Targeted RASSF1A expression inhibits proliferation of HER2-positive breast cancer cells *in vitro*

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Abstract. Human epidermal growth factor receptor 2-positive (HER2+) breast cancer, which accounts for 15-20% of all breast cancer, is associated with tumor recurrence and poor prognosis. RAS association domain family protein 1 subtype A (RASSF1A) is a tumor suppressor that is silenced in a variety of human cancers. The present study aimed to investigate the role of RASSF1A in HER2+ breast cancer and the therapeutic potential of RASSF1A-based targeted gene therapy for this malignancy. RASSF1A expression in human HER2+ breast cancer tissues and cell lines was evaluated by reverse transcription PCR and western blot analysis. The associations between tumorous RASSF1A level and tumor grade, TNM stage, tumor size, lymph node metastasis and five-year survival were examined. HER2+ and HER2-negative (HER2-) breast cancer cells were transfected with a lentiviral vector (LV-5HH-RASSF1A) that could express RASSF1A under the control of five copies of the hypoxia-responsive element (5HRE) and one copy of the HER2 promoter (HER2p). Cell proliferation was evaluated by the MTT and colony formation assays. It was found that tumorous RASSF1A level was negatively associated with tumor grade (P=0.014), TNM stage (P=0.0056), tumor size (P=0.014) and lymph node metastasis (P=0.029) and positively associated with five-year survival (P=0.038) in HER2+ breast cancer patients. Lentiviral transfection of HER2+ breast cancer cells resulted in increased RASSF1A expression and decreased cell proliferation, especially under hypoxic conditions. However, lentiviral transfection of HER2-breast cancer cells did not affect RASSF1A expression. In conclusion, these findings verified the clinical significance of RASSF1A as a tumor suppressor in HER2+ breast cancer and supported LV-5HH-RASSF1A as a potential targeted gene therapy for this malignancy.

Introduction

Breast cancer is a heterogeneous malignancy that is categorized into clinically relevant molecular subtypes based on the expression of molecular markers, such as the estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER-2, also called Neu) (1-4). HER2-positive (HER2+) cancers, which account for ~15-20% of all primary breast carcinomas, tend to grow more aggressively and have a worse prognosis (5,6). Despite the success of HER2-targeting therapies, HER2+ breast cancer remains a major challenge in clinical practice (7-9).

RAS association domain family protein 1 subtype A (RASSF1A), which belongs to the family of RAS effectors, is a tumor suppressor that is frequently silenced in a number of cancers through promoter hypermethylation (10). In fact, RASSF1A methylation might serve as a potential diagnostic biomarker for breast cancer (11). In addition, RASSF1A suppresses the growth of estrogen receptor α-positive (ERα+) breast cancer cells by keeping ERα expression and function under control via mechanisms involving the Hippo-Kinases LATS1 and 2 (12,13). However, the function of RASSF1A in HER2+ breast cancer remains to be elucidated.

Gene therapy is used to treat genetic and hereditary disorders by expressing a foreign gene in the host cells to produce desired therapeutic effects (14). The study of the molecular features of breast cancer has established gene therapy as a promising approach for this cancer (15). A key challenge of cancer gene therapy is the tumor-targeting efficiency, which means that the therapeutic gene should be specifically expressed in tumor cells, thereby reducing the damage to normal cells (16). The techniques to achieve tumor-specific gene expression include distinct delivery systems and expression driven by tumor-specific promoters (17). While tumor-targeting delivery systems have been extensively explored over the last two decades (18), the study of using a specific promoter to drive gene expression in tumor cells but not normal cells is relatively rare (19).

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HER2 is a cell-specific oncogene that has low levels of expression in normal cells, but is highly expressed in a number of cancers, including HER2+ breast cancer (20). Thus, the HER2 promoter (HER2p) is exploited for targeted gene expression in HER2+ cancer cells (21).

Local hypoxia is a hallmark of a number of solid tumors and 25-40% of invasive breast cancers exhibit hypoxic regions (22). Hypoxia leads to increased activity of hypoxia-inducible factors, which bind hypoxia-response elements to promote the expression of genes involved in cell adaptations to hypoxia (23). The present study first investigated the clinical significance of RASSF1A in human HER2+ breast cancer. Next, an expression vector was constructed carrying RASSF1A under the control of HER2p and five copies of the HRE, aiming to selectively overexpress RASSF1A in HER2+ breast cancer cells, especially under hypoxic conditions. The effects of RASSF1A overexpression on the proliferation of HER2+ breast cancer cells was then assessed. The results of this study could help the future development of new targeted gene therapy strategies for HER2+ breast cancer.

Materials and methods

Patients and tissue samples. A total of 54 treatment-naïve patients with HER2+ breast cancer who underwent surgery at Shaanxi Provincial Cancer Hospital (Xi'an, Shaanxi, China) between January 2016 and December 2016 were included in this study. Patients who received any preoperatively adjuvant chemotherapy, radiotherapy or hormone therapy were excluded. The information on patient survival was obtained from a 5-year follow-up by telephone or outpatient examination. The overall survival rate was calculated as the percent of patients still alive at a specific time from the date of surgery during the 5-year follow-up period. The tumor stage, TNM stage, tumor status and nodal status were classified according to international standards for staging breast cancer (24) and the grouping in Table I was categorized according to clinical practice, which was in line with most papers (25,26). Tumor and adjacent non-tumor tissues were collected during surgery and stored at -80°C until analysis. HER2 overexpression was confirmed by pathohistological examination of tumor tissues. The median relative RASSF1A mRNA level was used as the cutoff value for the definition of high and low RASSF1A expression in tumor tissues. The present study was approved by the Ethics Committee of Shaanxi Provincial Cancer Hospital. All participants provided written informed consent.

Cell lines and culture. The AU565 (HER2+), SKBR-3 (HER2+), MCF-7 (HER2-) and BT474 (HER2+) human breast cancer cell lines and the MCF-10A human normal breast cell line were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO_2. To study the effects of hypoxia, cobalt (II) chloride ($CoCl_2$), a well-known hypoxia mimic agent, was added to the medium to create hypoxia-like state *in vitro*.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from $1x10^4$ cells using TRIzol reagent (Thermo Fisher Scientific, Inc.) as previously described (27) and RNA was reverse-transcribed into the complementary DNA (cDNA) using a reverse transcription PCR (RT-PCR) kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. PCR amplification with the specific primers (Table II) was performed with the miScript SYBR Green PCR Kit (Qiagen GmbH) in triplicates at 98°C for 2 min; 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 1 min and a final 10 min extension at 72°C. The mRNA levels were calculated using the $2^{-\Delta\Delta Cq}$ method (28). Data were analyzed with the RealPlex analysis system (Eppendorf).

Western blot analysis. Western blot analysis was performed as described in our previous report (24). In brief, cells were lysed in RIPA lysis buffer (MilliporeSigma) in the presence of protease inhibitors and quantified using a BCA Protein Assay kit. Proteins (30 μg in each lane) were resolved with 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Following blocking with 5% fat-free dry milk at room temperature for 1 h, the membranes were incubated with anti-RASSF1A (1:1,000; cat. no. ab126764; Abcam), anti-β-actin (1:1,000; cat. no. ab8226; Abcam), or anti-HER2 (1:1,000; cat. no. ab134182; Abcam) antibody at 4°C overnight, followed by goat anti-rabbit IgG (1:3,000, cat. no. ab150077; Abcam) at room temperature for 1 h. Protein bands were detected using a chemiluminescence detection system (Beyotime Institute of Biotechnology) and densitometry analysis was performed using QuantityOne version 4.5.0 (Bio-Rad Laboratories, Inc.). Data were normalized to β-actin.

Lentivirus production. The full-length human RASSF1A cDNA (NM_007182) and HER2p were PCR-amplified from pcDNA-RASSF1A (Shanghai GeneChem Co., Ltd.) and genomic DNA from HER2+ breast cancer tissues, respectively, using specific primers shown in Table II. The HER2p product was digested with *Hin*dIII/*Pst*I and subcloned into pLEGFP-N1-5HRE-CEAp (29) at the *Hin*dIII and *Pst*I sites to replace CEAp, generating pLEGFP-N1-5HRE-HER2p. The 5HRE-HER2p fragment was subsequently subcloned into the pLVX-EGFP-3FLAG lentiviral vector to replace the CMV promoter, generating pLVX-5HRE-HER2p-EGFP-3FLAG. Next, the RASSF1A product was subcloned into pLVX-5HRE-HER2p-EGFP-3FLAG to replace EGFR, generating pLVX-5HRE-HER2p-RASSF1A-3FLAG (LV-5HH-RASSF1A). All primers were synthesized at Sangon Biotech Co., Ltd. The pLVX-5HRE-HER2p-3FLAG vector (LV-5HH) served as negative control. The recombinant lentiviral vectors were verified by restriction endonuclease digestion and DNA sequence analysis at Sangon Biotech Co., Ltd.

Lentiviral infection of breast cancer cells. Lentiviruses carrying LV-5HH-RASSF1A or LV-5HH ($1x10^8$ pfu) were custom-prepared by GeneChem. For lentiviral infection, breast cancer cells were seeded in 24-well plates ($5x10^4$ cells/well), cultured overnight and then infected for 72 h at 37°C with corresponding lentiviruses at a MOI of 10 in the presence of

Table I. Relationships between tumorous RASSF1A mRNA level and clinicopathologic characteristics of HER2+ breast cancer patients.

Characteristics	Cases (n=54)	Number of patients		χ^2 (P-value[b])
		High (n=27)	Low (n=27)	
Age (years)				1.1868 (0.2760)
≤50	26	15	11	
>50	28	12	16	
Stage[a]				6.0331 (0.0140)
I-II	25	17	8	
III	29	10	19	
TNM stage[a]				7.6704 (0.0056)
I+II	22	16	6	
III+IV	32	11	21	
Tumor status[a]				6.0000 (0.0143)
T1	27	18	9	
T2-T4	27	9	18	
Lymph nodal status[a]				4.7472 (0.0294)
N0	28	18	10	
N1-N3	26	9	17	

[a]Tumor stage, tumor status and nodal status were classified according to the international standards for staging breast cancer. [b]P-values were determined by the Chi-square test or Fisher's exact test. RASSF1A, RAS association domain family protein 1 subtype A; HER2, human epidermal growth factor receptor 2.

Table II. Prime sequences used in the present study.

Gene	Primer	Base sequence	PCR product (bp)
Her2p	Forward	5'-CAAGCTTGTCGCGAGCAGGCAACCCAGGCGTCCCG-3'	176
	Reverse	5'-GCTGCAGCCTCCCCTGGTTTCTCCGGTCCCAA-3'	
5HRE+Her2p	Forward	5'-CGACGCGTCGATT ATGCTAGTCCAC-3'	397
	Reverse	5'-CCCTCGAGGGGCCTCCCCTGGTTTCTCCGGTCCCAA-3'	
RASSF1A	Forward	5'-CGGAATTCCGATGTCGGGGGAGCCTGAGCT-3'	943
	Reverse	5'-CGGGATCCCGGTCCCAAGGGG GCAGGCGT-3'	
β-Actin	Forward	AATCTGGCACCACACCTTCTA	170
	Reverse	ATAGCACAGCCTGGATAGCA	

The underlining in the sequences indicates the endonuclease recognition sites. RASSF1A, RAS association domain family protein 1 subtype A; HER2, human epidermal growth factor receptor 2.

polybrene. Following infection, the cells were selected with 10 μg/ml puromycin for two weeks. The surviving cells were used in subsequent experiments.

MTT assay. Cell viability was determined with the MTT assay. In brief, cells were seeded in 24-well plates (5×10^4 cells/well) and cultured for 7 days at 37˚C. To test the effects of hypoxia, cells were cultured for 7 days in the presence of 50 μmol/l $CoCl_2$ (30). After repeated washing with serum-free DMEM to remove cell debris, the cells were incubated with 20 μl of 5 g/l 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; MilliporeSigma) for 4 h. The resulting formazan crystals were dissolved in 200 μl DMSO and the absorbance at 490 nm was recorded on a Victor 3 microplate reader (PerkinElmer, Inc.).

Colony formation assay. Cells were seeded in 60-mm cell culture dishes (200 cells/well) and incubated for 7 days. To test the effects of hypoxia, cells were incubated for 7 days at 37˚C in the presence of 50 μmol/l $CoCl_2$. Then, the cells were fixed with methanol for 15 min at room temperature and stained with Giemsa for 30 min at room temperature. Colonies containing >50 cells were counted with an inverted microscope (Olympus Corporation; magnification, x10).
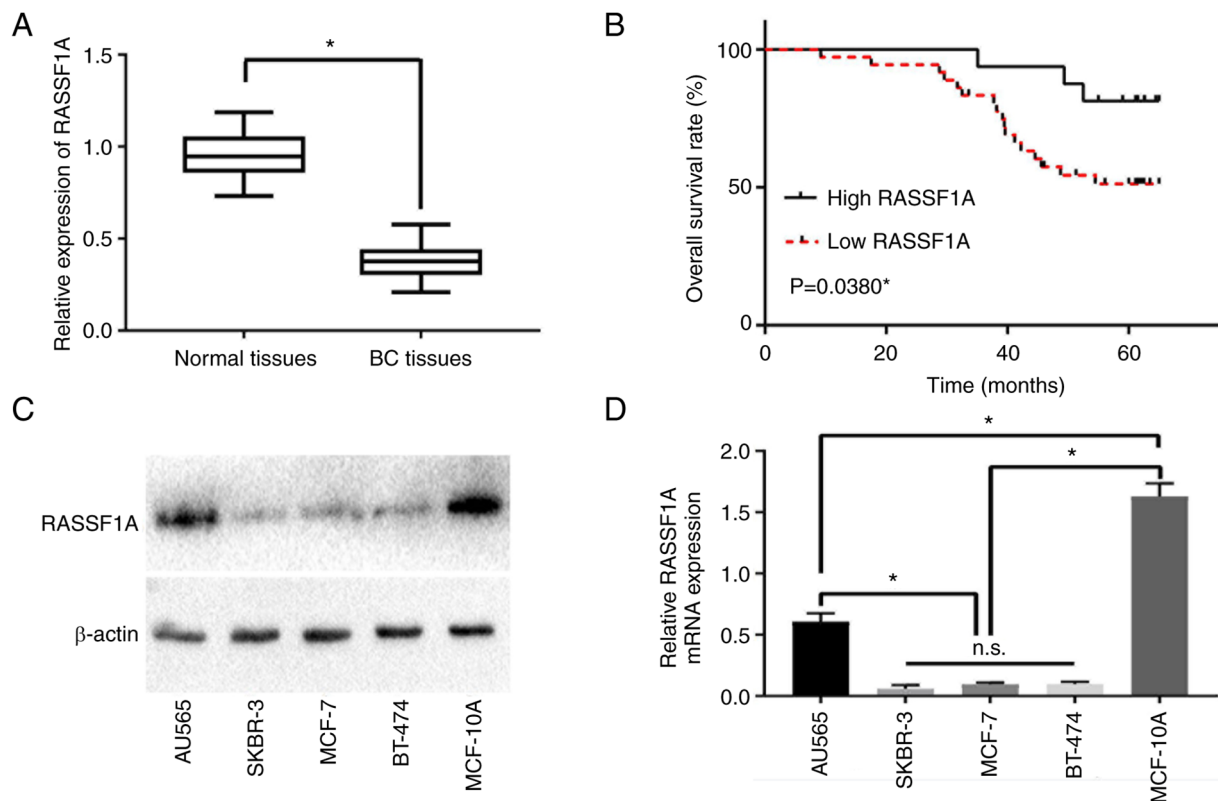
Figure 1. RASSF1A expression in HER2+ breast cancer tissues and breast cancer cell lines. (A) Relative mRNA levels of RASSF1A (normalized to β-Actin) in tumor and adjacent normal tissues of HER2+ breast cancer patients by RT-PCR. n=54, *P<0.05. (B) Kaplan-Meier overall survival curves of HER2+ breast cancer patients with high (n=27) or low (n=27) RASSF1A mRNA level. The median relative RASSF1A mRNA level (0.335) was used as the cutoff value for the definition of high (>0.335) and low (<0.335) RASSF1A mRNA expression. RASSF1A (C) protein and (D) mRNA levels in AU565, SKBR-3, MCF-7 and BT474 human breast cancer cells and MCF-10A human normal breast cells by western blot analysis and RT-PCR, respectively. n=3; *P<0.05. RASSF1A, RAS association domain family protein 1 subtype A; BC, breast cancer.

Statistical analysis. All results are presented as the mean ± standard deviation (SD) from three independent experiments. Data analysis was performed with SPSS 22.0 (IBM Corp.) and GraphPad Prism 6.0 (Dotmatics). The RASSF1A mRNA levels in tumor and adjacent normal tissues from HER2+ breast cancer patients were compared using the paired t-test. Other data from different groups were compared using one-way ANOVA followed by Bonferroni multiple comparisons test. The data from groups that were separated by two independent variables (i.e. cell group and normoxia/hypoxia) were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test. The relationships between tumorous RASSF1A level and tumor grade, TNM stage, tumor size and lymph node metastasis were interpreted using the Fisher's exact test or chi-square test. Survival analysis was performed using the Kaplan-Meier method or log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

Tumorous RASSF1A expression is negatively associated with disease progression and poor prognosis in HER+ breast cancer patients. The RASSF1A mRNA levels in tumor and adjacent normal tissues from 54 HER2+ breast cancer patients were determined by RT-PCR. The tumor tissues exhibited a significantly lower RASSF1A level than adjacent normal tissues (P<0.05, Fig. 1A). Kaplan-Meier survival analysis revealed a positive association between tumorous RASSF1A level and five-year overall survival (P<0.05, Fig. 1B). In addition, hierarchical cluster analysis showed that tumorous RASSF1A was negatively associated with tumor grade, TNM stage, tumor size and lymph node metastasis (P<0.05, Table I). Meanwhile, no significant correlation was detected between RASSF1A expression and age (P>0.05; Table I). These data supported the hypothesis of RASSF1A as a tumor suppressor in HER2+ breast cancer.

RASSF1A is downregulated in human breast cancer cells. The RASSF1A mRNA and protein levels in AU565, SKBR-3, MCF-7 and BT474 human breast cancer cells and MCF-10A human normal breast cells were determined by RT-PCR and western blot analysis, respectively. Similar to the clinical data, all four types of breast cancer cells exhibited lower RASSF1A mRNA and protein expression than MCF-10A normal breast cells (P<0.05, Fig. 1C and D). Among the four types of breast cancer cells, SKBR-3 showed the highest HER2 expression, while MCF-7 showed the lowest (Fig. 2A-C). Based on these results, SKBR-3 and MCF-7 cells were used as representative HER2+ and HER2-breast cancer cells, respectively, in subsequent experiments.

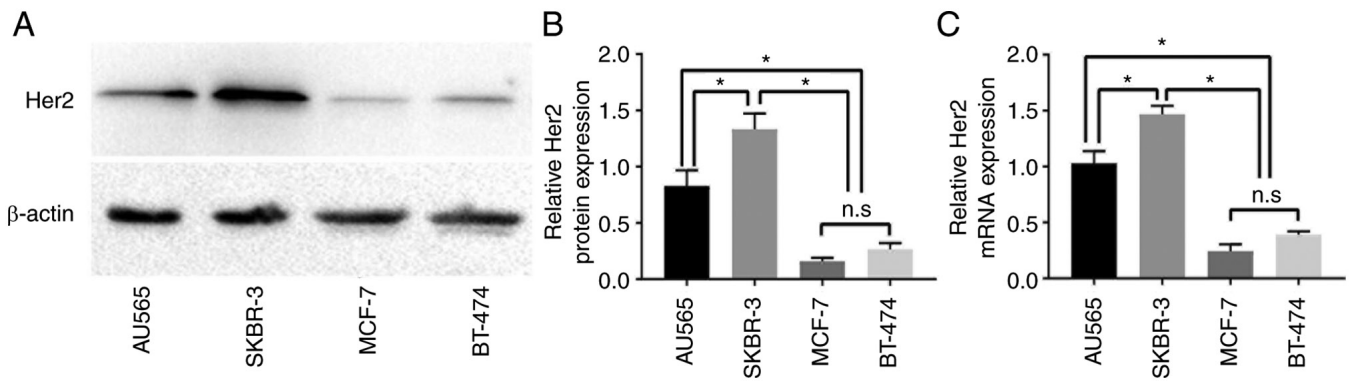*5HH drives RASSF1A expression in HER2+ but not HER2-breast cancer cells*. For targeted RASSF1A expression

Figure 2. HER2 expression in human breast cancer cell lines. (A and B) HER2 protein levels in AU565, SKBR-3, MCF-7 and BT474 human breast cancer cells by western blot analysis. n=3, *P<0.05. (C) HER2 mRNA levels in AU565, SKBR-3, MCF-7 and BT474 human breast cancer cells by RT-PCR. n=3, *P<0.05. HER2, human epidermal growth factor receptor 2.
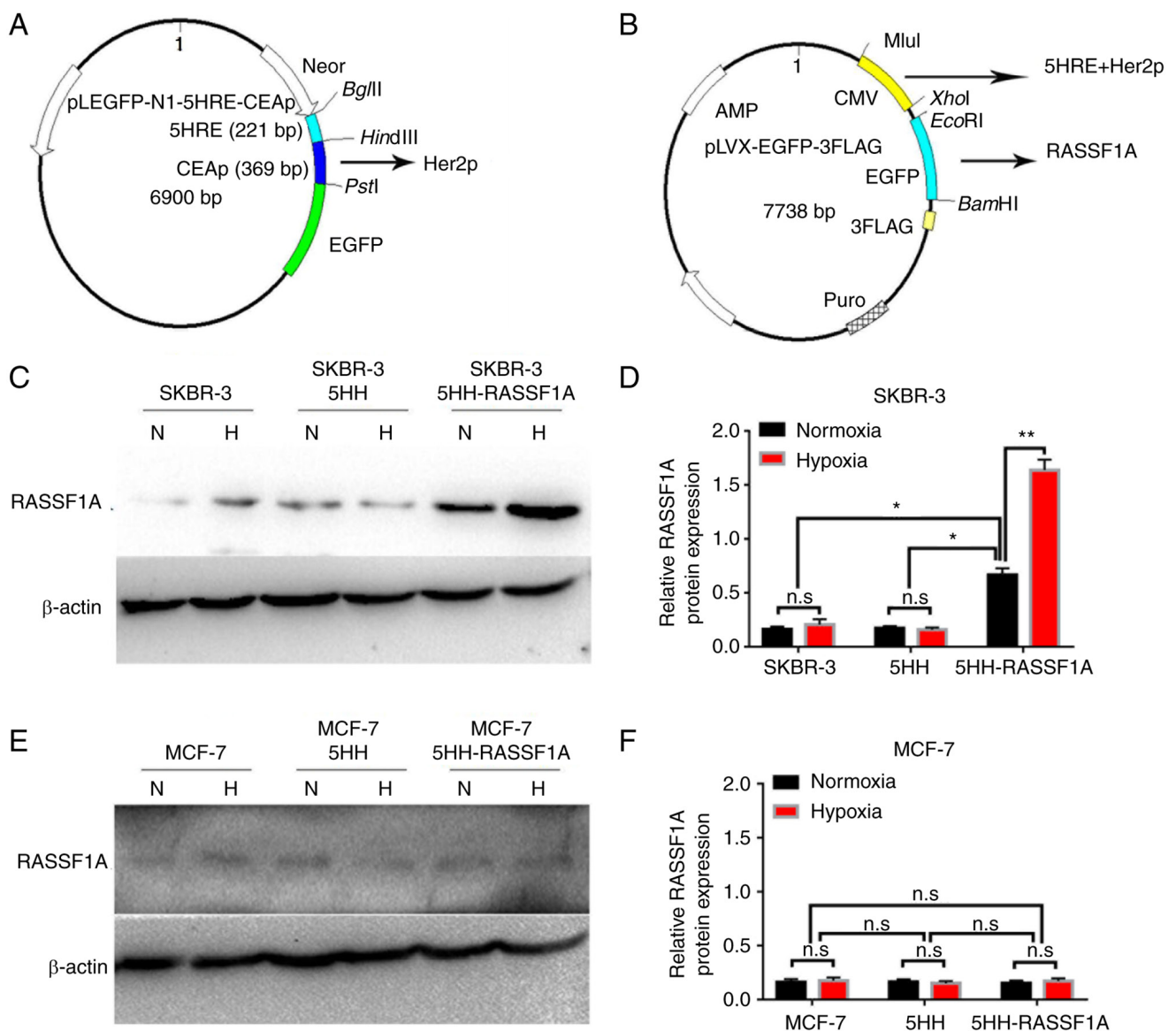

Figure 3. 5HH-driven RASSF1A expression in HER2+ breast cancer cells. (A) Schematic chart of the pLEGFP-N1-5HRE-HER2p recombinant plasmid. (B) Schematic chart of the pLVX-5HRE-HER2-RASSF1A-3FLAG (LV-5HH-RASSF1A) recombinant plasmid. (C and D) SKBR-3 cells transfected with LV-5HH-RASSF1A or vector control (LV-5HH) were cultured under normoxia (N) or hypoxia (H) for 24 h. RASSF1A protein levels were determined by western blot analysis. The untransfected cells were included as control. n=3, *P<0.05, **P<0.01, ns=not significant. (E and F) MCF-7 cells transfected with LV-5HH-RASSF1A or vector control (LV-5HH) were cultured under normoxia (N) or hypoxia (H) for 24 h. RASSF1A protein levels were determined by western blot analysis. The untransfected cells were included as control. n=3, ns=not significant. RASSF1A, RAS association domain family protein 1 subtype A; HER2, human epidermal growth factor receptor 2.

in HER2+ breast cancer cells, especially under hypoxic conditions, a lentiviral expression system (LV-5HH-RASSF1A) was constructed that could express RASSF1A under the control of a promoter composed of five copies of HRE and one copy of HER2p (5HH; Fig. 3A and B). The 5HH-driven RASSF1A expression was confirmed by western blot analysis in the HER2+ SKBR-3 cells transfected with LV-5HH-RASSF1A (P<0.05; Fig. 3C and D). In keeping with hypoxia-induced activation of HRE-mediated transcription, more pronounced expression was detected under hypoxic conditions (P<0.01; Fig. 3C and D). By contrast, no 5HH-driven RASSF1A expression was detected in the HER2-MCF-7 cells transfected with LV-5HH-RASSF1A, either under normoxia or hypoxia (Fig. 3E and F). These results were in line with the low transcriptional activation activity of HER2p in HER2-breast cancer cells. Thus, the 5HH promoter only drives RASSF1A expression in HER2+ but not HER2-breast cancer cells.

5HH-driven RASSF1A expression in HER2+ breast cancer cells inhibits cell proliferation. MTT and colony formation assays were used to evaluate the effects of 5HH-driven RASSF1A expression on HER2+ breast cancer cell proliferation. After 7 days of cultivation, LV-5HH-RASSF1A-transfected SKBR-3 cells showed significantly reduced viability compared with LV-5HH-transfected cells (P<0.05; Fig. 4A) and the reduction in cell viability was even more pronounced under hypoxic conditions (P<0.01, Fig. 4A). In addition, LV-5HH-RASSF1A-transfected SKBR-3 cells exhibited decreased colony formation capacity compared with LV-5HH-transfected cells, especially under hypoxic conditions (P<0.05, Fig. 4B and C). Together, these results indicated that 5HH-driven RASSF1A expression in HER2+ breast cancer cells inhibited cell proliferation.

Discussion

Currently, HER2+ breast cancer is treated with HER2-targeting monoclonal antibodies such as trastuzumab and pertuzumab and tyrosine kinase inhibitors such as tucatinib and lapatinib (31,32). Although positive clinical outcomes are well documented, the continuous usage of these drugs may induce drug resistance in some patients. Patients who develop resistance to multiple HER2-targeting therapies often have limited treatment options and thus suffer poor clinical outcomes (33). Therefore, there exists a need for new targeted therapies for HER2+ breast cancer. The present study found that RASSF1A was downregulated in human HER2+ breast cancer and its expression was negatively associated with disease progression and mortality, which underscored the clinical significance of RASSF1A as a tumor suppressor in this specific subtype of breast cancer. Moreover, by using a lentiviral expression system under the control of a hypoxia-inducible, HER2p-driven promoter (5HH), the present study successfully introduced the RASSF1A gene into HER2+ breast cancer cells to express the RASSF1A protein. The same expression system failed to express RASSF1A in HER2-breast cancer cells, in which HER2p has a low transcriptional activation activity. This targeted RASSF1A expression inhibited the proliferation of HER2+ breast cancer cells and, to a greater degree, under hypoxia. Together, these clinical and *in vitro* findings
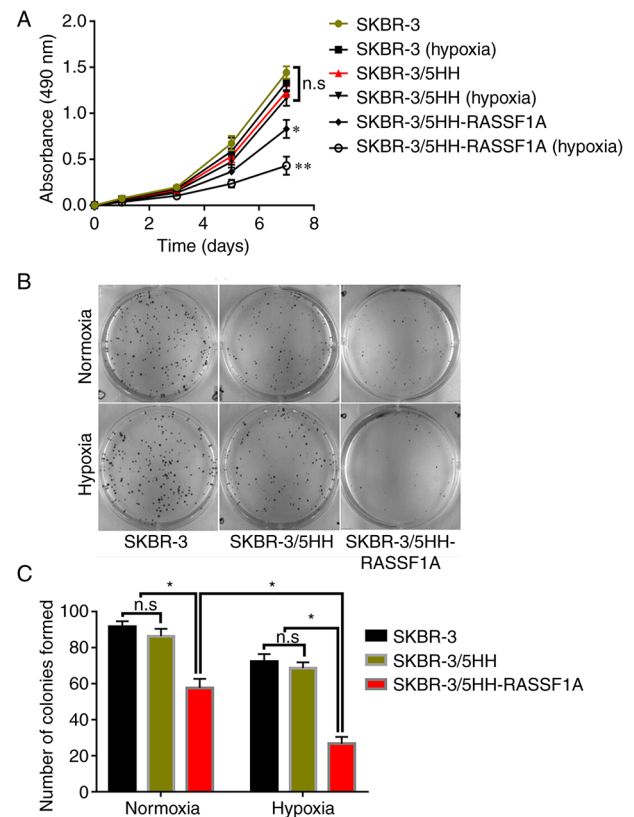

Figure 4. 5HH-driven RASSF1A expression in HER2+ breast cancer cells inhibits cell proliferation. SKBR-3 cells transfected with LV-5HH-RASSF1A or vector control (LV-5HH) were cultured under normoxia or hypoxia for up to 7 days as described in *Materials and methods*. The untransfected cells were included as control. (A) Cell viability at indicated time by the MTT assay. n=3, *P<0.05 vs. SKBR-3/5HH, **P<0.01 vs. SKBR-3/5HH (hypoxia), ns=not significant. (B and C) Cell growth on day 7 by the colony formation assay. n=3, *P<0.05, ns=not significant. RASSF1A, RAS association domain family protein 1 subtype A; HER2, human epidermal growth factor receptor 2.

supported 5HH-driven RASSF1A expression as a potential targeted gene therapy for HER2+ breast cancer.

Given the presence of a number of defective genes, breast cancer is an ideal candidate for gene therapy. At present, there are ~50 ongoing gene therapy clinical trials for breast cancer (34). These clinical trials target a variety of breast cancer susceptibility genes such as BRCA1, BRCA2, TP53 and PTEN and the genetic materials are transferred into cancer cells using both viral and non-viral vectors. The clinical trials have shown that gene therapy can be less toxic than conventional therapies, but this approach faces two key challenges to success: The persistent expression of anti-cancer gene products and a tumor-selective delivery system (35).

In recent years, universal tumor-specific promoters, such as the survivin and telomerase reverse transcriptase gene promoters and the hTERT promoter, have been exploited for cancer gene therapy (36-39). Hypoxia, caused by rapid tumor growth, has a key role in cancer progression and is the focus of a number of cancer treatment strategies (40). In 2000, Shibata *et al* (41) developed a hypoxia-responsive vector with five copies of HRE derived from the promoter region of the human VEGF (5HRE) for tumor-specific gene therapy. Since then, constructs with 5HRE have been successfully used to drive hypoxia-inducible gene expression in

tumor cells (42-44). The present study identified RASSF1A as a new anti-tumor gene for HER2+ breast cancer and, more importantly, achieved selective delivery of this gene to HER2+ breast cancer cells using a lentiviral expression system under the control of HER2p and the 5HRE promoter. The results from the present study should encourage the development of new targeted gene therapy strategies for HER2+ breast cancer.

The present study is limited by the lack of investigations on the molecular mechanisms mediating the anti-tumor function of RASSF1A. RASSF1A has been shown to inhibit ERα+ breast cancer by suppressing ERα expression and function via Hippo-Kinases (12,13). The molecular pathways affected by RASSF1A in HER2+ breast cancer remain to be elucidated. In addition, further studies are required to confirm the efficacy of 5HH-driven RASSF1A expression for HER2+ breast cancer *in vivo*. HER2 amplification is not limited to breast cancer, but occurs in a number of other solid tumors such as bladder, cervical, uterine and testicular cancers, wherein it drives disease progression (45). Correspondingly, 5HH-driven gene therapy may hold promise as a general therapeutic approach for all types of HER2+ cancers.

In conclusion, the present study verified RASSF1A as a tumor suppressor in HER2+ breast cancer and achieved selective delivery of this gene to HER2+ breast cancer cells using a lentiviral expression system under the control of HER2p and the 5HRE promoter. The selective delivery of RASSF1A resulted in growth inhibition of HER2+ breast cancer cells. These findings encourage the development of new targeted gene therapy strategies for HER2+ breast cancer and possibly, all HER2+ cancers.

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Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SH and JG contributed to the conceptualization and the design of the present study. SH, YH, LH, NC and XY performed the experiments and analyzed the data. HW and PH were responsible for the acquisition, analysis and interpretation of the data. YF, JZ and JYZ contributed to the drafting of the manuscript. SH and JG confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Waks AG and Winer EP: Breast cancer treatment: A review. JAMA 321: 288-300, 2019.
2. Iancu G, Serban D, Badiu CD, Tanasescu C, Tudosie MS, Tudor C, Costea DO, Zgura A, Iancu R and Vasile D: Tyrosine kinase inhibitors in breast cancer (review). Exp Ther Med 23: 114, 2022.
3. Ding S, Sun X, Lu S, Wang Z, Chen X and Shen K: Association of molecular subtype concordance and survival outcome in synchronous and metachronous bilateral breast cancer. Breast 57: 71-79, 2021.
4. Howlader N, Altekruse SF, Li CI, Chen VW, Clarke CA, Ries LA and Cronin KA: US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. J Natl Cancer Inst 106: dju055, 2014.
5. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235: 177-182, 1987.
6. Howlader N, Cronin KA, Kurian AW and Andridge R: Differences in breast cancer survival by molecular subtypes in the United States. Cancer Epidemiol Biomarkers Prev 27: 619-626, 2018.
7. Pallerla S, Abdul AURM, Comeau J and Jois S: Cancer vaccines, treatment of the future: With emphasis on HER2-positive breast cancer. Int J Mol Sci 22: 779, 2021.
8. Takada M and Toi M: Neoadjuvant treatment for HER2-positive breast cancer. Chin Clin Oncol 9: 32, 2020.
9. Choong GM, Cullen GD and O'Sullivan CC: Evolving standards of care and new challenges in the management of HER2-positive breast cancer. CA Cancer J Clin 70: 355-374, 2020.
10. Hesson LB, Cooper WN and Latif F: The role of RASSF1A methylation in cancer. Dis Markers 23: 73-87, 2007.
11. Li M, Wang C, Yu B, Zhang X, Shi F and Liu X: Diagnostic value of RASSF1A methylation for breast cancer: A meta-analysis. Biosci Rep 39: BSR20190923, 2019.
12. Thaler S, Schmidt M, Schad A and Sleeman JP: RASSF1A inhibits estrogen receptor alpha expression and estrogen-independent signalling: Implications for breast cancer development. Oncogene 31: 4912-4922, 2012.
13. Roßwag S, Sleeman JP and Thaler S: RASSF1A-mediated suppression of estrogen receptor alpha (ERα)-driven breast cancer cell growth depends on the Hippo-kinases LATS1 and 2. Cells 10: 2868, 2021.
14. Sun W, Shi Q, Zhang H, Yang K, Ke Y, Wang Y and Qiao L: Advances in the techniques and methodologies of cancer gene therapy. Discov Med 27: 45-55, 2019.
15. Hong R and Xu B: Breast cancer: An up-to-date review and future perspectives. Cancer Commun (Lond) 42: 913-936, 2022.
16. Sun W, Liu XY, Ma LL and Lu ZL: Tumor targeting gene vector for visual tracking of Bcl-2 siRNA transfection and anti-tumor therapy. ACS Appl Mater Interfaces 12: 10193-10201, 2020.
17. Montaño-Samaniego M, Bravo-Estupiñan DM, Méndez-Guerrero O, Alarcón-Hernández E and Ibáñez-Hernández M: Strategies for targeting gene therapy in cancer cells with tumor-specific promoters. Front Oncol 10: 605380, 2020.
18. Altwaijry N, Somani S and Dufès C: Targeted nonviral gene therapy in prostate cancer. Int J Nanomedicine 13: 5753-5767, 2018.
19. Chen C, Yue D, Lei L, Wang H, Lu J, Zhou Y, Liu S, Ding T, Guo M and Xu L: Promoter-operating targeted expression of gene therapy in cancer: Current stage and prospect. Mol Ther Nucleic Acids 11: 508-514, 2018.

20. Hurst HC: Update on HER-2 as a target for cancer therapy: The ERBB2 promoter and its exploitation for cancer treatment. Breast Cancer Res 3: 395-398, 2001.
21. Cui X, Chen H, Zhang Q, Xu M, Yuan G and Zhou J: Exploration of the structure and recognition of a G-quadruplex in the her2 proto-oncogene promoter and its transcriptional regulation. Sci Rep 9: 3966, 2019.
22. Lundgren K, Holm C and Landberg G: Hypoxia and breast cancer: Prognostic and therapeutic implications. Cell Mol Life Sci 64: 3233-3247, 2007.
23. Wu D, Potluri N, Lu J, Kim Y and Rastinejad F: Structural integration in hypoxia-inducible factors. Nature 524: 303-308, 2015.
24. Lakhani SR, Ellis IO, Schnitt SJ, Tan PH and van de Vijver MJ (eds): WHO classification of tumors of the breast. 4th edtion. Lyon, IARC Press, 2012.
25. You D, Wang D, Liu P, Chu Y, Zhang X, Ding X, Li X, Mao T, Jing X, Tian Z and Pan Y: MicroRNA-498 inhibits the proliferation, migration and invasion of gastric cancer through targeting BMI-1 and suppressing AKT pathway. Human Cell 33: 366-376, 2020.
26. Luo M, Hou L, Li J, Shao S, Huang S, Meng D, Liu L, Feng L, Xia P, Qin T and Zhao X: VEGF/NRP-1axis promotes progression of breast cancer via enhancement of epithelial-mesenchymal transition and activation of NF-κB and β-catenin. Cancer Lett 373: 1-11, 2016.
27. Chen N, He S, Geng J, Song ZJ, Han PH, Qin J, Zhao Z, Song YC, Wang HX and Dang CX: Overexpression of Contactin 1 promotes growth, migration and invasion in Hs578T breast cancer cells. BMC Cell Biol 19: 5, 2018.
28. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
29. Zhou PH, Zheng JB, Wei GB, Wang XL, Wang W, Chen NZ, Yu JH, Yao JF, Wang H, Lu SY and Sun XJ: Lentivirus-mediated RASSF1A expression suppresses aggressive phenotypes of gastric cancer cells in vitro and in vivo. Gene Ther 22: 793-801, 2015.
30. He S, Sun XJ, Zheng JB, Qi J, Chen NZ, Wang W, Wei GB, Liu D, Yu JH, Lu SY and Wang H: Recombinant lentivirus with enhanced expression of caudal-related homeobox protein 2 inhibits human colorectal cancer cell proliferation *in vitro*. Mol Med Rep 12: 1838-1844, 2015.
31. Kreutzfeldt J, Rozeboom B, Dey N and De P: The trastuzumab era: Current and upcoming targeted HER2+ breast cancer therapies. Am J Cancer Res 10: 1045-1067, 2020.
32. Murthy RK, Loi S, Okines A, Paplomata E, Hamilton E, Hurvitz SA, Lin NU, Borges V, Abramson V, Anders C, *et al*: Tucatinib, trastuzumab, and capecitabine for HER2-positive metastatic breast cancer. N Engl J Med 382: 597-609, 2020.
33. Tesch ME and Gelmon KA: Targeting HER2 in breast cancer: Latest developments on treatment sequencing and the introduction of biosimilars. Drugs 80: 1811-1830, 2020.
34. Arabi F, Mansouri V and Ahmadbeigi N: Gene therapy clinical trials, where do we go? An overview. Biomed Pharmacother 153: 113324, 2022.
35. Dastjerd NT, Valibeik A, Rahimi Monfared S, Goodarzi G, Moradi Sarabi M, Hajabdollahi F, Maniati M, Amri J and Samavarchi Tehrani S: Gene therapy: A promising approach for breast cancer treatment. Cell Biochem Funct 40: 28-48, 2022.
36. Xu Y, Hou J, Liu Z, Yu H, Sun W, Xiong J, Liao Z, Zhou F, Xie C and Zhou Y: Gene therapy with tumor-specific promoter mediated suicide gene plus IL-12 gene enhanced tumor inhibition and prolonged host survival in a murine model of Lewis lung carcinoma. J Transl Med 9: 39, 2011.
37. Fang L, Shanqu L, Ping G, Ting H, Xi W, Ke D, Min L, Junxia W and Huizhong Z: Gene therapy with RNAi targeting UHRF1 driven by tumor-specific promoter inhibits tumor growth and enhances the sensitivity of chemotherapeutic drug in breast cancer in vitro and in vivo. Cancer Chemother Pharmacol 69: 1079-1087, 2012.
38. Alekseenko IV, Pleshkan VV, Sass AV, Filyukova OB, Snezhkov EV and Sverdlov ED: A universal tumor-specific promoter for cancer gene therapy. Dokl Biochem Biophys 480: 158-161, 2018.
39. Liu Q, Kulak MV, Borcherding N, Maina PK, Zhang W, Weigel RJ and Qi HH: A novel HER2 gene body enhancer contributes to HER2 expression. Oncogene 37: 687-694, 2018.
40. Choe SS and Kim JB: Hypoxia-inducible factors: New strategies for treatment of obesity-induced metabolic diseases. Postgrad Med J 96: 451-452, 2020.
41. Shibata T, Giaccia AJ and Brown JM: Development of a hypoxia-responsive vector for tumor-specific gene therapy. Gene Ther 7: 493-498, 2000.
42. Zheng J, He S, Qi J, Wang X, Yu J, Wu Y, Gao Q, Wang K and Sun X: Targeted CDX2 expression inhibits aggressive phenotypes of colon cancer cells *in vitro* and *in vivo*. Int J Oncol 51: 478-488, 2017.
43. Zhu S, Ying Y, Ye J, Chen M, Wu Q, Dou H, Ni W, Xu H and Xu J: AAV2-mediated and hypoxia response element-directed expression of bFGF in neural stem cells showed therapeutic effects on spinal cord injury in rats. Cell Death Dis 12: 274, 2021.
44. Zhu S, Ying Y, He Y, Zhong X, Ye J, Huang Z, Chen M, Wu Q, Zhang Y, Xiang Z, *et al*: Hypoxia response element-directed expression of bFGF in dental pulp stem cells improve the hypoxic environment by targeting pericytes in SCI rats. Bioact Mater 6: 2452-2466, 2021.
45. Yan M, Schwaederle M, Arguello D, Millis SZ, Gatalica Z and Kurzrock R: HER2 expression status in diverse cancers: Review of results from 37,992 patients. Cancer Metastasis Rev 34: 157-164, 2015.