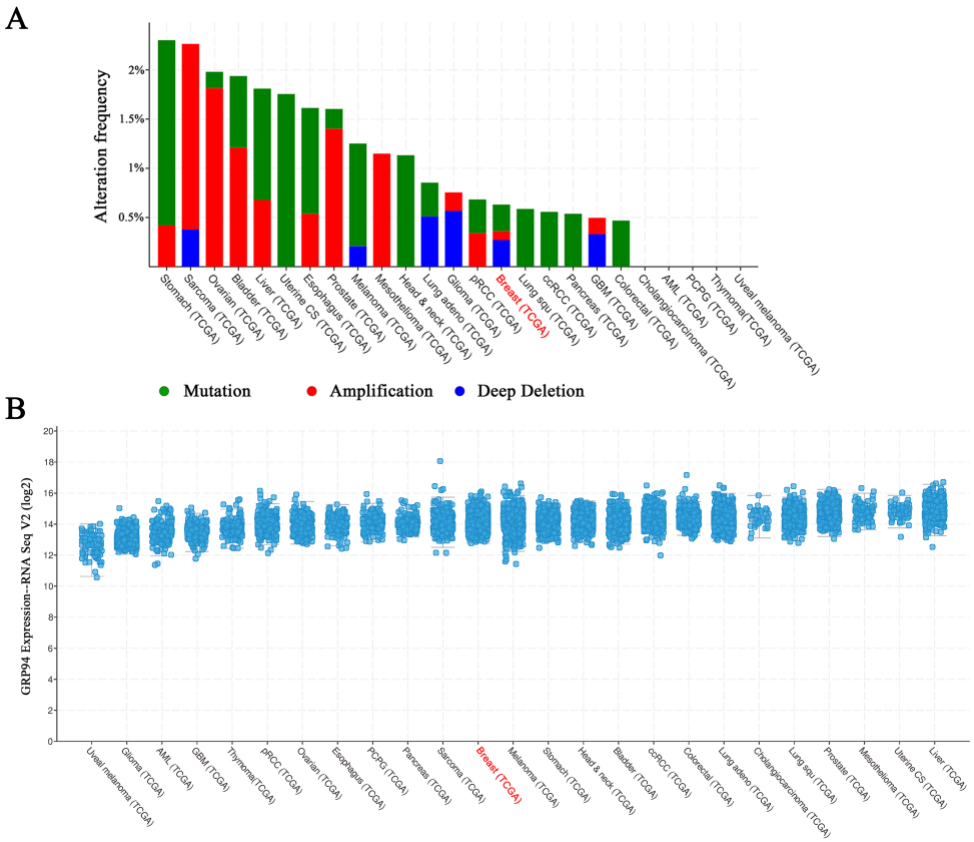
10,385 patients. GRP94 was mutated in most cancers, with an average frequency of 1.1%.

Comment 2: *Fig. 1a. The figure is confused for interpretation. Do the bars from the second line correspond to the 10,385 cases analyzed? Or are the numbers of changes found per tumor? I suggest that there is a fusion between the information in Fig. 1a and 1b. I suggest using the layout in Fig. 1b to exemplify the data. It would also be interesting to include in the legend or methodology the quantity of each tumor type of the 10,385 evaluated tumors.*

Reply 2: Yes, the bars from the second line correspond to the 10,136 cases analyzed (because of the update of cBioPortal database, the number of cases has changed), but we didn't display the whole picture of the patients, because there are too many patients to show. And the information in Fig. 1a and 1b. is similar so we decided to keep the Fig.1b and deleted the Fig.1a. (see Page 28, line 419-420). And we have listed the quantity of each tumor type of the 10,136 evaluated cases in the methodology as advised. (see Page 8, line 115-126)

Changes in the text:

(1) Figure 1:



(2) **Page 8, line 115-126:** Page 7, line 100-109: The mutation and expression patterns of GRP94 from 26 cancer types were obtained from the cBioPortal analysis platform (http://www.cbioportal.org/), which include Stomach Adenocarcinoma(440), Sarcoma(255), Ovarian Cancer(585), Bladder Urothelial Carcinoma(411), Liver Hepatocellular Carcinoma(372), Uterine Corpus Endometrial Carcinoma(Uterine CS, 529), Esophageal Carcinoma(559), Prostate Adenocarcinoma(494), Skin Cutaneous Melanoma(448), Mesothelioma(87), Head and Neck Squamous Cell Carcinoma(523), Lung Adenocarcinoma(566), Brain Lower Grade Glioma(514), Kidney Renal Papillary Cell Carcinoma(pRCC, 283), Breast Invasive Carcinoma(1084), Lung Squamous Cell Carcinoma(487), Kidney Renal Clear Cell Carcinoma(ccRCC, 512), Pancreatic Adenocarcinoma(184), Glioblastoma Multiforme(GBM, 592), Colorectal Adenocarcinoma(594), Cholangiocarcinoma(36), Acute Myeloid Leukemia(AML,

200), Pheochromocytoma and Paraganglioma(PCPG, 178), Thymoma(123), Uveal Melanoma(80).

Expression of GRP94 in breast cancer and Relationship between GRP94 and clinical pathological features

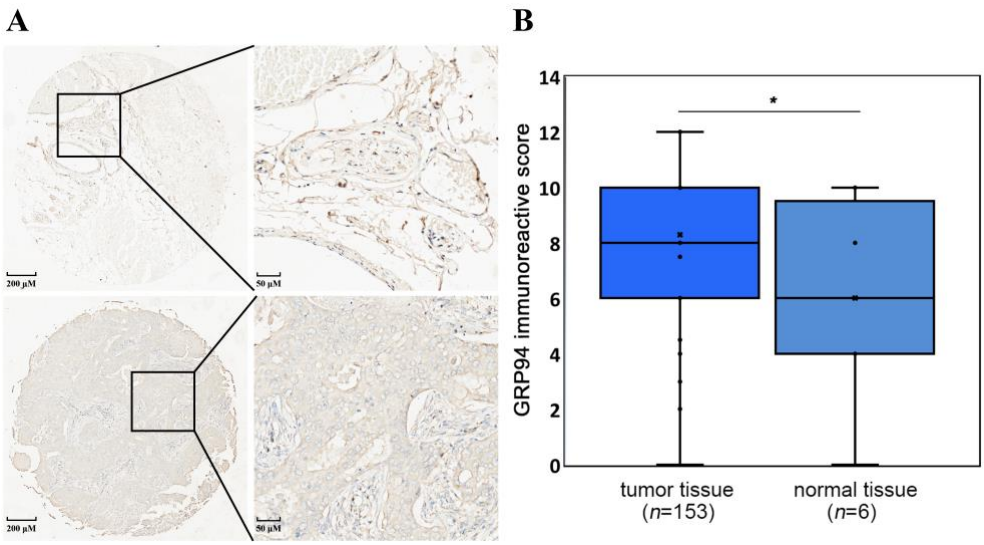
To confirm these results, GRP94 was evaluated by immunohistochemistry. GRP64 was mainly expressed in the cytoplasm and nucleus. According to immunohistochemistry scores, GRP94 expression was significantly higher in breast cancer than normal breast tissue (P <0.05).

***Comment 3**: Figure 3 - The background is saturating the visibility of the immunohistochemistry staining. I suggest replacing or improving the quality of the figure. The caption must contain the value of cytoplasmic staining intensity and the cell positivity defined by the authors as described in the methodology for the figure.*

Reply 3: We have replaced with another normal tissue cases and improved the quality of the figure, and added the IS (Immune score, which is the product of the cytoplasmic staining intensity and the percent of positive cells) as described in the methodology in the legend of the figure. (see Page 29, line 426-427 and Page 25, line 396-397)

Changes in the text:

(1) Figure 3:



(2) Page 25, line 396-397:

legend: Fig. 3 Expression of GRP94 in the TMA. (A) Top: normal breast tissue, IS=4 ; bottom: BC tissue, IS=12; (B) Box plot of the immunoreactive score of GRP94 in tumor tissue and normal tissue. *p<0.05

Comment 4: *Page 7, Lines 127-129. If 153 cases were evaluated, there was a loss of 7 cases from the total. The exclusion criterion, or methodological errors, must be presented and justified. Similarly, for the ten cases of normal breast tissue that were assessed, but only 6 were presented.*

Reply 4: We have explained in the methodology that there was a loss of 7 BC cases and 4 normal breast tissue cases because of immunohistochemistry detachment. (see Page 7, line 90-92)

Changes in the text: The TMAs were constructed with 1.5 mm cores from formalin-fixed paraffin-embedded tissue blocks, and 10 normal breast tissue cores were also included at the end of the TMAs, among which there was a loss of 7 BC cases and 4

normal breast tissues because of immunohistochemistry detachment.

Comment 5: *In the same way, the authors considered using a cutoff of 6 in the methodology, dividing the cases into two groups: high and low expression. The results divided according to the cutoff criterion of 6 must be presented, even if this information is present only in tables 1 and 2 (113 high and 40 low).*

Reply 5: We have modified our text as advised. (see Page 12, line 184-185)

Changes in the text: In the 160 cases contained within the TMA, the rate of positive GRP94 expression was 99.346% (152/153) in BC, and there were 113 cases with high expression of GRP94, 40 cases with low expression according to the cutoff IS criterion of 6.

Comment 6: *Another point, what was the statistical evaluation that the authors used to compare the expression of cases and controls? The statistical evaluation needs to be better clarified in the methods.*

Reply 6: We compare the expression of cases and controls by χ^2 test, and we have clarified the statistical evaluation in the methods. (see Page 9, line 144-149)

Changes in the text: The $\chi 2$ test was used to examine the expression difference between normal breast tissues and BC tissues. The spearman's rank correlation coefficients between expression of GRP94 and clinical pathological features were

analyzed and examined by IBM SPSS Statistics 22. The pictures were made using Adobe Photoshop CS6. The survival analysis was carried out using GraphPad Prism 7.

Relationship between GRP94 and clinical prognosis

Comment 7: *Page 7, Lines 145 - 146 - The evaluation of the 150 cases revealed that the high expression of GRP94 decreases the overall survival, although not statistically significant. Due to these results, the authors extended the analysis using data from an external dataset (KM-plotter). This analysis is not described in the methodology and needs to be better clarified, as well as the number of cases for each analysis. This information appears only in the legend of Fig. 4b and c with representative numbers of samples analyzed.*

Reply 7: We have described the survival analysis in the methodology as advised, as well as the number of cases for each analysis. (see Page 9, line 135-143)

Changes in the text: The Kaplan Meier plotter database (www.kmplot.com), was applied to evaluate the relationship between GRP94 and clinical prognosis(ref) in BC. And 1402 BC cases included in this database were used to analysis the OS of BC patients, and 3915 BC cases were used to analysis the relapse free survival (RFS). And we split the patients by using best performing expression value of GRP94 as cutoff into two groups (high vs. low). We evaluated the survival time of BC patients using a Kaplan-Meier survival plot. The hazard ratio (HR) with 95% confidence intervals and log rank P value were calculated and displayed on the plot.

Discussion

Comment 8: *The authors need to better elucidate the pathways involved with GRP94, in particular EGFR. Androgen receptor staining is common to triple negative breast tumors with high expression of EGFR, especially due to the increased risk of metastases in these subtypes. The authors do not discuss these findings.*

Reply 8: We have discussed more about the pathways involved with GRP94, and its relationship with EGFR and AR staining in the triple negative breast tumors in our text. (see Page 15, line 246-258)

Changes in the text: It is universally known that triple-negative breast cancer is aggressive high metastatic potential and have the worst prognosis and distant metastasis-free survival among all subtypes of BC(26). Few targeted therapies are available for the patients with triple negative breast cancer (TNBC)(27). And AR staining is common to TNBC with high expression of EGFR(28). According to our research, patients with high expression of GRP94 usually showed positive staining of AR. In the meanwhile, these patients have a higher positive rate of EGFR. Therefore, we inferred that GRP94 may play an important role in the development of TNBC patients with high expression of EGFR and AR staining. In human oropharyngeal carcinoma, EGFR support the radioresistance by activating endoplasmic reticulum stress signaling PERK- eIF2alpha-GRP94(29). Consequently, the high expression of GRP94 maybe associated with the metastasis and radioresistance of EGFR high expression TNBC patients. GRP94 is a potential prognosis predictor and therapeutic

target in EGFR positive metastatic TNBC.