CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1

Oestrogen Receptor α and β variants in clinical breast cancer

Oestrogens play a crucial role in a variety of physiologic processes in many different tissues and organs acting predominantly through the two nuclear receptors ERα and ERβ. Tissues regulated developmentally by oestrogen include the brain (McEwen *et al*, 1997), both female and male reproductive organs (Dupont *et al*, 2000; McPherson *et al*, 2008) and breast (Cheng *et al*, 2005b). Oestrogens have also been implicated in the pathogenesis of a number of disease states. ERα expression occurs more frequently in lung tissue of women than men and is displayed at a higher frequency in lung tumour tissue with the major ERα splice variant being ERαΔ7. ERαΔ4, ERαΔ3+4 and ERαΔ5 are also expressed in lung tumour tissue. ERβ is expressed equally in lung tumours of both genders. (Fasco *et al*, 2002). ER, and more specifically ERβ, has been shown to be inversely correlated with colon cancer (Slattery *et al*, 2001; Campbell-Thompson *et al*, 2001; Wada-Hiraike *et al*, 2006). In prostate cancer wild-type ERβ expression has been demonstrated to be lost at both the transcriptional and translational levels during tumour progression (Leav *et al*, 2001; Carruba, 2007) but the C-terminal truncated splice variant of ERβ (ERβcx) is significantly more expressed in high grade prostatic cancers with poor survival (Fujimura *et al*, 2001). In cancers

of the female reproductive organs ERα mRNA appears to be the main ER isoform expressed in malignant ovarian tumours and ERβ signaling in the ovary may play a more protective role in tumourigenesis (Lindgren *et al*, 2004; Yap *et al*, 2009). The ERαΔ4 splice variant has been implicated in the progression to advanced stages of ovarian cancer (Park *et al*, 1996) and in both ovarian and endometrial carcinomas the presence of the splice variant ERαΔ5 may be the cause of poor responsiveness to antiestrogen treatment (Bryant *et al*, 2005). The ERαΔ7 splice variant has also been found in moderate- to well-differentiated endometrial adenocarcinomas (Horvath *et al*, 2000). ERα is important in the development of uterine fibroids as an up-regulation of ERα over ERβ mRNA ratio has been observed during uterine fibroid development (Bakas *et al*, 2008).

ERα variants have been previously identified by a number of researchers in both breast tumours and breast cancer cell lines (Ferguson and Davidson, 1997; Murphy *et al*, 1998). Similarly ERβ variants have been described although to a lesser degree than ERα (Poola *et al*, 2002a). In this study the ERα variants were assessed according to clinical parameters such as tumour size, nodal involvement, presence or absence of metastases, stage of disease, menstrual status and ER status. The breast tumour samples were also compared with normal breast tissue obtained from women who had had elective breast reduction mammoplasty. As the samples were obtained as FNAs the quantity of available material was small and therefore only a few samples were available for ERβ analysis. The ERα and ERβ variants were further evaluated with respect to each other in order to assess whether they had any significant effect on each other. The menstrual status (Table 12) and nodal involvement (Table 9) of the patients were

not significantly influenced by the presence or absence of specific ERα exons, ERα exon variants or ERβ regions.

Wild type ERα2 and ERαΔ2 were observed simultaneously in the majority of the breast tumours (70%) and normal tissue (62%) examined. X^2 analyses of these results with respect to the clinical parameters tested were not significant indicating that the ERαΔ2 variant does not affect the wild type functioning of ERα.

All but one patient in this study expressed the complete ERα3. In this case ERαΔ3 was expressed simultaneously with the complete exon. This black South African premenopausal patient had presented with a Stage 4 infiltrating duct carcinoma of the breast and no information was available on her ERICA status. No significance was detected with respect to race although according to Poola *et al* (2002c) black women from both the United States and South Africa have breast tumours that are poorly differentiated or undifferentiated. Walker *et al* (2004) have indicated that, particularly in South African black women, the mean age of admission was young (54.1 +/- 10.9 years) compared to developed countries and that the disease was far advanced with 21.1% at Stage 3 and 63.1% presenting at Stage 4. This late presentation may be due to a lack of education in the population with respect to the disease. In our study other Stage 4 patients, however, did not exhibit ERαΔ3. The control samples only expressed the entire ERα3. This result is contrary to previous studies by Zhang *et al* (1996) where the variant ERαΔ3 was detected in the majority of ER-positive and PR-negative tumours (90%). Erenburg and co-workers (1997) indicated that breast cancer cells have an increase in ERαΔ3 compared to normal breast epithelium. The PCR primers used in the latter study,

however, were not designed to amplify the complete exon 3 nor were the resulting products sequenced.

In this study the presence ERα3 in its entirety was also observed in both T-47D clones, TCA3 and TCC1. This further indicates that ERα3 does not play a significant role in breast tumourigenesis.

As ERα4 encodes for the hinge domain as well as part of the ligand binding domain of ERα it plays an important role in influencing the DNA-binding properties of the receptor with the hinge region amino acids playing a critical role in stabilizing DNA binding of the ER (Mader *et al*, 1993). This region also includes the TAF-2 domain required for stimulating the transcriptional activity of the wild-type ERα protein. The variant, ERαΔ4, has an in-frame deletion of exon 4 and the ERαΔ4 protein, which would be lacking all but four amino acids of the hinge domain and the amino terminal of the ligand binding domain (Pfeffer *et al*, 1993) has been shown to be incapable of binding oestrogen and ERE (Koehorst *et al*, 1994). Although ERαΔ4 has lost its DNA- and hormone-binding capability and TAF-2 domain, it has the potential to dimerize with itself or form heterodimers with wild-type ERα resulting in the alteration of the DNA-binding capability of wild-type ER and the depletion of wild-type protein available for transcription activation. The ERαΔ4 protein may also alter the function of normal ERα by competing with other factors necessary for gene-specific ERα activity.

All Stage 1 patients expressed ERα4 and ERαΔ4 simultaneously. The percentage of patients expressing the complete ERα4 decreased with the increase in stage of

disease and in Stage 4 patients ERα4 was expressed in only 28% (5/18) of the samples analyzed while the variant, ERαΔ4, was expressed in 95% (17/18) of patients. There is thus an indication of statistical significance between presence of ERα4 and stage of disease (p=0.008) as well as presence of metastases (M) (p=0.002). No metastases were observed in 72% (31/43) of patients with the complete ERα4 whereas 77% (13/17) of M1 patients did not express ERα4 but largely its deletion variant ERαΔ4. These results suggest that a higher ERα4:ERαΔ4 ratio is associated with low grade breast cancer but metastases at distant sites become more prevalent as the ratio of ERαΔ4:ERα4 increases.

In contradiction to these results it was observed that although ERα4 and ERαΔ4 were expressed simultaneously in 49% (30/61) of the patients the variant ERαΔ4 was observed in all of the normal breast samples whereas the complete exon ERα4 was only present in 23% (3/13) of these samples. This suggests that the ERαΔ4 protein plays a role in normal cells perhaps by altering target specificity and thereby modulating ERα concentration which is important in regulating responsiveness to oestrogen. ERαΔ4 was observed in 90% (55/61) of diseased patients with the complete ERα4 present in 59% (36/61) of samples studied. Further investigation into the role of ERαΔ4 protein in both breast cancer cells and normal cells may give further insight into the progression of the disease.

ERα5 encodes part of the ligand binding domain of ERα together with exons ERα6 and ERα7. ERα5 was observed in all diseased and normal tissue analyzed with 57% of patients and 54% of controls coexpressing the ERαΔ5 variant. These results are supported by a number of groups as ERαΔ5 can form heterodimers

with wild-type ERα and produce a protein that remains constitutively active (Castles *et al*, 1993; Zhang *et al*, 1996). The ERαΔ5 variant and a smaller form, ERαΔ5plus, which may either be a PCR artifact, a degradation product or a truncated form of ERα, were not found to have any significant bearing on clinical parameters such as tumour size (T), nodal involvement (N), metastatic status (M), stage of disease, or menstrual status. ERαΔ5, however, was observed as significant (p=0.011) with respect to ER status and is present more predominantly in ERα positive patients. As ERα5 variants also show no significance with regard to survival or hormone response it may be suggested that variants of the ERα5 exon do not play an important role in the progression of disease or in resistance to hormone treatment. However, the ERαΔ5plus 'truncated' variant was observed in 15% (9/61) of patients, generally in the presence of ERαΔ5, but not in the control samples.

The ERα6 wild-type was found to be expressed simultaneously with 80% of patients exhibiting ERαΔ5plus. This may be due to the fact that this shortened variant is located at the 5' end of ERα exon 5 flanking ERα6 again indicating the possibility of it being a PCR artifact or a degradation product. If this is a truncated variant, previously unknown, further investigation into its protein expression is warranted.

Although most previous studies have reported ERαΔ6 as the least prevalent ERα mRNA variant (Poola *et al*, 2000) 15% (9/61) of the patients in this study were observed to lack ERα6, the least prevalent variant being ERαΔ3 which was observed in only one patient. ERαΔ6 was not expressed in the control group. The

ERαΔ6 variant is usually coexpressed with the wild-type suggesting heterodimerization and no correlation with nodal involvement, menstrual status and ER status was indicated. Metastases were observed in 67% (6/9) of patients exhibiting ERαΔ6 compared to 26% (11/51) of patients with wild-type ERα6 indicating that increasing presence of variant ERαΔ6 significantly increases metastatic potential of the cells (p=0.018). Stage 4 of the disease also showed a significant increase in ERαΔ6 (p=0.023) again indicating the potential relevance of ERαΔ6 with regard to the progression of disease. As the number of patients observed with ERαΔ6 was small in this study it would be beneficial to examine a greater patient population to determine whether a true correlation exists between the level of ERαΔ6 protein expression and prognosis. ERαΔ6 was not found to have any correlation with survival or hormone resistance.

The variants ERαΔ2 and ERαΔ6 were found to be statistically significant with respect to each other (Fisher exact p=0.007). In this study 80% (49/61) of patients expressed ERαΔ2 with wild-type ERα6. This is probably due to the fact that 97% (59/61) of all patients expressed wild-type ERα6. Only 8% (5/61) had the multiple exon deletion ERαΔ2-ERαΔ6. In the control group 77% (10/13) expressed ERαΔ2 and all expressed the wild-type ERα6. All the patients exhibiting ERαΔ6 exhibited ERαΔ4 simultaneously. This indicates the presence of multiple ERα exon deletions which have been found in previous studies (Pfeffer *et al*, 1995; Poola and Speirs, 2001). In contrast, the ERαΔ4 and ERαΔ6 multiple variant was not expressed in the control group. This multiple variant may therefore be significant in the progression of disease which is further indicated by the observation that both ERαΔ4 and ERαΔ6 are significantly indicated with regard to clinical parameters

involved in disease progression. ERαΔ2 was expressed simultaneously with ERαΔ4 in all control samples and in 87% (48/55) of patients expressing ERαΔ4 thus suggesting no significant role for this multiple variant in tumourigenesis. Both of these multiple variants involve the ERα exon 4 deletion and yet in the absence of ERα2 there seems to be no effect with regard to tumourigenesis but with ERαΔ6 tumourigenesis is indicated and further investigation into the ERαΔ4-ERαΔ6 multiple variant and the expression of its protein may provide a better understanding of its effect on tumour progression.

Other multiple exon deletions of prevalence included ERαΔ2-ERαΔ5 in 91% (32/35) of patients with ERαΔ5. In the control group 71% (5/7) of samples with ERαΔ5 expressed this multiple deletion suggesting that it is not involved in tumourigenesis. Similarly multiple deletions ERαΔ4-ERαΔ5 in 94% (33/35) of patients and in all controls exhibiting ERαΔ5; ERαΔ2-ERαΔ7 in 86% (48/56) of patients and 75% (9/12) controls exhibiting ERαΔ7; and ERαΔ4-ERαΔ7 in 93% (51/55) of patients and in all controls exhibiting ERαΔ7 are not indicated in tumourigenesis. The multiple deletion ERαΔ6-ERαΔ7 may be involved in tumour progression as while it is observed in 89% (8/9) of patients with the ERαΔ6 deletion it is not observed in the control group. This may again be a reflection on ERαΔ6 itself which is not found in the control group as discussed previously.

The ERαΔ7 variant was observed in 90% (55/61) of the patients and in 85% (11/13) of controls examined. As has been previously reported ERαΔ7 was the most frequently observed variant (Zhang *et al*, 1996; Poola *et al*, 2000). Wild-type ERα7 was expressed simultaneously with ERαΔ7 in 69% (42/61) of patients and in 77%

(10/13) of controls suggesting the formation of heterodimers. The ERαΔ7 variant showed no significance with respect to any of the clinical parameters analyzed but seems to show the dominant negative activity described by other researchers (Fuqua *et al*, 1992; Garcia-Pedrero *et al*, 2003) as the presence of both wild-type ERα7 and ERαΔ7 are indicated in increased tumour size. The presence of the ERαΔ7 variant did not show any significance with regard to hormone response as has been postulated by other groups (Fuqua *et al*, 1992).

Due to the small sample size obtained by FNA ERβ variants could not be examined in as much detail as the ERα variants. The two domains of ERβ, namely the DNA binding domain and the ligand binding domain were PCRed but not each exon individually. It has been suggested that the relative expression levels of ERβ *versus* those of ERα decrease during tumourigenesis (Leygue *et al*, 1998) and that this loss is due to promoter methylation in breast cancer cells (Rody *et al*, 2005; Zhao *et al*, 2003). This factor may also contribute to the difficulty of visualizing minute quantities of ERβ RT-PCR product. Both ERβDBD and ERβLBD showed no significance with respect to the clinical parameters assessed but these results are not truly representative as only 28 of the 61 patient samples had sufficient RNA to PCR for the ERβDBD and only 26 of the original 61 patient samples were assessed for the ERβLBD. The samples tested for ERβDBD and ERβLBD were not always from the same patients. The control group had a similar situation with very little or no material available for ERβ analysis. A more comprehensive study of the ratio of ERα:ERβ variants should be performed in order to gain a better understanding of tumour progression and the effects of the two types of ER. As promoter methylation is frequently observed in cancer (Garinis *et al*, 2002), the

loss of ERβ expression may be an indicator of breast cancer development (Chang *et al*, 2006) and ERβ may act as a tumour suppressor gene.

5.2

ERα variants and ER status assessment

ER status refers to the presence of ERα within the cells. The Abbott ER-ICA Monoclonal kit (Abbott Diagnostics, West Germany), one of the methods used for assessing ER status, utilizes the monoclonal antibody H222 which is of rat origin and is directed against the functional section of the ERα, coded for in ERα5 (Greene and Jensen, 1982). Another antibody which may be used for ER status assessment is DAKO® monoclonal mouse anti-human ERα Clone 1D5 (DAKO Corporation, CA, USA) which is directed to the N-terminal end of the ER (Al Saati *et al*, 1993). It is important to know which antibody is being utilized in these assessments as this may affect the success of treatment. In this study all the ERα positive samples, according to ER immunocytochemical tests, contained the complete ERα2 exon. This was largely simultaneous with ERαΔ2 expression but the presence of the complete ERα2, which is located at the N-terminal end of the ER and is partially responsible for coding the DNA binding domain of ERα, would be required for recognition by the DAKO® monoclonal ER immunocytochemical assay. These ER immunocytochemical tests were done by the routine pathology laboratory at the Charlotte Maxeke Academic Hospital in Johannesburg using the DAKO® monoclonal antibody.

The wild type ERα2 was also associated significantly with the presence of the complete ERα4 exon (Fisher exact p=0.005) and the ERαΔ5 deletion variant (Fisher exact p=0.042). As ERα4 also codes for part of the DNA binding domain as well as the hinge and part of the ligand binding domain of ERα it is not surprising that the presence of ERα4 also showed significance with respect to ERα status (p=0.046). It is interesting to note that the deletion variant ERαΔ5 was observed as significant (p=0.011) in ERα positive patients (77% (20/26) of ERα positive patients as compared to 38% (9/24) of ERα negative patients). ERαΔ5 was not observed in 63% (15/24) ERα negative patients. This is an indication that ERαΔ5 is present more predominantly in ERα positive patients as recognized by the DAKO® monoclonal antibody. Had the antibody used for immunocytochemical analysis of ERα been directed towards the ligand binding domain, such as the H222 monoclonal antibody, the ER status of the tumour may have been reflected as ER-negative. This again emphasizes the importance of the manner of ER status testing and the knowledge of the monoclonal antibody used as ER status often determines the type of treatment prescribed. This may also reflect on the observation of hormone responsiveness and development of hormone resistance. Both the DBD and LBD should be assessed with respect to ERα status in order to gain a comprehensive overview.

5.3

The effects of environmental factors on oestrogen responsiveness, ERα and ERβ expression in T-47D clones

The development and progression of cancer has long been ascribed to the clonal evolution of neoplastic cells (Nowell, 1976; Nowell, 1986; Shackney *et al*, 1989). In a model of breast cancer development normal ductal epithelium evolves to typical hyperplasia to atypical hyperplasia to carcinoma *in situ* to invasive carcinoma and, finally, to metastatic carcinoma (Dupont and Page, 1985). There is much evidence to support the hypothesis that oestrogens are important as they act as potent mitogens for normal breast epithelial cells and that the duration of breast epithelium exposure to oestrogen is a significant risk factor for breast cancer development. (Fuqua *et al*, 2000). The evolution of spontaneous variants within breast cells may, however, give rise to the progression of tumours that respond differently to hormones. In this study the cloning of T-47D cells resulted in five clones of which two were chosen for further study. These clones differed in their oestrogen responsiveness with the TCA3 parent clone exhibiting strong oestrogen and tamoxifen responsiveness. The TCC1 parent clone was only slightly oestrogen responsive and tamoxifen resistant. The ERα profiles of each of these clones also varied. Initially, TCA3 exhibited ERαΔ2, ERαΔ5 and ERαΔ7 simultaneously with their corresponding complete exons while TCC1 did not. In fact, the only exon deletion occurring within the TCC1 parent clone was ERαΔ4. ERβ expression has been shown to be low in T-47D breast tumour cells as compared with ERα (Ström *et* al, 2004). This was confirmed as ERβ expression in both clones remained low and unaltered throughout the experiment.

After ten passages under varying conditions both clones were reassessed. The TCA3 control cells remained unchanged with regard to ERα profiles. The cells remained oestrogen and tamoxifen responsive but it was observed that after 48 hours of growth with oestrogen followed by treatment with tamoxifen that there was an increase in cell proliferation indicating possible tamoxifen agonistic behaviour. Growth of TCA3 cells in 10^{-8}M oestradiol, tamoxifen, androstene-dione, oestriol, and cholesterol respectively all expressed similar growth patterns and ERα profiles as the parent clone. Growth of TCA3 and TCC1 cells in 10^{-8}M aminoglutethimide for ten passages showed similar hormone responses but TCA3 cells showed the complete loss of ERα exon 4 under these conditions. The nonsteroidal aromatase inhibitor, aminoglutethimide, binds reversibly to the haem portion of the enzyme, aromatase, thus interfering with the final step in the synthesis of oestrogen. ERα exon 4 constitutes the hinge domain of ERα, which contributes flexibility to the DNA- versus the ligand binding domain of the gene. The absence of this area of the gene, which influences the ability of the receptor to bind to DNA and ligand, thus lacks oestrogen-induced transcriptional activity and it is therefore not surprising that this did not affect the oestrogen responsiveness of the cells or their sensitivity to tamoxifen. It may therefore be proposed that ERαΔ4 plays no role in the development of tamoxifen resistance and that the presence of any of the oestrogen metabolites examined does not affect the genetics of the TCA3 cells.

The presence of 10^{-8}M tamoxifen was observed to slow down the growth of the TCA3 clone and 10^{-8}M cholesterol caused a change in phenotype with the cells becoming rounder and unable to attach and thus did not survive long enough to

perform oestrogen response tests possibly due to other factors involved with cholesterol metabolism. In all cases ERα3 and ERα6 were always expressed as complete and therefore seem unlikely to contribute to oestrogen responsiveness in the TCA3 clone.

Cholesterol and androstendione are oestrogen precursors, their presence within the growth medium increasing the concentration of oestrogen in the cells and thus stimulating cell growth. In the TCA3 clones this stimulation of growth was greater than the growth observed in medium containing 17β-oestradiol due to increased concentration of oestrogen present. Oestriol is a weak oestrogen metabolite which has also been questioned with regard to stimulation of breast cancer cells. In this study the presence of oestriol in the medium showed slightly lower growth stimulation than that of androstenedione. Oestriol, in this case, increases breast cell growth.

The TCC1 control cells, after ten passages, showed greater sensitivity to tamoxifen than the parent clone. This occurred in conjunction with the appearance of ERαΔ2, ERαΔ5 and ERαΔ7. These exon deletions occurred simultaneously with the presence of their respective complete exons. It seems, therefore, that a heterozygous genotype confers greater sensitivity to tamoxifen in the T-47D cell line.

Growth in 10^{-8}M oestradiol over 10 passages indicated less tamoxifen sensitivity and also exhibited the greatest growth stimulation. This coincided with the homozygous expression of ERα7. This was again reflected in TCC1(10) cells

grown in 10^{-8}M tamoxifen, aminoglutethimide, androstenedione, oestriol and cholesterol respectively. All these conditions exhibited tamoxifen resistant phenotypes which are observed in some clinical patients after prolonged treatment with tamoxifen.

ERα exon 7 is located in the hormone binding domain of the ERα gene. This domain forms a hydrophobic pocket which binds hormone specifically and masks the DNA-binding domain when unoccupied thus preventing transcription. The presence of tamoxifen should block the conformational change in the ER required to activate transcription. Growth with prolonged exposure to tamoxifen, however, may cause some change within this pocket that exposes the DNA binding domain thus activating transcription. The selective pressure leading to hormone resistance is triggered by the dual factors of genetic instability and mutations in genes that regulate growth (Graham *et al*, 1990). It may thus be suggested that a heterozygous ERα7/ERαΔ7 genotype could be responsible for greater tamoxifen sensitivity and that further investigation into ERα exon 7 and tamoxifen resistance is warranted.

5.4

CONCLUSIONS AND FUTURE DIRECTIONS

Menstrual status and nodal involvement (N) are not affected by variants of ERα or ERβ genes. ER status in this study relies on the complete expression of ERα2 and ERα4 to encode a protein that is recognized by the mouse antibody, Clone 1D5 (DAKO Corporation, CA, USA), in order to bind to the oestrogen receptor in the

immunocytochemical assay. The method and the monoclonal antibody used to assess ER status are therefore important with regard to clinical treatment implemented.

In this study the presence of ERαΔ2 did not affect the wild-type functioning of ERα in patients or tissue culture cells. Contrary to previous studies ERαΔ3 was the least prevalent variant observed and was not found to have a role in tumourigenesis. ERα5 and ERαΔ5 were present largely as heterodimers and although ERαΔ5 was more prevalent in ERα-positive patients there was no significance with respect to progression of disease or response to hormone treatment. ERαΔ5 was present in both T-47D clones examined after 10 passages although not in the parent TCC1 clone. The ERαΔ5plus fragment was considered to be a PCR artifact or degradation product due to its consistent presence with ERα6.

Although the presence of ERαΔ4 in normal cells suggested that the ERαΔ4 protein plays a role in normal cells perhaps by modulating ERα concentration, important in regulating responsiveness to oestrogen, an increase in the ratio of ERαΔ4 variant: wild-type ERα4 indicated an increase in metastatic potential of diseased tissue. As cell culture models may mimic the natural progression of solid tumours these *in vivo* results are interesting when compared the ER profiles of T-47D tissue culture clones, TCA3 and TCC1. ERα4 and ERαΔ4 heterodimers were found to be present in both T-47D clones and after 10 passages the TCA3 clone grown in 10^{-8}M aminoglutethimide indicated a complete loss of ERα4 although hormone responsiveness was unaltered. ERαΔ4 may play no role in the acquisition of

tamoxifen resistance but further investigation of this variant may provide a better understanding of the progression of the disease.

Another exon variant of particular interest was ERαΔ6. Previous studies have shown this to be the least prevalent variant but, although it was not found to be present in the T-47D clones or in the control breast mammoplasty group, 15% of patients in this study were observed to lack ERα6. There was an indication of ERαΔ6 influencing metastatic potential and progression of disease and it would be of interest to study the affects of this variant in greater detail and with a larger patient population.

The ERαΔ7 variant was the most frequently observed variant, as has been described by many groups (McGuire *et al*, 1991; Zhang *et al*, 1996; Poola *et al*, 2000). The presence of this variant did not show any significance with regard to hormone response *in vivo*. In the more tamoxifen resistant TCC1 clone, however, the presence of ERαΔ7 together with the complete exon ERα7 conferred a greater sensitivity to tamoxifen. Investigation into this variant both *in vivo* and *in vitro* may provide further information on the acquisition of hormone resistance in breast carcinoma.

Multiple exon deletions, particularly the ERαΔ4-ERαΔ6 variant, may also be significant with regard to disease progression and should be studied further.

In this study ERβ was not studied in sufficient depth mainly due to the lack of sample material. In order to make a valuable assessment of ERβ variants and

their potential relationships with ERα variants the ERα variants of particular interest, such as ERαΔ4, ERαΔ6 and ERαΔ7, should be studied more comprehensively with specific ERβ variants.

The *in vitro* study also confirmed the effects of oestrogen on the growth of breast cells. Growth in the oestrogens, oestradiol and oestriol, as well as in oestrogen precursors, androstenedione and cholesterol, over 10 passages exhibited growth stimulation in both T-47D clones.

Another factor, which affected the survival data, is the problem of patient follow-up. In Africa, particularly, patients are often unable to reach hospitals for follow-up treatment and this may give skewed results as far as survival analysis is concerned. This problem may be addressed by restricting the study to patients that are able to comply over the study period although this may also not be truly representative of the population.

Cancer evolves from a combination of genetic and epigenetic abnormalities resulting in dysregulated gene expression and function with the most common epigenetic modifications being increased methylation of CpG islands within gene promoter regions, which play an important role in controlling gene expression, and deacetylation and or methylation of histone proteins, which govern the structural status of chromatin and thus the transcriptional status of genes within a particular locus (Ellis *et al*, 2009). These modifications therefore affect chromatin structure and transcription of protein encoding mRNAs. The loss of ERα from initially ERα-

positive breast tumours may be due to such modifications (Giacinti *et al*, 2006; Herynk and Fuqua, 2007).

MicroRNAs (miRNAs), which are short, non-protein-coding RNAs of 20 to 25 nucleotides in length, are also known to alter gene expression at a post-transcriptional level. miRNAs lower protein levels by repressing target mRNA translation and/or by inducing the degradation of the mRNA (Filipowicz *et al*, 2008) by binding to the 3' untranslated region (UTR) of target mRNAs. There are a number of studies documenting the miRNA profiles of breast tumours (Iorio *et al*, 2005; Calin and Croce, 2006; Sempere *et al*, 2007; Verghese *et al*, 2008). As ERα mRNA has a long 3' UTR of about 4.3 kb it has been shown to be a target of miRNAs that repress ERα expression leading to a reduction in oestrogen signalling (Pandey and Picard, 2009). miRNA transcript signatures predictive of ER status have been generated from microarray analysis (Lowery *et al*, 2009) and include biologically relevant miRNAs that have previously been identified as dysregulated in breast cancer (Mattie *et al*, 2006; Blenkiron *et al*, 2007) and other cancers (Cheng *et al*, 2005a; Schetter *et al*, 2008; Huang *et al*, 2008). They are also involved in the regulation of cell functions such as growth, apoptosis, migration, and invasion. miRNA expression may be affected by genetic abnormalities as they are frequently located in chromosomal regions characterized by nonrandom abberations in human cancer (Calin *et al*, 2004). Whether ERα exon deletions have any effect on miRNA expression or *vice versa* have yet to be examined.