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# Reduced expression of E-cadherin is related to invasive disease and frequent recurrence in bladder cancer

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**Abstract** A series of 161 bladder cancer biopsy specimens (survival data available in 122 cases) was analysed immunohistochemically for the expression of E-cadherin (E-CD), the most important cell-to-cell adhesion molecule in epithelial cells. Altogether, 81% of the tumours were E-CD-positive, the staining being heterogeneous in nearly all tumours. Normal transitional epithelium was positive for E-CD while in carcinomas the expression was reduced or even absent (18%). Lower levels of E-CD were detected in rapidly proliferating high-grade muscle-invasive tumours. Reduced expression of E-CD was related to a dense inflammatory cell reaction in tumour stroma. The median clinical follow-up was 12.0 years. Short recurrence-free survival of Ta-T1 tumours ($P = 0.02$) was related to expression of E-CD fewer than 50% of cancer cells. In survival analysis the fraction of E-CD-positive cells ($P = 0.1$) and the expression intensity of E-CD ($P = 0.09$) showed a non-significant association to prognosis. Multivariate survival analysis indicated that expression of E-CD has no independent prognostic value over grade or stage while recurrence-free survival was related to E-CD expression.

**Key words** Bladder cancer · Cell adhesion molecule · E-cadherin

## Introduction

Adhesion molecules are important in cell–cell interactions (Wydie et al. 1979; Eidelman et al. 1989; Springer 1990; Takeichi 1990, 1991; Frixen et al. 1991; Mortarini and Anichini 1993) and they belong to four major groups: (a) the immunoglobulin family, (b) integrins, (c) selectins and (d) cadherins. Cadherins are responsible for cell-to-cell adhesion (Wydie et al. 1979; Takeichi 1990). These molecules are glycoproteins with an extracellular component having an adhesion site in the N-terminal region and several binding sites for calcium. The binding mediated by cadherins is homophilic, i.e. a cadherin molecule on one cell only binds to another cadherin molecule of the same type on the next cell. In adult epithelium, cadherins are present on the lateral cell surfaces (zonula adherens, desmosomes), and they are linked to the cellular cytoskeleton (Hirano et al. 1987; Eidelman et al. 1989; Takeichi 1990).

Cell-to-cell interactions play a key role in the regulation of cellular function and differentiation (Wydie et al. 1979; Takeichi 1990, 1991; Mortarini and Anichini 1993). Diminished expression of these molecules, due to genetic down-regulation, has been related to dissociation of epithelial cells, and experimental analyses suggest that their invasive potential is also increased (Frixen et al. 1991; Ruggeri et al. 1992).

Some bladder carcinomas have a high metastatic potential (Lipponen et al. 1993a,c) and recent analyses suggest that molecules mediating cellular interactions may be involved in the development of metastatic disease (Frixen et al. 1991). To date, the clinical significance of various adhesion molecules in bladder cancer has not been investigated in a long-term follow-up, while initial results suggest prognostic significance for E-cadherin (E-CD) expression (Bringuier et al. 1993). The present analysis was designed to elucidate the incompletely understood role of cell-to-cell adhesion molecule (E-CD) expression in bladder cancer in a cohort of 122 bladder cancer patients with a mean follow-up of more than 10 years.

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## Materials and methods

The present series consists of 122 almost consecutive (occasional cases were excluded because of insufficient biopsy specimens for immunohistochemistry) bladder cancer biopsy specimens in which clinical follow-up data were available for survival analysis (Table 1). The analysis also included an additional 39 cases in which TNM classification and histological data were available for analysis of clinicopathological correlations. Accordingly the total number of cases was 161. All the tumours were primary tumours from different patients; recurrent tumours were not included and information related to tumour multifocality was not available. The patient material was from the years 1970–1986 and it was clinically followed-up until 1991. The staging of tumours was done according to The International Union against Cancer (UICC) (1978). Staging was based on bimanual palpation, cystoscopy, urography, lymphography, chest and bone radiographs, ultrasonography and routine laboratoty tests reflecting bone and liver metabolism. Lymph node involvement was not assessed in superficial tumours unless node involvement was not suspected. The treatment of tumours was carried out according to generally accepted principles (Zingg and Wallace 1985) and the types of therapy used are listed in Table 1. The follow-up was conducted at 3-month intervals during the first year and twice a year thereafter for 5 years and then annually. If a recurrent tumour was detected the follow-up program was restarted.

**Table 1** Pertinent data of the patients in which clinical follow-up data were available

| | |
|---|---|
| *Clinical data* | |
| Male/female | 96/26 |
| Age (years), range, mean (SE) | (33–85) 67.0 (0.9) |
| Follow-up (years), median, mean (SE) | 12.0,12.0 (0.4) |
| G1/G2/G3 | 43/50/29 |
| Ta/T1/T2/T3/T4 | 20/30/37/23/12 |
| N0/N1–3 at diagnosis | 100/22 |
| M0/M1 at diagnosis | 114/8 |
| Died of bladder cancer/other | 47/36 |
| Therapy[a] | |
| Intravesical chemotherapy | 28 |
| Transurethral resection | 128 |
| Partial cystectomy | 14 |
| Cystectomy | 13 |
| Cystectomy and radiation therapy | 16 |
| Radiation therapy alone | 15 |

[a] Note that the same patient was treated by different methods during the follow-up

### Histological methods

The specimens from the primary tumours obtained at operation, were fixed in buffered formalin (pH 7.0), embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin. Histological grading of tumours was completed using the grading system described by Mostofi (1973). The counting of mitotic figures was done using an objective magnification of ×40 (field diameter 490 μm). The volume-corrected mitotic index (M/V) method was used, which expresses the number of mitotic figures per square millimetre of neoplastic epithelium in the section (Haapasalo et al. 1989). The density of tumour-infiltrating lymphocytes (TIL) was scored into three categories as described in detail recently (Lipponen et al. 1993b). The categories of TIL were (a) absent or weak (TIL 1), (b) moderate (TIL 2) and (c) dense (TIL 3).

### Flow cytometry and nuclear morphometry

Flow cytometry and nuclear morphometry were completed as described previously (Lipponen et al. 1993a, c). The DNA index (DI) was available in 101/122 (83%) cases and the S-phase fraction could be analysed in 84/122 (69%) cases. Tumours with a DNA index value below 1.00 were considered diploid, and tumours with a DNA index above 1.00 were aneuploid. Morphometric nuclear factors were determined by interactive measurement of cancer cell nuclei by using an IBAS 1&2 image analyser system. In this analysis the mean nuclear area, the SD of the nuclear area, the mean nuclear perimeter, the mean longest nuclear axis and the mean shortest nuclear axis are used.

### Immunohistochemistry

For detection of E-cadherin, 5-μm sections of formalin-fixed, paraffin-embedded specimens were cut onto poly-L-lysine-treated slides. Samples were deparaffinized and rehydrated before incubation for 5 min with 5% hydrogen peroxide to inhibit the endogenous peroxidase. The slides were treated with 0.1% pronase for 15 min in 37 °C without calcium. After treatment with the blocking goat serum (to reduce the non-specific binding of conjugated second antibody), the samples were incubated overnight at + 4 °C with the monoclonal mouse anti-E-cadherin (uvomorulin, L-CAM) antibody (IgG1; Sanbio, UK; diluted 1:10 in phosphate-buffered saline, PBS, pH 7.2, which contained 1% bovine serum albumin and 0.10% sodium azide). The antibody is raised by using hybridoma technology in a mouse myeloma cell line (P3-XG3-Ag8.653-BALB/c). The antibody reacts specifically with the 120-kDa and 80 kDa ARC-1 protein in Western blotting and, in normal epithelial cells, the antigen is localised on lateral cell borders (Frixen et al. 1991). After washes with PBS (pH 7.2), an incubation with biotin-conjugated goat anti-(mouse Ig) secondary antibody (Vector Laboratories Inc., Burlingame, Calif., USA) diluted to 1:200 in PBS was carried out for 30 min at room temperature. Slides were washed twice with PBS and incubated in preformed avidin–biotinylated-peroxidase complex (ABC, Vectastain Elite kit, Vector) for 40 min. The reaction product was visualized by immersing the slides in diaminobenzidine tetrahydrochloride (Sigma, Paisley, UK) and finally counterstained with Mayer's haematoxylin. Both positive (bladder cancer biopsy specimen) and negative (positive control processed without primary antibody) controls were added in each experiment and they were shown to be positive or negative respectively.

### Scoring of immunoreactivity

One section from each of the tumours was studied. All sections were first screened to disclose the areas with well-preserved tissue architecture and cell morphology for scoring of immunoreactivity. Necrotic tumour areas or areas with detoriation of tissue morphology due to processing were discarded in the analysis. Intensity of the membrane staining of cells in the selected areas was scored into four categories. If none of the cells was positive, the sample was scored negative (0). The sections with a distinct membrane staining were scored "strong" (3) (Fig. 1). Tumours with just identifiable weak membrane staining were scored "weak" (1), and the rest of cases were scored "intermediate (moderate)" (2). As a second parameter, the fraction of positive cells was estimated in the selected well-preserved areas with intact tissue architecture of the tumour sample.

Statistical analysis

In basic statistical calculations, the SPSS/PC + program was used in an IBM computer and the statistical tests used are indicated with the results when appropriate. Frequency distributions were tested by the $\chi^2$ test and Yate's correction was applied when appropriate. The differences between the means of continuous variables were tested by analysis of variance. The univariate survival analysis (log rank analysis, SPSS-X) was based on the life-table method with the statistics of Lee and Desu (1972). Multivariate survival analysis was done with the BMDP (2L) program package in a stepwise manner and continuous variables were used as absolute numbers in this analysis. The entry limit was $P < 0.1$ and the removal limit was $P > 0.15$.

## Results

The E-CD was expressed on cell membranes (Fig. 1) and the fraction of E-CD-positive cells as well as the expression intensity of E-CD were highly variable; 30/161 (19%) of the tumours were completely negative for E-CD. In normal transitional epithelium, E-CD was expressed at cell membranes particularly on lateral cell borders, and the umbrella cells showed a strong staining of cell membranes. The cytoplasm was positive in some of the tumours.

The fraction of E-CD-positive cells was significantly related to histological grade (Table 2) and clinical stage so that poorly differentiated invasive tumours had fewer positive cells than the well-differentiated ones. Tumours with pelvic lymph node metastasis did not differ from N0 tumours with respect to E-CD expression while M1 tumours expressed E-CD in a smaller fraction of cells than the M0 tumours. The density of tumour-infiltrating lymphocytes was inversely related to the fraction of E-CD-positive cells, so that tumours with dense TIL were negative for E-CD. E-CD was expressed in a larger fraction of cells in diploid tumours and slowly proliferating tumors than in rapidly proliferating aneuploid tumours (Table 3). Among the morphometric nuclear size and shape parameters, the standard deviations (SD) of the nuclear area ($R = -0.189, P < 0.05$) and of the nuclear perimeter ($R = -0.185, P < 0.05$) were inversely correlated to the fraction of E-CD-positive cells. The correlation between DI ($R = -0.255, P < 0.05$), M/V ($R = -0.280, P < 0.05$) and the fraction of E-CD-positive nuclei was also significant.

The expression intensity of E-CD was not significantly related to grade or stage (Table 4) while the relationship between density of TIL and E-CD expression intensity reached statistical significance (Table 4). E-CD expression intensity was independent of morphometric or flow-cytometric parameters.

Progression (increase in T category) of Ta-T1 tumours was not related to E-CD expression ($\chi^2 P = 0.4$). Recurrence-free survival of Ta-1 tumors (Fig. 2) was related to the fraction of E-CD-positive cells, while survival ($P = 0.3$) was independent of E-CD.

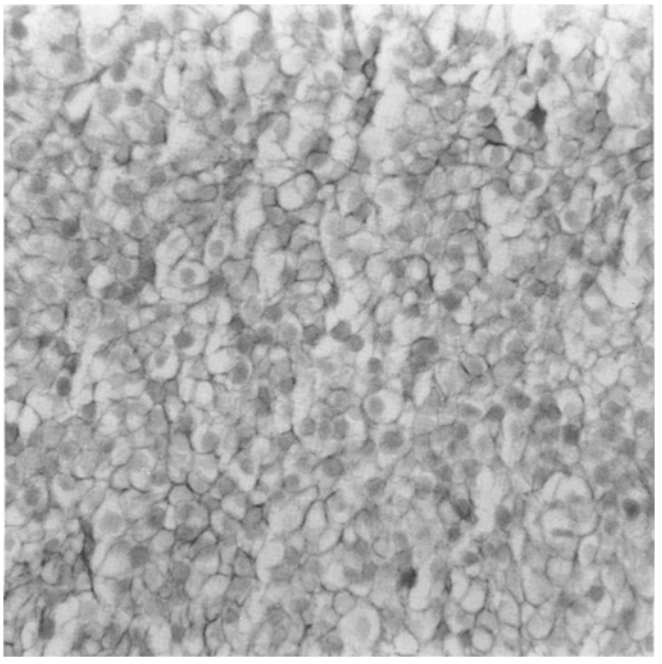

**Fig. 1** Expression of E-cadherin (E-CD) is located on cell membranes and shows considerable intratumour variation. The expression of E-CD was scored as being "strong" in this microscope image (magnification 250 ×)

**Table 2** Mean (SE) fraction of E-cadherin (E-CD)-positive cells as related to histological and clinical features. Two groups: two-tailed *t*-test; several groups: analysis of variance. Tumour-infiltrating lymphocyte (*TIL*) and NM classification were available in 122 cases

| Variable | Number | Mean (SE) | Statistics |
|---|---|---|---|
| Grade 1 | 56 | 57.6 (4.9) | |
| Grade 2 | 63 | 38.9 (4.3) | $F = 7.7, P < 0.001$ |
| Grade 3 | 42 | 30.3 (5.5) | |
| TIL 1 | 65 | 42.3 (4.3) | |
| TIL 2 | 32 | 41.9 (7.0) | $F = 3.9, P = 0.0228$ |
| TIL 3 | 25 | 19.6 (6.0) | |
| Ta | 23 | 53.6 (7.9) | |
| T1 | 49 | 58.7 (5.2) | |
| T2 | 47 | 32.5 (4.6) | $F = 5.1, P = 0.0006$ |
| T3 | 29 | 31.2 (7.0) | |
| T4 | 13 | 31.1 (8.6) | |
| N0 | 100 | 38.2 (3.6) | $t = 0.21$ |
| N1–2 | 22 | 36.3 (8.2) | $P = 0.835$ |
| M0 | 114 | 53.7 (11.4) | $t = -1.42$ |
| M1 | 8 | 36.8 (3.4) | $P = 0.192$ |

Progression of tumours in the entire cohort showed a significant relationship to the fraction of E-CD-positive cells (Table 5). In the entire cohort the fraction of E-CD-positive cells (Fig. 3) and the expression intensity of E-CD (Fig. 4) were not statistically significant prognostic factors, although a clear trend was present. Mitotic index $\chi^2 = 15.5$,

**Table 3** Mean (SE) fraction of E-CD-positive cells as related to flow-cytometric and morphometric features. Statistical analysis was by the two-tailed *t*-test; flow-cytometric data were not available in all cases. *SPF* S-phase fraction, *M/V* volume-corrected mitotic index, *NA* nuclear area, *SDNA* SD of nuclear area

| Variable | Number | Mean (SE.) | Statistics |
|---|---|---|---|
| Diploid | 47 | 41.6 (5.0) | $t = 1.48$ |
| Aneuploid | 54 | 31.2 (4.8) | $P = 0.141$ |
| SPF < 10% | 48 | 47.3 (5.1) | $t = 1.82$ |
| SPF > 10% | 36 | 33.2 (5.6) | $P = 0.072$ |
| M/V < 10/mm$^2$ | 63 | 45.7 (4.7) | $t = 2.51$ |
| M/V > 10/mm$^2$ | 59 | 29.6 (4.3) | $P = 0.013$ |
| NA < 80 μm$^2$ | 76 | 46.5 (3.6) | $t = 1.80$ |
| NA > 80 μm$^2$ | 48 | 35.1 (4.7) | $P = 0.074$ |
| SDNA < 25 μm$^2$ | 75 | 41.9 (4.3) | $t = 1.62$ |
| SDNA > 25 μm$^2$ | 47 | 31.4 (4.7) | $P = 0.108$ |

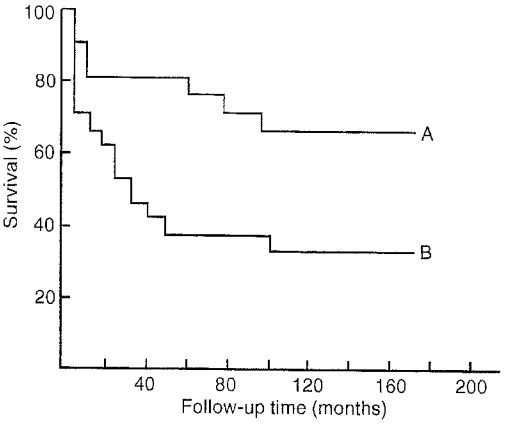


**Fig. 2** The recurrence-free survival of Ta-T1 bladder cancer patients categorised according to expression of E-CD. The curves are significantly different ($\chi^2 = 5.2$, $P = 0.0214$). *A* E-CD expression in over 50% of cells, $n = 25$; *B* E-CD expression in fewer than 50% of cells, $n = 25$

$P = 0.0001$), SD of nuclear area ($\chi^2 = 4.6$, $P = 0.031$), DNA ploidy $\chi^2 = 11.7$, $P = 0.0006$), S-phase fraction ($\chi^2 = 14.7$, $P = 0.0001$), grade ($\chi^2 = 23.6$, $P < 0.0001$), papillary status ($\chi^2 = 8.6$, $P = 0.0032$) and T category ($\chi^2 = 38.2$, $P < 0.0001$) were significant prognostic factors in univariate survival analysis. Multivariate survival analysis showed that T category ($\beta = 0.835$, $RR = 2.3$, $P < 0.001$) and grade ($\beta = 0.709$, $RR = 2.0$, $P = 0.001$) were independent prognostic factors. Recurrence-free survival was related to the M/V index ($\beta = 0.048$, $RR = 1.04$, $P = 0.01$) and fraction of E-CD-positive cells ($\beta = -0.01$, $RR = 0.99$, $P = 0.09$).

**Table 5** The fraction of E-CD-positive cells (means, SE) in progressing and non-progressing tumours. Statistical analysis was by two-tailed *t*-test

| TNM category | No. | E-CD$^+$ cells | Statistics |
|---|---|---|---|
| T category | | | |
| No progression | 79 | 44.0 (4.1) | $t = 1.47$ |
| Progression[a] | 43 | 31.4 (5.4) | $P = 0.145$ |
| N category | | | |
| No progression | 81 | 44.0 (4.1) | $t = 2.67$ |
| Progression[a] | 41 | 25.8 (5.0) | $P = 0.009$ |
| M category | | | |
| No progression | 84 | 42.6 (3.9) | $t = 2.16$ |
| Progression[a] | 38 | 27.5 (5.7) | $P = 0.033$ |

[a] Progression in T, N, or M category

## Discussion

Defects in cell-to-cell adhesion have long been suggested to be intimately involved in the metastatic behaviour of malignant cells (Zutter et al. 1990; Frixen et al. 1991; Ruggeri et al. 1992). In experimental conditions, several cell lines acquire invasive and metastatic properties when the cell adhesion molecules are not expressed or their expression is disturbed (Frixen et al. 1991; Ruggeri et al. 1992).

**Table 4** The expression intensity of E-CD related to histological grade, TIL grade and T category. Statistical analysis was by the $\chi^2$-test

| Variable | No. | Expression of E-CD | | | | Statistics |
|---|---|---|---|---|---|---|
| | | E-CD(0) | E-CD(1) | E-CD(2) | E-CD(3) | |
| Grade 1 | 56 | 8 | 30 | 16 | 2 | |
| Grade 2 | 63 | 12 | 25 | 14 | 12 | $\chi^2 = 11.1$, $P = 0.08$ |
| Grade 3 | 42 | 10 | 19 | 11 | 2 | |
| TIL 1 | 64 | 8 | 29 | 19 | 8 | |
| TIL 2 | 30 | 9 | 14 | 5 | 2 | $\chi^2 = 12.9$, $P = 0.044$ |
| TIL 3 | 24 | 10 | 10 | 4 | 0 | |
| Ta | 23 | 4 | 14 | 5 | 0 | |
| T1 | 49 | 4 | 20 | 18 | 7 | |
| T2 | 47 | 9 | 23 | 8 | 7 | $\chi^2 = 18.4$, $P = 0.102$ |
| T3 | 29 | 10 | 11 | 7 | 1 | |
| T4 | 13 | 3 | 6 | 3 | 1 | |

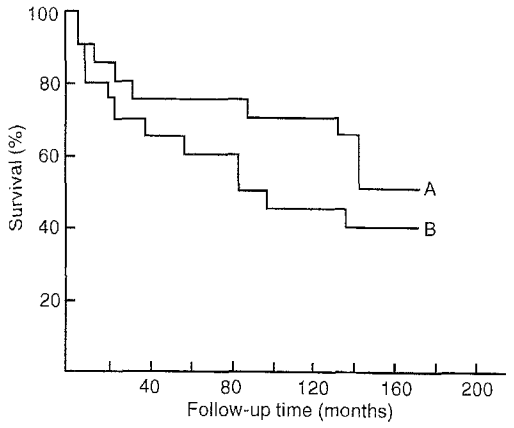


**Fig. 3** The survival of bladder tumours categorised according to expression of E-CD. The curves are not significantly separated ($\chi^2 = 2.2$, $P = 0.13$). *A* E-CD expression in more than 10% of cells, $n = 73$; *B* E-CD expression fewer than 10% of cells, $n = 49$

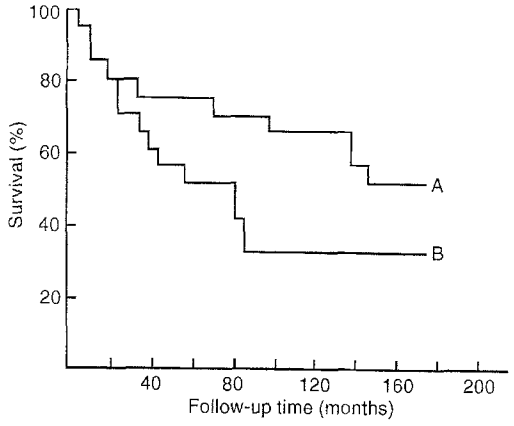


**Fig. 4** The survival of bladder cancer patients categorised according to the expression intensity of E-CD. The curves are not significantly separated ($\chi^2 = 2.8$, $P = 0.09$). *A* E-CD (1, 2, 3), $n = 93$; *B* E-CD (0), $n = 29$

In the normal bladder, transitional epithelium expresses E-CD, whereas the expression of cadherins in bladder carcinomas has been rarely studied (Bringuier et al. 1993). Bringuier et al. (1993) have established a correlation between reduced expression of E-CD and the differentiation grade in bladder cancer. The present results show a significantly lower fraction of E-CD-positive cells in grade-3 tumours, which is in line with the results of Bringuier et al. (1993). However, a review of the literature shows that the relationship between E-CD expression and malignancy grade is variable in different neoplasia (Shiozaki et al. 1991; Gamallo et al., 1993; Rasbridge et al. 1993; Dorudi et al. 1993).

Bringuier et al. (1993) reported close to 10% completely negative tumours while, in this series, 18% of the tumours were negative. The difference in results may be due to different stage/grade distributions of the tumours. Also the enzymatic pretreatment of paraffin-embedded material may reduce the staining as well. However, comparative studies between paraffin-embedded materials and frozen tissues with this same antibody showed that only the staining intensity was weaker in some of the tumours while the fraction a positive cells remained similar (Lipponen et al. 1994).

E-CD expression was related significantly to T category, which is also consistent with the results of Bringuier et al. (1993). We could not establish a significant relationship between NM classification and expression of E-CD although there was a clear trend between reduced expression of E-CD and distant metastasis. In colon carcinomas the expression of E-CD has been related to lymph node involvement (Dorudi et al. 1993), although some E-CD-negative primary tumours proved to be E-CD-positive when the lymph node metastases were analysed (Dorudi et al. 1993). Accordingly, the expression of E-CD in tumour metastases may be influenced by the heterogeneous expression of E-CD in the primary tumours, or other factors related to the growth environment of cancer cells (Mareel et al. 1991). In head and neck tumours expression of E-CD seems to be independent of stage (Mattijssen et al. 1993) also.

The cell adhesion system and tumour/host interaction are probably functionally interrelated (Tomita et al. 1993; Mortarini and Anichini 1993). Tomita et al. (1993) showed that intercellular adhesion molecule-1 is expressed in bladder tumours that are infiltrated by T lymphocytes but is not expressed in normal urothelium. T-cell tumour interaction also has prognostic significance since tumours with a dense lymphocyte infiltrate have a better prognosis (Lipponen et al. 1993). The present analysis shows that E-CD expression is lower in tumours with a dense TIL, suggesting that immunological defence mechanisms and E-CD are also interrelated. Alteration in the expression of adhesion molecules is not only limited to neoplastic disorders since in several inflammatory diseases their expression may be changed as well (Springer 1990; Wood et al. 1993; Mortarini and Anichini 1993).

The relationship between morphometric nuclear factors and expression of E-CD may be related to changes in the polarity of E-CD expression as observed in malignant tumours (Shiozaki et al. 1991). This concept is supported by the notion that E-CD molecules are associated with the cell cytoskeleton (Hirano et al. 1987; Ruggeri et al. 1992) and accordingly alternations in cell morphology are possible.

Expression of E-CD has been related to prognosis in epithelial neoplasia (Mattijssen et al. 1993) and, to date, there is one published report available on the prognostic value of E-CD in bladder cancer (Bringuier et al. 1993). This showed a significant relationship between increased risk of progression, death from bladder cancer and low expression of E-CD. Our survival analysis supports these previous results although the prognostic results were not so convincing as in their series (Bringuier et al. 1993). The staining for E-CD showed remarkable individual differences and the staining for E-CD showed a broad overlap between the different

patient groups. This finding also compromises the prognostic impact of E-CD expression for the individual patients. One should also note that, in this cohort, a substantial number of patients died of other diseases than bladder cancer, which may reduce the prognostic value of E-CD in survival analysis based on cancer deaths only. However, the recurrence-free survival was clearly shorter in tumours with a low fraction of E-CD-positive cells, which may be related to intraluminal dissemination of malignant cells resulting in elevated risk of recurrence. The results of multivariate analysis also suggests that a high fraction of E-CD-positive cells is protective for recurrence even in rapidly proliferating tumours although the risk of recurrence is mainly related to cell proliferation (Lipponen et al. 1993a,c).

The present analysis assessed the expression of E-CD, which is only a part of the cell-to-cell adhesion system of bladder cancer cells. The integrins mediating cell-to-matrix interactions are also important in the development of a malignant phenotype (Zutter et al. 1990). Accordingly, it seems unlikely that any single alteration in the cell adhesion system is sufficient for an invasive and metastatic phenotype in bladder cancer. Further analyses are certainly required to assess the functions of the entire adhesion system (Mortarini and Anichini 1993) in bladder cancer prognosis.

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