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ER regulates an evolutionarily conserved apoptosis pathway

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ABSTRACT

Estrogen receptor (ER) is regarded as a major causal factor in breast cancer and FoxA1, a winged-helix transcription factor belonging to the forkhead family, has been found to function as a pioneer factor in the recruitment of ER to several *cis*-regulatory elements in the genome. High throughput chromatin immunoprecipitation analyzed by hybridization to microarrays (ChIP-chip) can reveal ER and FoxA1 binding sites occupied by transcription factors. However, these results are blind to lineage-specific *cis*-regulatory elements. In this study, we identified ER and FoxA1 binding sites conserved in evolution by using Detection of LinEage-Specific Selection (DLESS) method. We also analyzed target genes close to conserved *cis*-regulatory elements by combining gene-expression data. A total of 7877 ER binding sites and 18,135 FoxA1 binding sites were identified in MCF-7 cells by performing an unbiased genome-wide ChIP-chip with False Discovery Rate (FDR) of 5%. Using DLESS method, we found target candidate genes closest to fully conserved *cis*-regulatory elements related to apoptosis according to gene ontology analysis. Furthermore, network analysis of apoptosis-related genes within 10 kb of fully conserved *cis*-regulatory elements was constructed using Ingenuity Pathway Analysis (IPA). Apoptosis genes in the network showed over- and under-expression in MCF-7 cell-line. And these apoptosis-related genes closest to fully conserved *cis*-regulatory elements in network showed strong correlation with ER in MCF-7 cells. These results elucidate that ER regulates an evolutionarily conserved apoptosis pathway. This opens up new perspectives in the apoptosis of human breast cancer from the evolution of *cis*-regulatory elements.
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1. Introduction

Estrogen receptor (ER) is considered as an essential regulator in female development and control of reproductive functions [1–3]. It has been shown as a major causal factor in breast and endometria cancer [4–6]. FoxA1 is a member of the forkhead class of transcription factors. Over-expression of FoxA1 has been commonly observed in ER-positive breast tumors due to initiation of cancers from luminal cells [7]. Recent studies have demonstrated that FoxA1 binding was essential for ER-chromatin interactions and subsequent expression of estrogen gene targets [8]. However, these results are blind to lineage-specific *cis*-regulatory elements. High throughput chromatin immunoprecipitation analyzed by hybridization to microarrays (ChIP-chip) can only reveal DNA binding sites occupied by transcription factors regardless of sequence conservation. Functional *cis*-regulated elements may be conserved across species under evolutionary constraints [9].

Detection of LinEage-Specific Selection (DLESS) is a powerful method to detect lineage-specific selection compared with other methods [10]. Most methods that allow for lineage-specific selection have required a priori specification of the branches of the tree on which the mode of selection may change. However, DLESS method does not require element boundaries to be determined a priori, making it particular useful for identifying *cis*-regulatory elements in noncoding sequences that are unusually conserved across species.

In the present study, we combined high throughput ChIP-chip analysis with lineage-specific selection method to study ER and FoxA1 *cis*-regulatory elements in the whole genome to explore regulatory mechanism in breast cancer.

2. Materials and methods

2.1. cis-Regulatory elements of ER/FoxA1

Whole-genome ChIP-chip raw data of ER/FoxA1 was performed as previously described [4,11]. MAT [12] was applied to ChIP-chip experimental data to predict *cis*-regulatory elements with False Discovery Rate (FDR) of 5%.

2.2. Conservation analysis of cis-regulatory elements of ER/FoxA1

Detection of LinEage-Specific Selection (DLESS) algorithm was applied in conservation analysis of ER/FoxA1 *cis*-elements. DLESS

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algorithm is based on a phylogenetic hidden Markov model (phylo-HMM). It is a generalization of the two-state phylo-HMM ("conserved" and "nonconserved") used by the phastCons program. DLESS works on the same principle, but also allows for conserved elements that have been "gained" or "lost" on any branch of the phylogeny. This algorithm is highly efficient and suitable for use with noncoding sequences.

2.3. Construction of gene network

Apoptosis-related gene network was constructed on the basis of Ingenuity Pathway Analysis (IPA). It is a system that transforms a list of genes of interest into a set of relevant networks based on structured content from Ingenuity's Pathways Knowledge Base (IPKB). This knowledge base contains over one million highly-structured findings manually curated from the scientific literature. The IPA algorithm uses a global molecular network of direct interactions observed among mammalian orthologs computed from the IPKB. It is a web-delivered application that enables the discovery, visualization, and exploration of molecular interaction networks in gene-expression data.

2.4. Correlation between ER/FoxA1 and apoptosis gene

Correlations between ER/FoxA1 and apoptosis gene were measured by Pearson's correlation coefficient based on cancer microarray data that are deposited in Gene-Expression Omnibus (GEO) and Oncomine. Pearson correlations were calculated to determine the correlation between a pair of genes a and b for all samples n:

$$r_{a,b} = \frac{\sum_n (x_{an} - \overline{x_a})(x_{bn} - \overline{x_b})}{\sqrt{\sum_n (x_{an} - \overline{x_a})^2}\sqrt{\sum_n (x_{bn} - \overline{x_b})^2}} \quad (1)$$

where x_{an} denotes the expression value for gene a in samples n and $\overline{x_a}$ is the mean of expression values for gene a.

3. Results

3.1. Identification of ER and FoxA1 cis-regulatory elements

We performed an unbiased genome-wide ChIP-chip to define ER and FoxA1 *cis*-regulatory elements in the MCF-7 breast cancer cell-line, as previously described [4]. A total of 7877 ER binding sites and 18,135 FoxA1 binding sites were identified in these cells using MAT algorithm [12] with False Discovery Rate (FDR) of 5%. The number of FoxA1 binding sites was more than twice the number of ER binding sites. Next, we mapped these FoxA1 and ER binding sites within the most recent available human genome. The mapping revealed a highly significant overlap with ER binding sites occurring on FoxA1 occupied sites. This co-binding indicated a functional relationship between ER and FoxA1. Therefore, ER and FoxA1 *cis*-regulated elements were analyzed together in the following research.

3.2. Lineage-specific analysis of ER and FoxA1 cis-regulatory elements

ER and FoxA1 *cis*-elements were classified into three kinds of events (gain, loss and conserved) by using DLESS. "Gain" means conserved in the subtree. "Loss" means conserved in all species except the subtree. And "conserved" means fully conserved in the whole species tree [10]. A total of 1763, 5124 and 30,721 subregions were identified as gain, loss and conserved events, respectively. The majority of subregions were identified as conserved, compared with gain and loss. In addition, the power of DLESS to detect fully conserved elements is excellent, even when element lengths are as small as 15 bp [10]. Moreover, cross-species conservation can be used as an effective filter for improving selectivity of detection of functional elements in the genome [13]. For these reasons, our analysis mainly focused on fully conserved *cis*-regulatory elements across species under evolutionary constraints. Furthermore, we made sure that different subregions in each *cis*-regulatory element had consistent evolutionary event. A total of 7416 conserved subregions were identified under this constraint. Finally, 5369 conserved subregions were selected for further analysis when subregions of less than 15 bp were excluded.

3.3. Ontological analysis of gene close to conserved ER and FoxA1 cis-regulatory elements

We searched for closest transcription start site (TSS) of RefSeq gene to each conserved subregions within a range of 10 kb. According to Gene Ontology (GO) analysis, annotation cluster with the largest enrichment score is related with apoptosis. However, this event did not occur in the *cis*-regulatory elements that belonged to gain or loss. Apoptosis did not appear in the first three ranked annotation clusters. The gene set belonged to apoptosis near conserved *cis*-regulatory elements was extracted for further analysis.

3.4. Apoptosis-related gene network

Network of gene set related to apoptosis was shown to contain 39 genes, of which 30 genes were included in our analysis (Fig. 1). The network described functional relationships among genes based on known interactions reported in literature. We analyzed the expression of these 30 apoptosis-related genes near *cis*-regulatory elements in MCF-7 cell-lines according to Gene-Expression Omnibus (GEO) and Oncomine database. Among these genes, *AIFM1*, *AIFM2*, *ANXA4*, *BAG3*, *BIK*, *CD3E*, *CLU*, *DNASE1*, *HDAC1*, *LYZ*, *MAL*, *NFKBIA*, *NGFR*, *RHOB*, *SOCS2*, *TIAF1*, *TNFRSF1A*, *TRAF4* and *ZBTB16* were regarded as under-expression in the MCF-7 breast cancer cell-line. In contrast, *BMF*, *CBX4*, *EGLN3*, *IER3*, *MAPK1*, *PAK1*, *PIM1*, *PTPRC*, *SIAH2*, *TP63* and *TPD52L1* exhibited over-expression in the MCF-7 breast cancer cell-line. The network recognized several important nodes with numerous interaction partners, including *MYC*, *TP53* and *HDAC1*. *MYC* could interact with *CLU*, *TPD52*, *CBX4*, *LYZ*, *ANXA4*, *IER3*, *PTPRC*, *NFkB*, *RHOB*, *ZBTB16*, *HDAC1*, *TP53*, *PIM1* and *Hsp70*. *TP53* could interact with *TP63*, *MAPK1*, *BIK*, *AIFM2*, *HDAC1*, *NFKBIA*, *IER3*, *MYC*, *Hsp70* and *CLU*. *HDAC1* could interact with *MYC*, *RHOB*, *NFkB*, *ZBTB16*, *NFKBIA*, *TP53* and *Hsp70*, which were all down-regulated in our 30 genes list. In addition, *AIFM2*, *TRAF4* and *NGFR*, which were functionally interacted with each other and formed a straight line in the network, were all down-regulated in breast cancer cells.

3.5. Correlation between ER/FoxA1 and genes in apoptosis gene network

We used the Pearson correlation coefficient and its absolute value as a measure of similarity between expression profiles of these genes and ER/FoxA1. There was a striking correlation between ER/FoxA1 and other genes. The absolute value of correlation coefficient was above 0.6 (Fig. 2). Compared with ER, there was a minor correlation between FoxA1 and other genes.

4. Discussion

FoxA1, which has documented function related to ER, was regarded as a "pioneer factor" responsible for the recruitment of ER to the genome [11]. Almost 45% of ER binding sites co-occurred in the same regions of FoxA1. Expression patterns of FoxA1 in normal breast and tumors were remarkably similar to those of ER [7]. Co-binding of ER and FoxA1 *cis*-regulated elements implied the

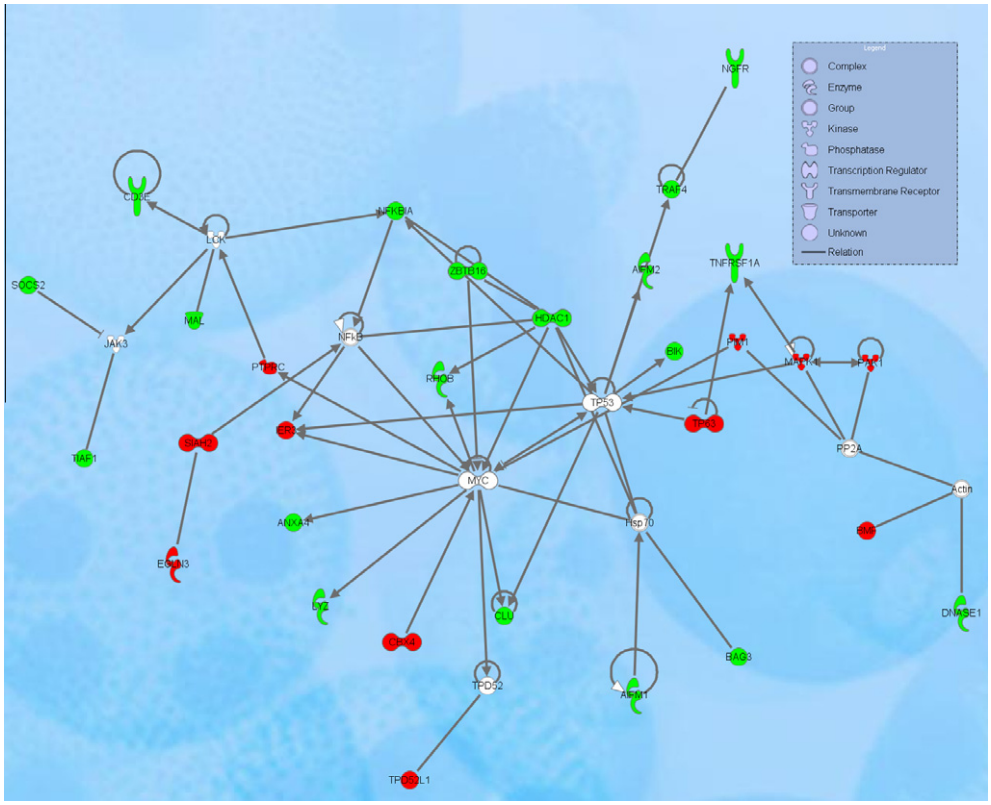

Fig. 1. Apoptosis-related genes network is shown. The gene network is constructed by Ingenuity software. Genes are colored according to gene expression value. Red gene symbols indicate up-regulation and green gene symbols indicate down-regulation. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

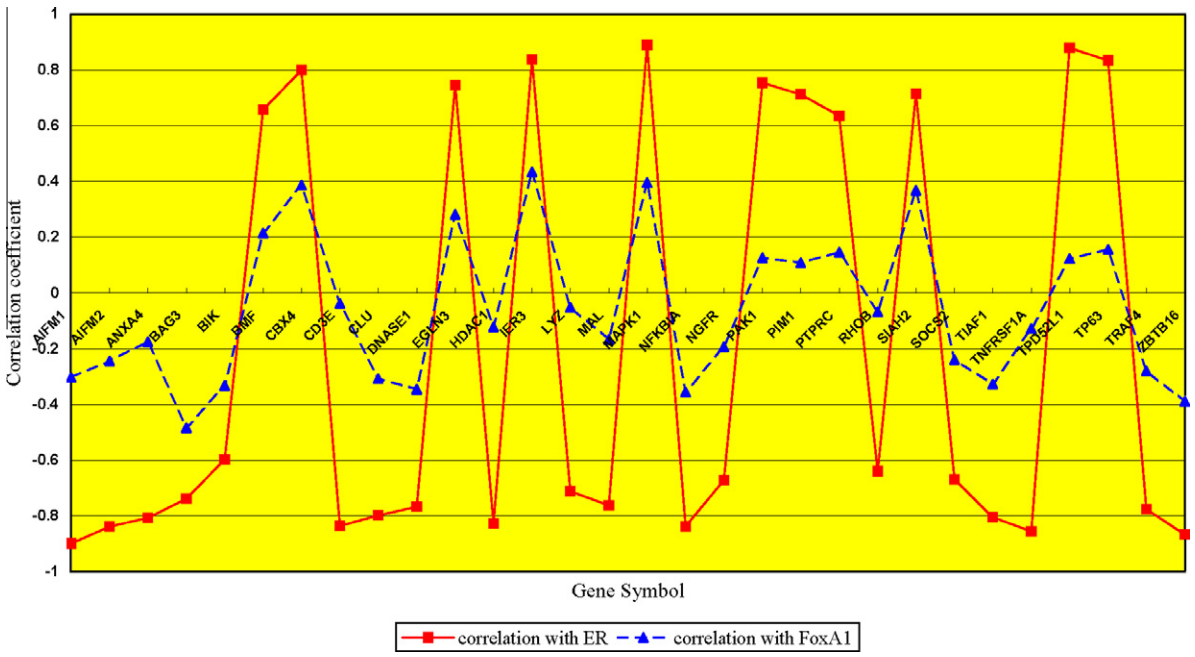

Fig. 2. Correlation between apoptosis-related gene and ER/FoxA1 is shown. The horizontal axis represents each gene symbol. The vertical axis represents the correlation between ER/FoxA1 and each gene in apoptosis network. Red solid line indicates correlation between apoptosis gene and ER. Blue dashed line indicates correlation between apoptosis gene and FoxA1. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

functional significance. Previous studies mainly focused on genome-wide distribution of ER/FoxA1 binding sites. Here, we placed our emphasis on conservation of ER/FoxA1 binding sites among different species. From a view of life-history evolution, fully conserved elements may take critical function. Therefore, fully conserved ER/FoxA1 binding sites were extracted separately for further analysis. The results showed that genes close to these fully conserved *cis*-regulated elements were related to apoptosis by GO

analysis. This means fully conserved ER/FoxA1 *cis*-regulated elements may control apoptosis-related genes. Several papers published by different labs independently support the conclusion. For example, *BIK* plays a critical role in promoting estrogen starvation or antiestrogen-induced apoptosis of human breast cancer cells using DNA Microarray, RT-PCR and Western blotting analysis [14]. *HDAC1* interacts with ER *in vitro* and *in vivo* and suppresses ER transcriptional activity [15,16]. The interaction of *HDAC1* with ER in breast cancer cells has been detected by a series of experiments involving RT-PCR [16,17]. *NFKBIA* was identified as the estrogen down-regulated gene in the microarray and reverse transcription-PCR analyses [18]. *RHOB* played a role in the negative regulation of ER transcriptional activity using the C3 exoenzyme [19]. *PAK1* might be involved in the progression of breast cancer and in the regulation of estrogen response through its interaction with ER [20–27]. The genes presented in these articles are included in the list of apoptosis-related genes from our study. Failure of apoptosis is one of the main contributions of tumor development. This indicates fully conserved ER/FoxA1 *cis*-regulated elements may contribute to pathogenesis of breast cancer.

We constructed apoptosis-related gene network to systematically study interactions among these 30 apoptosis-related genes. We have been curious to know why the remaining 9 genes in this gene network do not locate within a range of 10 kb from the center of conserved elements. The positions of these 9 genes in human genome were retrieved from UCSC genome browser database. The closest distance between each gene and the center of closest conserved elements was calculated, respectively. Because preliminary analysis [28] found that "*cis*-candidate region" lied 500 kb upstream of TSS and 500 kb downstream of transcription end site (TES), the distance was evaluated not only from transcription start sites, but also from transcription end sites. We took into account the closest conserved *cis*-regulatory elements to TSS/TES of each gene. The results showed that the distances between the center of closest conserved *cis*-regulatory elements and TSS/TES of each of these nine genes all exceeded 10 kb (Table 1). This was the reason why these 9 genes were excluded from apoptosis-related gene network. Four genes (*LCK*, *TPD52*, *PP2A* and *Actin*) were added into this network when criterion was extended to 100 kb. Only *NFkB* and *JAK3* still were excluded from this network when criterion was extended to 500 kb. This was basically in agreement with the definition of "*cis*-candidate region" [28]. *NFkB* plays a major role in oncogenesis and regulating the expression of genes involved in the development and progression of cancer such as proliferation, migration and apoptosis. More recently, *NFkB* activation has been connected with the control of apoptosis [29] and inhibition of *JAK3* induces apoptosis [30]. Long-distance regulatory interactions between evolutionarily conserved *cis*-regulatory elements and apoptosis-related genes deserve further investigation.

In addition, we noticed a comparative number of apoptosis-related genes that were up- and down-regulated in MCF-7 cells. *SOCS2* (suppressor of cytokine signaling-2) was regarded as under-expression in the MCF-7 breast cancer cell-line. *JAK3* exhibited outlier expression in leukaemia and brain in the Oncomine database. In this network, *SOCS2* showed inhibition of active *JAK3*. *TIAF1* interacted with *JAK3*. When transiently over-expressed, *TIAF1* induced apoptosis MCF-7 breast cancer cells [31]. *LCK* protein increased tyrosine phosphorylation of ITAM from *CD3E* protein. *CD3E* exhibited outlier expression in leukaemia in the Oncomine database. *MAL*, which was regarded as over-expression in breast cancer [32], interacted with *LCK*. *PTPRC*, which was regarded as under-expression in breast cancer [33], increased tyrosine kinase activity of *LCK*. *BMF* was up-regulated in MCF-7 cells [34]. *LYZ* was down-regulated in MCF-7 cells [35]. Recent findings demonstrated that apoptosis was induced by chemoresistant drugs in ER-negative breast cancer cells [36,37]. Weak correlation between these apoptosis-related genes and FoxA1 did not necessarily indicate that there was no relationship between FoxA1 and apoptosis. In this study, we just considered apoptosis-associated gene occurred near fully conserved *cis*-regulatory elements of ER/FoxA1 from an evolutionary point of view. Actually, a potential link between FoxA1 and the cell cycle machinery has been found by identifying $p27^{kip1}$ up-regulation on FoxA1 silencing [38].

Table 1
The distance between genes excluded from apoptosis network and conserved *cis*-regulatory elements of ER/FoxA1.

Gene symbol	Access number	Location	Closest distance from TSS (bp)	Closest distance from TES (bp)
MYC	NM_002467	chr8:128817497-128822860	113,374	108,011
TP53	NM_000546	chr17:7512445-7531588	168,374	149,231
NFkB	NM_003998	chr4:103422486-103538458	563,279	763,599
JAK3	NM_000215	chr19:17796593-17819841	802,726	825,974
LCK	NM_005356	chr1:32489427-32524353	70,670	35,744
TPD52	NM_005079	chr8:81109660-81246391	57,140	47,825
Hsp70	NM_002154	chr5:132415561-132468608	193,953	140,906
PP2A	NM_021131	chr9:130913065-130951044	82,473	44,494
Actin	NM_001101	chr7:5533305-5536758	13,484	16,937

TSS: transcription start site; TES: transcription end site.

5. Conclusions

This study confirms that candidate genes near fully conserved ER and FoxA1 binding sites across evolution are associated with apoptosis. These apoptosis-related genes exhibited over-expression or under-expression in the MCF-7 breast cancer cell-line. A strong correlation was shown to exist between apoptosis-related gene and ER. In contrast, the correlation between apoptosis-related gene and FoxA1 was weaker. Our results demonstrate that ER regulates an evolutionarily conserved apoptosis pathway. This opens up new perspectives in the apoptosis of human breast cancer from the evolution of *cis*-regulatory elements.

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