CHAPTER 8

The epidermal growth factor receptor pathway in relation to pelvic lymph node metastasis and survival in early stage cervical cancer

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ABSTRACT

Objective: To correlate the expression of EGFR components with clinical behaviour of early stage cervical cancer.

Patients and Methods: Tissue samples of 336 consecutive FIGO stage IB-IIA cervical cancer patients all treated primarily by radical surgery were collected. Clinico-pathological and follow-up data were prospectively obtained during standard treatment and follow-up. As representants for the EGFR pathway, expression of EGFR, pEGFR, PTEN, pAKT, and pERK was assessed by immunohistochemistry on tissue microarrays (TMAs).

Results: Positive immunostaining was observed for EGFR in 32.1%, for pEGFR in 21.0%, for PTEN in 38.3%, for pAKT in 5.3% and for pERK in 4.3% of tumour samples. Positive EGFR immunostaining was associated with squamous cell carcinoma of the cervix (OR=7.41, 95% CI=3.38-16.23, p<0.001), negative pEGFR immunostaining with poor differentiation (OR=0.39, 95% CI=0.20-0.73, p=0.004) and negative PTEN immunostaining with metastatic pelvic lymph nodes (OR=0.51, 95% CI=0.30-0.90, p=0.019). In multivariate analysis only pelvic lymph node metastasis (HR=6.11, 95% CI=3.46-10.77, p<0.001) and poor differentiation (HR=1.91, 95% CI=1.12-3.26, p=0.018) were related to disease specific survival.

Conclusion: In early stage cervical cancer loss of PTEN expression is associated with pelvic lymph node metastasis, suggesting PTEN to be one of the tumour suppressor genes affecting pelvic lymph node metastasis. However, expression of EGFR pathway components does not appear to have prognostic impact in surgically treated early stage cervical cancer.

INTRODUCTION

Early stage cervical cancer is generally treated by radical hysterectomy and pelvic lymph node dissection. In cases with poor clinico-pathological factors, adjuvant radiotherapy with or without chemotherapy is often administered. Conventional prognostic factors in early stage cervical cancer are: tumour size, depth of stromal invasion, lymphovascular space involvement, parametrial invasion and pelvic lymph node metastasis [1-4]. Pelvic lymph node metastasis appears to be the most important of these parameters [5], with a five-year survival approximating 90% in node negative early stage cervical cancer patients primarily treated with surgery and decreasing to approximately 65% in patients with pelvic lymph node metastasis [6]. In early stage cervical cancer, molecular markers could be helpful in selecting lymph node negative patients with an unfavourable prognosis for adjuvant treatment and might identify new targets for patient-tailored therapy.

The epidermal growth factor receptor (EGFR) is involved in the ErbB signalling network, which is often deregulated in cancer. Autophosphorylation of EGFR to pEGFR leads to activation of the Ras/Raf/MEK/ERK pathway and the PI3K/AKT pathway, both of which are involved in processes that are associated with carcinogenesis and tumour progression, such as inhibition of apoptosis, cell migration, cell growth, and angiogenesis [7]. PTEN (phosphatase and tensin homolog deleted on chromosome ten) acts as a tumour suppressor gene by inhibiting phosphorylation and thereby activation of AKT [8,9]. Only few data, obtained in small series of cervical cancer patients primarily treated by surgery, exist on EGFR or PTEN expression [10-13] and no published studies provide a comprehensive analysis of several EGFR pathway components simultaneously in a well-defined series of early stage cervical cancer patients.

Previous studies of molecular markers in early stage cervical cancer have been limited by the small number of patients evaluated and/or mediocre documentation of clinico-pathological parameters. The present study was designed to correlate expression of EGFR, pEGFR, PTEN, pAKT and pERK in relation to clinico-pathological parameters and disease specific survival in a large, well-documented series of patients with early stage cervical cancer and long-term follow-up.

PATIENTS AND METHODS

Patients and treatment

Since 1980, clinico-pathological characteristics and follow-up data of all cervical cancer patients referred to the Department of Gynaecologic Oncology of the University Medical Center Groningen have been prospectively entered into a computerized database. Clinical staging of each patient

is performed under general anaesthesia in accordance with the International Federation of Gynaecology and Obstetrics (FIGO) criteria. For the present study we selected all early stage cervical cancer patients, treated primarily by surgery between January 1980 and December 2004 from our database (n=336). All patients underwent type 3 radical hysterectomy and pelvic lymph node dissection. Patients with pelvic lymph node metastases, parametrial invasion or positive excision margins received adjuvant external beam radiotherapy with or without chemotherapy. Paraffin-embedded formalin-fixed primary tumour tissue was collected from each patient. Patients were only included in our analysis, if sufficient, representative tumour tissue was available for TMA construction. After completion of treatment, patients were followed up at the outpatient clinic for at least 5 years.

Institutional Review Board approval

In the University Medial Center Groningen clinico-pathologic and follow-up data are prospectively obtained during standard treatment and follow-up and stored in a computerized registration database. For the present study, all relevant data were retrieved from this computerized database into a separate, anonymous database. Patient identity was protected by study-specific, unique patient numbers. Codes were only known to two dedicated data managers, who also have daily responsibility for the larger database. In case of uncertainties with respect to clinico-pathologic and follow-up data, the larger databases could only be checked through the data managers, thereby ascertaining the protection of patients' identity. Using the registration database all tissue specimens were identified by unique patient numbers and retrieved from the archives of the Department of Pathology. Therefore, according to Dutch law no further Institutional Review Board approval was needed for this study (http://www.federa.org/).

Tissue Microarray (TMA) construction

As previously described, representative areas of tumour were marked on haematoxylin- and eosin- (H&E) stained slides of the paraffin-embedded tissue [14]. Areas of necrosis and/or heavy leucocytic infiltrate were avoided. The TMAs were constructed using a precision instrument (Beecher Instruments, Silver Spring, Maryland). Three 0.6 mm in diameter cores were punched from the marked area of the paraffin-embedded tissue (donor block) and transferred to a predefined location in a blank paraffin block (recipient block). After all the cores had been inserted, the recipient block was placed in an oven of 37°C for 15 minutes to attach the cores to the surrounding paraffin. Each TMA also contained benign (skin epithelia, normal cervical tissue and colon polyps) and tumour (breast, colon and ovarian carcinoma) tissue that served as controls for immunostaining and comparison of TMAs. In total 5 TMAs were constructed.

Immunohistochemistry

For immunohistochemistry, 4 µm sections were cut from the TMA and mounted on amino-propyl-ethoxy-silan (APES, sigma-Aldrich, Diesenhofen Germany)-coated glass slides. Immunohistochemistry for EGFR, pEGFR, PTEN, pAKT and pERK was performed as described previously [15]. Details of the antibodies used for immunohistochemistry and methods for antigen retrieval are summarized in table 1. The avidin-biotin-peroxidase method was utilized for all antibody detection, except pAKT for which the EnVision horseradish peroxidase system (Dako, Copenhagen, Denmark) was used. Slides were deparaffinised in xylene and rehydrated in ethanol. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxidase for 30 minutes. For stainings in which the avidin-biotin-peroxidase method was used, endogenous avidin and biotin activity was blocked using a blocking kit (Vector Laboratories, Burlingame, UK). Immunostaining was visualized by 3'3-diaminobenzidinetetrahydrochloride and counter immunostaining was performed with haematoxylin.

Table 1 | Antibodies utilized for immunostaining

Antigen	Antigen retrieval	Clone	Company	Dilution	Incubation time
PTEN	Citrate (pH 6)[1]	6H2.1	Cascade[2]	1:100	60 minutes
EGFR	Proteinase K 0.1%, 30 minutes	111.6	Neomarkers[3]	1:200	60 minutes
pEGFR	EDTA (pH 8)[1]	1H12	Cell Signaling[4]	1:200	60 minutes
pAKT 1/2	Citrate (pH 6)[1]	736E11	Cell Signaling[4]	1:50	overnight (4°C)
pERK 1/2	Citrate (pH 6)[1]	20G11	Cell Signaling[4]	1:50	overnight (4°C)

1. Sections were boiled in a microwave for 15 minutes
2. Cascade Bioscience, Winchester, USA
3. Neomarkers, Lab Vision Corporation, Fremont, USA
4. Cell signaling, Danvers, USA

Evaluation of immunostaining

Scoring was performed by two independent observers (JJHE, MGN) without knowledge of clinical data. A concordance of more than 90% was found. The discordant cases were reviewed and scores were reassigned on consensus of opinion. Immunostaining intensity was semi quantitatively scored. Only patients with at least two representative cores were included in the analysis. Tumours were considered positive for EGFR in when >10% positive membranous immunostaining was observed [12]. pAKT and pERK immunostaining were considered positive if >10% of a tumour showed cytoplasmic and/or nuclear immunostaining [15]. Positive expression

of PTEN was defined as >10% cytoplasmic immunostaining [16]. Positive pEGFR was defined as at least weak positive cytoplasmic immunostaining, as the activated EGFR is internalized [17].

Statistical analysis

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). Associations (Odds Ratios and 95% CI) between immunostaining intensity and clinico-pathological characteristics were assessed in a univariate logistic regression model using positive protein expression as dependent factor and the clinico-pathological characteristics as independent factors. Relations (Hazard Ratios and 95% CI) between disease specific survival, clinico-pathological features and immunostaining were calculated using both univariate and multivariate Cox proportional hazard analysis. In these analyses, factors with a p value >0.10 in the univariate analyses were excluded stepwise in multivariate analyses. P values of <0.05 were considered statistically significant.

RESULTS

Patients and tumour characteristics

In total 336 patients diagnosed with early stage cervical cancer (IB1: n=221 (66%); IB2: n=, 63 (19%); IIA: n=52 (15%)) and treated by radical hysterectomy and pelvic lymph node dissection were identified. In 310 cases sufficient pre-treatment tissue was available for TMA construction. Median age was 43 years (range 17-86 years) and median follow-up time was 5.5 years (range 0.31-18.60 years). Overall 5-year disease specific survival for the 310 patients was 82.4%. Additional patient and tumour characteristics are summarized in table 2.

Clinico-pathological factors in relation to EGFR, pEGFR, PTEN, pAKT and pERK

The proportion of patients included in the analysis based on two or more representative cores was 93.5% for EGFR, 93.5% for pEGFR, 91.0% for PTEN, 90.7% for pAKT and 89.1% for pERK. Figure 1 shows a representative negative and positive core for each staining. Positive EGFR immunostaining was observed in 93/290 (32.1%) patients, positive pEGFR immunostaining in 61/290 (21.0%) patients, positive PTEN immunostaining in 108/282 (38.3%) patients, positive pAKT immunostaining in 15/281 (5.3%) patients and positive pERK immunostaining in 12/276 (4.3%) patients. Positive pEGFR immunostaining correlated with PTEN (OR 2.454, 95% CI 1.367-4,403, p=0.003) and with pERK (OR 4.468, 95% CI 1.380-14.468, p=0.013). No additional correlations between the immunostains were found (data not shown).

Table 2 | Patients and tumour characteristics.

	N=310 N (%)
FIGO stage	
IB1	197 (64%)
IB2	63 (20%)
IIA	50 (16%)
Treatment	
WM	188 (61%)
WM + radiotherapy	106 (34%)
WM + chemo-radiotherapy	16 (5%)
Tumour type	
Squamous carcinoma	200 (65%)
Adenocarcinoma	87 (28%)
Other	23 (7%)
Differentiation grade	
Good/moderate	180 (58%)
Poor	124 (40%)
Unknown	6 (2%)
Lymphangioinvasion	
Yes	160 (52%)
No	149 (48%)
Unknown	1 (0%)

	N=310 N (%)
Depth of invasion	
0-10 mm	165 (53%)
≥10 mm	131 (42%)
Unknown	14 (5%)
Margins	
Negative	299 (96%)
Positive	11 (4%)
Lymph nodes	
Negative	222 (72%)
Positive	88 (28%)
Tumour diameter	
0-4 cm	223 (72%)
≥ 4 cm	87 (28%)
Recurrence	
No	245 (79%)
Local-regional	38 (13%)
Distance	14 (5%)
Progression	4 (1%)
Unknown	9 (5%)

WM= Wertheim Meigs

Table 3 shows clinico-pathological features in relation to immunostaining. Positive membranous immunostaining for EGFR was associated with squamous cell carcinoma of the cervix (OR=7.41, 95% CI=3.38-16.23, p<0.001), negative pEGFR cytoplasmic immunostaining was associated with poor differentiation (OR=0.39, 95% CI=0.20-0.73, p=0.004) and negative PTEN immunostaining was associated with metastatic pelvic lymph nodes (OR=0.51, 95% CI=0.30-0.90, p=0.019).

EGFR, pEGFR, PTEN, pAKT and pERK and disease specific survival

In univariate Cox Regression analysis, none of the immunostain results correlated with disease specific survival (table 4). In multivariate Cox Regression analysis only positive lymph nodes (HR=6.11, 95% CI=3.46-10.77, p<0.001) and poor differentiation (HR=1.91, 95% CI=1.12-3.26, p=0.018) were independent prognostic factors for disease specific survival.

Table 3 | Clinico-pathological parameters and immunostaining.

	EGFR positive			pEGFR positive			PTEN positive			pAKT positive			pERK positive		
	OR	***95% CI***	***P value***	***OR***	***95% CI***	***P value***	***OR***	***95% CI***	***P value***	***OR***	***95% CI***	***P value***	***OR***	***95% CI***	***P value***
Age	1.00	0.99-1.02	0.747	1.02	0.99-1.04	0.117	1.00	0.98-1.01	0.630	1.02	0.99-1.06	0.248	0.99	0.94-1.03	0.550
Stage ≥IB2	1.37	0.83-2.27	0.222	0.87	0.48-1.58	0.653	0.73	0.44-1.21	0.221	2.02	0.71-5.75	0.186	0.14	0.02-1.10	0.062
Squamous	**7.41**	**3.38-16.23**	**<0.001**	1.41	0.73-2.75	0.325	1.76	0.99-3.10	0.052	2.33	0.50-10.75	0.280	0.61	0.17-2.22	0.452
Poor differentiation	0.75	0.45-1.25	0.273	**0.39**	**0.20-0.73**	**0.004**	0.66	0.40-1.09	0.102	0.74	0.25-2.24	0.597	0.73	0.22-2.50	0.620
Lymphangioinvasion	1.18	0.72-1.94	0.517	0.99	0.56-1.75	0.967	0.83	0.51-1.34	0.445	1.02	0.36-2.90	0.970	0.66	0.20-2.12	0.483
Infiltration depth ≥10 mm	1.50	0.90-2.49	0.120	0.78	0.43-1.40	0.407	0.61	0.37-1.01	0.053	0.38	0.12-1.23	0.106	0.85	0.26-2.73	0.778
Positive lymph nodes	1.62	0.95-2.75	0.077	1.20	0.66-2.21	0.547	**0.51**	**0.30-0.90**	**0.019**	0.58	0.16-2.12	0.410	0.83	0.22-3.13	0.777
Tumour diameter ≥4 cm	1.36	0.80-2.34	0.260	0.81	0.42-1.54	0.513	0.73	0.42-1.25	0.247	1.30	0.43-3.92	0.645	0	0	0.997

OR = Odds ratio, 95%CI = 95% confidence interval

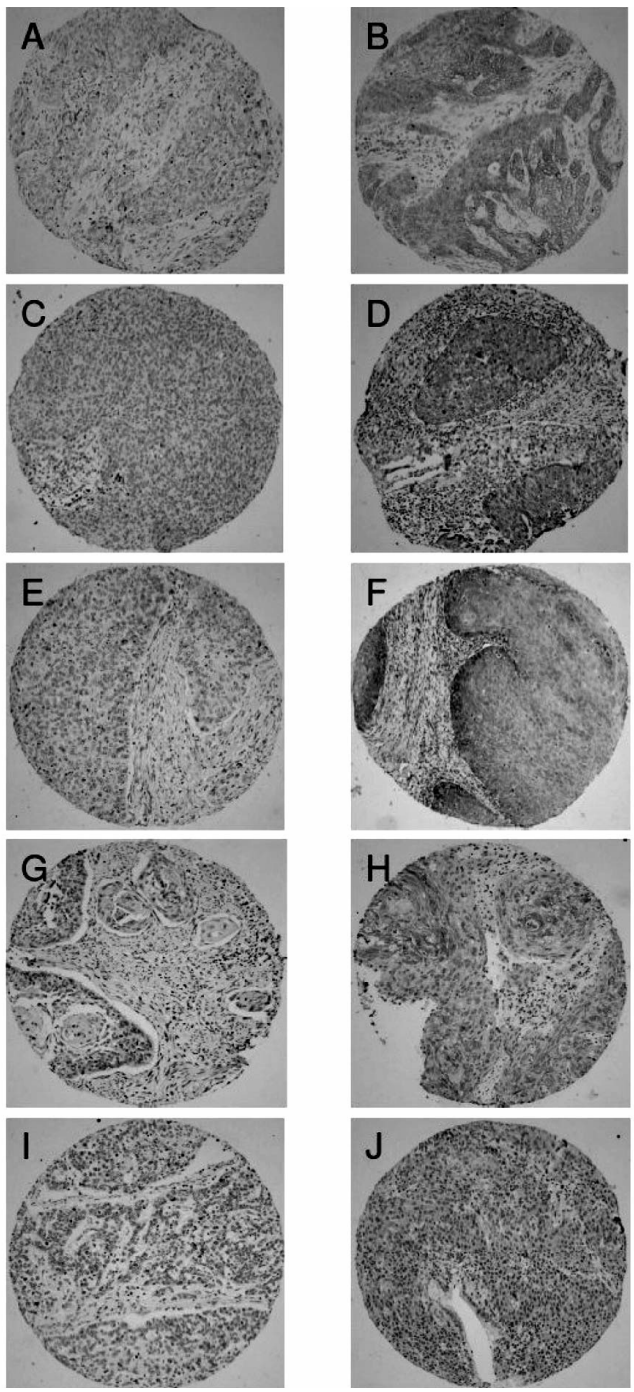

Figure 1 | Tumour microarray stained for EGFR, pEGFR, PTEN, pAKT, and pERK (100x). Negative staining (a, c, e, g, and i). Positive staining (b, d, f, h, and j).

Table 4 | Disease specific survival (Cox regression analysis)

	Univariate			Multivariate		
	HR	***(95%CI)***	***P value***	***HR***	***(95%CI)***	***P value***
Age	1.02	1.00-1.04	0.055	#		
Stage ≥IB2	**2.34**	**1.38-3.98**	**0.002**	#		
Squamous	0.72	0.40-1.29	0.266	#		
Poor differentiation	**1.73**	**1.01-2.95**	**0.045**	**1.91**	**1.12-3.26**	**0.018**
Lymphangioinvasion	**2.38**	**1.32-4.26**	**0.004**	#		
Infiltration depth ≥10 mm	**2.00**	**1.15-3.49**	**0.014**	#		
Positive lymph nodes	**5.76**	**3.30-10.04**	**<0.001**	**6.11**	**3.46-10.77**	**<0.001**
Tumour diameter ≥4 cm	**2.45**	**1.44-4.16**	**0.001**	#		
EGFR	1.24	0.69-2.22	0.468	#		
pEGFR	0.81	0.39-1.66	0.556	#		
PTEN	0.61	0.33-1.14	0.120	#		
pAKT	0.71	0.17-2.93	0.638	#		
pERK	0.05	0.0-15.02	0.298	#		

= not included in final analysis, HR = Hazard ratio, 95%CI = 95% confidence interval

DISCUSSION

Immunohistochemical expression of EGFR pathway components EGFR, pEGFR, PTEN, pAKT and pERK was evaluated in relation to clinico-pathological parameters and disease specific survival in a consecutive series of early stage cervical cancer patients. Our study indicates that loss of PTEN expression frequently occurs in early stage cervical cancer and is related to pelvic lymph node metastasis (OR=0.51, 95% CI=0.30-0.90, p=0.019), but not to survival. Only limited data exist on PTEN expression and its possible implications for the biologic behaviour of cervical cancer. In a study by Lee et al. reduced PTEN expression was identified in 17.6% (15/85) of surgically treated cervical cancer patients and was associated with decreased disease free and overall survival, but not with pelvic lymph node metastasis [13]. They reported a gradual reduction of PTEN expression along the continuum from normal epithelium through intraepithelial neoplasia to squamous cell carcinoma. Discrepancies between the data from Lee et al. and our study might be due to the number of evaluated patients (n=85 vs. n=310) and/or interpretation of immunostaining, since Lee et al. defined reduced PTEN expression by comparison with corresponding normal tissue [13]. In our opinion, a minimal percentage of positive cells should be taken into account when

assessing PTEN expression in a tumour. In a study comparing different immunostainings in TMA and full sections of vulvar cancer patients, a minimal percentage of positive cells was also taken into account and this study resulted in a good reproducibility of immunostaining on TMA [18].

Experimental data also indicate a role for loss of PTEN in determining the metastatic potential of tumours. In a study utilizing a benign melanocytic hyperplasia mice model, silencing PTEN lead to the development of melanoma and metastases to lymph nodes and lungs [19]. In a study of colorectal cancer patients, Sawai et al. observed an association between reduced PTEN expression and liver metastases [20]. Activation of receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor, Her2/neu and insulin-like growth factor receptor 1 results in recruitment of phosphoinositide 3-kinase (PI3K) [9]. The direct product of PI3K is phosphatidylinositol-3,4,5-triphosphate (PIP3) and PIP3 is the primary target of PTEN [21]. Loss of PTEN function results in accumulation of PIP3 and delete thereby activation of its downstream targets: the AKT pathway. Activation of the AKT pathway may cause cell cycle progression, cell survival, cell spreading and motility and angiogenesis [9]. In our study, no relation between loss of PTEN and activation of the AKT pathway was found. Our low percentage (5.3%) of pAKT positive cases is in contrast to previous studies in cervical cancer that used the same antibody and staining protocol but observed 29-94% pAKT positive cases [22-24]. A major difference between these studies and our study is that we assessed pAKT in a much larges series (310 versus 31) of early stage cervical cancer patients.

Loss of PTEN can be due to mutations, deletions, gene promoter methylation or microRNAs (miRs) [25-28]. Mutations and deletions of *PTEN* are rare events in cervical cancer [16,22,29]. In a study by Yang et al. *PTEN* methylation was observed in 20/127 (15.7%) cervical cancers, while Cheung *et al.* reported *PTEN* methylation in 36/62 (58%) of squamous cell cervical cancers. They found no PTEN expression in 3 of 10 *PTEN* methylation negative cases and in 0 of 10 *PTEN* methylation positive cases [16]. Preliminary data from our cases show *PTEN* gene promoter methylation in 4/19 (21%) cases. No PTEN immunostaining was observed in these 4 *PTEN* methylation positive cases (data not shown). Loss of PTEN expression could also occur via miRs. Several miRs, such as miR-21 and miR-214, can target PTEN [27,28]. Both miRs appear to be up-regulated in cervical cancer [30,31], but a relation with PTEN loss has not been reported. It might be that loss of PTEN expression can only partially be explained by down regulation via miRs.

A downstream component of the PI3K/AKT pathway, known as the mTOR pathway (mammalian Target of Rapamycin), is up regulated in many cancers. As a consequence of PTEN loss, activation of the mTOR pathway may occur. As shown in, *in-vitro* and *in-vivo* experiments, cells without PTEN are more sensitive to mTOR inhibitors (rapamycin) [32,33]. These mTOR inhibitors might provide efficacious additional therapy in cervical cancer patients with an unfavourable prognosis and loss of PTEN.

Our study indicates a strong relation between squamous cell carcinomas and EGFR staining, which was previously reported in cervical cancer by Kersemaekers et al. [11]. In their study, EGFR overexpression was observed in 54% (73/136) cervical cancer cases and was associated with reduced disease free and reduced overall survival in surgically treated cervical cancer patients [11]. These findings could not be confirmed in our large series of cervical cancer patients, where 32.1% of cases overexpressed EGFR. One explanation might be the use of a different antibody. Another possibility is that our study included a larger percentage of adenocarcinomas (28% versus 9%) and as is shown in our study, EGFR expression is highly associated with squamous cell carcinoma.

In conclusion, in early stage cervical cancer, loss of PTEN expression is associated with pelvic lymph node metastasis, suggesting that PTEN is one of the tumour suppressor genes affecting pelvic lymph node metastasis in early stage cervical cancer. Overall however, the EGFR pathway does not appear to have prognostic impact in surgically treated early stage cervical cancer.

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