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The Relationship Between Long Non-Coding RNA Expressions and Ponatinib in Breast Cancer

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BACKGROUND/AIMS
Breast cancer is the most common type of cancer in women and is among the leading causes of cancer-related deaths. Long non-coding RNAs (lncRNAs) play significant roles in cell proliferation, transcriptional regulation, cell cycle progression, apoptosis, carcinogenesis, and metastasis. Studies have shown that ponatinib has an antiproliferative effect in some types of cancer. The aim of the present study was to evaluate the effect of ponatinib on cytotoxicity and to determine changes in lncRNA expression levels with the use of ponatinib treatment in estrogen receptor (ER)-independent MDA-MB-231 and ER-dependent MCF-7 breast cancer cells.

MATERIAL and METHODS
The cytotoxic effects of ponatinib were determined by using the xCELLigence system. Changes in lncRNA expression profiles were determined using quantitative reverse transcription polymerase chain reaction to investigate the antiproliferative roles of ponatinib in breast cancer.

RESULTS
In human breast adenocarcinoma cell lines (MCF-7 and MDA-MB-231), the IC50 doses of ponatinib were determined to be 4.59 μM (72 h) and 1.41 μM (48 h), respectively. After ponatinib treatment, we observed changes in lncRNA expression profiles in ER-independent MDA-MB-231 and ER-dependent MCF-7 breast cancer cells compared with the control group.

CONCLUSION
The changes in the lncRNA expression profiles and the anti-cancer agent of ponatinib play roles in the definition of therapeutic target for new approach in breast cancer.

Keywords: Long non-coding RNAs (lncRNAs), ponatinib, MCF-7, MDA-MB-231, breast cancer

INTRODUCTION

Breast cancer is common in women and is characterized by high rates of malignancy and metastasis (1). Since breast cancer has a heterogeneous molecular structure, there is no common treatment strategy. Most patients develop resistance during treatment. Therefore, it has been found that alternative medicine sources are used as new options for breast cancer treatment (2).

Ponatinib, a strong tyrosine kinase inhibitor, targets BCR-ABL1 oncoprotein. It has antiangiogenic and antineoplastic activities. It has been applied in the treatment of hematological malignancies, such as Philadelphia chromosome-positive acute lymphoblastic leukemia and chronic myeloid leukemia (3). In addition, the antineoplastic effects of ponatinib on various cancer cells, such as endometrial, bladder, stomach, breast, lung, and colon cancers, have been demonstrated (4). Ponatinib has been shown to induce dose-dependent G2/M arrest in ovarian and MCF-7 breast cancer cells, but does not block the cell cycle in colon and SKBR3 breast cancer cells (5).

Long non-coding RNA (lncRNA) is a kind of non-coding RNA (ncRNA) molecule longer than 200 nucleotides (6, 7). lncRNAs play biological roles in cell proliferation, proliferation, metabolic functions and differentiation, and the development of many diseases. They also play a role in genomic imprinting, gene regulation, alternative splicing, chromatin organization, and genomic packaging. The abnormal regulation of lncRNAs is associated with the formation, development, and progression of different

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types of diseases, particularly cancer. Specific lncRNAs are considered as indicators of diagnostic, prognostic, or predictive therapeutic responses for various diseases (8). Various studies have been conducted on lncRNAs. For example, it was shown that HOXAII-AS inhibits the formation of cell colonies in the breast cancer cell line and arrests the cell cycle in the G0/GI phase (9).

In conclusion, in our study, the antiproliferative effects of ponatinib and lncRNA expression profile were found in breast cancer. Some lncRNA genes have been shown to have anti-cancer effects. We believe that ponatinib can be used as a candidate biomarker for future effective treatment of breast cancer.

MATERIAL and METHODS

Cell Culture

MDA-MB-231 and MCF-7 cell lines were obtained from ATCC (Guernsey, Ireland). MCF-7 cells were cultured with RPMI-1640 (Biological Industries, Beit-Haemek, Israel), and MDA-MB-231 cells were cultured with Leibovitz's L-15 (Biological Industries) media containing 1% L-glutamine (EMD Millipore, K0282, Darmstadt, Germany), 10% inactivated fetal bovine serum (Capricorn Scientific, FBS-11B, Ebsdorfergrund, Germany), and 1% penicillin/streptomycin (Biochrom, A2213, Berlin, Germany) in 5% CO_2 and 37 °C. Ponatinib (Selleckchem, Munich, Germany) was suspended in dimethyl sulfoxide (Sigma-Aldrich, Taufkirchen, Germany). Our study was conducted according to the Declaration of Helsinki.

Cytotoxicity Assay

MCF-7 and MDA-MB-231 (1×10^4 cells/well) were seeded in 96-well E-plates in triplicate to investigate the cytotoxic effects of ponatinib. The cells were incubated for 24 h before ponatinib treatment. MCF-7 cells were treated with ponatinib with concentrations of between 100 µM and 3.1 µM, whereas MDA-MB-231 cells were treated with ponatinib doses of 50 µM–1.5 µM. The cells were incubated for 48 h, and impedance was monitored every 15 min throughout the period using the xCELLigence system. Cytotoxicity was evaluated by comparing the viabilities of the ponatinib-treated cells to the untreated control cells using the xCELLigence RTCA software.

lncRNA Expression Profiling

Total RNA isolation and cDNA synthesis

For lncRNA expression profiling studies, RNeasy Mini Kit (Qiagen, Kat. No: 74134) was used to extract total RNA (including small RNAs) from the ponatinib-treated and -untreated MDA-MB-231 and MCF-7 cells (2×10^6 cells/mL). The concentration and purity of RNA samples were determined by measuring absorbance at wavelengths of 260/280 nm and 230/260 nm using the NanoDrop instrument (Thermo Scientific, Wilmington, DE, USA). For further analysis, RNA samples with A260/A280 and A230/A260 absorbance ratios >2.0 were used.

qRT-PCR analysis

For cDNA synthesis, an RNAQuant cDNA Synthesis Kit (System Biosciences, CA, USA) was used according to the manufacturer's instruction. The Disease-Related lncProfiler Array was used to investigate the antiproliferative roles of lncRNAs in breast cancer after ponatinib treatment. Relative quantitation of 83 lncRNAs was measured by using a Maxima SYBR Green qPCR Master Mix (Thermo Scientific) on LightCycler 480 II (Roche Life Science, Indianapolis, IN, USA). In addition to seven human housekeeping genes (ACTB, B2M, PGKI, GAPDH, HPRTI, RPLIA, and RPLI3A) and four small RNA transcript primers (7SL scRNA, 5.8S rRNA, U87 scaRNAU6, and smRNA) for normalization, the quantitative reverse transcription polymerase chain reaction (qRT-PCR) array plate included one genomic DNA control and one negative control. The relative expression of lncRNAs was determined by using the $2^{-\Delta\Delta CT}$ method. Fold changes of lncRNA expression levels after ponatinib treatment were evaluated by comparing with the untreated control groups. Log2 transformation was applied to the $2^{-\Delta\Delta Ct}$ values of the lncRNA expression in the control and ponatinib-treated groups. Fold changes for lncRNA expressions and their significance were calculated by Student's t-test using an online software (https://www.qiagen.com/jp/shop/genes-and-pathways/data-analysis-center-overview-page/) (GeneGlobe Data Analysis Center; Qiagen, Valencia, USA). Changes in lncRNA expression of ±2-fold were compared with the control group. A p value <0.05 was considered significant.

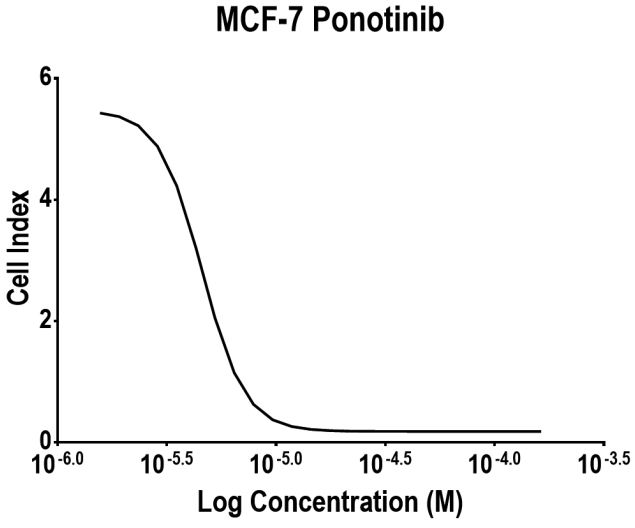

FIGURE 1. Cytotoxic effect of ponatinib in MCF-7 cell line

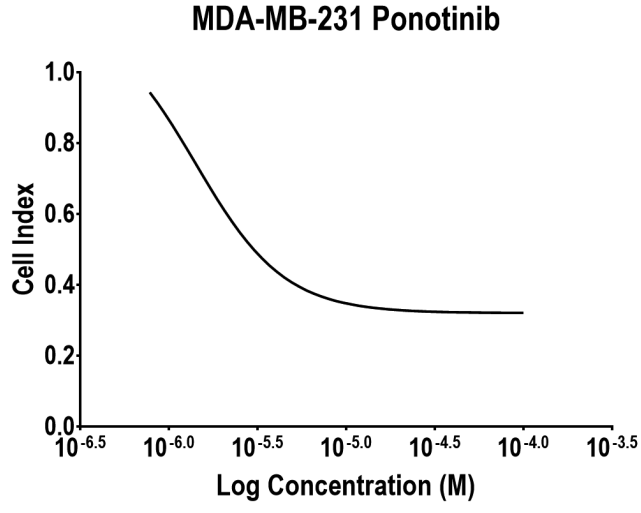

FIGURE 2. Cytotoxic effect of ponatinib in MDA-MB-231 cell line

RESULTS

Cytotoxic Effect of Ponatinib

MDA-MB-231 and MCF-7 cell lines revealed the cytotoxic effect of ponatinib by MTT analysis. The IC_{50} doses of ponatinib were determined to be 1.41 μM for MDA-MB-231 cell line (48 h) and 4.59 μM for MCF-7 cell line (72 h) at 48 h by using the xCELLigence system (Figure 1, 2). Ponatinib inhibited cell proliferation in a dose- and time-dependent manner compared with the untreated MDA-MB-231 and MCF-7 control cells.

Expression Profile Changes of lncRNA

Following ponatinib treatment, changes in lncRNA expression were found in MCF-7 and MDA-MB-231 cells compared with the control groups. According to log2 transformation, 17 lncRNAs (AAAI, aHIF, BC200, DISC2, EGO, HOXA11AS, MALAT1, MEG3, NEAT1, NCRMS, PCAT-1, PCAT-14, PCAT-43, SAF, SRA, WTI-AS, and ZEB2NAT) were downregulated, whereas 5 lncRNAs (CMPD, DGCR5, HI9-AS, HARIA, and LIT) were upregulated in MCF-7 cell lines ($p<0.05$) (Table 1).

TABLE 1. Exchange of lncRNAs expression in ponatinib treated MCF-7 cell line

lncRNA Symbols	Fold Change (Log2Transformed)	p
AAAI	-11.7	0.000124
aHIF	-4.79	0.000056
BC200	-2.13	0.000081
CMPD	2.44	0.00029
DGCR5	2.02	0.000234
DISC2	-3.6	0.000097
EGO	-2.79	0.00005
HI9-AS	2.29	0.000842
HARIA	5.38	0.000072
HOXA11AS	-6.05	0.000086
LIT	2.04	0.000616
MALAT1	-2.69	0.000043
MEG3	-3.68	0.000144
NEAT1	-2.85	0.000019
NCRMS	-3.06	0.000063
PCAT-1	-5.29	0.000045
PCAT-14	-5.02	0.000157
PCAT-43	-2.20	0.000114
SAF	-2.37	0.000106
SRA	-2.97	0.000069
WTI-AS	-7.86	0.000072
WTI-AS	-2.54	0.000432

lncRNAs: Long non-coding RNA; aHIF: Hypoxia Inducible Factor; CMPD: Cancer mutant proteom Database; DGCR5: DiGeorge Syndrome Critical Region Gene 5; DISC2: Disrupted in schizophrenia 2; EGO: Eosinophil Granule Ontogenesis; LIT: Late inhibitor of T4; MALAT1: Metastasis Associated Lung Adenocarcinoma Transcript 1; NCRMS: Non-coding RNA in Rhabdomyosarcoma; SAF: Serum Amyloid A- Activating Factor; SRA: Steroid Receptor Activator

In addition, according to log2 transformation, 22 lncRNAs (AAAI, aHIF, BC200, BCMS, DLG2AS, GAS5, HI9, HOXA11AS, IPW, LIPA16, LIT, MALAT1, MERIIC, NEAT1, PCAT-14, PCGEM1, RMRP, SOX2OT, TELOMERASE RNA, TMEVPG1, TUG1, UCA1) were downregulated, whereas 6 lncRNAs (IGF2AS, MEG3, PCAT-43, ST7OT2, ST7OT3, and WTI-AS) were upregulated in MDA-MB-231 cell lines ($p<0.05$) (Table 2).

DISCUSSION

In recent years, ncRNAs have been extensively studied in various biological processes and in human diseases, including cancer (10). Currently, a large number of human lncRNAs can act as biomarkers for cancer diagnosis and prognosis (11). Most of the lncRNAs are abnormally expressed in breast cancer; they act as tumor suppressors and oncogenes according to their function and expression patterns (12).

TABLE 2. Exchange of lncRNAs expression in ponatinib treated MDA-MB-231 cell line

lncRNA Symbols	Fold Change (Log2Transformed)	p
AAAI	-7.73	0.000312
aHIF	-2.66	0.000187
BC200	-6.16	0.000031
BCMS	-4.80	0.000146
DLG2AS	-2.90	0.000132
GAS5	-5.39	0.000082
HI9	-5.15	0.000066
HOXA11AS	-7.83	0.00012
IPW	-5.15	0.00006
IGF2AS	2.93	0.00013
LIPA16	-2.44	0.000147
LIT	-2.99	0.000435
MALAT1	-7.38	0.000015
MEG3	4.46	0.000065
MERIIC	-5.65	0.000055
NEAT1	-4.17	0.000016
PCAT-14	-2.93	0.000381
PCAT-43	3.54	0.00004
RMRP	-7.73	0.000007
SOX2OT	-5.28	0.000216
ST7OT2	3.33	0.000204
ST7OT3	2.67	0.000113
TELOMERASE RNA	-3.46	0.000539
TMEVPG1	-3.10	0.000714
TUG1	-3.48	0.000122
UCA1	-7.97	0.000073
WTI-AS	2.91	0.000211

lncRNAs: Long non-coding RNA; aHIF: Hypoxia Inducible Factor; BCMS: Gene associated with multiple splicing B-cell neoplasia; IPW: Imprinted Gene in Prader-Willi Sendrom; LIT: Late inhibitor of T4; MALAT1: Metastasis Associated Lung Adenocarcinoma Transcript 1; MEG3: maternally expressed 3 TMEVPG1: Theiler's mouse encephalomyelitis virus resume candidate gene; ITUG1: Taurine RNA arranged upward 1; UCA1: Associated with urethral cancer 1

Long non-coding RNA H19 is known to play an oncogenic role in breast cancer. Studies have shown that H19 plays a critical role in cell survival and proliferation in estrogen receptor (ER)-positive breast cancer cells (13). While silencing of H19 in breast cancer cells reduces proliferation, overexpression of H19 is suggested to accelerate cell cycle progression (14). Inhibition of H19 reduces the metastasis of pancreatic cancer in vivo. Therefore, they showed that H19 is a new candidate for treatment of pancreatic cancer (15). In our study, ponatinib was suppressed in ER-negative breast cancer cells of H19. We can associate the 5.15-fold reduction in H19 expression with the tumorigenesis process.

Chen et al. (16) have shown that IGF2AS is involved as an epigenetic tumor suppressor in human prostate cancer. In our study on breast cancer, it was evaluated that the expression of lncRNA IGF2AS had a 2.93-fold increase, playing a role as a tumor suppressor.

Wang et al. (17) showed that overexpressed PCAT-14 is associated with a poor prognosis in patients with hepatocellular carcinoma (HCC). PCAT-14 has been proposed as a new prognostic factor and therapeutic target because it regulates proliferation and cell cycle in HCC cells (17). Qiao et al. (18) showed that the downregulation of lncRNA PCAT-1 inhibits proliferation, blocks cell cycle passage, and suppresses cyclin and c-myc expression in colorectal cancer cells. In our study, the expression of lncRNA PCAT-14 and PCAT-1 decreased with the effect of ponatinib. This may suggest that they induce apoptosis.

In their study on breast cancer, Iranpour et al. found that SOX-2OT overexpresses tumor tissues compared with non-cancerous tissues. It has been observed that SOX2OT acts as oncogenesis, and that its expression is more negative for patients (19).

Farhangian et al. (20) found that SOX2OT expression shows a significant reduction compared with non-tumor tissues in gastric cancer (GC) samples. They showed that SOX2OT plays a tumor suppressor role with the downregulation of SOX2 in GC. It can also be a good biomarker in the diagnosis of the disease (20). In our study, at the expression level of SOX2OT, a 5.25-fold decrease was found in ER-negative breast cancer cells. The decrease in the expression level of SOX2OT in ponatinib-treated breast cancer cells reveals the antiproliferative effect of ponatinib. We think that studies on this subject can be used as a biomarker in the diagnosis and treatment of the disease.

In the present study, it was observed that the expression of BC200 increased in breast cancer. Among breast cancer tissues, it was observed that BC200 was expressed at a higher level in ER-positive tumors than in ER-negative tumors. BC200 has been shown to play a role as an oncogene in breast cancer. Therefore, BC200 can be shown as a prognostic marker and target to minimize irregular cell proliferation in ER-dependent breast cancer (21). In their study with carboplatin, Wu et al. (22) found that the expression of BC200 increases in ovarian cancer cell lines. It was observed that the cells decreased their sensitivity to the drug by inhibiting BC200 (22). We found that this lncRNA BC200, which is defined as an oncogene in breast cancer, is suppressed with ponatinib in hormone-sensitive and -independent breast cancer cells.

CMPDI showed an antiproliferative effect in GC cell lines MKN-45 and SGC790I. In addition, CMPDI induced time- and dose-dependent apoptosis in MKN-45 cell lines (23). In our study, we showed that ponatinib-treated MCF-7 cells regulated the expression of the tumor suppressor lncRNA CMPDI. As with GC, CMPDI may also contribute to the antiproliferative effect of ponatinib in breast cancer cells.

Fung et al. (24) found that DGCR5 expression is significantly lower in bladder cancer tissues than in healthy tissues. The increased expression of DGCR5 showed a high survival rate. In addition, it was found that the overexpression of DGCR5 inhibited proliferation, colony formation, and cell cycle progression (24). According to these data, after ponatinib treatment, the expression of DGCR5 is increased in ER-positive breast cancer cells. We investigated that ponatinib may suppress metastasis and induce apoptosis in tumorigenesis progression.

It has been observed that the decreased expression of PVTI and the increased expression of HARIA in patients with diffuse glioma increased the survival rate of patients receiving chemotherapy and radiotherapy. PVTI and HARIA can be used as biomarkers in the diagnosis and treatment of diffuse gliomas (25). In our study, we observed that the expression level of HARIA was increased in ER-dependent breast cancer cell lines. As a result, ponatinib may have an apoptotic effect on MCF-7 cell lines in cytotoxic and tumorigenesis.

TUGI was found to be abnormally expressed in cancer. lncRNA TUGI expression decreases in glioma and non-small cell lung cancer. However, it can act as a potential tumor suppressor by inhibiting cell proliferation and promoting apoptosis (26, 27). Fan et al. (28) found that TUGI expression decreases in various cancer tissues and cell lines. They observed that TUGI induces apoptosis and promotes cell cycle arrest in breast cancer cells (28). We have observed that TUGI is downregulated in ER-negative breast cancer cells after ponatinib treatment. We can suggest that TUGI can suppress proliferation, cell migration, and invasion.

Li et al. (29) found that the downregulation of UCAI reduces cell proliferation, cell migration, and invasion in esophageal squamous cell carcinoma (ESCC). We observed that UCAI decreased expression, such as ESCC cells, in MDA-MB-23I cell lines. As a result, in breast cancer cells treated with ponatinib, ponatinib can suppress metastasis and proliferation.

The downregulation of MALAT-I by SiRNA was found to inhibit prostate cancer cell growth, invasion, and migration in the G0/GI phases and inhibited the castration-resistant prostate cancer cell cycle (30). We observed a decrease in expression levels of MalatI in breast cancer cells treated with ponatinib. We suggest that the downregulation of Malat-I can play a role as a prognostic factor in breast cancer.

Li et al. (31) found that the expression levels of ZEBI ASI are higher in HCC tissues than in healthy neighboring tissues. They observed that ZEBI ASI expression increases with HCC metastasis. It was determined that survival time was shortened, and that recurrence rates were higher in patients with HCC who had high ZEBI ASI expression. ZEBI ASI supports the prolifer-

ation and metastasis of HCC; therefore, they asserted that they played an oncogene role in HCC (32). According to our results, lncRNA ZEB2NAT has a 2.54-fold decrease in the level of the hormone-sensitive breast cancer cells seen as oncogene.

Sun et al. (33) found that the expression of MEG3 is lower in breast cancer tissues than in adjacent tissues. The heterogeneous expression of MEG3 has been observed to induce proliferation by inducing the G0/GI proliferation phase and decreases cells in the S (mitotic) phase under in vitro conditions (33). In our study, MEG3 expression was decreased in ER-positive breast cancer cells. Therefore, we believe that apoptosis may be induced.

NEATI acts as an oncogene and tumor suppressor. While the expression of NEATI increases in most cancer tissues, there is a decrease in leukemia and multiple myeloma expression. Ghaforui-Fard et al. (34) reported a decrease in NEATI expression in various types of breast cancer, esophageal carcinomas, and gliomas. According to our data, the decrease in the expression of lncRNA NEATI determined as an oncogene was found to be significant. As a result, we think that target therapies can be considered as prognostic markers. In our study, the proliferation of the cells should be checked periodically.

We believe that ponatinib can be used as a candidate biomarker for future treatment of breast cancer. Such studies may be promising for a variety of diseases, allowing new lncRNA-based therapeutic strategies to be developed and the identification of new markers to make a diagnosis.

Ethics Committee Approval: Authors declared that the research was conducted according to the principles of the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" (amended in October 2013).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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Conflict of Interest: The authors have no conflicts of interest to declare.

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