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Sporadic loss of leucocyte-function-associated antigen-3 (LFA-3) in colorectal carcinomas *

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Summary. Leucocyte-function-associated antigen-3 (LFA-3) is a cell surface glycoprotein involved in T-cell/target cell interaction. The expression of LFA-3 on the cell surface was found to be inevitably associated with the expression of HLA molecules. Loss of LFA-3 or HLA proteins on tumour cells might result in ineffective T-cell/target cell interaction and a failure of immunological tumour surveillance. Immunohistochemistry revealed that LFA-3 is expressed in normal colonic epithelium; however, in a minor fraction of colonic adenomas and in 50.3% of colorectal carcinomas LFA-3 expression was reduced or even absent. To investigate whether the presence or absence of LFA-3 in colorectal carcinomas influences the relapse rate and time of tumour-related death, 149 patients who underwent putatively curative surgery were surveyed for a maximum of 65 months (mean 48 months). In contrast to the prognostic role of tumour stage and grade, the presence versus absence of LFA-3 was not correlated with recurrence or survival. Regarding survival and growth of residual tumour cells after potentially curative resection of the initial tumour burden, we conclude that the status of LFA-3 expression in colorectal carcinoma seems to be irrelevant in vivo.

Key words: Leucocyte-function-associated antigen-3 – Colorectal carcinoma

Introduction

Leucocyte-function-associated antigen-3 (LFA-3), also designated CD58 antigen, is encoded by a gene on chromosome 1 (Barbosa et al. 1986). LFA-3 is a heavily glycosylated, broadly distributed surface glycoprotein of 60–65 kDa, two forms of which a type I transmembrane and a phosphatidylinositol-anchored form have been described (Sanchez-Madrid et al. 1982; Barbosa et al. 1986; Dustin et al. 1987; Seed 1987). During cognate T-cell/target cell interaction, LFA-3 on the target cell specifically binds to the CD2 molecule on the T-cell (Shaw et al. 1986; Seed and Aruffo 1987; Takai et al. 1987), causing antigen-independent conjugate formation, T-cell activation through CD2 signalling and stabilization of the physical interaction of HLA proteins on the target with the T-cell antigen receptor which, in the presence of nominal antigen, produces the second activation signalling via the CD3 complex (Meuer et al. 1984; Hünig et al. 1987; Mentzer et al. 1987; Peterson and Seed 1987; Richardson et al. 1988; Recny et al. 1990).

There is evidence that during this process LFA-3 is physically associated with the particular HLA proteins. It also became obvious that antigen-specific binding is markedly decreased in the absence of LFA-3 (Krensky et al. 1983; Shaw and Ginther Luce 1987; Recny et al. 1990).

These data, combined with the finding that LFA-3 is down-regulated in various Epstein-Barr virus (EBV)-positive Burkitt's lymphoma lines (Billaud et al. 1987; Gregory et al. 1988) or even abrogated in sporadic cases of colonic carcinomas (Smith et al. 1989) and transitional cell carcinoma (Nouri et al. 1990), suggest that a loss of LFA-3 might be responsible for the likely escape of HLA-deficient tumours from cytotoxic T-cell attack (Smith et al. 1989).

The present immunohistochemical study investigates the expression of LFA-3 antigen in normal and neoplastic colorectal epithelium and in colorectal adenomas to determine the local distribution pattern in its natural context. To determine the influence of LFA-3 expression within the neoplastic population of colorectal carcinomas on disease-free survival and on the risk of tumour-related death, we conducted a prospective study surveying 149 patients who underwent putatively curative surgery.

* Dedicated to Prof. G. Seifert at the occasion of his 70th birthday

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Material and methods

Potentially curative (based on clinical, paraclinical and pathohistological grounds) surgical treatment for colonic carcinoma was carried out in 149 patients who were incorporated into this study from 1 January 1984 to 1 September 1985 (Table 1). A second malignancy was the only pre-operative exclusion criterion. All patients received standard follow-up examinations at 3-month intervals during the first 2 post-operative years, which was then reduced to a 6-month cycle. The data were subsequently reported to the tumour registry by means of a computer-aided reminder system archiving the information on all patients until death or until the end of the observation period (1 June 1989). The lethal disease complex and its dependency on the underlying neoplastic disease were also analysed on the basis of the patients' complete records.

Immediately after removal, the entire gut specimen was examined and representative samples of tumour tissue, together with normal mucosa and possible co-existing adenomas (20 specimens), were quick-frozen in liquid nitrogen for immunohistochemical investigation. The tumours whose primary site and metastatic distribution at the time of operation were well-documented were typed, graded, and staged according to the UICC classification (Dukes and Bussey 1958; Hermanek and Sobin 1987; Jass and Sobin 1989). The data of this documentation are listed in Table 1.

The mAb BRIC5 (IgG_{2a} isotype, kindly provided by D.J. Anstee, Oxford, UK) was used for immunohistochemical detection of CD58 antigen (Knapp et al. 1989). Binding of mAb was detected with a polyclonal biotinylated sheep antibody to mouse immunoglobulins and a streptavidin-biotinylated peroxidase complex (Amersham, High Wycombe, UK). 3-Amino-9-ethylcarbazole (AEC) and *N'N*-dimethylformamide (DMF) were obtained from Sigma (St. Louis, Mo., USA).

MAb was diluted 1:1000 in phosphate-buffered saline (PBS), pH 7.4. The biotinylated anti-mouse Ig antibody was diluted 1:50 in PBS and the streptavidin peroxidase complex 1:100. Incubation times were 1 h at room temperature for the primary antibody and 30 min for the second- and third-step reagents. Using AEC as chromogen (0.4 mg/ml in 0.1 M acetate buffer, pH 5.0, with 5% DMF and 0.01% hydrogen peroxide for 10 min), the peroxidase reaction caused an intensive red precipitate. The sections were rinsed in tap-water, counterstained with Harris' haematoxylin and mounted with glycerol gelatin.

Intrinsic positive controls for immunoreactivity in each section were small subsets of lymphocytes present in all carcinomas and normal colonic mucosa remnants present in many specimens. Each series of frozen sections contained a negative control without the primary reagent. In addition, a control using irrelevant isotype-matched mAb was carried out in a number of tissue sections. In both sets of negative controls, staining was observed in granulocytes whose endogenous peroxidase was not blocked for the benefit of optimal antigenicity. Also observed was a faint microgranular cytoplasmic colour precipitate in some epithelial areas caused by endogenous biotin; these reactivities were not assessed, nor was the staining in areas of tumour necrosis.

The tumour cell reactivity, regardless of staining intensity, was scored as either positive or negative. Many tumours contained stained and non-reactive tumour cells in varying amounts, which were assessed in a semi-quantitative fashion. To evaluate the data statistically, three categories were established: (a) LFA-3 expression within a tumour was regarded as *normal* when the entire neoplastic population was stained and no unreactive subsets were observed; (b) LFA-3 expression was regarded as *reduced* whenever a subset of unstained tumour cells was detectable; and (c) as *lost* when the tumour cell compartment was unreactive to anti-LFA-3 throughout.

Statistical analysis of the study was performed by a computer-based statistical analysis system. The observation period ended on 1 June 1989, i.e. 65 months after the first and 45 months after the last patient had entered the study. Recurrence-free and overall survival were calculated by the Kaplan-Meier procedure (Kaplan and Meier 1958); only tumour-related events were accepted for statistical analysis. To make up for differences between Kaplan/Meier curves, the *P*-value was calculated by a log-rank test (Mantel and Haenszel 1959). A chi-square test was applied to the analysis of contingency tables.

Table 1. Clinical and pathological characteristics of colon cancer patients with putatively curative resection

Clinical features		Observation ($n=149$)
Sex	Male	82
	Female	67
Mean age (years ± SD)		64.4±11.3
Recurrence during observation period (65 months maximum)		
	Male	16
	Female	11
Tumour-related deaths during observation period		
	Male	8
	Female	7
Pathological features		
Tumour localization		
Caecum		9
Ascending colon		22
Hepatic flexure		3
Transverse colon		4
Splenic flexure		2
Descending colon		4
Sigmoid colon		21
Rectum		84
Tumour type (ICD-O-DA)		
Adenocarcinoma		106
Mucinous carcinoma		40
Signet ring carcinoma		1
Undifferentiated carcinoma		2
Grade of differentiation (UICC)		
I	Well-differentiated	16
II	Moderately differentiated	110
III/IV	Poorly/undifferentiated	23
Tumour stage		
Dukes'	UICC	
A	I (T1–2,N0,M0)	52
B	II (3–4,N0,M0)	47
C	III (T1–4,N1–3,M0)	50
D	IV (T1–4,N1–3,M1)	0

Results

Immunohistochemical staining with anti-LFA-3 showed that normal colonic epithelium was consistently LFA-3-positive, that is to say, both goblet cells and columnar epithelium expressed the LFA-3 antigen in the cytoplasm and on the basolateral cell surface. There were no alterations in antigenic density from proximal to distal colon and between the basal parts of the crypts and the mucosal surface (Fig. 1a). LFA-3 was broadly distributed in the autochthonous structures of the gut wall and was strongly expressed in fibres of the visceral nervous plexus and in the vascular endothelium and weakly expressed in smooth muscle cells, in some fibroblasts as well as in some connective tissue fibrillar structures.

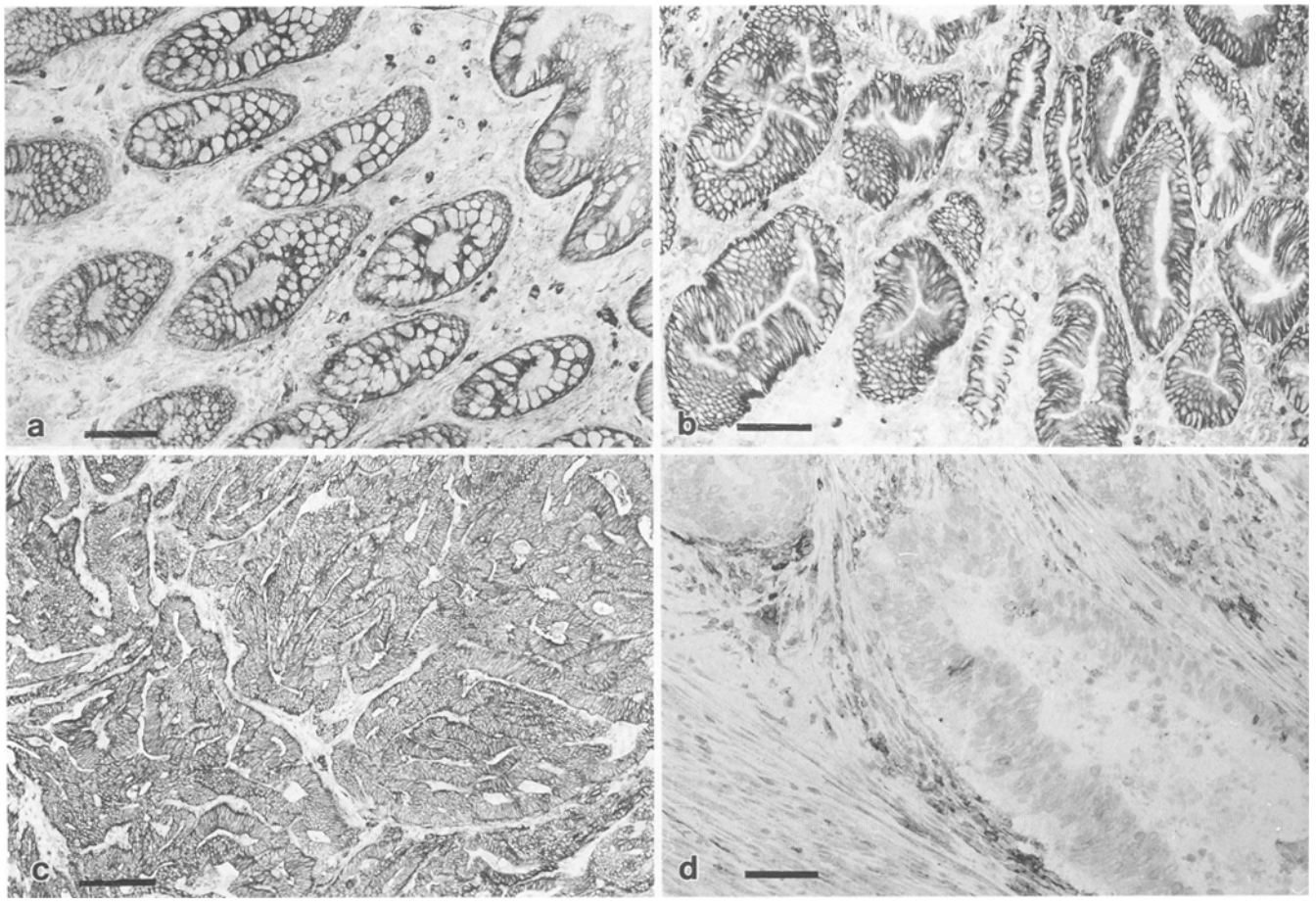

Fig. 1a–d. Expression of LFA-3 in normal colorectal mucosa, adenomas and carcinomas. **a** In the normal colonic epithelium, LFA-3 is expressed in basal and apical parts of the crypts, in columnar cells and in the cytoplasm of goblet cells. The dark stained cells in the mucosal stroma are granulocytes whose endogenous peroxidase was not blocked (*bar* = 80 μm). **b** This well-differentiated adenoma is entirely LFA-3 positive; the staining intensity is more prominent on the cell surface than in the cytoplasm (*bar* = 160 μm). **c** This moderately differentiated carcinoma expresses LFA-3 throughout the neoplastic epithelium (*bar* = 160 μm). **d** In contrast, this moderately differentiated carcinoma is completely devoid of LFA-3; the randomly localized lympho-histiocytic population and some fibroblasts are positive (*bar* = 80 μm)

Immunohistochemical staining of colonic adenomas revealed only minor heterogeneity. Out of 20 colonic adenomas tested, 18 had LFA-3-positive tumour cells with staining intensity corresponding to the adjacent normal mucosa (Fig. 1b); however, 2 well-differentiated adenomas contained areas with a markedly reduced antigenic density and even lacked the LFA-3 antigen in some foci.

In contrast, LFA-3 expression in colorectal carcinomas was markedly heterogeneous. Out of 149 colonic carcinomas (cf. Table 1), 74 tumours (49.7%) expressed LFA-3 in normal quantities (Fig. 1c), whereas 10 tumours (6.7%) were completely devoid of LFA-3 (Fig. 1d, Table 2). The remainder displayed a reduction of LFA-3 and/or mixed pattern of LFA-3 expression: 24 tumours (16.1%) contained only few LFA-3-negative cells, 11 (7.4%) were composed of LFA-3-positive and -negative cells, 19 (12.6%) were predominantly negative and 11 (7.4%) contained but small groups of LFA-3-positive tumour cells (Table 2).

Statistical analysis yielded no correlation between reduction/loss of LFA-3 and Dukes' stage or grade of differentiation or typing. The data of the survival analysis calculated on the basis of all 149 patients with putatively curative surgical treatment (Ro resection) are given in Table 3. Disease-free survival and the probability to succumb to tumour-related death was correlated with

Table 2. LFA-3 antigen expression in 20 colorectal adenomas and 149 colorectal carcinomas, as determined by immunohistochemistry

Score		Adenoma		Carcinoma	
		n	(%)	*n*	(%)
+	(1)	18	(90.0)	74	(49.7)
+ > −	(2)	2	(10.0)	24	(16.1)
+/−	(3)	0	0	11	(7.4)
− > +	(4)	0	0	19	(12.6)
− ≫ +	(5)	0	0	11	(7.4)
−	(6)	0	0	10	(6.7)

+, All epithelial cells positive; −, negative staining; A/B, pattern A and B in about equal proportions; A > B, pattern A clearly dominating pattern B; A ≫ B, pattern B only rarely observed

Table 3. Influence of prognostic variables on recurrence and tumour-related death, calculated on the basis of a follow-up ranging from 65 to 45 months

Parameter	Number of observations		Number of tumour recurrences		Number of tumour-related deaths	
	n	%				
Dukes' stage						
A,B	97	65.1	11		5	
C	52	34.9	16	$P=0.003$	10	$P=0.009$
Grade						
I	16	10.7	1		0	
II	110	73.8	19		9	
III/IV	23	15.5	7	$P=0.170$	6	$P=0.038$
Localization						
Caecum to descending colon	44	29.5	6		4	
Sigmoid colon	21	14.1	2		2	
Rectum	84	56.4	19	$P=0.184$	9	$P=0.846$
Type						
Non-mucinous	103	69.1	17		8	
Mucinous	46	30.9	10	$P=0.469$	7	$P=0.100$
LFA-3 expression						
Normal	74	49.7	12		8	
Reduced/lost	75	50.3	15	$P=0.653$	7	$P=0.515$

the tumour stage (log-rank test). Furthermore, the relative risk of tumour related death was significantly higher in cases of histopathological grade III (poorly differentiated) and IV (undifferentiated) carcinomas. No discriminating effect either both disease-free survival or risk of tumour-related death could be determined with respect to tumour localization and type. The degree of LFA-3 expression in colonic carcinomas did not influence disease-free survival or risk of tumour-related death (Fig. 2a–c).

Discussion

We have shown LFA-3 antigen to be constitutively expressed in normal colonic epithelium. In a minor fraction of colonic adenomas and in about half of the colorectal carcinomas, however, LFA-3 antigen density is considerably diminished and even completely abrogated in a small proportion of carcinomas. Our patients had undergone potentially curative surgical treatment, and their data on both the risk of tumour recurrence and tumour-related death were correlated with the Dukes' stage. Also, the histological grade of tumour differentiation had a significant impact on the survival rate. Thus, our cohort confirms the prognostic significance of the parameters that emerged from recent studies of colorectal carcinoma (Gastrointestinal Tumor Study Group 1984; Carlon et al. 1985; Chapuis et al. 1985; Jass et al. 1986; Griffin et al. 1987; Newland et al. 1987; Minsky et al. 1988; Wiggers et al. 1988a, b; Fisher et al. 1989; Stähle et al. 1989; Moertel et al. 1990).

The physiological role of LFA-3 on the target cell is to interact with the CD2 molecule on the T-cell (Shaw et al. 1986; Seed and Aruffo 1987; Takai et al. 1987), providing antigen-independent conjugate formation, T-cell activation and physical interaction with HLA proteins. Loss of LFA-3 on target cells might therefore weaken antigen-specific T-cell binding (Krensky et al. 1983; Shaw and Ginther Luce 1987; Recny et al. 1990). These findings suggest that a loss of LFA-3 on tumour cells might be responsible for the likely escape of tumours from cytotoxic T-cell attack (Smith et al. 1989). Based on animal models, it was proposed that the presence/absence of HLA molecules might modulate a potential anti-tumour T-cell response. Numerous studies have demonstrated that about 10–15% of colonic carcinomas completely lack HLA-A,B,C antigens and that about 20% display reduced amounts (Csiba et al. 1984; Gosh et al. 1976; Momburg et al. 1986; Durrant et al. 1987; Van den Ingh et al. 1987; Stein et al. 1988; Lopez-Nevot et al. 1989). However, induction of HLA-D molecules was also observed in (subsets) of tumour cells in about 50% of all colonic carcinomas (Gosh et al. 1986; Momburg et al. 1986; Degener et al. 1988). Despite these aberrations, we were recently able to show that the mode of HLA-A,B,C and -D expression in colonic carcinoma per se does not influence recurrence and survival (Möller et al. 1991).

LFA-3 (CD58) loss on tumour cells was first described by Billaud et al. (1987). They reported this antigen missing from 8 of 10 EBV-negative and from 2 of 13 EBV-positive Burkitt's lymphoma cell lines, but this feature could not be confirmed for EBV-induced lymphoblastoid B-cell lines. In yet another study on Burkitt's lymphoma cell lines (Gregory et al. 1988), LFA-3 absence was combined with an in vitro growth pattern as single-cell suspension, and LFA-3 presence with aggregate formation by homotypic adhesion. Gregory et al. (1988) in addition proposed that LFA-3 deficiency aided the escape of Burkitt's lymphoma from virus-specific T-cell surveillance. Although recombinant tumour necrosis factor-alpha and recombinant interferon-gamma have been reported to increase LFA-3 surface expression on melanoma cell lines and simultaneously to lower their proliferation rates (Mortarini et al. 1990), it is not yet known whether LFA-3 reduction/loss is brought about by some message signalling physiological down-regulation of this adhesion molecule or some regulatory malfunction within the tumour cell itself.

LFA-3 loss in colonic carcinomas has so far been shown only in a study based on a limited number of cases (Smith et al. 1989); a case cohort sufficient to satisfy statistical analysis requirements has still been outstanding. Correlating our original data on the degree of LFA-3 expression in colorectal carcinoma with our follow-up data, it has emerged that LFA-3 expression was neither related to the clinical or pathohistological parameter examined, nor with disease-free survival and the risk or tumour-related death. Although loss of LFA-3 expression seems to indicate malignancy of colonic

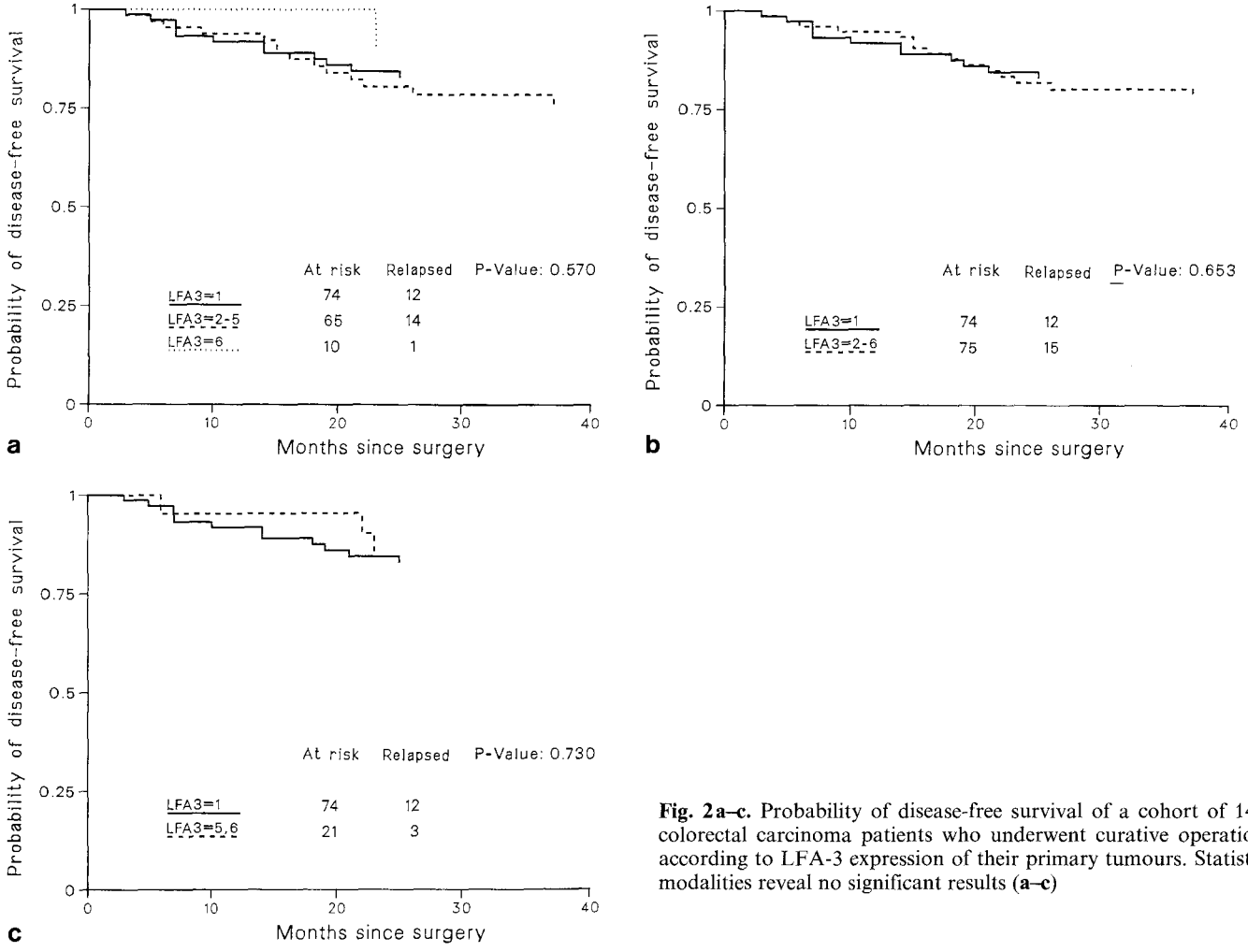

Fig. 2a–c. Probability of disease-free survival of a cohort of 149 colorectal carcinoma patients who underwent curative operation according to LFA-3 expression of their primary tumours. Statistic modalities reveal no significant results (**a–c**)

epithelium, the failure to express an antigen necessary for the interaction with T-cells was not coupled with the patient's prognosis. Thus, it is still obscure whether the failure to detect any prognostic effects is caused by the insignificant alteration of the anti-tumour T-cell response brought about by LFA-3 loss or because of the ineffective cytotoxic T-cell activity in colorectal carcinoma.

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