Oestrogen Receptor Mutations and Their Influence on Breast Cancer Growth.

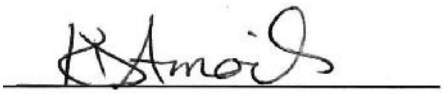
Karin Dagmar Amoils

A thesis submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy.

Plettenberg Bay, 2011

Declaration

I declare that this thesis is my own, unaided work. It has not been submitted before for any degree or examination at any other University. The information used in this thesis was obtained whilst I was employed by the Department of Medicine, University of the Witwatersrand.



K.D. AMOILS

19th day of September 2011.

This research was approved by the Committee for Research on Human Subjects, University of the Witwatersrand. (Human Ethics Clearance Number: M110134)

For

my beautiful daughter

Shannon

and my very patient husband

Johnny

“All rising to great places is by a winding stair.”

Buddha

ABSTRACT

Oestrogen receptor (ER) mutations have been identified for both ERα and ERβ in previous studies. The effects of the deletion variants due to splice mutations on clinical parameters, prognosis and treatment were examined in 61 breast carcinoma patients and 13 control samples from elective reduction mammoplasty procedures, respectively. RNA extracted from fine needle aspirates (FNAs) of breast tissue was reverse transcribed and using nested PCR and sequence analysis the presence of these variants elucidated. Using X^2 and Fisher's exact tests their significance with respect to clinical parameters such as tumour size, nodal involvement, stage, presence or absence of metastases, menstrual status and hormone responsiveness was examined. Kaplan-Meier survival analysis was also determined.

The T-47D breast cancer cell line was cloned with two clones being selected for further analysis, namely TCA3 (hormone sensitive) and TCC1 (hormone resistant). These clones were treated for ten passages with oestrogen metabolites, 17-β-oestradiol and oestriol; oestrogen precursors, androstenedione and cholesterol; an anti-oestrogen, 4-hydroxy-tamoxifen; and the aromatase inhibitor aminoglutethimide, respectively. RNA was extracted from the cells initially and after the tenth passage and the ERα and ERβ exon profiles were examined using RT-PCR and sequence analysis. After the tenth passage hormone response tests were performed every 24 hours (up to 96 hours) with cell number being determined using the MTT assay.

The results indicate that ERα and ERβ variants do not have any affect with respect to menstrual status and nodal involvement (N). Expression of ERα2 and ERα4 are required by the mouse monoclonal antibody (DAKO ® Clone 1D5) in the immunocytochemical assay used for the recognition of the protein in order to assess ER status and therefore show significance. ERαΔ2 and, contrary to previous investigations, the variant ERαΔ3 were not found to play a role in tumourigenesis. ERαΔ5 was observed to be more prevalent in ERα-positive patients and was usually co-expressed with the complete ERα5 indicating heterodimerization. ERαΔ5 showed no significance with respect to progression of disease or response to hormone treatment.

An increase in the ratio of ERαΔ4: wild-type ERα4 indicated an increase in metastatic potential of diseased tissue. ERα4 and ERαΔ4 heterodimers were 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 without altering hormone responsiveness. These results suggest that ERαΔ4 may play a role in progression of disease but not in the acquisition of tamoxifen resistance.

ERαΔ6 was observed in 15% of patients but not in the T-47D clones or the control samples. An increase in the expression of ERαΔ6 among patient samples significantly increased their metastatic potential (p=0.018). ERαΔ6 was also observed as significant with respect to stage of disease (p=0.023) indicating the possible relevance of ERαΔ6 in progression of the disease.

ERαΔ7 was the most frequently observed variant and did not show any significance with regard to any of the clinical parameters examined. The presence of ERαΔ7 did not show significance with regard to hormone response *in vivo* but *in vitro* the presence of this variant, expressed as a heterodimer with the wild-type ERα7, conferred greater sensitivity to tamoxifen in the tamoxifen resistant clone TCC1.

Multiple exon deletions of ERα were also observed. The two more significant multiple deletion variants were those involving ERαΔ4, namely, ERαΔ2-ERαΔ6 and ERαΔ4-ERαΔ6. The multiple variant ERαΔ4-ERαΔ6 may be involved in tumour progression.

ERβ variants were not examined in as much detail as ERα variants due to insufficient material available for analysis. The two domains, the DNA binding domain and the ligand binding domain, of ERβ were analyzed in a few of the patients and in the T-47D clones. They were not found to be significant with respect to the clinical parameters investigated and the ERβ profiles of the TCA3 and TCC1 clones remained unchanged after 10 passages under varying growth conditions.

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CONFERENCE PRESENTATION

IUBMB/SASBMB Special Meeting on The Biochemical and Molecular Basis of Disease, 19-23 November 2001, Cape Town, South Africa.

Title: “Oestrogen Receptor α Mutations and Their Influence on Clinical Breast Cancer.” K.D. Amoils, P. MacPhail, P. Ruff and R. Duarte.

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LIST OF ABBREVIATIONS

2n	diploid
4n	tetraploid
aa	amino acids
AF	activation function
AI	aromatase inhibitor
AMV	avian myeloblastosis virus
ATCC	American Tissue Culture Collection
bp	base pairs
^{o}C	degrees centigrade
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CoR	coregulator
DBD	DNA binding domain
DEPC	diethylene pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMEM	Earle's Minimal Essential Medium
ER	oestrogen receptor
ERα	oestrogen receptor alpha

LIST OF ABBREVIATIONS (CONTINUED)

ERβ	oestrogen receptor beta
ERE	oestrogen response element
ERICA	oestrogen receptor immunocytochemical assay
FCS	foetal calf serum
FNA	fine needle aspirate
FSH	follicle-stimulating hormone
h	hours
HCl	hydrogen chloride
hGR	human glucocorticoid receptor
HPRT	hypoxanthine phosphoribosyltransferase
HRT	hormone replacement therapy
hsp	heat shock protein
IGFR	insulin-like growth factor receptor
I.U.	international unit
kb	kilobase
KCl	potassium chloride
kDa	kiloDalton
LBD	ligand binding domain
LH	luteinizing hormone
M	molar
M-phase	mitotic phase
MA	megestrol acetate
MAPK	mitogen activated protein kinase

LIST OF ABBREVIATIONS (CONTINUED)

$MgCl_2$	Magnesium chloride
min	minute
MISS	membrane-initiated steroid signaling
ml	millilitre
mM	millimolar
MMP	matrix metalloproteinase
MPA	medroxyprogesterone acetate
miRNA	microRNA
mRNA	messenger ribonucleic acid
MTT	3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide
N	nodal involvement
NA	norethisterone acetate
NaOH	sodium hydroxide
ng	nanograms
NH_2	amino
NISS	nuclear-initiated steroid signaling
oes	oestradiol
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
PR	progesterone receptor
PRPP	phosphoribosyl pyrophosphate

LIST OF ABBREVIATIONS (CONTINUED)

RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RPMI-1640	Roswell Park Memorial Institute Medium 1640
RT-PCR	reverse transcribed polymerase chain reaction
s	seconds
SERM	selective oestrogen receptor modulator
SFCS	stripped foetal calf serum
SPF	S-phase fraction
STAR	Study of Tamoxifen and Raloxifene
T	tumour size
T_m	melting temperature
TAE	Tris-acetate EDTA
tamox	tamoxifen
Taq	*Thermus aquaticus*
TD	touchdown
TDLU	terminal ductal lobular units
TGF-α	transforming growth factor alpha
USF	upstream stimulatory factor
UTR	untranslated region
UV	ultraviolet
V	volts
wt	wild type