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# Concise approach for screening long non-coding RNAs functionally linked to human breast cancer associated genes

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ABSTRACT

Cancer research studies using next-generation sequencing have revealed a number of genes of which aberrant expression is associated with various cancers. Recently, long non-coding RNA (lncRNA) has been highlighted due to its tissue-specific expression and cell cancerization functions, such as the regulation of key tumor suppressors. In this study, we suggest a very efficient approach to survey lncRNAs putatively associated with breast cancer. We targeted lncRNAs linked with breast cancer associated genes (BCAGs) and analyzed their expression pattern in human breast cancer cell lines. A total of 337 BCAGs were retrieved from literature review and the existence of 121 lncRNAs were identified from the 15 kb up- and downstream regions of the list of genes. Twenty lncRNAs' expression were detectable in human breast cancer cell lines with different expression patterns. Interestingly, the expression of three lncRNAs, two up-regulated (RAD51C v.4, LOC105371849) and one down-regulated (LOC102724064), were closely correlated with adjacent BCAGs (RAD51C, HEATR6 and BRMS1) in breast cancer cell lines. We thus demonstrated association between the lncRNA and its adjacent BCAG using LOC105371849-HEATR6, of which the function and regulation in breast cancer are still unknown. Knockdown of LOC105371849 by siRNA decreased the expression of HEATR6 mRNA in the MCF7 human breast cancer cell line. In conclusion, this study provides a better understanding about the biological roles of lncRNAs in breast cancer and may be useful in the investigation of proper targets for diagnostic and/or therapeutic breast cancer markers using public databases.

## 1. Introduction

Breast cancer is the most commonly diagnosed cancer and has the second highest cancer-associated mortality in women (DeSantis et al., 2017). Over the last several decades, there have been numerous studies, projects and clinical researches aimed at demonstrating new mechanisms, prognostic and diagnostic markers, and therapeutic targets for breast cancer, resulting in a reduction in the risk of breast cancer (Van Poznak et al., 2015). Recent use of next-generation sequencing technology on various cancers has produced a large amount of data showing genomic aberrations that may directly link to various cancer mechanisms, such as BRCA1/2 gene mutations (single nucleotide polymorphism (SNP), copy number variation (CNV), insertion/deletion (Indel), and epigenetic modifications) (Goodwin et al., 2016; van de Vijver et al., 2002). Moreover, it is no longer surprising that the alteration of non-protein coding regions, where ~80% of the whole genome is actively transcribed without protein coding potential, has crucial roles in overall biogenesis and various pathological changes, including cancer (Ponting et al., 2009). The resulting large population of transcripts are referred to as Non-coding RNA (ncRNA), and has been highlighted over the last few decades (Mattick, 2004).

These ncRNAs can be classified into several groups according to their size, with those longer than 200 nucleotides defined as Long non-coding RNAs (LncRNA) (Mercer et al., 2009). Numerous lncRNAs (58,648) have been identified in the human genome (GRCh38.p12) (Iyer et al., 2015). Recent studies revealed that lncRNAs play an important role in regulating gene expression at various levels, including alternative splicing, regulation of protein activity, and alteration of protein localization, as well as chromatin modification, transcription, and posttranscriptional processing (Gonzalez et al., 2015). Additional studies explored the mechanisms of lncRNAs' contribution to important cancer phenotypes, including cell cycle regulation, survival and

*Abbreviations:* lncRNA, long noncoding RNA; LNA, locked nucleic acid; BCAG, breast cancer associated genes

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immune responses, and publicly shared the resulting expression profiles in diverse cancers including breast cancer (Quinn and Chang, 2016). There indeed are several publicly available databases that allow users to search for and download lncRNA sequences and functional characteristics, as well as expression profiles in various diseases, including cancers (Amaral et al., 2011; Li et al., 2015; Wapinski and Chang, 2011). Yet, despite growing *in silico* resources regarding expression profiles of lncRNAs in various cancers, the functions of most lncRNAs in breast cancer remain unclear. Thus, determining the target protein coding genes regulated by oncogenic lncRNA might provide a starting point for an extensive study.

The locally associated expression interference relationship between anti-sense RNAs and protein coding genes has been reported (Fu et al., 2015; Vance et al., 2014). The naturally occurring antisense RNAs can modulate the expression level of the sense mRNA through processes such as transcriptional interference, RNA splicing, editing and short RNAs. The antisense RNAs are often subdivided into cis- and trans-, depending on the regulation mechanism of the target gene's expression. Although potential regulatory mechanisms of both cis- and trans-antisense RNAs are unknown, cis- antisense RNA's mechanism used to be defined as the suppressive influence of one transcriptional process on an adjacent transcriptional process through the interfering RNA polymerase (RNAP) DNA trafficking (Wierzbicki, 2012). Moreover, antisense RNAs transcribed from opposite strands of the DNA tend to have a potential to bind with corresponding sense transcripts resulting in transcription attenuation, RNA degradation or translational inhibition (Zhang et al., 2018). Directly- or reversely-correlated lncRNA expressions with the expression of neighboring protein coding genes have been reported in several different processes of development and diseases (Lin et al., 2016; Xue et al., 2016). Thus, unravelling the profiles of these lncRNAs in cancer is essential and urgent.

The aim of this study was to identify putative oncogenic lncRNAs, of which the expression might be linked to adjacent genes that were previously documented as oncogenic driver genes or are associated with the tumorigenesis and prognosis of breast cancer. In this study, we surveyed lncRNAs closely located near the genes associated with breast cancer, and validated their expression in five different human breast cancer cell lines. Thus, this study and approach may give a new and efficient insight to initiate the functional study of lncRNA in breast cancer.

## 2. Materials and methods

### 2.1. In silico lncRNA survey

Breast cancer associated genes were identified from the literature review, using “breast cancer,” “gene,” or “gene expression” search terms in PubMed, UCSC and Ensembl. Public cancer gene expression databases, such as LNCpedia and Malacards, were also surveyed (https://lncipedia.org/), (https://www.malacards.org/). LncRNAs within a 15 kb up- and downstream region of the breast cancer associated genes were analyzed. The overall procedure and conceptual scheme were illustrated in Fig. 1, and breast cancer associated genes (BCAG) and adjacent lncRNAs found in this study were summarized in Table S1.

### 2.2. Cell culture

MCF-7, MDA-MB-231 and MDA-MB-436 cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (HyClone, GE Healthcare Life Sciences, PA, USA), containing 10% fetal bovine serum (FBS) (JCBIO Co.LTD, Korea) and 1% antimycotic antibiotic (Gibco, MA, USA), while MCF-10A was maintained in DME/ F12(HyClone) containing 10% FBS and 1% antimycotic antibiotic. SK-BR3 cell lines were maintained in RPMI with 10% FBS and 1% antimycotic antibiotic.

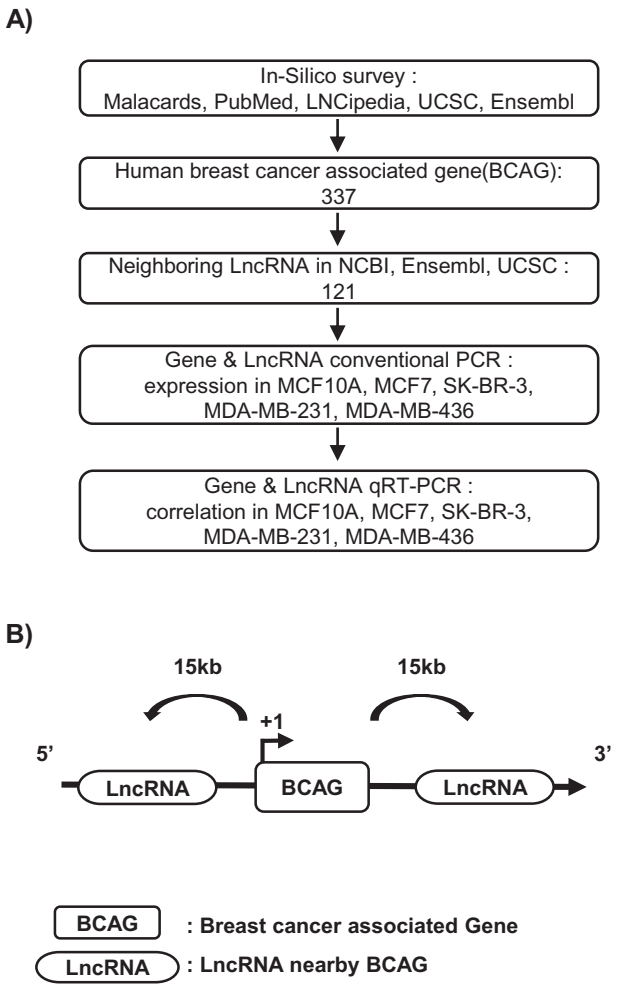


**Fig. 1.** Experimental Scheme. A) Overall procedure and conceptual scheme. B) Representative BCAGs and lncRNA structures in UCSC browser.

### 2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from breast cancer-related cell lines using the RNeasy Mini plus kit (Qiagen, Hilden, Germany). The chance of genomic DNA (gDNA) contamination was eliminated in two steps; gDNA was removed from the RNA isolation process by the gDNA elimination column provided in the RNeasy Mini plus kit (Qiagen), and additionally DNaseI digestion followed by heat inactivation was applied after RNA isolation. The RNA quality was assessed by 260/230 ratio using a Nanodrop Epoch Microplate Spectrophotometer (BioTek Instruments, VT, USA) and stored at −80 °C until used. By using an Omniscript RT kit with random 9(N)mer plus oligo-dT primers, following manufactures guidelines (Qiagen), cDNA was synthesized from 2 μg of total RNA. The reaction mixture was incubated at 42 °C for 1 h and then the enzyme was heat inactivated at 70 °C for 15 min.

### 2.4. Conventional and realtime PCR

Conventional PCR was performed on 2 μl cDNA using GO Taq polymerase and buffers (Qiagen). Primers for each breast cancer associated gene and lncRNA were listed in Table S2, with GAPDH as an internal control gene. After an initial incubation at 95 °C for 3 min, 35 cycles of the following temperature conditions were applied: 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s (depending on the amplicon size, 1 min was alternately used for elongation). A final extension at 72 °C for 5 min was performed. The final amplicon (5 μl) was electrophoresed in 1% agarose gel. Image analysis for densitometry was

performed by ImageJ software Ver. (https://imagej.nih.gov/ij/, ImageJ bundled with 64-bit Java 1.8.0_112). All procedures and reaction conditions were maintained for the realtime PCR analysis, except for the addition of SYBR and being performed in the realtime PCR machine (Bio-Rad CFX Manager, CA, USA). Relative expression levels were calculated by delta-delta CT method.

### 2.5. Knockdown of LOC105371849 lncRNA by siRNA

Three LOC105371849 siRNAs, with scrambled siRNA as a control (Ctrl siRNA), were designed and synthesized by Bioneer Inc. (Daejeon, Korea); siRNA-1 was designed in exon 1, siRNA-2 was designed spanning exon 1 and exon 2, and siRNA-3 was designed in Exon 2. Each siRNAs detailed information was put into Table S3. Following optimization, 225 pmol of siRNA-2 was solely used for further analysis. MCF7 cells were cultured in 6well plates and starved for 12 h prior to transfection. Seventy-five pmol/each or pooled siRNA was transfected into the MCF7 cells and total RNA was isolated at 24 h after transfection. Transfection was performed using the Lipofectamine 3000 kit (Invitrogen, CA, USA), following the manufacturer's manual.

### 2.6. KM plotter analysis

Kaplan-Meier plotter was used to employ 3951 breast cancer patients' data obtained from GEO, EGA and TCGA data (http://kmplot.com/analysis/) (Gyorffy et al., 2010). Heatr6 (218992_at) expression was tested in 8 different analytical subtypes. All default parameters and options were used except the auto select best cutoff option for splitting patients into low and high.

## 3. Results

### 3.1. Identification of lncRNAs locally linked to BCAGs

Literature review with keywords “breast carcinoma” in Malacard (Version 4.5) retrieved 337 breast cancer-associated genes (BCAGs). Neighboring lncRNAs were surveyed within the 15 kb up- and downstream range from each BCAG, and a total of 121 lncRNAs were identified from 94 BCAGs. The survey strategy is illustrated in Fig. 1A and BCAGs with neighboring lncRNAs are listed in Table S1. Most of the BCAGs have only one nearby lncRNA, but multiple lncRNAs were found for some BCAG regions. The FGF8 gene harbors 4 lncRNAs and each of the regions surrounding IGF1, GATA3, KDM5B and NTRK3 harbors 3 lncRNAs (Fig. 1B). In order to determine direct association, only 73, one to one paired lncRNA and BCAGs, were analyzed further.

### 3.2. BCAG and neighboring lncRNA expression in breast cancer-related cell lines

We investigated lncRNA expression in MCF7, and MDA-MB-231 human breast cancer cell lines, as well as MCF10A normal control cell lines. Out of 73 pairs of BCAG and lncRNAs, only 20 pairs were validated by RNA expression in the breast cancer-related cell lines The expression patterns of these BCAGs in the breast cancer related cell lines were diverse. MTOR, RAD51B, JUN, PELP1, IL6, TGFBR2, HEATR6, RAD51C and CDC25B showed high expression in breast cancer cell lines, but BRMS1, BARD1, KMD5B, HIF1A, BACH1, and MTA1 maintained a comparable gene expression level between normal and breast cancer cell lines. On the other hand, RAD51, PTK6, STAT1 and SMAD4 showed lower gene expression levels in cancer cell lines than the normal cell line. Very little CCNG1 expression was detected in all 3 breast cell lines (Fig. 2A).

Furthermore, the expression levels and patterns of neighboring lncRNAs were very varied. Eighteen lncRNAs validated in this study were up-regulated at least in one cancer cell line. Three lncRNAs, LOC105377700 (CCNG1), LOC105370547 (RAD51B), and LINC00189 (BACH1), were significantly expressed only in MCF7 but LOC541472 (IL6) and LOC105372117 (SMAD4) were only found in MBA-MD-231. LncRNAs, LINC01135 (JUN) and LOC101559451 (PELP1) were not detected in MDA-MB-231. The expression of lncRNAs, LOC105376736 (MTOR), LOC102724064 (BRMS1), LOC105373805 (STAT1), RAD51C v.4 (RAD51C), LOC105371849 (HEATR6), and LINC01730 (CDC25B) were up-regulated in both cancer cell lines, MCF7 and MBA-MD-231.The expression of the other five lncRNAs, LOC100507437 (MTA1), LOC101928103 (BARD1), RAD51-AS (RAD51) and PCAT6 (KDM5B) was found in all 3 cell lines without a clear distinguishing pattern between normal and cancer (Fig. 2B and Supplementary Fig. 1).

### 3.3. Correlation in the expression of BCAGs and neighboring lncRNAs

Quantitative realtime PCR was employed to confirm a correlation in RNA expression between BCAGs and their adjacent lncRNAs in 5 human breast cancer related cell lines (Fig. 3). The most interesting patterns and correlations were found in three pairs of BCGs (RAD51C, BRMS1 and HEATR6) and their adjacent lncRNAs respectively. The pair of RAD51C v.4 and RAD51C mRNA as well as that of LOC105371849 and HEATR6 mRNA were abundantly expressed only in MCF7. Correlation in RNA expression was also found in the LOC102724064 and BRMS1pair, which tended to not be dependent on normal *versus* cancer, but rather on cell types (Fig. 3A). However, no correlation was found in RNA expression patterns between lnRNAs LOC101559451, LINC01135 and LOC541472 and nearby BCAGs PELP1, JUN and IL6, respectively (Fig. 3B). The genomic structures of these pairs of genes and lncRNAs are line-drawn in Fig. 3C. HEATR6 and BRMS1 have an anti-sense lncRNA 378 bp and 2866 bp upstream of TSS, respectively, but RAD51C and RAD51C v.4 share RNA structures, TSS and exons.

### 3.4. Association between HEATR6 and adjacent lncRNA (LOC105371849)

We tested the concept that closely located lncRNAs and BCAGs may be functionally linked to each other. The LOC105371849 lncRNA and HEATR6 (HEAT repeat containing 6) gene are located on chromosome 17(17q23) in opposite directions. Since the lncRNA (LOC105371849) is 378 bp upstream from the TSS of HEATR6 and covers most of the HEATR6promoter region, the expression of either mRNA or antisense lncRNA, might influence the other. Although association of HEATR6 to breast cancer has been reported, the regulation of its gene expression and its function in both normal and cancer breast tissues are unknown (Fig. 4A). Thus, to knockdown the lncRNA expression in the MCF7 breast cancer cell line, we employed siRNAs targeting LOC105371849, listed in Table S3. By targeting the splicing junction of exon 2 and 3 of LOC105371849 with the siRNA, transcript levels were successfully decreased to 10% of the control level. Interestingly, HEATR6 gene expression was also influenced (~60% decreased) by the knockdown of LOC105371849 lncRNA (Fig. 4B).

### 3.5. Putative functions of HEATR6 in human breast cancer

The molecular markers of BCL2, MKI67 and CCND1 genes, which encode the BCL2, Ki-67 and cyclin D1 proteins, have frequently been used to test cancer cell apoptosis and proliferation (Penault-Llorca et al., 2008; von Minckwitz et al., 2008).The cell morphology and the expression of these genes were examined in MCF7 cancer cell line after the treatment of siRNA targeting anti-HEATR6 lncRNA, to cultivate a better understanding of the function of HEATR6 in breast cancer (Fig. 5). Twenty-four hours after transfection, the cells were examined by phase contrast microscopy and analyzed for cell apoptosis and proliferation using real-time PCR. at first, no difference in either morphology or cytotoxicity between untransfected cells and those transfected with negative control siRNA was noticed. However, some small but consistent morphological changes were then observed in siLOC105371849 lncRNA transfected cells(Fig. 5A). We examined for

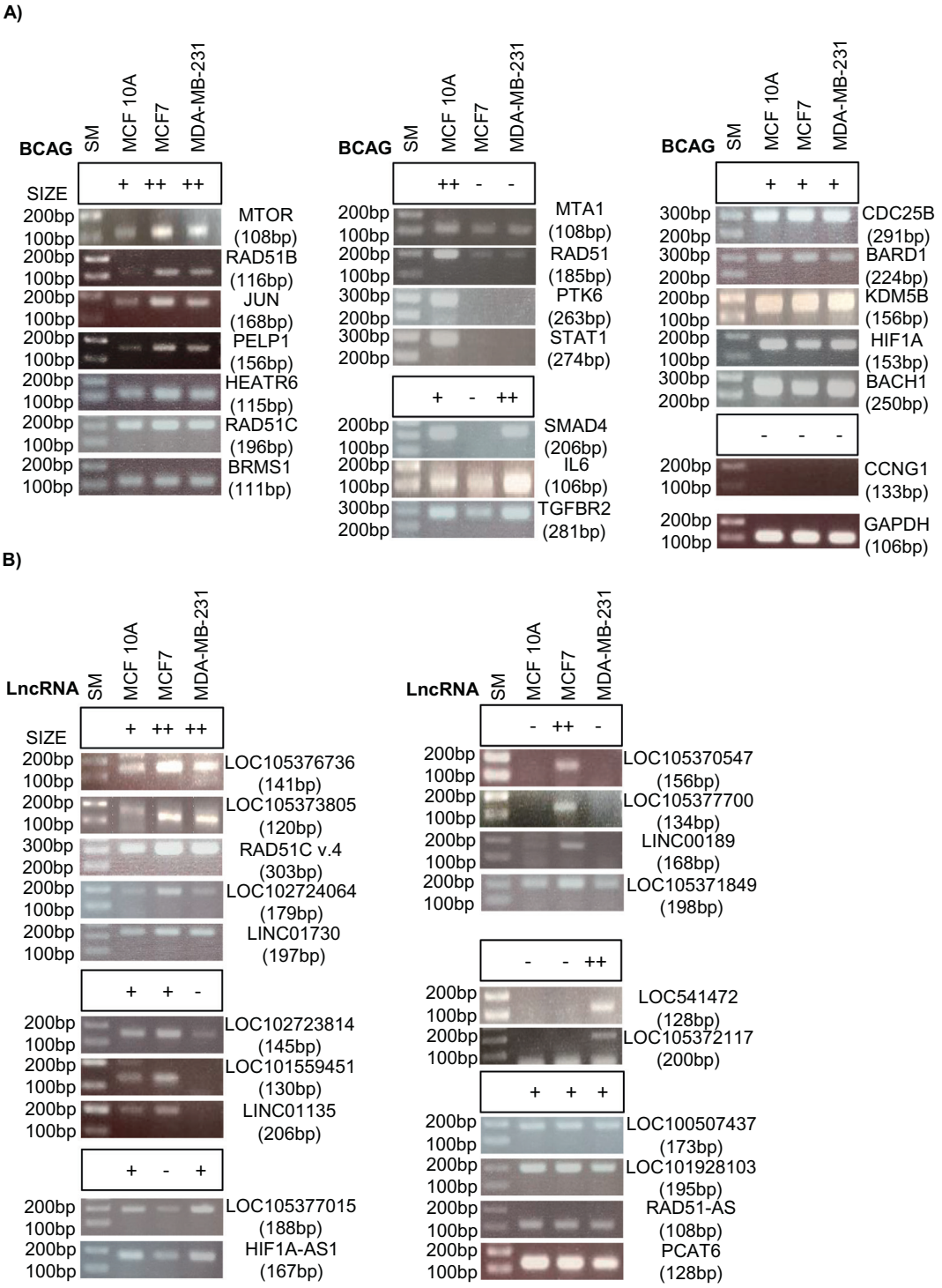


**Fig. 2.** Expression of BCAGs and adjacent lncRNAs in human breast cell lines (normal, (MCF10a); breast cancer, MCF7 and MDA-MB-231). A) BCAG expression. B) lncRNA expression. SM: size marker. −, +, ++: relative expression levels.

apoptosis using the gene expression level of BAX and BCL2, but their expression was not significantly altered by siLOC105371849 lncRNA transfection. Instead of the apoptotic markers, KI67 and Cyclin D1 expression was significantly decreased in siLOC105371849 lncRNA (Fig. 5B). To further assess the importance of HEATR6 and its gene expression regulation by anti-sense lncRNA LOC105371849 in breast cancer, we evaluated the prognostic value of HEATR6 across a large set of public cancer databases, such as TCGA using the Kaplan-Meier plotter. There were two HEATR6s (65493_at and 218991_at based on Affymetrix ID), but only 218991_at gene expression which is suggested probe was tested to be significantly associated with overall survival (OS) (Fig. 5C). The highest association between HEATR6 expression and OS was found in the ER- subtype(p-value: 3.7e−5), whereas less statistical value was found in the ER+ subtype (p-value: 0.0034). In addition, a higher association between HEATR6 expression and OS was observed at the second grade (Grade I [p-value: 0.34] instead of II [p-value:0.0029] or III [p-value:0.0031]).

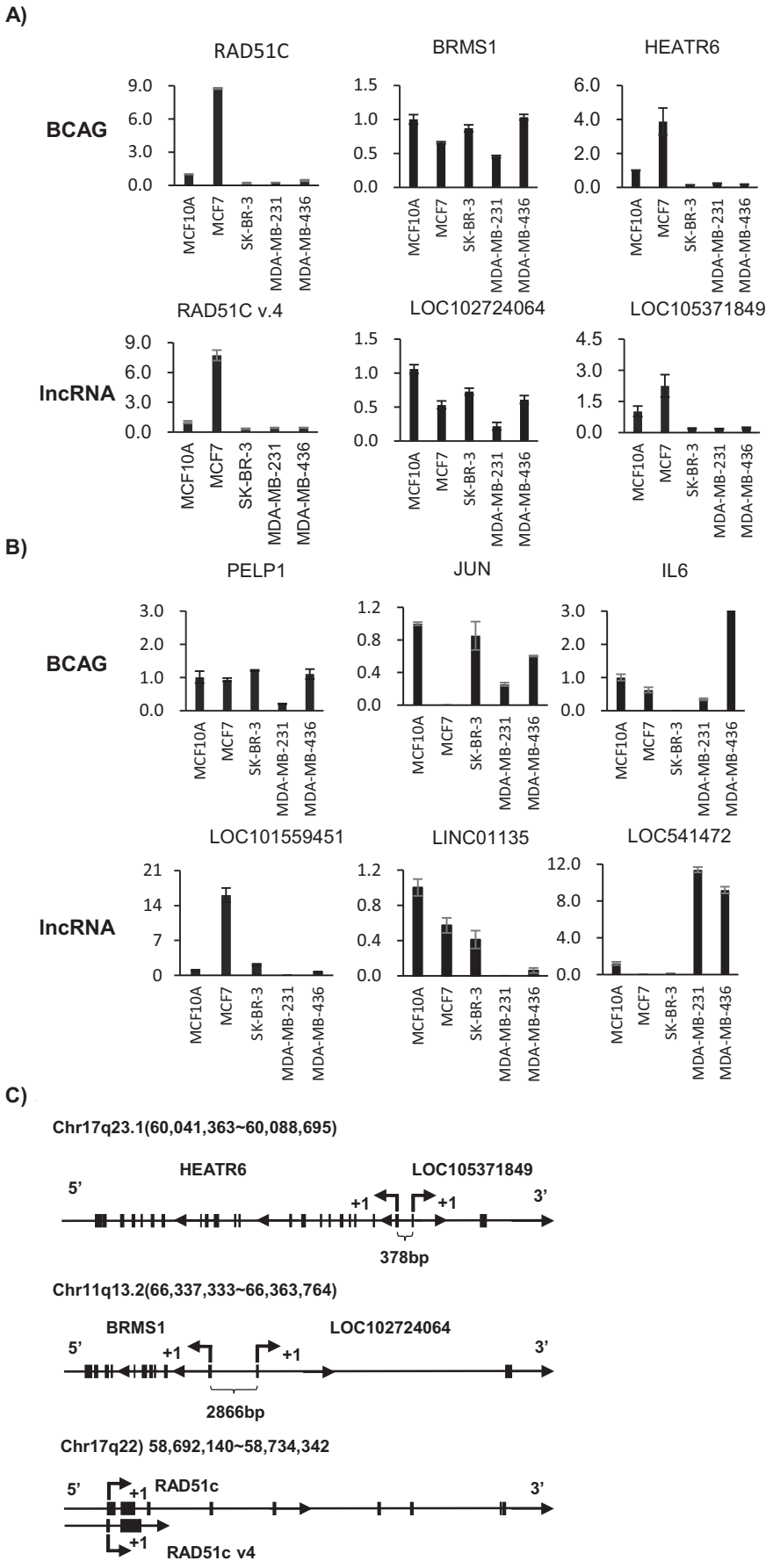


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**Fig. 3.** Quantitation of the six pairs of BCAG and adjacent lncRNAs. Relative RNA expression was measured by quantitative realtime RT-PCR. GAPDH was used as a housekeeping control and delta delta CT method was used for calculation. A) Three pairs of gene and lncRNAs whose expressions were correlated. B) Three pairs without correlation in expression. C) The genomic structures of the three pairs of gene and lncRNA with good RNA expression correlation.

## 4. Discussion

Over the past few decades, a large number of lncRNAs have been identified, more than the number of protein coding genes from the human genome, and much of this sequencing data was made publicly available. Although several tens of thousands of lncRNAs have been identified and their functional roles characterized in various diseases including several types of cancers, most are yet veiled.

In this study, we surveyed publicly open databases and retrieved a list of breast cancer associated genes (Hubbard et al., 2002; Rappaport et al., 2014; Volders et al., 2013). We then investigated the human genome to search for lncRNAs existing within 15 kb around the BCAGs, which are thought to be linked to the BCAGs in a cis- regulatory manner. The expression of lncRNA and BCAG pairs was analyzed by RT-PCR in human breast cancer cell lines. A total of 20 pairs of gene and adjacent lncRNAs were expressed in various levels and patterns in human breast cancer related cell lines. Most were amplified in a cell type specific manner without correlation in expression between the gene and lncRNA. However, we found three pairs of lncRNA and BCAGs (RAD51Cv.4/ RAD51C, LOC102724064/ BRMS1, LOC105371849/ HEATR6) of which the expression was highly correlated in various human breast cancer cell lines.

RAD51C which encodes a DNA double-strand break repair protein, has been reported to confer high-penetrance susceptibility to breast cancer (Sato et al., 2017; Wong et al., 2011). Although, association between its germline mutations and breast cancer has been recently investigated, the functional roles are still unknown (Tung et al., 2016). The RAD51Cv.4 lncRNA is a variant of the RAD51C transcript which shares its 5′ transcription machinery. Based on the structural comparison between RAD51C and its lncRNA and their correlated expression profiles, we assume very cautiously that RAD51C v. 4 acts as an enhancer RNA for RAD51C. However, this should be tested. As per its name, breast cancer metastasis suppressor-1 (BRMS1) is known as metastasis-suppressing gene that can inhibit tumor progression without blocking the growth of primary tumors, in breast cancers. Epigenetic controls of gene expression, such as promoter methylation, have been discovered for BRMS1 (Kong et al., 2015). However, there is no clear understanding about key molecules or pathways influencing BRMS1 gene expression. Thus, the investigation of epigenetic association between lncRNA, LOC102724064, and BRMS1 might be very important to fully understand the regulation of BRMS1 in breast cancer and metastasis. Although HEATR6 (HEAT repeat-containing protein 6) is known as an amplification-dependent oncogene, its molecular function and regulation are still unknown. LncRNA, LOC105371849, is located only 378 bp away from the 5′ end of HEATR6 and covers the promoter region in an antisense direction. Since, our strategy was designed for surveying lncRNAs in cis-regulation of BCAGs, these lncRNAs might functionally recruit regulatory complexes through RNA-protein interactions to influence the expression of nearby genes, but further study into the regulation mechanisms of these lncRNAs on the BCAGs in their vicinity is a necessity.

In this study, we demonstrated the correlation between lncRNAs and adjacent genes using the expression of HEATR6 and lncRNA, LOC105371849, which were tightly correlated in breast cancer cell lines. Knockdown of lncRNA LOC105371849 decreased the level of HEATR6 mRNA expression which resulted in some changes in cell

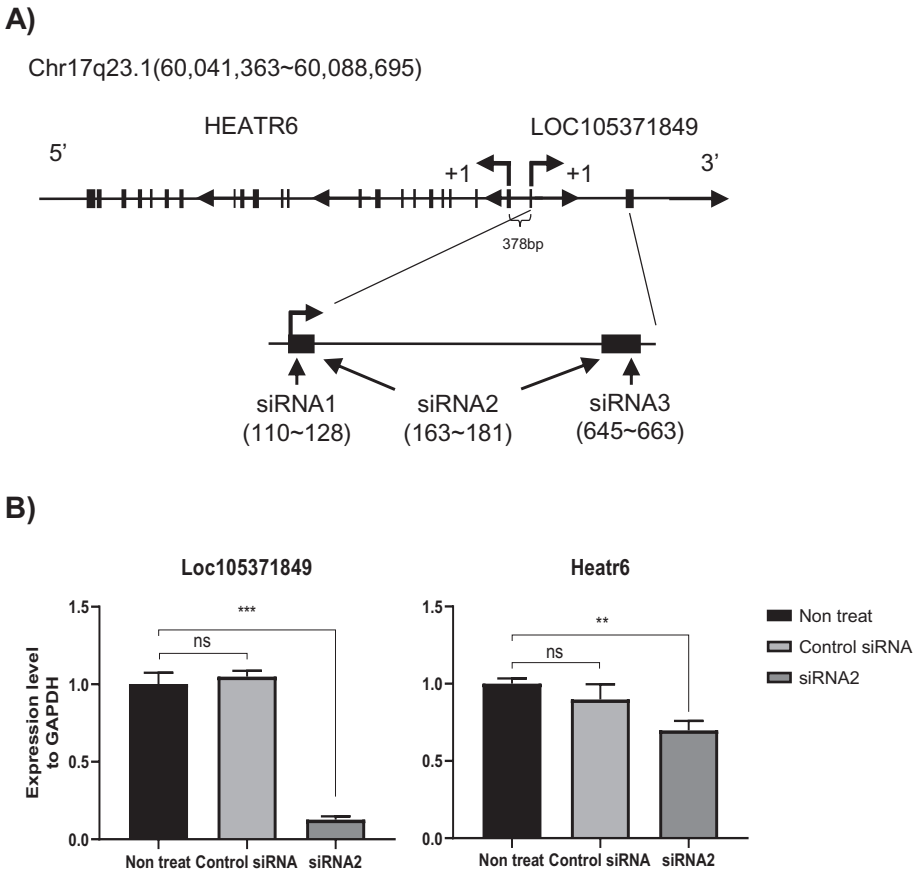


**Fig. 4.** Knockdown of lncRNA, LOC105371849 (Heatr6) by siRNA's influence on HEATR6 BCAG mRNA expression in MCF7 human breast cancer cell line. A) Chromosomal features of HEATR6 and LOC105371849 lncRNA and the loci of siRNA for knockdown. B) Transfection of siRNA targeting LOC105371849 decreased HEATR6 mRNA expression as well as LOC105371849 itself. * and ** indicates statistical significance in ANOVA statistics ($p < .05$ and $p < .01$, respectively).

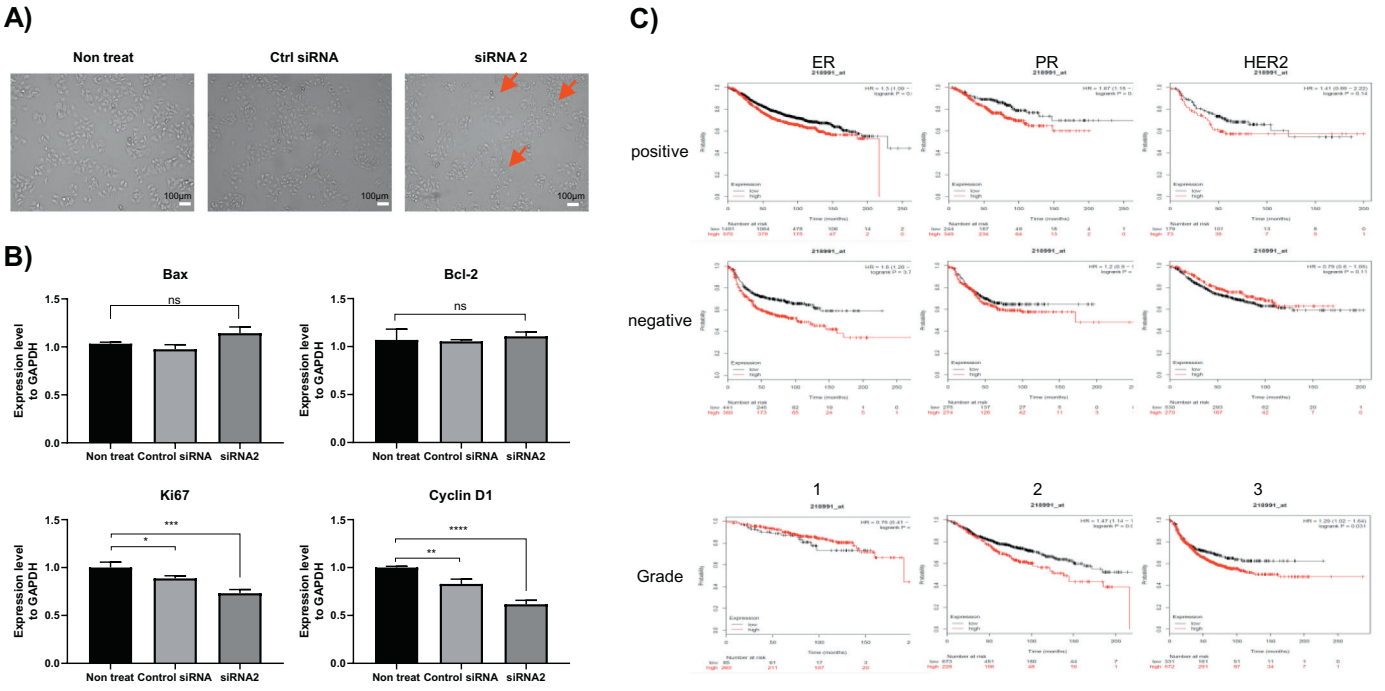


**Fig. 5.** Putative roles of HEATR6 in breast cancer A) Morphology of HEATR6 knockdown MCF7 cell with control cells. Scale = 100 μm. B) Relative gene expressions of cell apoptosis and proliferation markers. *, **, *** and **** indicates p-value < .05, 0.01, 0.001 and 0.0001. ns means not significant. C) KM-plot analysis using affymatrix probes (218991_at) for HEATR6. Survival data was depicted in three different molecular subtypes (ER, PR, HER2) and grades. Black: low expression, red: high expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

morphology. Since some cytotoxicity was found in cancer cell lines with a knockdown of lncRNA LOC105371849, we analyzed the level of BAX and BCL2 gene expression but no significant changes were found. However, the expression of proliferation markers (MKI67 and CCND1) was decreased in cancer cells transfected with siLOC105371849lncRNA. This result suggests that lncRNA LOC105371849 may be involved in cell proliferation in breast cancer *via* HEATR6 regulation. Furthermore, KM plotter analysis revealed that high expression level of HEATR6 in certain molecular subtype and cancer grade II and III is associated with low survival rate of breast cancer patients.

## 5. Conclusions

This study screened lncRNAs functionally linked to breast cancer and tested their potential role in the regulation of neighboring BCAGs. This might be an efficient way increase understanding of the regulation mechanisms of various genes in associated diseases. Using this procedure, we indeed demonstrated the association of HEATR6 and its adjacent lncRNA (LOC105371849), and putative HEATR6 functions in breast cancer proliferation in certain molecular subtypes and stages. It might be important for a better understanding of breast cancer biology and could potentially be a predictor for the prognosis of breast cancer in patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2019.04.003.

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