# A Comparative Study of Genome-Wide SNP, CGH Microarray and Protein Expression Analysis to Explore Genotypic and Phenotypic Mechanisms of Acquired Antiestrogen Resistance in Breast Cancer

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Running title: SNP/CGH arrays and protein expression in breast cancer.

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**Abstract**

Allelic imbalance is a common feature of many malignancies. We have measured allelic imbalance in genomic DNA from the breast cancer cell lines T47D, MDA-MB-231, two antiestrogen sensitive ($MCF7^N$ and $MCF7^L$) and two resistant MCF7 cell lines (MMU2 and LCC9) using single nucleotide polymorphism (SNP) oligonucleotide microarrays. DNA from $MCF7^L$ and MMU2 cells was also analysed by comparative genome hybridisation (CGH) to compare with SNP microarray data. Proteins previously determined to be involved in disease progression were quantified by Western blot and compared to array data. The SNP and CGH array both detected cytogenetic abnormalities commonly found in breast cancer: amplification of chromosomes 11q13-14.1, 17q and 20q containing *cyclin D1*, *BCAS1* and *3* (Breast Cancer Amplified Sequence) and *AIB1* (Amplified in Breast cancer) genes; losses at 6q, 9p and X chromosomes which included *ERα* (Estrogen Receptor alpha) and $p16^{INK4A}$ genes. However the SNP chip array data additionally identified regions of loss of heterozygosity (LOH) followed by duplication of the remaining allele – uniparental disomy (UPD). Good concordance between SNP arrays and CGH analyses was observed, however there was poor correlation between gene copy number and protein levels between the cell lines. There were reductions in ERα, cyclin D1 and p27 protein levels whilst p21 protein levels were elevated in antiestrogen resistant MCF7 cell lines. Although protein levels varied there was no difference in gene copy number. This study shows SNP and CGH array analysis are powerful tools for analysis of allelic imbalance in breast cancer. However the antiestrogen resistant phenotype was likely to be due to changes in gene expression and protein degradation rather than in altered gene copy number.

## Introduction

Breast cancer is the most common malignancy in women in the United Kingdom and United States with 1 in 9 expected to develop the diseases during their lifetime (**www.cancerreasearchuk.org**). Antiestrogens, in particular tamoxifen, have been the main treatment for hormone dependent breast cancers and still remain the treatment of choice in the pre-menopausal setting. However, resistance is a major clinical problem as one third of estrogen dependent breast cancers are resistant to antiestrogens (1). Additionally, while many breast cancers may initially respond to antiestrogens, resistance often develops. An understanding of the genetic and proteomic changes which develop within a cancer cell that may confer antiestrogen resistance is therefore critical to the effective management of breast cancer.

Several studies have characterised the genomes of breast cancer cell lines to detect common chromosomal aberrations in breast cancer and their possible implications in tumourigenesis. Allelic imbalances in tamoxifen resistant and sensitive breast cancers have been detected by karyotyping (2) and also comparative genomic hybridisation (CGH) (3, 4).

Although CGH can be used on a genome wide scale to detect chromosomal gains and losses it has low resolution and allows for detection of genetic aberrations in the range of 1 to 20 Mb. Moreover, it cannot detect copy number changes coincident with regions of loss of heterozygosity (LOH). Advances in the understanding of the molecular pathology underlying many forms of malignancy has come from the mapping of minimal regions of LOH as these have often been associated with the

presence of a mutated oncogene on the remaining allele. Combining detection of both copy number changes and LOH allows for the recognition of a situation whereby the loss of one allele is followed by duplication of another, possibly mutated, allele (uniparental disomy, UPD).

The more recent introduction of high density single nucleotide polymorphism (SNP) arrays, such as the Affymetrix 10 K GeneChip, enables genotyping of over 10,000 SNPs in one assay, with analysis at 105 Kb resolution. This array contains probe sets which are specific for 11,555 unique genomic loci. The 10 K GeneChip array platform has been shown to be extremely accurate (99.5%), with high reproducibility (99.5%) and call rate (95%) (5). In this study we initially identified common allelic imbalances in 3 breast cancer cell lines of different origin – MCF7 (antiestrogen sensitive), T47D (intermediate antiestrogen sensitivity) and MDA-MB-231 (antiestrogen resistant) (6) by SNP array analyses. Then, to investigate if any allelic imbalances could be detected which may have conferred the acquired antiestrogen resistance phenotype, we compared SNP chip data from parental MCF7 cells ($MCF7^N$ and $MCF7^L$) to that from MCF7 cells with acquired antiestrogen resistance (MMU2, LCC9). The abnormalities found by SNP chip analyses in two of these cell lines ($MCF^L$ and MMU2) were verified by comparison to those detected by CGH studies. Finally, in order to assess the relevance of genomic data to the antiestrogen resistance phenotype, we measured the expression of proteins associated with proliferation and poor prognosis in antiestrogen sensitive and resistant MCF7 cells.

## Materials and methods

*Cell lines and maintenance*

Antiestrogen sensitive MCF7 cells were routinely passaged at Newcastle University, Newcastle Upon Tyne and MCF7 and St James's University Hospital, Leeds, UK and were designated $MCF7^N$ and $MCF7^L$ respectively. Two antiestrogen resistant cell lines were used: MMU2 cells were derived from $MCF7^L$ cells which had been grown continuously in 0.1 µM 4-hydroxytamoxifen until rendered resistant (7,8). LCC9 cells were a gift from Robert Clarke (Georgetown University School of Medicine) and were derived from MCF7 cells passaged at Georgetown University grown in increasing concentrations of the antiestrogen ICI 182,780 (9). T47D and MDA-MB-231 cells were passaged at Newcastle University. All tissue culture and other reagents were of analytical grade and were purchased from Sigma-Aldrich Company Ltd (Dorset, UK) unless otherwise stated.

Cells were incubated in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 µg/ml) unless otherwise stated. MMU2 cells were grown in phenol red free RPMI 1640 medium and LCC9 cells in phenol red free Dulbeccos Minimum Essential Medium (Gibco Invitrogen). Medium was supplemented with dextran charcoal stripped 10% FCS and penicillin (50 U/ml) and streptomycin (50 µg/ml). Before an experiment MMU2 cells underwent 2 passages in full RPMI 1640 medium and LCC9 cells underwent 2 passages in full Minimum Essential Medium (Gibco Invitrogen. Paisley, UK). All cells were grown in fully supplemented media during experimental procedures.

*Single nucleotide polymorphism microarray analysis*

Total genomic DNA (minimum 250 ng) was extracted from thawed cell pellets using

the Nucleon™ method (Scotlab, Strathclyde, Scotland) and sent to Medical Research Council Geneservice (Hinxton, Cambridge, United Kingdom) where it was digested with Xba I restriction enzyme, DNA fragments were ligated to adaptors which recognise cohesive four base pair overhangs. Ligated DNA fragments were amplified by PCR using primers that recognize the adaptor sequence. Amplified DNA was fragmented, end-labeled with a fluorescent tag and hybridized to the GeneChip® Mapping arrays (Affymetrix UK Ltd, High Wycombe, UK). The Affymetrix GeneChip® 10 K array contains probe sets directed against 11,555 unique genomic loci. Each SNP is represented by 40 different 25 bp oligonucleotides. Thus, the 10 K array offers genome analysis at a resolution of approx 105 Kb. Hybridisation to each probe was assessed using a gene chip scanner (Affymetrix) and data were analysed using the GeneChip® Operating Software (GCOS, Affymetrix), the GeneChip® DNA Analysis Software (GDAS, Affymetrix) and the GeneChip® Chromosome Copy Number Tool Software. The Affymetrix(r) GeneChip(r) Chromosome Copy Number Analysis Tool was used to estimate the probability of continuous homozygote calls. Values of less than $1 \times 10^{-5}$ were considered significant, as previously described (10). Assessment of the intensity of hybridisation to the SNP and comparison to a reference set of 110 normal individuals were used to derive copy number estimates for an individual SNP, which have been integrated into the Chromosome Tool software programme (Affymetrix) (10). Data were exported to an Excel spreadsheet and probability of LOH and copy number plotted against chromosomal location.

*Comparative Genome Hybridisation (CGH) analysis*

Unless otherwise stated all reagents were provided in the Vysis Random Priming Labelling Kit and used according to the manufacturer's instructions (Vysis, Inc,

USA). Genomic DNA was extracted from $MCF7^L$ and MMU2 cells. DNA from male and female blood lymphocytes were used as negative controls. Test and reference DNA were hybridised and heated at 80°C for 10 minutes to denature the DNA. These were cooled before placing on a Genosensor™ Amplicon 300™ microarray for 72 hours at 37°C. Arrays were washed with 50% formamide, 2X SSC at $40^oC$, (3 x 10 minutes) then with 1XSSC at room temperature, (4 x 5 minutes) and rinsed in $dH_2O$. DAPI was applied and a coverslip fixed. After 45 minutes incubation, arrays were imaged using GenoSensor Reader Software. Normal female versus normal male DNA arrays were analysed and a mean hybridisation ratio (green: red fluorescence ratio) calculated for all 287 clones on the GenoSensor Array 300 using Genosensor™ software. The mean intensity of the resulting Cy-3:Cy-5 ratios was calculated for triplicate spots. The male female chip was analysed in duplicate and an average intensity of 1.0 calculated. For subsequent experiments in which DNA from $MCF7^L$ and MMU2 test DNA (all labelled green) was hybridised to male reference DNA (labelled red), ratios of > 1 represented a relative gain of DNA in the test sample and ratios < 1 a relative loss of test DNA. In addition, a CGH chip directly comparing DNA amplifications and deletions implicated in TAM resistance allowed quantification of the mean intensity of the ratio of Cy-3 labelled MMU2: Cy-5 labelled $MCF7^L$. A 99% confidence interval was used throughout this study to test the significance of differentially expressed genes. Chromosomes with amplifications or deletions >3 standard deviation from the mean of female/male reference were considered significant, since at the 99% confidence interval the value observed is outside the normal range (3).

*Western blotting*

Exponentially growing cells were harvested by scraping into ice cold lysis buffer (20% (v/v) glycerol, 4% (w/v) SDS, 100 mM Tris HCl, pH 6.8). Lysates were passed through a 25G needle and heated to 85$^{o}$C for 5 min before protein determination. Following the addition of an equal volume of sample buffer (0.001% (w/v) bromophenol blue, 100 mM dithiothreitol) 20 μg protein samples were resolved on 4-20% polyacrylamide Tris/glycine gels (Invitrogen) and blotted onto Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham) using Tris/glycine transfer buffer under recommended conditions. Blots were blocked in TTBS buffer (20 mM Tris, 140 mM NaCl, 0.1 % (v/v) Tween-20, pH 7.6) containing 5% (w/v) non-fat dried milk for 1 h and then incubated overnight in antibody buffer (1% (w/v) non-fat dried milk in TTBS) containing primary antibody (total pRb (1:500, G3-245, BD Pharmingen. Devon, UK); Cyclin D1 (1:500, DCS-6, Dako. Cambridgeshire, UK); ERα (1:250, 1D5, Dako; actin (1:1000, AC-40, Sigma. Dorset, UK); CDK4 (1:500, C-22, Santa Cruz. Wiltshire, UK); CDK2 (1:500, M2, Santa Cruz); Cyclin A (1:500, C-19, Santa Cruz); Cyclin E (1:500, C-19, Santa Cruz); p27 (1:500, C-19, Santa Cruz); p21 (1:200, AB-1, Oncogene research products. Cambridge, UK). After three 15 min washes in TTBS buffer, blots were incubated with 1:5000 peroxidase-conjugated swine anti-rabbit secondary antibody (Dako), with the exception of blots using cyclin D1 or ERα primary antibodies where 1:1000 peroxidase-conjugated goat anti-mouse secondary antibody (Dako) was used. Following three washes in TTBS, labelled proteins were detected using Supersignal West Dura extended duration enhanced chemiluminescence substrate (Pierce). Chemiluminescence was detected by placing blots into a dark box with a CCD camera (Fuji LAS 3000, Raytek) and quantified

using Aida image analyser software. ERα protein was standardised to a purified ERα protein (Sigma) standard curve and fmoles/mg of cell lysate determined.

## Results

*Common abnormalities in MCF7, T47D and MDA-MB-231 breast cancer cell lines detected by SNP chip array*

In order to investigate if any allelic imbalances were important for the development of breast cancer we compared the SNP chip data in all of the cell lines analysed. Regions of gain and LOH in all breast cancer cell lines and MCF7 sub-lines are shown in Figure 1 and the common regions of gain, LOH and UPD are given in Tables 1, 2 and 3. The majority of allelic imbalances detected were unique to the individual cell lines. However, there were also several aberrations which were common to all 3 breast cancer cell lines and were therefore likely to be more typical of those genetic abnormalities detected in breast cancer (Table 1). These included LOH and haploid copy number at 6q25-ter the region containing the *ERα* gene (6q25.1). All breast cancer cell lines analysed in this study contained a haploid copy of 9p which contains the gene locus for $p16^{INK4A}$ (9p21) CDK4/6 inhibitor. Deletion of one copy of the X chromosome was observed in all the breast cancer cell lines tested. LOH was detected at 16q12-22, however no loss of material had occurred indicating UPD (Table 1). All of the breast cancer cell lines had increased copy number of several regions which are typically amplified in breast cancers (Table 1/ Figure 1). Amplification of the 11q13.1-14.1 region which contained the *cyclin D1*, *FGF19*, *3* and *4* gene loci was also observed. The 17q23 locus which contained the *BCAS3* (Breast Cancer Amplified Sequence 3) gene and *ABC1* (Amplified Breast Cancer 1) gene loci also

had increased copy numbers. Likewise 20q12-13.2 was amplified which contained *BCAS1* and *AIB1* (Amplified in Breast) cancer genes. These data also correlated well with other previous analyses carried out by the Cancer Genome project (www.sanger.ac.uk).

*Common abnormalities in MCF7 antiestrogen sensitive and resistant breast cancer cell lines detected by SNP chip array*

Since the cell lines showed significant heterogeneity we investigated the genomic changes which were only seen in MCF7 cells (Table 2). In addition to those described above MCF7 cell lines had amplification of 1q arm areas which contained the *N-ras* oncogene (1p13.2) and also the *COX2* (1q31.1) gene. The *p44S10* (3p14.1), *c-myc* (8q24) and the *K-ras* (12p11.2) gene locations were also found to be amplified. The region containing the *CDK2* gene (12q13) was amplified which mediates the S phase entry. Also 15q21 which contains the *MEK1* gene (15q22.1-22.3) and the *P450arom* (15q21.1) gene which encodes the aromatase enzyme.

Reductions in copy number equalling haploid numbers were observed at the *Rb* gene locus (13q14.2). The CDK inhibitory protein $p57^{Kip2}$ (11p15) gene region had reduction in SNP copy number as did the tyrosine kinase receptor *EGFR* gene locus (7p12-14). Normal copy number with LOH indicating UPD had occurred in MCF7 cell lines at 3pter-14.1, 9q21.1, 18p and 21q22.12-22.3 (Table 2). No known obvious potential oncogenes linked with breast cancer tumourigeneis could be found in these regions.

*Common abnormalities in MCF7 tamoxifen resistant breast cancer cell lines detected by SNP chip array*

We next compared the SNP data from the antiestrogen resistant MCF7 cells with the parental MCF7 cell lines in an attempt to discover if any regions of genetic imbalance could be involved in producing the acquired antiestrogen resistant phenotype. There were very few differences that were common to LCC9 and MMU2 cells and not found in $MCF7^N$ and $MCF7^L$ cell lines. In general the genotype of all the MCF7 cell lines was very similar confirming their common origin. However, we did detect gains of material at 18p11.32 and losses at 4q34.2-35.2 and 5p15 which were unique to the antiestrogen resistant MCF7 cell lines (Table 3). Unfortunately no genes located in these regions could be found which could be implicated in breast cancer tumourigenesis or antiestrogen resistance based on current knowledge.

*Comparison of SNP data to CGH data*

In addition to comparing the SNP array data to that seen at **www.sanger.ac.uk** we further validated our results by comparing to SNP chip data to CGH array analyses in the tamoxifen sensitive $MCF7^L$ and resistant MMU2 cell lines.

Both the SNP chip and CGH array produced similar results (Figure 2). Losses at 1p, 9p and amplifications at 3p, 16p, 16q and 21q were detected by both techniques. However there were a small number of conflicting differences in the abnormalities detected. Losses of genetic material on the X chromosome by SNP chip was not reported with CGH, similarly losses on 4p detected by CGH were not observed in SNP chip data (Figure 2). In contrast to the CGH array, SNP chip analyses also

highlighted regions of LOH associated with normal or increased copy number (UPD) (Figure 3).

*Protein expression in tamoxifen sensitive and resistant MCF7 cells*

The genomic analyses of the MCF7 cell lines demonstrated little change between the tamoxifen sensitive and resistant cell lines and did not explain the acquired antiestrogen resistance phenotype. We therefore looked at the expression of proteins which have previously been described to be involved in the regulation of estrogen and antiestrogen cell cycle control including ERα, cyclin D1, p27, p21, CDK4, 2, cyclin A, E and total pRb and compared their protein expression to the SNP and CGH copy number in the relative gene location. Protein was extracted from asynchronous exponentially growing cell cultures. ERα protein was most abundant in the tamoxifen sensitive $MCF7^N$ cell line, while T47D, LCC9 and MMU2 contained significantly reduced levels of ERα protein in comparison (2.7, 8 and 17 fold reductions respectively, quantified by densitometry). ERα protein was undetectable in MDA-MB-231 cells (Figure 4b,c,d). Although there was variable ERα protein expression, all of the breast cancer cell lines tested had haploid copy number at the gene locus (6q25.1) by SNP and CGH chip analyses (Figure 4a).

Further investigation of protein levels focused on antiestrogen sensitive $MCF7^N$ cells and resistant MMU2 and LCC9 cells. These cell lines were from the same parental lineage and therefore were likely to be less genetically diverse, with differences in cell cycle protein levels more likely to be due to acquired antiestrogen resistance rather than differences in the parental origin of the cell lines. The levels of the CDK2

(12q13), cyclin E (19q12) and cyclin A (4q25-31) proteins were similar in each of the cell lines which also had the same gene copy numbers. However, there was considerable variation in cyclin D1 protein levels with LCC9 and MMU2 cells having around 2-fold reductions compared to the antiestrogen sensitive $MCF7^N$ cell line (Figure 5b; representative blot, Table 2 pooled densitometric analysis from 3 independent experiments) despite having similar amplification (4n copy number) of the *cyclin D1* gene locus (11q13) detected by SNP analyses (Figure 5a).

All cells grew at the same rate (doubling time = 32 hr) and had similar cell cycle profiles (Neil Johnson, unpublished data) it is therefore likely that differences in protein levels were not due to accumulation in different cell cycle stages.

In comparison to antiestrogen sensitive $MCF7^N$ cells both LCC9 and MMU2 cells had reduced p27 (2.3 and 1.5 fold reduction respectively) and increased p21 (3.4 and 2.8 fold increase respectively) protein levels (Figure 4b/Table 4). However, no changes in gene copy number could be found at the *p27* (12p13) and *p21* (6p21.2) gene loci. The levels of total pRb protein were highest in the parental MCF7 cell line and were comparatively reduced in both MMU2 and LCC9 cells (1.6 and 1.4 fold reduction respectively) (Figure 4b/ Table) even though all MCF7 subtypes were haploid for the Rb gene locus (13q14.2).

**Discussion**

This study demonstrated the 10 K SNP chip array is a valuable method of detecting allelic imbalance in breast cancer cells and correlates well both with CGH analyses of the same cell lines and with reported CGH studies in breast cancer cell lines (3, 11,

12). In addition to CGH, the 10 K SNP chip array detected further regions of abnormalities due to the increased resolution of the genome it measures. The CGH array used in this study covered 300 gene loci giving a less intensive genome analyses compared to the SNP chip array which covered 11,555 SNPs in the genome.
The main purpose of this study was to determine if genotypic changes detected by SNP and CGH array could explain the acquisition of the antiestrogen resistance phenotype. We compared array data from two parental and two antiestrogen resistant MCF7 cell lines. Analysing two parental and two resistant cell lines enabled us to discriminate between genetic changes caused by random genetic drift and those resulting from the antiestrogen treatment selective pressure. Genetic changes were also compared to protein levels which could be linked to the antiestrogen resistant phenotype.

There was an extensive range of allelic imbalances in every one of the breast cancer cell lines detected by SNP chip array analyses. It was clear that the MCF7, T47D and MDA-MB-231 cell lines were isolated from different parental origins and $MCF7^{N}$, $MCF7^{L}$, MMU2 and LCC9 were from the same lineage. There were 3 regions of common allelic imbalances found in the antiestrogen resistant MCF7 cell lines that were not present in the antiestrogen sensitive MCF7 cells (Table 3). Alterations common to LCC9 and MMU2 that were different from MCF7 did not harbour genes that on the basis of current knowledge could explain the antiestrogen resistant phenotype. There was a similar number of different allelic imbalances detected between $MCF7^{L}$ and $MCF7^{N}$ (5 differences) or MMU2 and LCC9 (3 differences). The lack of consistent differences in the abnormalities detected in MMU2 and LCC9 cell lines compared to parental MCF7 cells suggested the differences were likely to be due

to random genetic drift rather than a cause or consequence of antiestrogen resistance. This is supported by the demonstration of genetic variation in every one of 11 subpopulations of MCF7 cells analysed by CGH (14).

There were many examples where LOH and haploid copy number were detected in all cell lines but there was a wide variation in protein levels between the cell lines. This was evident in a number of cases. It was previously believed there was no relationship between LOH at the gene locus and *ERα* gene expression (15). We have confirmed this observation as the cell lines we studied contained varying levels of ERα protein despite all having LOH at the *ERα* gene locus. Likewise the *cyclin D1* gene was amplified in all of the cell lines to a similar degree, but the protein levels in the antiestrogen resistant MCF7 cell lines were reduced compared to the parental sensitive MCF7 cell line. The work reported here suggests acquired antiestrogen resistance in these cell lines was mediated at the mRNA and protein level rather than at the genomic level (Figure 6) confirming previous reports of increased mRNA and protein of growth factor signalling molecules as a primary feature of acquired antiestrogen resistance (16).

In conclusion, our study demonstrates that whole genome SNP and CGH analysis are valuable tools in characterising markers of malignant progression. However, it is clear that there can be considerable variation in protein expression, despite an equivalent gene copy number between cell lines. It was likely that acquired antiestrogen resistance in MCF7 cell lines is mediated by alterations in gene expression and/or protein degradation rather than at the genomic level.

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**Table 1. Common allelic imbalances present in all breast cancer cell lines.**

| Chromosome | Gains | Candidate genes[a] | LOH | UPD | Candidate genes[a] |
|---|---|---|---|---|---|
| **6** | 6p12.3-12.1 | | 6q25.2-ter | | *ERα, IGF2R* |
| **8** | | | 8p22 | | |
| **9** | | | 9p | | *p16* |
| **11** | 11q13-14.1 | *cyclin D1, FGF19, 3, 4* | | | |
| **13** | | | 13q22-34 | | |
| **14** | 14q11-22 | | | | |
| **16** | | | | 16q12-22 | |
| **17** | 17q23.2 | *BCAS3, ABC1* | | | |
| **20** | 20q13.2 | *BCAS1, AIB1, TOPO1* | | | |
| **X** | | | X | | |

[a] Genes which could be implicated in tumourigenesis

**Table 2. Common allelic imbalances present only in MCF7 cell lines**.

| Chromosome | Gains | Candidate genes[a] | LOH | UPD | Candidate genes[a] |
|---|---|---|---|---|---|
| **1** | 1p13.2, 1q31.1, 1q12-23 | *N-ras, COX2* | 1pter-21.1, 1q44 | | *p58, SRC2* |
| **3** | 3p25, 3p14.1, 3q26 | *P44S10* | | 3pter-14.1 | |
| **7** | 7p14.1 | | 7pter-14.3, 7p12.1 | | *EGFR* |
| **8** | 8q21-ter | *C-myc* | 8p | | |
| **9** | 9q21.1 | | | 9q21.1 | |
| **11** | | | 11pter-15.3, 11q12.1, 11q14.2-ter | | *p57* |
| **12** | 12p11.2, 12q12-21 | *K-ras, MDM2, CDK2* | | | |
| **13** | 13q12.1-14.2 | *BRCA2* | 13q14.2-ter | | *Rb* |
| **14** | 14q | *ERβ* | | | |
| **15** | 15q21.1-21.3 | *MEK1, CYP19* | 15q11.2-21.1 | | |
| **16** | 16q22-ter | | 16q12-q22 | | |
| **18** | | | 18q | 18p | |
| **20** | 20q | | | | |
| **21** | 21q22.12-22.3 | | 21q11.2-22.13 | 21q22.12-22.3 | |

[a] Genes which could be implicated in tumourogenesis

**Table 3. Common allelic imbalances present in tamoxifen resistant MCF7 cells and not present in tamoxifen sensitive MCF7 cells**.

| Chromosome | Gains | Candidate genes | LOH | UPD | Candidate genes |
|---|---|---|---|---|---|
| **4** | | | 4q34.2-35.2 | | |
| **5** | | | 5p15 | | |
| **18** | 18p11.32 | | | | |

**Table 4. Fold changes in protein expression in antiestrogen-resistant variants of MCF7.**

| Protein | MMU2 | LCC9 |
|---|---|---|
| CDK4 | 1 ± 0.1 | 0.96 ± 0.07 |
| CDK2 | 1.25 ± 0.36 | 0.95 ± 0.12 |
| Cyclin A | 0.93 ± 0.17 | 0.84 ± 0.18 |
| Cyclin E | 1.05 ± 0.26 | 0.85 ± 0.9 |
| Cyclin D1 | 0.5 ± 0.15 | 0.47 ± 0.05* |
| p27 | 0.64 ± 0.15 | 0.43 ± 0.04* |
| p21 | 2.84 ± 0.43* | 3.38 ± 0.63* |
| total pRb | 0.70 ± 0.11 | 0.62 ± 0.08* |

Data are mean +/- standard deviation of densitometric anaysis of 3 independent experiments of the type shown in figure 5b expressed as a fraction of the expression in parental $MCF7^N$ cells. * Significantly different from $MCF7^N$ $p < 0.05$

**Figure legends**

**Figure 1**

SNP chip analysis of the genomes of breast cancer cell lines. Composite diagram showing chromosome gains and losses in $MCF7^N$ (A), $MCF7^L$ (B), MMU2 (C), LCC9 (D), T47D (E) and MDA-MB-231 (F) cell lines. Gains are shown to the right of the chromosome and losses to the left.

**Figure 2**

Comparison of SNP chip analysis to CGH**.** Diagram showing comparative chromosomal gains and losses in $MCF7^L$ (A) and MMU2 (B) detected by SNP chip (S) and CGH array (C). Gains are shown to the right of the chromosome and losses to the left.

**Figure 3**

Example of UPD detected by SNP chip analyses. Chromosome 21q11.2-22.13 deletion with LOH of 21q. Amplification of 21q22.12-22.3 region with LOH indicating the occurrence of UPD in MCF7 cell line detected using 10K SNP arrays. a) representations of the areas of chromosome loss (to the left of 0) and gain (to the right of 0), 0 = normal copy number b) the probability of LOH along the chromosome. A value of 1 represents a probability of less than 1 in $10^5$ that a sequence of heterozygous calls will have happened by chance alone.

**Figure 4**

ERα gene copy number changes and protein expression. (a) SNP copy number at the ERα gene location (6q25.1 – white box) in $MCF7^N$ and MMU2 cell lines. 0 = 2n, -1 = 1n, -2 = 0n, 2 =4n, 4 = 6n. (b and c) ERα protein levels were measured by western

blot in (b) $MCF7^N$, T47D and MDA-MB-231, (c) $MCF7^N$, LCC9 and MMU2 cells, representative blots shown. (d) Densitometric analyses of ERα protein levels, mean and S.D fmols/mg from 3 independent experiments was determined by comparing values to a purified ERα standard curve on the same membrane. * Significantly different from $MCF7^N$ cells $p < 0.05$.

**Figure 5**

Cyclin D1 gene copy number changes and cell cycle proteins expression. (a) SNP copy number at the cyclin D1 gene location (11q13 – white box) in $MCF7^N$ and MMU2 cell lines. 0 = 2n, -1 = 1n, -2 = 0n, 2 =4n, 4 = 6n. (b) Protein expression of CDK4, CDK2, cyclin A, cyclin E, cyclin D1, p27, p21 and pRb measured by western blot in $MCF7^N$, MMU2 and LCC9 cells. Representative blots shown, mean and S.D values from 3 independent experiments shown in Table 4.

**Figure 6**

Possible mechanisms of acquired antiestrogen resistance in breast cancer. Alterations in the levels and activity of cellular proteins is ultimately responsible for the development of tamoxifen resistance. Amplification or deletion of gene copies, alterations in transcriptional and translational regulation may contribute to altered protein production. Protein function or rate of degradation may be altered by changes in post-translational modifications. It is likely all of these factors contribute to produce tamoxifen resistance.

**Figure 1**

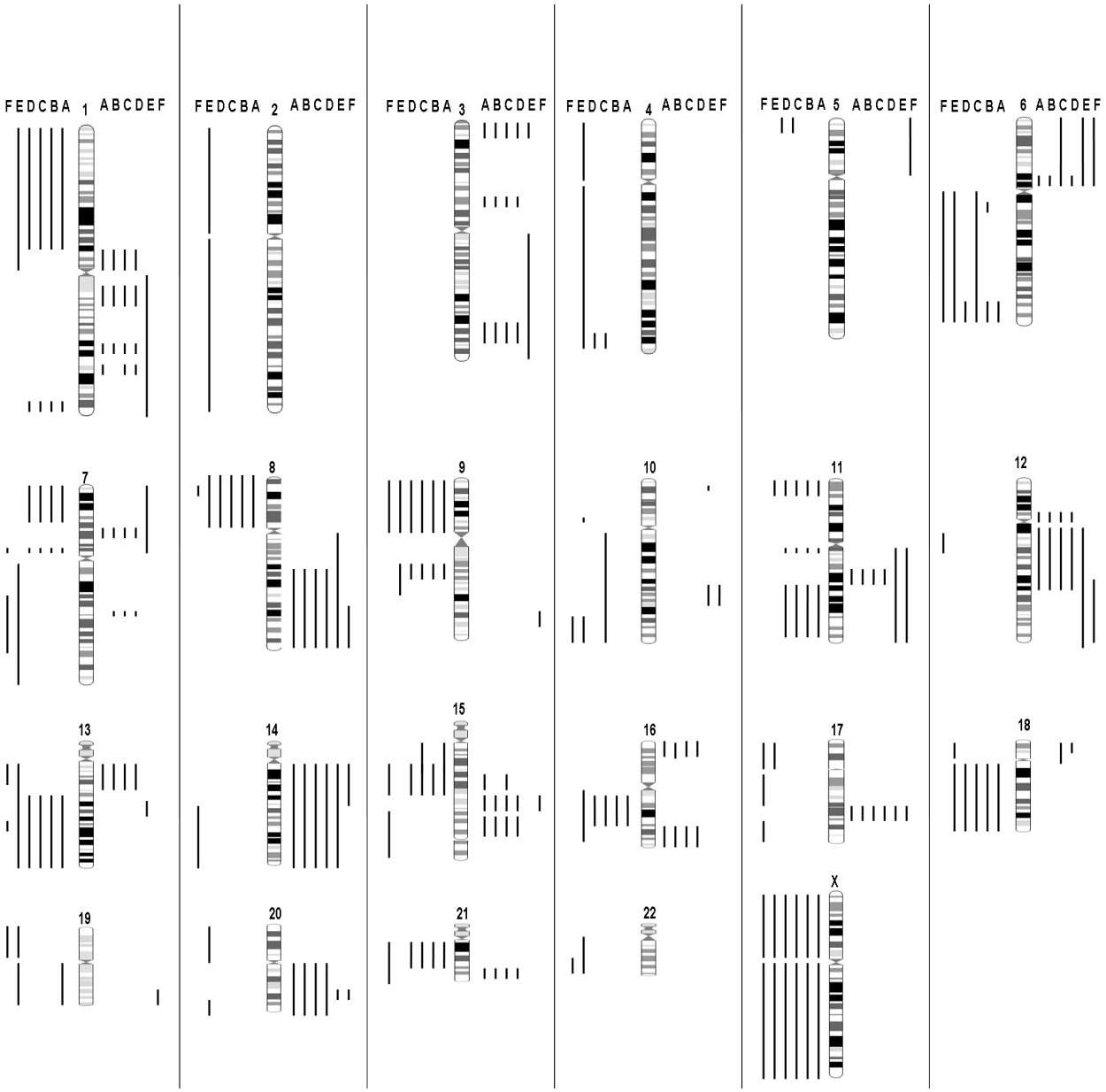

FEDCBA 1 ABCDEF
FEDCBA 2 ABCDEF
FEDCBA 3 ABCDEF
FEDCBA 4 ABCDEF
FEDCBA 5 ABCDEF
FEDCBA 6 ABCDEF
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X

**Figure 2**

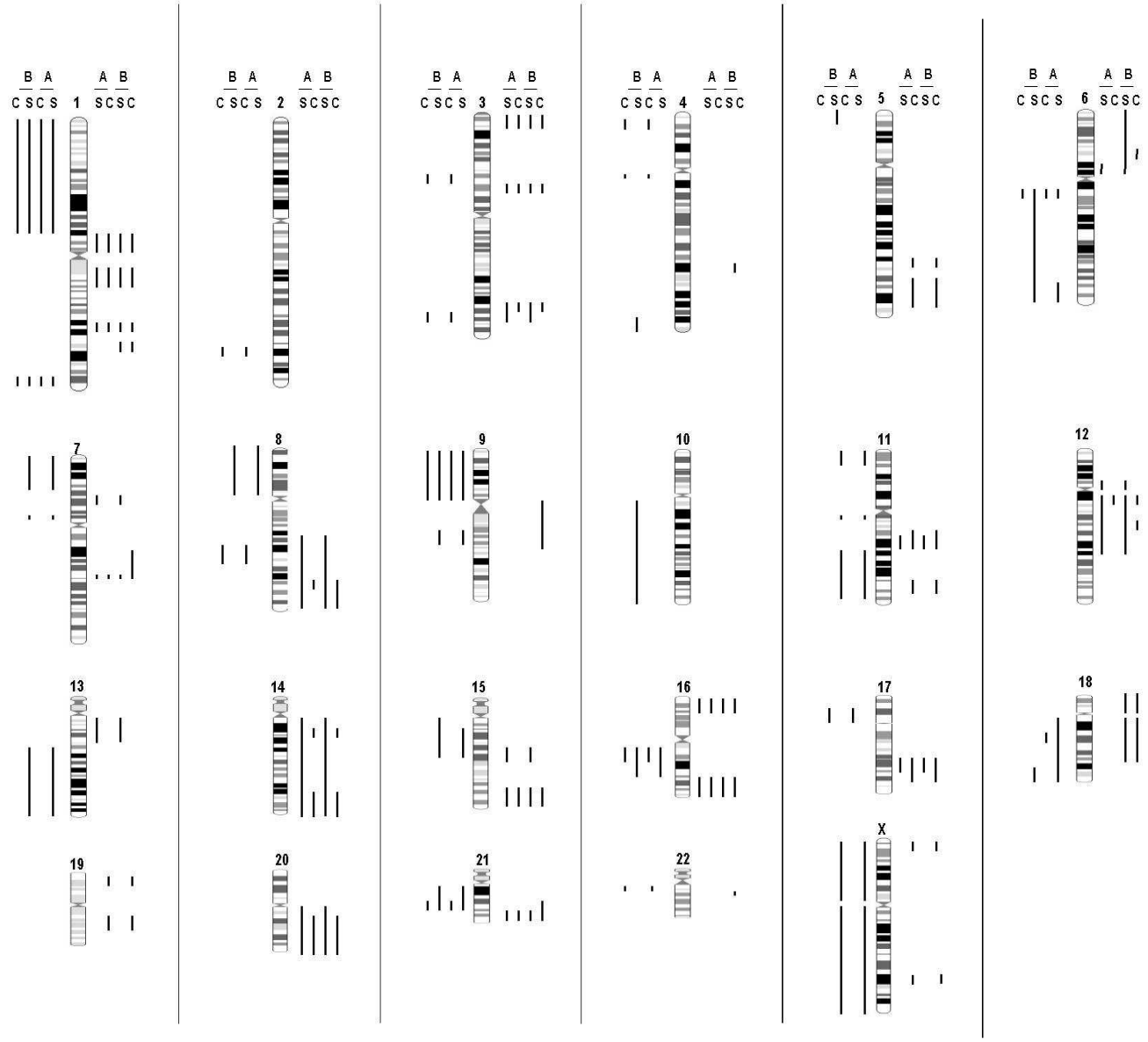

B A A B
C S C S 1 S C S C
B A A B
C S C S 2 S C S C
B A A B
C S C S 3 S C S C
B A A B
C S C S 4 S C S C
B A A B
C S C S 5 S C S C
B A A B
C S C S 6 S C S C
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X

**Figure 3**

(a) (b)

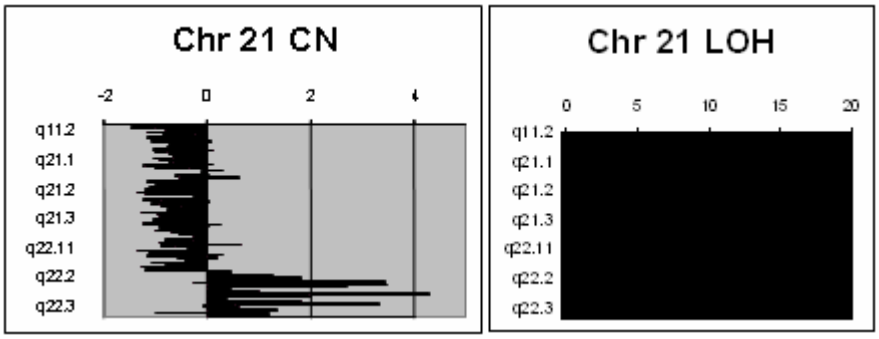
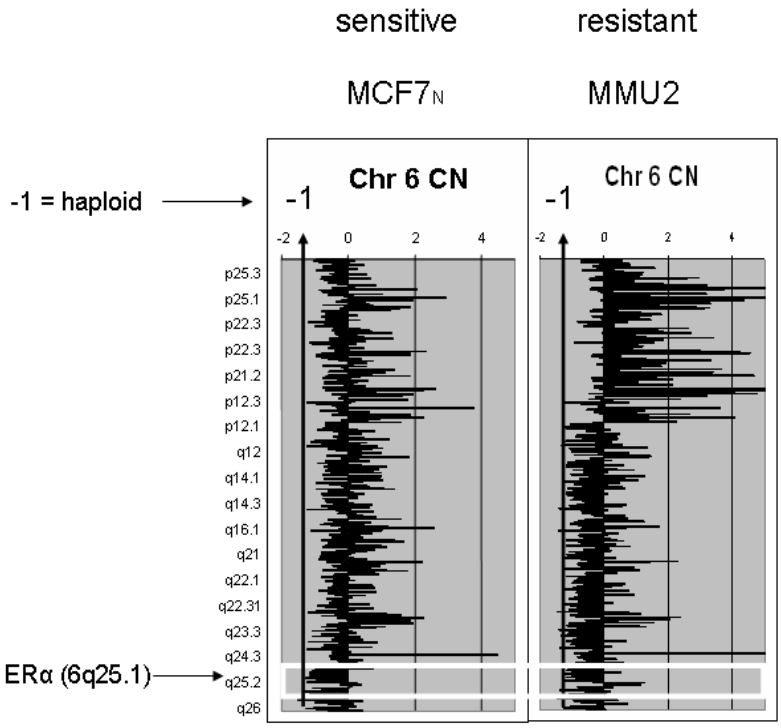
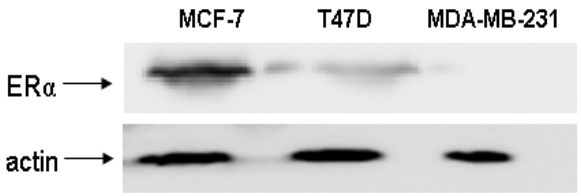

Chr 21 CN
-2
0
2
4
q11.2
q21.1
q21.2
q21.3
q22.11
q22.2
q22.3
Chr 21 LOH
0
5
10
15
20
q11.2
q21.1
q21.2
q21.3
q22.11
q22.2
q22.3

**Figure 4**

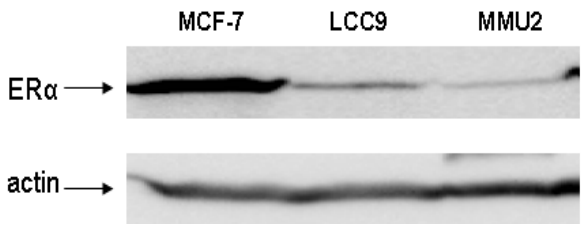
(a)


sensitive
resistant
MCF7N
MMU2
-1 = haploid
Chr 6 CN
-1
Chr 6 CN
-1
-2
0
2
4
p25.3
p25.1
p22.3
p22.3
p21.2
p12.3
p12.1
q12
q14.1
q14.3
q16.1
q21
q22.1
q22.31
q23.3
q24.3
ERα (6q25.1)
q25.2
q26

(b)


MCF-7
T47D
MDA-MB-231
ERα
actin

(c)


MCF-7
LCC9
MMU2
ERα
actin

(d)

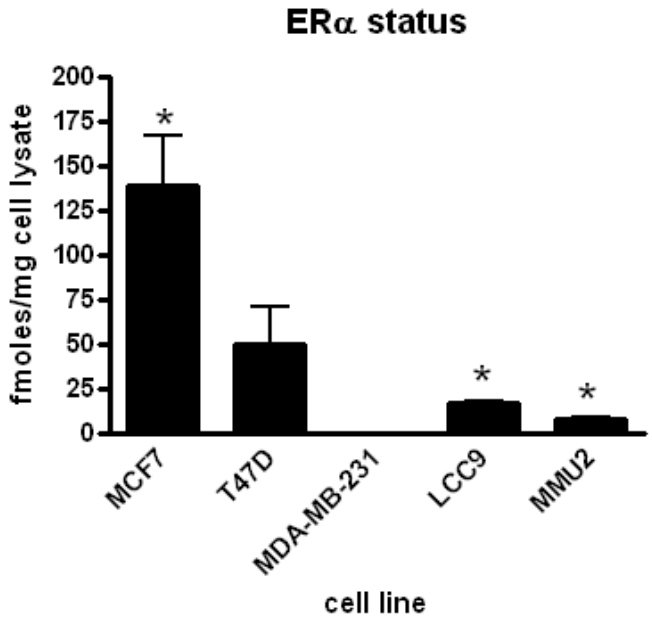
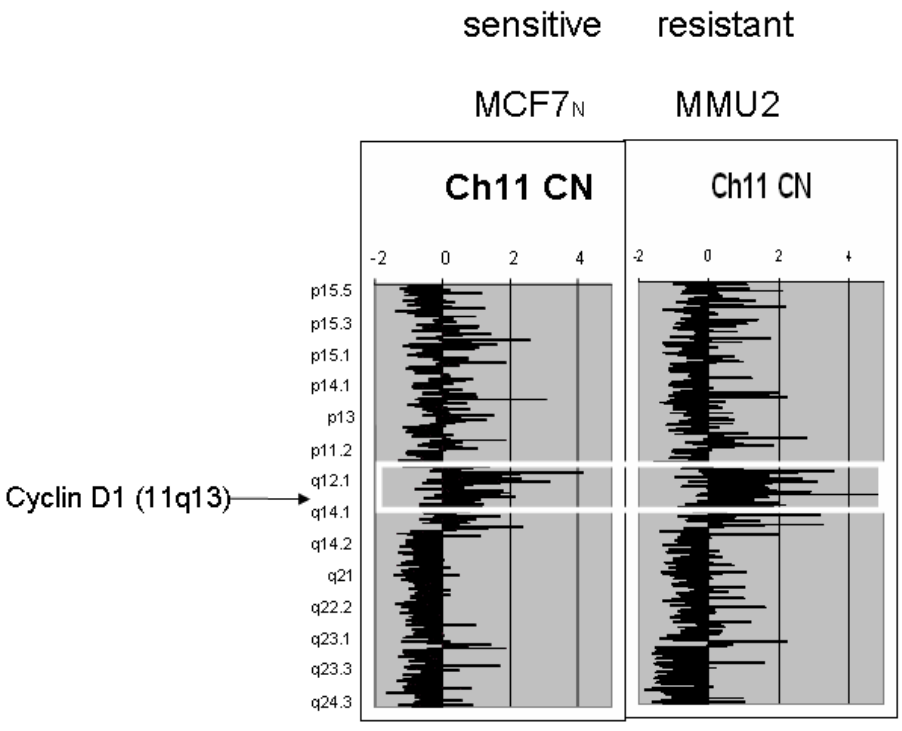

ERα status
fmoles/mg cell lysate
200
175
150
125
100
75
50
25
0
*
*
*
MCF7
T47D
MDA-MB-231
LCC9
MMU2
cell line

**Figure 5**

(a)


sensitive
resistant
MCF7N
MMU2
Ch11 CN
Ch11 CN
-2
0
2
4
-2
0
2
4
p15.5
p15.3
p15.1
p14.1
p13
p11.2
q12.1
q14.1
q14.2
q21
q22.2
q23.1
q23.3
q24.3
Cyclin D1 (11q13)

(b)

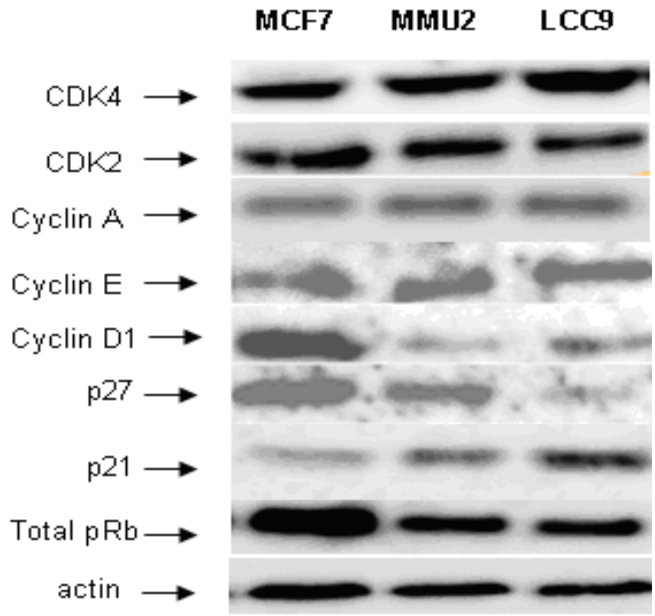
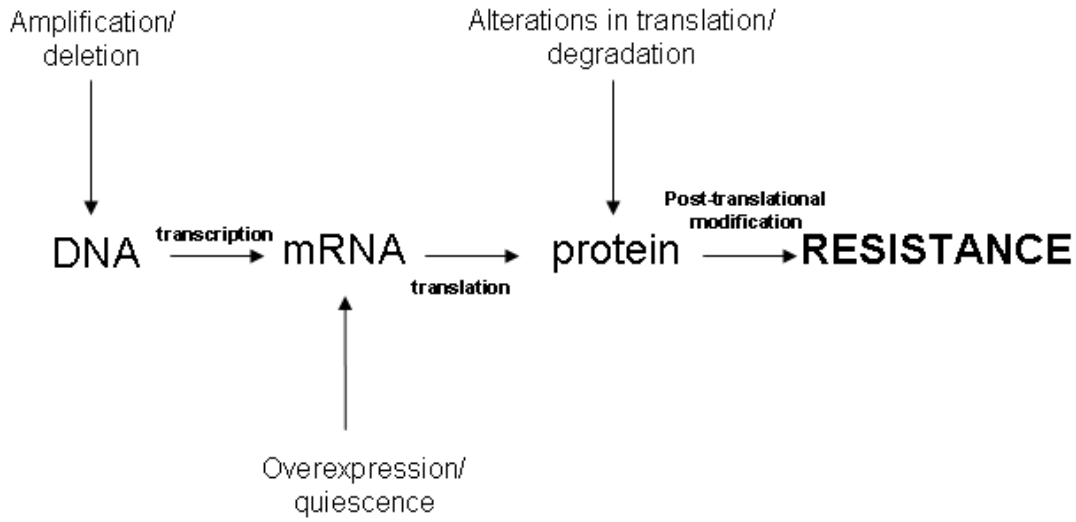

MCF7
MMU2
LCC9
CDK4
CDK2
Cyclin A
Cyclin E
Cyclin D1
p27
p21
Total pRb
actin

**Figure 6**


Amplification/
deletion
Alterations in translation/
degradation
DNA
transcription
mRNA
translation
protein
Post-translational
modification
RESISTANCE
Overexpression/
quiescence