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EXPRESSION OF OESTROGEN AND PROGESTERONE RECEPTORS, Ki-67, p53 AND BCL-2 PROTEINS, CATHEPSIN D, UROKINASE PLASMINOGEN ACTIVATOR AND UROKINASE PLASMINOGEN ACTIVATOR-RECEPTORS IN CARCINOMAS OF THE FEMALE BREAST IN AN AFRICAN POPULATION
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EXPRESSION OF OESTROGEN AND PROGESTERONE RECEPTORS, Ki-67, p53 AND BCL-2 PROTEINS, CATHEPSIN D, UROKINASE PLASMINOGEN ACTIVATOR AND UROKINASE PLASMINOGEN ACTIVATOR-RECEPTORS IN CARCINOMAS OF THE FEMALE BREAST IN AN AFRICAN POPULATION

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ABSTRACT

Objective: **To determine the expression of oestrogen (ER) and progesterone receptor (PgR), Ki-67, p53, bcl-2 proteins and the proteolytic enzymes cathepsin D (CD), urokinase-plasminogen activator (uPA) and its receptor (uPA-R) in primary carcinomas of the breast from indigenous Tanzanian female patients by immunohistochemistry.**
Design: **Prospective cross-sectional study.**
Setting: **Muhimbili Medical Centre, Dar es Salaam, Tanzania.**
Subjects: **Sixty patients admited between 1995 and 1997.**
Results: **Markers were found to be expressed as follows: ER (33.3%), PgR (18.3%), p53 (30%), bcl-2 (43.5%) and the median proliferation rate of Ki-67 was 15%. Proportion of tumours positive for ER, PgR and bcl-2 initially decreased to 12 months disease duration, after which it increased. The observed proliferation rate approaches that reported in developed countries. p53 expression did not influence the proliferation rate nor did bcl-2 expression. ER, PgR and bcl-2 were strongly co-expressed. CD was predominantly expressed in stromal macrophages than in cancer cells.**
Conclusion: **The low expression of ER and PgR and their strong co-expression with bcl-2 might negatively influence response to hormonal therapy. The influence of bcl-2 on tumour response to anti-cancer therapy in patients with long disease duration requires urgent clarification. Determination of CD in stromal macrophages rather than in cancer cells may have greater prognostic significance in patients of this region.**

INTRODUCTION

Carcinoma of the female breast is the second most frequent malignancy after carcinoma of the uterine cervix among the sub-Saharan African population(1-3). In African females this malignancy has been reported to have distinct peculiarities. The disease occurs predominantly in pre-menopausal women diagnosed in stages III and IV and is of higher histological grade than reported in females with this disease in developed countries(1-6). Furthermore, this malignancy was reported as diploid in 80% of the cases in this geographical region while in the Western countries it varied between 20 and 50% only(7,8). Except for the ploidy status, these peculiarities indicate that breast cancer may be more aggressive in the sub-Saharan female than in her Western counterpart.

The aggressiveness of a tumour is dependent upon several factors. These include tumour size, histological type and grade, oestrogen and progesterone receptor status, proliferation rate, DNA ploidy and disease stage. Furthermore, oncogene expression, apoptotic rates, and expression of proteolytic enzymes related to tumour invasion, have also been shown to be of prognostic importance(9-14). In breast cancer the hormonal receptors, ER and PgR are well established prognostic and predictive markers. Recently, the tumour suppressor genes p53 and bcl-2 over expression have been emphasised to be independent markers of poor prognosis in patients with this disease(15-18). Further, increased expression of the tumour proteolytic enzymes Cathepsin D (CD) and urokinase-type of plasminogen activator (uPA) as well as the latter's receptor (uPA-R) have also been shown to be independent markers of increased tumour aggressiveness (19,20).

To the best of our knowledge the expression of hormonal markers, oncogenes, tumour supressor genes and proteases of tumour invasion have not been previously investigated in tropical African patients with breast cancer. The possible contribution of these markers to the peculiarities reported in the female with breast cancer in this geographical region are therefore unknown. Furthermore, the management of these females is based on the extrapolation of the experience accrued in the developed countries to the African patient. Probable differences in the biology of the disease are not considered.

In order to understand better the biology of breast cancer in the sub-Saharan female, this study assessed the frequency and pattern of expression of the following markers: ER, PgR, Ki-67, p53, bcl-2, CD, uPA and uPA-R by using immunohistochemistry. Results are discussed in respect to the biology of the disease, and their probable influence on its management.

MATERIALS AND METHODS

Fresh biopsy material from 60 female breast cancer patients treated at Muhimbili Medical Centre in Dar es Salaam, Tanzania, between 1995 and 1997 formed the basis of this study. This is a tertiary care hospital and is also the national referral hospital. The tumour material was collected prospectively from routine biopsies. The specimens were obtained after local anaesthesia by lignocaine tissue infiltration in superficial tumours, under general anaesthesia in deeper located lesions, as well as from mastectomy specimens. Demographic data including patients' age, age at menarche, parity, menopausal status, and disease duration were concurrently collected.

Tissues were collected in a chilled container at 4°C, from which samples of about 0.5 x 0.5 cm were snap frozen in liquid nitrogen within 20 minutes after biopsy and stored at 70° C until required. The remaining tissue was fixed in formal saline, processed and paraffin embedded in the usual manner. For immunohistological processing, 4 micrometer sections were cut, and fixed in acetone for 10 minutes at 4°C. Where necessary, slides were wrapped in parafoil and stored at -70° C until required. All tumours were histologically classified according to the WHO classification(21) using formalin fixed paraffin embedded sections stained with haematoxylin and eosin. In addition, tumour grade was assessed using the criteria of Elston *et al* (22) which gives a score of 3 to 9. A score of 3 to 5, 6 to 7, 8 to 9 qualifies a carcinoma as grade I, II, and III, respectively.

Immunohistochemistry was carried out by the standard ABC method(23) employing antibodies shown in Table 1. In brief, sections were hydrated from xylene through graded alcohols to phosphate-buffered saline, then endogenous peroxidase quenching was effected by incubation with 3% H_2O_2 for 30 minutes, endogenous biotin quenching using the avidin-biotin kit (Dako) according to the manufacturer's recommendations. Non-specific staining was blocked by normal mouse and rabbit serum for monoclonal and polyclonal primary antibodies (Dako), respectively. Negative controls were carried out by omiting the primary antibody. The reaction was visualised using streptavidin-biotin-immunoperoxidase system (Dako AS), with diaminobenzidine as chromogen substrate. Sections were then briefly counterstained with haematoxylin. Table 1 shows characteristics of antibodies which were utilised, dilutions, buffer pH, and incubation durations.

For ER, PgR, Ki-67 and p53, an unequivocal nuclear staining was recorded, while for bcl-2 cytoplasmic staining was assessed. Cathepsin D was scored positive when there was a clear coarse granular cytoplasmic, while for uPA and uPA-R, a fine granular cytoplasmic staining was found, in tumour or stromal cells. ER, PgR, Ki-67 p53 and bcl-2 were scored using a modification of the immunoreactive score (IRS)(24). This semiquantitative and subjective method considers both the intensity of staining and the proportion of positive cells. Intensity was recorded as 0 (no staining) to 3 (strong staining), while percentage of positive cells was scored as 0 (no tumour cells positive), 1 (positive in <10% of tumour cells), 2 (positive in 10-50% of tumour cells), and 3 (positive in >50% of tumour cells). The product of staining intensity and percentage of positive cells was defined as the staining index, giving a score of 0-9. This score was further categorised as follows: 0= negative, 1-2 = weak positivity, 3-4 = moderate positivity, 6-9= strong positivity. A score of 3 or above was considered positive. For Ki-67, the percentage of positive tumour cell nuclei was scored by counting about 1000 cells in those fields showing the highest positivity (hot spots) at 1000 magnification power. Basing on median percentage of positive cells of 15%, tumours 0-15% positive cells were considered low proliferating, while those expressing 15 or more percent high. Staining intensity of positive nuclei was also noted as for p53 and bcl-2.

For statistical analysis the SPSS 7.5 (Statistical Package for the Social Sciences, Munich, Germany) was used. The chi-square test was used to investigate the relationships between categorical variables. Differences between proportions were assessed using the Chi-square test. Associations between quantitative variables were assessed using correlation co-efficients.

RESULTS

The mean age of the patients was 52 years, mean tumour size 8 cm., disease duration 12.3 months and mean parity 4.2 children. Further, 67% of the patients were postmenopausal, 93% stage II and III disease, while 86.7% tumours were of histological grade II and III. The mean proliferation rate for Ki-67 was 15% (Table 1).

The hormone markers ER and PgR were scored positive in 33.3% and 18%, respectively, while p53 and bcl-2 showed a positive score in 30% and 43% of the cases. The proteases CD and uPA and the latter's receptor, uPA-R, were found to have a positive score in 6.7%, 23.3% and 33% of the tumours, respectively in cancer cells (Table 1).

ER and PgR showed a statistically significant decrease in proportion of tumours scoring positive for these markers to 12 months disease duration. The proportion of tumours scoring positive then increased. Bcl-2 positive score showed a similar relationship to disease duration as ER and PgR (Table 1).

Table 1

Expression of ER, PgR, Ki-67, p53, bcl-2, uPA and uPA-R in relation to patients' demographic and clinical characteristics

Characteristic	No. of cases	ER positive %	PgR positive %	CD positive %	UPA positive %	UPA-R positive %
All patients	60	33.3	18.3	6.7	23.3	33.3
Disease duration (months)	45					
1-6	16	12.5	–	43.8	25.0	31.3
7-12	20	35.0	30.0	50.0	35.0	35.0
13-18	1	– (r=0.389)	- (r=0.327)	– (r=0.007)	– (r=0.045)	– (r=-0.037)
19-24	4	50.0 (p=0.010)	– (p=0.030)	75.0 (p=0.964)	50.0 (p=0.764)	50.0 (p=0.809)
31-36	2	50.0	–	100.0	50.0	50.0
43-48	2	100.0	100.0	100.0	–	50.0
TNM disease stage						
II	4	25.0 (r=0.21)	25.0	–	25.0	50.0
III	38	34.2 (p=0.871)	23.7 (r=0.198)	57.8 (r=0.247)	34.2 (r=0.019)	34.2 (r=0.105)
IV	18	33.3	5.6 (p=0.129)	50.0 (p=0.058)	38.9 (p=0.885)	27.8 (p=0.418)
Tumour size	60					
2.1-5.0	18	50.0 (r=0.00)	– (r=0.310*)	55.6 (r=0.029)	38.9 (r=0.017)	38.9 (r=-0.077)
5.1>	42	66.6 (p=0.612)	26.2 (p=0.012*)	47.0 (P=0.653)	33.3 (P=0.588)	31.0 (P=0.378)
Histology (invasive)						
Ductal	47	27.7	17.0	53.2	34.2	27.7
Lobular	9	55.6 (r=-0.200)	33.3 (r=0.037)	66.7 (r=0.029)	44.4 (r=-0.120)	55.6
Cribriform	2	1 (p=-0.125)	- (p=0.774)	50.0 (p=0.824)	50.0 (r=-0.154)	50.0 (r=-0.154)
Papillary	1	–	–	100.0	–	100.0 (P=0.238)
Mucinous	1	100.0	–	100.0	–	–
Histological grade						
I	15	40.0	13.3	46.7	26.7	33.3
II	28	28.6 (r=-0.032)	17.9 (r=0.096)	53.6 (r=-0.012)	39.3 (r=-0.026)	25.0 (r=0.069)
III	17	35.2 (p=0.804)	25.0 (p=0.459)	70.6 (p=0.925)	35.3 (p=0.845)	47.1 (p=0.598)

Table 2

Correlation coefficient (Pearson's) between biological markers

	ER		PgR		Ki-67		p53		bcl-2		Cathepsin D		uPA	
Marker	Corr.	P	Corr.	P	Corr.	P	Corr.	P.	Corr.	P.	Corr.	P	Corr.	P
ER	–	–												
PgR	0.396**	0.004	–	–										
Ki-67	0.141	0.206	0.302*	0.021	–	–								
p53	0.000	0.622	-0.028	0.570	0.73	0.389	–	–						
bcl-2	0.452**	0.001	0.455**	0.001	0.135	0.217	0.015	0.566	–	–				
Cath D	0.094	0.407	0.046	0.566	0.000	0.694	-0.029	0.653	0.036	0.586	–	–		
UPA	0.195	0.119	0.248	0.069	0.158	0.180	-0.017	0.392**	0.003	0.169	0.230	–	–	
uPA-R	0.100	0.312	0.122	0.273	0.141	0.206	0.154	0.184	0.238	0.059	-0.047	0.593	0.446**	0.001

*Correlation significant at the 0.05 level
**Correlation significant at the 0.01 level

Table 2 shows statistical correlation between the investigated markers of tumour aggressiveness. The following markers are found to be positively related with statistical significance: ER and PgR, ER and bcl-2, PgR and Ki-67, uPA and bcl-2 expression in cancer cells.

DISCUSSION

In the present study the majority of the breast cancer patients, 93%, were observed in stages III and IV, while 70% of these tumours were larger than 5 cm and in 47% they were of histologic grade III. This is in contrast to predominantly stage I and II cancers, encountered in developed countries where the disease is detected early by mammographic imaging. This locally advanced stage and the high histological grade are characteristic for breast cancers encountered in this geographical region and are implicated to reflect the reported higher aggressiveness of this malignancy in this region. Though these features may in part reflect the late presentation of the patients to the tertiary health care center, other inherent factors, including race have been implicated(6).

The proportion of tumours which scored positively for ER (33%) and PgR (18%) observed in this series was remarkably low when compared with reports from developed countries where these proportions constitute 56-78% and 41-56% for ER and PgR, respectively(25,26).

Important factors which have been implicated to favour low expression of hormone receptors include age, menopausal status, higher parity, tumour size, disease

stage, histological type and race respectively(25-36). In concurrence with these reports, results of this study showed high proportion of premenopausal patients (40%), higher number of children (4.2), large tumour size (8 cm) and predominance of invasive ductal histological types of (78%), all of which favoured expression low proportions of hormone receptors. Further, association between race and breast cancer as is reflected by the low incidence rates of breast cancer in oriental Japanese females compared to Western females is well documented(37). Recently, several studies have reported on the lower expression of hormone receptors in African-Americans in comparison to their Caucasian counterparts(35,36). In view of the even lower proportions of expression of the hormone receptors by indigenous African patients in this study compared to the African-Americans, it is our opinion that race played a significant role in the poor expression of hormone receptors in the studied patients.

In this study the proportion of tumours with a positive ER and PgR score were observed to decrease during 12 months disease duration. Surprisingly, after this duration the proportion of these tumours increased. At 36 months disease duration, only tumours with a positive score were encountered. This suggests an existence of an ER and PgR expressing sub-clone which expanded over time in a proportion of the studied tumours.

PgR is an ER responsive protein and its expression reflects the functional potency of ER in the respective tumour. Oestrogen has been reported to have a mitogenic effect on breast cancer cell lines expressing oestrogen receptors *in vitro*(38,39). The statistically significant association between PgR positive score and proliferation rate (Ki-67) observed in this study would suggest that this hormone exerts a significant influence on proliferation in the tumours which express its dependent receptor, PgR.

The mean proliferation rate of 15% observed in the current study approaches that of 16% determined previously by flow cytometry(7). These rates do not differ from 10-18% reported by other investigators in developed countries(40-42). P53 protein overexpression, which reflects its gene's mutation, has been related to higher proliferation rates of the tumours in which the alteration occurs(43,44). This is because the mutant p53 fails to induce tumour cells with damaged genome to undergo apoptosis, allowing them to proliferate(45,46). The proportion of tumours overexpressing p53, and which are presumed to have p53 mutation observed in the current study (30%), also did not differ from 14-58% reported in other studies(16,47-49). Contrary to results of other investigators, this study could not establish an association between p53 overexpression and proliferation rates(50,51). Consistent with findings of other investigators, however, we did not observe a relationship between p53 overexpression and bcl-2(51). The effect of bcl-2 of overriding apoptosis has been implicated in facilitating the appearance of clones with unstable genome by allowing such cells with damaged genome to survive instead of eliminating them by apoptosis(52) . In this study no correlation was found between bcl-2 positive score and histological grade (Table 2).

The proportion of tumours expressing the anti-apoptotic protein bcl-2 observed in the current study (43%) approached that reported by other researchers(50). This study also confirmed reports of previous investigators that the expression of this marker was associated with that of ER and PgR (53,54). Among the 33% and 18% of cases which were positive score for ER and PgR, respectively, 25% co-expressed ER and bcl-2 and 16% PgR and bcl-2. In the present study bcl-2 and hormonal receptors positive scores were, in addition, observed to be similarly related to disease duration, such that at 36 months disease duration only tumours with a positive score for ER and bcl-2 were encountered. In view of these observations it is our opinion that the anti-apoptotic effect of bcl-2 may play an important role in the selection and survival of a tumour clone expressing bcl-2 over the disease duration. This might have in turn favoured the survival and expansion of the clones expressing hormone receptors with which bcl-2 was co-expressed. This association between the co-expression of bcl-2, ER and PgR on the one hand, and their increase in proportion after 12 months' disease duration on the other, in breast cancer has not been previously reported. This is most likely because reports from developed countries on these markers are based on breast cancers which are usually discovered through mammographic imaging or have a disease duration which does not exceed 12 months.

In the present study low proportions of tumours scoring positively for CD (6.7%) were observed compared to reports of other investigators 48%(20), 66%(55) 59%(56). Similarly, we observed a low proportion tumours scoring positively for uPA in cancer cells(23%) in comparison to reports which have ranged from 33 to 58%(20,57). uPA-R expression was found in 33% of the cases in cancer cells in the current study. This proportion was higher than 14% reported previously(58).

Although there was a higher proportion of cancer cells scoring for uPA compared to those scoring for CD in this study, these cells were localised within cancer cell clusters, and such cells could not be demonstrated in areas of stromal degradation. A similar behaviour was observed for uPA-R. In areas where cancer cell invasion and stromal degradation were evident, stromal macrophages expressing CD were observed to be the predominant participants of the degradation process. Further, in these areas cancer cells did not express either uPA or uPA-R. Also, stromal macrophages expressing these markers were not found in serial sections of areas which expressed CD. In view of these findings we think that stromal macrophages rather than cancer cells were the main modulators of stromal degradation in the studied tumours. Further, the higher frequency of tumours containing stromal macrophages expressing CD observed in the current study in comparison to other reports would suggest this to be a characteristic feature of breast cancers encountered in this geographical region. Furthermore, a strong stromal CD reaction has been reported to be related

with aggressive behaviour of mammary carcinomas(59,60). The observed over-expression of CD by stromal macrophage observed in this study might be an important contributing factor to the reported aggressive clinical picture of breast cancer in African patients(4,5).

Bcl-2 showed a strong association with uPA expression in epithelial cells. Though uPA was also strongly related to CD epithelial expression, bcl-2 and CD expression were unrelated. Further, although CD is reported to be oestrogen regulated *in vitro*(61), we have found no relationship between the expression of this marker and that of hormone receptors. This is similar to the observations of other investigators(62).

Anticancer therapies including hormone therapy are reported to effect their cytotoxicity by the induction of endogenous apoptotic pathways(63-66). Also, bcl-2 has been reported capable of blocking apoptosis induced by radio- and chemotherapy *in vitro*(67,68). Clinical studies, however, have observed that a combined positive expression of ER and bcl-2 was associated with a better response to hormonal therapy and a longer recurrent and metastatic free interval than for cases negative for these markers. In this study, of the 43% of the tumours which had a positive score for bcl-2, 25% also co-expressed ER. It suggests that this proportion of tumours would respond to hormone therapy. Another peculiarity of the tumours in this study which may have a bearing on response to therapy is their histological grade which takes into account differentiation, proliferation rate and anaplasia(22). High grade tumours which are likely to have an unstable genome were likely to be chemosensitive and radiosensitive. Recent studies, however, have found no correlation between histological grade and response to radiotherapy(69,70). Histopathological therapeutic effects of chemotherapy, however, have been found to be related to histological grade(71,72,73). Only 30% of cases which were of histological grade I and II encountered in this study are expected to benefit from chemotherapy. These patients are however expected to be less responsive to radiotherapy.

In conclusion, this study showed a low frequency of hormone receptors and an increase in proportion of tumours co-expressing ER, PgR and bcl-2 from 12 month's disease duration. It also showed that stromal macrophages expressing CD were the predominant modulators of breast cancer invasion. These features suggest to be characteristic of this malignancy in this geographical region. Further, the role of stromal macrophages in facilitating tumour invasion might be a significant contributing factor to the reported more aggressive behaviour of this disease in African females. In addition, assessment of hormone receptors, bcl-2 and histological grade, would identify patients who might benefit most to standard treatment modalities. Studies to confirm these observations are urgently required.

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