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Immunohistochemical evaluation of alpha-catenin expression in human gastric cancer

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Abstract E-cadherin (E-cad) plays a major role in the maintenance of cell-cell adhesion in epithelial tissues, and impaired E-cad expression correlates with tumour invasion and metastasis. Alpha-catenin (α-cat), an undercoat protein of adherens junctions, binds to the cytoplasmic domain of E-cad and is essential for linking E-cad to actin-based cytoskeleton. We investigated E-cad and α-cat expression in 60 human gastric cancers immunohistochemically. The 60 gastric cancers were classified into 18 (30%) in which α-cat expression was preserved, and 42 (70%) reduced cases. The reduction of α-cat expression was significantly related to dedifferentiation, depth of invasion, infiltrative growth and lymph node metastasis. We also examined the co-expression of α-cat and E-cad. Seventeen (28%) tumours preserved both molecules [α-cat(+)/E-cad(+)] and 33 (55%) tumours reduced both [α-cat(−)/E-cad(−)], whereas 9 (15%) tumours exhibited α-cat(−)/E-cad(+). The frequency of lymph node metastasis in α-cat(−)/E-cad(+) tumour (67%) was significantly higher than that in α-cat(+)/E-cad(+) tumours (24%) and was close to that in α-cat(−)/E-cad(−) tumours (82%). The frequency of haematogenous liver metastasis in α-cat(−)/E-cad(+) tumours (44%) was significantly higher than that in α-cat(+)/E-cad(+) tumours (6%) or α-cat(−)/E-cad(−) tumours (9%). Thus, in all E-cad(+) tumours, the frequency of lymph node and liver metastasis was higher in α-cat(-) tumours than in α-cat(+) tumours. α-Cat expression is apparently better at predicting tumour invasion and metastasis than E-cad expression.

Key words Gastric cancer · Alpha-catenin
Immunohistochemistry · E-cadherin · Cancer invasion

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Introduction

Cadherins are a family of transmembrane glycoproteins responsible for calcium-dependent intercellular adhesion by homophilic interaction (Takeichi 1988). They bind cells so tightly that inactivation of other adhesion systems has little effect on cell-cell adhesion when cadherins are functioning (Duband et al. 1987; Larjava et al. 1990). More than ten subclasses of cadherins have been identified, such as E-, P-, N-cadherin (Hatta and Takeichi 1986; Nagafuchi et al. 1987; Nose et al. 1987), L-CAM and others (Gallin et al. 1987; Heimark et al. 1990). Their amino acid sequences show a high degree of homology especially in the cytoplasmic domain (Nose et al. 1990), where cadherins are bound to some undercoat proteins of intercellular adherens junction, including α-(102kDa), β-(88kDa), and γ-catenin (80kDa) (Vestweber and Kemler 1984; Peyrieras et al. 1985; Ozawa et al. 1989). Recent studies suggested that cadherins require anchoring to the actin-based cytoskeleton through catenins in order to exhibit cell-cell binding function (Nagafuchi et al. 1991; Tsukita et al. 1992). This is supported by the observations that cells transfected with truncated cadherin, which does not bind to α-cat, did not form tight intercellular adhesions (Nagafuchi and Takeichi 1988, 1989; Ozawa et al. 1989, 1990) and one human lung cancer cell line which did not show tight cell-cell adhesion strongly expressed E-cad, but did not express α-cat (Shimoyama et al. 1992).

Detachment of tumour cells from a primary lesion is the initial step of invasion ad metastasis (Nicolson 1988; Fidler 1989), and involves disruption of cell-cell adhesion. We have previously demonstrated the existence of reduced E-cad expression in human cancers. (Shiozaki et al. 1991), and a significant relationship between reduced E-cad expression and aggressive clinicopathological factors, such as dedifferentiation or infiltrative growth in gastric cancer and breast cancer (Oka et al. 1992, 1993). These findings were confirmed by several investigators (Schipper et al. 1991; Umbas et al. 1992). Thus, impaired E-cad expression was correlated with histopatho-

logical features indicating reduction of intercellular adhesion. A number of authors have observed carcinomas that expressed E-cad but invaded sparsely, not forming close contacts (Oka et al. 1992; Shimoyama and Hirohashi 1991) a phenomenon which suggests that a mechanism which interferes with cadherin function may exist in such tumours, even in the existence of E-cad.

In this study, we investigated E-cad and α-cat expression in surgically resected human gastric cancer tissue, in order to evaluate intercellular adhesion more precisely, using immunohistochemical staining. Furthermore, we analysed the relationship between intercellular adhesiveness and clinicopathological features, especially tumour invasion and metastasis.

Materials and methods

The surgical specimens were obtained from 60 patients with gastric cancer from November 1990 to December 1992 in the Department of Surgery II, Osaka University Medical School. The age of the patients ranged from 28 to 82 years (mean, 59.8). No patient had received anticancer therapy prior to the operation. Samples were taken from representative cancerous lesions including adjacent non-neoplastic mucosa. In 2 of the 60 patients, tissue samples of metastatic liver tumours were also examined.

Immunostaining for α-cat and E-cad was performed by the avidin-biotin-peroxidase complex method (Hsu et al. 1981) as described previously (Shiozaki et al. 1991). In brief, fresh samples were immediately frozen in dry ice acetone, cut into 4-μm-thick sections, and mounted on slides. The sections were then fixed with 3.6% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min. Two mAbs, α18 rat IgG against α-cat (Nagafuchi and Tsukita 1994) or HECD-1 mouse IgG against human E-cad (Shimoyama et al. 1989) were applied and incubated overnight at 4 °C sequentially followed by biotinylated goat anti-rat IgG or horse anti-mouse IgG, and avidin combined in vitro with horseradish peroxidase (Vectastain ABC kit, Vector, Burlingame, Calif., USA). Slides were developed using diaminobenzidine supplemented with 0.02% hydrogen peroxide for 4 min. The sections were counterstained with haematoxylin, dehydrated and mounted.

The expression of α-cat and E-cad for cancer cells was compared with that of normal epithelial cells in the same sample, which always express both molecules strongly. The cancer cells whose staining was as strong as that of normal epithelial cells were defined positive. The grade of α-cat and E-cad expression of the tumours was evaluated according to the proportion of positive cells. When more than 90% of the cancer cells were positive, the tumours were evaluated as preserved (+) and when less than 90% as reduced (−). The reduced tumours were subdivided into heterogenously reduced [hetero(−)] with 10%–90% of positive cells and homogeneously reduced [homo(−)] with less than 10% of positive cells.

A consecutive section from each specimen was stained with haematoxylin and eosin for histological evaluation. The clinicopathological terminology is derived from the general rules proposed by Japanese Research Society for Gastric Cancer (1981), exceptionally, histological type is classified according to TNM classification of malignant tumours (UICC). There were 19 primary tumours consisting of a mixture of histologically different tumour components. In these tumours, the correlation between α-cat expression and histological types was evaluated for each tumour component, and the other clinicopathological factors were compared with α-cat expression of the dominant histological type. The correlation between grading of α-cat expression and the various histological findings was analysed for statistical significance by Spearman's rank correlation coefficient. Correlation between α-cat/E-cad co-expression and the frequency of various routes of metastasis (peritoneal dissemination, lymph node or liver metastasis) was assessed by the chi-square test or Fisher's exact probability test. A P value of less than 0.05 was accepted as statistically significant.

Results

α-Cat was strongly expressed in all the non-cancerous epithelium at cell-cell boundaries, and was also detected weakly in fibroblasts and smooth muscle cells (Fig. 1A, B). Sixty gastric cancers were classified into 18(+) and 42(−) [17 hetero(−) and 25 homo(−)] for α-cat expression. As shown in Fig. 1C, α-cat was expressed at the cell-cell boundaries in α-cat(+) tumours as well as in normal epithelium, and α-cat(+) tumours formed tight intercellular adhesion. α-Cat hetero(−) tumours were composed of a mixture of α-cat positive and negative cells, and positive expression of α-cat was sometimes observed not only at the cell-cell boundaries but also in the cytoplasm of cancer cells. Furthermore, the α-cat positive cells in the α-cat hetero(−) tumours seemed to adhere to each other and build gland-like formations (Fig. 1D). As shown in Fig. 1E, α-cat homo(−) tumour expressed a trace amount of α-cat. In α-cat homo(-) tumour, cancer cels invaded sparsely and did not form close contacts.

Table 1 shows the relationship of α-cat expression to histological findings. The frequency of α-cat(+) was higher in well-differentiated carcinoma (59%, 13/22) than in moderately (14%, 3/22) or poorly differentiated carcinoma (14%, 5/36). Thus, the reduction of the α-cat expression was negatively correlated with differentiation ($P<0.01$). Eighty percent of tumours (12/15) with mucosal or submucosal invasion were α -cat(+), while only 8% of tumours (3/36) with extension beyond muscle invasion were α-cat(+). Sixty-three percent of tumours (5/8) with expansive growth (INF α) were α-cat(+), whereas only 5% of tumours (1/21) with infiltrative growth (INF γ) were α-cat(+). Thus, the reduction of α-cat was significantly correlated with depth of invasion and infiltrative growth by Spearman's rank correlation coefficient ($P<0.01$).

The relationship between metastasis and α-cat expression is summarized in Table 2. The frequency of α-cat(+) in tumours with lymph node metastasis was 11% (4/37), and this value was significantly lower than that in tumours without lymph node metastasis (61%, 14/23) ($P<0.05$). However, α-cat expression showed no significant correlation with peritoneal dissemination or liver metastasis.

Co-expression of α-cat and E-cad was also examined (Table 3). All the non-cancerous epithelial cells strongly expressed E-cad molecules at the intercellular borders as well as α-cat. Sixty tumours were classified into 26(+) and 34(−) [19 hetero(−), 15 homo(−)] for E-cad expression. In the majority of the tumours (50/60, 83%), the expression of these two molecules was at an equivalent level, based on our classification. Thus, the correlation between these molecules was strong and statistically sig-

Table 1 Relationship between α-catenin expression and histological findings

	Expression of α-catenin				
		Reduced			
	Preserved[a]	Hetero[b]	Homo[c]	Total	P value r_s
Differentiation grade					
well differentiated	13	9	0	22	
moderately	3	11	8	22	$P<0.01$
poorly	5	5	26	36	0.602
Depth of invasion[d]					
m, sm	12	1	2	15	
pm	3	1	5	9	$P<0.01$
ss, se, si	3	15	18	36	0.478
Growth pattern[e]					
INF α	5	0	3	8	
INF β	12	11	8	31	$P<0.01$
INF γ	1	6	14	21	0.395

[a] >90% cells were preserved
[b] 10–90% cells were preserved
[c] <10% cells were preserved
[d] *m, sm, pm* and *ss,* spread to mucosa, submucosa, muscularis propria and subserosa, respectively; *se,* exposing on the serosal surface; *si,* infiltrating the neighbouring tissue
[e] INF α, grows expansively; INF β, intermediate between α and γ; INF γ, grows infiltratively

Table 2 Relationship between α-catenin expression and metastasis

	Expression of α-catenin				
		Reduced			
	Preserved	Hetero	Homo	Total	P value r_s
Peritoneal dissemination					
P (−)	17	14	22	53	
P (+)	1	3	3	7	NS
Lymph node metastasis					
N (−)	14	1	8	23	$P<0.05$
N (+)	4	16	17	37	0.282
Liver metastasis					
H (−)	17	14	21	52	
H (+)	1	3	4	8	NS

Table 3 Relationship between α-catenin and E-cadherin expression in primary tumours

		α-cat expression	
		(+)	(−)
E-cad expression	(+)	17	9
	(−)	1	33
		$P<0.01$	

nificant ($P<0.01$). However, the reduced expression of α-cat was observed more frequently than that of E-cad (70% versus 57%).

With regard to histological type, 13 (76%) out of the 17 α-cat(+)/E-cad(+) tumours were differentiated type, and these two molecules were expressed on their neoplastic tubules and papillae. This type of tumour formed strong intercellular adhesion (Fig. 2A, B). However 33 out of 42 tumours with α-cat(−) were also E-cad(−), and 27 (80%) out of the α-cat(−)/E-cad(−) tumours were of the undifferentiated type whose cancer cells were scattered (Fig. 2C, D). Furthermore, a discrepancy in the expression of these two molecules was observed in 10 tumours. Nine of 10 showed α-cat(−)/E-cad(+). The majority (7/9, 78%) consisted of moderately or poorly differentiated adenocarcinoma with non-scirrhous growth pattern. They did not form tight cell-cell adhesion and exhibited intermediate adhesiveness between [α-cat(+)/E-cad(+)] and [α-cat(−)/E-cad(−)] tumours (Fig. 2E, F). The remaining tumour, which was a signet-ring cell carcinoma, showed α-cat(+)/E-cad(−) pattern (Fig. 2G, H). Signet-ring cell carcinomas, which usually invade sparsely, expressed these molecules in various fashion; 4 cases were α-cat(+)/E-cad(+) (Fig. 2I, J), 2 cases were α-cat(−)/E-cad(+) and 5 cases were α-cat(−)/E-cad(−). In these tumours, α-cat was detected not only at their cell-cell boundaries but also in their cytoplasm.

The relationship between co-expression pattern and metastasis is summarized in Table 4. The frequency of lymph node metastasis in α-cat(−)/E-cad(+) tumours (6/9, 67%) was significantly higher than that in α-cat(+)/E-cad(+) tumours (4/17, 24%) by the chi-square test ($P<0.05$) and was close to that in α-cat(−)/E-cad(−)

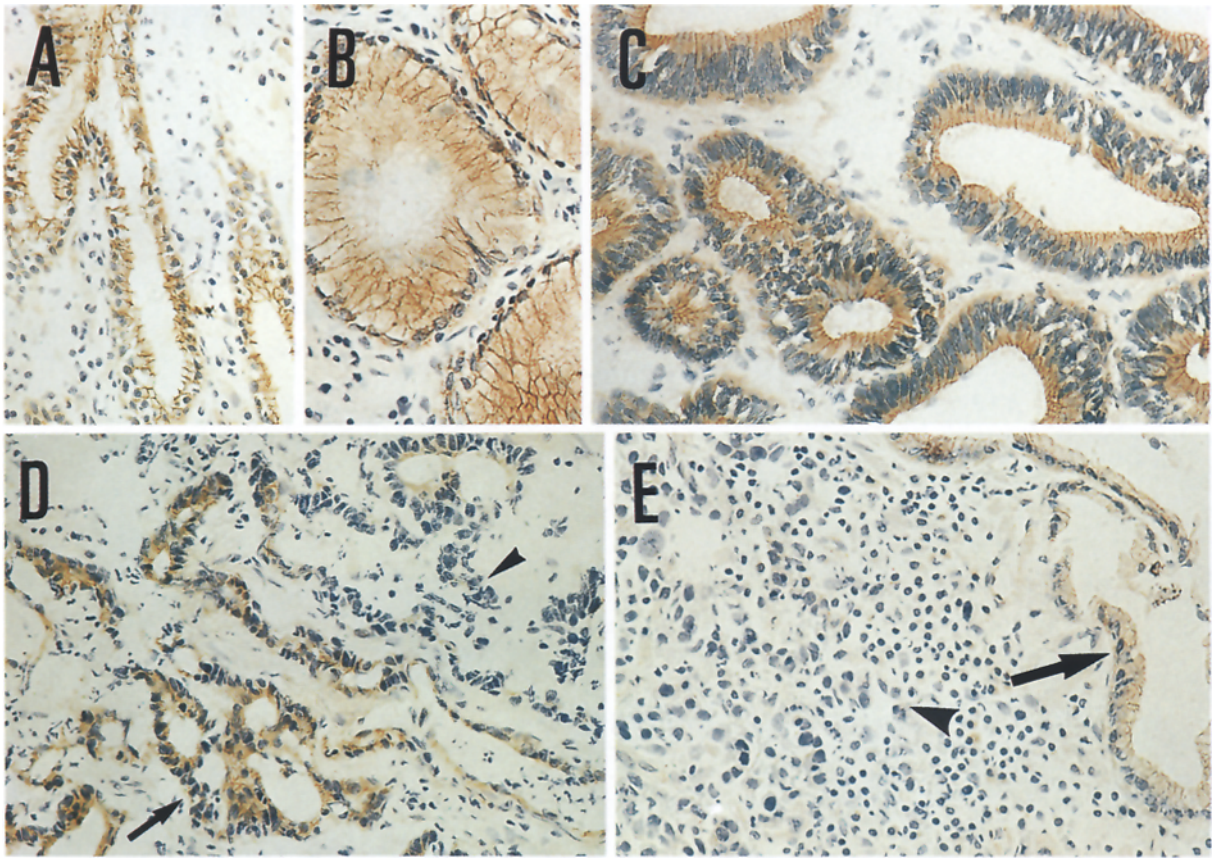

Fig. 1A–E Immunohistochemical reactivity of alpha-catenin (α-cat) in human gastric carcinoma. **A, B** Normal gastric glands. All of the epithelial cells express α-cat strongly on cell-cell boundaries; **A**× 66, **B**×100. **C** Preserved α-cat expression (+): well-differentiated adenocarcinoma. All of the cancerous cells strongly express α-cat as well as normal epithelium ×100. **D** Heterogenously reduced expression: moderately differentiated adenocarcinoma. Its α-cat expression differs from cell to cell. *Arrow,* α-cat positive cell; *arrowhead,* α-cat negative cell. **E** Homogenously reduced expression: poorly differentiated adenocarcinoma. α-Cat expression of all tumour cells is negative. *Arrowhead* indicates α-cat negative cells, and *arrow* indicates the cells in non-cancerous epithelium, ×66

tumours (27/33, 82%). Concerning haematogenous liver metastasis, α-cat(−)/E-cad(+) tumours metastasized more frequently (4/9, 44%) than α-cat(+)/E-cad(+) tumours (1/16, 6%) or α-cat(−)/E-cad(−) tumours (3/33, 9%), and the frequency of liver metastasis in α-cat(−)/E-cad(+) tumours was statistically significant by Fisher's exact probability test ($P<0.05$). We examined both the primary tumour and its metastatic tumour of liver in two cases. In one case, tumours in both lesions showed α-cat(−)/E-cad(+), while in another case, primary tumour showed α-cat(+)/E-cad(+) but metastatic tumour showed α-cat(−)/E-cad(+) (Fig. 3A–D). There was no association between co-expression pattern and peritoneal dissemination.

Table 4 Relationship between α-cat/E-cad co-expression and metastasis; ( ) percentage of metastasis in subgroup divided by α -cat/E-cad co-expression

		α-cat/E-cad				
		+/+ n=17	+/− n=1	−/+ n=9	−/− n=33	Total
Peritoneal dissemination	P (−)	16	1	8	28	53
	P (+)	1 (6)	0 (0)	1 (11)	5 (15)	7
Lymph node metastasis	N (−)	13	1	3	6	23
	N (+)	4(24)	0 (0)	6 (67)	27 (82)	37
Liver metastasis	H (−)	16	1	5	30	52
	H (+)	1 (6)	0 (0)	4 (44)	3 (9)	8

* $P<0.05$
** $P<0.01$

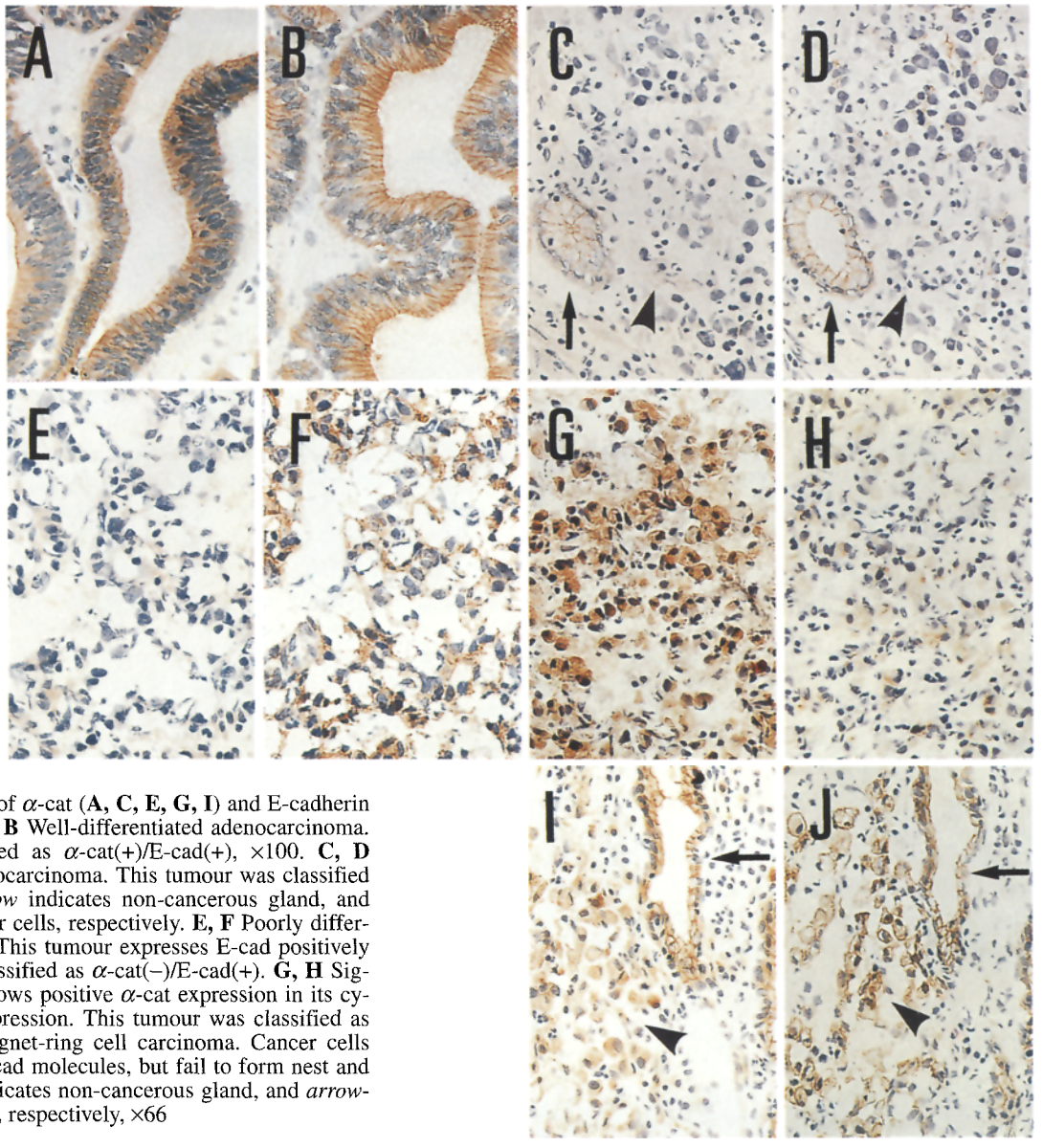

Fig. 2A–J Co-expression of α-cat (**A, C, E, G, I**) and E-cadherin (E-cad; **B, D, F, H, J**). **A, B** Well-differentiated adenocarcinoma. This tumour was classified as α-cat(+)/E-cad(+), ×100. **C, D** Poorly differentiated adenocarcinoma. This tumour was classified as α-cat(–)/E-cad(–). *Arrow* indicates non-cancerous gland, and *arrowhead* indicates cancer cells, respectively. **E, F** Poorly differentiated adenocarcinoma. This tumour expresses E-cad positively but not α-cat, and was classified as α-cat(–)/E-cad(+). **G, H** Signet-ring cell carcinoma shows positive α-cat expression in its cytoplasm but not E-cad expression. This tumour was classified as α-cat(+)/E-cad(–). **I, J** Signet-ring cell carcinoma. Cancer cells express both α-cat and E-cad molecules, but fail to form nest and invade sparsely. *Arrow* indicates non-cancerous gland, and *arrowhead* indicates cancer cells, respectively, ×66

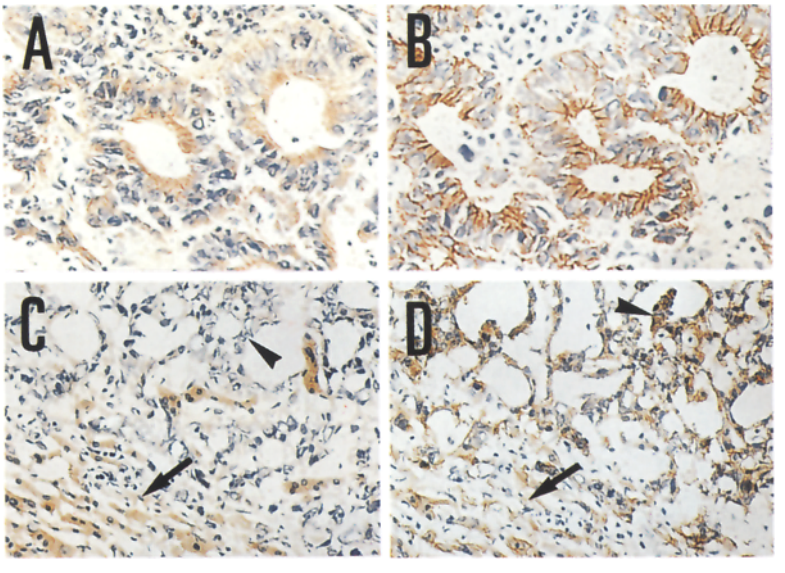

Fig. 3A–D Co-expression of α-cat (**A, C**) and E-cad (**B, D**) in primary tumour and its metastatic liver tumour. **A, B** Primary tumour expresses both α-cat and E-cad molecules positively, ×66. **C, D** Its metastatic liver tumour expresses E-cad but not α-cat. *Arrow* indicates non-cancerous hepatocytes which express these two molecules positively. *Arrowhead* indicates cancer cells, ×33

Discussion

α-Cat is an E-cad-associated protein which binds directly to the cytoplasmic domain of E-cad, forms a linkage to actin and regulates E-cad function. In this study, the reduction of α-cat expression was significantly correlated with histological findings which were affected by intercellular adhesion, including dedifferentiation, depth of invasion, infiltrative growth and lymph node metastasis. Since reduction of α-cat, as well as that of E-cad, induces reduction of intercellular adhesion, it is reasonable to conclude that these results for α-cat were similar to our previous report concerning E-cad (Oka et al. 1992).

Both α-cat and E-cad were preserved in 17 tumours (28%), and both were reduced in 33 tumours (55%). Thus, there existed a strong correlation between α-cat and E-cad expression. This correlation is explained by the experiment in vitro. L cells, which did not have endogenous cadherin, expressed the mRNA of α-cat but had only a trace amount of α-cat protein. When exogenous normal cadherin gene was introduced, not only cadherin but also a high amount of α-cat protein was induced without affecting the amount of its mRNA. However, when carboxyl terminus-truncated E-cad, which could not bind α-cat, was introduced, α-cat expression did not increase (Nagafuchi et al. 1991). These results indicate that α-cat protein is unstable unless connected with cadherin, possibly because of digestion in the cytoplasm. Thus, reduction of α-cat expression in vivo as well as in L cells could be a result of reduction of E-cad expression. These two molecules, therefore, demonstrated a strong correlation in the present study.

However 9 cases (15%) showed reduced expression of α-cat with preserved E-cad. This type of disorder with selective reduction of α-cat is possibly caused by the disorder of α-cat itself and was observed in vitro. One human lung cancer cell line, PC9 cells, did not show tight cell-cell adhesion, even though it expressed E-cad strongly; no α-cat protein was expressed due to homozygous deletion in part of the α-cat gene (Shimoyama et al. 1992). Furthermore, Hirano et al. (1992) described PC9 cells showing tight intercellular adhesion when αN-catenin, a subtype of α-cat, gene was transfected.

In our observations, these α-cat(−)/E-cad(+) tumours did not form tight cell-cell adhesions, and metastasized to lymph nodes more frequently than α-cat(+)/E-cad(+) tumours, resembling the behaviour of α-cat (−)/E-cad(−) tumours. Thus, intercellular adhesion of these tumours in vivo was considered to be disrupted to some extent, like PC9 in vitro. In a previous study on E-cad, we observed some tumours with positive E-cad expression, but they did not form tight cell-cell adhesions. This phenomenon can be explained by the existence of α-cat(−)/E-cad(+) tumours. As mentioned above, the reduction of α -cat expression is caused by the reduced amount of E-cad molecules as well as the disorder of α-cat itself. Thus, immunohistochemical detection of α-cat may more directly mirror E-cad-mediated cell-cell adhesiveness than the detection of E-cad itself. It is thus useful to evaluate not only E-cad but also α-cat expression in cancer tissues.

The frequency of liver metastasis in α-cat(−)/E-cad(+) tumours was higher than that in α-cat(+)/E-cad(+) tumours or in α-cat(−)/E-cad(−) tumours, and we observed a tumour which showed conversion of expression pattern from α-cat(+)/E-cad(+) in primary tumour to α-cat(−)/E-cad(+) in metastatic liver tumour. This result is not identical to that in lymph node metastasis, and cannot be fully explained at this stage. Generally the frequency of haematogenous liver metastasis is much lower than that of lymph node metastasis, and differentiated carcinomas are more often associated with liver metastasis than with lymph node metastasis (Duarte and Llianos 1981; Rhomberg and Gruber 1989; Esaki et al. 1990). We assume that disruption of intercellular adhesion is necessary but not sufficient for metastasis, especially to the liver, and that α-cat(−)/E-cad(+) tumours exhibiting intermediate intercellular adhesion may be more likely to produce liver metastasis than the other phenotype because of their unstable intercellular adhesion.

In the present study, a signet-ring cell carcinoma expressing α-cat(+)/E-cad(−) pattern was observed. This phenomenon seems not to be consistent with the finding that α-cat is unstable unless connected with cadherins. We presume that P-cadherin might be expressed in this tumour, because α-cat binds not only to E-cad but also to other types of cadherin (Nagafuchi et al. 1991) and P-cadherin was frequently detected in human signet-ring cell carcinomas (Shimoyama et al. 1991).

Signet-ring cell carcinomas showed various staining patterns of α-cat and E-cad co-expression, but grew infiltratively; the cancer cells were scattered in any co-expression pattern. So we should consider another disorder other than α-cat and E-cad expression change in these tumours. Recently tyrosine phosphorylation of β-catenin (Matsuyoshi et al. 1992; Behren et al. 1993) or of both cadherin and catenin (Hamaguchi et al. 1993) were reported to suppress cadherin function in vitro. Moreover, many unidentified proteins of signet-ring cell carcinoma or poorly differentiated carcinoma expressed high levels of tyrosine phosphorylation in vitro (Takeshima et al. 1991 and our unpublished observations). Therefore, tyrosine phosphorylation in this system may affect the intercellular adhesion of signet-ring cell carcinomas in vivo.

E-cad-mediated intercellular adhesion is involved in a number of undercoat proteins at the adherens junction, including β-, γ-catenin, 220-kDa protein (Itoh et al. 1991) and cytoskeletal protein of actin filament. These molecules constitute a huge and complicated system of intercellular adhesion. Any disorder in this system can thus induce abnormal intercellular adhesion. Intercellular adhesion mediated by cadherin is not stable, and undergoes changes in various ways. Even though disruption of cell-cell adhesion is only one step in the complicated process of tumour invasion and metastasis, we believe it may play a major role in the process.

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