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ORIGINAL PAPER

Cell signaling events differentiate ER-negative subtypes from ER-positive breast cancer

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Abstract Currently available markers routinely used in clinical practice are of limited value to patients with estrogen receptor-negative (ER^-) breast cancer [basal-like and HER2neu-positive (HER^+)], an aggressive subtype. Our aim was to uncover molecular pathways and signaling networks exposed by differentially methylated genes informative of the biology of ER^- breast cancer (BC) subtypes versus ER-positive (ER^+). Whole-genome methylation array analysis was carried out using the Illumina Infinium HumanMethylation27 BeadChip on 14 primary BC: five ER^+, four triple-negative (TNBC), and five ER^-HER2^+. Degree of methylation was calculated as a β-value (ranging from 0 to 1), and M-values [log (β/(1 − β)] were used for significance tests. To identify methylated genes associated with ER^- subtypes (TNBC and ER^-HER2^+) and distinct from ER^+, a weighted algorithm, developed to increase statistical rigor, called out genes in which methylation changed dramatically between ER^+ and ER^- subtypes. Differentially methylated gene lists examined using Ingenuity Pathway Analysis called out canonical pathways and networks with clues to biological distinctiveness as well as relatedness between ER^- subtypes as compared to ER^+ BC. The study highlights the interplay of ER^- subtype-specific genes and their signaling pathways as potential putative fingerprints in refining classification of BC subtypes and as potential biological markers designed to hit multiple targets.

Keywords Breast cancers subtypes · Differential methylation · Canonical pathways · Networks

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Introduction

Despite advances in breast cancer (BC) treatment and outcome over the last two decades, women continue to relapse and die of advanced disease. Currently available markers routinely used in clinical practice are of limited value to patients with estrogen receptor-negative (ER^-) BC [basal-like and HER2neu (HER2)-positive (HER^+)], an aggressive subtype. Gene expression studies using DNA microarrays have identified subtypes of BC that were not apparent using traditional histopathologic methods [1, 2]. Four common subtypes have been identified: two of these are derived from ER^- tumors (basal-like and $HER2^+$) and two are derived from ER-positive (ER^+) tumor subtypes of luminal A and B [2, 3]. Though the ''basal-like'' category of breast tumors (BLBC) is composed almost entirely of ''triple-negative'' breast cancers [4] (TNBC) [i.e., tumors that are ER^-, progesterone receptor-negative (PR^-), and HER2-negative ($HER2^-$)], TNBC is a term based on immunohistochemistry (IHC) assays for ER, PR, and HER2, whereas basal-like is a molecular phenotype initially defined using cDNA microarrays [1, 2]. Though most TNBC cluster within the basal-like subgroup, these terms are not synonymous; there is up to 30 % discordance between the two groups [5–8].

Up to now, the major BC subtypes have been characterized as distinct entities based on genomic, transcriptomic, and proteomic levels [1, 2]. The biological significance of DNA methylation in the regulation of gene expression and

its role in cancer are increasingly recognized. Utilizing global approaches, the goal was to identify aberrantly methylated genes that underlie the pathogenesis of ER^-BC ($ER^-PR^-HER2^-$ and $ER^-PR^-HER2^+$) and to uncover molecular pathways and signaling networks exposed by differentially methylated genes (DMG) informative of the biology of ER^-BC subtypes vs ER^+BC.

Materials and methods

Patient cohort

The study cohort comprised fresh-frozen samples from 14 primary BC: five ER-positive (ER^+), four triple-negative (TNBC), and five ER^-HER2^+. DNA was extracted according to the manufacturer's protocol (Qiagen Inc, Chatsworth, CA, USA). This study was approved by the Henry Ford Health System Institutional Review Board.

Genome-wide methylation

Whole-genome methylation array analysis was carried out using the Illumina Infinium HumanMethylation27 BeadChip to quantify the proportion of methylated cytosines (5mC) to total cytosines at 27,578 different CpG dinucleotides located in more than 14,000 gene promoters. The methylation score for each CpG is represented as a beta (β) value according to the fluorescent intensity ratio. Every β value is accompanied by a detection p value. β values may take any value between 0 (non-methylated) and 1 (completely methylated) and were determined using the GenomeStudio (Illumina, San Diego, USA). Probes are discarded if this detection p value is more than 0.05. The only corrections that are made to the data are background subtraction and normalization. The resulting beta values were exported into Microsoft Excel and JMP (SAS Institute, USA) for data analysis. All genome-wide comparisons were corrected for multiple comparisons using the method of Benjamini and Hochberg [9, 10].

Data analysis

Data were analyzed by GenomeStudio software (Illumina). Degree of methylation was calculated as a β-value (ranging from 0 to 1), and M-values [$\log(\beta/(1-\beta))$] were used for significance tests. To identify methylated genes associated with ER^- subtypes (TNBC and ER^-HER2^+) and distinct from ER^+, a downsizing algorithm utilizing a three-tiered approach was developed to call out genes in which methylation changed dramatically between ER^+ and ER^- subtypes was used. Tier 1 computed adaptive FDR (aFDR) [9] values for all CpGs/(or their averages for each gene) to be 0.05 or lower. Tier 2 included Tier 1 CpGs/genes with a twofold change (ratio $\geq$ 2.0 or ratio $\leq$ 0.5). Tier 3 required an additional restriction to include Tier 2 CpGs/genes with an absolute difference between the mean β s of $\geq$0.2. Tier 3 differentially methylated gene lists between ER^+ and ER^- groups/subtypes were examined using Ingenuity Pathway Analysis (IPA) software.

Pathway analysis

To determine the biological processes enriched within genes differentially methylated in our comparisons, gene lists were uploaded into Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc.). IPA integrates genes and molecules which are part of the same biological functions or regulatory networks interacting together. Core analyses of statistically significantly differentially methylated Tier 3 gene lists were undertaken for comparison groups ER^+ versus ER^-, TNBC versus ER^+, and ER^-HER2^+ versus ER^+ using IPA's "genes only knowledge base," with default parameters including validation options like "experimentally validated" and "highly predicted."

Results

Differentially methylated genes

Genes along the X and Y chromosomes were removed for a total of 13,890 genes [11]. Data analysis accounted for DMG among four separate groups to include (1) ER^- versus ER^+, (2) TNBC versus ER^+, (3) ER^-HER2^+ versus ER^+, and (4) TNBC versus ER^-HER2^+. Differentially methylated genes for comparison groups at each Tier level are provided in Table 1.

At Tier 3, there were 207 DMG for ER^- versus ER^+, 191 DMGs for TNBC versus ER^+, and 99 DMGs for ER^-HER2^+ versus ER^+. For TNBC versus ER^-HER2^+, only two Tier 3 genes, *CYBA* and *COL21A1*, were noted.

IPA pathway analysis

IPA individual reports for each comparison group/subtype analyses for ER^+ and ER^- samples provided the most enriched canonical pathways with enrichment p values and ratio (number of molecules from a dataset with total number of molecules in that particular pathway) and associated networks with score in each analyses.

The top canonical pathways (Table 2) and networks (Table 4) called out by the differentially methylated Tier 3 genes among comparison subtypes for ER^- versus ER^+ are detailed in Tables 2 and 4. The two Tier 3 DMG between

Table 1 Differentially methylated genes (*X*, *Y* chromosomes removed; total genes = 13890)

Comparison group	Differentially methylated genes	Hypermethylated	Hypomethylated
ER^-(all) versus ER^+			
Tier 1	1782	1070	712
Tier 2	331	28	303
Tier 3	207	19	188
TNBC versus ER^+			
Tier 1	1995	1157	838
Tier 2	305	41	264
Tier 3	191	27	164
ER^-HER2^+ versus ER^+			
Tier 1	761	428	333
Tier 2	144	59	85
Tier 3	99	31	68
TNBC versus ER^-HER2^+			
Tier 1	432	208	224
Tier 2	35	3	32
Tier 3	2: (*CYBA*, *COL21A1*)	1(*COL21A1*)	1(*CYBA*)

Table 2 Comparison of Top Canonical pathways (with pathway *p* value ranking and ratio of # of differentially methylated genes among total pathway genes)

ER^- versus ER^+: 207 differentially methylated genes	TNBC versus ER^+: 191 differentially methylated genes	ER^-HER2^+ versus ER^+: 99 differentially methylated genes
Axonal Guidance Signaling 1.12E−03, 13/464 (0.028)[a]	Axonal Guidance Signaling 4.85E−03, 11/464, (0.024)[a]	Tryptophan Degradation III (Eukaryotic) 5.25E−03, 2/21, (0.095)
CDK5 Signaling 3.09E−03, 5/90, (0.056)	Transcriptional Regulatory Network in Embryonic Stem Cells 7.95E−03, 3/40, (0.075)[a]	Transcriptional Regulatory Network in Embryonic Stem Cells 1.83E−02, 2/40, (0.05)[a]
Protein Kinase A Signaling 3.78E−03, 11/387, (0.028)	GABA Receptor Signaling 1.24E−02, 3/47, (0.064)	Axonal Guidance Signaling 2.42E−02, 6/464, (0.013)[a]
Erythropoietin Signaling 6.75E−03, 4/74, (0.054)	Human Embryonic Stem Cell Pluripotency 1.24E−02, 5/152, (0.033)	P2Y Purigenic Receptor Signaling Pathway 2.56E−02, 3/129, (0.023)
Dopamine-DARPP32 Feedback in cAMP Signaling 1.02E−02, 6/167, (0.036)	CDK5 Signaling 1.28E−02, 4/90, (0.044)	CMP-N-acetylneuraminate Biosynthesis I (Eukaryotes) 2.57E−02, 1/5, (0.2)

[a] shared canonical pathways

Table 3 Shared canonical pathway genes (bolded)

Comparison Groups/# genes/total pathway genes	Axonal guidance signaling pathway genes: total 464 genes in pathway
ER^- versus ER^+/13/464	***PAPPA2***, ***WNT3A***, ***PRKCQ***, ***ADAM12***, ***UNC5A***, ***WNT9B***, ***NTRK3***, *EPHB2*, ***ARHGEF7***, ***PRKAR1B***, ***NGF***, ***ADAMTS2***, ***PRKCB***
TBNC versus ER^+/11/464	***PAPPA2***, ***WNT3A***, ***WNT9B***, ***ADAM12***, ***NTRK3***, ***ARHGEF7***, *ADAM19,* ***NGF***, ***ADAMTS2***, WNT2
ER^-HER2^+ versus ER^+/6/464	*GNG4*, ***WNT3A***, *RHOD*, ***NGF***, *HKR1*, ***ADAMTS2***
	Transcriptional Regulatory Network in Embryonic Stem Cells: total 40 genes in pathway
ER^- versus ER^+/2/40	***ISL1***, ***HAND1***
TBNC versus ER^+/2/40	***ISL1***, ***HAND1***
ER^-HER2^+ versus ER^+/3/40	***ISL1***, ***HAND1***, *GSX2*

Bolded shared genes among comparison groups

Un-bolded unique genes

TNBC versus ER^-HER2^+ *CYBA* and COL21A1 were excluded from IPA analysis (due to inadequate number of genes).

There was some overlap of top canonical pathways among ER^-BC groups as compared to ER^+ (Table 2). For example, the axonal guidance signaling pathway was the top-ranked pathway in ER^- versus ER^+ BC and in TNBC versus ER^+ and ranked second in ER^-HER2^+ versus ER^+. Of the 464 genes in this pathway, 13 of the 207 DMG between ER^- and ER^+ were represented in this canonical pathway (Table 3). Of these 13 genes, 9 genes *PAPPA2*, *WNT3A*, *PRKCQ*, *ADAM12*, *WNT9B*, *NTRK3*, *ARHGEF7*, *NGF*, and *ADAMTS2* were common to TNBC versus ER^+ and three genes *WNT3A*, *NGF*, and *ADAMTS2* to ER^-HER2^+ versus ER^+ (Table 3). For TNBC versus ER^+, of the 11 genes in the pathway, two were unique to the TNBC subtype (*ADAM19*, *WNT2*); for the ER^-HER2^+ versus ER^+ group, of the six genes in the pathway, three were unique (*GNG4*, *RHOD*, and *HKR1*).

Similarly, the Transcriptional Regulatory Network in Embryonic Stem Cells pathway common to ER-negative subtypes TNBC versus ER^+ and ER^-Her2^+ versus ER^+ also showed similar ranking. Of the 40 genes in this pathway, *ISL1* and *HAND1* were common to both comparison groups ER^-HER2^+ versus ER^+ (Fig. 1a highlights 2/40 genes) and TNBC versus ER^+ (Fig. 1b highlights 3/40 genes: *ISL1*, *HAND1*, and *GSX2*). This pathway was not ranked among the top five for ER^- versus ER^+.

Comparison of top networks among the three comparison groups indicates distinct networks. However, several biological activities spelled out within a network's annotation such as cellular development are common among the three comparison groups (Table 4).

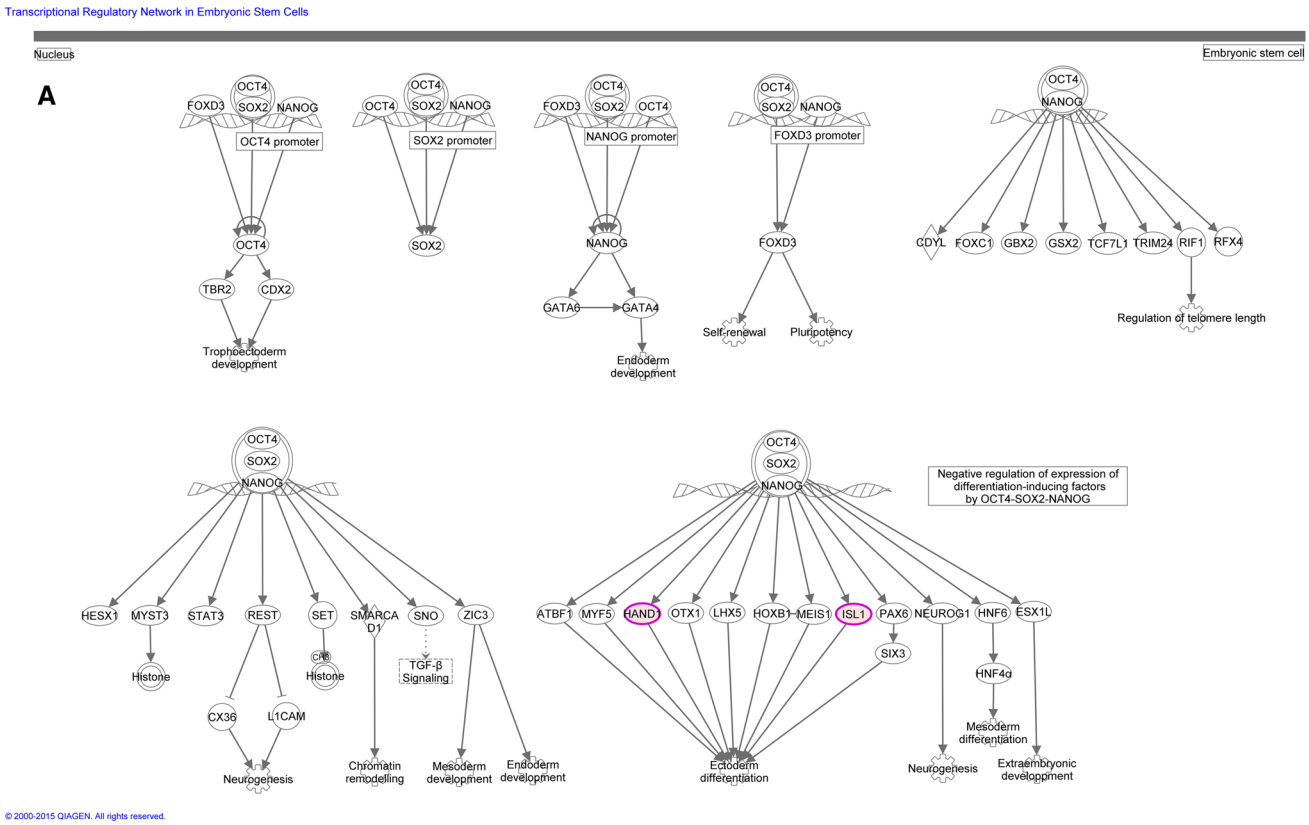
Discussion

Most cancers represent complex diseases characterized by multiple- rather than single-gene alterations. The complexity of BC is further compounded by its high heterogeneity making progress for improved diagnosis and treatment a major challenge. Current clinical methods based on anatomic staging (tumor size and node status) and on a few immunohistochemical markers are unable to accurately classify subtypes and develop appropriate treatment plans. Because of availability and price, IHC is commonly used to infer tumor subtype; however, it is less accurate than gene expression profiling. Genomics has shown that BC is a spectrum of disease with distinct molecular alterations accounting for differences in treatment response and outcome [12–14]. This information has allowed us to improve prognostic accuracy in early-stage BC and has redefined how we manage estrogen receptor-positive disease [15, 16]. Conversely, estrogen receptor-negative BC is more aggressive than ER^+ with an overall worse prognosis and few targeted treatment options. Subtype classification by the PAM50 gene expression signature shows at least two distinct subtypes of ER^-, referred to as basal-like and HER2-enriched (HER2E). >90 % of Basal-like tumors are triple-negative (ER^-/PR^-/$HER2^-$); however, approximately one-third of triple-negative cancers are something other than basal-like. The cancer genome atlas (TCGA) network using multiple platforms to investigate BC subgroups concluded that diverse genetic and epigenetic alterations converge phenotypically into four major expression-only breast tumor subgroups, i.e., luminal A, luminal B, HER2-positive, and triple-negative [17].

Fig. 1 Canonical pathway: Transcriptional Regulatory Network in Embryonic Stem Cells **a** ER^-HER2^+ versus ER^+: 2/40 genes in pathway: *ISL1*, *HAND1*, **b** TNBC versus ER^+: 3/40 genes in pathway: *ISL1*, *HAND1*, and *GSX2*

Recent cancer genome sequencing efforts, including those of BC, have led to the identification of novel cancer genes and previously unrecognized signatures of mutational processes [18, 19]. These studies highlight acquired mutations often affecting genes involved in regulating chromatin dynamics or the processing of epigenetic marks as seen in various cancer types [17, 19], supporting the importance of the epigenome in cancer development. However, there is a dearth of knowledge about the contribution of epigenetic changes to the development of biologically distinct BC subtypes. This is mainly due to a lack of comprehensive profiling technologies or a lack of validated clinical relevance in independent patient cohorts [17, 20].

We report on DMG not only between ER^+ and ER^- but also between ER-negative subtypes of TNBC and ER^-HER^+ subtypes using the Illumina 27 K platform and provide a glimpse of their biological activities based on pathway analysis. With the advent of high-throughput platforms, large-scale structure of genomic methylation patterns is available through genome-wide scans and tremendous amount of DNA methylation data have been recently generated. However, sophisticated statistical methods to handle complex DNA methylation data are very limited. For this study, we proposed a weighted model-based approach of a 3-Tier strategy. Our goal for a 3-Tier system was to provide a framework to increase statistical rigor in the detection of biologically relevant methylation markers. The latter achieves two outcomes: (1) exclusion of CpGs likely to increase the risk of false discoveries and (2) serves as a strategy to reduce the number of genes/CpGs for confirmation especially for study cohorts with DNA and RNA sources challenged by formalin fixation or


Transcriptional Regulatory Network in Embryonic Stem Cells
Nucleus
Embryonic stem cell
A
OCT4
SOX2
NANOG
FOXD3
OCT4 promoter
SOX2 promoter
NANOG promoter
FOXD3 promoter
TBR2
CDX2
Trophoectoderm development
GATA6
GATA4
Endoderm development
Self-renewal
Pluripotency
CDYL
FOXC1
GBX2
GSX2
TCF7L1
TRIM24
RIF1
RFX4
Regulation of telomere length
HESX1
MYST3
STAT3
REST
SET
SMARCAD1
SNO
ZIC3
Histone
CAB
TGF-β Signaling
CX36
L1CAM
Neurogenesis
Chromatin remodelling
Mesoderm development
Endoderm development
Negative regulation of expression of differentiation-inducing factors by OCT4-SOX2-NANOG
ATBF1
MYF5
HAND1
OTX1
LHX5
HOXB1
MEIS1
ISL1
PAX6
NEUROG1
HNF6
ESX1L
SIX3
HNF4α
Mesoderm differentiation
Ectoderm differentiation
Neurogenesis
Extraembryonic development
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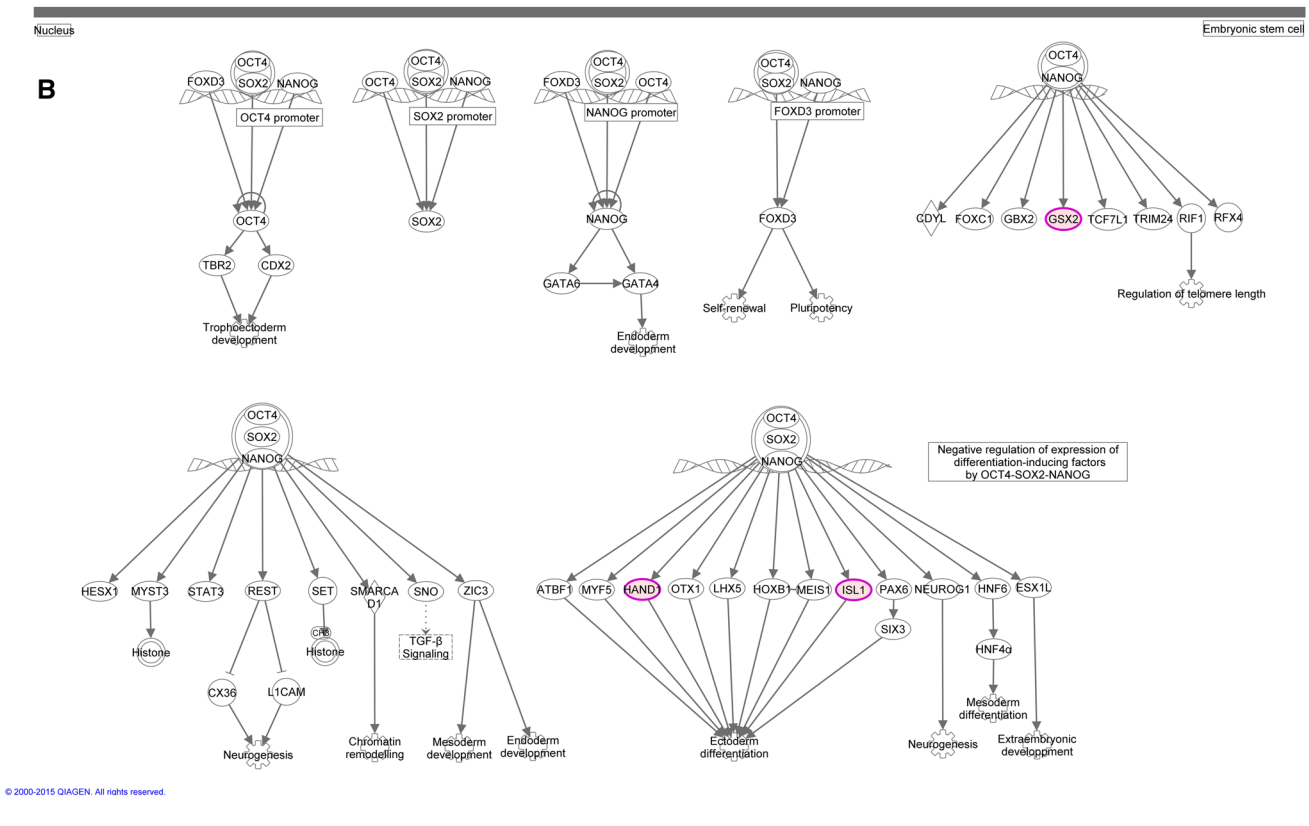
Transcriptional Regulatory Network in Embryonic Stem Cells
Nucleus
Embryonic stem cell
B
OCT4
SOX2
NANOG
FOXD3
OCT4 promoter
SOX2 promoter
NANOG promoter
FOXD3 promoter
TBR2
CDX2
Trophoectoderm development
GATA6
GATA4
Endoderm development
Self-renewal
Pluripotency
CDYL
FOXC1
GBX2
GSX2
TCF7L1
TRIM24
RIF1
RFX4
Regulation of telomere length
HESX1
MYST3
STAT3
REST
SET
SMARCAD1
SNO
ZIC3
Histone
CAB
TGF-β Signaling
CX36
L1CAM
Neurogenesis
Chromatin remodelling
Mesoderm development
Endoderm development
Negative regulation of expression of differentiation-inducing factors by OCT4-SOX2-NANOG
ATBF1
MYF5
HAND1
OTX1
LHX5
HOXB1
MEIS1
ISL1
PAX6
NEUROG1
HNF6
ESX1L
SIX3
HNF4α
Mesoderm differentiation
Ectoderm differentiation
Neurogenesis
Extraembryonic development
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meager amounts of tissue availability for molecular characterization.

Pathway analysis has become the first choice for extracting and explaining the underlying biology for high-throughput molecular measurements, as it reduces complexity and has increased explanatory power. A pathway-level understanding of genomic perturbations to better decipher changes observed in cancer cells is supported by recent pilot studies by TCGA and others [21, 22], demonstrating that even when patients harbor genomic alterations or aberrant expression in different genes, these genes often participate in a common pathway [23].

A limitation of this study was the small number of samples particularly for the ER-negative subtypes. Regardless, DMG met the Tier 3 bar among all comparison groups. Top-ranked canonical pathways and networks suggest biological distinctiveness as well as relatedness between ER^- subtypes as compared to ER^+ BC. The latter is illustrated for axonal guidance signaling and the Transcriptional Regulatory Network in Embryonic Stem Cells canonical pathways.

Axon guidance is integral to organogenesis, regeneration, wound healing, and other basic cellular processes [24, 25]. This signaling pathway is comprised of genes with important regulatory roles in normal neuronal migration and positioning during embryonic development. More recently, axon guidance pathway genes have been implicated in cancer cell growth, survival, invasion, and angiogenesis [26, 27]; however, the incidence of aberrations in these genes in cancer is largely unknown. In pancreatic cancer, the widespread genomic aberrations observed in axon guidance genes suggest involvement of axon guidance genes in pancreatic carcinogenesis [26]. The DMG observed here in axon guidance suggests that they may have a role in ER^-BC, particularly with respect to providing further differentiation among ER^- subtypes of TNBC and ER^-HER2^+.

The Transcriptional Regulatory Network in Embryonic Stem Cells canonical pathways also ranked highly for ER^- subtypes TNBC versus ER^+ and ER^-HER2^+ versus ER^+. Pluripotency and self-renewal are two defining properties of embryonic stem (ES) cells. Pluripotency is the capacity to generate all cell types, while self-renewal is the capacity to maintain ES cells in a proliferative state for extended periods [28]. Breast cancer stem cells were first identified as a $CD44^{hi}/CD24^{lo}$ population with enhanced ability to initiate tumor growth when xenografted into immunocompromised mice [29]. The $CD44^{hi}/CD24^{lo}$ phenotype has been found to be associated with the basal-like BC tumor subgroup [30] and BRCA1 tumors [30, 31], suggesting that it might be indicative of aggressive molecular subtypes.

For TNBC versus ER^-HER2^+, only two Tier 3 genes, *CYBA* and *COL21A1*, were noted and were not interrogated in IPA due to a paucity of genes. The *CYBA* gene regulates immune system cells and autophagy through phagocytic clearance and was recently reported in pathways associated with pancreatic cancer [32]. *COL21A1* was among 14 genes in the pancreatic cancer study whose expression correlated strongly and universally with that of heme oxygenase-1 (HO-1) [33]. HO-1 is expressed in many cancers and promotes growth and survival of neoplastic cells and has been implicated in tumor cell invasion and metastasis [34]. Deregulation of collagen genes including *COL21A1* has been reported in ovarian cancer [35].

Recent studies show that genes differentially methylated between clinically important tumor subsets play roles in differentiation, development, and tumor growth and may be critical to establishing and maintaining tumor phenotypes and clinical outcomes [36]. A 2014 study assessing

Table 4 Comparison of top networks (with score in parenthesis)

ER^- versus ER^+: 207 differentially methylated genes	TNBC versus ER^+: 191 differentially methylated genes	ER^-HER2^+ versus ER^+: 99 differentially methylated genes
Cellular development, nervous system development and function, neurological disease (48)	Nervous system development and function, organ morphology, cellular movement (49)	Tissue morphology, cardiovascular system development and function, organismal development (45)
Nutritional disease, cancer, cardiovascular system development and function (41)	Cellular development, nervous system development and function, tissue development (49)	Cellular assembly and organization, nervous system development and function, cellular growth and proliferation (21)
Psychological disorders, cell morphology, cancer (34)	Cellular compromise, cardiovascular disease, organismal injury and abnormalities (26)	Organismal development, cell-to-cell signaling and interaction, cellular growth and proliferation (19)
Cell-to-cell signaling and interaction, infectious disease, cell death and survival (18)	Cellular development, gene expression, nervous system development and function (18)	Gene expression, organismal survival, organ morphology (19)
Cellular development, gene expression, organismal development (16)	Cell morphology, skeletal and muscular system development and function, cell death and survival (18)	Drug metabolism, lipid metabolism, small molecule biochemistry (17)

differentially methylated CpGs supports distinct mechanisms leading to changes in CpG methylation states operative in different BC subtypes [37], where DNA methylation patterns (CpGs) were linked to the luminal B subtype characterized by CpG island promoter methylation events.

In our study, the biological processes enriched within genes differentially methylated at the Tier 3 level in our comparisons become clearly delineated in top-ranked canonical pathways and networks. Signaling events in the cell play a critical role in the execution of key biological functions providing clues to how complex cellular signaling cascades and networks may be programmed in ER^- subtypes as compared to ER^+ BC. This pilot study highlights the interplay of ER^- subtype-specific genes and their signaling pathways as potential putative fingerprints in refining classification of BC subtypes and as potential biological markers designed to hit multiple targets.

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Conflict of interest None declared by the authors.

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