G9A performs important roles in the progression of breast cancer through upregulating its targets

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Abstract. Breast cancer (BC) is the most common type of malignancy in females worldwide, however, its underlying mechanisms remain poorly understood. The present study aimed to investigate the mechanisms behind the development and progression of BC and identify potential biomarkers for it. The chromatin immunoprecipitation-DNA sequencing (ChIP-Seq) dataset GSM1642516 and gene expression dataset GSE34925 were downloaded from the Gene Expression Omnibus database. Affy and oligo packages were used for the background correction and normalization of the gene expression dataset. Based on Limma package and the criteria of a fold change >1.41 or <0.71, and a false discovery rate adjusted P-value <0.05, differentially-expressed genes (DEGs) in euchromatic histone lysine methyltransferase 2 (*G9A*) -knockout (KO) breast samples compared with control samples were identified. The Database for Annotation, Visualization and Integrated Analysis was used for the functional enrichment analysis of the DEGs. Bowtie 2 and model-based analysis of ChIP-Seq version 14 (macs14) were used for the mapping of raw reads and the identification of G9A binding sites (peaks), respectively. In addition, overlapping genes between the DEGs and genes in the peaks located in -3000 to 3000 bp centered in the transcription start sites (conpeaks) were screened out and microRNAs (miRNAs) believed to regulate those overlaps were identified through the TargetScan database. A total of 217 DEGs were identified in G9A-KO samples, which were mainly involved in the biological processes and pathways associated with the inflammatory response and cancer progression.

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A total of 10,422 peaks, containing 1,210 conpeaks involving 1,138 genes, were identified. Among the 1,138 genes, 15 were overlapped with the DEGs, and 35 miRNAs were identified to regulate those overlaps. Insulin-induced gene 1 was regulated by 9 genes in the miRNA-gene regulation network, which may indicate its importance in the progression of BC. The present study identified potential biomarkers of BC that may be useful in the diagnosis and treatment of patients with the disease.

Introduction

Breast cancer (BC), is a type of malignancy that poses the greatest threat to female health worldwide, despite its relatively long 5-year survival rate (~58.75% between 1972 and 2011 in China) (1). BC is one of the most common types of cancer in Asia, where ~39% of all worldwide cases are diagnosed (2). The treatment and care for patients with BC is a large economic burden in developed and developing countries (3-5). Considering the health and economic effects of BC, numerous studies have been conducted to investigate the pathogenesis, and to identify therapeutic targets and potential factors that may contribute its progression. However, the mechanisms behind the development and progression of BC remain poorly understood, and additional studies are required.

G9A [also known as euchromatic histone lysine methyltransferase 2 (*EHMT2*)], encodes a methyltransferase that methylates lysine residues of histone H3. The dysregulation of *G9A* is associated with numerous abnormal biological processes and the emergence of diseases, including cancer. For example, the downregulation of *G9A* could trigger DNA damage responses and inhibit the progression of colorectal cancer (6). In head and neck squamous cell carcinoma, *G9A* dysregulation has been associated with epithelial-mesenchymal transition (EMT)-mediated metastasis and the maintenance of cancer stem cell-like characteristics (7). Furthermore, it has been identified that *G9A* performs important roles in the transition of acute-to-chronic pain (8). In BC, the aberrant expression of *G9A* could affect the stability of SRY-box 2 (SOX2), a tumor suppressor gene, by inducing its epigenetic silencing (9). Furthermore, a study by Si *et al* (10) revealed that *G9A* performed important roles in the dysfunction of the reciprocal feedback loop between GATA binding protein 3 and zinc finger E-box binding homeobox 2, and

that it contributed to the progression of BC. The combined analysis of its genome-wide profiles and the gene expression data following *G9A*-knockdown (KO) or overexpression in BC could improve our understanding of its regulatory functions, and has not previously been investigated.

MicroRNAs (miRNAs/miRs) are small non-coding RNA molecules, 22-25 nucleotides in length. miRNAs are formed from the precursor miRNA, a double-stranded hairpin RNA or even the intergenic region, and perform important roles in the regulation of gene expression at the post translation level by silencing mRNA translation or inducing protein degradation (11). Numerous miRNAs have been shown to contribute to the progression of a number of cancer types. For example, the downregulation of *miR-711* could inhibit the cellular proliferation of BC, and *miR-711* has been shown to be an independent prognostic factor for BC (12). *miR-27a/b* and *miR-494* may regulate the expression of tissue factor pathway inhibitor α (*TFPIα*) in its estrogen-mediated downregulation in BC (13).

In the present study, through the combined analysis of genome-wide profiles of *G9A* obtained through chromatin immunoprecipitation-DNA sequencing (ChIP-Seq) and the gene expression dataset [from the Gene Expression Omnibus (GEO)] following *G9A*-KO in BC, potential biomarkers were identified for the treatment of BC. miRNAs that may regulate those biomarkers were screened out and the core miRNAs and genes were obtained. The findings of the present study may be valuable for the diagnosis and treatment of BC.

Materials and methods

ChIP-Seq and gene expression dataset. In the present study, the ChIP-Seq and gene expression datasets were comprehensively obtained from the GEO database (http://www.ncbi.nlm.nih.gov/geo). The ChIP-Seq dataset (GSM1642516) was deposited from the study by Si *et al* (10), which used the G9A-specific antibody to capture the DNA fragments in MCF-7 cells and sequenced those fragments based on the GPL11154 Illumina HiSeq 2000 (*Homo sapiens*). Furthermore, an input control sample, which extracted all DNA in the human breast adenocarcinoma MCF-7 cell line, was adopted for the background correction in the identification of *G9A* binding sites (peaks). The gene expression dataset GSE34925 (14) contained 3 *G9A*-KO and 3 control BC samples, which were based on GPL6244 (HuGene-1_0-st) Affymetrix Human Gene 1.0 ST Array [transcript (gene) version] (Affymetrix, Inc., Santa Clara, CA, USA).

Differential expression analysis. The raw CEL data (data storage format) were imported to R based on the affy package (15). Background correction and expression normalization was conducted using the oligo package (16). Probe IDs were converted to gene symbols via the annotation package of the microarray platform and the expression values were summarized that corresponding to multiple probe sets. A paired Student's t-test was conducted between logarithmic transformed expression values of *G9A*-KO samples and control samples. Differentially-expressed genes (DEGs) with a fold-change expression value of >1.41 or <0.71, and a false discovery rate corrected P-value of <0.05 were screened out.

Functional enrichment analysis. The Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov/) (17) was used for the functional analysis of the DEGs. Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.kegg.jp/) pathways that satisfied the threshold of P<0.05 were screened out.

Genome-wide binding analysis of G9A. Bowtie 2 (18), an ultrafast genome aligning tool, was used for the mapping of the raw reads to UCSC hg19 genome with a maximum of 2 mismatches in every read. Model-based analysis of ChIP-Seq version 14 (macs14) (19) was used for the identification of the binding sites of *G9A* (also known as call peak) with a P-value of <1x10^{-5}. Peaks located in -3000 to 3000 bp centered in the transcription start sites (TSS) were considered to be conpeaks. Based on the ChIPseeker package (20), the nearby genes, genome features (including promoter and 5'-untranslated region) and distances to the nearest TSS were assigned to the peaks.

Construction of miRNA-gene network. The overlapping genes between DEGs and genes located in the conpeaks were screened out. miRNAs that may regulate those overlaps were identified using the TargetScan database (http://www.targetscan.org/) (21). The miRNA-gene regulation network was visualized through Cytoscape (http://www.cytoscape.org/).

Results

DEGs. A total of 217 DEGs were identified in the *G9A*-KO BC samples, and the distribution of these DEGs is demonstrated in Fig. 1. The supervised clustering (Fig. 2) based on those DEGs was able to separate the *G9A*-KO and control samples well.

Enriched functions. A total of 172 GO terms and 3 KEGG pathways were identified to be enriched in DEGs, and were mainly involved in the cell process, the inflammatory response and cancer progression, for example, the extracellular region part. The top 10 GO terms according to P-value and KEGG pathways are presented in Tables I and II, respectively.

Genome-wide binding profile of G9A. A total of 10,422 binding sites (peaks) of *G9A* were identified, including 1,210 conpeaks located in -3000 to 3000 bp centered in the TSS. The distribution of all peaks across the genome is shown in Fig. 3. In addition, 1,138 genes were identified to be located in the 1,210 conpeaks and 15 of these genes overlapped with DEGs (Table III).

miRNA-gene regulation network. Based on TargetScan, a total of 35 miRNAs were identified to regulate the 15 overlapping genes, and 65 miRNA-gene pairs were obtained among them. The miRNA-gene regulation network is shown in Fig. 4. The top 10 genes according to their connectivity in the miRNA-gene network are listed in Table IV. In the network, insulin-induced gene 1 (*INSIG1*) was regulated by 9 miRNAs and had the highest connectivity, which indicated that it was a hub gene. Furthermore, two other public

Table I. Top 10 GO terms according to the P-value.

Category	GO term	Count	P-value
CC	Extracellular space	27	5.22×10^{-8}
CC	Extracellular region part	32	9.50×10^{-8}
BP	Organ development	44	1.04×10^{-6}
BP	Anatomical structure development	53	1.44×10^{-5}
BP	System development	50	1.59×10^{-5}
BP	Integrin-mediated signaling pathway	8	1.70×10^{-5}
CC	Extracellular region	44	1.95×10^{-5}
BP	Inflammatory response	15	2.81×10^{-5}
BP	Blood vessel development	13	3.09×10^{-5}
BP	Defense response	21	3.32×10^{-5}

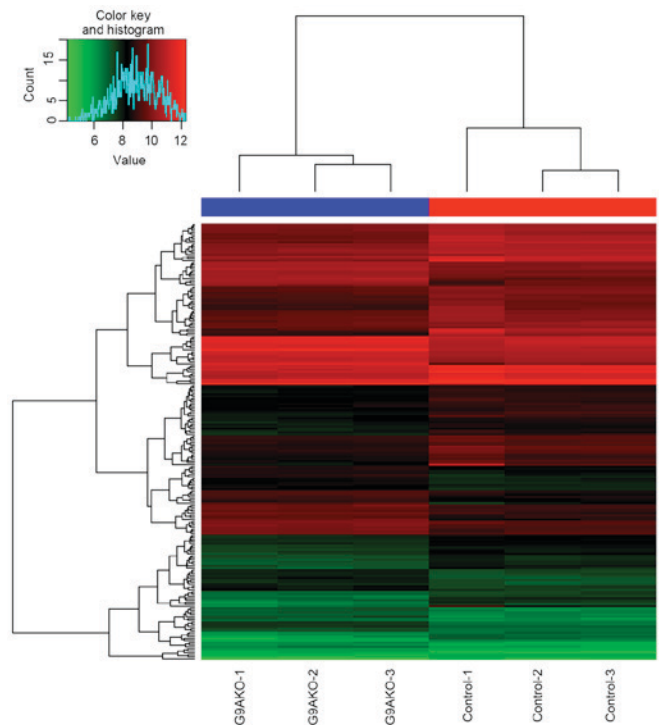GO, gene ontology; CC, cellular component; BP, biological process.

Figure 2. Two-way supervised clustering of differentially-expressed genes and samples using R software. *G9A*, euchromatic histone lysine methyltransferase 2; KO, knockout.
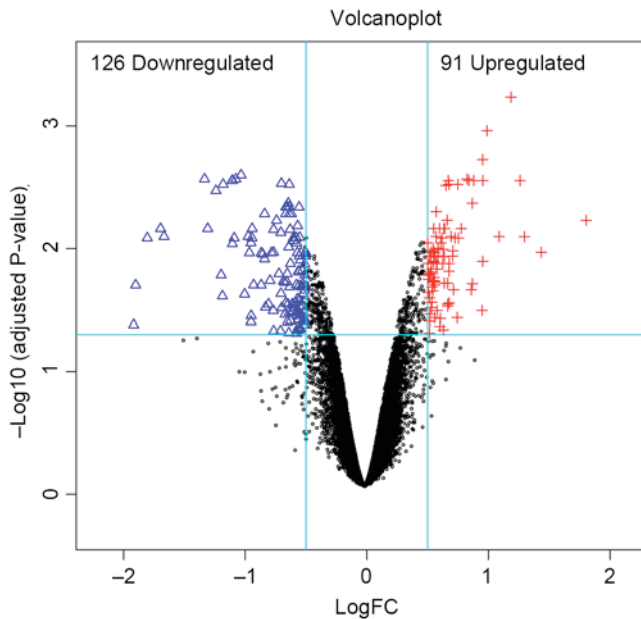

Figure 1. Distribution of DEGs in euchromatic histone lysine methyltransferase 2-knockout samples. The red plus signs and blue triangles represent upregulated and downregulated genes, respectively. The black circles represent non DEGs. FC, fold-change.
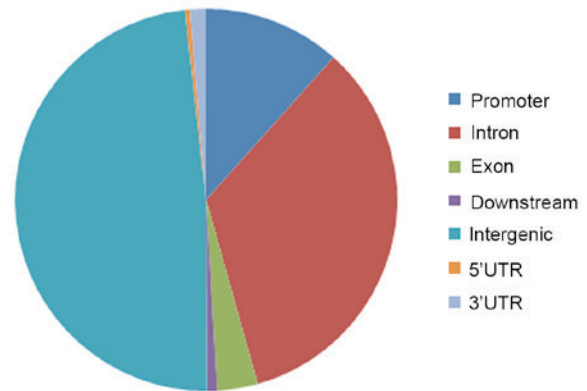

Figure 3. Distribution of peaks of euchromatic histone lysine methyltransferase 2 across the genome. UTR, untranslated region.

datasets (GSE29044 and GSE36774) were downloaded from the GEO database, which including data from breast cancer and normal tissue samples. These two datasets were background corrected and quantile normalized using the preprocessCore package of R (http://www.bioconductor.org/packages/release/bioc/html/preprocessCore.html). The Pearson and Spearman's correlation indices were respectively calculated using R between relative INSIG1 and G9A mRNA levels. The results indicated a significantly positive correlation (Fig. 5) between *G9A* and *INSIG1*, which may demonstrate their important roles in the progression of BC. The top 10 genes according to their connectivity in miRNA-gene network are presented in Fig. 4.

Discussion

BC is a complicated disease, and numerous factors may contribute to its initiation and progression, including occupational exposure (22), variations in expression level or mutations of specific genes (23,24). The traditional treatment options for BC mainly involve surgical resection, chemotherapy and radiotherapy, which have been shown to be effective but rarely eradicate the disease. With the development of next-generation sequencing technology, numerous biomarkers of BC have been identified and certain novel therapeutics have been proposed (25). Immunotherapy is one of the most popular therapeutic treatment options (26,27). The identification of novel biomarkers may therefore improve the prognosis for patients with BC.

Table II. Enriched Kyoto Encyclopedia of Genes and Genomes pathways.

Pathway name	Count	P-value	Genes
Cytokine-cytokine receptor interaction	13	2.99×10^{-4}	*CSF2, TNFSF4, IL24, IL7R, CXCL11, TNFSF18, CXCL10, KDR, INHBB, VEGFC, CXCL16, IL1A, GHR*
Focal adhesion	11	5.24×10^{-4}	*VEGFC, ITGB8, LAMA5, ITGB4, ITGA2, RELN, BIRC3, VAV1, MYLK, KDR, SPP1*
ECM-receptor interaction	6	6.80×10^{-3}	*ITGB8, LAMA5, ITGB4, ITGA2, RELN, SPP1*

ECM, extracellular matrix.

Table III. Overlapping genes between differentially-expressed genes and genes located in conpeaks.

Gene	logFC
SLCO4C1	-0.65617
ARHGAP24	-0.66706
ANK2	-0.55143
TMX4	0.54535
LARP4	-0.50197
TC2N	-0.78340
TMTC1	0.52521
SLFN5	-0.62773
CYTIP	0.67537
PLCB4	-0.55811
ATF7IP2	-1.19761
CAMK2D	-0.50487
TLR4	0.85905
INSIG1	-1.18712
GHR	-0.60367

FC, fold-change.

Table IV. Top 10 genes according to their connectivity in the microRNA-gene network.

Gene	Connectivity
INSIG1	9
ARHGAP24	8
CAMK2D	8
GHR	8
ANK2	7
TLR4	6
TMX4	6
CYTIP	4
miR-141	4
miR-145	4

Figure 4. miRNA-gene regulation network. Rectangles show genes, whilst miRNAs are shown in ovals. Arrows represent the interactions between these genes and miRNAs.

In the present study, the genome-wide profiles of *G9A* in genome wide were identified using ChIP-Seq. DEGs in *G9A*-KO BC samples compared with the control samples were screened out. The combined analysis obtained potential direct targets of *G9A*, the majority of which were downregulated in the *G9A*-KO BC samples, which may indicate its upregulated functions in BC.

DEGs in *G9A*-KO samples are primarily involved in biological processes or pathways associated with cellular processes, inflammatory responses and cancer progression, including extracellular region part, cytokine-cytokine receptor interaction and extracellular membrane (ECM) receptor interaction, all of which are closely associated with the development of BC. In particular, inflammatory triple-negative BC, a type of rare and aggressive BC with a significantly poorer 5-year survival rate compared with other types of BC, is believed to mainly be attributed to an abnormal inflammatory response (28,29). Previous studies have considered that the ECM-receptor interaction pathway may be associated with the progression of BC, which could indicate the repeatability of the present study (30,31).
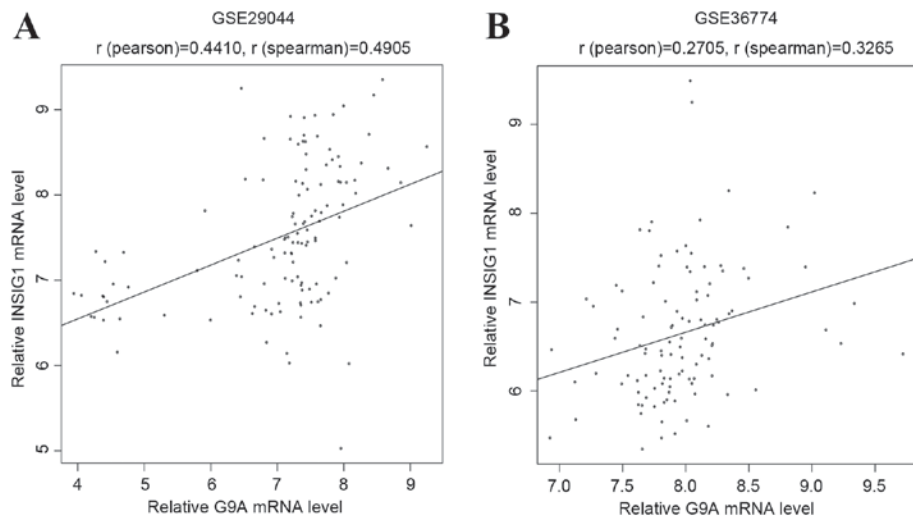
Figure 5. (A and B) Expression values of *G9A* were plotted against that of *INSIG1* based on the other two public datasets (GSE29044 and GSE36774). *G9A*, euchromatic histone lysine methyltransferase 2; *INSIG1*, insulin-induced gene 1.

Among the 1,138 genes distributed in the 1,210 peaks located between -3000 to 3000 bp centered in the TSS, 15 were identified to overlapped with the DEGs in the *G9A*-KO BC samples. Furthermore, 11 of those overlaps were revealed to be downregulated in *G9A*-KO samples compared with the control samples, which may indicate that *G9A* is mainly involved in the upregulation of its target genes. The positive correlation between the expression values of *G9A* and *INSIG1* was verified using two public datasets (Fig. 5). Furthermore, *INSIG1* possessed an intimate connectivity pattern in the miRNA-gene regulation network (Fig. 4). Thus, this suggests that *G9A* and *INSIG1* serve important roles in the progression of BC. *INSIG1*, which is downregulated in *G9A*-KO BC, was closely associated with the progression of several types of cancer via the regulation of glucose metabolism and hypoxia-induced EMT (32,33). BC had a strong association with insulin resistance, which was mediated by *INSIG1* (34). In previous studies, *INSIG1* was also demonstrated to be differentially expressed in BC (35,36). Combined with the results of the present study, this indicates the important roles of *INSIG1* in BC.

miR-203 regulated 3 genes in the miRNA-gene regulation network, including *INSIG1*, which has been reported as a tumor suppressor in numerous types of cancer, including BC (37-39). The activity of *miR-203* was also affected by a number of other factors, including kallistatin (40). Therefore, it would be useful to study the upstream and downstream regulation loops of *miR-203* for the diagnosis and treatment of BC, as well as other types of cancer. Other nodes (miRNAs or genes) with high connectivity in the miRNA-gene regulation network may also be potential biomarkers for BC, which should be additionally verified through molecular biological experiments.

In conclusion, the present study conducted the combined analysis of ChIP-Seq and gene expression profiles in BC, and identified potential biomarkers via screening. The present results may aid the development of novel diagnostic or treatment methods, including immunotherapy, and therefore improve the prognosis of BC.

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