RESEARCH ARTICLE

Sex Steroids Regulate Expression of Genes Containing Long Interspersed Elements-1s in Breast Cancer Cells

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Abstract

Long interspersed elements-1s (LINE-1s) are dispersed all over the human genome. There is evidence that hypomethylation of LINE-1s and levels of sex steroids regulate gene expression leading to cancer development. Here, we compared mRNA levels of genes containing an intragenic LINE-1 in breast cancer cells treated with various sex steroids from Gene Expression Omnibus (GEO), with the gene expression database using chi-square analysis (http://www.ncbi.nlm.nih.gov/geo). We evaluated whether sex steroids influence expression of genes containing an intragenic LINE-1. Three sex steroids at various concentrations, 1 and 10 nM estradiol (E2), 10 nM progesterone (PG) and 10 nM androgen (AN), were assessed. In breast cancer cells treated with 1 or 10 nM E2, a significant percentage of genes containing an intragenic LINE-1 were down-regulated. A highly significant percentage of E2-regulated genes containing an intragenic LINE-1 was down-regulated in cells treated with 1 nM E2 for 3 hours ($p<3.70E-25$; OR=1.91; 95% CI=2.16-1.69). Similarly, high percentages of PG or AN-regulated genes containing an intragenic LINE-1 wwere also down-regulated in cells treated with 10 nM PG or 10 nM AN for 16 hr (p=9.53E-06; OR=1.65; 95% CI=2.06-1.32 and p=3.81E-14; OR=2.01; 95% CI=2.42-1.67). Interestingly, a significant percentage of AN-regulated genes containing an intragenic LINE-1 was up-regulated in cells treated with 10 nM AN for 16 hr (p=4.03E-02; OR=1.40; 95% CI=1.95-1.01). These findings suggest that intragenic LINE-1s may play roles in sex steroid mediated gene expression in breast cancer cells, which could have significant implications for the development and progression of sex steroid-dependent cancers.

Keywords: Estrogen - progesterone - androgen - LINE-1s - gene experession - breast cancer cells

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Introduction

In eukaryotic cells, activator and repressor proteins exploit chromatin structure to activate or repress genes. Gene expression is regulated by both intrinsic and extrinsic factors to the cell. Cell-intrinsic factors include DNA methylation and histone modification, meanwhile cell-extrinsic factors include small molecules, secreted proteins, temperature and oxygen (Ralston and Shaw, 2008). Sex hormones (cell-extrinsic factors) have been defined by their role in normal reproductive function. Sex hormone levels showed different effect to target organs and sex phenotype. In women, estrogen (E2) and progesterone (PR) are two main important hormones to control reproductive organ function meanwhile androgen (AN) is very important for men. These hormones have been defined in normal reproductive function control including growth, proliferative, reproductive and sex appearance. Levels of sex hormones can control the activating or inhibiting function of target organ (Wierman, 2007). An example of this mechanism can be found in the menstrual cycle which presents different levels in each phase to prepare or break down endothelial lining of the uterus. Moreover, low levels of androgen in men may cause an abnormal sex phenotype and cause some diseases (Kato et al., 2005).

Controlling of gene expression by sex hormones can act as transcription factor and helps bind to their specific receptors. There are 3 different kinds of receptors. First, the estrogen receptor (ERα, ERβ) can bind to estrogen like estradiol, estrone and estriol. Second, progesterone receptor (PR-A, PR-B), can bind to progesterone and other progestogens. Third, the androgen receptor, which can bind to androgens such as testosterone and dihydro-testosterone

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(DHT) (Maggiolini and Picard, 2010). It is known that the most steroid hormone biological effects exert via their receptors at gene regulation levels (Stanisic et al., 2010). Hormone response elements (HREs), the specific DNA sequences in target genes, are one of the most important regions of nuclear receptors for regulating transcription. HREs can be found in 5'-flanking region of the target genes, near the core promoter and also in enhancer regions several kilobases upstream of the transcriptional initiation site (Aranda and Pascual, 2001). However, it's also found in the binding of steroid receptor complexes to negative HREs which can repress gene expression (Puzianowska-Kuznicka et al., 2013). Thus, sex hormone receptors are sequence-specific transcription factors because they can interact at further upstream regions of specific sequences. This mechanism attracts RNA polymerase II in promoter transcription (Quigley et al., 1995). Sex hormones inducing gene expression is one of the most important mechanisms. It is very complicated and more knowledge is still needed to explain their expression control in many types of sex hormone regulated genes.

Retrotransposons are transposable elements which can reverse transcribed into DNA and integrated into the genome at a new location. Long interspersed nuclear element-1s (LINE-1s) is the one family of non-LTR retrotransposons that presents more than 500,000 copies of repetitive sequences in the human genome (Brouha et al., 2003). LINE-1s can encode protein for retrotransposition process to propagate themselves throughout the genome via RNA intermediates. In human transcription unit, 79% of genes found at least one segment of LINE-1s (Han et al., 2004). LINE-1s have been related in many functions through the evolutionary process which depends on their insert position. These include the correlation of LINE-1s elements to the spreading of the X inactivation, intragenic LINE-1s hypomethylation as a cause of cancer and are associated with down-regulation of gene expression during early human and mouse embryogenesis (Aporntewan et al., 2011; Ngamphiw et al., 2014). LINE-1s inserting intron can affect the target gene expression and function. This can cause exon skipping, alternative splicing (Ding et al., 2006). Abnormality activation of LINE-1s such as hypomethylation of LINE-1s may have a role in genome instability and may spontaneously cause disease, especially cancer, in approximately 1 out of every 1,000 humans, (Kazazian and Moran, 1998; Esnault et al., 2000; Xiao-Jie et al., 2016; Khowutthitham et al., 2012).

Our previous report found that genes with intragenic LINE-1 had a related multiple regulatory mechanism according to the following function of differentiation control of cells, cell proliferation, especially in the hormonal response process. Furthermore, down-regulation in genes containing LINE-1 were affected to cancer and even some hormone-related diseases (Wanichnopparat et al., 2013). Active intragenic LINE-1s evolution correlates with gene regulation pathways. This pathway is also initiated by sex hormones. It is interesting how LINE-1s are activated by sex hormones and regulate gene expression. Therefore, the aim of this study was to investigate the correlation between sex hormones and genes containing LINE-1 expression.

Materials and Methods

Data collection

Intragenic LINE-1s (from LINE-1 base) were identified according to their genome based on NCBI Reference Sequence (RefSeq) annotation (Penzkofer et al., 2005) which was shown in our previous report (Aporntewan et al., 2011). The expression profiles from microarray experiments of sex steroid hormones were performed. These were available from Gene Expression Omnibus (GEO datasets: http://www.ncbi.nlm.nih.gov/gds) (Edgar et al., 2002). Five experiments from public array expression were analyzed. Breast cancer cell lines which were selected in these studies include MCF-7 (for E2 study), T-47D (for PG study) and ZR-75-1 (for DHT study). Cancer cell lines were treated by E2 with 1 nM at 3 hours (GSE11506 , Lin et al., 2007a), 10 nM at 6 hours (GSE30931, Prenzel et al., 2011) and 10 nM at 12, 24, 48 hours (GSE11352, Lin et al., 2007b). For PR (GSE62243) and DHT (GSE61368) (Need et al., 2016) were also treated with 10 nM at 16 hours. GEO sample numbers (GSMs) in each experiment was separated into control (non treated hormone) and experimental groups (treated hormone). A significance level of 0.05 was used in each experiment. Genes containing LINE-1 lists were collected from L1Base (http://line1.bioapps.biozentrum.uniwuerzburg.de/l1base.php) (Penzkofer et al., 2005).

Statistical analysis

The means of mRNA levels in control and experimental groups from each experiment was performed by student's t-test. To evaluate the influence of genes containing LINE-1s to host gene expression, genes were divided into two categories, either containing LINE-1s or not containing LINE-1s. Pearson's chi-squared test was used to evaluate the distribution in up or down-regulated genes which

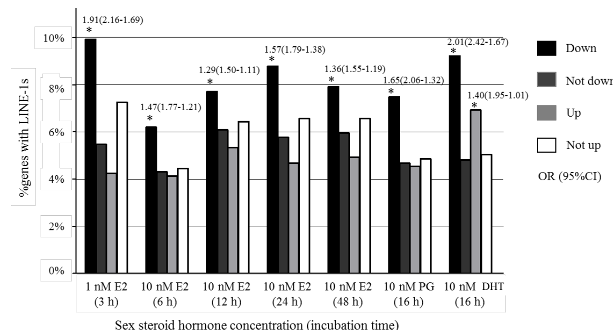

Figure 1. Percentages of Up- and Down-regulated Genes Containing an Intragenic LINE-1 Regulated by Sex Steroid Hormones. Estrogen (E2) regulated genes were included in GSE11506 (1 nM (3h)), GSE30931 (10 nM (6h)) and GSE11352 (10 nM (12, 24 and 48h)). Progesterone (PG) and androgen (AN) regulated genes were included in GSE62243 (10 nM (16h)) and GSE61368 (10 nM (16h)), respectively. E2-regulated genes were highly and significantly regulated down regulated by treatment of 1 nM E2 for 3 hours (p-value (odd ratio) =3.70 E-25(1.91)). Pearson's Chi-square test was performed to calculate statistical significant differences ($P<0.05$). Down represents "down-regulated genes", whereas Not down represents "unchanged" or "up-regulated genes". Up represents "up-regulated genes", whereas Not up represents "unchanged" or "down -regulated genes".

presented the intragenic LINE-1 (Aporntewan et al., 2011). Moreover, the probability of up or down-regulation in genes containing LINE-1 was calculated by dividing the probability of an event happening and probability of an event not happening which is shown as odds ratio. Odds ratios that presented more than 1.0 indicated a strong difference from chi-square. The expression of each gene was classified into four groups based on p-value. The genes containing LINE-1 which were either up or down regulated were included in group A. Meanwhile, genes not containing LINE-1 were included in group B. Group C was composed of nonsignificant up or down-regulated genes containing LINE-1. The last, group D, was composed of nonsignificant up or down–regulated genes not containing LINE-1. Percentages of up or down-regulated and not up or not down-regulated genes containing LINE-1 which were induced by sex steroid hormones were analyzed. These were calculated by using the number of genes containing LINE-1 in up or down-regulated divided by the total number of up or down-regulated genes with and without LINE-1. Similarly, the number of not up or not down-regulated genes were calculated.

Results

We analyzed microarray expression data of sex hormones in breast cancer cells and investigated changes in expression of genes containing intragenic LINE-1. To evaluate the expression of genes containing LINE-1s, genes from expression arrays were classified into four groups depending on gene regulation and the presence of intragenic LINE-1s. These groups are 1) up 2) down 3) not up and 4) not down-regulated genes with LINE-1 (Figure 1).

We found that down regulation of genes containing intragenic LINE-1 were correlated with E2 concentrations and time of E2 treatment. Significant percent of E2-regulated genes and containing intragenic LINE-1 was down-regulated in MCF-7 breast cancer cells treated with 1nM E2 for 3 hr (p=3.70E-25; OR (95% CI) = 1.91 (2.16-1.69) (Figure 1 and Table 1). We next analyzed data obtained from MCF-7 breast cancer cells treated with 10 nM E2 for 6, 12, 24 and 48 hours. Significant percent of E2-regulated genes and containing intragenic LINE-1 were down-regulated at 6, 12, 24 and 48 hours of E2 treatment (p=6.87E-05; 1.47(1.77-1.21), p=9.22E-04; 1.29(1.50-1.11), p=1.06E-11; 1.57(1.79-1.38), p=6.63E-06; 1.36(1.55-1.19), respectively) (Figure 1 and table 1). No statistically significant difference was observed when comparing E2 up-regulated genes with or without intragenic LINE-1s in MCF-7 cells treated with either 1 or 10 nM E2 (Figure 1 and Table 1).

Moreover, significant percent of PG-regulated genes and containing intragenic LINE-1 was down regulated

Table 1. Sex Steroids Regulate Expression of Genes Containing Intragenic LINE-1

Sex steroid hormones	GSE	Concentration (Incubating time)	Gene regulation	LINE-1	No LINE-1	Odd ratio	95% CI	p-value
Estrogen	11506	1 nM (3 hours)	Down	390	3541	1.91	Lower: 1.69	3.70E-25
			Not down	910	15771		Upper: 2.16	
			Up	275	6192	0.57	Lower: 0.50	2.30E-16
			Not up	1025	13120		Upper: 0.65	
	30931	10 nM (6 hours)	Down	125	1893	1.47	Lower: 1.21	6.87E-05
			Not down	1263	28047		Upper: 1.77	
			Up	82	1902	0.93	Lower: 0.74	5.06E-01
			Not up	1306	28038		Upper: 1.16	
	11352	10 nM (12 hours)	Down	220	2635	1.29	Lower: 1.11	9.22E-04
			Not down	1080	16678		Upper: 1.50	
			Up	134	2374	0.82	Lower: 0.68	3.41E-02
			Not up	1166	16939		Upper: 0.99	
		10 nM (24 hours)	Down	322	3345	1.57	Lower: 1.38	1.06E-11
			Not down	978	15968		Upper: 1.79	
			Up	131	2674	0.70	Lower: 0.58	1.25E-04
			Not up	1169	16639		Upper: 0.84	
		10 nM (48 hours)	Down	300	3491	1.36	Lower: 1.19	6.63E-06
			Not down	1000	15822		Upper: 1.55	
			Up	158	3047	0.74	Lower: 0.62	4.84E-04
			Not up	1142	16266		Upper: 0.88	
Progesterone	62243	10 nM (16 hours)	Down	91	1126	1.65	Lower: 1.32	9.53E-06
			Not down	967	19710		Upper: 2.06	
			Up	57	1202	0.93	Lower: 0.71	6.03E-01
			Not up	1001	19634		Upper: 1.22	
Dihydrotestos-terone	61368	10 nM (16 hours)	Down	139	1369	2.01	Lower: 1.67	3.81E-14
			Not down	1151	22781		Upper: 2.42	
			Up	40	538	1.40	Lower: 1.01	4.03E-02
			Not up	1250	23612		Upper: 1.95	

Influence of sex steroid to genes containing intragenic LINE-1 expression. Significant p-value and odd ratio were shown in the comparison between up or down-regulated genes. Down-regulated genes containing LINE-1 showed significant results in all GSE. Low concentration (1 nM) of E2 at 3 hours showed the highest significance and number of genes. Pearson's Chi-square test was performed to calculate significant differences (p<0.05). Down represents "down-regulated genes", whereas Not down represents "unchanged" or "up-regulated genes". Up represents "up-regulated genes",

in T-47D breast cancer cells treated with 10 nM PG for 16 hr (p=9.53E-06; 1.65(2.06-1.32) (Fig 1. and table 1). No statistically significant difference was observed when comparing PG up-regulated genes with or without intragenic LINE-1s in T47D cells treated with 10 nM PG. Finally, significant percent of AN-regulated genes and containing intragenic LINE-1 was found to be down regulated in ZR-75-1 breast cancer cells treated with 10 nM DHT for 16 hr (p=3.81E-14; OR (95% CI) = 2.01 (2.42-1.67) (Figure 1 and table 1). Interestingly, significant percent of AN-regulated genes and containing intragenic LINE-1 was up-regulated gene in ZR-75-1 breast cancer cells treated with 10 nM DHT for 16 hr (p= 4.04 E-02; OR (95% CI) = 1.40 (1.95-1.01) (Figure 1 and Table 1).

Discussion

In our previous report, we found that hypomethylation of LINE-1 is one mechanism which plays an important role in gene regulation (Aporntewan et al., 2011). The degree of LINE-1 hypomethylation increases in more advanced cancers and is also related to tumor size and high grade tumors (Tangkijvanich et al., 2007; Florl et al., 1999; Kitkumthorn and Mutirangura, 2011). Breast cancer is an example which also shows high LINE-1 hypomethylation as compared to their normal tissue surrounding tissues (Chalitchagorn et al., 2004; Cho et al., 2010). LINE-1s can control gene expression by producing unique RNA sequences (combination of pre-mRNA of host gene and RNA of LINE-1). These sequences can transcribe from 5' UTR LINE-1 promoter which can transcribe in both forward and reverse directions (Matlik et al., 2006; Weber et al., 2010). For the reverse direction of unique RNA sequences, they can regulate RNA-induced silencing complex (RISC) and AGO2 protein. This mechanism can abolish host gene mRNAs and results in repression gene expression (Aporntewan et al., 2011). Here we found that all three sex steroids down-regulated genes containing intragenic LINE-1 in breast cancer cells. Therefore, in cancer, sex steroids may help initiate LINE-1s retrotransposition by promoting hypomethylation process and cause down-regulated genes with intragenic LINE-1.

Interestingly, DHT was found to stimulate genes containing intragenic LINE-1 under the same condition which was found to repress genes containing intragenic LINE-1. This phenomenon may be explained by another type of LINE-1 unique RNA sequence which transcribes in the forward direction. Promoter of LINE-1s can act as another promoter to generate these unique transduction sequences. Some of LINE-1 transcriptions (isoform) can produce from LINE-1 position and continue to the end of the host gene sequence (Kitkumthorn and Mutirangura, 2011). Production of these isoforms may be initiated by 10 nM DHT and results in increase mRNA and cause up-regulated genes containing LINE-1.

Breast cancer has been reported in both male and female. Endogenous sex hormone levels especially in E2 have been associated with increased breast cancer risk in both sexes (Sieri et al., 2009; Brinton et al., 2015). High expression of ERα was found associate to the size of tumor in breast carcinomas (Hosseini et al., 2014). Even though breast cancer is a leading cause of death in female, approximately 0.2% of all malignancies were found in male (Buzdar, 2003; Yang et al., 2004). There was a report that found that the increasing risk of ER+ /PR+ and ER+ /PR cancers was associated with high levels of testosterone (Sieri et al., 2009) This dominant hormone in male can convert to estrogen by aromatase enzyme which directly stimulates tumor cell proliferation (Dickson and Stancel, 2000). High rates of ER and PR expression in male were found while lower ER and PR were found in female (Giordano et al., 2004; Nadji et al., 2005; Shandiz et al., 2015). Together, these data suggest that sex steroids play important roles in the etiology and progression breast cancer. The high sex hormone receptor expression in male breast cancer may relate to worse prognosis as compared than in female breast cancer.

In conclusion, our findings suggest that intragenic LINE-1 may play an important role in sex steroid mediated gene expression in breast cancer cells. In addition, sex steroids activation of LINE-1 mRNA through genes containing intragenic LINE-1 could provide a novel mechanism for sex steroids induced cancer. However, more studies will be needed to investigate a possible role of sex steroid induced LINE-1 mRNA in sex steroid induced carcinogenesis.

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Connection up-or down-regulation expression analysis of microarrays extension (CU-DREAM Ex) is a bioinformatics program which is used to intersect microarray expression data with genes containing LINE-1 library. The CU-DREAM Ex program and user guideline can be downloaded at http://pioneer.netserv.chula.ac.th/~achatcha/cu-dream.This research has been supported by the Thailand Graduate Institute of Science and Technology (TGIST), National Science and Technology Development Agency (NSTDA) research fund (TG01-55-003) to SC and the Rachadaphiseksomphot Endowment Fund 2013 of Chulalongkorn University (CU-56-355 HR) to VB.

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