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13. ABSTRACT (Maximum 200 Words) In this project, we attempt to establish the utility of an antisense iron-responsive element (AS-IRE)-mediated gene expression system to targeting HER-2/neu-overexpressing breast cancer cells. During the first two years of funding, we have finished the proposed goal stated in Task 1 by identifying the "optimal" HER-2/neu antisense IRE, i.e., AS-IRE4. We demonstrated that AS-IRE4 could interact with IRP-1 and behave as a translational inhibitor when placed in the 5'UTR of a gene. In addition, we showed that, using hTERT-AS-IRE4-luc, AS-IRE4 can be regulated by iron in low HER-2/neu-expressing MDA-MB-468 cells but such regulation is impaired in high HER-2/neu-expressing MDA-MB-453 cells. Thus, this observation is consistent to our working hypothesis that AS-IRE4/IRP interaction would be abolished HER-2/neu-overexpressing cells. Therefore, our results strongly suggest that AS-IRE4 behaves as a functional IRE. Importantly, we showed that AS-IRE4 could preferentially direct gene expression in HER-2/neu-overexpressing breast cancer cells. In that, we demonstrated a preferential reporter gene expression of hTERT-AS-IRE4-luc. Moreover, we showed preferential cell killing in HER-2/neu-overexpressing MDA-MB-453 cells using hTERT-AS-IRE4-Bax as opposed to in low HER-2/neu-expressing MDA-MB-468 cells (Task 2). The identification of AS-IRE4 also facilitates our progress toward the final goal of this project, i.e., to test the therapeutic efficacy of the hTERT-AS-IRE4-luc in a pre-clinical gene therapy model (Task 3). We will use adenoviral vector and liposome as delivery systems to deliver hTERT-AS-IRE4-luc into the tumor-bearing mice. Treatment efficacy will be measured in terms of tumor-specific as well as HER-2/neu overexpression-specific targeting, tumor size, survival, and metastatic lesions. Treatment efficacy between liposome- and adenovirus-mediated gene therapy will also be compared.
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INTRODUCTION

The overexpression of HER-2/neu proto-oncogene has been found in a variety of human cancers. In particular, amplification and overexpression of the HER-2/neu gene were found in 20-30% of patients with breast cancer. These patients had decreased survival and increased relapse rates. Therefore, HER-2/neu overexpression has been used as a poor prognostic indicator for patients suffering from this disease. To develop an expression system that targets the breast cancer cells overexpressing HER-2/neu mRNA, a novel approach is described that combines the antisense principle and the biochemical property of a translation regulator, an iron-responsive element (IRE). IRE, when placed 5' to a gene, functions as a negative translation regulator in that IRE interacts with iron-regulatory proteins (IRPs) and this protein-RNA complex blocks translation (1). One way to alleviate this translation inhibition is to prevent the IRE/IRP interaction by disrupting the IRE stem-loop structure via a sense-antisense hybrid. Thus, a HER-2/neu antisense IRE (AS-IRE) possessing the IRE consensus sequence and functioning as a translation inhibitor was generated. When placed 5' to a reporter gene, AS-IRE could direct the reporter gene expression in breast cancer cells that overexpress HER-2/neu mRNA, since the IRE-mediated translation inhibition can be overcome by the overexpression of HER-2/neu mRNA. In this project, we attempt to establish the utility of this novel approach to targeting HER-2/neu-overexpressing breast cancer cells. Our goals are: (1) to obtain an optimal AS-IRE that directs the maximum expression of the reporter gene in HER-2/neu overexpressing breast cancer cells; (2) to demonstrate a preferential killing of HER-2/neu overexpressing breast cancer cells by using the optimal HER-2/neu antisense IRE to direct the expression of a toxin gene encoding diphtheria toxin A-chain (DT-A); and (3) to test the therapeutic effect of the AS-IRE-mediated DT-A expression vector *in vivo* by treating the mice that bear tumors with or without the overexpression of HER-2/neu gene.

BODY

(Figures are attached in the Appendices)

Task 1: To obtain an optimal HER-2/neu antisense IRE.

To identify potential AS-IREs that could direct preferential gene expression in HER-2/neu-overexpressing cancer cells, we scanned the antisense sequence of HER-2/neu cDNA and searched for regions that contain an IRE consensus sequence, 5'CNNNNNCAGUG^{3'}. Five elements on the HER-2/neu antisense sequence fulfilled the IRE consensus requirements, i.e., 458-479 (AS-IRE-1), 825-847 (AS-IRE-2), 1729-1750 (AS-IRE-3), 2904-2924 (AS-IRE-4), and 3193-3215 (AS-IRE-5). (The nucleotide numbering is based on the HER-2/neu cDNA sequence (2).) To test if AS-IREs could bind to IRP, we performed a competition assay in which the control was a radioactive IRE (cIRE) derived from the IREs located in 3'UTR of transferrin receptor mRNA (1), and that forms a complex with endogenous IRP in cell lysate isolated from MDA-MB-453 breast cancer cells (3, 4) (Fig. 1A, indicated by an arrow). This RNA/protein complex is indeed IRE/IRP complex since it can be competed away by excess (50-fold) amount of cold cIRE but not by mutant IRE (mcIRE) in which both bulge C in the stem and G at the fifth position in the loop are deleted. When excess amount of cold *in vitro* synthesized AS-IREs were added to the cIRE/IRP binding reactions, the cIRE/IRP complex was readily competed. This result suggests that these AS-IREs have maintained the integrity of both the sequence and stem-loop structure that are necessary for IRE/IRP interaction *in vitro* (5).

To functionally demonstrate an AS-IRE/IRP interaction-mediated translational inhibition, we placed the AS-IREs in the 5'UTR of a luciferase (luc) reporter gene to generate AS-IRE1 ~ 5-luc driven by the bacterial phage T7 promoter. We tested the *in vitro* translational inhibitory function of AS-IREs by adding recombinant IRP-1 to a transcription and translation coupling system (TNT) in wheat germ extract (WGE) that does not naturally contain IRP (6, 7). Like the positive control, cIRE-luc (Fig. 1B), a functional AS-IRE should allow translation of luc mRNA in WGE without IRP-1, but not with IRP-1. Although AS-IREs direct different level of basal luc activities, our result clearly shows that most AS-IRE-directed translation can be inhibited by IRP-1 with exception of AS-IRE2 and AS-IRE3 (Fig. 1B). The addition of IRP-1 has little effect on luc, the negative control. Interestingly, AS-IRE4 appears to be most sensitive to IRP-mediated translational inhibition with more than 18-fold reduction in translational efficiency (Fig. 1B). Therefore, we chose AS-IRE-4-Luc to further characterize the system.

Another prerequisite for a functional IRE is its ability to be regulated by iron. For example, IRE/IRP-directed translation is up-regulated in the presence of iron source, e.g., Hemin, but is down-regulated when iron is scarce, e.g., in the presence of an iron chelator, Desferrioxamine (Desf) (8). To test if AS-IRE4 is iron-regulated, we placed AS-IRE4-luc under the control of human telomerase reverse transcriptase (hTERT) gene promoter (9) that has been used to direct tumor-specific gene expression *in vitro* and *in*

vivo (10) and subsequently generated hTERT-AS-IRE4-luc and the control, hTERT-luc, that lacks AS-IRE4. We then transfected both constructs into a low HER-2/neu-expressing human breast cancer cell line, MDA-MB-468, with (Hemin) or without (Desf) iron, followed by a luciferase assay. As shown in Fig. 2, in the presence of iron, luciferase activity is about 1.5 fold higher than that in the absence of iron. As expected, hTERT-luc is not regulated by iron. Together, our results suggest that AS-IRE4 behaves as a canonical IRE in that it interacts with IRP-1 and is regulated by iron.

To test if AS-IRE4 could preferentially direct gene expression in a HER-2/neu overexpression-specific manner *in vivo*, we transfected hTERT-AS-IRE4-luc into either low (e.g., MDA-MB-468) or high (e.g., MDA-MB-453 which overexpresses 12~16-fold HER-2/neu mRNA (11)) HER-2/neu-expressing human breast cancer cell line. At first, since our model predicts a disruption of AS-IRE stem-loop structure in high HER-2/neu-expressing cells (12), we reasoned that the iron regulation would be subsequently impaired in AS-IRE-directed translation. To test that hypothesis, we transfected hTERT-AS-IRE4-luc into MDA-MB-453 cells with or without excess iron. As shown in Fig. 2, in contrast to that in MDA-MB-468 cells, no significant difference in AS-IRE4-directed luciferase activity with or without iron was observed in MDA-MB-453 cells. Again, hTERT-luc is not regulated by iron. Thus, this result supports the idea that AS-IRE/IRP interaction is disrupted in cells that overexpress HER-2/neu mRNA. Furthermore, our model also predicts a preferential gene expression on the translational level directed by AS-IRE in HER-2/neu-overexpressing cells (12). To test that possibility, we transfected hTERT-AS-IRE4-luc (or hTERT-luc) into MDA-MB-468 and MDA-MB-453 cells. Consistent to the translational inhibitory function of IRE, AS-IRE4 drastically reduces gene expression in either cell line, i.e., < 1% in MDA-MB-468 and < 5% in MDA-MB-453 (Fig. 3A). However, the relative luciferase activity of hTERT-AS-IRE4-luc in MDA-MB-453 is 6-fold higher than that in MDA-MB-468. The above observation suggests that AS-IRE4 is able to direct a preferential gene expression in HER-2/neu-overexpressing breast cancer cells.

Task 2: To obtain an optimal HER-2/neu antisense IRE-regulated toxin gene (DT-A).

With the identification of the optimal AS-IRE, i.e., AS-IRE4, we are in a position to generate a therapeutic gene expression vector directed by AS-IRE4. Due to the fact that many breast cancer cells are resistant to 5-FU, we have countered difficulties by using pro-drug approach such as YCD described in our previous progress report. Therefore, we chose, Bax (a pro-apoptotic protein in the Bcl-2 protein family (13)) as a potential therapeutic gene to achieve anti-tumor effect on both *in vitro* and *in vivo* cancer model systems (10). First, we tested if AS-IRE4 could direct Bax gene expression in such a way that would result in a preferential killing of HER-2/neu-overexpressing breast cancer cells. To do that, we placed Bax gene under the control of hTERT promoter with or without AS-IRE4 in 5'UTR to generate hTERT-AS-IRE4-Bax and hTERT-Bax, respectively. We transfected these constructs into either MDA-MB-468 or MDA-MB-453 cells, followed by TUNEL assay (14) to monitor the resulting apoptosis. Consistent

with the result from the reporter assay shown in Fig. 3A, we observed a significantly low Bax-induced cell killing by hTERT-AS-IRE4-Bax as compared with that by hTERT-Bax, i.e., ~10% in MDA-MB-468 and ~24% in MDA-MB-453 cells (Fig. 3B). Again, it supports the translational inhibitory function of AS-IRE4. However, there is more than 2-fold apoptosis caused by hTERT-AS-IRE4-Bax in MDA-MB-453 than that in MDA-MB-468 cells. This observation supports the hypothesis that hTERT-AS-IRE4-Bax could be used to achieve a preferential cell killing in breast cancer cells that overexpress HER-2/neu mRNA.

Task 3: To demonstrate the therapeutic effect of the HER-2/neu antisense IRE-mediated gene expression.

With a successful demonstration of preferential killing in HER-2/neu-overexpressing cells shown above (see **Task 2**), we will test the therapeutic efficacy of hTERT-AS-IRE4-Bax gene therapy treatment in an orthotopic breast cancer xenograft model. Two gene delivery systems will be employed: liposome and adenoviral vector. hTERT-AS-IRE4-Bax/liposome complex and adenovirus expressing hTERT-AS-IRE4-Bax will be delivered into the tumor-bearing mice. Following administration, we will measure the tumor size, survival, and metastatic lesions to determine the therapeutic efficacy of the AS-IRE-mediated gene therapy. Treatment efficacy between liposome- and adenovirus-mediated gene therapy will also be compared.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of AS-IRE4 as the "optimal" AS-IRE.
- Construction of hTERT-AS-IRE4-luc.
- Demonstration of iron-regulated gene expression using hTERT-AS-IRE4-luc.
- Demonstration of HER-2/neu overexpression-specific expression using hTERT-AS-IRE4-luc.
- Demonstration of HER-2/neu overexpression-specific cell killing using hTERT-AS-IRE4-Bax.

REPORTABLE OUTCOMES

- A manuscript describing the above observation has been submitted and is currently under review.

CONCLUSIONS

During the first two years of funding, we have finished the proposed goal stated in **Task 1** by identifying the “optimal” HER-2/neu antisense IRE, i.e., AS-IRE4. We demonstrated that AS-IRE4 could interact with IRP-1 and behave as a translational inhibitor when placed in the 5'UTR of a gene. In addition, we showed that, using hTERT-AS-IRE4-luc, AS-IRE4 is regulated by iron in low HER-2/neu-expressing MDA-MB-468 cells but such regulation is impaired in high HER-2/neu-expressing MDA-MB-453 cells. Thus, this observation is consistent to our working hypothesis (12) that AS-IRE4/IRP interaction would be abolished HER-2/neu-overexpressing cells. Therefore, our results strongly suggest that AS-IRE4 behaves as a functional IRE. Importantly, we showed that AS-IRE4 could preferentially direct gene expression in HER-2/neu-overexpressing breast cancer cells. In that, we demonstrated a preferential reporter gene expression of hTERT-AS-IRE4-luc. Moreover, we showed preferential cell killing in HER-2/neu-overexpressing MDA-MB-453 cells using hTERT-AS-IRE4-Bax as opposed to in low HER-2/neu-expressing MDA-MB-468 cells (**Task 2**). The identification of AS-IRE4 also facilitates our progress toward the final goal of this project, i.e., to test the therapeutic efficacy of the hTERT-AS-IRE4-luc in a pre-clinical gene therapy model (**Task 3**). We will use adenoviral vector and liposome as delivery systems to deliver hTERT-AS-IRE4-luc into the tumor-bearing mice. Treatment efficacy will be measured in terms of tumor-specific as well as HER-2/neu overexpression-specific targeting, tumor size, survival, and metastatic lesions. Treatment efficacy between liposome- and adenovirus-mediated gene therapy will also be compared.

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APPENDICES

Legends to figures

Fig. 1. (A) AS-IREs interact with cellular IRP. The radioactive-labeled cIRE probe (2 ng) incubated with the MDA-MB-453 cell lysate (5 μ g) serves as the positive control (0). The cIRE/IRP complex resolved on a 6% native PAGE is indicated by an arrow. The competition assay was performed by incubating fifty-fold (100 ng) of cold cIRE, mcIRE, or AS-IRE1 ~ 5 with the positive control. (B) Recombinant IRP-1 inhibits AS-IRE-mediated translation *in vitro*. One microgram of linearized luciferase reporter gene (luc, AS-IRE1~5-luc, or cIRE-luc) driven by T7 promoter was used in the *in vitro* transcription and translation (TNT) system (Promega) in wheat germ extract with or without purified recombinant IRP-1 protein (1.6 μ g). The luciferase activity was measured by a luminometer. The relative luciferase activity is shown here and it was determined by setting the activity of luc without IRP-1 at 100%.

Fig. 2. AS-IRE4-mediated translation is regulated by iron. hTERT-IRE4-luc (IRE4-luc) or hTERT-luc (luc) was transfected into either MDA-MB-453 or MDA-MB-468 cells in the presence of 50 μ M Hemin or 50 μ M Desferrioxamine (Desf). The luciferase activity was measured 48 h after transfection. The internal control, pRL-TK, was used to normalize transfection efficiency. The relative luciferase activity of hTERT-IRE4-luc or hTERT-luc in the presence of Hemin was measured against that in the presence of Desf (100%) in each cell line.

Fig. 3. (A) AS-IRE4 mediates preferential gene expression in HER-2/neu-overexpressing breast cancer cells. hTERT-luc and hTERT-IRE4-luc were transfected into either MDA-MB-453 or MDA-MB-468 cells. The luciferase activity was measured 48 h after transfection and the internal control, i.e., pRL-TK, was used to normalize the transfection efficiency. The relative luciferase activity of hTERT-IRE4-luc was measured against the activity of hTERT-luc (100%) in each cell line. (B) AS-IRE4 mediates preferential cell killing in HER-2/neu-overexpressing breast cancer cells. hTERT-Bax and hTERT-IRE4-Bax were transfected into either MDA-MB-453 or MDA-MB-468 cells (1×10^6 cells per 60 mm dish). Forty-eight hours after transfection, cells were harvested into a cytospin chamber. TUNEL assay was then performed to score the apoptotic cells under 40 \times magnification from ten random fields with total more than 300 cells. Each sample was counted twice. The percentage of apoptotic cell per field was calculated and the average of ten fields is presented. The relative cell killing of hTERT-IRE4-Bax is measured against that of hTERT-Bax (100%) in each cell line.

Fig. 1

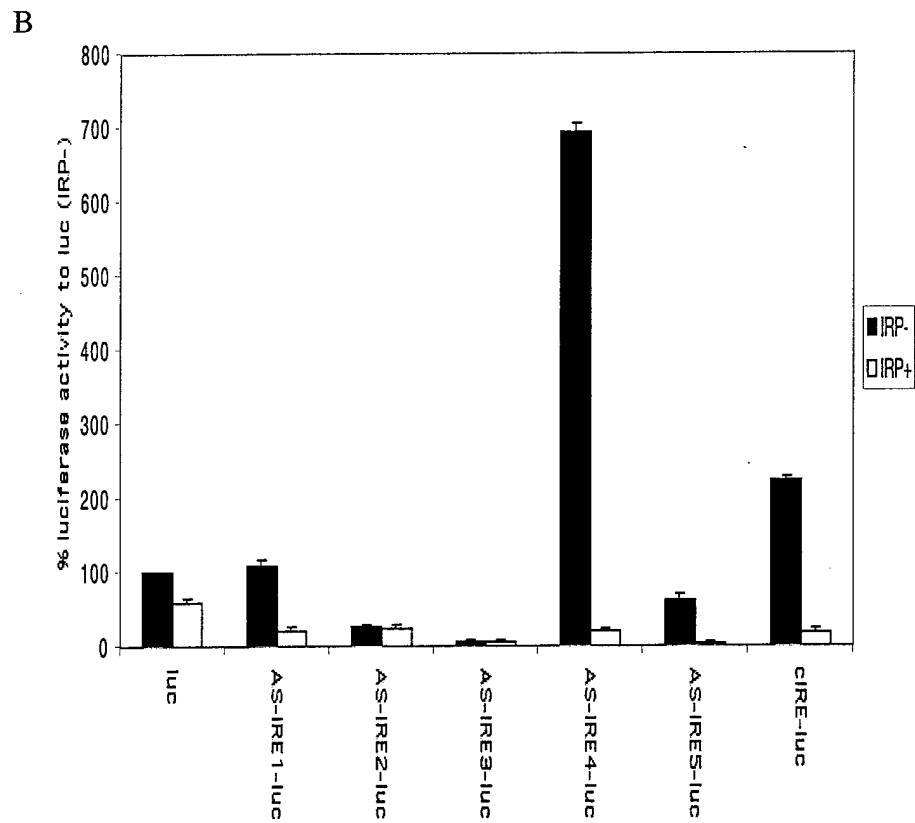
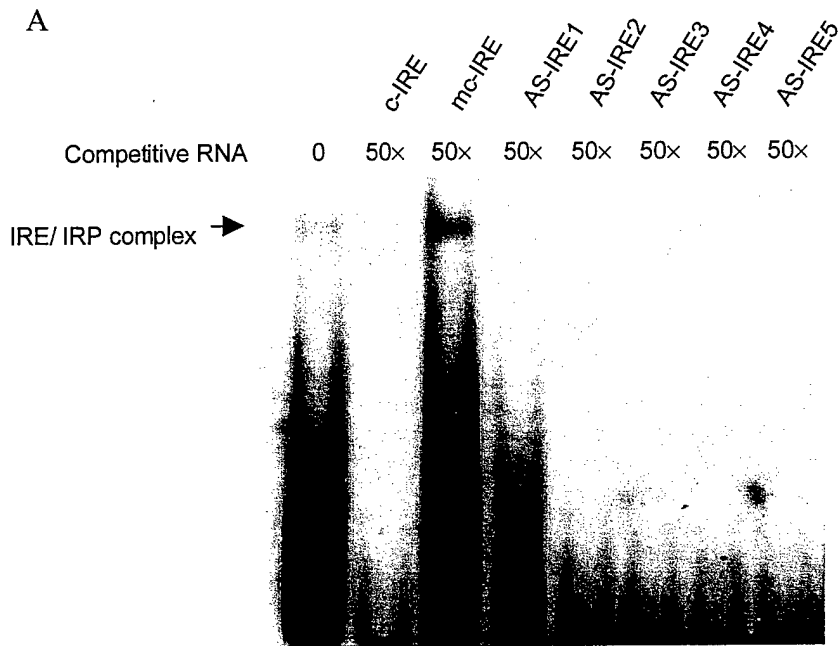


Fig. 2

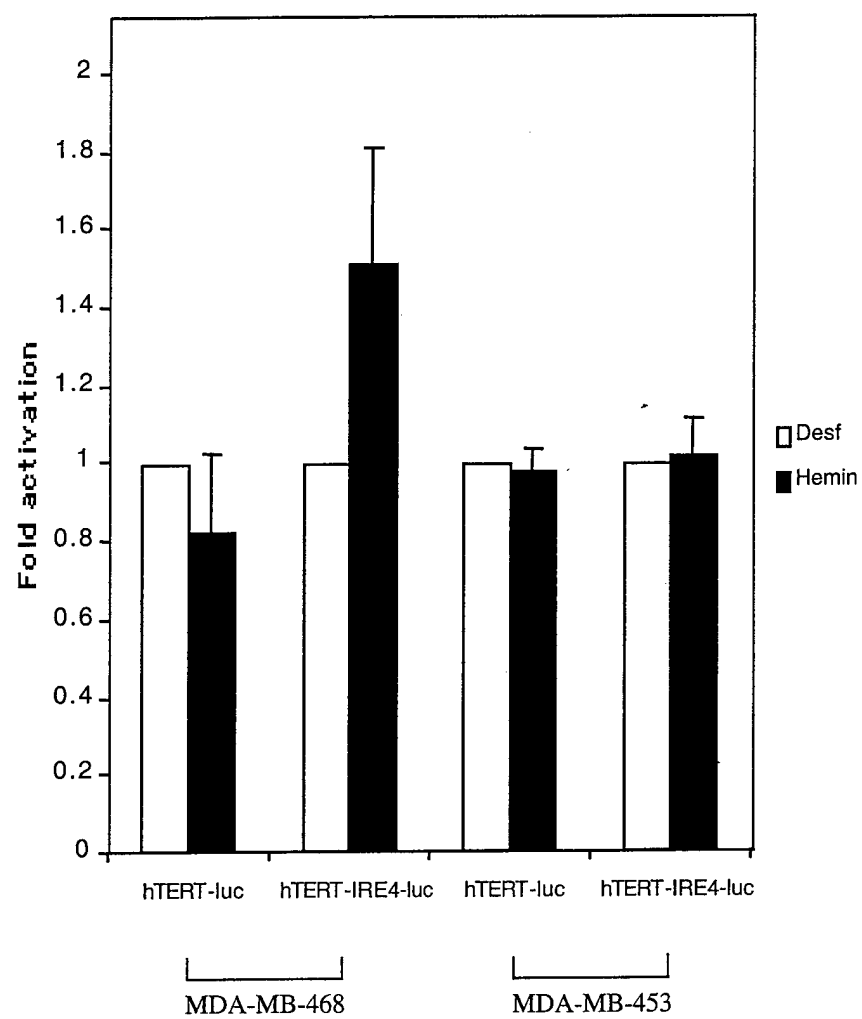


Fig. 3

