

Intracellular Delivery of ERBB2 siRNA and p53 Gene Synergistically Inhibits the Growth of Established Tumor in an Immunocompetent Mouse

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Abstract

Breast cancer is one of the leading causes of deaths worldwide in women with hormone therapy, chemotherapy, targeted therapies, or their combinations being the current options for treating the disease at the different stages (stages I-III) with associated side-effects or increasing life-span at the advanced stage (stage IV). Small interfering RNA (siRNA) as an effective tool to selectively knockdown of a particular gene could be harnessed in combination with plasmid DNA (carrying a gene of interest) and conventional anti-cancer drugs for precisely treating breast cancer with minimal side effects. However the limitation of the naked siRNA and DNA in penetrating the plasma membrane and their sensitiveness to nuclease-mediated cleavage render the technology rather complex in therapeutic intervention. Recently, we have developed pH-sensitive carbonate apatite as a potential nano-carrier to efficiently deliver siRNA or DNA across the cell membrane and facilitate them to escape endosomal acidic compartment resulting in specific cleavage of a particular mRNA transcript or expression of a desirable protein, respectively. Moreover, we demonstrated nanoparticle-assisted delivery of the siRNAs targeting cyclin B1, PLC-gamma-2/calmodulin1, NFκB1/NFκB2, ABCG2/ABCB1 and cROS1 mRNAs sensitizes cervical adenocarcinoma and breast cancer cells towards traditional anti-cancer drugs. Here, we report that co-delivery of the siRNA targeting HER2/ErbB2 gene transcript and p53 gene with the help of carbonate apatite nanoparticles synergistically induces inhibition of growth/proliferation of breast cancer cell lines as well as regression of the breast tumor induced in Balb/c mice. Additionally, combined delivery of nanoparticle-associated HER2/ErbB2 siRNA and p53 gene apparently slows down the growth of the established tumor in presence of doxorubicin or paclitaxel compared with the individual free drugs. Thus, the combination of HER2/ErbB2 knockdown and restoring of normal p53 function could be a highly promising approach necessitating further studies through pre-clinical trials with different models of breast cancer to establish the therapeutic role of this combination therapy prior to conducting clinical trials in breast cancer patients.

Keywords: Carbonate apatite; siRNA; Gene expression; Transfection; Breast cancer; HER2/ErbB2; p53; Doxorubicin; Paclitaxel; Cisplatin; Chemosensitivity

Abbreviations: Dulbecco's Modified Eagle's Medium (DMEM); Small interfering RNA (siRNA); Fetal bovine serum (FBS); Extracellular Signal-Regulated Kinase (ERK); Mitogen-activated protein kinase (MAPK); Phosphatidylinositol 3-kinases (PI3Ks); Receptor tyrosine kinases (RTKs); Multidrug resistance protein 1 (MDR1); Phosphoinositide 3-kinase (PI3K)

Introduction

Breast cancer, one of the most common cancers with 450,000 annual deaths world-wide, is clinically categorized on the basis of the existence of estrogen receptor (ER) [1,2], the amplification of HER2/ErbB2 gene [3] and the absence of three receptors, such as ER, progesterone receptor (PR) and HER2/ERBB2 (Triple Negative) [4,5]. While for the first two groups of breast cancer receptor-specific therapy is applied, cytotoxic chemotherapy remains the mainstay of treatment for triple negative breast cancer (TNBC). Like other types of cancers, breast cancer is the result of gain-of-function mutations in proto-oncogenes stimulating cell growth and survival and/or loss-of-function mutations in tumor suppressor genes allowing unrestrained growth of cancer cells [6]. Oncogenic proteins and tumor suppressors play the key roles in ensuring a critical balance for regulation of the key cell signaling pathways that ultimately control cell proliferation and survival [6]. The ErbB2, phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K), c-Myc, and cyclin D1 oncogenes are frequently overexpressed in breast cancer. Mutations in tumor suppressor genes, such as BRCA1, BRCA2,

p53 and PTEN are responsible for both hereditary and acquired breast cancer [7,8].

Over expression of HER2/ErbB2, a member of the EGFR family of receptor tyrosine kinases (RTKs) takes place in approximately 25–30% of human breast cancers predominantly as a result of amplification of the *c-erbB-2* proto-oncogene [3,9,10] leading to increased level of metastases, resistance to endocrine therapy and poor survival [3,9-13]. The primary mechanism by which ErbB2 induces mammary tumorigenesis in human breast cancer is through activation of PI3K/Akt and MAP kinase pathways ultimately affecting transcription factors and the machinery that controls the cell cycle, such as cyclin D1 [14] as well as Cdk4, a known cyclin D1 partner [15,16]. On the other hand, p53 gene is found mutated in ~23% of breast cancer samples with

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~70% of the mutations being the missense type [17]. In a normal cell, p53 protein is maintained at very low level by promoting proteasomal degradation of p53 through p53-interacting protein, MDM2, an E3 ubiquitin ligase. Cellular stress-induced posttranslational modification of p53 and MDM2 stabilizes and activates p53 [18] enabling it to bind DNA in a sequence-specific manner and regulating the expression of hundreds of genes for cycle checkpoint activation, cellular senescence and apoptosis [19]. Centrosome amplification in p53 mutant cells [20] leads to chromosome instability, a hallmark of solid tumors whereas loss of p53 results in aberrant asymmetric cell divisions of mammary stem cells of ErbB2-associated mouse mammary tumors [21]. Mutations in p53 gene as well as overexpression of HER2/ErbB2 were also found to be linked to chemoresistance [22-25].

Considering the crucial roles of HER2/ErbB2 overexpression and p53 mutations in development, progression and chemoresistance of breast cancer which consists of a heterogeneous population of cells, strategies in silencing the overexpressed gene and providing the wild-type p53 gene could be pivotal in effective treatment of the cancer. A number of existing non-viral vectors are available for intracellular delivery of siRNA and plasmid DNA to silence the target mRNA of a particular gene and to express a protein of interest, respectively with limitations in proper condensation, cellular uptake and endosomal escape, leading to a decrease in overall performance of the delivered siRNA or DNA [26,27]. Recently, we have developed pH-sensitive carbonate apatite as a potential nano-carrier to efficiently deliver siRNA or DNA across the cell membrane and facilitate them to escape endosomal acidic compartment finally leading to knockdown of a specific mRNA transcript or expression of a desirable protein, respectively [28,29]. Moreover, we demonstrated nanoparticle-assisted delivery of the siRNAs targeting cyclin B1, PLC-gamma-2/calmodulin 1, NFκB1/NFκB2, ABCG2/ABCB1 and cROS1 mRNAs sensitizes cervical adenocarcinoma and breast cancer cells towards traditional anti-cancer drugs [30-34]. Here, we report that combined delivery of the siRNA targeting HER2/ErbB2 gene transcript and p53 gene with the help of carbonate apatite nanoparticles synergistically induces death or growth inhibition of breast cancer cell lines as well as the breast tumor induced in Balb/c mice. Moreover, intratumoral delivery of nanoparticle-associated HER2/ErbB2 siRNA and p53 gene apparently slow down the growth of the established tumor in presence of doxorubicin or paclitaxel compared with the individual free drug.

Materials and Methods

Reagents

Dulbecco's modified Eagle medium (DMEM) was purchased from BioWhittaker (Walkersville, USA). DMEM powder, foetal bovine serum (FBS) and trypsin-ethylenediaminetetraacetate (trypsin-EDTA) were obtained from Gibco BRL (California, USA). Calcium chloride dehydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), sodium bicarbonate, dimethyl sulphoxide (DMSO) and thiazolyl blue tetrazolium bromide (MTT) were from Sigma-Aldrich (St Louis, MO, USA). The chemotherapy drugs, doxorubicin, paclitaxel and cis-diammineplatinum (II) dichloride are from Sigma Aldrich (St.Louis, USA). Both doxorubicin and cis-diammineplatinum (II) dichloride were dissolved in distilled water and 2mM stock solutions were prepared whereas paclitaxel was dissolved in DMSO and 10mM stock solution was prepared.

siRNA design and sequence

The validated anti-ERBB2 siRNA was purchased from

QIAGEN (California, US) with the target sequence of 5'-AACAAAGAAATCTTAGACGAA-3'. siRNA was supplied in lyophilised form and upon delivery, the siRNA (1 nmol) was reconstituted with RNase-free water to obtain a stock solution of 20 μM . The siRNA solution was then allocated into multiple reaction tubes for storage as repeated thawing might affect the silencing efficiency of siRNAs. The siRNAs were stored at 20°C as recommended by QIAGEN.

Isolation of p53

E coli DH5 α cells harboring p53 plasmid were grown in LB media overnight at 37°C (Kanamycin was added at a concentration of 50 $\mu\text{g}/\text{ml}$ culture). Next day small colonies were picked and grown individually in 50 ml of LB broth with kanamycin for 16 hour. The p53 plasmids were isolated and purified from the bacteria using 'Qiagen Plasmid Mega' kit. The extracted DNA was subjected to spectrophotometric quantification by taking absorbance at 260 nm while a ratio of absorbance at 260 and 280 nm at 1.8 or more was considered to be of high purity. Additionally, purified DNA was run on 0.8% agarose gel and the DNA bands were visualized under ultraviolet transilluminator.

Cell culture and seeding

MCF-7 and 4T1 cells were grown in 25 cm² culture flask in DMEM supplemented with 10% heat-inactivated FBS in a humidified atmosphere containing 5% CO₂ at 37°C. Exponentially growing MCF-7 and 4 T1 cells were trypsinised and following addition of fresh medium, the cell suspension was centrifuged at 10,000 rpm for 5 min and the supernatant was discarded. Fresh medium was added to resuspend the pellet and the cells were counted using haemocytometer. Appropriate dilutions were made using culture medium to produce a cell suspension with concentration 5.0 x 10⁴ cells/ml. One ml of the prepared cell suspension was subsequently added into each of the wells in 24-well plate and allowed to attach for overnight at 37°C and 5% CO₂ before siRNA transfection.

Formulation of carbonate apatite complexes of ERBB2 siRNA and/or p53 plasmid and transfection of MCF-7 and 4T1 cells

On the day of siRNA transfection, 100 mL of DMEM was prepared using 1.35 g of DMEM powder and 0.37 g of sodium bicarbonate with the pH subsequently adjusted to 7.4 using 0.1 M hydrochloric acid. The prepared DMEM solution was filtered using 0.2 μM syringe filter in laminar flow hood, followed by transferring 1 ml of the filtered medium into 1.5 ml microcentrifuge tubes. 4 μl of 1 M calcium chloride was then added into the microcentrifuge tubes, followed by addition of siRNA (40 nM) and p53 plasmid DNA (100 ng) and incubation at 37°C for 30 min. After the incubation, 10% FBS was added into each microcentrifuge tube. Culture medium from the wells seeded one day before was aspirated and replaced with 1 mL of the prepared medium containing siRNA-loaded carbonate apatite nanoparticles in presence or absence of free drugs. Plates were then incubated at 37°C and 5% CO₂ for two consecutive days.

Cell viability assessment with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Following two days of siRNA transfection, the fraction of viable MCF-7 and 4T1 cells was determined using MTT assay. Briefly, 50 μL of MTT (5 mg/mL in PBS) was added aseptically into each of the wells in siRNA transfected-plate, followed by incubation at 37°C and 5% CO₂ for 4 h. After the incubation, medium containing MTT

was aspirated and the purple formazan crystals at the bottom of each well were dissolved by mixing with 300 μ L of DMSO solution. Absorbance of the resulting formazan solution was then determined spectrophotometrically at wavelength 595 nm using microplate reader (DynexOpsys MR, US) with reference to 630 nm. Each experiment was performed in triplicates and the data were plotted as mean \pm standard deviation (S.D.) of three independent experiments.

Data analysis

The cell viability in the treated wells was expressed as a percentage and was calculated using the absorbance values obtained from MTT assay by using the following formula:

$$\% \text{ cell viability} = \frac{\text{Absorbance of treated sample}}{\text{Absorbance of control}} \times 100\%$$

Tumour induction in Balb/c mice

The IMU animal ethics committee approved all the procedures used in the project with the approval no. being IMU 220/2010. Five-weeks-old female Balb/c mice were purchased from Institute for Medical Research (IMR) and were housed under standard conditions according to appropriate animal care guidelines. The animals were housed in plastic cages with 3 mice placed in each cage inside a well-ventilated room at $22 \pm 2^\circ\text{C}$ with a 12-hr L: D cycle. The mice groups were fed with regular ad libitum. All the animals had free access to standard diet and water. 1×10^5 cells murine breast cancer 4T1 cells were injected subcutaneously into the mammary fat pad. When tumors reached around 75 mm^3 , the mice were randomly assigned to different groups for further study or treatments with six mice per group.

Intratumoral delivery of nanoparticle-associated ErbB2 siRNA/p53 plasmid in Balb/c mice with 4T1 mammary carcinoma

In order to estimate gross body weight and subsequently, tumor volume regression, following induction of 4T1-induced mammary tumors, nanoparticle complex of ErbB2 siRNA and 53 plasmid was directly injected into the tumor. For a comparative study, in addition to untreated control, nanoparticles alone and free anti-cancer drugs injected in both of the studies, other injected samples in gross tumor volume estimation was nanoparticle-loaded ERBB2 siRNA/p53 plasmid in presence of the individual anti-cancer drug. Depending on the grouping, the mice were treated with 100 μ L of the particle suspension originally formulated in 1 ml of DMEM with 7 mM of Ca^{2+} in presence or absence of ERBB2 siRNA (800 nM) plus p53 plasmid (100 ng) and/or anticancer drugs (100 nM), through intratumoral injection and the tumor volumes were estimated according to the modified ellipsoidal formula: $1/2(\text{length} \times \text{width}^2)$.

Statistical analysis

Statistical analysis was done using the SPSS statistical package (version 18.0 for Windows). LSD post-hoc test for one-way ANOVA was used to analyze and compare the significant difference between treated and non-treated samples. Data is presented as mean \pm SD and $P < 0.05$ is considered as statistically significant.

Results and Discussion

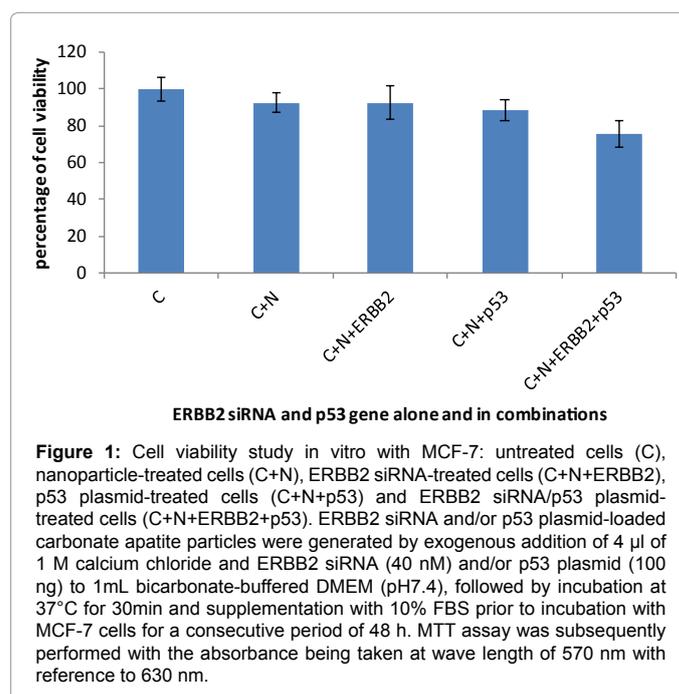
Intracellular delivery of HER2/ErbB2 siRNA and p53 gene into MCF-7 and 4T1 breast cancer cells

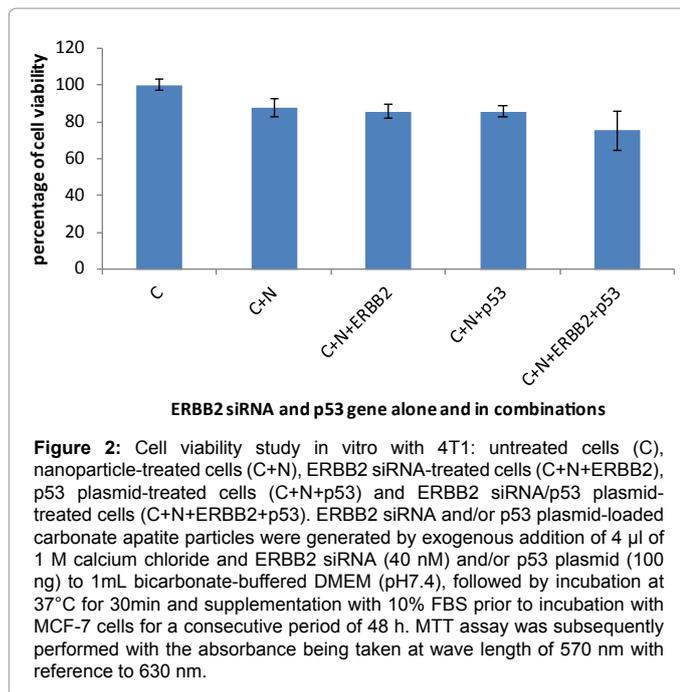
Since both 'gain of function' in the form of overexpressed

oncogene(s), such as HER2/ErbB2 and 'loss of function' by mutated tumor suppressor genes, such as p53, contribute to the development of a variety of breast cancers, silencing of HER2/ErbB2 expression and providing wild-type p53 into breast cancer cells might block the cell proliferation- and survival-related signaling cascades influenced by the particular RTK as well as mutated p53. Carbonate apatite nanoparticles were used to complex with HER2/ErbB2 siRNA (constructed and validated by Qiagen) and/or p53 plasmid DNA prior to the 48 h incubation with MCF-7 (human breast cancer cell line) and 4T1 (murine breast cancer cell line). As shown in Figure 1, neither HER2/ErbB2 siRNA nor wild-type p53 gene when intracellularly delivered by nanoparticles could kill MCF-7 cells and inhibit the cell growth in comparison with the nanoparticles. However, when both the siRNA and the p53 plasmid were delivered together, they showed synergistic effect. Although MCF-7 cells have low expression level of HER2/ErbB2 [35,36] and possess wild type p53 gene [37-39], an increase in p53 expression beyond the basal level might promote cell proliferation/survival in absence of the RTK. A similar finding was observed in 4T1 cells (Figure 2) which also possess low level of HER2/ErbB2 [40,41], but are deficient of p53 [42-44], indicating that expression of wild-type p53 could suppress in absence of HER2/ErbB2 the growth/proliferation of the tumor cells originally established by losing p53 expression [45]. Cell viability assessment using 'All Stars Negative Control siRNA', having no target sequence within the cellular mRNA pool (designed and synthesized by Qiagen) resulted in no cytotoxic effect irrespective of the siRNA doses used (not shown here), indicating the role of siRNA specificity in gene knockdown and consequential change in any cellular fate.

Intratumoral delivery of HER2/ErbB2 siRNA and wild-type p53 gene

In order for further evaluation of potential synergistic effect *in vivo* of HER2/ErbB2 knockdown and wild-type p53 expression in inducing death or inhibiting growth of tumor cells as observed *in vitro* in human and murine breast cancer cell lines irrespective of the presence or absence of normal p53, nanoparticle formulation of HER2/ErbB2





siRNA and wild-type p53 gene was directly injected into the tumor of 4T1 cells established in mammary fat pad of Balb/c mice (an immune-competent model of aggressive breast cancer). As shown in Figure 3, although there was virtually no change in the average body weights of the mice treated intratumorally with either nanoparticles alone or combination of ErbB2siRNA and wild-type p53 plasmid compared with untreated control group, following induction of the tumors of measurable size using subcutaneously injected 4T1 cells, there was a noticeable and statistically significant reduction in tumor growth ($p < 0.05$) over the period of observation in the particular group of mice receiving intratumoral injection of nanoparticle-associated ErbB2 siRNA and wild-type p53 gene (Figure 4), suggesting a synergistic effect on tumor growth regression similarly as the earlier in vitro outcome. The rationale behind the absence of total inhibition of tumor growth could be due to the injection frequency of the sample with combined siRNA and plasmid DNA, which was once in our current experimental set-up and therefore the combined effect was very likely to subside in the later phase owing to the degradation of the therapeutic molecules. Although the nanoparticles seemed to inhibit the tumor growth in the beginning, there was no substantial difference at the later phase between the control group (untreated) and the nanoparticle-treated group, suggesting that the nanoparticles of carbonate apatite might have negligible effect of toxicity.

Influences of HER2/ErbB2 knockdown and wild-type p53 expression on traditional anti-cancer drug-induced tumor regression

Conventional anti-cancer drugs, such as cisplatin, doxorubicin and paclitaxel with known therapeutic efficacy in malignant breast cancer [43-48] have limitations due to their toxic effects on normal cells and therefore the dosage of the drugs finally given to the patients is therapeutically insufficient leading to chemoresistance and tumor recurrence [49]. As a matter of fact, the majority of initially chemoresponsive tumors develop resistance to once effective chemotherapeutic agents [50] and a switch to other chemotherapy

regimens is ineffective because of the tumor's cross-resistance to multiple chemotherapy drugs [51]. Silencing of an oncogene and/or expression of a normal tumor suppressor gene could either play an additive role or exert a synergistic effect through a potential 'cross-talk' with an anti-cancer drug, augmenting the overall therapeutic efficacy. In order to explore whether the intratumoral delivery of nanoparticle-embedded ErbB2 siRNA and wild-type p53 gene could synergize with the anti-tumor effects of the conventional drugs, cisplatin was injected directly into the tumors growing in the mammary fat pad of Balb/c mice either in free form or together with the nanoparticle complex of ErbB2 siRNA/p53 plasmid. As shown in Figure 5, although free cisplatin demonstrated higher cytotoxic response than the nanoparticle complex of ErbB2 siRNA/p53 plasmid, however, cisplatin when treated along with the complex could not further enhance the effect ($p < 0.05$), suggesting a possible additive effect on the same population of tumor cells subjected to growth arrest or apoptosis. On the contrary, as for doxorubicin (Figure 6) as well as paclitaxel (Figure 7), the

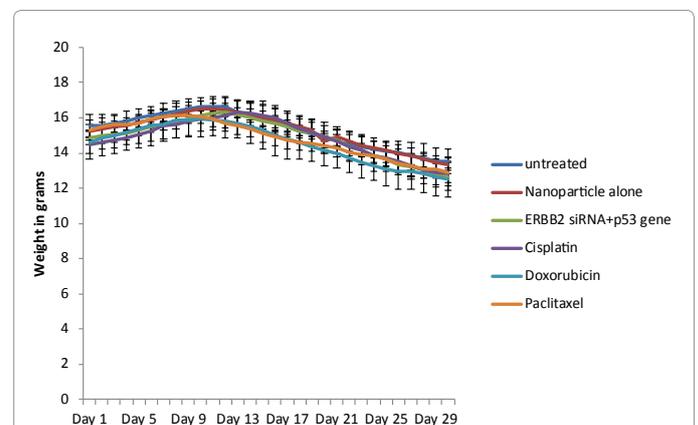


Figure 3: Effects on gross body weights of the mice group after intratumoral injection of nanoparticles alone, nanoparticle-loaded ERBB2 siRNA/p53 plasmid, cisplatin, doxorubicin and paclitaxel.

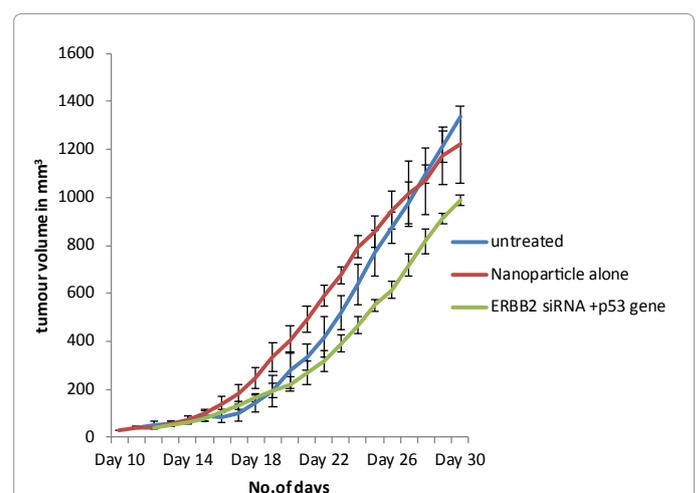


Figure 4: Effect on gross tumor volume regression following intratumoral injection of nanoparticle-associated ERBB2 siRNA and p53 plasmid. The mice bearing 4T1-induced mammary tumor were treated with 100 μ l of the particle suspension originally formulated in 1 ml of DMEM with 7 mM of Ca^{2+} in presence or absence of ERBB2 siRNA (800 nM) plus p53 (100 ng) or anticancer drugs (100 nM), through intratumoral injection and the body weight was measured throughout the experiment from day 1 to day 29.

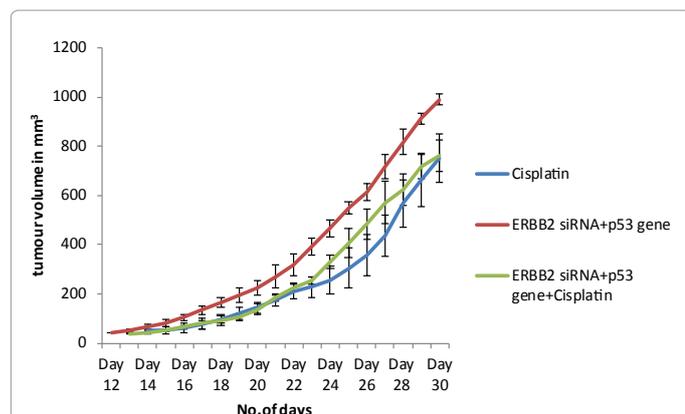


Figure 5: Effect on gross tumor volume regression following intratumoral injection of nanoparticle-associated ERBB2 siRNA and p53 plasmid in presence or absence of cisplatin. The mice bearing 4T1-induced mammary tumor were treated with 100 μ l of the particle suspension originally formulated in 1 ml of DMEM with 7 mM of Ca^{2+} in presence or absence of ERBB2 siRNA (800 nM) plus p53 (100 ng) and/or cisplatin (100 nM), through intratumoral injection and the tumor volumes were estimated according to the modified ellipsoidal formula: $1/2(\text{length} \times \text{width}^2)$.

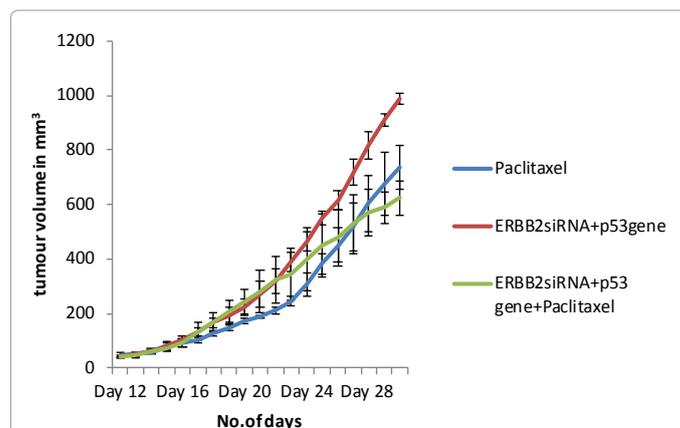


Figure 7: Effect on tumor volume reduction following intratumoral injection of nanoparticle-associated ERBB2 siRNA and p53 plasmid in presence of paclitaxel. The mice bearing 4T1-induced mammary tumor were treated with 100 μ l of the particle suspension originally formulated in 1 ml of DMEM with 7 mM of Ca^{2+} in presence or absence of ERBB2 siRNA (800 nM) plus p53 (100 ng) and/or paclitaxel (100 nM), through intratumoral injection and the tumor volumes were estimated according to the modified ellipsoidal formula: $1/2(\text{length} \times \text{width}^2)$.

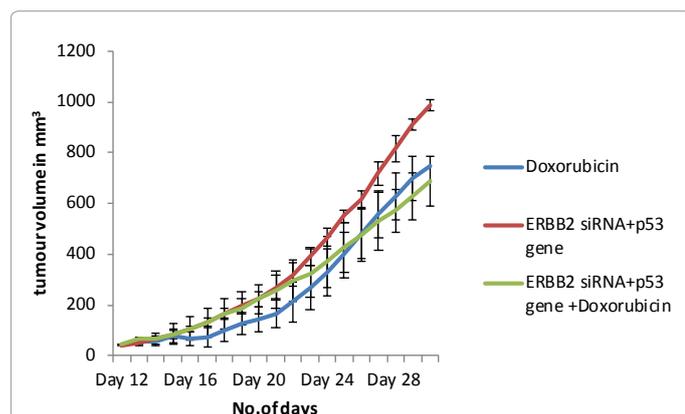


Figure 6: Effect on tumor volume reduction following intratumoral injection of nanoparticle-associated ERBB2 siRNA and p53 plasmid in presence of doxorubicin. The mice bearing 4T1-induced mammary tumor were treated with 100 μ l of the particle suspension originally formulated in 1 ml of DMEM with 7 mM of Ca^{2+} in presence or absence of ERBB2 siRNA (800 nM) plus p53 (100 ng) and/or doxorubicin (100 nM), through intratumoral injection and the tumor volumes were estimated according to the modified ellipsoidal formula: $1/2(\text{length} \times \text{width}^2)$.

nanoparticle complex of ErbB2siRNA/p53 plasmid apparently showed an inhibition on the growth of the tumors particularly at the later stage of the experimental period compared with the free individual drugs, suggesting a concerted role of ErbB2 knockdown and p53 expression in regression of tumor growth. This could be explained by the earlier findings that loss of p53 function in concert with ErbB2 expression (and/or lack of expression of bcl-2) might contribute to doxorubicin resistance [25] and MAP kinase cascades that can be stimulated by HER2/ErbB2 are essential for apoptotic response to paclitaxel-induced cell death while p53 could act as a survival factor in breast cancer cells treated with paclitaxel by blocking cells in G2/M phase of the cell cycle [52].

Conclusion

We have demonstrated that knockdown of ERBB2 gene and

expression of normal p53 gene could be an attractive approach in synergistic inhibition of the growth of the established tumor and even could make the tumor more sensitive to the conventional drugs, such as doxorubicin and paclitaxel.

Acknowledement

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Disclosure

The authors report no conflicts of interest in this work.

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