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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Steroid hormones play pivotal roles in the control of breast cancer proliferation and differentiation. The biological effects of these hormones are mediated through intracellular receptor proteins, e.g. the estrogen receptor (ER) and through interaction of these receptors with specific co-activators. Of particular interest is the co-activator AIB1 (amplified in breast cancer 1). The AIB1 gene was found to be amplified in 5-10% of primary breast tumor specimens and AIB1 mRNA was found to be overexpressed in many breast tumor specimens. It has been shown that AIB1 promotes the activity of the ER and the growth of human MCF-7 breast cancer cells. Our hypothesis was that AIB1 determines activation or repression of a vast number of genes important for breast cancer growth. Our goal in this study was to identify these target genes.
We generated MCF-7 breast cancer cell lines in which we could specifically downregulate AIB1 levels utilizing tetracycline-regulatable ribozymes directed against AIB1. Utilizing these cells, we found, after estrogen and growth factor stimulation, that gene expression profiles differed significantly from cells with high AIB1 levels when compared to cells with significantly reduced AIB1 levels by microarray analysis. Several of the identified AIB1 target genes might have implications for breast cancer therapy.

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Introduction

Small fat soluble hormones such as steroids, retinoids and vitamin D3 play pivotal roles in the control of breast cancer proliferation and differentiation. The biological effects of these molecules are mediated through intracellular receptor proteins. Estrogens stimulate proliferation of estrogen receptor (ER) positive breast cancer cells and the ER status of a breast tumor is predictive of the outcome of the disease. Therapy (such as tamoxifen) targeted at reducing the estrogenic stimulus to the breast has been shown to be effective. Conversely retinoids and vitamin D3 are strongly growth inhibitory in breast tumor cells and analogues of these compounds are currently being tested for efficacy in breast cancer. Recently the discovery of proteins known as steroid receptor co-activators has led to another level of complexity to our understanding of how hormones exert their effects [1,2]. Of particular interest is the co-activator **AIB1 (amplified in breast cancer 1)**, which interacts with the estrogen receptor (ER) [3]. The AIB1 gene was found to be amplified in 5-10% of primary breast tumor specimens, AIB1 mRNA was found to be overexpressed in many breast tumor specimens and AIB1 protein expression is significantly elevated in about 10% of primary breast tumors compared to normal breast tissue. Amplification of the AIB1 gene correlates with estrogen and progesterone positivity of primary breast tumors as well as with tumor size. It has been shown that AIB1 promotes the activity of the ER and estrogen-dependent growth of human MCF-7 breast cancer cells. Therefore, it has been hypothesized that AIB1 may contribute to the development of breast cancer.

Based on these data, **our hypothesis was that AIB1 determines activation or repression of a vast number of genes important for breast cancer growth. Our goal in this study was to identify these target genes.** To accomplish this goal, we generated human breast cancer cell lines (e.g. MCF-7) in which we specifically downregulated AIB1 levels utilizing tetracycline-regulatable ribozymes directed against AIB1 (tet-off system). Utilizing these cells, **we wanted to compare, after estrogen and growth factor stimulation, gene expression profiles from cells which have high AIB1 levels with gene expression profiles from cells that have significantly reduced AIB1 levels by microarray analyisis, in order to identify AIB1 target genes that might have potential implications for breast cancer therapy.**

Proposal Body

In the approved Statement of Work the following major task was outlined.

Task 1 & 2: Using tetracycline regulated ribozymes [13] we aimed to identify gene expression profiles and AIB1 target genes that might have potential implications for breast cancer therapy utilizing these cells by comparing gene expression profiles from cells which have high AIB1 levels with gene expression profiles from cells that have significantly reduced AIB1 levels by microarray analyisis.

The following report summarizes the data obtained with the cDNA array analysis.

Task 1: **The goal of our studies was to perform microarray analysis in order to identify gene expression patterns dependent on AIB1 levels and to identify AIB1 target genes that might have potential implications for breast cancer therapy.**

For this we used cell lines in which we could regulate AIB1 levels using ribozymes [13].

A.) Description of the microarry technology used in this study

The type of array used was the GeneChip® Human Genome U95 set from *Affymetrix, Inc.* This array contains transcript coverage of the entire human genome. Using the U95 set allowed us to study the expression level of more than 60,000 full-length genes and ESTs (expression tagged sequences) using quantitative microarray technology. Represented sequences are derived from sequence clusters in Build 95 of the UniGene database (sequences in UniGene build 95 are from GenBank 113 and dbEST/10-02-99).
Critical specifications of the GeneChip® Human Genome U95 set:

- number of arrays in the set is five,
- the array format is a standard format,
- the feature size is 20 μM,
- the oligonucleotide probe length is a 25-mer,
- the probe pairs/ sequence is ~16
- Detection sensitivity is 1:100,000* (* As measured by detection in a comparative analysis between a complex target containing spiked control transcripts and a complex target with no spikes).
- Control sequences included:
  - Hybridization controls: *bioB, bioC, bioD* and *cre*
  - Poly-A controls: *da, lys, phe, thr,* and *trp*
  - Housekeeping/ Control Genes: GAPDH, beta-Actin, Transferrin receptor and ISGF-3

The DNA-Chip analyzer (dChip) [14] is a software package used for expression analysis of oligonucleotide arrays and high-level analysis procedures, such as hierarchical clustering. Hierarchical clustering uses standard statistical algorithms to arrange genes according to similarity in their pattern of gene expression [15]. Relationships among genes are expressed as a tree whose branch length represents the degree of similarity between genes. Groups of genes with similar expression patterns are put next to each other. Each sample or cell line can also be expressed as a tree and each branch also represents the degree of similarity between samples. For this analysis both samples and the genes were clustered together to form a single dendrogram. The left side of the dendrogram represents relationships among each genes, while the top of the dendrogram represents the relationships among the samples to each other.
The expression values for a gene across all samples are standardized (linearly scaled) to have mean 0 and standard deviation 1. These standardized values are used to calculated

correlations between genes and samples and serve as the basis of merging nodes. Joining two genes creates a node, and then a gene expression profile is computed for the node by averaging observation for the joined genes. Before the hierarchical clustering was performed, genes were initially excluded that showed little variation across the samples or their signal was absent from the majority of the samples. This process is termed filtering genes. This was the criteria we used to filter genes when the 20 samples were analyzed together:

- Variation across samples: 0.5 < standard deviation / mean between < 10.00
- P (present) call % in the array used >= 90%
- Replicate variation: 0.00 < Median (standard deviation / mean) < 0.50)
- The expression level >= 20.00 in >= 100% samples

Using these criteria, 169 genes out of a probe set of 12,600, satisfied this filtering criterion. These 169 genes were then analyzed, comparing the AIB1 ribozyme on/off cells with the different types of treatments. Her2 ribozyme cells [21] were used as a control to determine differences in the gene expression profile in the AIB1 ribozyme cells.

B) Results of the microarray analysis

The result of the microarray analysis is graphically summerized in Fig.1 (see Appendix; on the bottom of the cluster image is the color scale: red represents the expression level above mean expression of a gene across all samples, white represents mean expression and blue represents lower than the mean expression).

The dChip program can catagorize genes into "functional category classification.' Genes are classified according to molecular function, biological processes and cellular components using GeneOntology terms (http://www.geneontology.org) After hierarchical clustering is performed, dChip searches all branches with at least 4 functionally annotated genes to assess whether a local cluster is enriched by genes having a particular function.

Significant GeneOntology clusters found in our analysis are:

- Found 13 "RNA binding" genes in a 116-cluster (all: 328/8100, P value: 0.000791)
  In other words, this cluster has 116 functionally annotated genes (genes without annotation are not counted), of which 13 are RNA binding genes; considering there are all together 328 RNA binding genes in the 8100 functionally annotated genes on the array, this cluster is significantly enriched by RNA binding genes. A p value of 0.000791 is calculated)
- Found 12 "structural protein of ribosome" genes in a 116-cluster (all: 84/8100, P value: 0.000000)
- Found 5 "cell cycle regulator" genes in a 116-cluster (all: 71/8100, P value: 0.003391)
- Found 15 "structural protein" genes in a 116-cluster (all: 440/8100, P value: 0.001458)
- Found 6 "calcium binding" genes in a 116-cluster (all: 109/8100, P value 0.004637)

- Found 15 "transcriptional regulation" genes in a 116-cluster (all: 395/8100, P value: 0.000483)
- Found 16 "protein biosynthesis" genes in a 116-cluster (all: 175/8100, P value: 0.000000)
- Found 16 "cell cycle" genes in a 116-cluster (all: 544/8100, P value: 0.004499)
- Found 19 "biosynthesis" genes in a 116-cluster (all: 343/8100, P value: 0.000000)
- Found 18 "macromolecule biosynthesis" genes in a 116-cluster (all: 221/8100, P value: 0.000000)
- Found 7 "pathogenesis" genes in a 116-cluster (all: 104/8100, P value: 0.000697)
- Found 20 "protein metabolism and modification" genes in a 80-cluster (all: 1078/8100, P value: 0.003365)
- Found 6 "cell cycle control" genes in a 36-cluster (all: 363/8100, P value: 0.004836)
- Found 5 "mitotic cell cycle" genes in a 36-cluster (all: 253/8100, P value: 0.004878)
- Found 9 "cell proliferation" genes in a 36-cluster (all: 363/8100, P value: 0.000669)
- Found 4 "transcription factor" genes in a 7-cluster (all: 789/8100, P value: 0.002458)
- Found 15 "nucleic acid binding" genes in a 41-cluster (all: 1486/8100, P value: 0.004398)

Significant cytoband clusters found in our analysis:

- Found 15 "6p" genes in a 158-cluster (all: 441/11263, P value: 0.001340)
- Found 16 "6" genes in a 116-cluster (all: 684/11263, P value: 0.001683)
- Found 5 "6p21" genes in a 47-cluster (all: 263/11263, P value: 0.004604)
- Found 10 "19" genes in a 55-cluster (all: 723/11263, P value: 0.002340)

Genes found in the array study that are of particular interest in understanding what genes are regulated by the steroid receptor coregulator, AIB1, in a breast cancer cell line treated with different growth factors and hormones.
The hierarchical clustering analysis was able to distinguish the Her2 ribozyme cells from the AIB1 ribozyme cells having distinct gene expression patterns. The expression pattern of AIB1 ribozyme cells in comparison to Her2 ribozyme cells can be separated into 3 general sections. In the first section of significant genes, Her2 ribozyme cells generally have a lower expression level compared to AIB1 ribozyme cells.

1. *"survivin or baculoviral IAP repeat-containing 5"* – Survivin was of particular interest to examine from the first section of the dendrogram. Survivin is prominently expressed in human cancers of the lung, colon, pancreas, prostate and breast. Survivin is also expressed in ~50% of high-grade non-Hodgkin's lymphomas, but not in low grade lymphomas [16]. Survivin or baculoviroal inhibitor of apoptosis protein repeat-containing 5 has a role in regulating apoptosis. Survivin is expressed in G2/M phase of the cell cycle. Survivin associates with microtubules of the mitotic spindle. If the interaction with microtubules is interrupted, the loss of survivin's anti-apoptosis function and an increase in caspase-3 activity, a mechanism involved in cell death, occurs [17]. Survivin expression in the AIB1 ribozyme cells is significantly decreased compared to the Her2 ribozyme cells.

The second section, both AIB1 ribozyme cells and Her2 ribozyme cells have similar levels of expression. AIB1 (+/Her) or AIB1 treated with doxcycline and Heregulin was the least related to the other AIB1 ribozyme samples. 75 of 169 genes were significantly overexpressed in the AIB1 (+/Her) compared with all 19 other cell lines.

2. *"breast cancer metastasis-suppressor 1"*- Of particular interest to study further was the gene, breast cancer metastasis-suppressor 1 or BRMS-1. BRMS-1 has been found to reduce the metastatic capacity of human breast carcinoma cell lines [18]. AIB1 (+/Her) cell line expresses BRMS-1 at a high level compared to the other 19 cell lines. The significance of this differential expression needs to be investigated further.

The third and last section has the most differences in expression between the AIB1 and Her2 ribozyme cells. The Her2 ribozyme cells are all generally down regulated, while the AIB1 ribozyme cells have differential level of expression dependent on the AIB1 status. The AIB1 + cells generally seem to have no change in expression in these genes, while the AIB1 – cells generally have an increased expression in these genes.

3. *"breast carcinoma amplified sequence 1"*- Amplification in chromosome region 20q13 is associated with a large number of tumor types and aggressive tumor behavior. Also, increased copy number in the 20q13 region is found in 40% of breast cancer cell lines and 18% of primary breast tumors [19]. *NABC1 or breast carcinoma amplified in sequence 1* was increased in expression in the AIB1(-) or ribozyme off cell lines compared with AIB1(+) or ribozyme on.

4. *"breast cancer 1, early onset"* – BRCA1 or breast cancer1 early onset is associated with genetic susceptibility to breast cancer. BRCA1 has been found to have a role in the maintaining genomic stability and transcriptional regulation. BRCA1 is located on chromosome 17q, an area frequently amplified in breast cancer [20]. Her2/neu (ERBB2) proto-oncogene is also located on 17q Her ribozyme cells all had a decrease in the expression of BRCA1 in comparison to the AIB1 ribozyme and did not have a significantly different level of expression within the treatments. The AIB1 ribozyme cells, however, expression of BRCA-1 was different depending the status of AIB1. The AIB1(-) ribozyme cells generally had a higher expression of BRCA1 compared to AIB1 (+) ribozyme cells.

Key Research Accomplishments

- ✓ We found 169 genes (out of 12,600 screened) that fullfilled stringent screening criteria after Affymetrix microanalysis. These genes were analyzed, comparing the AIB1 ribozyme on/off cells with the different types of treatments. Her2 ribozyme cells were used as a control to determine differences in the gene expression profile in the AIB1 ribozyme cells.

- ✓ We identified 4 genes (survivin, breast cancer metastasis-suppressor 1, breast carcinoma amplified sequence 1, breast cancer 1, early onset) that were specifically regulated by AIB1 in human MCF-7 breast cancer cells and which might have potential implications for breast cancer therapy and are currently being studied further in the lab.

Reportable Outcomes

List, H-J., Oh, A., and Riegel, A.T. "Finding AIB1 regulated target genes using microarray-based gene expression profiling." Manuscript in preparation.

Conclusions

During this project, we wanted to identify AIB1 target genes that might be important for breast cancer growth. To accomplish this goal, we used human breast cancer cell lines in which we can specifically downregulate AIB1 levels utilizing tetracycline-regulatable ribozymes directed against AIB1 [13]. Utilizing these cells, we wanted to compare gene expression profiles from cells which have high AIB1 levels with gene expression profiles from cells that have significantly reduced AIB1 levels by microarray analyisis, in order to identify AIB1 target genes that might have potential implications for breast cancer therapy.

During this project Affymetrix microarray studies in MCF-7 human breast cancer cells identified gene expression patterns that were different in cells which have high AIB1 levels from gene expression profiles from cells that have significantly reduced AIB1 levels. In addition, we were able to identify four AIB1 target genes that might have potential implications for breast cancer therapy. We anticipate that the results from this study will give valuable insights into the biological significance of AIB1 as well as its potential role as a therapeutic target in breast cancer.

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Figure 1

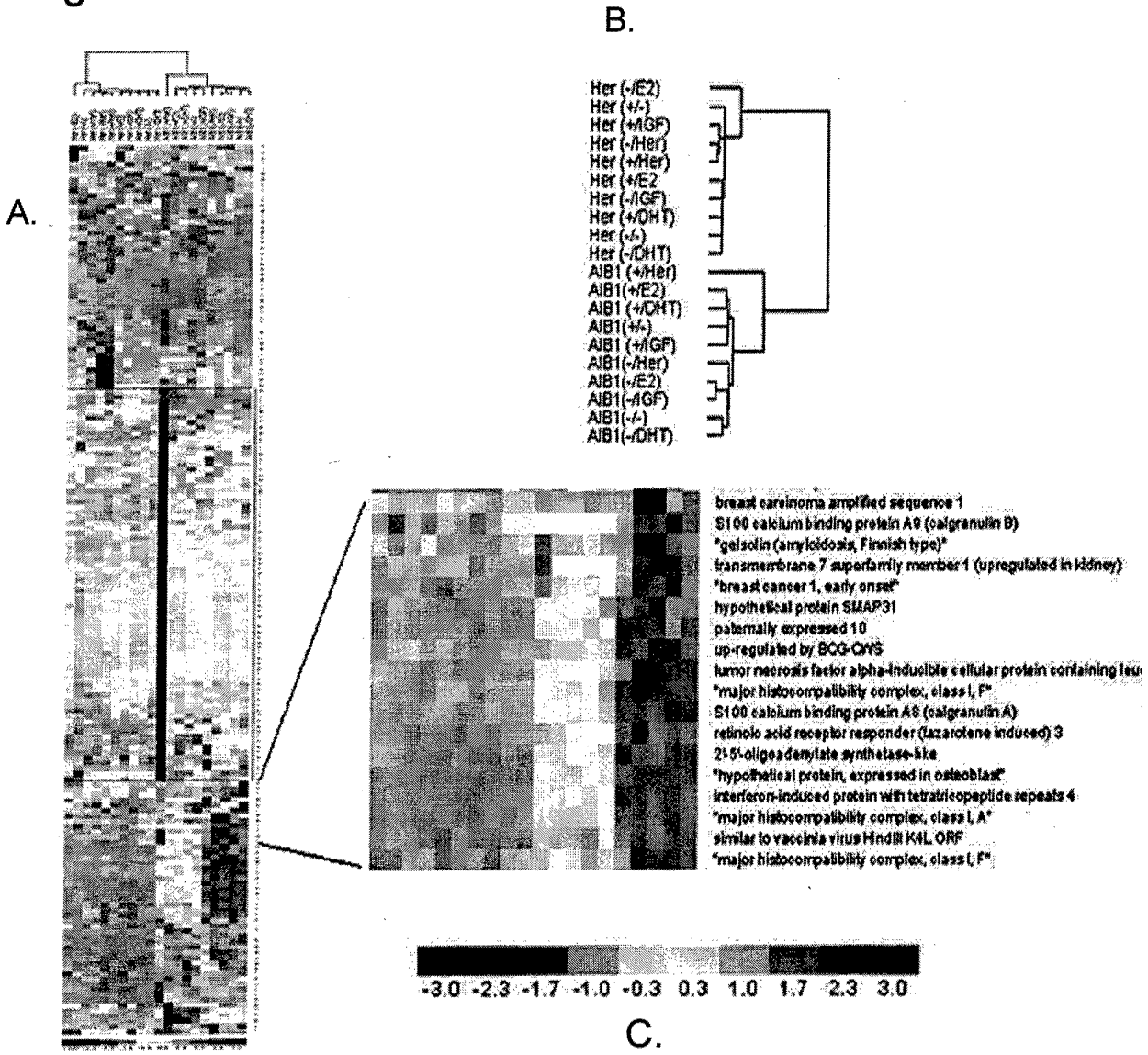

Figure 1 – The final dendogram containing the 169 genes found to be significantly expressed between 20 samples. **(A.),** each row in the dendogram represents the expression level of a gene, while each column represents the expression pattern of the different genes in each sample. Using hierarchical clustering, the dCHIP analysis software was able to group each gene and sample based on degrees of similarity in expression. The genes are not grouped according to any functional similarity. A portion of the final dendogram was enlarged. **(B.),** represents a hierarchical tree of how the samples grouped together based on their gene expression profile. The Her2 ribozyme cells were different from the AIB1 ribozyme cells. Within the AIB1 ribozyme group, AIB1 (+/- Her) or ribozyme off and heregulin treated cells had a gene expression profile that separated it from the other growth factor treated AIB1(+) cells. AIB1 (-/E2, IGF, -, DHT) or ribozyme on, had little differences in their gene expression profiles. (C.), represents a color scale: red represents the expression level above the mean expression of a gene, white represents mean expression, and blue represents a level lower than the mean expression level.