18th World IMACS / MODSIM Congress, Cairns, Australia 13-17 July 2009
http://mssanz.org.au/modsim09

Oncomine meta-analysis of breast cancer microarray data identifies upregulation of NRF-1 expression in human breast carcinoma

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Abstract: We have conducted a study where Oncomine microarray data was meta-analyzed to evaluate NRF-1 gene expression in human breast tumors. Our initial analyses indicated that NRF-1 gene is differentially expressed in breast cancer patients based on their estrogen receptor (ER) status. Therefore, in our first meta-analyses 18 studies were grouped separately for ER+ and ER- patients. It was observed that NRF-1 gene expression is up-regulated in ER + patients compared to ER- patients. Further, two separate meta-analyses on ER+ and ER- patients were conducted to associate NRF-1 gene expression levels for prognosis and for histological grades of breast tumors. In a combined 15 microarray studies, ER- patients with down regulated NRF-1 expression either died or had a relapse of cancer after the treatment. ER+ patients with increased levels of NRF-1 expression however, survived or had no relapse after treatment. Interestingly, in our third set of meta-analyses conducted on 15 studies, it was observed that NRF-1 gene expression also increased significantly with the progression of tumor grades from 1 to 3. Findings of this study have a major implication for the role of NRF-1 in the breast cancer, suggesting that NRF-1 possibly participates in the growth of breast cancer by altering its activity.

Keywords: *Breast cancer, NRF1, estrogen receptor, Oncomine*

1. INTRODUCTION

NRF1/α-PAL (nuclear respiratory factor-1/α-palindrome-binding protein) is a transcription factor (Evans and Scarpulla, 1989; Virbasius et al., 1993; Gopalakrishnan and Scarpulla, 1995). It belongs to the NRF1/Ewg family. The optimal NRF1 binding site is (T/C)GCGCA(C/T)GCGC(A/G). It is widely expressed, and the strongest expression is in skeletal muscle. This transcription factor activates the expression of several key genes regulating cell growth and development, nuclear genes required for respiration, heme biosynthesis, and mitochondrial DNA transcription and replication (Gopalakrishnan and Scarpulla, 1995; Efiok et al., 1994). A genome-wide analysis has revealed that NRF1 binding elements are present in genes involved in DNA replication, mitosis, and cytokinesis, suggesting that NRF1 plays an important role in cell cycle regulation (Efiok et al.,1994). Similarly, computation analysis of NRF1 gene regulation by querying the TRANSFAC database revealed that the TGCGCATGCGCA motif of the consensus NRF1 binding site is present in genes encoding proteins regulating cell growth, and DNA repair. NRF1 overexpression has been observed in hepatoma and thyroid oncocytoma (Dong et al., 2002; Savagner et al., 2003). The role of NRF1 in human breast cancer is the least studied of all other transcription factors. In contrast, however, there were more than 100 Oncomine studies with NRF1 gene expression. In this study the Oncomine cancer microarray database was primarily employed for NRF1 expression analyses in breast cancer.

2. METHODS

We used Oncomine's gene search function to locate microarray studies expressing NRF1 in breast cancer. Oncomine processes and normalizes each dataset used in these analyses independently. For the differential expression analysis, they use t-statistics with false discovery rates as a corrected measure of significance. After searching for the gene of interest in Oncomine, we sorted the results based on each class of analysis, noted the significant studies produced, and then created a boxplot with the results of this analysis. We then performed a meta-analysis of this data by combining all the normalized values of NRF1 in association with estrogen receptor (ER) status, histological grade and prognosis. These microarray values were pre-processed for normalization by

Oncomine. Although many analytical methods are used for analysis of microarray data, we chose to use t-statistics to measure the difference in expression in NRF1. Y-axis units are normalized expression values (standard deviations above or below the median per array).

3. RESULTS

3.1. NRF1 expression in ER negative (ER-) breast tumors vs. ER positive (ER+) breast tumors

We first examined expression of NRF1 based on ER status of breast cancer tumors. The distribution of Oncomine samples by ER and NRF1 status is shown in **Table 1**. NRF1 was significantly overexpressed in ER+ tumors compared to ER- tumors in four separate studies within Oncomine (**Figure 1**). Several other studies also showed median expression of NRF1 to be overexpressed in ER+ tumors compared to ER- tumors, though this overexpression was not significant. We then performed a meta-analysis of this data by combining all the normalized values of the 18 studies into one single comparison of NRF1 expression in ER- and ER+ tumors. A Mann-Whitney two-sample rank-sum test showed that NRF1 was significantly overexpressed in ER+ tumors compared to ER- tumors ($p < 0.127$) (**Table 2**). A box-plot of this data which compared medians also shows the data to be significant (**Figure 2**).

Table 1. Distribution of Oncomine breast tumors by ER and NRF1 status.

ER and NRF1 Status	n
ER+ NRF1 Total	1205
ER- NRF1 Total	616
ER+ NRF1+	555
ER+ NRF1-	576
ER- NRF1+	261
ER- NRF1-	355

Table 2. Results of Mann-Whitney test on meta-analysis of NRF1 expression by estrogen receptor status of breast tumors.

	N	Rank Sum	Mean Rank	U
ER- NRF1 Total	616	534716.5	868.05	397599.5
ER+ NRF1 Total	1205	1124214.5	932.96	344680.5
Mean Difference (95% CI)		-0.069110 (-0.123870 to -0.014870)		
Mann-Whitney's statistic		397599.5		
Z statistic (2-tailed p)		-2.49 (0.0127)		

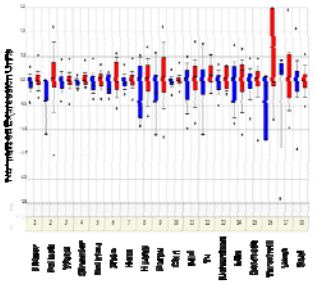


Figure 1. NRF1 expression in breast cancer by estrogen receptor status. Studies showing significant overexpression of NRF1 in ER+ tumors are Bittner (P = .004), Pollack (P = .02), Wang (P = .037), and Ginestier (P = .046). ER- tumor boxplots are denoted in blue; ER+ tumor boxplots are denoted in red.

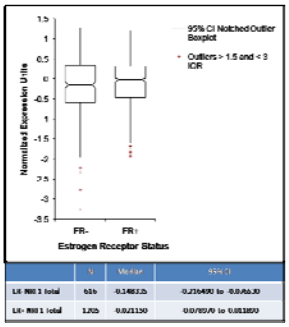


Figure 2. Boxplot results of meta-analysis of NRF1 expression by estrogen receptor status of breast tumors.

3.2. NRF1 overexpression by grade in breast tumor tissue

We also examined NRF1 expression in Grade 1, 2, and 3 breast tumors. Three studies found significant overexpression of NRF1 as Grade increased (**Figure 3**). A meta-analysis was then conducted on 13 studies. This study found NRF1 to be significantly overexpressed as Grade increased among breast tumors (P = 0.0001) (**Table 3**). A box-plot of this data which compared medians also shows the data to be significant with histological grade of breast tumors (**Figure 4**).

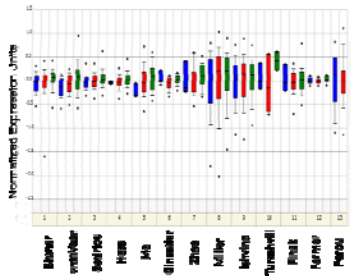
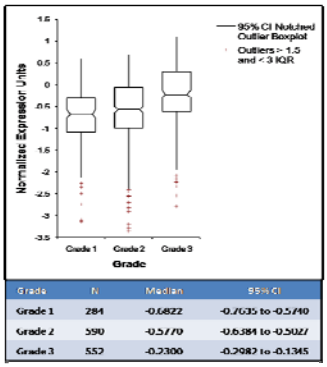


Figure 3. NRF1 expression in breast tumors by grade. Studies showing significant overexpression of NRF1 as grade of breast cancer increased were Bittner (P=.002), vantVeer (P=.003), and Sotiriou (P=.008). Grade 1 tumors are blue, Grade 2 red, and Grade 3 green. The study by Perou compared Grade 2 vs. Grade 3.



Grade	N	Median	95% CI
Grade 1	284	-0.6822	-0.7635 to -0.5740
Grade 2	590	-0.5770	-0.6384 to -0.5027
Grade 3	552	-0.2330	-0.2982 to -0.1345

Figure 4. Boxplot results of meta-analysis of NRF1 expression by breast tumor grade. Note: Perou study was excluded due to the study only having data for Grade 2 and Grade 3 tumors.

Table 3. Median test on meta-analysis results of NRF1 expression per Grade of breast tumor.

Grade	N	Less or Equal	Greater	Median
Grade 1	284	182	102	-0.6822
Grade 2	590	342	248	-0.5770
Grade 3	552	189	363	-0.2330
Overall Median (p value) = 0.4602914 (<0.0001)				

3.3. NRF1 overexpression in breast tumor tissue by survival status

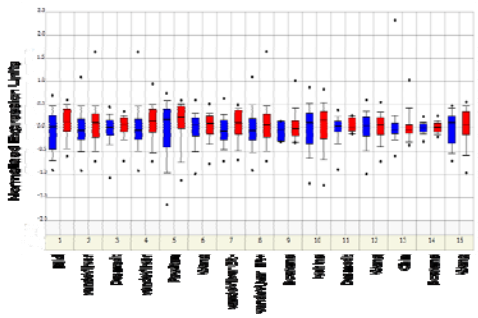


Figure 5. NRF1 expression in breast cancer tissue by survival status. The study by Bild showed significant overexpression of NRF1 among breast cancer patients who died (P=.014). Alive/No Disease patients are blue, Dead/Disease Relapse patients are red.

Our final study using Oncomine data examined the association between NRF1 expression and survival status of breast tumor patients. One study showed significant overexpression of NRF1 among those who survived or had no relapse after treatment for breast cancer compared to patients who died or relapsed after treatment for breast cancer (**Figure 5**). However, several studies that showed overexpression of NRF1 among patients who died or relapsed approached significance, and thus we decided to combine the studies in two separate meta-analysis. The first meta-analysis of 15 studies, which compared patients who survived vs. patients who died after treatment for breast cancer, found NRF1 to be significantly overexpressed in patients who survived after treatment for breast cancer (P = 0.0043) (**Table 4 and Figure 6**). A second meta-analysis, comparing patients who had no relapse after treatment for breast cancer to those who did relapse after treatment, found no significant difference between the groups (P = 0.6986) (**Table 5 and Figure 7**).

Table 4. Results of Mann-Whitney test on meta-analysis of NRF1 expression by survival (Alive vs. Dead).

Survival Status	N	Rank Sum	Mean Rank	U
Alive	914	594680.0	650.63	143375.0
Dead	350	204800.0	585.14	176575.0
Mean Difference (95% CI)		0.105580 (0.033290 to 0.177860)		
Mann Whitney's statistic		143375.0		
Z statistic (2 tailed p)		2.85 (0.0043)		

Table 5. Results of Mann-Whitney test on meta-analysis of NRF1 expression by survival (No Disease vs. Relapse).

Survival Status	N	Rank Sum	Mean Rank	U
No Disease	553	[illegible]	444.06	[illegible]
Relapse	329	143836.5	437.19	92385.5
Mean Difference (95% CI)		0.012750 (0.049330 to 0.074090)		
Mann-Whitney's statistic		[illegible]		
Z statistic (2 tailed p)		0.39 (0.6986)		

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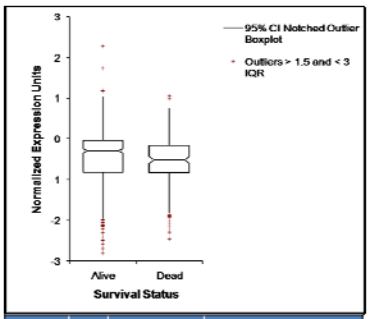
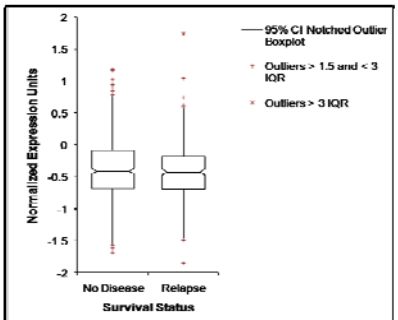


Survival Status	N	Median	95% CI
Alive	914	-0.287155	-0.343800 to -0.243030
Dead	350	-0.516230	-0.501820 to -0.432300

Figure 6. Boxplot results of meta-analysis of NRF1 expression by survival (Alive vs. Dead).



Survival Status	N	Median	95% CI
No Disease	553	0.414280	0.451070 to 0.361580
Relapse	329	-0.432370	-0.465740 to -0.363740

Figure 7. Boxplot results of meta-analysis of NRF1 expression by survival (No Disease vs. Relapse).

DISCUSSION

Major findings that emerged from meta-analysis in this study are that: 1) NRF1 was significantly overexpressed in ER+ tumors compared to ER- tumors; 2)) NRF1 was significantly overexpressed as breast tumor's grade increased; and 3) A significant overexpression of NRF1 was observed among those who survived or had no relapse after treatment for breast cancer compared to patients who died or relapsed after treatment for breast cancer. The findings of this study have a major implication for the role of NRF-1 in breast tumors, however, it should be interpreted cautiously, because most of the micro-array studies did not provide information about variables, such as age and, ethinicity, and confounding factors, such as smoking status, alcohol use, and epidemiologic health disparity information such of socio-economic status.

In addition to regulating mitochondria biogenesis and respiration, cell growth, and cell cycle genes, NRF1 binds to the gene promoters of cysteine proteases (CAPNS1 and CASP2), chemokines (CXR5, CKLF), the putative breast adenocarcinoma marker BC2, BRCA2, BCCIP, and tumor suppressors (putative tumor suppressor, 101F6 and tumor suppressor deleted in oral cancer-related 1) (Roy and Tamuli, 2008). These NRF-1 target genes control cell adhesion, cell spreading, migration, proliferation, apoptosis, and tumor invasion. NRF-1 overexpression has been observed in hepatoma and thyroid oncocytoma (Dong et al., 2002; Savagner et al., 2003). NRF1 regulatable human TOMM34 gene is upregulated frequently in colorectal tumors (Blesa et al., 2008). Motifs bound by ELK1, E2F, NRF1 and NFY positively correlate with malignant progression of breast cancer (Niida et al., 2008). The above studies, along with findings of the present study showing increased NRF1 expression in ER+ breast tumors compared ER- breast tumors, and statistical association with both histological grades and prognosis of breast cancer, provide strong support to our concept that NRF1 may play an important role in the development of breast cancer.

The mechanism by which NRF1 may play a role in breast cancer is not clear. NRF1 is a redox sensitive transcription factor (Felty et al., 2005a). Some of the same mitogenic pathways that are sensitive to oxidant levels and carcinogenic levels of estrogen are also directly regulated by NRF1. For example, the expression of CDC25C, which is required for progression of the cell cycle, is regulated by both E2 and reactive oxygen species (ROS) and its promoter contains an NRF1 binding motif. The expression of cyclin D1 is also regulated by both E2 and ROS. There are several estrogen-regulatable genes, which are also regulated by ROS. Cell cycle regulation by the cyclin-dependent kinases and cyclins is dependent upon cell adhesion mediated by integrins, which control expression of cell cycle genes via ROS. Many of the genes associated with high-risk breast tumors appear to participate in cell cycle regulation, including those encoding CDC2 and PRC1. As noted above, both genes are NRF1 regulatable. Importantly, in human breast cancer cells, the expression of almost 15% of the genes significantly affected by E2 contain the NRF1 binding element, and the NRF1 binding signature is significantly enriched in the promoters of genes induced by estrogen treatment. NRF1 and CREB elements significantly co-occur on promoters of cell cycle-regulated genes. We have recently shown that inhibitors of mitochondrial oxidant production prevent E2-induced expression of cell cycle genes containing NRF1 binding sites (cyclin B1, PCNA, and PRC1), decrease E2-induced NRF1 expression, and delay growth (Felty et al., 2005b). E2 control stimulates NRF1 expression (Mattingly et al., 2008). NRF1 responds to redox signaling pathways through post-translational modifications and through its specific interaction with transcriptional co-activators. These findings show that E2 may stimulates NRF1 expression and cell cycle progression of ER+ breast cancer cells through ROS, possibly by altering NRF1 activity.

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