March 2002

Epidermal Growth Factor Receptor (EGFR) as a Prognostic Marker: an Immunohistochemical Study on 315 Consecutive Breast Carcinoma Patients

S. A. Aziz
Aga Khan University

Shahid Pervez
Aga Khan University

N. Kayani
Aga Khan University

M. H. Rahbar
Aga Khan University

S. Khan
Aga Khan University

Epidermal Growth Factor Receptor (EGFR) as a Prognostic Marker: an Immunohistochemical Study on 315 Consecutive Breast Carcinoma Patients

S. A. Aziz,S. Pervez,N. Kayani,M. H. Rahbar (Departments of Pathology,and Community Health Sciences, The Aga Khan University, Karachi.)
S. Khan (Departments of Surgery and Community Health Sciences, The Aga Khan University, Karachi.)

Abstract

Objective: To assess the independent and interdependent prognostic value of epidermal growth factor receptor (EGFR) in carcinoma of breast in female population. The Type I family of growth factor receptors includes epidermal growth factor receptor (EGFR also known as EGFRI).
Methods: The expression of EGFR protein was analysed immunohistochemically on 315 tumour specimens of infiltrating ductal carcinoma of breast. These patients also had axillary lymph nodes sampling.
Results: Overexpression and/or amplification of EGFR was observed in 70 (22.00%) tumours. Eleven (16%) were grade I, 43 (61%) grade II and 16 (23%) grade Ill tumours. Axillary lymph node metastasis had significant correlation with intensified positivity of EGFR ($p< 0.05$). Significant number of EGFR positive patients developed local recurrence and distant metastases to brain, liver and bone ($p< 0.05$). EGFR positivity showed significant correlation with the disease free and overall survival ($p< 0.05$). At a median follow-up of48 (4 years) months in EGFR positive patients, the overall survival was 3.39 years and disease free survival was 2.86 years. EGFR negative tumour patients showed a better survival. In this group the overall survival was 4.62 years and the disease free survival was 4 years.
Conclusion:EGFR analysis can be a useful indicator for the selection of patients who are at the high risk, for hormonal therapy decisions and can be useful as a target for new treatment modalities (JPMA 52:104; 2002).

Introduction

In an attempt to reliably predict biological behaviour of various malignant tumors including breast cancer, several novel prognostic markers were conceived and rigorously tested in the last two decades. Epidermal growth factor receptor (EGFR) was among the first in this plethora of new prognostic markers.
EGF is a growth-promoting agent found in human milk and high plasma and tissue levels are found at the times of breast development and maturation. EGF stimulates cell proliferation by high affinity binding to a specific EGF receptor. The EGFR is a 170-kDa-membrane protein[1] comprising an external domain, a transmembrane domain, and a cytoplasmic domain[2]. One of the actions of EGFR is to stimulate auto-phosphorylation of the membrane receptor, an action similar to that of the oncogenic viral-coded proteins

such as pp6O.
EGFR antibody reacts with the squamous cell carcinoma arising from the squamous epithelium of skin of the cervix and metaplastic squamous areas like lung cancer[3-5]. EGFR has also been identified in both breast cancer cell lines and primary breast cancer specimens[3,6-9]. EGFR status has been shown to be an important risk factor for recurrence of breast carcinoma and predictor of both relapse free and overall survival[9].
Over-expression of the EGFR is a hallmark of numerous solid tumours, thus providing means of selectively targeting therapeutic agents. The type 1 receptor family comprises the prototype EGFR (also known as EGFR 1) and proteins encoded by C-erbB-2, C-erbB-3, and C-erbB-4 genes. Overexpression of EGFR I and C-erbB-2 proteins has been described in various human carcinomas and generally reported as an adverse prognostic marker[10].
This study was done to assess the independent and interdependent prognostic value of EGFR in carcinoma of breast.

Material and Methods

A sample of 315 patients with histologically proven diagnosis of invasive ductal carcinomas (IDC) of breast with lymph nodes sampling from January 1992 to December 1997 were included in this study. Based on available information we assumed a difference of 1.5 years in survival time with EGFR positive and EGFR negative patients. The sample size of 315 was expected to detect this difference with a power of at least 90% at 5% level of significance.
Morphological variables like age, grade, carcinoma type, vascular / lymphatic invasion, lymph node status and tumour size were recorded. Other variables like age menopausal status, parity, distant metastasis; treatment protocol and survival details were retrieved from their medical records. Fixation and processing was done by routine method. After processing, the tissues were embedded in paraffin using the Histocenter 2 from Shandon. 5U thick sectioning was done by Microtome AS 325 from Shandon. The same breast tumour paraffin blocks were used to make further sections for immunohistochemistry. The sections were cut and picked on poly-L-Lysine coated slides. Expression of EGFR protein was evaluated using mouse Anti-Human EGFR protein monoclonal antibody EGFR (DAKO, Denmark) diluted at 1/25, following pre-treatment of sections in a microwave oven (5x3 min. at 630 W) using PAP technique. A breast carcinoma section expressing EGFR was used as a positive control. Same case omitting the primary antibody was used as a negative control with each staining procedure.
The percentage of EGFR positive tumour cells was estimated semi-quantitatively and they were graded on a scale of 0-3 (0%, <10%, 10-50% and >50%) and the intensity of reaction (-, +, ++, +++ or ++++)12. To minimise the subjectivity, slides were scored on a double-headed microscope (Olympus BX5O) separately by two senior histopathologists. Immunostaining was repeated on equivocal cases and consensus was achieved between the two pathologists in all cases.

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 Kaplan Meier estimator is an important tool for analysing 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.
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 95% CI were used to describe the relationship between each prognostic variable and the outcome variable.
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 95% CI was used to describe the relationship between the set of prognostic variables and the outcome variable.

Results

Table 1 provides the descriptive statistics about the sample. Analysis was done on a total number of 315 observations, with 36.2% survivals till the end of this study, i.e. May 1999.

Table 1. Summary survival data for prognostic factors associated with survival in patients with breast carcinoma.

Variables		Total cases		Median Survival time	(SE)	Mean survival time	(SE)
		No.	%				
Age	< 49 years	161	51.11	3.58	(.18)	3.35	(.13)
	≥49 years	154	48.99	3.00	(.17)	3.17	(.14)
Grades	I	45	14.28	3.17	(.25)	3.11	(.22)
	II	214	67.93	3.33	(.16)	3.41	(.13)
	III	56	17.77	2.67	(.27)	2.91	(.23)
Tumour size	<2 cms.	68	21.58	3.50	(.18)	3.26	(.17)
	2-5 cms.	175	55.55	3.33	(.16)	3.32	(.13)
	> 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)
EGFR graded on a scale	Negative	245	77.71	3.00	(.18)	4.44	(.13)
of 0-3 (0/+/++/+++)	Mild +ve	28	8.88	3.33	(.16)	3.52	(.34)
	Moderate +ve	38	12.06	3.00	(.17)	3.11	(.18)
	Strong +ve	4	1.26	3.17	(.25)	3.06	(.39)
Stages	Stage I	38	12.00	3.58	(.27)	3.45	(.24)
	Stage II	114	36.20	3.75	(.39)	3.65	(.16)
	Stage III	128	40.63	2.50	(.12)	2.89	(.14)
	Stage IV	35	11.11	3.08	(.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)
Chemotherapy	None	80	25.4	3.00	(.17)	3.11	(.15)
	Yes	235	74.6	3.33	(.16)	3.32	(.12)
Metastasis	None	211	67	3.58	(.14)	3.50	(.13)
	Yes	104	33	2.42	(.13)	2.86	(.15)

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 occut at a relatively younger age (approx. 10 years earlier than the Western world) with the incidence being 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 (SE0.14). 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.

Histological grading showed a median survival time of 3.17 years and 3.33 years for the subjects with grade 1 and grade II Tumors, with corresponding mean survival time of 3.11 years and 3.41 years (SE0.22). Those with grade Ill tumours had median survival times of 2.67 years (mean 2.91). When the prognostic marker EGFR was absent, the mean and median survival time was found to be significantly better. Mean overall survival in EGFR negative patients were 4.62 years compared to when this markers was strongly positive 3.39 years. For staging we used the TNM (tumour, node, metastasis)

classification. A better survival for the subjects in an early stage is seen, with a mean survival of 3.45 years (SE=0.24) and median survival time 3.58 years for stage I in comparison to the mean survival of 2.86 years (SE=0.28) and median survival time 3.08 years for stage IV. The chemotherapy and hormonal therapies both appear to improve the prognosis by improving survival, since better mean and median survival times in subjects were observed among patient who are on these therapies in comparison to those who did not receive the intervention. The presence of metastatic lesions in any organ of the body was negatively associated with the prognosis as median survival time for subjects with metastasis was 2.42 years (mean 2.86) when compared to the median survival time for subjects without metastasis 3.58 years (mean 3.5). Similarly with increasing tumour size, the prognosis appears to worsen, with better mean and median survival times among subjects with smaller lesions. Controlling all potential contributors, the effect of EGFR on survival time was still significant.

Clinical, histopathological and immunohistochemical characteristics

The histopathological characteristics of the tumours are listed in Table 2.

Table 2. EGFR overexpression and histological characteristics.

Parameters	Total patients		EGFR protein over expression graded on a scale of 0-3 (0/+/++/+++)				P- value
			Negative (245)		Positive (70)		
Grade	No.	%	No.	%	No.	%	
I	45		34	14	11	16	0.0012
II	214		171	70	43	61	
III	56		40	16	16	23	
Tumour size							
< 2 cm.	68	21.5	22	09	46	66	0.0236
2-5 cm.	175	55.5	166	68	09	13	
> 5 cm.	72	22.8	57	23	15	21	
Axillary lymph nodes							
Negative	145	46.0	116	47	27	39	0.0163
Positive	170	54.0	129	53	43	62	
ER/PR status (Negative)	112		60	24	52	74	0.0326
ER/PR (Positive)	203		185	75	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

All of them were IDC. Regarding histological grade, 45 (14%) tumours were well differentiated, 214 (68%) were moderate, and 56 (18%) were poorly differentiated carcinomas. According to size, tumours were divided into three categories of <2 cm 68 (21.5%), 2-5 cm 175 (55.5%) and > 5 cm 72 (23%), in diameter. Positive axillary lymph nodes status was observed in 170 (54%), while negative axillary lymph node status was observed in 145 (46%) subjects.

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 2. Stain intensified positivity was dominated by ++ moderate 38 (54%) followed

by + mild positive 28 (40%) and +++ or ++++ strong positive 4 (6%) (Figure 1).

Figure 1. Section of the Carcinoma breast stained with a monoclonal antibody against EGFR. Note strong (+++) membrane staining of all tumour cells. Mag = 20X.

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), tumour 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). Brain, liver and bone metastases were seen in strong EGFR positive cases with a p value of 0.0041, 0.0482 and 0.0352 respectively. Vascular/lymphatic invasion was identified in 37 (53%) of EGFR positive cases. There was significant difference with a p value of 0.0162.

Survival analysis

After a median follow-up of 48 months (range 3 to 73 months), the overall survival of breast cancer patients amounted to 75%. In univariate as well as multivariate analyses EGFR overexpression had a significant influence on survival (Tables 3 and 4).

Table 3. 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
Tumour 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	0.0309	0.7618

Table 4. Prognostic value of EGFR protein overexpression in tumour subset analysis (multivariate p value /Hazard rates, 95% confidence limits are only given if EGFR remained an independent factor in Cox's analysis).

Factor	EGFR protein overexpression	
	Univariate analysis P value	Multivariate analysis (p value)/Hazard rate (95% confidence limit)
Grade		
I	NS	
II	NS	
III	0.0380	
Tumour size		
< 2cm	NS	
>2 cm	0.0126	0.0007/1.481 (1.13-1.97)
Axillary lymph nodes		
Negative	NS	
Positive	0.0001	0.0341/1.406 (1.04-1.91)
ER/PgR positive	0.0721	
ER/PgR negative	0.0326	
ER positive	NS	0.0361/1.456 (1.03-2.18)

Overall survival rates amounted to 78% and 43% in patients with negative and positive EGFR protein overexpression in tumours (Figure 2).

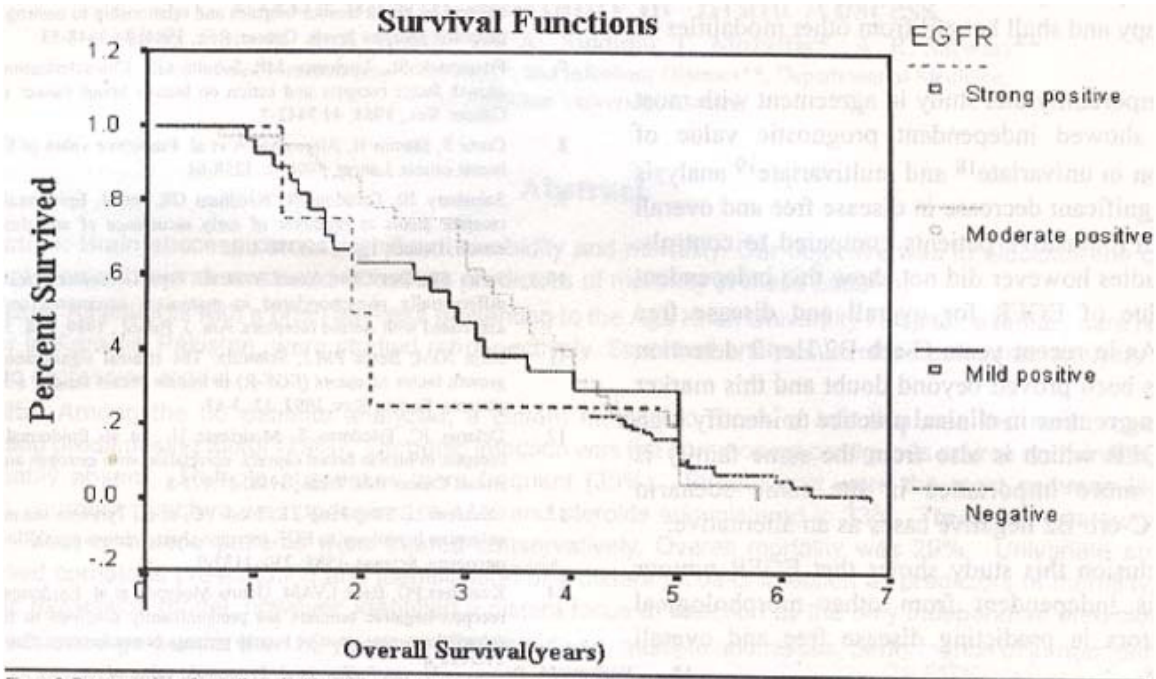

Figure 2. Representation of overall survival in relation to EGFR status correlate with the significant finding. The intensity of positivity reveals a ladder formation with 75% EGFR positive patients died with an overall survival of 3.39 years.

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 tumour 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 4), tumour size larger than 2 cm and ERJ PgR negativity.

Axillary lymph nodes negativity and EGFR positivity was seen in 30 (43%) cases, with a mean tumour size of 3.6 cm, 57% cases were negative for ERIPgR, with an overall survival of 2.9 years and disease free survival of 2.5 years. Whereas axillary lymph nodes positivity and EGFR positivity was seen in 40 (57%) cases, with a mean tumour size of 5.0 cm, 68% cases were negative for ER/PgR, with an overall survival of 2.5 years and disease free survival of 2.3 years. Statistically there was a significant correlation with a p value of 0.0431.

Multiva riate analysis of survival

In a Cox proportional hazard model of all patients there was a significant influence on overall survival for EGFR positivity (p value 0.0045), tumour size (p value 0.0026) and histological grade 111 (p value 0.0012) when assessed with axillary lymph nodes and ERJPgR status. In multivariate analysis, the independent prognostic factors for breast cancer patients were tumour size, axillary lymph nodes invo1vement, histological grade, EGFR overexpression and ER /PgR status. In axillary lymph nodes positive group for overall survival, EGFR positivity, and tumour size >2cm and hormonal status are significant (p value 0.0012). When examining the axillary lymph node negative subgroup, we find EGFR to be a 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 (Figure 2).

Discussion

The distribution of EGFR (EGFR 1) in breast cancers has diversely been reported in the literature. In our study we found that 22% (n=70/3 15) of the tumours were EGFR positive. Based on 40 separate studies comprising 5232 patients[11], the mean percentage of EGFR positivity reported in breast cancer is 45% (range 14-91%). Overall, there is no clear difference in results between radioligand binding assays, immunological methods, autoradiography, and measurement of EGFR transcripts although EGFR positivity by immunological methods tends to be lower[12]. Nonetheless, the rate of EGFR positive tumours may vary and likely depend upon the method used. The C-erbB-2 oncogene is related to but distinct from the EGFR gene. The EGFR gene is located on the band p1 i-p 13 of chromosome 7 and the C-erbB-2 gene on q21 of chromosome 17[13].
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[14]. No relationship was found between the tumour EGFR immuno-reactivity and the patient's age, in agreement with a previous report[15]. We did observe like others,[16] significant correlation between the tumour EGFR immuno-reactivity and the tumour size. In our study there was also a significant correlation between positive lymph nodes and positive EGFR (Table 2), this is in consensus with most studies but in contrast to some other studies reported previously[17].
Estrogens are involved in the release of growth factors and may mediate the tumour cells response to growth factors such as EGF. A significantly higher proportion of EGFR positive tumours are estrogen receptor negative and fails to respond to endocrine therapy as evident by the significant poor overall and disease free survival. In this study we did find significant correlation with EGFR positivity and hormonal negativity, most of the other studies have demonstrated the same[6,11,14]. Therefore, the evaluation of tumour 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[18] and multivariat[19] 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 and disease free survival[20,21]. As in recent years C-erb B2/Her 2 detection importance has been proved beyond doubt and this marker is now becoming routine in clinical practice to identify high risk group, EGFR which is also from the same family is gaining even more importance in the same scenario particularly in C-erb B2 negative cases as an altemative.
In conclusion this study shows that EGFR tumour 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 tumour 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.

References

1.Cohen 5, Ushiro H, Stoscheck C, et al, A native 170,000-epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. J. Biol. Chem., 1982; 257:1523-31.
2.Downward J, Mayea E, Scrace 0, et al. Close similarity of epidennal growth factor receptor and V-erb-B oncogene protein sequences. Nature, 1984; 307: 521-27.
3.Ozanne B, Richards C5. Overexpression of the EGF receptor is a hallmark of squamous cell carcinomas. J. Pathol., 1986; 149: 9-14.
4.Tungekar MF, Turley H, Dunnill MS, et at. Interleukin 4 receptor expression on human lung tumours and normal lung. Cancer. Res., 1991; 51: 261-4.
5.Cerny T, Barnes DM, Hasleton P, et al. Expression of epidermal growth factor (EGF-R) in human lung tumours. Br, J. Cancer, 1986; 54:265-9.
6.Fitzpatrick SL, Brightwell J, Wittliff JL, et al. Epidermal growth factor binding by breast tumour biopsies and relationship to oestrogen receptor and progestin receptor levels. Cancer. Res., 1984; 44:3448-53.
7.Fitzpatrick 5L, Lachance MP, Schultz OS. Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture, Cancer, Res., 1984; 44:3442-7.
8.Costa 5, 5tamm H, Almendral A et al. Predictive value of EGF receptor in breast cancer, Lancet, 1998; 1: 1258-64.
9.Sainsbury JR, Farndon JR, Needham GK et al. Epidermal growth factor receptor status as predictor of early recurrence of and death from breast cancer. Lancet, 1987; 1: 1398-402.
10.5arah SB, Dot Chin, Yosef Y et at. Type I-receptor tyrosine kinases are differentially phosphorylated in mammary carcinoma and differentially associated with steroid receptors. Am. J. Pathol., 1989; 148: 549-58.
11.Kltjn JGM. Bcrns PM.J, Schmitiz, The clinical significance of epidermal growth factor receptors (EGF-R) in human breast cancer: a review on 5232 patients. Endoer. Rev, 1992; 13: 3-17.
12.Delarue JC, Friedman 5, Mouriesse H, et al. Epidermal growth factor receptor in human breast cancers: correlation with estrogen and progesterone. Breast Cancer Res. Treat., 1988; 11:173-8.
13.Coussens L, Yang-Feng TI. Liao YC, et al. Tyrosinc kinase receptor with extensive homology to EGF receptor shares chromosomal location with ncu oncogene. Scicnce, 1985; 230: 1132-9.
14.Koenders PG. Beex LVAM, Gcurts-Mocspot, et al. Epidermal growth factor receptor-negative tumours are predominantly confined to the subgroup of estradiol receptor-positive human primary breast cancers. Cancer. Res., 1991; 51:4544-8.
15.Foekens JA, Portengen H, van Putten, et al. Prognostic value of receptor for insulin-like growth factor 1, somatostatin, and epidermal growth factor in human breast cancer, Cancer. Res., 1989 49: 7002-9.
16.Wright C, Aiigus B, Nicholson S, et al. Expression of EGFR 1 oncoprotcin: a prognostic indicator in human breast cancer, Cancer. Res., 1989; 19: 2087-90.
17.Pirinen R, Lipponen P, Syrjanen K. Expression of epidennal growth factor receptor (EGFR) in breast cancer as related to clinical, prognostic and cytometric factors.

Anticancer Res., 1995; 15: 2835-40.
18.Ncwby JC, AHern RP, Leek RD. et al. lmmunohistochcmical assay for epidermal growth factor receptor on paraffin-embedded sections: validation against ligand-binding assay and clinical relevance in breast cancer. Br. J. Cancer,l995; 71: 1237-42.
19.Torregrosa D, Bolufer P, Lluch A, et al. Prognostic significance of c-erbB2/nen amplification and cpidermal growth factor receptor (EGFR) in primary breast cancer and their relation to estradiol receptor (ER) status. Clin. Chim. Ada., 1997; 262: 99-119.
20.Mon 1, Monmoto 1, Komaki K, et al. Comparison of estrogen receptor and epidermal growth factor receptor content of primary and involved nodes in human breast cancer. Cancer, 1991; 58: 532-37.
21.Gerstein ES, Muaviia MA, Letiagin VP, et al. Prognostic significance of epidermal growth factor receptors in stage I-li breast cancer: results of a six-year follow-up. Vopr Onkol., 1998; 44: 383-9.