SELENOF is a New Tumor Suppressor in Breast Cancer

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Abstract

Epidemiological data has shown a protective role for selenium and cancer, including breast cancer. Selenium gets incorporated into selenoproteins, which are thought to mediate the protective effects of selenium. While some selenoproteins are not affected by selenium status, SELENOF (formerly referred to as SEP15) levels are responsive to selenium dietary intake and availability. Thus, lower levels of SELENOF due to low selenium status may be of consequence in breast cancer. Among the 25 human selenoproteins, SELENOF is implicated in breast cancer based on our initial observation of loss of heterozygosity at the SELENOF locus in tumor samples from breast cancer patients. These data included here indicate that SELENOF is a new candidate tumor suppressor in breast cancer.

Objectives

- Identify SELENOF mRNA levels in aggressive late-stage breast tumors and observe SELENOF levels prediction of patient outcome.
- Identify SELENOF levels by ectopic expression significance in breast cancer cell viability, and in vivo tumor growth.
- Indicate that SELENOF is a new candidate tumor suppressor in breast cancer.

Materials & Methods

Reagents: Doxycycline (Dox), 4-hydroxytamoxifen (4OHT) and fulvestrant (ICI), propidium idiodide and Annexin V from Sigma. Antibody for SELENOF from AbCam. Antibody for β-actin from Sigma. siRNA for SELENOF and lipofectamine RNAiMAX from ThermoFisher Scientific.

Cell lines, culture conditions and treatments: Several types of cells were used for this study, including MCF-7, T47D, BT474, LTED, MDA-MB-231, MDA-MB-468, and MCF-7 SELENOF cells. ER+ breast cancer cells were maintained in RPMI 1640 media with phenol red supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 2 mmol/l L-glutamine, 1% antibiotics penicillin-streptomycin and 6 ng/ml insulin. MDA-MB-468 cancer cells were maintained in RPMI 1640 media with phenol red supplemented with 10% FBS, 1% nonessential amino acids, 2 mmol/l L-glutamine and 1% penicillinstreptomycin. MDA-MB-231 cancer cells were maintained in RPMI 1640 media with phenol red supplemented with 5% FBS, 1% non-essential amino acids, 2 mmol/l L-glutamine, and 1% antibiotics penicillinstreptomycin.

Clonogenic assay: Cells were seeded at 1,000 cells per well in 6-well plates, treated with Dox for 2 weeks, harvested and fixed using Crystal Violet staining solution, and imaged using ImageJ software.

Cell viability: Cells were seeded at 20,000 cells per well in 24-well plates, treated with Dox, for 10 days, and then fixed using Crystal Violet staining solution. Fixed cells were later solubilized in 1% Sodium Dodecyl Sulfate (SDS) Solution, and absorbance was measured at 562 nm.

FACS analysis: Cell Cycle Analysis: After overnight attachment, cells were treated with 1 ug/ml Dox for 5 days. The cells were then fixed in 70% ice-cold methanol and sorted by FACS Canto.

Measurement of Cell Death: Live cells were stained with annexin V-Alexa-488-conjugated antibody and PI, and analyzed on a FACS Canto instrument.

Western blot: Whole cell extracts were prepared using the M-PER reagent, run on a 4-12% gradient gel using PAGE electrophoresis, transferred to nitrocellulose membranes, blocked overnight in TBS/T buffer containing 5% non-fat dry milk, and incubated overnight with appropriate primary and secondary antibodies

RT-quantitative PCR (QPCR): Total RNA was isolated from cells using TRIzol and reverse transcribed using M-MLV reverse transcriptase. The resulting cDNA was mixed with SYBR Green Master mix and forward and reverse primers, and amplifications were performed using a QuantStudio3 instrument.

In vivo Studies: In mice, 5 million MCF-7 cells were injected into the thoracic mammary glands and mice were supplemented with estrogen pellets. Mice were randomized into either vehicle control (n = 4 mice, 8 tumors) or doxycycline treatment (n = 4 mice, 8 tumors), and tumor size was measured 3 times per week with an electronic caliper.

Statistical analysis: Data from three independent determinations were analyzed using GraphPad Prism 9.0 (GraphPad Software).

Results

SELENOF expression is reduced in breast tumors and predicts poor clinical outcome

Figure 1.

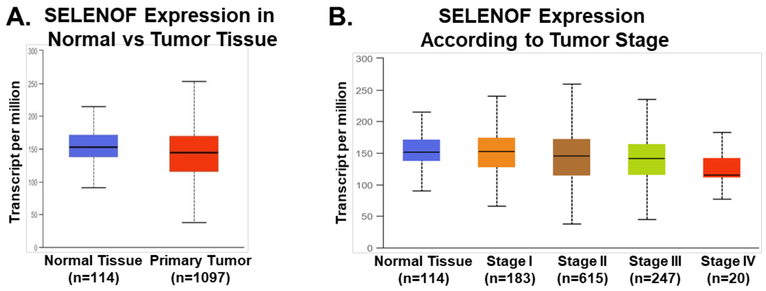

Figure 1. SELENOF expression is lower in breast tumors and inversely correlates with tumor stage. (A) SELENOF expression in breast tumors was examined using the Cancer Genome Atlas (TCGA) database. SELENOF mRNA expression is lower in primary breast tumors vs normal breast tissue, p=7.69E-2. (B) SELENOF mRNA expression according to breast tumor stage is indicated. Normal tissue vs tumor stage I, p=4.21E-1; Normal tissue vs tumor stage II, p=2.26E-1; Normal tissue vs tumor stage III, p=3.43E-2; Normal tissue vs tumor stage IV, p=2.92E-3.

Results

Figure 2.

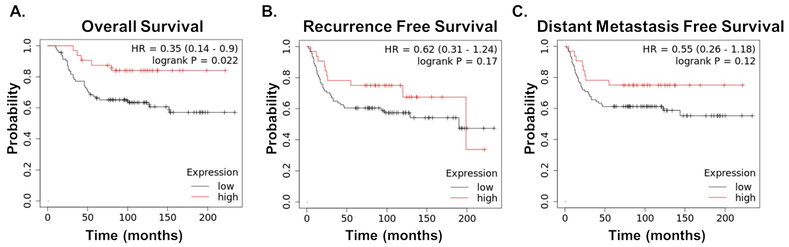

Figure 2. SELENOF levels are inversely associated with poor clinical outcome. Kaplan-Meier curves for (A) overall survival, (B) recurrence-free survival and (C) distant metastasis-free survival were generated using KMplotter, n=126 samples (kmplot.com).

SELENOF overexpression attenuates cell viability, reduces clonogenic survival, and enhances therapeutic response

Figure 3.

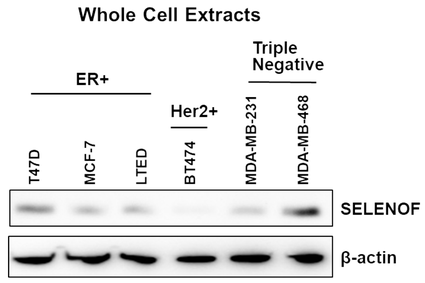

Figure 3. SELENOF is low in human breast cancer cell lines. SELENOF levels were profiled by western blotting in whole cell extracts from various human breast cancer cell lines. β-actin served as a loading control.

Figure 4.

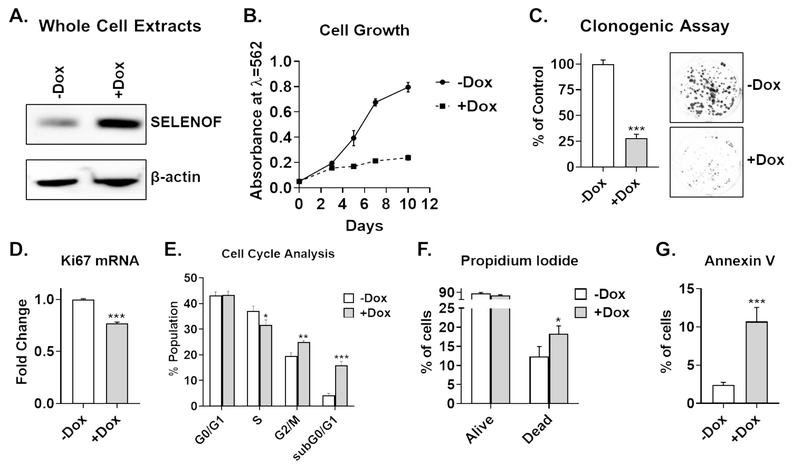

Figure 4. SELENOF overexpression attenuates breast cancer cell growth by reducing proliferation and inducing cell death. (A) Western blot for SELENOF levels in MCF-7 SELENOF cells treated -/+ doxycycline (Dox, 1μg/ml) for 72hrs. MCF-7 cells were stably transfected with a Dox-inducible SELENOF expression construct. β-actin served as a loading control. (B) Viability assay of MCF-7 SELENOF cells treated -/+ Dox for 10 days. Cells were fixed, stained with crystal violet, solubilized in 1% SDS, and quantified as absorbance at 562nm. (C) Clonogenic assay was conducted in MCF-7 SELENOF cells treated twice weekly -/+ Dox for 2 weeks. Dissociated single cells were seeded at clonogenic density. Colonies were fixed and stained with crystal violet and quantified by ImageJ. Data was normalized to no treatment controls shown as 100%. Representative pictures are shown (right panel). (D) The proliferation marker Ki67 was measured by RT-QPCR in cells treated as in (A). (E-G) FACS analysis was conducted in MCF-7 SELENOF cells treated -/+Dox for 5 days to measure (E) the cell-cycle distribution of each gated sub-G0/G1, G0/G1, S and G2/M phases , and (F) cell death measured by PI staining, and by (G) Annexin V staining. * p<0.05, ** p<0.01, *** p<0.001.

Results

Figure 5.

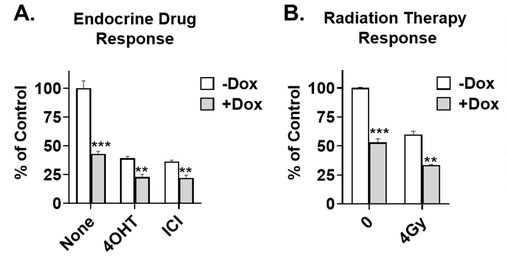

Figure 5. SELENOF overexpression sensitizes breast cancer cells to endocrine therapy and radiotherapy. MCF-7 SELENOF cells were treated in estrogenized media with -/+Dox for 2 days prior to adding (A) 4OHT or ICI, 1μM each, or (B) 4Gy radiation, single dose, and then incubated for another 3 days. Cells were fixed and stained with crystal violet. Data was normalized to no treatment controls shown as 100%. ** p<0.01, *** p<0.001.

SELENOF overexpression blocks xenograft tumor growth

Figure 6.

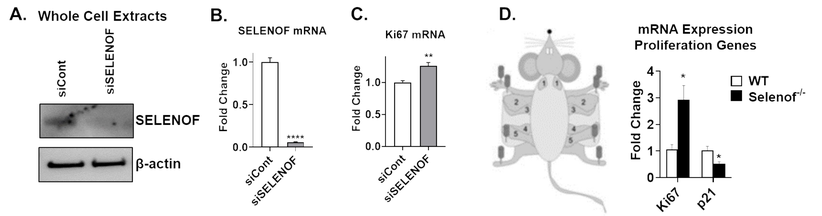

Figure 6. Silencing SELENOF produces the opposite effect to SELENOF overexpression. (A-B) SELENOF (A) protein level by western blot and (B) gene expression by RT-QPCR in MDA-MB-468 cells transfected with 10nM SELENOF siRNA or siControl for 48 hrs are shown. (C) The proliferation index Ki67 was measured by RT-QPCR in cells treated as (A). (D) The ki67 and p21 mRNA levels in Selenof$^{-/-}$ murine mammary glands vs age-matched C57Bl5 wild type (WT) murine mammary glands (n=4 per group) were measured by RT-QPCR. * p<0.01, ** p<0.005, *** p<0.001.

Figure 7.

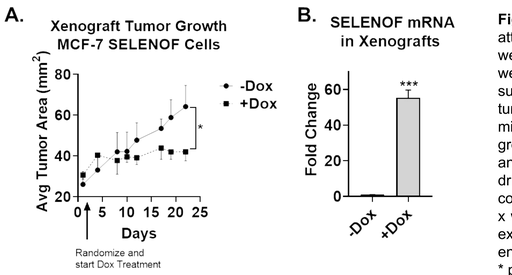

Figure 7. SELENOF overexpression attenuates xenograft tumor growth. (A) Cells were injected into the mammary glands of 5-week old athymic nude female mice supplemented with estrogen pellets. Once tumors were established (~100 mm^3 in size), mice were randomized into two treatment groups (n=4 mice, 8 tumors each): Control and Dox, which was administered in the drinking water (2mg/ml). End-point comparison of mean tumor volumes (length/2 x width^2 x π) is shown. (B) SELENOF mRNA expression in xenograft tumors excised at the end of study measured by RT-QPCR.
* p<0.01, *** p<0.001.

Conclusions

- Using *in vitro* cell models and an *in vivo* xenograft model, data was generated indicating that enhancing the expression of SELENOF attenuates cell and tumor growth by reducing proliferation and increasing cell death.
- Annexin V staining and cell cycle analysis indicated that the observed cell death was due, at least in part, to increased apoptosis.
- SELENOF ectopic expression enhanced the therapeutic responses to endocrine drugs or radiation.
- Altogether, our data indicate that SELENOF loss is likely to promote breast cancer progression and SELENOF can function as a tumor suppressor.

Future Research

SELENOF and racial disparity in breast cancer

- Lower selenium status among African Americans and genetic predisposition to reduced SELENOF levels together may contribute to aggressive disease and increased mortality experienced by this population. As a result, strategies to restore SELENOF expression can be exploited to elicit anti-tumor activities in a subset of breast cancers, particularly affecting African American women.