

# Detection of mRNA for Nuclear Receptor Co-Activators 1 and 3 in Archival Breast Cancer Tissue and Surrounding Stroma: A Tissue Expression Study

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**Abstract:** Before the age of 75 years, approximately 10% of women will be diagnosed with breast cancer, one of the most common malignancies and a leading cause of death among women. The objective of this study was to determine if expression of the nuclear receptor coactivators 1 and 3 (*NCoA1* and *NCoA3*) varied in breast cancer grades. RNA was extracted from 25 breast tumours and transcribed into cDNA which underwent semi-quantitative polymerase chain reaction, normalised using 18S. Analysis indicated that an expression change for *NCoA1* in cancer grades and estrogen receptor alpha negative tissue ( $P= 0.028$  and  $0.001$  respectively). *NCoA1* expression increased in grade 3 and estrogen receptor alpha negative tumours, compared to controls. *NCoA3* showed a similar, but not significant, trend in grade and a non-significant decrease in estrogen receptor alpha negative tissues. Expression of *NCoA1* in late stage and estrogen receptor alpha negative breast tumours may have implications to breast cancer treatment, particularly in the area of manipulation of hormone signalling systems in advanced tumours.

**Keywords:** Breast cancer, stromal tissue, gene expression, nuclear receptor co-activator genes.

## INTRODUCTION

Breast cancer is one of the most common forms of cancer, causing much morbidity and mortality worldwide. Current health estimates indicate that around 10% of women in industrialised countries like the USA and Australia will develop breast cancer at some time in their lives [1, 2]. The growth of breast tumours and benign breast tissue is driven by several cellular signalling methods, of which the most important is considered to be estrogen [3]. The effects of estrogen are primarily modulated through the estrogen receptors alpha and beta ( $ESR\alpha$  and  $ESR\beta$ ), members of the nuclear receptor superfamily, which have genomic and non-genomic mechanisms [3, 4]. In order to activate specific functions, estrogen binds to its receptors, which dimerise and recruit additional cofactors before moving on to affect metabolism or gene expression [4]. Among the estrogen cofactors are the nuclear receptor co-activator (*NCoA*) family of co-enzymes. This family is involved in the function of most known hormone receptors [5]. Members of the *NCoA* family have additional names according to their functions or protein associations, notably *NCoA3*, with the alternative name *AIB1*, or Amplified in Breast Cancer. As this implies, the over-expression of *NCoA3* and other *NCoA* family members has been found to increase the transactivation function of several nuclear receptors, and evidence suggests that they are required for the function of nuclear receptors [6]. The *NCoA* proteins interact with ligand binding domains

of nuclear receptors, binding both receptors in a dimer through LXXLL amino acid motifs present on both the *NCoA* and nuclear receptor (NR) proteins [6, 7]. Once bound to the activated nuclear receptor, the *NCoA* protein is able to recruit the CBP/p300 and p/CAF protein complexes, both of which have an acetyltransferase activity, which results in the acetylation of histone proteins, which is required for the de-repression of the genes controlled by the nuclear receptors [6, 8]. Members of the *NCoA* family are also known to have a weak histone acetyltransferase activity, and there is some evidence that *NCoA* family members may cooperate in other signalling pathways [9]. Binding to the other cofactors required for NR function also appears to be mediated by the LXXLL motifs, reinforced by crystal structure analysis and the conservation of these motifs in the genes, indicating that they perform some important function in the overall receptor/coactivator complex [6, 8, 10].

It is currently poorly understood if *NCoA* proteins are specific for certain nuclear receptors, as well as whether or not they control additional cofactor recruitment, and thus the final function of the NR complex. However, some evidence from peroxisome proliferator-activated receptor gamma binding assays indicates that the specific *NCoA* bound to a NR complex does affect the binding of other cofactors [11]. The specificity of the *NCoA* proteins may be mediated *via* amino acids bordering LXXLL motifs that bind NRs [6, 7, 11]. Other studies support this, indicating that *NCoA1* activity is restricted to the nuclear receptors that accept hormone signals [8]. However, studies of *NCoA* ablation have shown that loss of *NCoA1* does not remove the normal responses to hormonal signals. Cells losing *NCoA1* often respond by up-regulation of *NCoA2*, indicating that the *NCoA* family may not be entirely locked into receptor

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specificity, which is perhaps mediated by *NCoA* isoform splicing [6, 8]. More recent studies have indicated that NCoA recruitment to receptor complexes may be mediated by phosphorylation and other post translational modifications of the NCoA proteins, functions modulated by specific kinases [12]. While other research has investigated protein expression of the *NCoA* genes in breast cancer, this study aimed to determine how the mRNA expression levels of the nuclear receptor coactivators 1 and 3 (*NCoA1* and *NCoA3*) varied in different breast cancer grades as a potential reflection of underlying transcriptional pressures.

**MATERIALS AND METHODOLOGY**

**Tissue Population**

The tissue population for this study was comprised of 25 archived breast tissue sections embedded in paraffin and fixed with 10% buffered formalin on slides. 10 micron thick sections were supplied with haematoxylin and eosin (H&E) stained slides as a reference for tumour location. All tumour samples were diagnosed as infiltrating ductal carcinoma. There were 6 samples from tumour grade 1, 7 samples each from grades 2 and 3 and 5 samples of benign breast tissue taken from unaffected patients as a control population. The average age of the individuals from whom the biopsies were obtained were 56.88 years, 59.18 years, 60.45 years and 55.93 years for the control and grades 1, 2 and 3, respectively. The archival breast tissue samples were obtained through collaboration with Gold Coast Hospital Pathology, with relevant ethical approvals (Approval numbers HSC/04/03/HREC and 9702 for Griffith University and Gold Coast Hospital, respectively). For consistency, cancer grading for each sample was confirmed by a single pathologist from the Gold Coast Hospital's Pathology Department (SRW). There was a variety of pathological data available for the population, including immunohistochemical staining to detect ESR $\alpha$  proteins. ESR $\alpha$  status was determined by standard clinical criteria for treatment. A summary of the population's ESR $\alpha$  status can be found in Table 1.

**Table 1. Population ESR $\alpha$  Immunohistochemical Staining Status**

Tumour Grade	ESR $\alpha$ Positive	ESR $\alpha$ Negative	Total
Control	5	0	5
Grade 1	6	0	6
Grade 2	5	2	7
Grade 3	2	5	7

**RNA Extraction**

RNA extraction and cDNA synthesis was performed as outlined in previous publications by Smith *et al.* [13]. Tumour tissue was first separated from the surrounding tissue by microdissection under an Olympus BX60 microscope (Olympus Optical Co., Tokyo, Japan), using supplied H&E slides as a guide to tumour location. All non-malignant tissue was removed as a single unit for separate analysis. Paraffin was removed with xylene, the tissue homogenised by passage through an 18G needle and then

subjected to 1 hour of treatment with TRIzol reagent. RNA was separated from DNA and protein through the use of chloroform and centrifuging. Extracted RNA was treated with Rnasin and Dnase I. Finally, RNA was further purified using a Qiagen Rneasy Mini Kit. cDNA was generated in a 25mL reaction using 2ng of total RNA, Superscript III from Invitrogen (0.2 $\mu$ L/reaction), random hexamers (9 $\mu$ g/reaction), 5mM dNTPs (0.5 $\mu$ L/reaction) and 1x Superscript buffer (Invitrogen).

**Expression Assay**

cDNA was made using the method outlined above underwent PCR with fluorescently labelled primers to amplify cDNA corresponding to mRNA for NCoA1 and 3. Individual samples underwent semi-quantitative PCR in triplicate for each gene before results were pooled and analysed. Primers for the *NCoA1* and *NCoA3* genes in this experiment were intron spanning, however, because the introns were kilobases in length and unlikely to amplify in a PCR optimised for small fragments, intronic primers previously used for the glucocorticoid receptor were multiplexed into the *NCoA* PCRs to detect genomic DNA present. No genomic DNA contamination was observed in any sample. NCoA genes were multiplexed with primers for ribosomal 18S RNA, as a ubiquitous control. Amplicons for *NCoA* genes were approximately 100 base pairs long, allowing cDNA derived from partially degraded RNA to be identified. Amplicons produced by contaminating genomic DNA present were 150bp in length. 18S amplicons were approximately 70bp long. Primers appear in Table 2.

**Table 2. Primer Compositions**

Primer Name	Sequence (5'-3')
NCoA1-F1*	CATTCCCCGGGAAGCTAC
NCoA1-R1	TTCACACCTGGGAGACTTCTT
NCoA3-F1*	TGGTTAGCCAGTTGCTGATGT
NCoA3-R1	GCAATTTGCGTTTTCTGTGA
Glucocorticoid receptor-F*	GAGTACCTCTGGAGGACAGA
Glucocorticoid receptor-R	ATGTCCATTCTTAAGAAACAGGA
18S-A#	CTTAGAGGGACAAGTCGCG
18S-B	GGACATCTAAGGGCATCACA

\*= Primer labelled with TET at 5' # = Primer labelled with HEX at 5'

PCR for both genes was carried out for 25 cycles in a mixture of 5.5 $\mu$ l Master Amp Premix C or L, for NCoA1 and 3, respectively (Epicentre, Madison, USA), 2 $\mu$ l of each of the two primers, 1 $\mu$ l of the two reference gene primers, 2 $\mu$ l of the cDNA sample (18-20ng/ $\mu$ l), 0.2 $\mu$ l Taq polymerase and 0.3 $\mu$ l of water. Prior to initiating PCR, the reaction mixture was heated at 95 $^{\circ}$ C for five minutes to dissociate any secondary structures. The parameters for one cycle of the PCR reaction were denaturation at 95 $^{\circ}$ C for one minute, annealing at 57 $^{\circ}$ C for one minute and thirty seconds and extension at 72 $^{\circ}$ C for one minute. Gene expression was quantified by expressing data as a ratio of *NCoA* to 18S, using an ABI 310 Genetic Analyzer, utilizing peak height as the measure of expression.

### Statistical Analysis

Averaged expression data for individual samples obtained for the *NCoA1* and 3 genes was normalised using the expression data obtained for the 18S gene. The normalised data were then analysed using one-way analysis of variance (ANOVA) to determine whether there was a significant difference between tumour grades and surrounding tissues or between tissues based on ESR $\alpha$  status. For ESR $\alpha$  status, expression values for the surrounding tissue for each tumour was included in the same category as the primary tumour, as a measure of the broad, tissue specific effect of tumour ESR $\alpha$  status on *NCoA* transcription. Control tissue was included in this analysis and forms part of the ESR $\alpha$  category. Appropriate *post hoc* tests were subsequently performed to elucidate any differences found.  $P < 0.05$  was considered statistically significant. The software package SPSS version 10.1 was employed for all statistical analyses (SPSS Inc., Chicago, IL, USA).

### RESULTS

The expression levels for the *NCoA1* and *NCoA3* genes were determined in all samples, both tumour and stroma. Comparisons were then made using the grade of tumour and the tissue's ESR $\alpha$  protein status as grouping variables. Expression ratios (*NCoA1*/18S) in different grades of tissue can be seen in for *NCoA1* in Fig. (1), while the *NCoA3* results can be seen in Fig. (2). The expression ratios for the ESR $\alpha$  status categories can be seen for both *NCoAs* in Fig. (3).

Despite the appearance of some changes in *NCoA* expression in different tissue categories, after ANOVA analysis, the differences was significant only for *NCoA1*. The p value for ANOVAs conducted on *NCoA1* were 0.028 for cancer grade and 0.001 for ESR $\alpha$  status. By contrast, the p values for *NCoA3* were 0.322 for the cancer grade analysis

and 0.365 for the ESR $\alpha$  protein analysis. Post-hoc tests on *NCoA1* data indicated that the expression of *NCoA1* was elevated in grade 3 tumours, but only in comparison to grade 1 tumours. For ESR $\alpha$  protein status, the expression of *NCoA1* significantly increased in ESR $\alpha$  negative tissues, both in tumour and stroma derived from tumours, compared to ESR $\alpha$  positive tissues. Expression data for *NCoA1* and *NCoA3* was normally distributed, with p values from the homogeneity of variances test being 0.089 and 0.107, respectively.

### DISCUSSION

The *NCoA* family are transcription factors that bind to signalling proteins and recruit further proteins to the signalling complex, modulating the effects of incoming messengers, including steroid hormones and others, such as transforming growth factor  $\beta$  [6, 7, 14]. As such, they have a role to play in multiple processes, including cellular growth, function and differentiation, making their regulation important in the development and progression of cancer [6, 7, 15]. The results of these studies showed that the mRNA expression of *NCoA1* is significantly affected by both cancer grade and ESR $\alpha$  protein status in the test population, while *NCoA3* mRNA expression remains unchanged in all classes of tissue.

For *NCoA3*, the data indicated that there was no significant association between the mRNA expression of this gene and either cancer grade, or ESR $\alpha$  protein status. In part, this was an unexpected result, since *NCoA3* has been found to have increased expression in breast cancer in cell lines and biopsies [15, 16]. This trend is not universal, however, and other studies have found that the expression of *NCoA3* remains unchanged in some cell lines [15, 17]. It is worth noting that the general level of expression for *NCoA3* in this study was higher than *NCoA1* and expression of *NCoA3* in grade 3 tumours is higher than in control tissues, though this

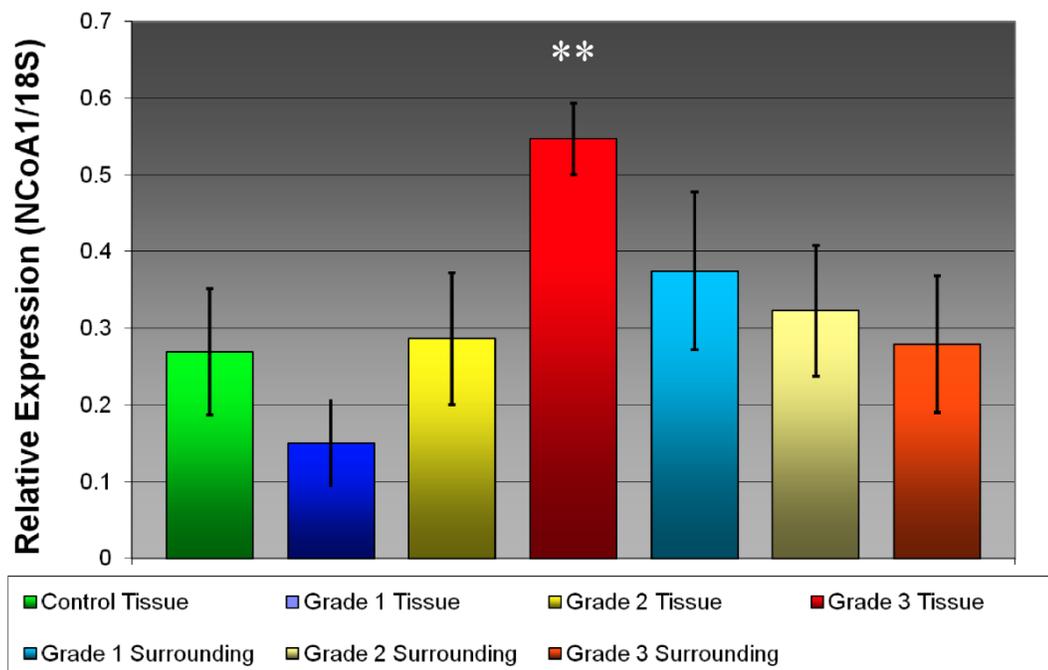


Fig. (1). Expression of *NCoA1* in tissue derived from different tumour grades and surrounding stroma.

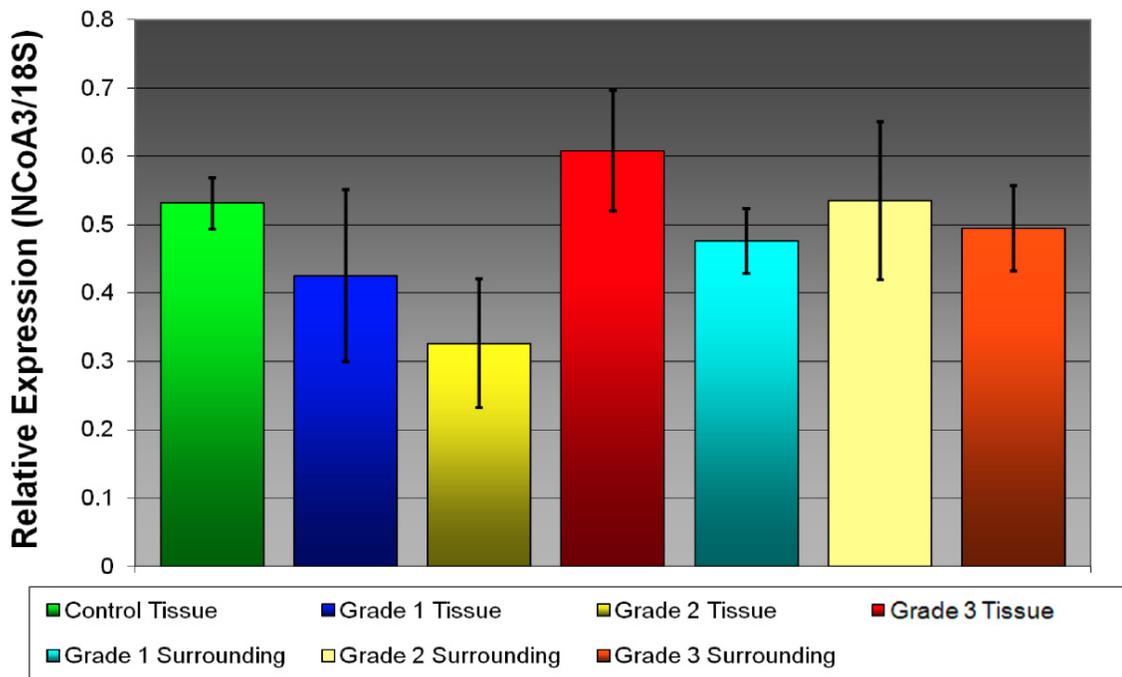


Fig. (2). Expression of NCoA3 in tissue derived from different tumour grades and surrounding stroma.

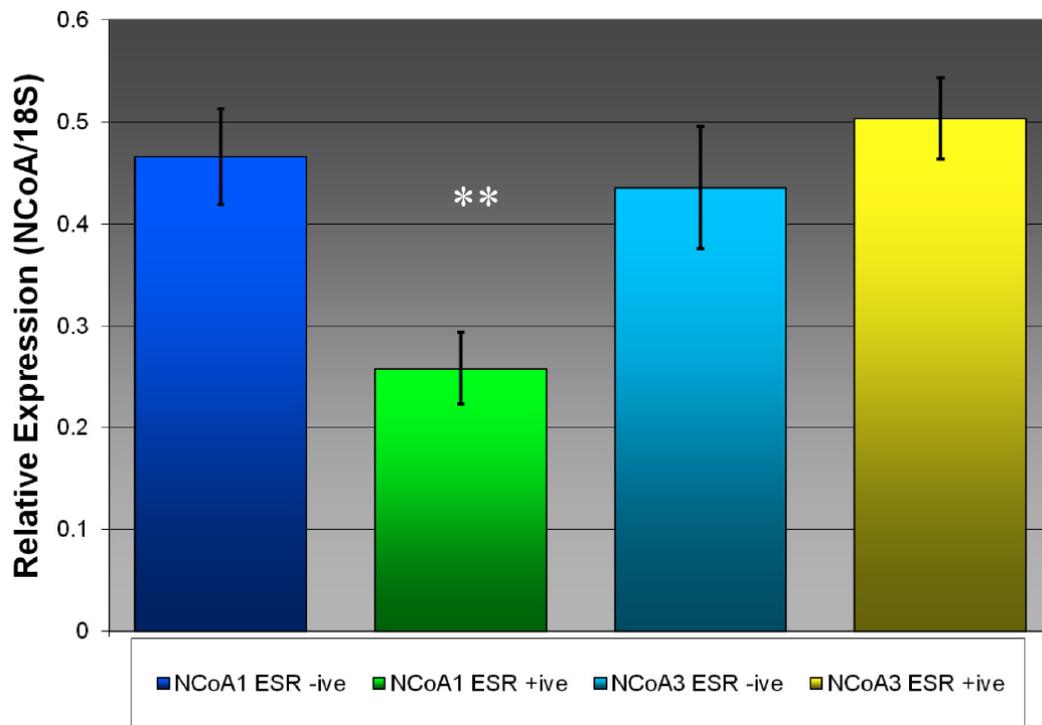


Fig. (3). Expression of NCoA1 and NCoA3 in Tissue Derived From Different Tumour Grades and Surrounding Stroma, Compared by ESR $\alpha$  status.

was not significant. *NCoA3* may not be over-expressed in the tumours because the particular alterations to growth induction pathways that these tumours are using do not require *NCoA3*. It is also possible that the cells are not experiencing a significant increase in *NCoA3* expression because the NCoA requirement of the cells is being fulfilled by the increased *NCoA1* expression or other cofactors. It is also possible that post transcriptional modification of the *NCoA3* mRNA modulates its function, removing the

necessity to increase expression to achieve a specific function. Finally, there is the possibility that the relatively low number of tumours available, or the semi-quantitative nature of this PCR do not allow this study sufficient resolution to identify which tumours overexpress *NCoA3*, and why.

The results for *NCoA1* indicated that the expression of that gene is increased in late stage breast cancer. This result

was not entirely unexpected, as the mRNA expression of *NCoA* family members, have been found to be up-regulated in many cancers, including breast tumours and cell lines [15-17]. Such up-regulation may increase the activity of a large range of target genes and can affect cells in many ways. Proteins that promote and repress cellular division are assisted in their function by NCoA1, which is often associated with the steroid hormones. This means that an increase in expression may not have specific effects on the tumour cells, contributing to cellular behaviour in concert with other co-activators and the signalling milieu the cells are in. The results for *NCoA1* also indicated that the expression was increased in grade 3 tumours in comparison to grade 1 tumours only, which are not significantly different to any other class of tumour, nor are they different to control or stromal tissue. Hence, although the expression of *NCoA1* was high in grade 3 tumours, it was not significantly higher than the expression of *NCoA1* in control tissue. This is perhaps not a reflection of a difference between grade 1 tumours and grade 3, but rather simple variation between the tumours, as the error bars in Fig. (1) demonstrate considerable differences within the grades. This, however, is consistent with results from other studies, which indicate that over-expression of the *NCoA* family is not universal for all cell lines or tumours, though it does occur [15-17]. More likely to be the result of a specific effect was the outcome of the ESR $\alpha$  protein analysis, which indicated that the mRNA expression of *NCoA1* was higher in tissues that are ESR $\alpha$  negative. With a p value of 0.001, this association was stronger than that of the cancer grade (p= 0.028) and has lower variation in the groups, reinforcing the correlation between the loss of the ESR $\alpha$  protein and an increase in *NCoA1* expression. It is also possible, however, that the difference observed was because nearly all ESR $\alpha$  negative tissues in this study were grade 3 tumours.

These results are more interesting in the light of a recent study by Green *et al.*, who subjected a large series of breast tumours to immunohistochemical detection of a large number of proteins, including NCoA1 and 3. Their study indicated that the reverse of this relationship was taking place at the protein level, that NCoA1 expression was higher in lower grade, less malignant and ESR $\alpha$  negative cancers [18]. In addition, they linked higher NCoA1 expression with less aggressive cancers and longer disease free survival. At first glance, it would seem counterintuitive that there should be so great a discordance between the results for mRNA and protein levels. However, earlier work in the population in this study has shown a very similar disconnect, as ESR $\alpha$  mRNA remains detectable and nearly identical to control tumour tissue in all samples, including those negative for the ESR $\alpha$  protein [13]. Likewise, earlier work in this population has indicated that the mRNA for numerous nuclear receptors inhibiting growth, such as the androgen, glucocorticoid and progesterone receptors increases in later stage breast cancers, though no direct protein comparisons are yet available [13, 19]. Experience in breast cancer tissues would indicate that the protein expression of these receptors reduces in later stage cancers [20].

The differences between the results of protein based studies, such as those of Green *et al.* and mRNA studies such as this one may reflect fundamental shifts in the way that hormone receptor and co-factor genes like the *NCoAs* are

regulated as breast cancer grows and progresses. Advanced and more aggressive tumours may be expressing the mRNA for growth inhibiting proteins as part of a pre-existing and still functional growth feedback pathway, but some other mechanism is preventing the protein from being produced. Potential mechanisms for this include post-translational modification of the final protein or production of alternative protein isoforms, perhaps with alternative functions. Both of these mechanisms have the potential to confuse the immunohistochemical methods used by Green *et al.* due to epitope shifts, while different mRNA isoforms may also lead to misleading results from PCR based approaches, such as this study. It is also possible that miRNA expression is ablating mRNA species responsible for production of *NCoA1* and related genes that produce mRNA, but for which proteins are lost in late stage breast cancers.

## CONCLUSION

Taken together with previous research from this tissue population and the results of protein based studies, these results indicate that growth braking mechanisms in cancer cells may still be partially functional, driving tumours to produce anti-growth mRNA, but that some as yet unknown mechanism, perhaps miRNA or protein modifications, prevents the production of significant quantities of complete protein. If there is such a translation changing mechanism at play in breast cancer cells and it could be blocked, normal hormone receptor systems and response to hormonally based treatment may be restored. Understanding which cofactors are favoured by cancers in particular pathological states and how they affect signalling may aid in designing targeted hormone therapies after responsiveness is restored. This complex state of affairs reinforces the need to examine the biology of cancer cells at all available levels, as what may seem relatively simple associations for individual markers with clinical parameters may be part of larger shifts in complex cellular biology that can be detected when all aspects are considered. Further research should confirm the expression levels of these proteins directly and attempt to identify if alternative mRNA isoform proportions and miRNA expression using a more directly quantitative methodology, as well as modified proteins, are associated with clinical parameters, as these may give clues to the larger story unfolding within breast cancer cells.

## ACKNOWLEDGEMENT

This study was made possible through internal funding at Griffith University.

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Received: January 14, 2009

Revised: January 19, 2010

Accepted: February 10, 2010

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