UNIVERSITY OF CALIFORNIA SAN DIEGO

Long non-coding RNA 152 Protects Breast Cancer Cell from DNA Damage

A Thesis submitted in partial satisfaction of the requirements

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in

Biology

by

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The Thesis of Jiani Wang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2020

EPIGRAPH

I am among those who think that science has great beauty.
A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. We should not allow it to be believed that all scientific progress can be reduced to mechanisms, machines, gearings, even though such machinery has its own beauty.

Marie Curie

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LIST OF ABBREVIATIONS

PARP	Poly-ADP-ribose polymerase
DSBs	Double-strand DNA breaks
MRN	MRE11/RAD50/NBS1 complex
ATM	Ataxia-Telangiectasia Mutated
γH2AX	Phosphorylated Histone H2AX at Serine 129
53BP1	p53 Binding Protein
BRCA1	Breast Cancer Type 1 Susceptibility Protein
CHK2	Checkpoint Kinase 2
NHEJ	Nonhomologous End-joining
HR	Homologous Recombination
Ku70-Ku80	ATP-dependent DNA Helicase 2 Subunit Ku70/80-like Proteins
LncRNA	Long non-coding RNA

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This thesis in full is unpublished material that will be prepared for submission for publication of the material. Chunyu Jin, Jiani Wang. The thesis author was the primary investigator and author of this material.

ABSTRACT OF THE THESIS

Long non-coding RNA 152 Protects Breast Cancer Cell from DNA Damage

by

Jiani Wang

Master of Science in Biology

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Professor Michael G. Rosenfeld, Chair
Professor Stephen M. Hedrick, Co-Chair

Genomic instability is a hallmark of cancer. Inducing severe DNA damage and augmenting apoptosis to destroy cancer cells is thus a major chemotherapy approach, specifically for Triple Negative Breast Cancer cell (TNBC cell). We have uncovered a previously unrecognized role for a specific long non-coding RNA 152 (*lncRNA152*) induced by DNA damage reagent and function in protect genome stability, significantly

overexpressed in TNBC cell. Our studies provide preliminary data that *lncRNA152* overexpressed in TNBC cell and could potentially serve as a TNBC diagnostic biomarker. *lncRNA152*-depleted cells are hypersensitive to the DNA damage inducer induced by the topoisomerase II inhibitor doxorubicin, possibly by interfering with DNA damage repair pathway. *lncRNA152* expression levels increase in response to DNA damage and are significantly higher in TNBC, potentially prevented apoptosis in these tumor cells.

We conclude that *lncRNA152* could serve as a TNBC diagnostic biomarker and potentially protects TNBC cells from DNA damage, perceptively providing a powerful approach to improve chemotherapy response.

Introduction

Genomic instability is a hallmark of cancer, and inducing severe DNA damage and augmenting apoptosis to destroy cancer cells is thus a major chemotherapy approach. DNA-damage-based chemotherapy is one of the major successful strategies for treating TNBC and other type of metastatic breast cancers that are no longer sensitive to endocrine therapy; It also is proving to be an adjuvant therapeutic strategy for many ER-positive breast cancer patients after surgery [1-3]. Indeed, PARP inhibitors, one of the main drugs developed for breast cancer, also functions in inhibiting repair system to augment DNA damage. Poly-ADP-ribose polymerase 1 (PARP-1) and PARP-2 are DNA damage sensors that are most active during S-phase of the cell cycle and function in DNA damage repair[4, 5]. PARP inhibitors are particularly effective in breast cancer patients with BRCA1 or BRCA2 mutations [6, 7]. Collectively, inducing severe DNA damage and augmenting apoptosis to destroy cancer cells is thus a major approach for treating TNBC cells[8].

Double-strand DNA breaks (DSBs) are known as the most fatal type of DNA damage. In the cells, the stress responsive pathway which is induced after DSBs are called DNA damage response (DDR). Following DNA damage, DSBs are sensed by the MRE11/RAD50/NBS1 (MRN) complex that recruits and activates a principal kinase named ataxia-telangiectasia mutated (ATM). The activation of ATM will auto-phosphorylate itself and leads to the phosphorylation of histone H2AX at serine 129(γH2AX). γH2AX will recruit additional phosphorylated ATM and other DDR factors such as p53 binding protein(53BP1), breast cancer type 1 susceptibility protein (BRCA1) and checkpoint kinase 2 (CHK2). The accumulation of DDR factors results in generation of cytological detectable nuclear foci [9]. There are two main repair pathways responding to DSBs: the error-prone pathway called non-homologous end-joining (NHEJ) and the more faithful pathway called homologous

recombination (HR). For NHEJ pathway, the ATP-dependent DNA helicase 2 subunit Ku70/80-like proteins (Ku70-Ku80) heterodimer (Ku) hold the DSB ends in close vicinity to enhance their direct ligation. The clamp-like complex is arranged and then DNA-dependent protein kinases, catalytic subunits (DNA-PKcs) are recruited to the damaged site. A number of other proteins, such as Artemis, DNA ligase IV, X-Ray repair cross complementing 4 (XRCC4) and XRCC4-like factor (XLF), gather with the Ku80, Ku70 and DNA-dependent protein kinase (DNA-PK) complex and participate in the DNA repair pathway. For HR, this more precise repair mechanism recruited when the DSB is excised by nucleases and helicases, making 3' single-stranded DNA (ssDNA) overhangs onto which the RAD51 recombinase accumulates. This construction can attack homologous duplex DNA to employ it as a template for DNA repair [10, 11]. Overall, NHEJ participates in DSB repairing preliminarily in G1 phase while HR is more active in S phase and G2 phase (Figure 1).

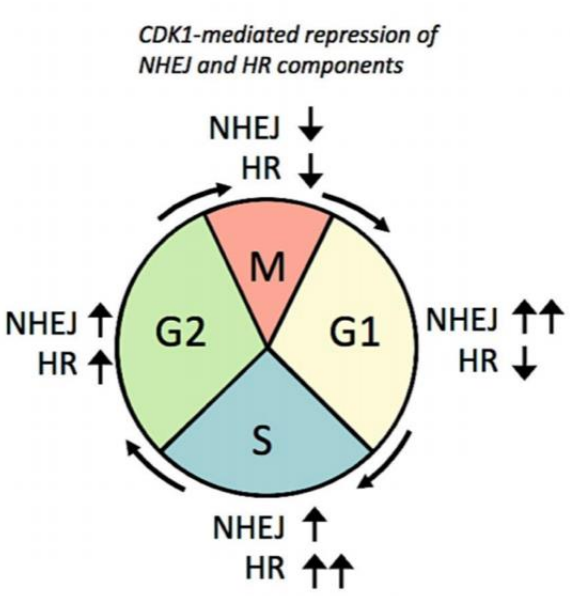


Figure 1: Double Strand DNA Repairing Method Activity in Different Phase. Homologous recombination (HR) repair activates the DNA damage checkpoint. Double-strand breaks formed in S or G2 phases are processed for HR repair. NHEJ is most activated in G1 phases.

From previous studies, we discovered many protein mechanisms involved in DNA

damage response. Lately, some scientists found some mRNA-like transcripts that do not code for any proteins are functioning in the DNA damage response. In general, most of mRNA-like transcripts are over 200 nucleotides long in length and have undiscovered functions such as regulating gene expression, telomere length, chromatin rearrangement, histone modification, modification of alternative splicing, dosage compensation, genomic imprinting and cell differentiation [11-14]. Their functions make them different from classic non-coding RNA, so they are categorized into a new group of RNA, called long non-coding RNA (*lncRNA*). Long non-coding RNAs have now been extensively proven to serve as functionally important molecules that some of them are involved in DNA damage response (DDR). The first research that discovered the relations between long non-coding RNA and DNA damage response claimed that long non-coding RNA can detect the transcript produced upstream of the cyclin D1 promoter. This detection follows DNA damage and binds to a DNA repairing factor that is RNA-binding protein, results in inhibiting histone acetyl transferase CREB-binding protein (CBP)/p300 and down-regulation of CCND1, a cell cycle regulator [15]. Following the first discovery of long non-coding RNA in DDR response, many studies have proved the role of long non-coding RNA in DSB repairing.

We have now identified a long non-coding RNA (*lncRNA152*), induced by DNA damage reagent and function in protect genome stability, significantly increased in breast cancer. Oxidative DNA damage has been suggested to be a biomarker of cancer risk[43-45], and numerous studies indicate *lncRNAs*, *mRNA* or *piRNA* could serve as cancer biomarker[46-73], in particular, the long non-coding RNA we study in this proposal, *lncRNA152*, was recently found to be a novel biomarker for diagnosis and monitoring of non-small-cell lung cancer[74]. Here, we will investigate the possibility of this long non-coding RNA as a biomarker for breast cancer, and as a potential therapeutic target. We have

preliminary data that *lncRNA152* functions to protect genomic stability and inhibit rearrangement. Indeed, knockdown of *lncRNA152* enhances doxorubicin induced cell death. *lncRNA152* expression levels increase in response to DNA damage, and are significantly higher in TNBC, which is likely to be a mechanism favoring cancer cell survival. We can suggest that this specific long non-coding RNA, *lncRNA152* functions to attenuate DNA repair / genome stability maintenance recommending its inhibition as a chemotherapy sensitizer strategy [16].

Results

TNBC-associated *lncRNA152* potentially serve as a TNBC diagnostic biomarker

To identify the potential functional non-coding RNAs involved in breast cancer disease, we mined microarray data of the 70 pairs of breast tumor/ normal tissue gene expression from the Cancer Genome Atlas (ATCC), and clustered the correlated long non-coding RNA according to the expression fold change value of tumor vs. normal in each pair. The most significant positive correlated *lncRNA* appeared in this analysis was *lncRNA152* (p=9.81E-08), regardless of ER, PR, or Her2 status (Figure 2).

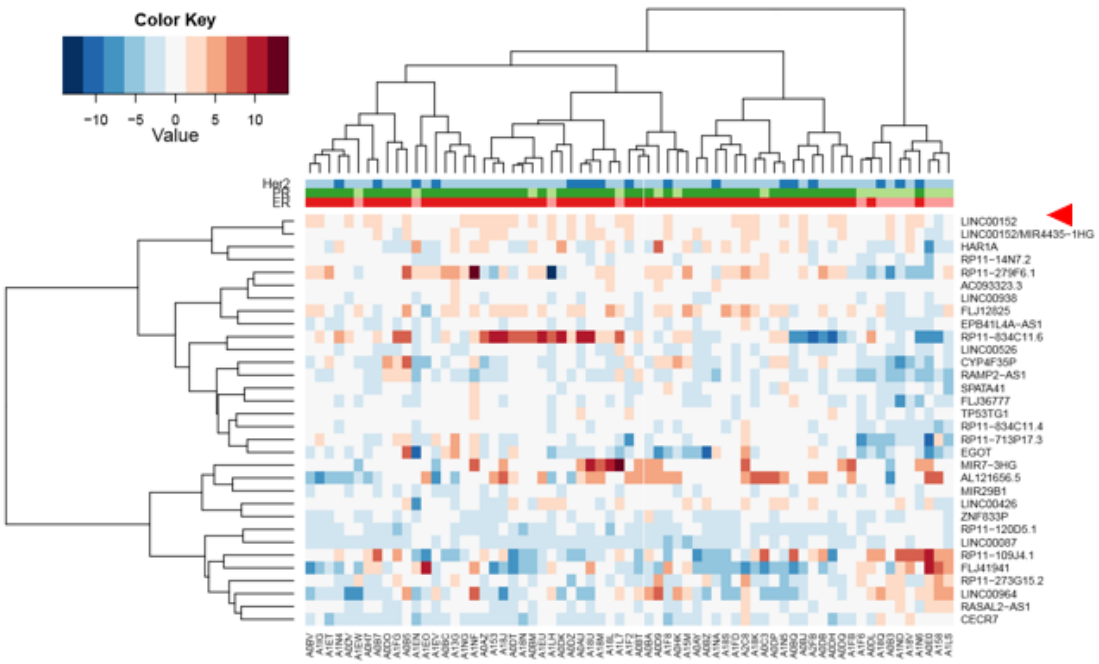


Figure 2: Heatmap of *lncRNA* Expression Microarray Data
Long non-coding RNAs expression level from 70 pairs of breast cancer vs normal tissue microarray data from The Cancer Genome Atlas (TCGA).

lncRNA152 is located at 2p11.2, which harbors three annotated transcript:NR_024204 (806bp), NR_024205 (642bp), and NR_024206 (496bp), sharing a common start and end sequence, allowing us to detect the relative abundance of each isoform by PCR amplification in MDA-MB-231 triple negative breast cancer cell line. NR_024206 (496n) is by far the dominant form. In agreement of more aggressiveness of triple negative type, we found *lncRNA152* expression was significantly higher in triple negative MDA-MB-231 cells compared to breast fibrocystic disease or ER+ breast cancers (Figure 3A). In addition, we compared the expression level with that in prostate cancer cell lines LNCaP, LNCaP-cds1, and CWR22Rv1, and find that they were all much lower than that in MDA-MB-231 cells, indicating that *lncRNA152* is a triple negative breast cancer cell line overexpressed long non-coding RNA(Figure 3B).

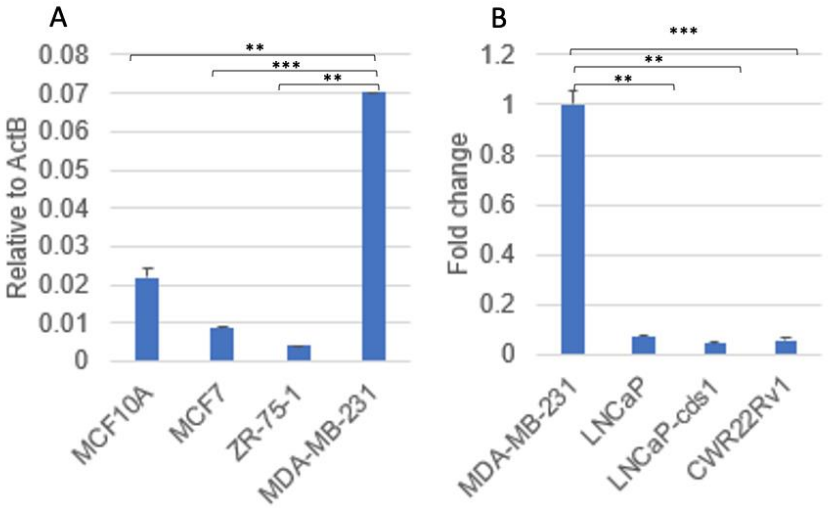


Figure 3: Robust Expression of *lncRNA152* MDA-MB-231 TNBC Cell Line.
(A) RT-qPCR of examining *lnRNA152* expression level in non-tumorigenic epithelial cell line MCF10A and breast cancer cell lines: MCF7, ZR-75-1, and MDA-MB-231. The expression levels are normalized relative to expression level of actin. (B) RT-qPCR of *lnRNA152* expression level in breast cancer cell line MDA-MB-231 and prostate cancer cell line LNCaP, LNCaP-cds1, and CWR22Rv, normalized by fold change of expression level in MDA-MB-231. Data represent mean ± STEV. *P<0.05; **P<0.01, ***P<0.001.

LncRNA152 Functions in Genome Stability Protection

A central question is whether *lnc*RNA152 will be a manipulabile determinant of DNA damage and apoptosis in breast cancer therapy. We performed RT-qPCR to measure the expression level of *lncRNA152* in MDA-MB-231 cells after 24 hours of 1uM doxorubicin treatment (Figure 4). Doxorubicin was used to generate DNA damage since it is a type of chemotherapy drug that damages DNA by inhibiting topo isomerase II. Comparing the normal condition without doxorubicin treatment, the expression level of *lnRNA152* is significantly high relatively to the expression level of actin in the MCF7 cells. Isoform NR_024204 of *lncRNA152* was also measured in this experiment, and the expression level after doxorubicin treatment did not increase as *lnRNA152* did (Figure 4). A positive control group was set up by measuring the expression level of GADD45a, in which GADD45a is a (Growth Arrest and DNA Damage Inducible Alpha) is a protein coding gene that normally gets higher expression after DNA damage.

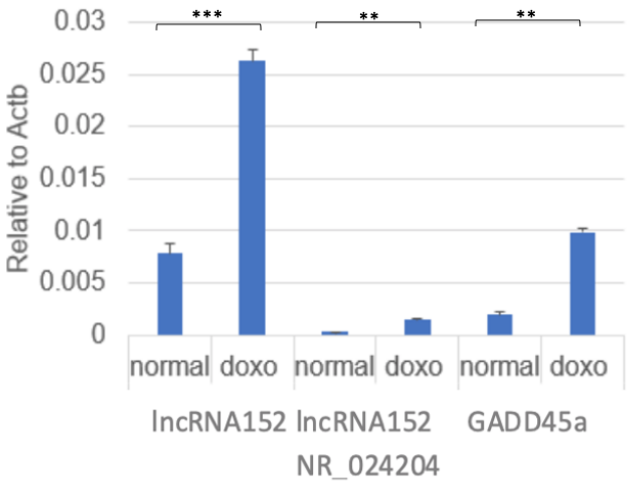


Figure 4: Increased Expression of *lnRNA152* Induced by DNA Damage.
lncRNA152 and its isoform NR_024204 are induced by doxorubicin(doxo), measured by RT-qPCR of -/+1uM doxo-treated MCF7 cells for 24h. Positive control with measuring GADD45a was set up. Data represent mean ± STEV. *P<0.05; **P<0.01, ***P<0.001.

To initiate investigation of *lncRNA152*'s role in DNA damage, MDA-MB-231 cells were treated with 1nM doxorubicin for 6 hours, following pre-transfection of *lncRNA152* siRNA or control siRNA, and gammaH2AX levels were detected by western blot. As expected, knockdown *lncRNA152* resulted in increased level of γH2AX (Figure 5A). To confirm this result, we evaluated the time course following treatment of doxorubicin, and found that γH2AX level was consistently higher 4 hours and 8 hours following *lncRNA152* knockdown condition compared to control (Figure 5B), suggesting it protects against DNA from damage from genotoxic stress. We also use 2 siRNAs to confirm this conclusion (Figure 5C, 5D).

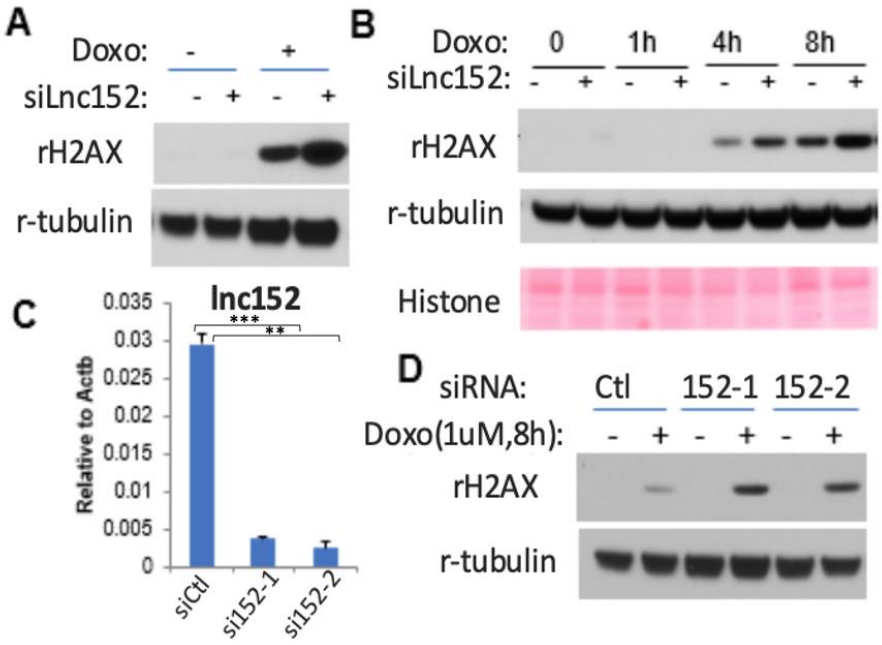


Figure 5: Knockdown of *lncRNA152* Increases DNA Damage in DNA-damaging Reagent-treated Condition in MDA-MB-231 cells.
(A) Western blot to detect γH2AX level with or without Doxorubicin (1nM) treating for 6h, upon siCtrl or siLnc152 knockdown in MDA-MB-231 cell line. (B) Time course detection of γH2AX level with or without Doxorubicin (1nM) treatment, upon siCtrl or siLnc152 knockdown. (C)RT-qPCR to detect the knockdown efficacy of the two siRNAs. Data represent mean ± STEV. *P<0.05; **P<0.01, ***P<0.001. (D) Western blot to detect γH2AX level upon 2 siRNAs in indicated condition.

To further investigate if lnRNA152 involved in DNA damage response pathways, we used the MCF7 cells breast cancer line where E2 itself can induce E2 responsive damage [17]. Instead of using doxorubicin to generate DNA damage, we treated MCF7 cells with E2 to damage oxidative DNA damage. RT-qPCR was performed to measure the expression of *lncRNA152* under E2 treatment for MCF7 cell line. The results indicated that *lncRNA152* is transcriptionally repressed by E_2 (Figure 6). Then, we transfected an *lncRNA152* overexpression plasmid to rescue the induced E_2 repression using empty vector as a control, and treated these stripped MCF7 cell with E_2 for 24 hours, then we performed immunostaining with γH2AX. We observed 15.3+/-2.8% cells were γH2AX positive in empty vector transfection group. However, there were only 6+/-1.8% cells positive in *lncRNA152* overexpression group. This result demonstrated a protection by *lncRNA152* against DNA damage (Figure 7), where we can confirm that *lncRNA152* plays a role in promoting DNA genomic stability.

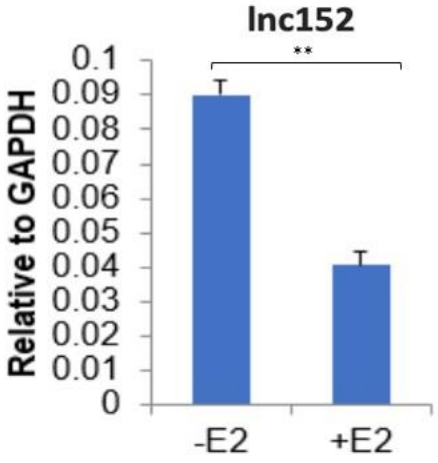
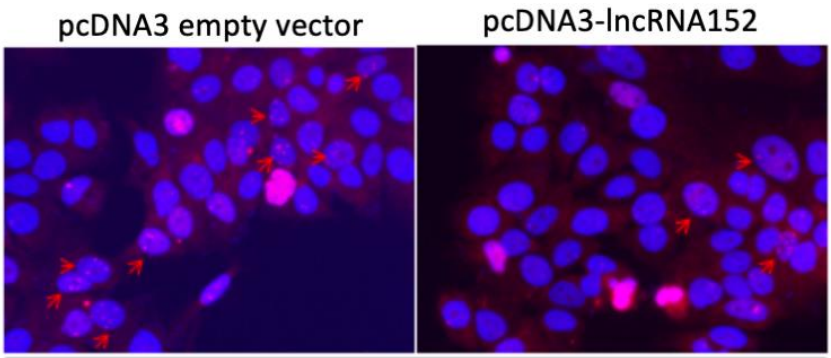


Figure 6: *lncRNA152* is Transcriptionally Repressed by E_2.
lncRNA152 is a E2-repressed RNA, measured by RT-qPCR of -/+10nM E2-treated MCF7 cells for 24h. Data represent mean ± STEV. *P<0.05; **P<0.01, ***P<0.001.



	γH2AX positive	Total	% of positive	Average± SD %	χ^2 test
pcDNA3	8	44	18.2	15.3±2.8	
	6	42	14.3		
	8	47	17		
	7	59	11.9		
pcDNA3-152	3	52	7.7	6.6±1.8	0.008579
	4	45	8.9		
	2	44	4.5		
	1	17	5.9		
	2	25	8		

Figure 7: *lncRNA152* Plays a Role in Promoting DNA Genomic Stability. Immunostaining of γH2AX (red) in E2-treated MCF7 cells for 24h after stripped for 2 days, with DAPI (blue) staining to locate nuclei. Arrows indicate γH2AX positive cells.

Discussion

Genomic instability is a hallmark of cancer, but DNA damage is best considered to be a double-edged sword, given that the major current anticancer therapeutics either directly damage DNA or target basic cell division mechanisms to block cell proliferation. Cancer cells that escape from these attacks are likely to have an enhanced DNA damage protection mechanism. We have uncovered a previously unrecognized role for a specific *lncRNA* overexpressed in breast cancer, which promises to uncover a chemotherapy "sensitizer" to enhance chemotherapy-induced cancer cell killing efficacy. Specifically, we propose here to identify this hypothesized genome integrity protection mechanism utilized by breast cancer cells. DNA damage induced *lncRNA152* could potentially serve as TNBC biomarker, based on the possibility that increased apoptosis of these cells would be of therapeutic benefit. Targeting *lncRNA152* might be tested for ability to provide and improve chemotherapy response by attacking TNBC cancer cells survival defense.

Robust expression of *lnRNA152* in breast cancer cell lines, specifically in the triplet negative breast cancer cell lines. Thus, we wonder whether *lncRNA152* could potentially serve as a triple negative breast cancer diagnostic biomarker. Many researchers have been studying the role of long non-coding RNAs in DNA damage response, which complements our study that has verified that *lnRNA152* plays a role in promoting genomic stability, at least in model cell lines. Relating *lnRNA152* to DNA damage, we have generated DNA damage by DNA damage regents and investigated effects of knockdown or overexpression of *lnRNA152*, with the intent of determining the recruitment of this *lncRNA* sites of induced DNA damage. Double-stranded DNA breaks are the main type of DNA damage we are focusing on in our study. The detection of γH2AX, the DNA damage biomarker, were frequently used in our study to determine if *lncRNA152* were involved in DNA damage

response pathways. Following the detection by γH2AX, we would next seek to determine the relationship between *lncRNA152* and downstream factors of DNA damage response pathways. First, we will assess which of the two main repair pathways responding to DNA damages. Non-homologous end-joining and homologous recombination are modulated by *lncRNA152*. Second, the most intriguing aspect of this project is whether *lncRNA152* might be a previously unrecognized component of multiple DNA damage repair complexes. Our initial approach is to use a vivo fluorescence-based system, named DNA repair reporter [18], to try to label this RNA as recruited component of repair complexes at sites cleaved by endonucleases. Specifically, this DNA repair reporter system allows the quantitative comparison of cells go for NHEJ or homologous recombination through the repair of restriction enzyme Sce1 cutes. Repair through NHEJ or HR leads to expression of GFP or mCherry respectively [18].Our approach is to introduce the system to TNBC cell lines following the knock down *lncRNA152*. Thus, we could find out which repair pathway *lnRNA152* potentially involved in. Thereby also linking this to the specific DDR complexes recruited to the damage site. Additionally, we want to identify which protein *lncRNA152* binds at these DNA damage sites. The molecular basis mechanism of *lncRNA152* in protecting genomic stability will thus potentially provide further insights into basic DDR mechanisms.

Materials and Methods

Cloning of Knockdown and Overexpressed Plasmids

To knock down *lnRNA152*, siRNA was designed to target *lncRNA152* (Table 1). siRNAs were annealed under 95 °C for 5 minutes and cooled down slowly. The vector pLKO.1 puro (Plasmid #8453, Addgene) were digested by EcoRI and BshT1, and then ligate with the annealed siRNAs. To overexpress *lnRNA152*, cDNA was synthesized by RNA extraction, RT-PCR. PCR was performed to amplified cDNA. The amplified cDNA was then digested by EcoRI and BshT1, and inserted into the digested vector pLKO.1 puro (Plasmid #8453, Addgene).

Table 1: Sequences of siRNAs Targets *lnRNA152*

	Target Sequence
siRNA-lnc152-1	AAGCTCTATGACACACTTGAT
siRNA-lnc152-2	CTATGTGTCTTAATCCCTT

RNA preparations, cDNA Synthesis and Quantitative RT-PCR

Total RNA from cell cultures was isolated using Thermo Fisher Scientific Zymo RNA Kit. 1ug of RNA was reverse transcribed using Invitrogen™ SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase in a 10µl reaction. The cDNA was diluted 10 fold in nuclease-free water. 1 µl of the diluted cDNA, along with 5 µM of each primer and SYBR Green PCR master mix (Applied Biosystems) were used for the Quantitative PCR reaction. Quantitative PCR reactions were performed in triplicate for each sample in a StepOnePlusTM Real-Time PCR System (Applied Biosystems) with a one-step program: 95°C, 10 min; 95°C, 10 s, 60°C, 1 min, for 40 cycles. The expression level of GAPDH was used as the control reference for all samples. The sequences of Quantitative PCR primers for *lncRNA152* genes are listed in Table 2.

Table 2: Sequence of Primers Used for Quantitative PCR

F, Forward; R, Reverse

Gene Name	Primer Sequence
lncRNA152	F,GCTGGAAGTGCAAAAGGAAG R, TCACTGGGGACATCACAGAA
lncRNA152 (NR_024204)	F, CAGAAGACCGAAAGGATGGA R, ATCTCTGTCGTCGTCCTCGT
GADD45a	F, TACGCTGATCCAGGCTTTCT R, AACAGGCTGAGCTTCTCCAA
GAPDH	F,GAAGGTGAAGGTCGGAGTCAAC R,CAGAGTTAAAAGCAGCCCTGGT

Western blotting

Washed cells were pelleted by centrifugation at 700 g for 5 min at room temperature and then lysed in 1% Triton X-100, 0.5% SDS, in 1X TBS supplemented with protease inhibitor and EDTA. The protein content was in microfuge tubes for 30 min at 4°C, and then sonicated. The protein content was heated on 95 °C with loading buffer for 5mins. A total of 20 µg of total protein was separated on a 10% denaturing polyacrylamide gel and electrophoretically transferred to PVDF membranes. Membranes were blocked with either 5% non-fat dried milk or 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% (v/v) Tween-20, followed by incubation with the primary antibody. Primary antibody directed against gH2AX was from Millipore; Antibody directed against Beta-Tubulin (T5326) was from Sigma-Aldrich Prestige Antibodies®. Secondary antibody Goat Anti-Rabbit IgG (H+L) Cross-Adsorbed secondary antibody (Catalog# G21234, Invitrogen) was used for incubation.

Immunofluorescence

Chambered cover glass was fixed using 4% paraformaldehyde/PBS for 20 min at room

temperature, and then rinsed by PBS for 3 times. Samples were block and permeabilized in PBS + 2% fish gelatin + 0.1% Triton® X-100. Chambered cover glass was incubated with diluted primary antibody (gH2AX, from Millipore) in blocking buffer on a wet chamber overnight. Samples were washed with PBS-T for three times, and then incubated with diluted secondary antibody (Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594, Catalog # A-21207, Invitrogen) for 2 hours. Samples were washed with PBS-T for three times. Samples were mounted in fluorescence ant-fade mounting media, stained with DAPI (D9542, Sigma).

This thesis in full is unpublished material that will be prepared for submission for publication of the material. Chunyu Jin, Jiani Wang. The thesis author was the primary investigator and author of this material.

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