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Oestrogen receptor mutations and their influence on breast cancer growth

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CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1

Oestrogen Receptor ± and ² variants in clinical bre

Oestrogens play a crucial role in physiological processes in a variety of different tissues and organs acting predominantly through receptors ER± and ER². The ERs are regulated developmentally by oestrogens in the brain (McEwen et al 1997), both female and male reproductive (Dupont et al 2000; McPherson et al 2002) and breast (Cheng et al 2005). Oestrogens have also been implicated in the pathogenesis of a number of disease states. ER± expression occurs more frequently in lung tissue than ER² and is displayed at a higher frequency in lung tumour tissue, with the ER±"7 splice variant being the most abundant. ER±"4, ER±"5 and ER±"3 are also expressed in lung tumour tissue. ER² is expressed equally in lung tumour and normal tissue (Fasco et al 2002). ER, and more specifically ER², has been shown to be inversely correlated with tumour grade in breast cancer (Speirs et al 2001; Carruba et al 2006; Thomas et al 2001; Waidelich et al 2006). In prostate cancer, however, ER² expression has been demonstrated to be lost at both the transcriptional and protein levels during tumour progression (Leav et al 2001; Carruba et al 2007). The C terminal truncated splice variant of ER² (ER²cx) is significantly expressed in high grade prostatic cancers when compared to normal tissue (Fujimoto et al 2001). In breast cancers

of the female reproductive organs ER± mRNA appears to be expressed in malignant ovarian tumours and ER² signaling in the o a more protective role in tumorigenesis (Lumachi et al 2004, Shapiro et al 2009). The ER±"4 splice variant has been implicated in the progression stages of ovarian cancer (Parrott et al 1996a) and in both ovarian and endometrial carcinomas the presence of the splice variant ER±"5 may alter responsiveness to antiestrogen treatment (Bryant et al 2005). The ER±"7 splice variant has also been found to inversely correlate with differentiated endometrial adenocarcinoma (Honma et al 2000). ER± is important in the development of uterine fibroids - an upregulation of ER± over ER² mRNA ratio is observed during uterine fibroid development (Bulun et al 2008).

ER± variants have previously been identified by a number of research groups in breast tumours and breast cancer cell lines (Ferguson and Davidson, 1993, Murphy et al 1998). Similarly ER² variants have been described to a lesser degree than ER± (Poola et al 2002). In this study the ER± variants were assessed according to clinical parameters such as tumour involvement, presence or absence of metastases, stage of disease, menopausal status and ER status. The breast tumour samples were analysed with normal breast tissue obtained from healthy donors who had undergone breast reduction mammoplasty. As the samples were obtained as FNAs the quantity of material was small and therefore only a few samples were available for analysis. The ER± and ER² variants were further evaluated to see if they had any significant effect on each other in order to assess whether they had any significant effect on each other in order to assess whether they had any significant effect on each other in order to assess whether

not significantly influenced by the presence or absence of specific ER± ER± exon variants or ER² regions.

Wild type ER±2 and ER±"2 were observed simultaneously in breast tumours (70%) and normal tissue (62%) samples. Statistical analyses of the results with respect to clinical parameters tested were not significant, indicating that the ER±"2 variants do not affect the wild type functioning of ER±2.

All but one patient in this study expressed the ER±"3 variant. This case was ER± was expressed simultaneously with the ER±2 variant. This black South African premenopausal patient had presented with a Stage 4 infiltrating carcinoma of the breast and no information was available on her family history. No significant difference was detected with respect to race although Parker et al. (2000) and Jack et al. (2001) have indicated that women from both the United States and South Africa have been diagnosed with poorly differentiated or undifferentiated tumours. Walker et al. (2004) have indicated that particularly in South African black women, there is a younger age of onset (54.1-10.9 years) compared to developed countries and that patients present far advanced with 21.1% at Stage 3 and 63.1% at Stage 4. Late presentation may be due to a lack of education in the population regarding the disease. Our study other Stage 4 patients, however, did not express ER±"3. The control samples only expressed ER±2. This result is contrary to previous studies by Zhang et al. (1999) where the variant ER±"3 was detected in the majority-positive and ER-negative tumours (90%). Erenburg and co-workers (1997) indicated that breast cancer cells have an increased S² fraction compared to normal breast epithelium. The PCR primers used in this

however, were not designed to the complete exon 3 sequence of the resulting products sequenced.

In this study the presence of ER±3 in its entirety was also observed in 7 of 40 clones, TCA3 and TCC1. This further indicates that ER±3 does not play a significant role in breast tumourigenesis.

As ER±4 encodes the hinge domain as well as part of the ligand binding domain of ER± it plays an important role in mediating the properties of the receptor, with the hinge region amino acids playing a role in stabilizing DNA binding of the ER (Fuqua et al., 1991). This region also includes the TAF-2 domain required for stimulating the transcriptional activity of wild type ER± (Tora et al., 1989). The variant, ER±"4, arises as a deletion of exon 4 from the ER±"4 protein which would be lacking a small proportion of the hinge domain and the amino terminal of the ligand binding domain (Kumar et al., 1987). This domain has been shown to be incapable of binding oestrogen (Koenstein and Gustafsson, 1994). Although ER±"4 has lost its DNA binding capability and the TAF-2 domain, it has the potential to dimerize with itself and with wild-type ER± resulting in the alteration of the DNA binding properties of wild type ER and the depletion of wild-type protein available for activation. The ER±"4 protein may also alter the function of normal ER± by competing for factors necessary for specific ER± activity.

All Stage 1 patients expressed ER±4 and ER±"4 in a heterogeneous manner. The percentage of patients expressing ER±4 decreased with the increase in stage

disease and in Stage 4 patients ER±4 was expressed in only 28% (5/18) of the samples analyzed while the variant ER±"4 was expressed in 95% (17/18) of patients. There is an indication of statistical significance in the presence of ER±4 and stage of disease (p=0.008) as well as presence of metastases (M1) (p=0.002). No metastases were observed in patients with complete ER±4 whereas 77% (10/13) of M1 patients did not express ER±4 largely as a result of deletion variant ER±"4. The results suggest that a higher ER±:4 ER±"4 ratio is associated with low grade tumors that are confined to the breast and distant sites become more prevalent when ER±"4 is expressed in excess.

In contradiction to these results it was found that both ER±4 and ER±"4 were expressed simultaneously in 94% (16/17) of the normal breast samples whereas the variant ER±"4 was observed in all of the normal breast samples whereas ER±4 was only present in 23% (3/13) of these samples. This suggests that the ER±"4 protein plays a role in normal cells perhaps by altering target gene expression and thereby modulating ER± concentration which is important in the responsiveness to oestrogen. ER±"4 was observed in 95% (19/20) of diseased patients with complete ER±4 present in 35% (6/17) of samples studied. Further investigation into the role of the ER±"4 protein in normal cells and tissues may give further insight into the progression of disease.

ER±5 encodes part of the ligand binding domain of ER± together with exons 7 and ER±7. ER±5 was observed in all diseased and normal tissues with 57% of patients and 54% of controls coexpressing both ER± variants. These results are supported by a number of groups who have also found ER±5 ca

with wild-type ER± and produce a protein that remains con
(Castles et al, 1993; Zhang et al, 1996). The ER±"5 variant a smaller form,
ER±"5plus, which may either be a PCR generated artefact or a product
truncated of form ERs were not found to have any significant bear
parameters such as tumour size (T), nodal involvement (N)
stage of disease, menstrual status. ER±"5, however, was observed
significantly (p=0.011) with tumour ER status, being present more predominant
in ER± positive. As the ER±"5 variants also show no significance
to survival or hormone response it may be suggested that
exon 5 does not play an important role in the progression of disease or in resistan
hormone treatment. However, the ER±"5plus truncated variant was observed i
15% (9/61) of patients, generally in the presence of both ER±"5 and the contro
samples.

The ER± wild-type was found to be expressed in a similar high proportion of
patients exhibiting ER±"5plus. This may be due to the fact that the
variant is located at the 5 end of the ER± exon 5 flanking
possibility of it being an artefact of the PCR amplification process. This truncated
variant, previously unknown, further investigation into its expression and protein
warranted.

Although most previous studies have reported the presence of an ER±"6 variant ER
mRNA variant (Pfeffer et al, 2000), 15% (9/61) of the patients studied were
observed to lack the ER±"6 exon. The most prevalent variant, ER±"3, which was
observed in only one ER± positive was not expressed in the control

ER±"6 variant is usually coexpressed with the wild type ER±"6 heterodimerization or correlation with nodal, invasive and ER status was found. Metastases were observed in 67% (4/6) of patients exhibiting ER±"6 compared to 15% (4/26) of patients without ER±"6 indicating that increasing presence of ER±"6 significantly increases metastatic potential (p=0.0148). Results (4 of the disease also show significant increase (p=0.0123) in ER±"6 again indicating the potential role ER±"6 with regard to the progression of disease and patient outcome observed with ER±"6 alone. Within this study it would be beneficial to greater patient population to determine whether a true correlation exists the level of ER±"6 protein expressed by ER±"6 and was found to have any correlation with survival or hormone resistance.

The variants ER±"2 and ER±"6 were found to be statistically significant respect to each other (Fisher exact p=0.0017) this study 100% (6/6) of patients expressed ER±"2 with the wild type ER±"6. This is probably due to the fact that (59/61) of all patients expressed ER±"2 only 85% (52/61) had the multiple exon deletion-ER±"62. In the control group, 37% expressed ER±"2 and all expressed both the ER±"6. the patients using ER±"6 exhibited ER±"4 simultaneously. This indicates the presence of multiple deletions which have been found in previous studies (Pfeffer and Speirs, 2000). In contrast, the ER±"4 and ER±"6 multiple variants expressed in the control group. This multiple variant may thereby the progression of disease which is further indicated by the ER±"4 and ER±"6 are significantly indicated with regard to

invo dd in ease progres ER±"2 was expressed simultaneous
ER±"4 in all control samples 48/48) and in patients 97%
thus suggesting no significant role for this multiple variant in tum
of these multiple variants ER±"4 exon 4 deletion and yet in th
of ER±2 there seems to be no effect with regard to tum
ER±"6 tumourigenesis and further investigation into the ER±"4
ER±"6 multiple variant and the expression provides a better
understanding of its effect on tumour progression.

Other multiple exon deletions of prevalence ER±"5 in 93.8% (
of patients with ER±"5. In the control samples 31% with
expressed this multiple deletion suggesting that it is not invo
tumourigenesis. Similarly multiple deletion ER±"5 in 94.3% (33/35) of
patients and controls exhibiting ER±"2 and ER±"7 in 84.8% (56 of
patients and 9/15 (62%) controls exhibiting ER±"4 and ER±"7 in 93%
(51/55) of patients and controls exhibiting ER±"7 not indicated in
tumourigenesis. These multiple deletions ER±"7 may be involved in tum
progression as while it is observed in 83% with the ER±"6 de
itself is not observed in the control group. This may again be a
itself which is not found in the control group as discussed

The ER±"7 variant was observed in 90% (patients and 11/13 in 85% (
of controls examined. As previously reported ER±"7 was
frequently observed variant (19/26; Prota 2000) with wild-type ER±7
was expressed simultaneously with ER±"7 in 69% (42/61) and in 77%

(10/13) of controls suggesting a low incidence of this variant. The ER±"7 variant showed no significance with respect to any of the clinical p seems to show the dominant negative activity described by (Fuqua et al 1992; Garcia-Prieto et al 2003) the presence of both wild ER±7 and ER±"7 are indicated in tumour. The presence of the ER±"7 variant did not show any significance with regard to has been postulated by other groups (Fuqua et al 1992).

Due to the small sample size of NAT ER² variants could not b examined in such detail as the ER± variants. The two, mainly of E the DNA binding domain and the ligand binding domain were exon individually. It has been suggested that the expression levels of E versus those of ER± decrease during tumourigenesis (Leygue et al 1998) and that this loss is due to promoter methylation (Rody et al 2005; Cancer c Zhao et al 2003). Factors may also contribute to the visualizing minute quantities of ER² mRNA. Both ER²DBD and ER²LBD show no significance with respect to pathological parameters assessed but these r are not truly representative as only 28 of the 61 participant RNA to PCR for the ER²DBD and only 26 of the original 61 assessed for the ER²LBD. These were tested for ER²DBD and ER²LB not always from the same patients. The control group had a similar situ very little information available for ER² and early comprehensive study of the ratio of ER² variants could be performed in order to gain understanding of tumour progression and the two types of promoter methylation is required to address (Cai et al 2002), the

loss of ER² expression may be an indicator of breast cance
et al., 2006) and ER² may act as a tumour suppressor gene

5.2

ER± variants and ER status assessment

ER status refers to the presence or absence of ER± in the tissue. The Abbott-ICE ER Monoclonal kit (Abbott Diagnostics, one of the commercial methods for assessing ER status, utilizes the monoclonal antibody H222 which and is directed against the functional domain of the ER±5 (Greene and Jensen, 1982) antibody which may be used for assessment is DAKO® monoclonal mouse anti-ER clone 1D5 (DAKO Corporation, CA, USA) is directed to the N-end of the ER± (Abbas et al., 1993). It is important to know which antibody is being assessments as this may affect the success of the treatment. positive samples, according to ER immunocytochemical t complete ER±2 exon 5. This is largely simultaneous with ER±"2 exp the presence of the complete ER±2, which is located at the ER and is partially responsible for coding the DNA binding be required for recognition by the DAKO® monoclonal ER immunocytochemical assay. These ER immunocytochemical tests were done by the laboratory of Charlotte Maxeke Academic Hospital, Johannesburg using the DAKO® monoclonal antibody.

The wild type ER±2 was also significantly associated with the presence complete ER±4 exon (Fisher exact $p=0.005$) and the ER±"5 deletion v (Fisher exact $p=0.042$). As ER±4 also codes for part of the DNA as well as the hinge and part of the ligand binding dom surprising that the presence of ER±4 also showed significa status ($p=0.046$). It is interesting to note that the deletion of variant ER±"5 w observed as significantly ($p=0.011$) ER± positive patients (72% of ER± positive patients as compared to 38% of ER± negative patients) was not observed in ER± negative patients. This is an ind ER±"5 is present more predominantly in ER± positive patie the DAKO monoclonal antibody H222 used for immunocyto analysis of ER± been directed towards the ligand binding H222 monoclonal antibody, the ER status of the tumour would as ER-Negative. This again emphasizes the importance of ER status testing and the knowledge of the mechanism of ER status often determines the type of treatment prescribed. This m observation of hormone responsiveness and development of Both the DBD and LBD should be assessed with respect to gain a comprehensive overview

5 3

The effects of environmental factors on responsiveness, ER± and ER² expression in MCF-7D cells

The development and progression has long been ascribed to evolution of neoplastic cells (Nowell, 1976; Nadkarni, 1986; S a model of breast cancer development normal ductal epithe hyperplasia to atypical hyperplasia to invasive carcinoma an finally, to metastatic carcinoma (Dupont and Page, 1985). ce to support the hypothesis that oestrogens are important a mitogens for normal breast epithelial cell proliferation and the breast epithelium exposure to oestrogen is a significant risk fa development. (Feigelson, 2000). The evolution of spontaneous variants breast cancer may give rise to the progression of tumours t different hormone. In this study the MCF-7D cells resulted in fi clones of which two were chosen for further study. These oestrogen responsiveness with the TCA3 parent clone exhib and tamoxifen responsiveness. The TCC1 parent clone wa oestrogen responsive and tamoxifen resistant. Sub-clones of each of t clones also differed initially, TCA3 exhibited ER±"2, ER±"5 an simultaneously with their corresponding wild type exons while TCC1 did no fact, the only exon deletion occurring within the TCC1 par ER² expression has been shown to be elevated in tumour cells compared with normal (Speirs et al., 2004). This was confirmed in both clones removed and cultured throughout the experiment.

After ten passages under varying conditions both clones w
TCA3 control cells remained unchanged ER profile. The cells
remained oestrogen and tamoxifen responsive it was observed that afte
hours of growth with oestrogen followed by treatment with ta
was an increase in cell proliferation indicating possible
behaviour. Growth of TCA3 cells in 10^{-8}M oestradiol, tamoxifen, 4-hydroxy
oestriol, and cholesterol resulted in similar growth patterns a
ER± profiles the parent clone of TCA3 and TCC1 cells in 10^{-8}M
aminoglutethimide for ten passages showed similar hormone sensitivities
cells showed the complete loss of ER± exon 4 sequences. These
nonsteroidal aromatase inhibitor, aminoglutethimide reversibly to the ha
portion of the enzyme, aromatase, thus interfering with
synthesis of oestrogen. ER± exon 4 constitutes the hinge domain of
contributes flexibility between the DNA and ligand binding domains of
The absence of this area of the gene, which influences the
bind to DNA and thus lacks oestrogen-induced transcriptional activity
is therefore not surprising that it did not affect the oestrogen respon
the cells or their sensitivity to tamoxifen. It may therefore be proposed that
plays no role in the mechanism of tamoxifen resistance and that th
any of the oestrogen metabolites examined affect the genetics of
TCA3 cells.

The presence of 10^{-8}M tamoxifen was observed to slow down the growt
TCA3 clone and 10^{-8}M cholesterol caused a change in the cell type
becoming rounder and unable to adhere to the substratum survive long enough

perform oestrogen response tests possibly due to other
cholesterol metabolic processes. ER±3 and ER±6 were expressed as
complete and therefore likely to contribute to oestrogen responsive
the TCA3 clone.

Cholesterol and androstendione are oestrogen precursors,
the growth medium increasing the concentration of these steroids
stimulating cell growth. In the TCA3 clones this stimulation
than the growth observed in medium containing 10^{-8} M 17^{2}-oestradiol
concentration of oestrogen present. Oestriol is a weak oestrogen
has also been questioned with regard to stimulation of breast
study the presence of oestriol in the medium showed growth
stimulation that of androstendione, oestriol, in this case,
cell growth.

The TCC1 control cells, after ten passages, showed greater sensitivity
tamoxifen than the parent cell line. This occurred in conjunction with the
of ER±"2, ER±"5 and ER±"7. The deletions occurred simultaneously
with the presence of their respective complete exons. It seems, therefore, that
heterozygous genotype confers greater sensitivity to tamoxifen
line.

Growth in 10^{-8} M 17^{2}-oestradiol over 10 passages indicated less tamoxifen
and also exhibited greater basal growth stimulation. This coincided with the
homozygous expression of ER±7 again reflected in TCC1(1

grown in 10^{-8}M tamoxifen, aminoglutethimide, oestrone, androstenedion
cholesterol respectively. Active oestrogen metabolites and tamoxifen resistant
phenotypes have been observed in clinical patients after prolonged treatment
with tamoxifen.

ER± exon 7 located in the hormone binding domain of the
domain forms a hydrophobic pocket which can bind oestradiol and hormones s
the DNA binding domain when unoccupied thus preventing t
presence of tamoxifen should block the conformational cha
to activate transcription. Growth with prolonged exposure to tamoxifen, h
may cause some change within this pocket that exposes the
thus activating transcription. The selective pressure leadin
is triggered by the dual factors of genetic instability and
regulate growth (Gottardis et al, 1990). It may thus be suggested that a
heterozygous ER±7/ER±"7 genotype could be responsible for greater tam
sensitivity and that further investigation into ER± exon 7 a
is warranted.

5.4 CONCLUSIONS AND FUTURE DIRECTIONS

Menstrual status and nodal involvement (N) are not affected by ER± or
ER² genes status. Further studies on the complete expression of
ER±4 to encode a protein that is recognized by the monoclonal antibody H2
(DAKO Corporation, CA, USA) in order to bind to the oestrogen rece

immunocytochemistry. The method and the monoclonal antibod
assess ER status therefore important with regard to clinic
implemented.

In this study the presence of ER±”2 did not affect the outcome of ER
in patients or tissue culture cells. Contrary to previous studies ER±”3
least prevalent variant observed and was not found to
tumourigenesis. ER±”5 and ER±”5 were largely as heterodimers
although ER±”5 was more prevalent in patients there was
significance with respect to progression of disease or r
treatment. ER±”5 was present in 47D but not T cells examined after 10 pas
although not in the parent. The ER±”5 complex fragment was consi
to be a PCR artifact or degradation product due to its co
ER±6.

Although the presence of ER±”4 in normal breast cells suggests that
plays a role in normal cells perhaps by modulating, with ER±, the
regulating responsiveness, a role observed in the ER±”4 variant
wild-type ER±4 did not increase metastatic potential. As of disea
cell culture models may mimic the natural progression of solid tu
vivo results are interesting when compared to the ER±”4 profiles
clones, TCA3 and TCA8. ER±”4 and ER±”4 heterodimers were four
present in 47D clones and after stage 8 the TCA3 grown in
10^{-8}M aminoglutethimide indicated a complete loss of ER±4
responsiveness was ER±”4 may play no role in the acqui

tamoxifen resistant breast cancer but investigation of this variant may pr
understanding of the progression of

Another exon variant of particular interest was ERα6. P
shown this to be the least prevalent variant was not found to
present in 47% of clones or in control breast mammoplasty group
patients in this study observed to lack ERα6. This was an indication
ERα6 influencing metastatic potential and progression of
of interest to study the affects of this variant in with a la
patient population.

The ERα7 variant was the most frequently observed variant
described by many (McGuire et al, 1991; Zhang et al, 1997; Poole et al,
2000). The presence of this variant is significantly with regard
hormone responsive on the more tamoxifen resistant TCC1 clo
the presence of ERα7 together with the complete exon ERα
sensitivity to tamoxifen. Investigation into this variant with may
provide further information on the acquisition of hormone
carcinoma.

Multiple exon deletions, especially ERα7 and ERα8 variant may also be
significant with regard to disease progression should be studied f

In this study ERα8 was studied in sufficient depth mainly due
sample material. In order to make a valuable assessment

their potential relationship with the ER± variants of particular interest, such as ER±”4, ER±”6 and ER±”7 be studied more comprehensively with specific ER² variants.

The in vitro study also confirmed the effects of oestrogen on the cells. Growth in the oestrogen oestradiol oestriol as well as the oestrogen precursors, androstenedione and dehydroepiandrosterone (DHEA) exhibited growth stimulation in both T-47D clones.

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

Cancer evolves from a combination of genetic and epigenetic changes resulting in dysregulated gene expression. The most common epigenetic modifications being increased methylation of CpG islands in the promoter regions which play an important role in controlling gene deacetylation and or methylation, which in turn affect the structure status of chromatin and thus the transcriptional activity of gene loci (Ellis et al 2009). These modifications can affect chromatin structure and transcription of protein encoding genes. The loss of ER± from initially

positive breast tumours may be due to such modifications (Lehmann 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 the post-transcriptional level. miRNAs lower protein levels by repressing target translation and/or by inducing the degradation of the mRNA (Lewis et al., 2003) by binding to the 3' untranslated region (3'UTR) of the target mRNA. A number of studies documenting the miRNA profiles of breast cancer (Calin and Croce, 2006; Sempere et al., 2007; Vergoulis et al., 2008) have been published. As ER± mRNA has a long 3'UTR (about 4 kb) it has been shown to be a target for miRNAs that repress ER± expression leading to a reduction in ER signalling (Pandey and Picard, 2009). miRNA transcript signatures predictive of ER status have been generated from microarray data analysis (Lu et al., 2005) and include biologically relevant miRNAs that have previously been dysregulated in breast cancer (Iorio et al., 2005; Blenkiron et al., 2007) and other cancers (Cheng et al., 2005; Schetter et al., 2008; Huang et al., 2008). They are also involved in the regulation of cell functions such as proliferation, migration, and invasion. miRNA expression may be affected by chromosomal abnormalities because they are frequently located in chromosomal regions affected by nonrandom abberations in human cancers (Calin et al., 2004). Whether ER± exon deletions have any effect on miRNA expression has yet to be examined.