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Clinicopathological implication of cripto expression in early stage invasive cervical carcinomas

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Abstract

This study evaluates the expression of cripto (CR-1) protein in matched sets of non-neoplastic cervical epithelium, primary cervical carcinoma and metastatic tumours in the lymph nodes to investigate its role in uterine cervical cancer development and progression. Ninety-four primary cervical carcinomas in an early clinical stage and having the same surgical treatment modality were analysed. Immunoreactivity in the primary tumour was compared with that of non-neoplastic cervical epithelium and metastatic lymph nodes. The conventional clinicopathological prognostic variables for cervical carcinomas such as grade, tumour size, depth of invasion, parametrial and endometrial extension, lymphovascular space involvement and lymph node metastasis status were also compared with CR-1 expression of the primary tumour. Strong CR-1 immunopositivity was significantly correlated with tumour size and lymphovascular space involvement ($P < 0.05$). Furthermore, a significant relationship was found between CR-1 immunoreactivity and endometrial extension as well as parametrial involvement ($P < 0.05$). Interestingly, the CR-1 expression level was increased in metastatic lymph nodes compared with their primary tumours. These results suggest that CR-1 may contribute to disease progression in cervical carcinomas.

Keywords: Cripto; Oncogene; Cervical neoplasms; Carcinoma; Immunohistochemistry; Microscopy; Pathology; Prognosis

1. Introduction

Recent molecular studies have provided insight into the probable mechanisms by which oncogenic human papilloma viruses (HPVs) contribute to cervical neoplasia, resulting in the loss of control of cell proliferation [1]. However, this model for cervical cancer pathogenesis cannot fully explain the development of cervical cancer and it seems that additional genetic alterations are necessary, consistent with the multistep model of carcinogenesis. Many investigators have studied the changes in function and expression of cellular genes controlling cell growth and differentiation in cervical carcinomas [2–6].

The epidermal growth factor (EGF) family consists of peptide growth factors that stimulate cellular proliferation by binding to a cell membrane receptor. Cripto-1 (CR-1) protein is a recently described member of this family. Its gene was originally cloned from the human teratocarcinoma cell line and encodes a 2.2 kb mRNA [7] which is translated into a 188 amino acid CR-1 protein. Unlike other members of the EGF family, it does not contain a hydrophobic signal peptide domain and transmembrane component. Its 37 amino acid central portion has structural homology with other EGF peptides [7,8]. Saccone and colleagues localised the *CR-1* gene to chromosome 3p21 by fluorescence *in situ* hybridisation [9]. This region is one of the most frequent sites where rearrangements occur during malignancies.

CR-1 gene overexpression by gene transfer studies results in *in vitro* transformation of mouse fibroblasts and mammary epithelial cells, indicating that CR-1 functions as a dominantly acting oncogene [7,10,11]. Additionally, recombinant CR-1 stimulates some human breast cancer cell lines [12]. Expression of the

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CR-1 protein was reported in 82% of human breast carcinomas [11], in 70–79% of colon carcinomas [13–15], 35–57% of gastric cancers [16–18] and 43% of pancreatic ductal adenocarcinomas [19]. Our study is the first in evaluating CR-1 expression in uterine cervical carcinomas.

There is a need to identify markers of tumour aggressiveness in patients with cervical carcinoma and define groups either with a favourable prognosis requiring no further treatment or where the outcome is likely to be poor and more intensive treatment might be beneficial. In the early stages of cervical carcinoma, one of the most important prognostic indicators is lymph node status [20]; however, in absolute terms, an equal number of deaths from the disease occurs in patients with or without lymph node metastases [21]. Therefore, determination of high-risk factors is still the subject of many investigations. In the present study, we analysed the expression of cripto-1 protein in early stage primary cervical carcinomas, in adjacent non-neoplastic cervical epithelium as well as metastatic foci in the lymph nodes, and evaluated its role in the pathogenesis and prognosis of cervical cancers.

2. Patients and methods

Amongst the patients diagnosed as having cervical carcinoma in the Obstetrics and Gynecology Department of Hacettepe University Hospital between 1980 and 1993, 94 cases in the early clinical stages (1B and 2A) and receiving the same treatment modality (type III hysterectomy + bilateral salpingo-oopherectomy + bilateral pelvic–paraaortic lymphadenectomy) were selected.

Pathology reports were reviewed and all the sections were re-evaluated to determine tumour size, depth of invasion, nuclear grade, presence of parametrial and endometrial extension, lymphovascular space involvement, status of lymph node metastases, number, location and size of the lymph nodes involved and diameter of the metastatic foci in them. The WHO-1992 classification [22] was used to categorise tumours histologically.

2.1. Immunohistochemistry

Six-micrometre tissue sections were obtained from formalin-fixed paraffin-embedded tissue blocks from 94 primary cervical carcinomas, representative for tumour and adjacent non-neoplastic cervical epithelium, as well as from 23 lymph nodes which had metastatic foci. Sections were deparaffinised and rehydrated. Antigenic retrieval was accomplished using a microwave oven, then endogenous peroxidase activity was blocked. After several phosphate buffered saline (PBS) washes and non-specific protein blockage, sections were incubated overnight with a 1/400 diluted rabbit anti-CR-1 antibody (kindly provided by Dr Tahara) in a humidified chamber at room temperature. The anti-CR-1 antibody was generated against a 17mer peptide corresponding to amino acid residues 97–113 of the human CR-1 protein, which represents the COOH-terminus of the 37 amino acid EGF-like region, the specificity of which has previously been described [13]. Sections were washed three times with PBS and then stained with the ABC method using a Histostain SP kit (Zymed, South San Francisco, CA, USA).

2.2. Evaluation of immunoperoxidase staining

Specific staining with primary antibody was graded from I to III taking into account both the intensity and extent of positive staining. First, a number from 1 to 3 was given according to staining intensity. Then the number of positive cells per slide was stratified into three groups based on the percentage of positive cells: group I, $<33\%$; group 2, 33–67%; group 3, $>67\%$. Scores ranging from 1 to 9 for specific staining for each case were obtained by multiplying the staining intensity by the number of the group that represented the percentage of positive cells as previously described [23]. A score of zero represents no specific staining observed, the scores of 1 and 2 were accepted as grade I (1+), 3 and 4 as grade II (2+), and 6 and 9 as grade III (3+).

2.3. Statistical analysis

The immunohistochemical findings and clinicopathological parameters were analysed using the Chi-square test and Student's *t*-test, with $P<0.05$ taken as the level of significance. Survival curves were computed by the method of Kaplan–Meier and analysed by the log-rank test.

3. Results

The mean follow-up period was 36.6 months, ranged between 2 and 134 months. The number of deaths was 15, all caused by neoplastic disease. The interval between diagnosis and the time of death ranged from 3 to 47 months. There were 29 cases with recurrence (31%) and 21 distant metastases (22%).

Out of 94 cervical carcinomas, 68 (72%) were histologically classified as squamous cell carcinoma, 15 (16%) as adenosquamous carcinoma, 10 (11%) as adenocarcinoma and 1 (1%) as undifferentiated carcinoma. CR-1 expression was present in 61 (65%) of the 94 primary cervical carcinomas. Specific staining was graded as (1+) in 36 (38%), as (2+) in 20 (21%) and (3+) in 5 (5%) cases. Staining was diffuse throughout the cytoplasm (Fig. 1). 43 (63%) of the squamous cell cancers, 6 (60%) of the adenocarcinomas, 11 (73%) of the

adenosquamous carcinomas and the 1 case (100%) of undifferentiated carcinoma were immunoreactive for CR-1 protein ($P > 0.05$). The pattern of staining was heterogeneous in some tumours, with increased intensity in deep and invading areas of the neoplasm (Fig. 2). In the non-neoplastic cervical epithelium evaluated in 72 cases, 38 (53%) were positively stained. However, staining was generally only weakly positive. Therefore, those tumours with strong expression in grades of (2+) or (3+) were regarded as overexpressing CR-1.

There was a statistically significant relationship between strongly positive (2+ and 3+) CR-1 immunoreactivity and the largest tumour diameter that ranged from 0.7 to 6 cm. Whilst the mean tumour diameter was 2.4 cm in the group that showed negative or weak (1+) expression, it was 3.4 cm in the strongly CR-1 protein expressing group (data not shown, $P = 0.012$). The frequency of overexpression was 21% in tumours with the largest diameter of less than or equal to 2 cm, and it rose to 36% in those between 2.1 and 4 cm and to 67% in tumours larger than 4 cm ($P = 0.046$) (Table 1).

In the presence of endometrial extension, strong immunoreactivity was seen in 41% of tumours whilst in its absence strong staining was observed in 20% ($P = 0.046$) (Table 1). A similar statistically significant correlation was found between parametrial spread and CR-1 overexpression ($P < 0.001$) (Table 1).

Out of 72 tumours showing lymphovascular permeation, 23 (32%) overexpressed CR-1. This ratio was 10% when lymphovascular involvement was not present ($P = 0.041$) (Table 1).

Pelvic and/or paraaortic lymph node metastases were observed in 29 cases (31%), 7 (24%) of which were bilateral. There were 6 cases with paraaortic metastasis. In five metastatic lymph nodes each belonging to a different patient there was pericapsular invasion by the tumour. Metastatic lymph node number was between one and three in 18 cases, four and ten in 8 cases and above 10 in 3 cases. There was no difference between primary carcinomas with and without lymph node metastasis with regard to CR-1 protein expression. Metastatic lymph node number and diameter did not show correlation with CR-1 expression. However 4 of the 5 which showed pericapsular lymph node invasion, expressed CR-1. Rates of bilateral lymph node involvement, as well as recurrence and distant metastasis were increased as CR-1 protein expression in primary tumour upgraded to 3+ (Table 2).

With regard to CR-1 immunoreactivity in the metastatic foci of the lymph nodes, in all of the 7 cases that

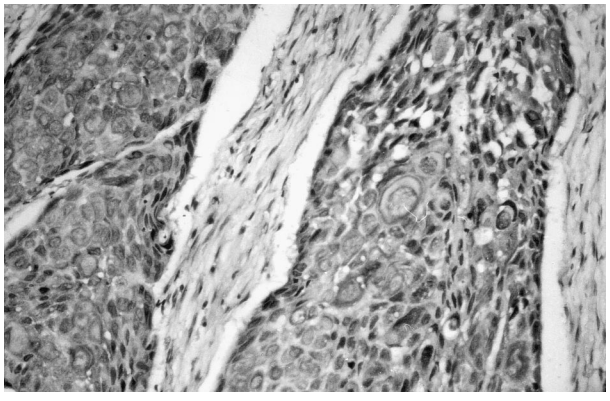

Fig. 1. Diffuse cytoplasmic expression of CR-1 by neoplastic cells (immunoperoxidase, anti-CR-1 antibody, ABC×215).

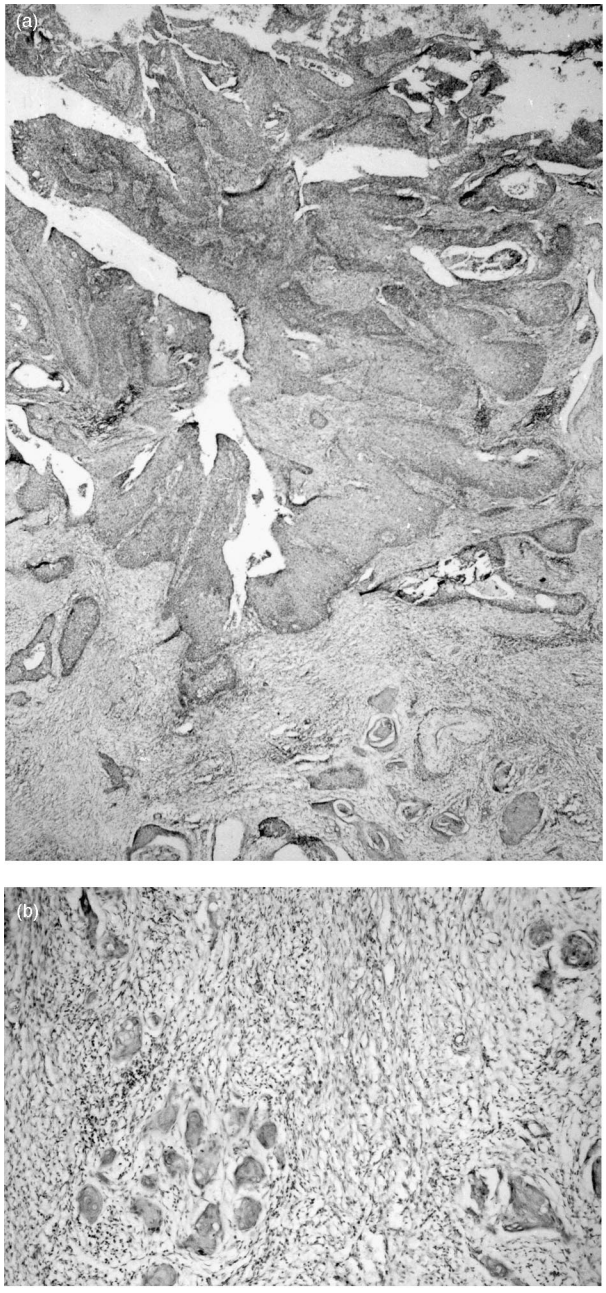

Fig. 2. (a) Primary cervical carcinoma expressing CR-1 protein at a low intensity (immunoperoxidase, anti-CR-1 antibody, ABC×29). (b) Deeply invasive areas of the same tumour. Expression level increases to 3+ (immunoperoxidase, anti-CR-1 antibody, ABC×115).

Table 1
Comparison of the degree of CR-1 expression with tumour diameter, endometrial extension, parametrial involvement and lymphovascular invasion in cervical carcinoma

Parameter		Cripto (CR-1) expression			*P* value[b]
		Negative or weak *n* (%)	Strong *n* (%)	Total *n* (%)	
Tumour diameter $n=55$ [a]	0–2 cm	19 (79)	5 (21)	24 (44)	0.046
	2.1–4 cm	14 (64)	8 (36)	22 (40)	
	>4.1 cm	3 (33)	6 (67)	9 (16)	
Endometrial extension $n=93$[a]	No	51 (80)	13 (20)	64 (69)	0.046
	Yes	17 (59)	12 (41)	29 (31)	
Parametrial involvement $n=88$[a]	No	56 (85)	10 (15)	66 (75)	<0.001
	Yes	7 (32)	15 (68)	22 (25)	
Lymphovascular invasion $n=93$[a]	No	19 (90)	2 (10)	21 (23)	0.041
	Yes	49 (68)	23 (32)	72 (77)	

[a] Number of cases available for evaluation.
[b] Chi-square Pearson test.

showed bilateral lymph node metastasis and also in all of 6 cases in which para-aortic lymph node involvement was present, metastatic tumour foci in the lymph nodes expressed the CR-1 protein. Furthermore, when CR-1 positivity was detected in metastases, the mean metastatic lymph node number was higher than those where CR-1 was not detected: in the first group, the frequency of metastases in more than four lymph nodes was 53% and in more than 10 was 18%, these ratios were 33% and 0% in the cripto negative group (data not shown).

When CR-1 expression of metastatic lymph nodes was compared with its primary tumour (could be done in 21 of 29 cases), a tendency for increased CR-1 expression in the metastatic foci was observed. Although immunoreactivity became negative in 3 cases in which primary tumours were positive (at the level of 1+) and remained at the same level in 7 (33%) cases, it revealed an increase in the remaining 11 (52%) cases (Chi square kappa value < 0.039). Unfortunately, the number of cases was insufficient for statistical confirmation of these data.

3.1. Survival analysis

There was a difference between the survival curves of the primary cervical carcinomas which overexpressed CR-1 and those with a negative or weak expression, but this difference did not reach a statistically significant level (data not shown).

Table 2
Proportion of cases in each CR-1 subgroup showing bilateral lymph node involvement, recurrence and distant metastasis

CR-1 expression degree	Bilateral lymph node metastasis	Recurrence	Distant metastasis
% of −	22	32	17
% of 1+	25	37	29
% of 2+	29	29	28
% of 3+	33	50	33

4. Discussion

It is apparent that some growth factors synthesised by normal and neoplastic cells regulate cellular growth autocrine, juxtacrine or paracrine pathways and may contribute to cervical carcinogenesis. *CR-1* is an oncogene from the EGF family. It is a potent mitogen for mammary epithelial cells as are EGF-like peptides. These peptides increase the intrinsic tyrosine kinase activity by binding to EGFR [24]. The mechanism of action of CR-1 protein is different. It does not bind directly to or activate the tyrosine kinases associated with the EGFR, c-erb B-2, c-erb B-3 or c-erb B-4 alone or in pairwise combinations [25]. Chemical cross-linking of ^{125}I-CR-1 to several cell lines has identified a novel receptor which differs from the other erb B receptors in size and subunit composition [26]. However, CR-1 does indirectly stimulate specific tyrosine phosphorylation of c-erb B-4. Inhibition of c-erb B-4 activity results in abrogation of CR-1-induced activation of MAPK (mitogen-activated protein kinase) by CR-1 [26].

Expression of CR-1 in normal and neoplastic epithelium shows variance between different tissues. In the study of Saeki and colleagues, CR-1 expression was correlated with the degree of atypia in adenomas, and, with the Duke's stage in carcinomas, it has been proposed that its expression can be useful as a marker to predict malignant changes occurring in adenomas [13,15].

In the immunohistochemical study of Qi and associates 82% of the breast carcinomas were immunopositive for CR-1 whilst only 13% adjacent non-involved breast epithelium was reactive to anti-CR-1 antibody, and it has been suggested that CR-1 may serve as a potential breast tumour marker [27]. In our study, CR-1

immunoreactivity was 53% in non-neoplastic cervical epithelium, and 65% in the primary cervical carcinomas. The expression of CR-1 and its high frequency in normal epithelium suggest that CR-1 is not tumour-specific, and is a growth factor synthesised by normal and non-neoplastic cells. Similarly, it is not cancer-specific in the pancreas, and has been shown to be expressed in most acinar and ductal cells [19]. However, according to our results, strong expression in the grades of 2+ and 3+, only found in tumour, is specific to neoplastic tissue rather than normal cervical tissue.

The importance of CR-1 as a prognostic factor has been studied in a limited number of human tumours. In pancreatic carcinomas, CR-1 correlates with advanced stage, but not with grade or survival, suggesting a limited role in disease progression [19]. However, findings are different in other gastrointestinal tumours: CR-1 immunoreactivity in colorectal tumours correlates with the degree of invasion, lymph node metastases and higher recurrence rate [28].

Stage is the most important prognostic factor in uterine cervical carcinomas. However, as in other organ malignancies, distinct biological behaviours are being observed for the tumours, even in the same stage. Tumour size, lymph node metastases, parametrial extension, lymphovascular space involvement and deep stromal invasion appear to be prognostic parameters in tumours at a certain stage. Many investigators agree on determining the high-risk group that will benefit from adjuvant treatment. In the present study, CR-1 overexpression demonstrated prominent association with parametrial ($P<0.001$) and lymphovascular space involvement ($P=0.041$), and tumour diameter, as well as endometrial extension ($P=0.046$). An interesting observation was made when CR-1 showed intense expression at the invasive border of the tumour. This was a rare finding, but in all of these tumours we noticed deep invasion and lymphovascular permeation. Thus, CR-1 overexpression appears to be a marker of unfavourable prognosis in cervical cancer.

In this study, we did not observe a statistically significant correlation between CR-1 protein expression detected by immunohistochemistry in cervical carcinomas and recurrence, distant organ metastases or length of survival. However, the frequency of tumour recurrence, bilateral lymph node and distant metastases increased with the grade of CR-1 immunoreactivity. Furthermore, when compared with the primary cervical tumour, metastatic areas in lymph nodes showed a rise in the degree of CR-1 immunoreactivity in most cases. These findings suggest that CR-1 overexpression accompanies disease progression in early stage cervical carcinomas.

Our results suggest that CR-1 overexpression may confer an additional growth advantage to cervical cancer cells and it may play a role in disease progression since it was significantly correlated with important unfavourable prognostic risk factors, namely lymphovascular space involvement, parametrial invasion, endometrial extension, tumour size and, because of its tendency to show more intense staining in tumours associated with recurrence, bilateral lymph node and distant organ metastases.

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