Author's response to reviews

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A Her2-let-7-β2-AR circuit affects prognosis in patients with Her2-positive breast cancer

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Running title: β2-AR predicts prognosis in Her2-positive breast cancer

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Abstract

Background: Our previous studies show that β2-adrenergic receptor (β2-AR) is highly expressed in most Her2-overexpressing breast cancers. However, the mechanisms underlying upregulation of the β2-AR expression in Her2-overexpressing breast cancer cells are not fully understood. The clinical significance of the β2-AR overexpression in breast cancer is unclear.

Methods: MCF-7 and MCF-7/Her2 cells were transfected with the let-7 mimics or inhibitors. The expression of β2-AR was analyzed by Western blot. The β2-AR status in primary and metastatic sites of breast cancer was analyzed by immunohistochemistry. The correlation of lymph node metastasis with the β2-AR level and the clinical prognostic significance of the β2-AR overexpression were evaluated.

Results: Ectopic expression of Her2 in breast cancer cell line MCF-7 (MCF-7/Her2) represses the expression of microRNA let-7f, which is previously identified to regulate baseline β2-AR expression. The treatment with MEK1 inhibitor PD98059 effectively restored the let-7f level, suggesting that Her2-overexpression-mediated ERK constitutive activation inhibited let-7f, leading to the upregulation of the β2-AR expression. The transfection with the let-7f mimics markedly downregulated the β2-AR level, whereas the let-7 inhibitor significantly upregulated the β2-AR expression in both parental MCF-7 and MCF-7/Her2 cells. In addition, treatment of MCF-7/Her2 cells with isoproterenol resulted in a concentration-dependent reduction of the let-7f expression, demonstrating that the inhibitory effect of Her2 overexpression on let-7f can be reinforced by agonist-triggered β2-AR activation. We further demonstrate that high level of β2-AR associates with lymph node metastasis and poor outcome in the patients with Her2-positive breast cancer.
Conclusions: The mutual and reciprocal interaction between Her2, β2-AR, and let-7f may maintain a high level of β2-AR in breast cancer cells. Our data suggest that β2-AR may be a new useful biomarker for predicting prognosis in Her2-positive breast cancer and may also be a promising selective therapeutic target for the aggressive subtype of breast cancer.

Keywords: β2-AR; Her2; let-7f; breast cancer; prognosis
**Background**

Breast cancer is the most common malignancy and the second leading cause of cancer death in women. During recent decades, the incidence of breast cancer among women has been increasing throughout the world. In approximately 25% of breast cancers Her2 is overexpressed. Activation of Her2 through homodimerization or heterodimerization upon ligand binding triggers a cascade of its downstream events, eventually leading to activation of multiple signaling pathways including Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/Akt, which critically regulate proliferation and/or migration of tumor cells and confer resistance to the anticancer agents in breast cancer [1, 2].

Her family protein-mediated signaling can integrate heterologous signaling network. Our previous studies reveal that crosstalk of Her2 and β2-adrenergic receptor (β2-AR), an important member of seven transmembrane G protein-coupled receptors (GPCRs) [3, 4], triggers a stronger or more sustained biological effect in response to catecholamine stimulation. Activation of β2-AR by catecholamine promotes the expression of numerous pro-survival, invasion, angiogenesis, and metastasis genes, such as matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), hypoxia inducible factor-1α, MUC4, and CD44 through transactivating multiple signaling pathways [5-7].

Several studies including ours showed that β2-AR is overly expressed in a variety of tumor tissues, including ovarian, breast, prostate, and gastric cancers and catecholamines manipulate the biobehaviors of tumor cells mainly through activation of the β2-AR-mediated signaling pathways [3, 4, 8-11]. In our previous study, we demonstrated that chronic catecholamine stimulation induces the Her2 expression via activating STAT3 and promoting its binding to the Her2 promoter. We also showed
that excessive phosphorylation of ERK in Her2-overexpressing breast cancer cells upregulates the level of β2-AR. The interplay between β2-AR and Her2 may result in an enhanced mitogenic effect [3]. However, the molecular mechanisms underlying the β2-AR upregulation by Her2 are not fully understood.

In the present study, we demonstrated that constitutive activation of ERK downregulates the expression of let-7f in the Her2-overexpressing breast cancer cells, resulting in upregulation of the β2-AR level. Our data reveal a novel mechanism of the β2-AR overexpression in the Her2-positive breast cancer. We further evaluated clinical significance of the β2-AR expression in prediction of prognosis in the patients with Her2-overexpressing breast cancer and demonstrated that high level of β2-AR is associated with lymph node metastasis and poor prognosis in Her2-positive breast cancer patients.

Methods

Cell culture and treatment

Human breast cancer cell line MCF-7 is obtained from the American Type Culture Collection. The MCF-7/Her2 cells stably overexpressing Her2 were established in our laboratory as described previously [12]. For the treatment with the β2-AR agonist, the cells were incubated overnight in a serum-free medium and then treated with 2.5 µM isoproterenol (ISO) (Sigma) for the indicated time points.

Transient transfection

MCF-7 and MCF-7/Her2 cells were transfected with the let-7 mimics or inhibitors using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer’s instructions.
Western blot

The following antibodies were used for immunoblotting: the antibodies against Her2 (4290, Cell Signaling), p-ERK (4370, Cell Signaling), ERK (4695, Cell Signaling), β2-AR (sc-569, Santa Cruiz), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Sungene Biotech). All experiments were performed in duplicate.

Real-time RT-PCR

The expression of let-7f was detected by real-time RT-PCR following the manufacturer’s instruction (Genepharma). The experiments were performed three times independently.

Immunohistochemistry

Immunohistochemical staining was performed as previously described [3]. To analyze the β2-AR status in primary and metastatic sites of breast cancer, the human breast cancer tissue microarrays, containing 49 primary tumors and 50 metastatic lymph node tissues, were purchased from the US Biomax company. Each case on the tissue microarray comprises of 2 cores and the mean scores of 2 cores were taken.

To investigate the correlation between the level of β2-AR and prognosis of the patients with breast cancer, the primary invasive breast cancer tissues from 29 patients with Her2 overexpression and prognosis-related information were obtained from 307 Hospital of People's Liberation Army. The rates of disease-free survival (DFS) and overall survival (OS) were determined using the Kaplan-Meier analysis.

To determine the correlation of lymph node metastasis (LNM) with the β2-AR level, immunohistochemical staining for β2-AR was performed on 59 primary tumor
tissues from the patients with Her2-positive breast cancer.

The degree of staining was scored as follows: no staining (−), week to moderate staining (+ – ++), and strong staining (+++). The expression of β2-AR was analyzed by the rabbit polyclonal antibodies against β2-AR (Abcam). The staining was assessed microscopically by two independent pathologists. Images were taken on an Olympus BX51 microscope (Olympus) using the Spot insight image capture system CCD camera. Written informed consents were obtained from the patients for the use of the tumor tissue samples in this research. The study was approved by the ethics and scientific committee of the Affiliated Hospital of the Academy of Military Medical Sciences.

**Statistical analysis**

For *in vitro* assays, the data were analyzed by ANOVA test and Student’s unpaired t-test. The survival was estimated by the Kaplan-Meier method and survival characteristics were compared using log rank tests. DFS was determined as an interval between the first day of therapy and the date of the development of progressive diseases. OS was measured from the date of therapy to the date of death or last follow-up. *P*<0.05 was considered statistically significant. The distribution of LNM and no LNM cases in two groups was analyzed by Chi-square test.

**Results**

**β2-AR is highly expressed in Her2-positive breast cancer**

In our previous study, we demonstrate that enforced overexpression of Her2 in breast cancer cells upregulates the expression of β2-AR at both mRNA and protein levels [3], raised questions as to how the expression of β2-AR is modulated by Her2 in breast
cancer. We interrogated the relative mRNA expression of *ADRB2* (β2-AR) in human breast cancer tissue samples by searching a publicly available database Oncomine (www.oncomine.org). In the majority (52/53) of the breast cancer tissue samples collected, Her2 is overexpressed. Coincidently, the levels of β2-AR mRNA are also high in these tumor tissues (Fig. 1A and 1B). Coexpression of Her2 and β2-AR at protein levels was further confirmed by immunohistochemistry on a human breast cancer tissue microarray consisting of 49 tumor tissues from breast cancer patients. Among the Her2-overexpressing tumor tissues, ~96% (27/28) was β2-AR-positive (Fig. 1C). However, in Her2-negative tumors only ~29% (6/21) was β2-AR-positive. 15 tumor tissues were double negative. The results were consistent with the findings in our previous study [3].

**Let-7f regulates β2-AR expression in breast cancer cells**

A recent study indicated that microRNA (miRNA) let-7f regulates baseline β2-AR expression [13]. In human airway epithelial cells, let-7f inhibits the β2-AR expression through a direct interaction with the 3′ UTR of the gene encoding β2-AR (*ADRB2*) that harbors a conserved 8-nucleotide seed region of let-7 family [13]. To determine whether let-7f regulates the expression of β2-AR and how the expression of β2-AR is upregulated in Her2-overexpressing breast cancer cells, we first established MCF-7/Her2 cells [12], which stably overexpress Her2 (Supplementary Fig. S1). Then parental MCF-7 and MCF-7/Her2 cells were transfected with synthetic mimics or inhibitors of let-7f. Fig. 2A and 2B show that the treatment with the let-7 inhibitors caused a concentration-dependent increase of the β2-AR expression in both MCF-7 and MCF-7/Her2 cells (upper panel). In contrast, the transfection with the let-7f mimics exhibited a marked inhibitory effect on the β2-AR expression in a concentration-dependent manner (lower panel).
**Her2 overexpression inhibits let-7f via constitutive activation of ERK**

Several recent studies indicated that the expression of the let-7 family is significantly downregulated in human cancers, including breast cancer. It has been reported that the copy number of let-7 family genes is reduced in breast cancer [14, 15]. Comparison of miRNA expression profiles using clinical breast cancer biopsies revealed that the expression of let-7f is significantly lower in Her2-positive than Her2-negative breast cancer [16].

The expression of let-7 can be inhibited by mitogenic signaling-mediated ERK activation [17]. Our previous studies showed that Her2 transcription is upregulated by β2-AR-mediated Stat3 activation and that Her2 and its downstream signaling can be transactivated by β2-AR in response to catecholamine stimulation [3, 18], implicating that constitutive ERK activation in the Her2-overexpressing cells may upregulate the expression of β2-AR by repressing the let-7f level. As shown in Supplementary Fig. S1, the overexpression of Her2 was accompanied by constitutive activation of ERK in MCF-7/Her2 cells. We examined the let-7f expression in MCF-7/Her2 cells by real-time RT-PCR. Fig. 3A demonstrates that the let-7f level was remarkably reduced approximately 3 folds, compared with the parental cells. The treatment with MEK1 inhibitor PD98059 effectively restored the let-7f level (Fig. 3B), suggesting that Her2-mediated ERK activation inhibited the expression of let-7f. We next investigated whether the β2-AR signaling interferes with the expression of let-7f by treating MCF-7/Her2 cells with 2.5 μM ISO. The treatment resulted in a time-dependent reduction of the let-7f expression (Fig. 3C). The data indicate that Her2 overexpression-induced ERK activation enhances the β2-AR expression by downregulating the level of let-7f and that the inhibitory effect of Her2 can be
reinforced by agonist-triggered β2-AR activation. The mutual and reciprocal interaction between Her2, β2-AR, and let-7f may maintain a high level of β2-AR and a low level of let-7f in breast cancer cells.

β2-AR overexpression correlates with DFS in breast cancer patients

Although Her2 overexpression represents a highly aggressive phenotype of breast cancer, the prognosis of the patients with Her2-overexpressing breast cancers may vary somehow. The biomarkers that can predict clinical outcome of the patients with Her2-overexpressing breast cancer are currently unknown [19]. It has been demonstrated that crosstalk between GPCRs and epidermal growth factor receptor (EGFR) contribute to cancer malignant progression [20-22]. Therefore, we evaluated the clinical prognostic significance of the β2-AR overexpression in the patients with Her2-positive breast cancers by retrospectively investigating the relationship between the level of β2-AR and DFS or OS of the patients. The expression of β2-AR in the primary tumors from 29 Her2-positive breast cancer patients was assessed by immunohistochemistry. Immunostaining was scored as high (3+++ ) and low/moderate (0 – 2++) according to the rate of positive cells and staining intensity (Supplementary Fig. S2). The rates of DFS and OS were determined using the Kaplan-Meier analysis. The level of β2-AR was high in 17 tumors. The patients with β2-AR-overexpressing tumors had a significantly lower DFS rate (P = 0.003, log-rank test; Fig. 4A). The OS rates at 5 years were 58.2% for the low/moderate β2-AR group and 31.6% for the high β2-AR group, but the difference between two groups was not statistically significant (P = 0.151, long-rank test; Fig. 4B). This could be due to the relatively short follow-up time and small sample size in this study. Nevertheless, these data implicate that the β2-AR overexpression correlates with poor prognosis in
Her2-positive breast cancer.

**β2-AR overexpression correlates with LNM in breast cancer patients**

The previous studies indicate that aberrant activation of the β2-AR-mediated signaling pathways promotes the malignant progression of cancer. Compelling evidence demonstrates that migrative, invasive, and metastatic capacities of cancer cells are critically regulated by the β2-AR-mediated signaling [6]. Thus, we examined the expression of β2-AR and Her2 in metastatic lymph nodes using a tissue microarray containing 50 metastatic lymph nodes from breast cancer patients. In agreement with the findings that Her2 and β2-AR were coexpressed in primary breast cancer tissues, the expression of β2-AR was also detected in most Her2-overexpressing metastatic lymph nodes (23/25, 92%) as shown in Fig. 4C. We further evaluated the correlation of LNM with the expression of β2-AR in 59 Her2-overexpressing breast cancer patients. The incidence (28/33, 85%) of LNM was significantly higher in the patients with high expression of β2-AR than in those patients with low/moderate expression of β2-AR (15/26, 58%; P<0.05; Fig. 4D). The data demonstrate that the β2-AR level significantly correlates with lymph node metastasis in Her2-positive breast cancer patients.

**Discussion**

It is becoming increasingly clear that the β2-AR-mediated signaling plays a key role in the malignant progression of cancer [6, 11]. Catecholamines can stimulate the expression of multiple molecules involved in tumor cell proliferation, migration, invasion, adhesion, and metastasis, influencing biological behaviors of tumor cells [23]. It has been reported that the level of catecholamines is high in tumor
microenvironment. Both tumor and nontumor cells may contribute to the increase of
catecholamine level in tumor microenvironment [6, 24, 25]. In tumor cells, β2-AR,
which functions as an intermediary in transmembrane signaling pathways, mediates
the effects of catecholamines.

Increasing evidence indicates that crosstalk between GPCR and growth factor
receptors profoundly affect pathophysiological consequences of tumor progression.
The findings in this study show that the β2-AR protein is overly expressed in most
Her2-positive breast cancer tissues. The β2-AR mRNA level was also high in
Her2-positive breast cancer. Our previous study shows that catecholamines promote
β2-AR/Her2 complexation and induce β2-AR-mediated Her2 transactivation [18],
implicating that reciprocal influence between Her2 and β2-AR may occur at
transcriptional and posttranscriptional levels. Let-7f is a recently identified inhibitor
of β2-AR. Analysis of miRNA expression profiling reveals that let-7f is significantly
downregulated in Her2-positive breast cancer [16]. Our data demonstrate that
constitutive ERK activation in the Her2-overexpressing breast cancer cells repressed
the level of let-7f and that the inhibitory effect could be enhanced by the β2-AR
agonist, indicating a novel mechanism of the β2-AR expression upregulation in
Her2-overexpressing breast cancer. The interplay of the β2-AR- and Her2-mediated
pathways synergistically abrogates the regulatory functions of the oncogene
suppressor let-7 and maintains a high level of β2-AR in breast cancer.

Human breast cancer is a clinically heterogeneous disease, consisting of a variety of
distinct subgroups of tumors with varying levels of gene and protein expression,
which endow human breast cancer with different clinical characteristics, disease
courses, and responses to specific treatments [26]. Based on genomic profiling, breast
cancers are divided into several molecularly defined subtypes, including luminal A
(ER/PR+, Her2-), luminal B (ER/PR/Her2+), Her2 (mostly Her2 amplified and ER-),
normal-breast-like (the highest expression of the genes known to be expressed by
adipose tissue and other nonepithelial cell types), and basal-like types (mostly ER-).
These molecular subtypes allow for a more rational, patient-specific approach to
therapy and prediction of clinical courses. We observed that high level of β2-AR was
closely associated with LNM and poor DFS in Her2-positive breast cancer patients,
indicating that β2-AR is a potential prognostic biomarker for survival and tumor
recurrence in Her2-overexpressing breast cancers. A recent study showed that single
nucleotide polymorphisms of the β2-AR gene were associated with LNM, poor
prognosis, and high expression levels of β2-AR, EGFR, VEGF, and MMP-2 [27]. The
β2-AR expression was also associated with poor prognosis, tumor-node-metastasis
stage, and Edmondson stage in hepatocellular carcinoma patients [28]. However, there
is a contradictory report showing that strong β2-AR expression was an independent
favorable prognostic factor for oral squamous cell carcinoma patients [29]. Further
investigations are needed to determine whether β2-AR as a prognostic predictor is
dependent upon certain types of cancers.

Combinations of different markers allow for the identification of tumors susceptible
to targeted treatments. Generally, the subgroups with the Her2 expression have the
shortest relapse-free and overall survival. However, Her2-positive breast cancers
receive benefit from targeted therapies such as the monoclonal antibody trastuzumab,
which binds to Her2 [30, 31]. Our recent study demonstrated that
catecholamine-induced β2-AR activation mediates desensitization of gastric cancer
cells to trastuzumab [4]. Several retrospective studies reported that β-blocker use
reduced distant metastasis, tumor recurrence, and cancer specific mortality [32-35].
These data implicate that β2-AR may be used as a new therapeutic target to improve
existing targeted therapies.

Conclusions

β2-AR is predominantly expressed in most Her2-overexpressing breast cancers. Her2-mediated activation of ERK represses miRNA let-7f, leading to the upregulation of the β2-AR expression. High level of β2-AR associates with lymph node metastasis and poor outcome. β2-AR may be a new useful biomarker for predicting prognosis in Her2-positive breast cancer and may also be a promising selective therapeutic target for the aggressive subtype of breast cancer.

Authors’ contributions

DL and LS participated in all experiments, TW evaluated the clinical data, ZY and CH performed pathological examination, LG and QD performed immunohistochemical staining, YL performed real-time PCR, MS and NG provided grant supports, designed study, and wrote manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Fig. 1. β2-AR is highly expressed in Her2-positive breast cancer tissues. A and B, The relative mRNA expression of Her2 (A) and ADRB2 (B) in human breast cancer (n=53) and normal breast (n=6) tissue samples was analyzed by searching a publicly available database Oncomine (www.oncomine.org). C, The expression of Her2 and β2-AR was detected by immunohistochemistry on a human breast cancer tissue microarray consisting of 49 tumor tissues from breast cancer patients. Bar = 1000 µm (low-power field) or 100 µm (high-power field).

Fig. 2. Let-7f regulates β2-AR expression in breast cancer cells. A and B, MCF-7 (A) and MCF-7/Her2 cells (B) were transfected with synthetic inhibitors or mimics of let-7f and the expression of β2-AR was analyzed by Western blot. These experiments were repeated twice.

Fig. 3. Her2 overexpression inhibits let-7f via constitutive activation of ERK. A, The expression of let-7f in MCF-7 and MCF-7/Her2 cells was detected by real-time RT-PCR. B, The cells were pre-treated with 25 µM PD98059 for 24 h and the expression of let-7f was analyzed. C, MCF-7/Her2 cells were treated with 2.5 µM ISO and the expression of let-7f was analyzed by real-time RT-PCR. These experiments were repeated at least twice. *P<0.05; **P<0.01

Fig. 4. β2-AR overexpression correlates with DFS and LNM in breast cancer patients. A and B, The rates of DFS (A) and OS (B) in the patients with Her2-positive metastatic breast cancer according to the expression level of β2-AR were determined by the Kaplan-Meier analysis. C, The expression of Her2 and β2-AR
was analyzed using a tissue microarray containing 50 metastatic lymph nodes from breast cancer patients by immunohistochemical staining. The middle and right panels are the magnifications of the square regions in the left and middle panels, respectively. Bar = 1000 µm (low-power field), 200 µm or 100 µm (high-power field). D, The relationship between LNM and β2-AR expression was evaluated in Her2-overexpressing breast cancer.
Supplementary figure legends

Supplementary Fig. S1
The expression of Her2 and phosphorylation of ERK in parental MCF-7 and MCF-7/Her2 cells were analyzed by Western blot.

Supplementary Fig. S2
The expression of β2-AR in the primary tumors from Her2-positive breast cancer patients was assessed by immunohistochemistry with the antibody against β2-AR. H, high expression; M, moderate expression; L, low expression; Bar = 100 µm