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517

Analysis of Morphological Immunohistochemical and Genetic Prognostic determinants in predicting Disease. 01-7-1998-2062 AD

FINAL REPORT

517

TABLE OF CONTENTS

FINAL REPORT

TABLE OF CONTENTS

SUMMARY

Breast cancer is an increasingly important cause of illness and death among women worldwide. In Pakistan also breast cancer is not only the commonest malignancy among women but it is occurs in a younger age as compared to western population. This study was conducted on 315 consecutive human breast biopsy and mastectomy specimen with lymph node sampling and histologically proven ductal carcinoma breast. The objective was to assess the utility of novel establish prognostic markers that will reliably assess the outcome of disease in breast carcinoma. Established parameters and prognostic variables of breast cancer like age of the patient, menstrual status, tumor size, histological grade of tumor, axillary lymph node status (distant metastases if any), Hormones Receptor (estrogen and progesterone) status were studied in parallel to novel prognostic markers like amplification of growth promoting genes / deletion of tumor suppresser genes (C-erb B-2, EGFR and p53), tumor proliferative index (PCNA), and Cathepsin-D. Analysis was done on a total number of 315 patients. The mean age was found to be 49 years while median was 48 years. Axillary lymph nodes positivity was seen in 170 (54%) cases. Mean tumor size was 4.16 cms (range 0.5-22 cms). Histologically grade II tumors comprised 214 (68%) cases, followed by grade III 56 (18%) and grade I 45 (14%) cases. Vascular /lymphatic invasion was seen in 150 (48%) cases.

A statistically significant difference was found in overall and disease free survival of patients when there was an amplification and/or over expression of C-erbB-2, EGFR, p53 and with PCNA reactivity of >25%. p53 positivity was seen in 55.23% of the cases, C-erbB-2 in 39.36%, EGFR in 22%. P53, C-erbB-2 and EGFR showed significant correlation with histological grade, tumor size, axillary lymph nodes, local recurrence,

distant metastases and ER/PgR status ($p < 0.05$). C-erbB-2 positive patients also showed resistance to chemotherapy ($p<0.05$). p53 and EGFR also showed significant correlation with vascular/lymphatic invasion ($p<0.05$). PCNA >25% was significantly correlated with histological grade, tumor size, vascular/lymphatic invasion and distant metastases. Cathepsin-D protein over expression was seen in 39% of the cases. Its over expression did not show any significant correlation with overall survival or disease free survival.

In this study we have shown that C-erbB-2, EGFR, p53 are independent prognostic markers in both univariate and multivariate analysis. According to our study Cathepsin-D over-expression was not a good prognostic marker if only expression in tumor cells is taken into account.

DETAILED REPORT

PROJECT TITLE

ANALYSIS OF MORPHOLOGICAL, IMMUNOHISTOCHEMICAL AND GENETIC PROGNOSTIC DETERMINANTS IN PREDICTING DISEASE FREE SURVIVAL OF BREAST CARCINOMA PATIENTS

PROJECT PERIOD

JULY 01, 1998 TO DECEMBER 15, 2000

INTRODUCTION

Breast cancer is an increasingly important cause of illness and death among women. (Sandra et al 1992). In most part of the world breast cancer is the major thereat to life of women, including Pakistan. Breast cancer is by far the most frequently diagnosed type of malignant neoplasm among women, and it claims tens of thousands of lives annually in the United States (Douglas WT 1994). Till 1993 lung cancer was the leading cause of death among women of United States, but since than breast cancer has taken over (Douglas JM 1993 and Reginald CS 1993). Breast cancer claims 45,000 lives of American women each year. This mortality persists despite the gains that have been made in mammography, surgery, radiation therapy, and chemotherapy. Population migration studies suggest environmental factors. The Hawaii-Japan studies show that, when the Japanese migrated from Japan to Hawaii, the incidence of breast cancer in this population increased. The westernisation of this people brought on more cancer. It can be speculated on a number of factors that may be responsible because life is so different between the East and West. However, one compelling correlation is the fat consumed in the diet. The traditional Japanese diet has less than 50% of the fat content of the diet in the United States. The positive relationship between dietary fat and breast cancer seems also to hold true for a number of other countries around the world (Reginald CS 1993). In the United Kingdom, where the age standardized incidence and mortality is the highest in the world, there are more than 15,000 deaths each year due to breast cancer. Breast cancer remains the commonest malignancy in women and comprises 18% of all female cancers worldwide (K McPherson 1994). Breast cancer is the most common malignancy in Saudi Arabia, 12.8% and in other countries of Middle East (Adnan Ezzat 1997).

I) Prognostic markers

The discovery of the role-played by oncogenes and tumor suppresser genes in the genesis and progression of many cancers have opened opportunities to explore their possible role as predictors of tumor behaviour. However, at present most of these have limited clinical decision-making impact and are still generally regarded as research procedures.

Breast cancer is an important health problem throughout the world. Emphasis in early diagnosis has resulted in prolonged survival but the overall survival has remained unchanged. There is a concept that prophylactic adjuvant chemotherapy may improve the survival of patients with early-stage breast cancer. But because of the potentially toxic effects of such therapy, it is important to select carefully those patients who should receive treatment and those who should be spared. Although several reliable prognostic indicators have been established, many of the new markers that have been studied have yield controversial results and their routine application needed further workup.

At the time of initial diagnosis and treatment of patients with malignant disease, determination of the prognosis is of paramount importance. Traditionally a number of variables have been used to predict the prognosis in early stage breast cancer. Recently a number of new prognostic markers, e.g., tumor proliferative index, and amplification or over expression of growth promoting factors (Oncogenes) have been identified as potential predictors of out come in patients with breast cancer (Tesch et al 1993).

II) Established Prognostic Determinants:

Established parameters and prognostic variables includes:

Age of the patient and menstrual status, tumor size, histological grading of tumor, axillary lymph node status (distant metastases if any), Hormones Receptor (estrogen and progesterone) status.

III) Novel prognostic determinants:

Novel prognostic determinants, includes:

Amplification of growth promoting genes / deletion of tumor suppresser genes (EGFR, C-erb B-2 and p53), Cathepsin-D ,tumor proliferative marker (PCNA), S-phase fractions, and DNA ploidy.

IV) Epidemiology of breast cancer in Pakistan

There is although no combined study done so far regarding the incidence of breast cancer or any other cancer, nor there is a countrywide cancer registry. A cancer registry has been established in Province of Sindh, but at present it is operating only in Southern district of Karachi, according to this cancer registry and few studies done, in Pakistan breast cancer is number one malignancy among women and it tends to occur more frequently in younger age groups as compared to Western series (Jaffery and Zaidi 1987

In our institution alone every month there is 25 to 30 newly diagnosed cases of breast cancers are done, which come to 20 to 25% of all the malignancies diagnosed each month. While the Epidemiology of breast cancer has been extensively studied and reviewed in the west, very few similar attempts have been made in Pakistan and other developing countries.

EXPERIMENTAL PROCEDURES

I) Sample size

In this study 315 consecutive human breast biopsy and mastectomy specimens from the registered patients of our university hospital were included. Time span extended from 1992 to 1998. A questionaire was designed and information obtained on that questionaire was statistically analysed on commercially available program SPSS (Statistical Program for Social Sciences) version 8.

II) Methodology

After routine processing the tissues were embedded in paraffin. The sectioning and H & E staining was performed by routine lab protocol. The same breast tumor paraffin blocks were used to make further sections for immunohistochemistry. The sections were cut and picked on poly-L-Lysine coated slides. Each immunohistochemical and genetic prognostic determinants was detected on a separate slide.

III) Histological grading of tumor

Tumors were graded according to Nottingham modification of the Bloom-Richardson system

IV) Immunohistochemical methods

A) Antibodies:

Panel of antibodies used in our study are as follows:

Antisera	Dilution	Monoclonal / Polyclonal	Antigen Retrival Procedure	Source
Anti-Human Estrogens Receptor	1:100	Mono	Citrate Treatment	DAKO; Code No M 7047
Rabbit Anti-human progesterone receptor	1:100	Poly	Citrate Treatment	DAKO; Code No.A 0098
Anti-human p53 protein	1:25	Mono	Citrate Treatment	DAKO; Code No. M 7001
Anti-human Epidermal growth factor receptor	1:10	Mono	Citrate Treatment	DAKO; Code No. M 0886
Anti-proliferating cell nuclear antigen	1:100	Mono	Citrate Treatment	DAKO; Code No.M 879
Rabbit Anti-human C-erbB-2 oncoprotein.	1:25	Poly	Citrate Treatment	DAKO; Code No.A 485
Rabbit Anti-Cathepsin-D	1:25	Poly	--	DAKO; Code No. A 561

- ***Secondary antibodies*** for primary monoclonal antibodies against (ER, p53, EGFR and PCNA).
- ***Rabbit Anti-Mouse Immunoglobulins***. (DAKO; Code No. Z 0109)
- ***Peroxidase-Conjugated Swine Anti-Rabbit Immunoglobulins***.(DAKO; Code No. P 217)

B) Immunostaining Procedure:

Immunostaining was done with using 3 steps PAP (Peroxidase antiperoxidase) technique for monoclonal antibodies and 2-step approach for polyclonal antibodies. Positive and negative controls were run with each batch Briefly sections were deparaffinized with xylene and rehydrated through graded alcohol. Antigen retrival (if required) was done through microwave oven technique. Before start of the staining for ER, p53, PCNA and EGFR, the sides were washed with Tris buffer. Excess tris was then removed and

endogenous peroxidase activity was blocked by 3%H_2O_2 in methanol for about 30 minutes. Slides were then washed in tris buffer and each slide was layered with normal swine serum (NSS) for 5-10 min to block the non-specific binding, excess. NSS was then removed and primary antibody was layered drop wise on each slide (including control) and incubated for 90 min at room temperature in a humidified chamber. The mouse antihuman & Rabbit anti human receptor antibodies were diluted in NSS. After washing with tris buffer 3 x 5 minutes each. The tissue section for PR, Cat-D, C-erb-B2 antibody were then incubated with secondary antibody i.e., Peroxidase-Conjugated Swine Anti-Rabbit Immunoglobulins at a dilution of 1:100 for 30 minutes. The ER, p53, PCNA and EGFR slides were then incubated with secondary antibody i.e., rabbit anti mouse immunoglobulin at a dilution of 1:100 for 30 minutes. After washing with tris buffer 3 x 5 minutes. The ER, p53, PCNA and EGFR slides were incubated with Peroxidase anti Peroxidase complex (mouse) for 30 minutes. Finally for colour development the slides were incubated with 3,3 diaminobenzidine tetrachloride (DAB) [BDH] for 5-7 minutes washing was done by Phosphate buffer saline (PBS). Brief counter staining was done with hematoxylin and then slides were washed in tap water for few minutes.The slides were then dehydrated in graded ethanol, clear in xylene and mounted in commercially available Distrene Dibutyl phthalate xylene (DPX)

V) Statistical analysis:

Our main interest was to estimate the survival time for breast cancer patients and look into the relationship between survival time and their prognostic variables. The death of the patients was considered as an event. The data was examined carefully, and decisions were made that how a variable is going to be analyzed. Either a continuous variable could be analyzed as such, or categorized according to cut off levels, which are biologically plausible.

The Kaplan Meier estimator is an important tool for analyzing censored data. The Survival curves, the mean (Standard error for mean), median Survival time (Standard error for median) along with the 25th and 75th percentiles were estimated for each prognostic variable using this method.

A) Univariate analysis was done to examine the relationship of each prognostic factor with the survival time using the Cox proportional hazard model or Log rank test. For qualitative variables, if more than two categories existed, then dummy variables were introduced. Hazard ratios along with there 95% CI were used to describe the relationship between each prognostic variable and the outcome variable.

B) Multivariate analysis was done to identify a subset of prognostic variables that relate significantly to the hazard, and consequently the survival of the patient. The model fitting was aimed to fit the most parsimonious model, which was biologically able to explain the data. The multivariate analysis also helped us to control for the confounding and study effect modification. An adjusted hazard ratio along with there 95% CI was used to describe the relationship between the set of prognostic variables and the outcome variable.

RESULTS

I) Descriptive Analysis

Table 1. Provides the descriptive statistics about the sample. Analysis was done on a total number of 315 observations, with 36.2% survived till the end of this study i.e. May 1999. Four censored observations as they died due to causes other than breast cancer. The mean and median survival times were calculated using the Kaplan Meier technique. Since in our country carcinomas of breast occur at a relatively younger age (approx. 10 years earlier than the western world) with the incidence more common in the reproductive age group, we dichotomised age at a cut off level of 49 years thus 51% of the subjects were in the reproductive age group, with a mean survival time of 3.35 years (standard error {SE}=0.13) in contrast to the 49% in the post-menopausal age with a mean survival time of 3.17 years (SE=0.14). On an average 25% of the subjects in the pre-menopausal group are surviving more than 4.67 years, in contrast to the 4.16 years, in the postmenopausal group. The median survival time was also better among the pre-menopausal group, with 50% of the subjects surviving more than 3.58 years, in contrast to the 3.00 years median survival time for post-menopausal group.

TABLE 1.Descriptive analysis showing the summary of survival data for prognostic factors associated with survival in patients with breast carcinoma

Variables		Total cases n (%)	Median Survival time (SE)	Mean survival time (SE)
Age	< 49 years	161 (51.11)	3.58 (.18)	3.35(.13)
	≥ 49 years	154 (48.99)	3.00(.17)	3.00(.14)
Tumor Grades	I	45 (14.28)	3.33(.16)	3.41(.13)
	II	214 (67.93)	3.17(.25)	3.11(.22)
	III	56 (17.77)	2.67(.27)	2.91(.23)
Tumor size	≤ 2 cms.	68 (21.58)	3.50(.18)	3.32 (.13)
	2-5 cms.	175 (55.55)	3.33(.16)	3.26 (.17)
	> 5 cms.	72 (22.85)	3.00(.17)	3.06 (.21)
Vasc Lymp. Invasion	Negative	165 (52.3)	3.33(.16)	3.27(.13)
	Positive	150 (47.7)	3.00(.17)	3.24(.14)
Tumor Stages	Stage I	38(12.00)	3.75(.39)	3.45(.24)
	Stage II	114(36.20)	3.58(.27)	3.65(.16)
	Stage III	128(40.63)	2.50(.12)	2.89(.14)
	Stage IV	35(11.11)	2.46(.36)	2.86(.28)
Family History	No	267 (84.7)	3.25(.13)	3.22(.11)
	Yes	48 (15.3)	3.50(.28)	3.43(.26)
Hormonal Therapy	None	114(36%)	2.92(.19)	2.88(.16)
	Yes	201(64%)	3.58(.27)	3.47(.12)
Chemo-Therapy	None	80(25.4)	3.00(.17)	3.11(.15)
	Yes	235(74.6)	3.33(.16)	3.32(.12)
Metastasis Distant	None	211(67%)	3.58(.14)	3.50(.13)
	Yes	104(33%)	2.42(.13)	2.86(.15)
P53	Negative	141(45)	4.00(.18)	4.44(.13)
	Mild +ve	43(25)	3.00(.16)	3.52(.34)
	Moderate	93(53)	2.89(.17)	3.11(.18)
	Strong +ve	38(22)	2.5(.25)	3.06(.39)
C-erb-B2	Negative	191(60.6)	4.36(.18)	4.44(.13)
	Positive	124(39.3)	3.33(.16)	3.00(.13)
EGFR graded on a scale of 0-3 (0/+/++/+++)	Negative	245(77.71)	3.00(.18)	4.44(.13)
	Mild +ve	28(8.88)	3.33(.16)	3.52(.34)
	Moderate	38(12.06)	3.00(.17)	3.11(.18)
	Strong +ve	4(1.26)	3.17(.25)	3.06(.39)
PCNA	< 0.25	149(47.3)	3.16 (.24)	3.46 (.25)
	> 0.25	166(52.7)	2.70 (.27)	3.12 (.23)
Cathepsin-D	Negative	195 (62)	3.40(.18)	3.50(.13)
	Mild +ve	45(14.2)	3.33(.16)	3.52(.34)
	Moderate	63 (20.0)	3.00(.17)	3.11(.18)
	Strong +ve	10 (3.17)	3.17(.25)	3.06(.39)

II) Clinical, Histopathological and Immunohistochemical Characteristics.

A) p53

p53 protein overexpression was observed in 174 (55.23%) out of 315 patients tumors. Its relationship to histopathological and other immunohistochemical characteristics is shown in table 2. The difference in P53 expression between patients aged <49 years and >49 years was not statistically significant (p= value 0.4368). By univariate analysis p53 expression was significantly correlated with histological differentiation, (p 0.0361), tumor size (p 0.0158), and axillary lymph nodes metastases (p 0.0116). Brain, liver, lung and bone metastases were seen in strong p53 positive cases with p values of 0.0235, 0.0300, 0.0001 & 0.0262 respectively. 85 (46%) out of 174 p53 positive cases showed ER or /and PgR positivity, a significant but inverse relationship between p53 overexpression and ER content was observed (p 0.0575).

B) C-erbB-2

C-erbB-2 protein over expression was observed in 124 (39.36%) patients out of 315 cases. Its relationship to histopathological and other immunohistochemical characteristics is shown in table 3. The difference in C-erbB-2 expression between patients aged <49 years and >49 years was not statistically significant (p= value 0.4368).C-erbB-2 over expression was significantly correlated with histological differentiation, (p 0.0361), tumor size (p 0.0158), and axillary lymph nodes metastases (p 0.0216).

C) EGFR

EGFR protein overexpression was observed in 70 (22.00%) patients out of 315 cases. Its relationship to histopathological and other immunohistochemical characteristics is shown in table 4.The difference in EGFR expression between patients aged <49 years and >49 years was not statistically significant (p= value 0.4368).

By univareate analysis EGFR overexpression was significantly correlated with histological differentiation, (p 0.0012), tumor size (p 0.0236), and axillary lymph nodes metastases (p 0.0163). Out of 70 EGFR positive only 18 (26%) cases showed ER or ER/PR positivity, therefore a significant but inverse relationship between EGFR overexpression and hormonal status was observed (p 0.0326).

D) PCNA

According to the pathological grading lowest mean PCNA clear reactivity to PCNA followed by grade II 30% and grade III 33%. The difference in PCNA expression between patients aged <49 years and >49 years was not statistically significant (p= value 0.4368). PCNA categorical expression was significantly correlated with histological differentiation, (p 0.0216), tumor size (p 0.0001). Brain, and bone metastases were seen in >25% positive cases with a p value of 0.0023 and 0.0062respectively. No significant difference was seen with axillary lymph nodes metastases in any of the PCNA category with a p value of 0.2116. There was no significant difference between either category of PCNA and ER/PgR status with a p value of 0.575. Vascular/lymphatic invasion was identified in 62(41%) of <25%PCNA and 88 (59%) of >25% PCNA positive cases. There was a significant difference with a p value of 0.0216. (Table 5)

E) Cathepsin-D

CD protein overexpression was observed in 120 (38.09%) patients out of 315 cases. Its relationship to histopathological and other iHC characteristics is shown in table 2. Stain intensified positivity was dominated by ++ moderate 63 (53%) followed by + mild positive 45 (38%) and +++ or ++++ strong positive 10 (12%). The difference in CD expression between patients aged <49 years and >49 years was not statistically significant (p= value 0.4368).

By univareate and multivariate analysis CD overexpression was significantly correlated with histological differentiation, (p 0.0312) and tumor size (p 0.0254). No significant correlation was seen with axillary lymph nodes metastases (p 0.6163), and hormonal status (p 0.3261). Bone metastases was significantly correlated with CD positive cases (p value 0.0052). Vascular/lymphatic invasion was identified in 63 (53%) of CD positive cases. There was significant difference with a p value of 0.0462. (Table 6)

TABLE 2 p53 expression and histological characteristics.

Parameters	**Total No. of patients**	**p53 protein expression**		**P value**
		Negative (141)	**Positive (174)**	
Grade				
I	45	24 (53%)	21 (47%)	0.0361
II	214	103 (48%)	111 (52%)	
III	56	14 (25%)	42 (75%)	
Tumor size				
≤ 2 cm.	68	50 (74%)	18 (26%)	0.0158
2-5 cm.	175	84 (48%)	91 (52%)	
> 5 cm.	72	29 (39%)	46 (61%)	
Axillary lymph nodes				
Negative	143	75 (52%)	68 (48%)	0.0116
Positive	172	68 (40%)	104 (60%)	
ER/PgR negative	130	56 (43%)	74 (57%)	0.0575
ERand/orPgR positive	185	100 (54%)	85(46%)	
Distant metastases				
Brain	38	17 (45%)	21 (55%)	0.0235
Liver	18	08(44%)	10 (56%)	0.0300
Lung	29	03 (10%)	26 (90%)	0.0001
Bone	73	29 (40%)	44 (60%)	0.0262

TABLE 3 C-erbB-2 over expression and histological characteristics.

Parameters	**Total No. of patients**	**C-erbB-2 protein over expression**		**P value**
		Negative (191) positive (124)		
Grade				
I	45	10 (23%)	35 (77%)	0.0361
II	214	141 (66%)	73 (34%)	
III	56	42 (75%)	14 (25%)	
Tumor size				
≤ 2 cm.	68	45 (66%)	23 (34%)	0.0158
2-5 cm.	175	118 (67%)	57 (33%)	
> 5 cm.	72	59 (82%)	13 (18%)	
Axillary lymph nodes				
Negative	143	85 (59%)	58 (40%)	0.0216
Positive	172	106 (62%)	66 (38%)	
ER/PgR negative	130	72 (55%)	58 (45%)	0.0575
ER/PgR positive	89	53 (60%)	36(40%)	
Only ER positive	96	66 (69%)	30 (31%)	
Distant metastases				
Brain	38	17 (45%)	21 (55%)	0.0235
Lung	29	03 (10%)	26 (90%)	0.0001
Bone	42	29 (69%)	13 (31%)	0.7562

TABLE 4 EGFR overexpression and histological characteristics.

Parameters	**Total No. of patients**	**EGFR protein over expression graded on a scale of 0-3** (0/+/++/+++)		**P value**
		Negative (245)	**positive (70)**	
Grade				
I	45	34 (14%)	11 (16%)	0.0012
II	214	171 (70%)	43 (61%)	
III	56	40 (16%)	16 (23%)	
Tumor size				
< 2 cm.	68	22 (09%)	46 (66%)	0.0236
2-5 cm.	175	166 (68%)	09 (13%)	
> 5 cm.	72	57 (23%)	15 (21%)	
Axillary lymph nodes				
Negative	143	116 (47%)	27 (39%)	0.0163
Positive	172	129 (53%)	43 (62%)	
ER/PR status (Negative)	112	60 (54%)	52 (74%)	0.0326
ER/PR positive	203	185 (60%)	18 (26%)	
Distant metastases				
Brain	38	17 (45%)	21 (55%)	0.0041
Liver	18	06 (33%)	12 (66%)	0.0482
Bone	42	13 (31%)	29 (69%)	0.0352

TABLE 5 PCNA expression and histological characteristics. (Univariate Analysis)

Parameters	Total no. Of patients	PCNA protein expression		P value
		<25% (149)	>25% (166)	
Grade				
I	45	26 (58%)	19 (42%)	0.0216
II	214	103 (48%)	111 (52%)	
III	56	20 (36%)	36 (64%)	
Tumor size				
≤ 2 cm.	68	48 (71%)	20 (29%)	0.0001
2-5 cm.	175	42 (24%)	133 (76%)	
> 5 cm.	72	09 (12%)	63 (88%)	
Axillary lymph nodes				
Negative	143	74 (52%)	69 (48%)	0.2116
Positive	172	75 (44%)	97 (56%)	
ER/PgR negative	130	62 (48%)	68 (52%)	0.5751
ER/PgR positive	89	41 (46%)	48(54%)	
Only ER positive	96	46 (48%)	50 (52%)	
Distant metastases				
Brain	38	13 (34%)	25 (66%)	0.0023
Bone	31	10 (32%)	21 (68%)	0.0062

TABLE 6 CD overexpression and histological characteristics.

Parameters	**Total No. of patients**	**CD protein overexpression**		**P value**
		Negative (195)	**positive (120)**	
Grade				
I	45	32 (16%)	13 (11%)	0.0312
II	214	119 (61%)	95 (79%)	
III	56	44 (23%)	12 (10%)	
Tumor size				
< 2 cm.	68	57 (29%)	11 (09%)	0.0254
2-5 cm.	175	90 (46%)	85 (70%)	
> 5 cm.	72	48 (25%)	24 (20%)	
Axillary lymph nodes				
Negative	143	88 (45%)	55 (46%)	0.6163
Positive	172	107 (55%)	65 (54%)	
ER/PgR negative	130	89 (46%)	41 (34%)	0.3026
ER/PgR positive	89	44 (23%)	45 (38%)	
Only ER positive	96	63 (32%)	33 (28%)	

III) Survival Analysis:

After a median follow-up of 48 months (range 3 to 73 months), the overall survival of breast cancer patients amounted to 65%.

A) p53

In univariate as well as in multivariate analyses p53 overexpression had a significant influence on survival. p53 mutation when correlated with overall survival, show significant correlation between p53 positivity and overall survival with a p value of 0.0523. At a median follow-up of 48 months, 65% of p53 positive patients died with an overall survival of 3.00 years and disease free survival of 2.5 years. Among p53 negative patients overall survival was 3.8 years and disease free survival was 3.3 years. In p53 positive patients irrespective to axillary lymph nodes status overall survival and disease free survival was poor compared to p53 negative patients.

In multivariate analysis, the independent prognostic factors for breast cancer patients were tumor size, axillary lymph nodes involvement, histological grade, p53 overexpression and ER /PgR status (table 7).

TABLE 7 Independent variables related to prognosis (Cox multivariate analysis).

Variable	**Coefficient**	**Standard error**	**P value**	**Hazard ratio**
Axillary lymph nodes positive(0/1-3/4 +)	0.6369	0.117	0.0001	1.891
Tumor size	0.5357	0.119	0.0001	1.709
Grade (I, II, III)	0.7352	0.242	0.0002	2.086
P53 (0/+/++/+++ or ++++)	0.2841	0.134	0.034	1.329
ER /PgR negative/ER/PgR positive or ER positive	0.2897	0.144	0.039	0.7418

B) C erb B2

In univariate as well as multivariate analyses C-erbB-2 over expression had a significant influence on survival. Overall survival rates amounted to 72% and 38% in patients with negative and positive C-erbB-2 protein over expression in tumors. C-erbB-2 positivity when compared with the overall survival is statistically highly significant with a p value of 0.020. At a median follow-up of 48 months, the overall survival was 3.0 years and disease free survival of 2.5 years. C-erbB-2 negative tumor patients showed a far better survival with the overall survival of 4.44 years and disease free survival of 3.78 years. By univariate analysis C-erbB-2 showed significant correlation with axillary lymph nodes positivity (table 8), tumor size larger than 2 cm and ER/ PgR negativity.

TABLE 8 Independent variables related to prognosis (Cox multivariate analysis).

Variable	**Coefficient**	**Standard error**	**P value**	**Hazard ratio**
Axillary lymph nodes positive (0/1-3/4 +)	0.6369	0.117	0.0001	1.891
Tumor size	0.5357	0.119	0.0001	1.709
Grade (I, II, III)	0.7352	0.242	0.0002	2.086
C-erbB-2 (0/+)	0.2841	0.134	0.034	1.329
ER /PgR negative/ER/PgR positive or ER positive	0.2897	0.144	0.039	0.7418

C) EGFR

EGFR positivity when compared with the overall survival was statistically significant with a p value of 0.0045. At a median follow-up of 48 months, the overall survival was 3.39 years and disease free survival of 2.86 years. EGFR negative tumor patients showed a far better survival with the overall survival of 4.62 years and disease free survival of 4.00 years. By univariate analysis EGFR showed significant correlation with axillary lymph nodes positivity (table 9), tumor size larger than 2 cm and ER/ PgR negativity. In a Cox proportional hazard model of all patients there was a significant influence on overall survival for EGFR positivity (p value 0.0045), tumor size (p value 0.0026) and histological grade III (p value 0.0012) when assessed with axillary lymph nodes and ER/PgR status In axillary lymph nodes positive group for overall survival, EGFR positivity, and tumor size >2cm and hormonal status are significant (p value 0.0012). When examining the axillary lymph node negative subgroup, we find EGFR to be significant predictor for overall survival and disease free survival (p value 0.0328). About 10% of EGFR patients had 5 years or less disease free survival, while 20% had 3 years or more disease free survival.

TABLE 9 Independent variables related to prognosis (Cox multivariate analysis).

Variable	**Coefficient**	**Standard error**	**P value**	**Hazard ratio**
Axillary lymph nodes positive (0/1-3/4 +)	0.5349	0.116	0.0001	1.791
Tumor size	0.5237	0.115	0.0001	1.809
Grade (I, II, III)	0.7162	0.246	0.0002	2.066
EGFR (0/+/++/+++)	0.2281	0.122	0.0034	1.339
ER /PgR negative/ER/PgR positive or ER positive	0.2237	0.134	0.0369	0.7618

D) PCNA

In univariate as well as in multivariate analyses PCNA categorical expression had a significant influence on survival (table 10). Overall survival rates amounted to 31% and 69% in patients with <25% PCNA and patients with >25% expression in tumors. PCNA expression when correlated with overall survival, show significant correlation between categorical PCNA with a p value of 0.0123. At a median follow-up of 48 months, 66% of<25 PCNA positive patients died with an overall survival of 3.16 years and disease free survival of 2.5 years, among >25% PCNA positive patients 77% died with an overall survival 2.7 years and disease free survival was 2.2 years.

By univariate analysis PCNA of either category showed no significant correlation with axillary lymph nodes positivity and ER/ PgR positvity.

TABLE 10 Independent variables related to prognosis (Cox multivariate analysis).

Variable	Coefficient	Standard error	P value	Hazard ratio
Axillary lymph nodes positive (0/1-3/4 +)	0.6369	0.117	0.0001	1.891
Tumor size	0.5357	0.119	0.0001	1.709
Grade (I, II, III)	0.7352	0.242	0.0002	2.086
PCNA (<25% and >25%)	0.2841	0.137	0.034	1.329
ER /PgR negative/ER/PgR positive or ER positive	0.2897	0.144	0.039	0.7418

E) Cathepsin-D

In univariate as well as in multivariate analyses CD overexpression had no significant influence on survival. CD positivity when compared with the overall survival was not statistically significant with a p value of 0.0875. At a median follow-up of 48 months, the overall survival for CD negative patients was 3.50 years and disease free survival was 2.93 years, while the overall survival of CD positive patients was 3.17 years and disease free survival was 2.67 years

Axillary lymph nodes negativity and CD positivity was seen in 55 (46%) cases, with a mean tumor size of 3.08 cm, 25% cases were negative for ER/PgR, with an overall survival of 3.18 years and disease free survival of 2.5 years. Whereas axillary lymph nodes positivity and CD positivity was seen in 65 (54%) cases, with a mean tumor size of 4.47 cm, 42% cases were negative for ER/PgR, with an overall survival of 2.7 years and disease free survival of 2.4 years. Statistically there was no significant correlation with a p value of 0.0764.

DISCUSSION / CONCLUSION

I) p53

In this study conducted on Pakistani women, we did find p53 as an independent prognostic factor as its association with several pathological variables and disease outcome was significantly correlated. The association between p53 oncoprotein and axillary lymph nodes metastases was significantly attributed to p53 positivity as significant number of p53 positive patients showed axillary lymph nodes metastasis compared to p53 negative tumors. Others like Sirvant JJ et al 2001 has reported similar findings. Likewise in our study p53 expression was significantly but inversely correlated with ER/PR status, larger tumor size and higher histological grade. . (Ferrero JM et al 2000, Ceccarelli C et al 2001 and Meenakshi A et al 1999)

In our study the correlation between p53 immune-expression, OS & DFS was also significant. This is in consensus with several other studies using univariate and multivariate analysis. . (Sirvant JJ et al 2001, Ferrero JM et al 2000, Ceccarelli C et al 2001, Meenakshi A et al 1999 and Chariyalertsak S et al 1998)

However in another study after 30 years of follow up p53 did not appear to have an independent prognostic value. (Reed W et al 2000)

In summary current data suggests that in spite of numerous studies examining p53 utility in breast cancer, overall picture remains still somewhat unclear. This is largely due to lack of unanimity of results in various studies. These differences may be due to technique, study design, subjective interpretation of results and above all due to racial differences of the population under study.

In conclusion the above finding have reinforced the view that p53 immunohistochemical detection will be of help in the clinical evaluation of breast carcinoma patients. This may be of particular relevance for the node negative patients with invasive ductal carcinomas, where p53 expression may help in decisions on adjuvant chemotherapy.

II) C-erbB-2

C-erbB-2 over-expression usually measured by immunohistochemistry has been shown to be an independent prognostic factor in several studies on either node-positive and node-negative patients, or studies on all patients in multivariate models. (Tandon AK et al 1989, Tsuda H et al 1989, Salmon DJ et al 1987,Wright C 1989,Gullick WL et al 1991, Cobleigh MA et al 1998, Salmon D et al 1998 and Jakic Razumovic J et al 2000)

The frequency of C-erbB-2 gene over-expression in the cohort of patients reported in our study (39.36%) is consistent with that of previously reported by others (10%-40%). In our patients we found significant association between C-erbB-2 over-expression and other biological parameters like histological grades, tumor size and axillary lymph nodes, as reported by others in this region. (Aryandono T et al 2000 and Looi LM et al 1998)

According to tumor subsets, by mean of univariate analysis C-erbB-2 was recognised as a significant prognostic factor for axillary lymph nodes positivity tumor size larger than 2 cm and ER/ PgR negativity.

In multivariate analysis, the independent prognostic factors for breast cancer patients were tumor size, axillary lymph nodes involvement, histological grade, C-erbB-2 over expression and ER /PgR content.

In our study, overall survival and disease free survival were significantly shorter and poorer for patients with C-erbB-2 oncoprotein expression than for those without such expression.

As reported by Bernard et al 1994, Sharma et al 1999 and Shimizu et al 2000, C-erbB-2 positive tumors show resistance to adjuvant chemotherapy, we have also evaluated C-erbB-2 with this mode in mind. C-erbB-2 positive patients did show resistance to chemotherapy when compared for recurrence and distant metastases following surgery. The mechanisms responsible for drug resistance with C-erbB-2 over-expression are unclear but seem to be independent of the multi-drug resistance-1 system. It is thought that C-erbB-2 signalling might alter the sensitivity to chemotherapeutic agents by acting on genes controlling drug-activating enzymes (Bernard et al 1994 and Shimizu et al 2000). Although more C-erbB-2 positive cases diagnosed were in stage II, compared to C-erbB-2 negative cases, a good proportion of which was in stage III, still poor overall survival and disease free survival in C-erbB-2 positive cases may be due to the resistance of these tumors to adjuvant chemotherapy.

Above findings suggest that it would be worthwhile to detect the over-expression of C-erbB-2 in all breast carcinoma patients with or without axillary lymph nodes positivity.

III) EGFR

In our study significant correlation was found with histological grade III and EGFR positivity. Several other reports also showed significant correlation with grade I and III (Sainsbury JRC 1985 and Koenders PG 1991). No relationship was found between the tumor EGFR immuno-reactivity and the patient's age, in agreement with a previous

report (Foekens JA et al 1989). We did observe significant correlation between the tumor EGFR immuno-reactivity and the tumor size. In our study there was also a significant correlation between positive lymph nodes and positive EGFR, this is in consensus with most studies but in contrast to some other studies reported previously (Pirinen R et al 1995). Estrogens are involved in the release of growth factors and may mediate the tumor cells response to growth factors such as EGF. In this study we did find significant correlation with EGFR positivity and hormonal negativity, most of the other studies have demonstrated the same (Sainsbury JRC et al 1985, Fitzpatrick Sl et al 1984, Klijn JGM et al 1992 and Koenders PG et al 1991). Therefore, the evaluation of tumor EGFR and hormonal status enables us to identify groups of patients who may not respond to hormonal therapy and shall benefit from other modalities of treatment.

Most importantly this study in agreement with most other studies showed independent prognostic value of EGFR detection in univariate (Newby JC et al 1995) and multivariate (Torregrosa D et al 1997) analysis, as there was significant decrease in disease free and overall survival in EGFR positive patients compared to controls. Some other studies however did not show this independent prognostic value of EGFR for overall & disease free survival (Pirinen R et al 1995 and Gerstein ES et al 1998).

In conclusion this study shows that EGFR tumor cell content is independent from other morphological prognostic factors in predicting disease free and overall survival, can easily be detected by immunohistochemistry which is a reliable method even on formalin fixed paraffin embedded breast tumor tissue. In addition EGFR analysis can be a useful indicator for the selection of patients for hormonal therapy and can be useful as a target for new treatment modalities particularly in C-erb B2 negative patients.

IV) PCNA

Immunohistochemical staining for PCNA in biopsy specimens of breast carcinoma tissue has the potential to demonstrate the proportion of cells in late G1 and S phases, and may provide prognostic information similar to that obtained from the flow cytometry studies.

It is of interest that most cases of PCNA- positive tumors were found to have a mean of 30% PCNA positive nuclei, considerably more than what has been reported in tissue culture cells (20% to 50%) and hematopoietic malignancies (11% to 36%) (Mathews MB et al 1984 and Celis JE et al 1985). The most likely explanation for these differences is that most of the patients in this study were histologically graded as II.The quality and range of staining with PCNA reported here is comparable to data reported elsewhere although the mean and median in this series are more than those found by Pinder SE et al 1994 in a different series of patients. This may in part be due to different means of assessing immunostaining in two studies (Muhammad G et al 1997).

PCNA intensity of > 25% was of great value to determine prognosis as most of the distant metastases and /or recurrences were seen in this group. Less overall survival and less disease free survival was also seen in this category. A positive correlation was seen with the increase in the survival if > 25% category patients were subjected to chemotherapy. There is a significant increase in the overall survival and disease free survival of these category patients.

In conclusion PCNA according to our study has proved to be a useful prognostic indicator, but before PCNA is critically examined as a potential prognostic indicator in breast carcinoma, appropriate monoclonal antibody has to be considered because

literature is full of such investigations with every commercially available antibody but which one is the best needs to be evaluated.

V) Cathepsin-D

The prognostic value of CD has been evaluated in several investigations, in which more than 4000 patients were studied. CD over-expression has been identified as a factor of poor prognosis in node-negative (Isola J et al 1993), node-positive (Riley LB et al 2000 and Romain S et al 1990) and in all stages of breast carcinoma (Foekens JA et al 1999, Rudland PS et al 2000, Spyratos F et al 1989 and Tandon AK et al 1990).

Numerous reports have provided evidence for a correlation between CD over-expression and an increased tendency towards invasive growth and metastases with poor clinical outcome (Duffy MJ et al 1992,Pujol P et al 1993, Thorpe SM et al 1989, Vignon F et al 1986 and Westley B et al 1980). Henry, et al in 1990 using IHC demonstrates that CD expression was associated with better prognosis. In another study Domagala, et al in 1992 by using IHC suggested that CD over-expression may be due to direct transcriptional regulation by activated estrogen receptors, inducing better prognosis, or may be due to autocrine growth factors, leading to poorer prognosis.

Since In our study, CD immunostaining in cancer cells was associated with no significant difference in overall and disease free survival. These results support the hypothesis that reactive fibroblasts around cancer cells might facilitate metastatic dissemination when expressing CD. Johnson MD et al 1993 provide evidence that CD secretion by cancer cells might not be involved in the invasive phenotype of breast cancer, and suggest that the enzyme derived from reactive stromal cells might be important. In summary we

conclude that CD over-expression is not a good prognostic marker if only expression on tumor cells is taken into account by IHC.

GENERAL DISCUSSION

Breast cancer, the most feared cancer among women, is an unpredictable disease. Whereas some patients enjoy a disease-free life for many years after the diagnosis of breast cancer, there are patients who suffer from a rapidly progressive disease with a fatal outcome. This difference in the natural history of breast cancer so far has been one of the main limiting factors in the development of a unified therapy regimen that can be used for all patients with breast cancer. As a result, despite an earlier clinical detection due to the improved diagnostic techniques, the overall survival of patients with breast cancer in the last 20 years has remained almost the same. Thus, the present challenge is to enhance our understanding of the events leading to the development of breast cancer and subsequently in better management of the disease.

However during the last two decades few years, some important developments have occurred. These include the development of several new (novel) prognostic markers and the availability of the new chemotherapeutic agents. In addition, the treatment of breast cancer has undergone a revolutionary change from radical surgery to a more conservative surgical approach, leading to preservation of the breast.

Considering the importance of accurately classifying breast cancer patients according to their relative risk for recurrence, eventually clinicians need to depend on the available prognostic factors to design their therapeutic approach. This will allow an informed discussion with the individual patient and will guide the therapy. Prognostic factors are especially important in node-negative breast cancer patients when many patients may be treated needlessly for the benefit of a few. The use of prognostic factors in node-positive locally advanced and metastatic breast cancer may also benefit from lesser aggressive

therapies.

At present time, the role of the currently available prognostic factors needs to be further clarified. Much research still needs to be done to identify panels of factors with independent prognostic significance to justify the expense and the time involved in performing these rests.

It is hoped that, eventually with the availability of new biological markers, advances in computer science and standardization of the technology, the reliability of prognostic factors in breast cancer will be enhanced. Nevertheless, currently, there are prognostic factors that can help in selecting breast cancer patients for different therapeutic modalities.

According to our study C-erbB-2 over expressing patients not only showed poor overall survival, but also C-erbB-2 over expression proved to the marker of drug resistance. The potential of detection of C-erbB-2 after the approval of drug 'herceptin' by FDA in 1998, has increased tremendously. EGFR expression in tumor cell is also an independent marker particularly for the selection of patients for hormonal therapy, specially in C-erbB-2 negative patients, and can be useful as a target for new treatment modalities. In our study, the correlation between p53 immuno-expression overall and disease-free survival was also significant.Cathepsin D was found of no value in our study while PCNA estimation had shown some promise to assess the aggressiveness of breast cancer.

LIST OF PUBLICATIONS

1 Pervez S, Aziz SA, Khan S, Kayani N & Rahbar MH. Relationship of p53 expression with nodal metastases and disease out come. A prospective study on 315 consecutive breast carcinoma patients. 2000.

2 Pervez S, Aziz SA, Khan S, Kayani N, Azam SI & Rahbar MH. Significance of immunohistochemical C-erbB-2 product localisation pattern for prognosis in human breast cancer.2000

3 Pervez S, Aziz SA, Khan S, Kayani N & Rahbar MH. Epidermal growth factor receptor (EGFR 1) as a prognostic marker: An Immunohistochemical Study on 315 consecutive breast carcinoma patients.2000

4 Pervez S, Aziz SA, Khan S, Kayani N & Rahbar MH.Immunohistochemical Analysis of Proliferating Cell Nuclear Antigen (PCNA) in Infiltrating Ductal Carcinoma Breast: Comparison with Clinical and Pathological Variables.2000

5 Pervez S, Aziz SA, Khan S, Kayani N & Rahbar MH. Immunohistochemical Cathepsin-D Expression in Breast Cancer: Correlation with Established Pathological Parameters and Survival.2000

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April 30, 2001

Immunohistochemical Cathepsin-D Expression in Breast Cancer ...

Dear Doctor Pervez:

Thank you very much for sending us your revised manuscript. Having re-examined it once again, I think that it is now suitable for publication in our journal. It will now be sent into print in its present form. Please check the galley proofs carefully when they come to you.

Yours sincerely

Albert Roessner, M.D.
(Executive Editor)

shahid.pervez

From: Looi Lai Meng [looilm@ummc.edu.my]
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Subject: Manuscript - p53 breast cancer

Dr. Shahid Pervez
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3 June 2001

Dear Dr. Pervez

Re: Manuscript: Relationship of p53 expression with clinicopathological variables and disease outcome. A prospective study on 315 consecutive breast carcinoma patients

I am pleased to inform you that reviewers have advised that your paper "Relationship of p53 expression with clinicopathological variables and disease outcome. A prospective study on 315 consecutive breast carcinoma patients" be considered favourably for publication in this Journal pending revision to answer the points raised by the reviewer. For the purpose of revision, I am enclosing the comments of the reviewer and a copy of your manuscript with some suggested corrections made (this will be sent by airmail). Please also indicate what CHS (the place of work of the last author) stand for.

I will be grateful if your revised manuscript can be accompanied by a cover letter indicating the revisions undertaken and the position (page numbers) in the text where these have been inserted. Furthermore, to facilitate Editorial undertakings, I would appreciate resubmission of the revised manuscript both as a hard copy and in electronic form in a computer floppy diskette, written preferably in Microsoft Word. In the interest of time, you can also submit it electronically through e-mail to the following address: looilm@ummc.edu.my

I hope to receive your revised manuscript as soon as possible so that it can be processed with minimal delay. Thank you for your support of this Journal.

Yours sincerely

Professor Dr. L.M. Looi
Editor

REVIEWER'S COMMENTS

Relationship of p53 expression with clinicopathological variables and disease outcome. A prospective study on 315 consecutive breast carcinoma

The Indian Journal of Pathology And Microbiology

Official Organ of Indian Association of Pathologists and Microbiologists

REVIEWER SHEET

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Address : 4/11 BMK St, T. Nagar, Chennai- 17
or
Consultant Pathologist, DDGH + RC
Andhra Mahila Sabha, Chennai - 28

Date of Despatch : 9.10.00

Paper No. : 134/00

Title of the Paper : Immunohistochemical ... pathological variables.

	ACCEPTABLE	TO BE MODIFIED	NOT ACCEPTABLE
1. Format		✓	
2. Tables			
3. Photographs			
4. Charts			
5. References			

Special comments The paper is acceptable but must be abbreviated to half its size (text) as there is too much of theoretical data which is not needed.

Final opinion Acceptable | Not acceptable | To be modified

Date : 7.10.00

SIGNATURE

★ Please note the remarks of the editorial office also (turn page) to assist you in review.

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Ref: JPMA/154/2000 Dated: 27-1-2001

Dr. Shahid Pervez,
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Department of Pathology,
The Aga Khan University Medical Centre,
Karachi.

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR 1) AS A PROGNOSTIC MARKER: AN IMMUNOHISTOCHEMICAL ..

Dear Dr. Shahid Pervez,

Your above cited article was reviewed and following comments are offered by the reviewer:

Although not truly original but it is first large study of its kind in our population

Introduction may be asked to be rewritten.

Results of survival correlation with EGFR positive have not been compared with other reported series.

A figure of EGFR as well as figures showing different grades and intestines of positive PAP might have been helpful. In the last graph of survival functions, all four groups have been represented by similar marks spo discrimination in this photocopy is impossible.

Last reference is redundant. Some more references need to be added comparing survival rates.

You are advised to revise your article based on the above comments and submit it to JPMA in duplicate alongwith floppy (M.S. Word version 6) at an early date.

Yours sincerely,

(Dr. SARWAR J. ZUBERI)
Editor

shahid.pervez

From: Timar Jozsef [jtimar@oncol.hu]
Sent: Wednesday, April 25, 2001 4:55 PM
To: shahid.pervez
Subject: POR article

Card for Timar Jozsef

Dr. Pervez

Thank you for submitting your article "Significance of immunohistochemical c-erbB2..." to Pathology Oncology Research.
The review process was terminated and the following issues were raised, answering what could promote the acceptance.
1./ Why you dont use for evaluation of cerbB2 expression the FDA approved 1-2-3+ system?
2./ It would be wise to document the various expression categories on figures: negative, cytoplasmic or membraneous labeling...

Hope you can respond quickly to these issues

Sincerely

Joseph Timar
Editor, POR

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FINAL TECHNICAL REPORT

PAKISTAN SCIENCE FOUNDATION PROJECT

No:P-TICR/BIO(145)

COLLECTION AND RECORD OF REPTILIAN FAUNA OF TABLELAND POTWAR, PUNJAB, PAKISTAN

Principal investigator:

M. S. Khan

Department of Zoology

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Rabwah 35460

Co-Principal investigator:

K.J. Baig

Research Associate

Pakistan Natural History Museum

Islamabad

Principal investigator: Institutional head :

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137 198

TABLE OF CONTENTS

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I N T R O D U C T I O N

Herpetological collections from Potwar Plateau, Salt Range, Punjab Pakistan, were made under Pakistan Science Foundation project No.P-TICR/BIO (145). The objective maily was to collect and put to record the reptilian fauna of the area.

The collection from the area was made from 1987 to 1990, and three annual reports were submitted: first (1987), second (1988) and the third (1990). Present report is final technical report, its data is based on the three reports already submitted to Pakistan Science Foundation.

During the running of the project 12 collection tours, each of 12-15 days of duration, were made to 88 different localities, scattered throughout the Potwar tableland (see Map at the end). Majority of the localities were visited thrice to ensure round the year collection. Day and night collection schedule was followed daily. It was done to ensure our collection as representative as possible.

As a conservation measure, and to least disturb the natural population of reptiles, only two to three specimens of each species were collected. The species collected from localities, if found in the other, its presence was noted without collecting a specimen. Ten to 12 specimens were collected of those species which were new to science. It was not possible for us to collect more secretive and agile species living in thick cover. Their record is based on sight.

The animals caught were photographed, so as to record their natural colour. An album is presented at the end of present report. The collection localities are listed at the end of the text according to the numbring recorded in the map.

OBJECTIVES ACHIEVED BY THE PROJECT

The reported project provided us with an opportunity to conduct a herpetological and ecological survey of the Potwar tableland. This large chunk of geologically interesting land was unknown herpetologically, except short occasional reports (Anderson, 1871; Stoliczka, 1872; Hora, 1926). Attempts to study zoogeography of Pakistani herpetofauna (Khan, 1980a), are always jeopardized by such large areas of terra incognita, scattered throughout Pakistan.

Following were our aims and objectives while conceiving the project, which are fairly met with:

1. To prepare a checklist of the reptilian species inhabiting the tableland. A part of the checklist has already been published (Khan and Baig, 1988).

2. To search for the species, once reported from the area with no subsequent record. They were:-

Bufo viridis, an element of Palearctic fauna. It has been also reported from the area, which appear to be based on misidentifications or some stray individuals washed down by the Kabul River. No distinct population of this toad has been found in the tableland.

Gymnodactylus montiumsalsorum, it has been reported from Salt Range, and is known now by a single much broken specimen in the British Museum (N.H.), London. We found it quite common in the Salt Range. Our findings about this gecko have appeared in a publication (Khan, 1989).

Gymnodactylus fedtschenkoi had been reported from Salt Range, and Balochistan (Hora, 1926; Minton, 1966; Mertens, 1969). Our collections show that its Pakistani record is based on misidentified specimens (Khan, 1991).

3. During herpetological study of the area we have discovered that recently described geckos Tenuidactylus indusoani Khan, 1988

and T. rohtasfortai Khan and Tasnim, 1990 are common in the area and have a much wider distribution.

4. Cyrtodactylus dattanensis Khan 1988, described from Hazara Division, NWFP, is common in alpine Punjab.

5. Hemidactylus persicus, which is a common gecko in Balochistan and lower Sindh, is caught from Rohtasfort, Jhelum. Discontinous distribution of this gecko reflects the historic movements of armies from Punjab-Palochistan-Persia in the days of Moughals. Geckos are notorious of being carried by human beings.

6. Varanus bengalensis has been found to differ ecologically from V. griseus. Former lives solitarily, when availables lives under rock rather than in burrows. On the other hand V. griseus are gregarious, living in burrows made in hard ground with sparse grass. It seldom extends in the rocky terrain.

7. Liolopisma himalayanum is found to abound in the alpine part of Punjab, not extending in the plains.

8. Agama agrorensis, A. melanura and Coluber rhodorachis are confined to the rocky area of the tableland not descending in the plains.

9. Agama agilis, Uromastyx hardwickii and Coluber ventromaculatus are plain forms occuring in the plain part of the plateau, not ascending into the rocky outcrops.

LIST OF PUBLICATIONS RESULTING FROM THE PROJECT

Following publications have resulted from the findings of the project, and have been published in internationak jornals of herpetology.

1. Checklist of the amphibians and reptiles of District Jhelum, Punjab, Pakistan. The Snake, 20:156-161, 1988.
2. Rediscovery and redescription of the highland ground gecko Tenuidactylus montiumsalsorum (Annandale, 1913). Herpetologica, 45:46-54, 1989.
3. A new gecko of the genus Tenuidactylus from northeastern Punjab, Pakistan, and southwestern Azad Kashmir. Herpetologica, 46:142-148, 1990.

Submitted for publication:

1. Checklist of herpetofauna of tableland Potwar, Punjab, Pakistan. The Snake.
2. Reproductive behaviour of the male Calotes versicolor. Herpetological Review.

C H E C K L I S T

In the following checklist the animals are systematically arranged. The collection localities are reffered by numbers in the map at page 21 . Brief notes on the status, ecology and morphology of each species are added.

Family TRIONYCHIDAE

Lissemys punctata punctata

Localities:2,10,19,20,17,27,31,32,40,45,53,55.

Status: fairly common.

Ecology & Natural history notes: It frequents side pools of rivers and canals with thick vegetation. It breeds during April-May. Hatchlings are seen from May to August.

Chita indica

Localities: 19.

Status: aboundant.

Ecology & natural history notes: It is common river turtle in River Jhelum. It seldom leaves the river. It basks during winter. Mating season extends from April to June. Young are seen during July-August.

Family EMYDIDAE

Kachuga tecta

Localities:2,10,17,19.

Status: rare.

Notes: The saw-back turtle lives in quite pools with vegetation. Breeding season extends from March to June, youngs are seen during August-September.

Family GECKKONIDAE

Eublepharis macularius (Fig. 1)

Localities: 34,47,48,50,58,72,75,77,80,81.

Status: frequent.

Notes:It is a ground large gecko, living in the holes and crevices in earth or in the piles of stones and holes in stone-walls. Breeding season extends from April to June, young are seen from July to August.

Hemidactylus flaviviridis (Fig.2)

Localities:1,2,4,9,17,19,23,24,30,31,32,35,42,43,46,51,52,53,54, 58,59,62,66,68,73,77,80,84,87.

Status: aboundant.

Notes: Common yellow-belled-house- gecko, shows metachromatic color changes. It breeds during April-May, young are seen by June-July.

Hemidactylus brooki

Localities:8,9,10,12,13,14,15,16,17,18,19,22,25,28,29,32,36,40,60, 61,66,72,85.

Status: Common.

Notes: It is mostly found in the tilled and green parts of the table-land. It do not extend in human inhabited houses if H. flaviviridis is around. It lives in holes and crevices in ground, piles of wood, debris and under logs. Breeding season extends from March to June, young are seen from July-August.

Hemidactylus persicus (Fig.3)

Locality:17.

Status:rare.

Notes: Found in remote parts of Rohtas Fort, near the mosque. Apparently, cannot compete with H. flaviviridis. Only three specimens sighted, two were caught. Much more common in Baluchistan (Khan, 1987; Khan and Ahmed, 1987).

Tenuidactylus indusoani (Fig. 4)

Localities:6,35, 46.

Status: rare.

Notes:The sandstone gecko was reported first from District Mianwali (Khan, 1988). Breeding occurs during March to May, youngs are seen June to August.

Tenuidactylus rohtasfortai

Localities:7,59,63,64,65,68.

Status:quite common in stated localities.

Notes:First recorded from Rohtas Fort, Jhelum. It is quite common in the wooded rocky areas down the Murree hills. Breeding takes place from April to June, youngs are seen from July-August.

Tenuidactylus montiumsalsorum (Fig. 5)

Localities:4,17,25.

Status:quite common in the localities.

Notes: Present collection of this gecko from Salt Range is after 134 years of the first collection by Theobald in 1851. This gecko is redescribed by Khan (1989). Breeding takes place from April to May, youngs are seen from June to September. It is a ground gecko.

Tenuidactylus watsoni (Fig.6)

Localities:25,35,37,38,45,56.

Status:rare in the localities mentioned.

Notes:This gecko is found in different situations, under logs, under bark of trees, in crevices and holes in soil etc. Reproduction starts from April to June, youngs are seen up to August.

Cyrtodactylus dattanensis (Fig. 7)

Locality: 68.

Status: Common in the locality.

Notes: First described from Hazara Division. It is first record

of this gecko from Alpine Punjab.

Family SCINCIDAE

Eumeces taeniolatus (Fig. 8)

Localities:17,18,23,43,53,58,84.

Status: rare.

Notes: Found in the roots of vegetation along the sandy fields round the major water courses. At the time of flooding, these scincid lizards come out and make for the hard ground.

Leiolopisma himalayanus (Fig. 9)

Localities:43,62,68.

Status:abounds in the localities mentioned.

Notes:This alpine skink abounds along the grassy margins of hilly torrents. They are most active during afternoon. Breeding season appers to be May to June, youngs are seen during August-September.

Mabuya dissimilis (Fig. 10)

Localities:10,19,20,27,31,32,55,59,60,66,69,75,82.

Status:common in grassfields.

Notes:The grassfield skink is common in tilled areas, along the marginal grass, in the plains. It do not extend in the hilly tracts and alpine part of the tableland. Breeding season extends from April to July, youngs are seen by August-September.

Ablepharis pannonicus (Fig. 11)

Localities:10,31,32,55,60,62,64.

Status:not frequent in the localities mentioned.

Notes:This skink is characteristic of hilly grasslands, where it hides under the stones. Breeding season appers to extend from April to May, while youngs are seen during July to September.

Family LACERTIDAE

Acanthodactylus cantoris (Fig. 12)

Localities: 17,2o,23,30,31,32,33,18,24,35,43,85,88.

Status: quite abounda**n**t in the mentioned localities;

Notes: It is common sand lizard in the sandy areas around major water channels. Breeding season extends from March to June, young are seen during August-September.

Acanthodactylus micropholis

Localities:18,24,35,43,83,85,88.

Status: common.

Notes:This sand lizard almost occupies same type of habitat as A cantoris. No observation on its reproduction.

Eremias guttulata (Fig.13)

Localities:11,12,14,19,28,32,33,35,86,88.

Status: Common in the localities mentioned.

Notes:It burrows in hard silty soil with sparse vegetation. Young are seen in April-May.

Ophisops jerdoni (Fig.14)

Localities:9,10,16,17,25,27,31,32,33,40,43,54,55,56,67,77,75,85.

Status: Common.

Notes:It frequents green fields, with stones and vegetation cover. Breeding season extends from March to June, youngs are around by July-September.

Family AGAMIDAE

Agama melanura

Localities:35,38,45,47,59,61,62,63,68,69.

Status:Abounds in the localities mentined.

Notes:This agama lives in crevices and holes among the rocks. Breeding season starts from March to May, youngs are seen during July-August.

Agama agilis (Fig.16)

Localities:2,17,19,31,32,33,35,46,79,88.

Status:Common.

Notes:It lives in long grass and low bushes in the plains, usually around the tilled fields. It climbs high in the branches of the bushes to see around and to bask.Breeding appear to start by March-May, youngs are out by May-August.

Calotes versicolor

Localities:2,4,6,17,19,24,34,37,39,43,45,54,58,78,81,86.

Status:Common.

Notes:Breeding males guard a herem of seven to 12 females on a tree, from March to June. Youngs are out by July-August.

Uromastyx hardwickii (Fig.17)

Localities:1,4,8,9,20,22,23,26,31,32,34,44,50,56,76,76,77,87,88.

Status:Very common in the mentioned localities.

Notes: Breeding activity starts by late February , eggs hatch by June-July.

Family VARINIDAE

Varanus bengalensis (Fig.18)

Localities:1,25,32,35,40,53,54,59,72,77,78,85.

Status: Common in the locality.

Notes:This varanid lives in holes and crevices among the rock, while in plains it excavates burrows in ground. Breeding activity starts by April-May, youngs are seen by June-July.

Varanus griseus

Localities:2,6,8,9,13,19,26,34,35,40,47,87,88.

Status:rare.

Notes:This varanid is gregarious, burrows in hard sandy soil, with sparse vegetation. Reproductive activity starts during March-April,

and youngs are active by June-July.

Family TYPHLOPIDAE

Typhlops braminus (Fig. 19)

Localities:8,9,10,24,27,30,59,64,67.

Status: common.

Notes: This earth snake is common along the sandy areas running along the banks of major water tracts. They are collected from the roots of the hedges growing in the area.

Family LEPTOTYPHLOPIDAE

Leptotyphlops macrorhynchus

Localities:76.

Status: rare.

Notes:Its ecology is like T. braminus.

Family BOIDAE

Eryx johnii

Localities:2,19,35,39,50,59,67,70,72,87.

Status:common.

Notes: The sand boa is common around sandy areas in the localities recorded. Breeding season starts by April, youngs are seen by August-September.

Family COLUBRIDAE

Boiga trigonata (Fig.20)

Localities:9,16,17,35,36,50,80.

Status: Not so common.

Notes: The common cat snake is arboreal in habits. It is often found in the wooded part of the area, with low bushes and trees. It hibernates on ground, ending the trees in summer. Breeding appear to take place by June-July, youngs are seen by August-September.

Coluber rhodorachis (Fig.21)

Localities:2,6,17,35,44,50,69,80,87.

Status:common.

Notes:The common cliff racer inhabits crevices and holes in the rocky part of the area. It do not extend in the plains. Breeding takes place by April-June, youngs are seen by July-August.

Coluber ventromaculatus

Localities:1,2,19,23,87,88.

Status:common.

Notes:The common plain racer do not extends in the rocky terrain. Breeding period starts from April-July, youngs are seen by June to September.

Amphiesma stolata (Fig.22)

Localities:19,24,30,31,33,45,75,84,86,88.

Status:common.

Notes:Rough-back common snake lives in tilled areas. Breeding appears to start by May to June, youngs are seen during August and September

Xenochrophis piscator (Fig.23)

Localities:10,17,20,19,31,32,45,58,59.

Status:common.

This water snake abounds in the different water channels in the tableland . Reproductive period of this snake starts from April to June, young are active by June.

Spalerosophis diadema (Fig.24)

Localities:1,2,10,16,19,20,23,35,36,39.

Status:common.

Notes:The diademe snake is the common rat snake of the area. It confines itself around human sattlements, attracted by rats and mice.Breeding season starts by March-April, young are active by

July-August.

Ptyas mucosus

Localities:4,16,20,30,33,35,38,45,50,55, 80,82,84,87,88.

Status:common.

Notes: Like S. diadema, this snake also live close to the human sattlements. Breeding starts with the onset of summer, young are active by June-July.

Oligodon arnensis

Localities:2,79,87.

Status: rare.

Notes: The Kukri snake is rare in the area. Breeding snakes are observed during May-July, young are seen during August-September.

Lycodon striatus (Fig. 25)

Localities:31,32,36,82,87.

Status: rare.

Notes: The wolf snake is seen breeding during April-May, young are active by June-July.

Psammophis schokari

Localities:32,34,35,84.

Status: rare.

Notes: This sand snake breeds during April-May, young are seen during July-August.

Family ELAPIDAE

Bungarus caeruleus (Fig.26)

Localities:10,19,30,31,36,54,57,58,66,81,28,29,30.

Status: common.

Notes: The common krait lives in the damp area around tilled fields Reproductive activity takes place by April-June, young are to be seen during July-August.

Naja naja (Fig. 27)

Localities: 31,86,88.

Status: rare.

Notes: The black cobra, lives around damp irrigated land. It is attracted by domesticated birds, and often killed in houses. Breeding pairs are seen from April to June, young are seen by July, which are all black.

Naja oxiana (Fig. 28)

Localities: 2,4,10,17,37,42,45,47,52,73,76,88.

Status: common.

Notes: The variegated cobra is quite common in the area . It lives in barren land with sparse vegetation. Breeding pairs are seen during March-May, young are active by May-September, which have cross striped pattern on body.

Family VIPERIDAE

Echis carinatus (Fig. 29)

Localities: 6,8,13,23,26,33,35,43,53,74,83,84.

Status: common.

Notes: The saw scaled viper inhabits barren areas with sparse vegetation. Breeding takes place during winter. Young ones are born by March-April.

Vipera russelli (Fig. 30)

Localities:75,78,81,83.

Status: rare.

Notes: This large chain viper lives in humid areas along main water courses in the upper part of the tableland. Young are born by July-August.

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LIST OF COLLECTION CITES

Number against localities refer to the position in MAP 1.

Locality	No.
Lilla	1
Goalpur	2
Haranpur	3
Pind Dadan Khan	4
Khewara	5
Dandot	6
Gharibwal	7
Buchal Kalan	8
Choa Saidan Shah	9
Kallar Khar	10
Bhawn	11
Dhudial	12
Phagwal	13
Kariala	14
Duman	15
Jogi Tilla	16
Rohtas Fort	17
Dina	18
Jhelum	19
Mangla Dam	20
Tarki	21
Sohawa	22
Chakwal	23
Cha Ganga	24
Nurpur	25
Duelmial	26

Locality	No.
Katas	27
Jandala	28
Rehana	29
Mahmood Abad	30
Hazro	31
Attock City	32
Hasan Abdal	33
Bahtar	34
Feteh Jhang	35
Jaffer village	36
Jhangir Dhari	37
Malal	38
Khanda	39
Nara	40
Basal	41
Pind Sultan	42
Pindi Ghaib	43
Fort Sarang	44
Dhok Patan	45
Talakang	46
Chinji	47
Darnal	48
Timmin	49
Pachand	50
Jebbi	51
Torab	52

Lakri Mar	53
Khor	54
Attock Khurd	55
Lawrence pur	56
Rawalpindi	57
Islamabad	58
Tret	59
Ghora Gali	60
Murree	61
Bann	62
Surba	63
Kotli Rattian	64
Lahtrar	65
Pahjar	66
Karor	67
Kahuta	68
Choa Khalsa	69
Salgran	70
Kallar	71
Chal Lala	72
Qazian	73
Golra	74
Rawat	75
Mendra	76
Changrilla	77
Gujar Khan	78
Kallar	79
Taxilla	80
Murat	81
Chontra	82
Banda	83
Sakkhu	84
Jatli	85
Chakkari	86
Chak Beli Khan	87
Sayyed Kasran	88

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LEGEND TO THE PHOTOGRAPHS

Photograph No.	Legend
1.	Eublepharis macularius
2.	Hemidactylus flaviviridis
3.	H. Persicus
4.	Tenuidactylus indusoani
5.	T. montiumsalsorum
6.	T. watsoni
7.	Cyrtodactylus dattanensis
8.	Eumeces taeniolatus
9.	Leiolopisma himalayana
10.	Mabuya dissimilis
11.	Ablepharis pannonicus
12.	Acanthodactylus cantoris
13.	Eremias guttulata
14.	Ophisophis jerdoni
15.	Agama melanura
16.	A. agilis
17.	Uromastyx hardwickii
18.	Varanus bengalensis
19.	Typhlops braminus
20.	Boiga trigonata
21.	Coluber rhodorachis

22. Amphiesma stolata
23. Xenochrophis piscator
24. Spalerosophis diadema
25. Lycodon striatus
26. Bungarus caeruleus
27. Naja naja
28. N. oxiana
29. Echis carinatus
30. Vipera russellii

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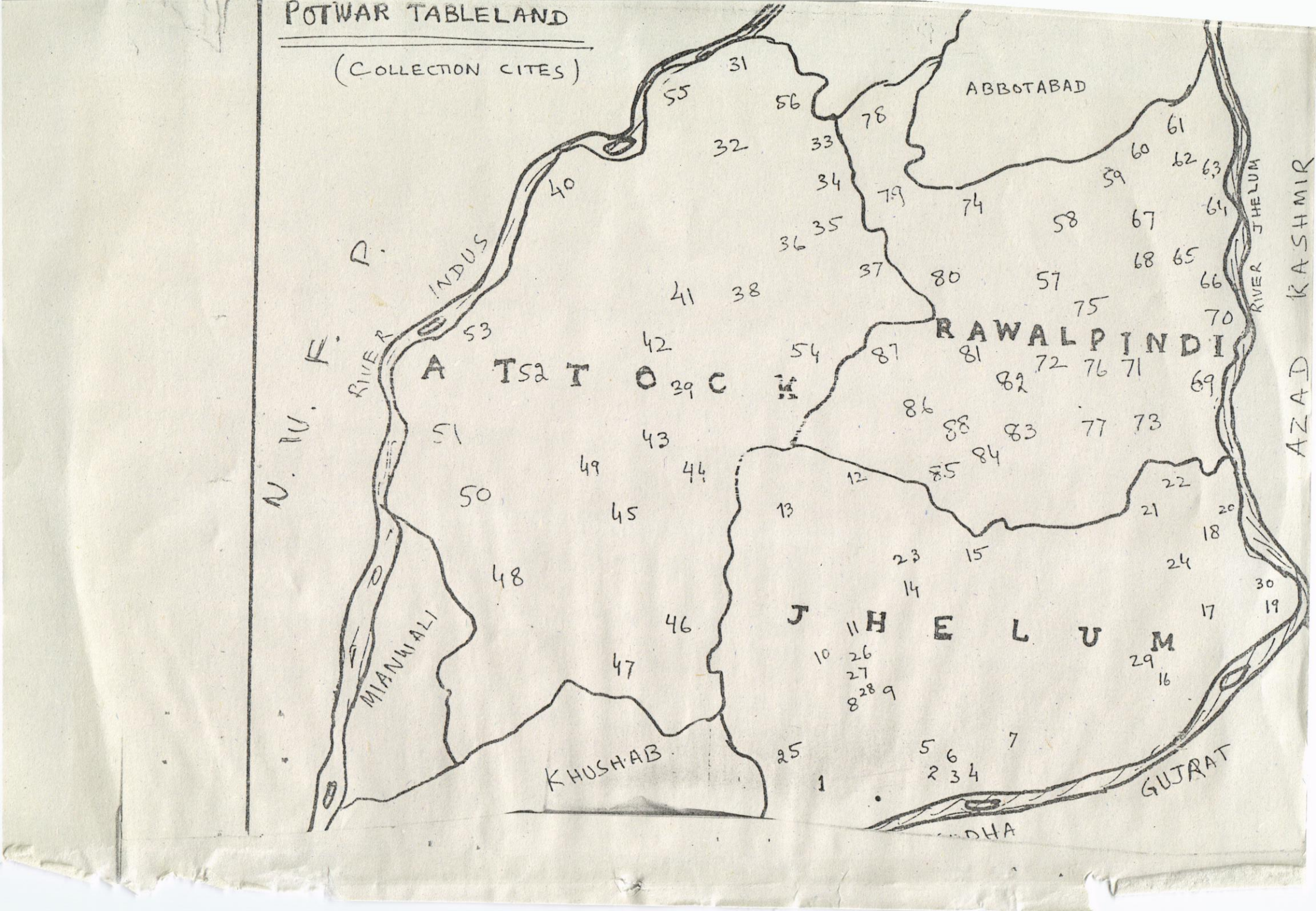
POTWAR TABLELAND
(COLLECTION CITES)
ABBOTABAD
RIVER JHELUM
AZAD KASHMIR
N. W. F. P.
RIVER INDUS
ATTOCK
RAWALPINDI
JHELUM
MIANWALI
KHUSHAB
GUJRAT