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Original Paper

Co-ordinated Changes in Expression of Cell Adhesion Molecules in Prostate Cancer

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The expressions of E-cadherin, the integrin subunits β_1, β_2, β_3, CD44 and α-catenin were studied in parallel by immunohistochemistry in a series of 40 prostate biopsies comprising one normal, 11 benign prostatic hyperplasia (BPH), and 28 prostatic adenocarcinomas. As reported by others, there was a consistent loss of E-cadherin expression with increasing tumour grade and de-differentiation. However, a significant proportion of losses occurred at earlier grades than previously reported. The parallel nature of this study showed, for the first time in human prostate carcinoma, a reciprocal expression pattern of E-cadherin and β_1 integrin in the higher grades of prostate cancer. A reciprocal expression pattern was also found for E-cadherin and CD44 between moderately and poorly differentiated tumours. α-Catenin expression was downregulated only in those cells which had previously lost E-cadherin expression, and β_2 and β_3 integrin were rarely expressed in prostate tumours. A loss of expression of the luminal epithelial specific keratins CK8 and CK18 was also observed in advanced stage, poorly differentiated carcinomas. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: prostate cancer, adhesion molecules, integrin, cadherin, catenin, CD44

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INTRODUCTION

PROSTATE CANCER (CaP) is an increasingly important disease in Western countries and is a major factor in male cancer mortality. Despite this, knowledge of the molecular events associated with the disease is relatively poor compared to the other common cancers. The oncogenes *C-MYC* and *ERBB2* and the tumour suppressor genes *TP53* and *RB* have been implicated to varying degrees and genetic lesions on chromosomes 8p, 10p and q, 16q, 13q, 18q, 9p and 6q have been identified, suggesting the presence of tumour suppressors at these loci [1, 2]. Some of these loci are associated with cell adhesion molecules which have been implicated as tumour suppressors [3]. It is, therefore, possible that these and other adhesion molecules might play a significant role in CaP development.

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E-cadherin is the epithelial-specific cadherin involved in the maintenance of epithelial sheet integrity and in developmental processes [4]. It has been described as an invasion or metastasis suppressor because its expression is lost from epithelial cells as they progress to advanced stage tumours and metastases, and re-introduction of E-cadherin DNA into invasive L-cells and RAS-transformed MDCK cells causes suppression of their invasive phenotype [4]. Because E-cadherin expression is lost with increasing grade and stage in prostate cancers [5] and a significant proportion of patients show allelic loss on chromosome 16 at 16q22.1-qter [6] (possibly including the E-cadherin gene at 16q22.1), E-cadherin is an attractive prognostic marker in CaP.

α-Catenin is one of three catenins which bind the cytoplasmic surface of E-cadherin and link it to the cytoskeleton [7]. Aberrant expression of α-catenin in other tumours [8] and in the PC3 prostatic carcinoma cell line [9] produce a breakdown in focal adhesion assembly which suggests that α-catenin loss or mutation may also be an important event

in CaP progression. However, to date, there has been no study of the levels of α-catenin during tumour progression *in vivo*.

The integrins are a large family of heterodimeric adhesion molecules involved in a wide variety of cell-to-cell, cell-matrix and cell-soluble factor adhesion events. Their expression has been studied in the progression of many tumours and cell lines, although the data are often conflicting [10]. β_1 integrin has a widespread distribution and is known to complex with 10 different α-subunits which have variable binding specificities for different matrix components and either prevent or promote cell migration through tissue. Inappropriate upregulation of β_2 integrins (leucocyte-specific) on tumour cells might cause them to mimic leucocytic properties, e.g. circulation, homing and extravasation [11]. Similarly, β_3 integrins (platelet-specific) can be upregulated on tumour cells and promote metastasis, e.g. $\alpha_v\beta_3$ in melanoma [11].

In prostate, studies of integrin expression in human carcinoma have largely focused on cell surface distribution rather than on the changes in levels of expression with progression [12–15]. Studies in prostate cell lines have given conflicting results [16–18], but suggest a pattern of changing expression with different *in vitro* characteristics which may be indicators of increasing tumour progression.

CD44, a hyaluronate receptor, is widely expressed and known to exist in at least 12 different splice variant forms. In tumours these, and the normal epithelial form, are often upregulated and increase the cells' propensity to metastasise [19]. The expression of CD44 isoforms has only been studied in prostate tumour cell lines [20–22].

This paper describes the patterns of expression of the six adhesion molecules described above in human carcinoma and plots the changes in their expression with progression.

MATERIALS AND METHODS

Tissues

Biopsies were taken from 39 tumour patients, with full consent, by transurethral resection of the prostate (TURP) at York District Hospital. Biopsies were immediately frozen to −20°C and subsequently stored at −70°C. The patients were chosen to reflect an even spread of grade of disease as determined by the initial histopathological reports (i.e. 39 patients' biopsies, of which 11 were BPH, 5 G1, 11 G2, 1 was classified as well differentiated (G1/G2) and 11 G3). Tissues were embedded in OCT and 7 μm thick sections were cut using a Leica cryocut 1800 freezing microtome. Grade and integrity of the sections were checked by haematoxylin/eosin staining before immunohistochemistry. The normal prostate tissue was obtained, *post mortem*, from a man aged less than 40 years, by radical prostatectomy at Hôpital Saint Louis, Paris.

Immunohistochemistry

Antibodies. The monoclonal antibodies shown in Table 1 were used.

Immune detection. Duplicate serial sections were stained with each antibody tested using the Vectastain ABC peroxidase kit and developed using the colour reagent amino-9-ethyl carbazole (AEC) (Sigma Cat. No. A-5754). All sections were counterstained with haematoxylin.

Assessment of grade and staining. Because prostatic tumours are histopathologically heterogeneous, a single section from each biopsy often contains areas of more than one tumour grade. Conventional Gleason grading [24] normally determines the individual grades within a section of tissue and takes the sum of the highest and lowest grades to arrive at a 'score' for the tumour. However, because different graded areas within a single section displayed different staining patterns for each antigen, the individual areas were recorded as independent data points. Therefore, the 39 biopsies produced many more data points for each grade as follows.

Normal tissue. Four areas from the normal tissue were analysed after being verified as being histologically normal.

Benign disease. Because the biopsies were obtained by TURP they came from periurethral regions, probably the transitional zone. Therefore, most of the benign biopsies were classed histologically as BPH, although the presence of small areas of prostatic intraepithelial neoplasia (PIN1-3) cannot be excluded. In total, 37 regions of benign histology from 15 patients (including 7 patients with diagnosed CaP) were analysed.

Well-differentiated tumours. Only 1 area of clear G1 and 10 areas of G2 were observed from a total of 5 patients' biopsies. The data were designated as 'well' differentiated.

Moderately differentiated tumours. A total of 31 areas possessing a distinct G3 morphology from 11 patients' biopsies were found and designated as 'moderately' differentiated.

Poorly differentiated tumours. 21 areas displayed the features of G4 from 8 patients' biopsies and were designated as 'poorly' differentiated.

Very poorly differentiated tumours. 22 areas of significant G5 were found from 10 patients' biopsies with distinguishable staining patterns. These showed markedly different

Table 1. Monoclonal antibodies used

Antibody	Antigen	Source
LCAM	E-cadherin	Novocastra Laboratories Ltd, The Netherlands (supplied by Euro-Path Ltd, Devon U.K.)
NCL-CD18	β^2 integrin	
NCL-CD61	β_3 integrin	
NCL-CD44	CD44	
α-18	α-catenin	Dr Tsukita, Department of Information Physiology, National Institute for Physiological Sciences, Okasaki, Japan [8]
β_1 integrin	β_1 integrin	Professor Ian Hart, ICRF Richard Dimbleby Cancer Research Unit, St Thomas' Hospital, London, U.K. (personal communication)
M20	Cytokeratin 8	Sigma Cat. No. C-5301
CY90	Cytokeratin 18	Sigma Cat. No. C-8541
	HPV16 E1–E4	Dr John Doorbar, Department of Pathology, University of Cambridge, Cambridge, U.K. [23]

staining patterns from G4 and were, therefore, placed in a separate category of 'very poorly' differentiated tumours.

The level of staining of the luminal epithelium within these areas was assessed relative to the level of staining of a constant normal control which was included in every batch of sections stained. The degree of stain of duplicate sections was assessed on a five-point scale in which 0 represented no staining and 1–4 represented increasing levels of staining. Grades of the sectioned biopsies were determined by three independent observers.

Because serial sections were used for a study comprising nine antigens, there was not always sufficient sections of small tumour areas for the whole study and, therefore, there are more data points for some antigens and some grades than others. For parallel stainings, the same serially sectioned graded area was analysed for the two antigens compared, so the parallel staining profiles were derived from exactly the same graded area, but from adjacent 7 μm sections in the block. Therefore, there are not as many data points in this analysis as in the single-antigen analysis.

RESULTS

Change of expression of adhesion molecules with loss of differentiation in tumour sections

Figure 1 shows examples of staining for E-cadherin, β_1 integrin, CD44, α-catenin and CK8 of tissue areas representing decreasing differentiation. E-cadherin expression was reduced (Figure 1(A)–(F)), whereas β_1 integrin staining (Figure 1(G)–(L)) increased with decreasing differentiation. CD44 expression (Figure 1(S)–(X)) increased gradually through to moderately differentiated graded areas, increased markedly in poorly differentiated tumour areas and equally rapidly reduced again in very poorly differentiated tumour areas. α-Catenin (Figure 1(M)–(R)) stained moderately in normal and BPH areas and was maintained in all graded areas up to moderately differentiated tumours. In poorly and very poorly differentiated tumours, expression was reduced but was never absent. Unexpectedly, there was a consistent loss of staining of the luminal specific cytokeratin, CK8, with increasing de-differentiation (Figure 1(Y)–(DD)).

The proportion of areas staining strongly (levels 3 and 4) in the luminal epithelium for each antigen studied is shown in Figure 2 for each grade of tumour. In each panel the data for E-cadherin are superimposed. The plots for E-cadherin, β_1 integrin, CD44, α-catenin and CK8 are in agreement with the patterns seen in Figure 1.

Half the normal/benign group (20/41) stained strongly for E-cadherin. This level of expression was maintained in the well-differentiated areas. However, 86% (37/43) of the poorly and very poorly differentiated areas were poorly (2, 1) or negatively (0) stained for E-cadherin, i.e. there was significant loss in expression between moderately and poorly differentiated areas. In total, some degree of loss of E-cadherin staining occurred in 87% (110/126) of the tumour areas studied.

Figure 2(a) shows the change of expression of the integrin subunits relative to E-cadherin. β_1 integrin expression increased with reduced differentiation. Only one tumour showed a high level of staining for β_2 integrin in a G3 area and no case gave strong staining for β_3 integrin. However, 19.6% (11/56) of all tumour areas showed weak staining for β_2 integrin and 25.8% (15/58) showed weak staining for β_3 integrin (data not shown).

Figure 2(b) shows the pattern of expression of CD44 relative to E-cadherin. The CD44 antibody is pan-specific and therefore does not discriminate between the various splice variants of this antigen. A marked increase in the high staining population was observed in the poorly differentiated areas (78, 7/9) followed by a dramatic decrease in the proportion of high staining CD44 positive areas in the very poorly differentiated tumours (36%, 5/14).

Figure 2(c) shows the pattern of staining of α-catenin relative to E-cadherin. A marked reduction in the percentage of high staining areas between the well and moderately differentiated grades was observed. However, there were only four data points in the well differentiated group compared to 13, 12 and 10 for the other three categories which may have given a falsely high proportion of graded areas showing high expression. Therefore, a true decrease in the number of highly staining areas may only be considered for the poorly differentiated grades. There were no cases where α-catenin staining was completely absent (data not shown).

Figure 2(d) shows the relative staining of the controls, CK8 and CK18 (positive) and HPV16 (negative). Surprisingly, the proportion of areas that stained strongly for the two cytokeratins reduced with increasing grade (except in very poorly differentiated tumour areas where CK8 expression was partially regained). The HPV16 E1–E4 protein was not detected in any of the biopsies.

Co-ordinate expression patterns

The data in Figure 2 indicate that a more detailed analysis of the co-ordinated expression of E-cadherin relative to some of the other antigens was warranted. The expression of pairs of antigens in serial sections of the same biopsy are shown in Figure 3. Areas that stained strongly (3–4) for both antigens were defined as high/high and those that stained weakly (0–2) were defined as low/low. Similarly, there are high/low and low/high groups.

E-cadherin and β_1 integrin [*Figure 3(a)*]. 37% (7/19) of the areas of normal and benign prostate tissues examined had a high/low pattern of expression. This changed to a low/high pattern with increasing grade. Therefore, the same cells which lost E-cadherin gained β_1 integrin expression in 7 of the tumour areas studied. Most of the remainder of the tumour areas (37%, 7/19) retained their low/low expression throughout.

E-cadherin and CD44. For E-cadherin and CD44 (Figure 3(b)), there was a reciprocal trend from the high/low to low/high expression pattern between moderately and poorly differentiated tumour areas. Between poorly and very poorly differentiated tumour areas, the low/low population dominated as CD44 expression was lost.

E-cadherin and α-catenin. For E-cadherin and α-catenin (Figure 3(c)), the normal/BPH and well differentiated tumour areas were predominantly high/high. In the moderately differentiated to poorly differentiated tumour areas, there was a loss of the high/high population which was replaced initially by a low/high population and then by the low/low population in very poorly differentiated tumour areas. This suggests that some loss of E-cadherin precedes the loss of α-catenin, but that the loss of α-catenin is from those cells that have already lost or are losing their E-cadherin. There are no areas that lose α-catenin expression that have not already lost E-cadherin.

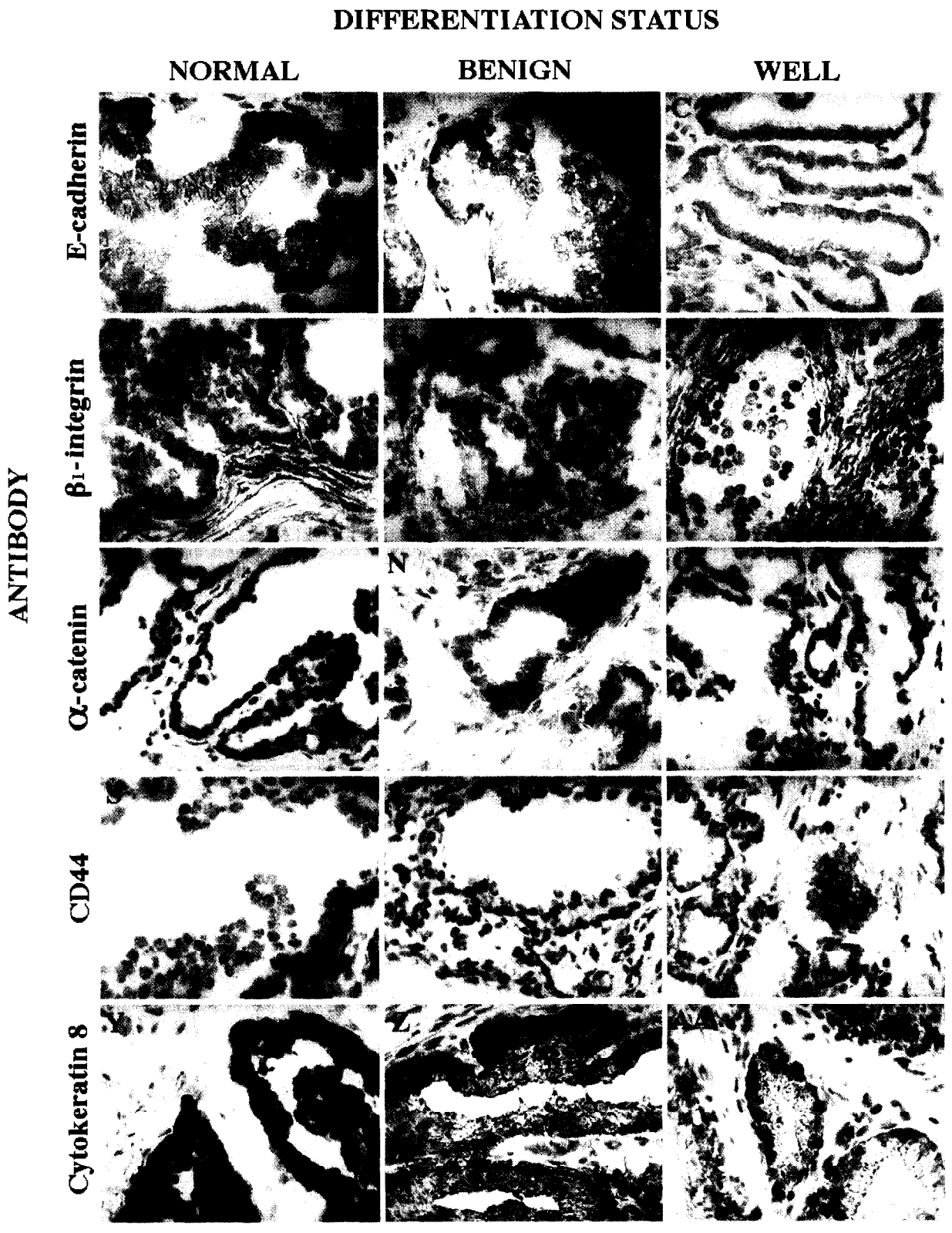

Figure 1. Continued opposite.

E-cadherin and cytokeratins 8 *and* 18. Between well and poorly differentiated states, there was a parallel loss of E-cadherin and CK8 and CK18; 56% (5/9) of the well differentiated areas and only 5.6% (1/18) of the poorly differentiated areas had high/high expression (Figure 3(d)). The majority of the poorly differentiated tumour areas had a low/low phenotype (67%, 12/18).

Intracellular location of the antigens

The intracellular location of each of the antigens was investigated by high-power microscopy. Figure 4 shows examples of normal and a tumour tissue stained for E-cadherin, β_1 integrin, α-catenin and CK8.

E-cadherin. As the tumour grade increased, there was a reduction in the intensity of E-cadherin staining and an

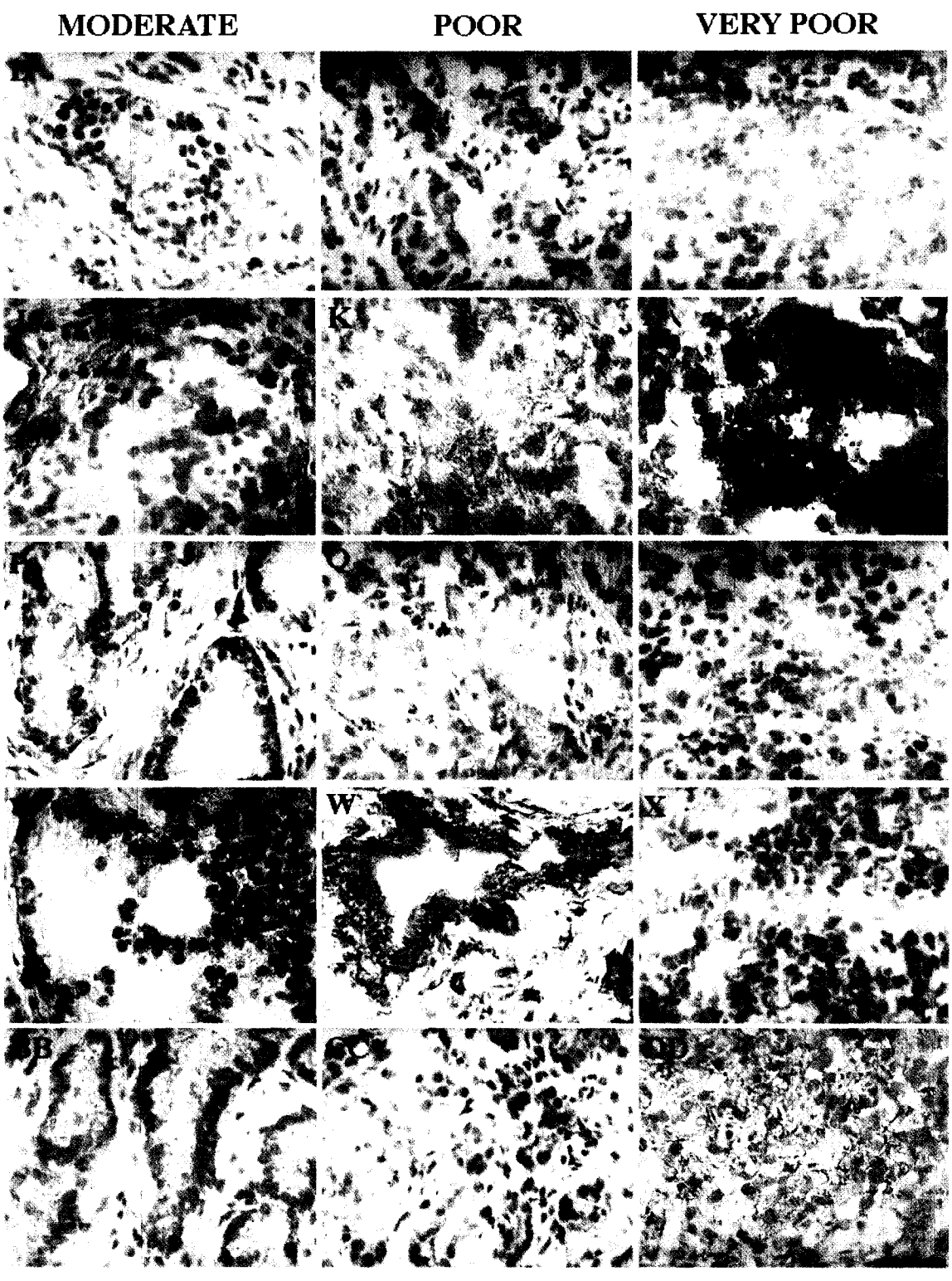

Figure 1. Immunohistochemical staining of E-cadherin, β_1 integrin, CD44, α-catenin and cytokeratin 8 during prostate cancer progression. (A)–(F) E-cadherin staining, (G)–(L) β_1 integrin staining, (S)–(X) CD44 staining, (M)–(R) α-catenin staining and (Y)–(DD) cytokeratin 8 staining. For each antigen, a progression from normal through to very poorly differentiated tissue is shown. All the sections are shown at 125× magnification with the scale bar representing 50 μm of tissue.

increased heterogeneity in its distribution within the epithelium with some cells showing a cytoplasmic distribution of the E-cadherin stain (Figure 4(A), (B)).

β_1 Integrin. β_1 integrin staining in normal luminal prostate epithelium was weak and diffusely cytoplasmic, although there was a polarised staining in the basal surface of the

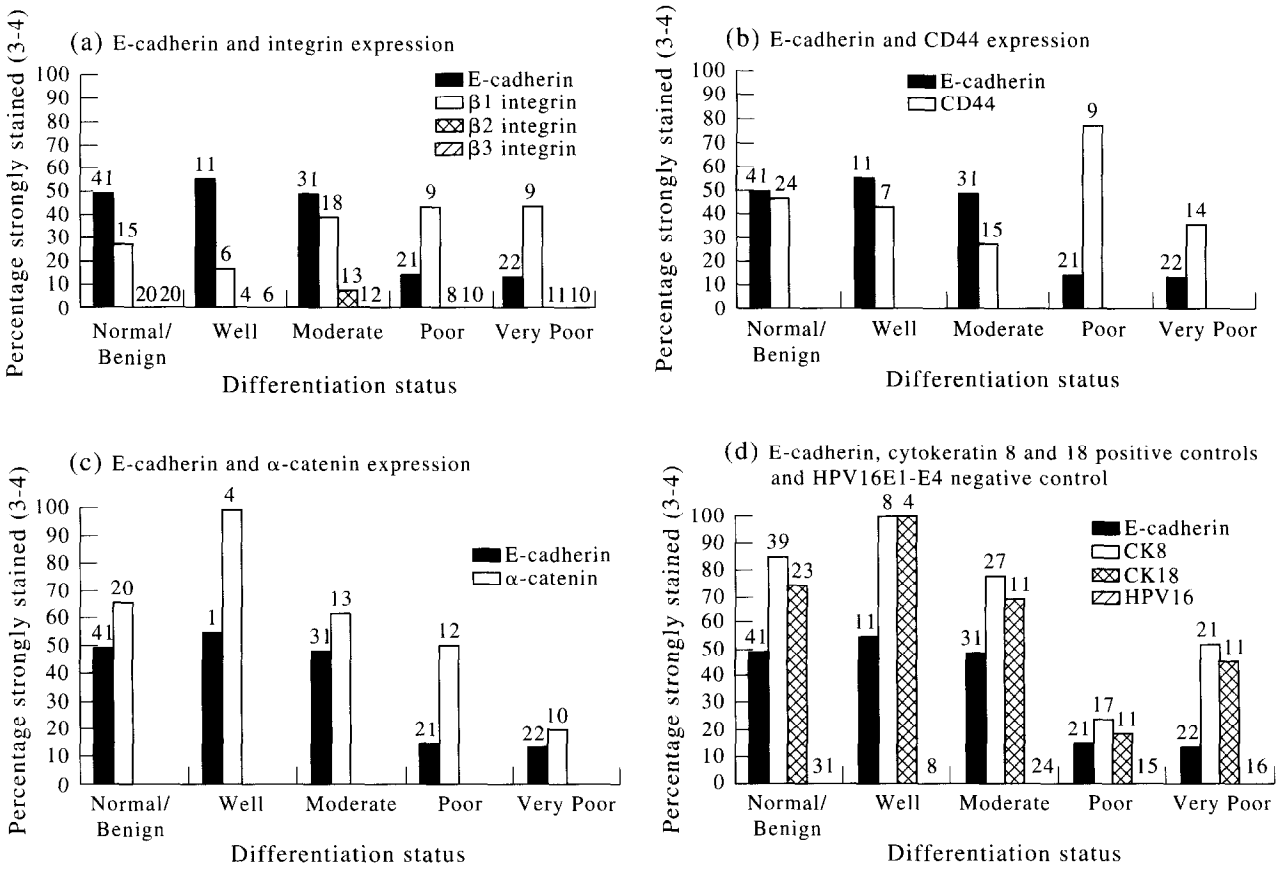

Figure 2. Individual antigen expression during progression. The numbers above each bar represent the total number of areas analysed for each antigen.

basal cells (Figure 4(C), arrowed). As luminal differentiation was lost, the cytoplasmic expression was maintained or increased, there was an increase in the surface-associated stain in the luminal cells, and the polarised basal stain was disrupted with the progressive loss of basal cells. Figure 4(D) shows an example of a G5 tumour where there are no basal cells and the remaining higher levels of stain are both cytoplasmic and surface associated.

α-Catenin. The weak/moderate surface-associated α-catenin staining of luminal cells in normal and benign epithelium (Figure 4(E)) became more heterogeneous: the intensity was reduced slightly and in some cells the staining was less surface-associated at the higher grades (Figure 4(F)).

CD44. CD44 expression was weak and cytoplasmic in the normal luminal epithelium and became increased and more surface-associated until in very poorly differentiated graded areas the expression was lost (data not shown).

CK8. CK8 staining was reduced and became less surface-associated as the differentiation pattern broke down between moderately and poorly differentiated tumour areas (Figure 4(G, H)). Figure 4(H) shows an example of a G5 tumour area where CK8 expression is almost completely lost.

DISCUSSION

E-cadherin was progressively and consistently lost from prostatic epithelium with loss of differentiation. Some degree of loss occurred in 87% (110/126) of the tumour areas studied. This compares with 50% (46/92) and 47% (42/89) found in the two studies of Umbas and associates [5, 25]. The values obtained in our study are higher because the tissue was microscopically graded instead of being averaged by using the system of Gleason scores. In addition, we found that the losses in expression occur at an earlier grade than previously suggested. We believe that this is consistent with the function of E-cadherin in the maintenance of epithelial sheet integrity. None of these patients received treatment prior to biopsy, ensuring that the losses observed were not a result of any drug effect.

No tissue showed α-catenin loss without having previously lost or reduced its E-cadherin expression. Further experiments are required to determine if these are interdependent events. However, mutations in the α-catenin gene, such as that found in the PC3 cell line, might lead to non-functionality of E-cadherin due to an inability to form focal adhesion plaques, i.e. the loss of expression of one gene product as well as the mutation of a downstream product could produce a tumour suppressor phenotype.

The upregulation of β_1 integrin observed in relatively dedifferentiated cells and the increase in its surface association are consistent with the endogenous pool of β_1 integrin being brought to the surface before there is any need for *de novo* synthesis. This result suggests that there may be upregulation of other β_1 integrin-associated α-subunits in the later stages of CaP progression.

This is the first time that CD44 expression has been followed in human prostate carcinoma and correlated with

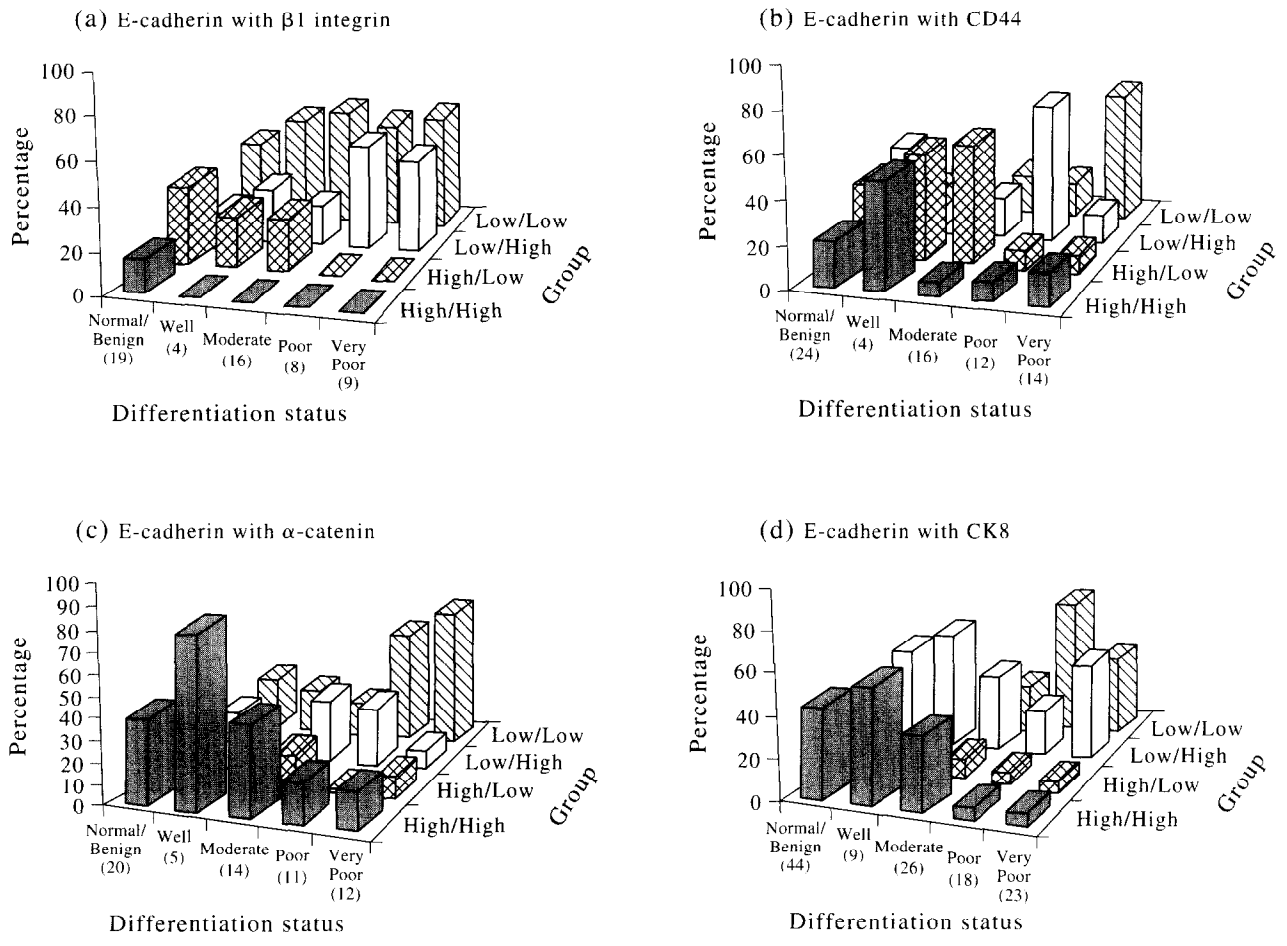

Figure 3. Co-ordinated expression of E-cadherin with β_1, CD44, α-catenin and cytokeratins during progression. The numbers on the *x*-axis represent the total numbers of areas analysed in each grade.

disease progression. The pan-specific CD44 antibody determined the overall changes in expression of CD44 in prostate cancer and showed a consistent increase in CD44 levels in the poorly differentiated tumours. The subsequent dramatic reduction in expression in the very poorly differentiated tumours appears to be inconsistent with those models of CD44 expression increasing and diversifying into many variant forms with increasing propensity to metastasise. A study on human gastric carcinomas showed a similar pattern of expression, i.e. levels of CD44 increased in invasive stages, but was reduced in more metastatic areas and in the lymph nodes [26]. Human chromosome 11p11.2-13 has been shown to suppress the metastatic ability of the Dunning rat prostatic carcinoma cell line and the gene for CD44 is located in this region, so it is possible that this loss of expression might be due to gene losses at the later stages [27]. It is difficult to understand how an absence of CD44 expression could promote metastasis. However, since CD44 is able to transduce intracellular signals and to link to cytosolic components, CD44 could function as a tumour suppressor [19] in an analogous manner to E-cadherin. Perhaps there is another metastasis suppressor in this locus and that the loss of CD44 expression is just fortuitous. A putative tumour suppressor gene, *KAI1*, has been identified in this locus whose product may also have an adhesive function [28].

The most striking result of this parallel study is the reciprocal expression of E-cadherin and β_1 integrin. Similar reciprocal expression of E-cadherin and β_1 integrin has been observed in cultured keratinocytes when the external Ca^{2+} concentration was altered, rendering the E-cadherin non-functional. This causes the keratinocytes to switch from a high/low to a low/high pattern and adopt a less differentiated phenotype [29]. Dunning rat prostate cancer cells injected into Copenhagen rats produced tumours which also displayed this phenotype [30]. This paper presents the first evidence that this phenomenon occurs in human carcinomas and can be correlated with disease progression. It is increasingly clear that there is cross-talk between different cell adhesion molecules involving as yet undetermined signal transduction pathways. Hodivala and Watt suggest that there is a downregulation of β_1 integrin expression by E-cadherin in keratinocytes [29]. Release of the repression would, therefore, allow expression of β_1 integrin and produce a more invasive phenotype. As described above, the timing we have found for the loss of E-cadherin expression suggests that E-cadherin is a local invasion suppressor rather than a metastasis suppressor as others have suggested, and that the co-ordinated upregulation of integrins might subsequently be the more likely candidates for promoting further invasion and/or metastasis. Clearly, the triggers for and controls over this change in expression require fuller investigation.

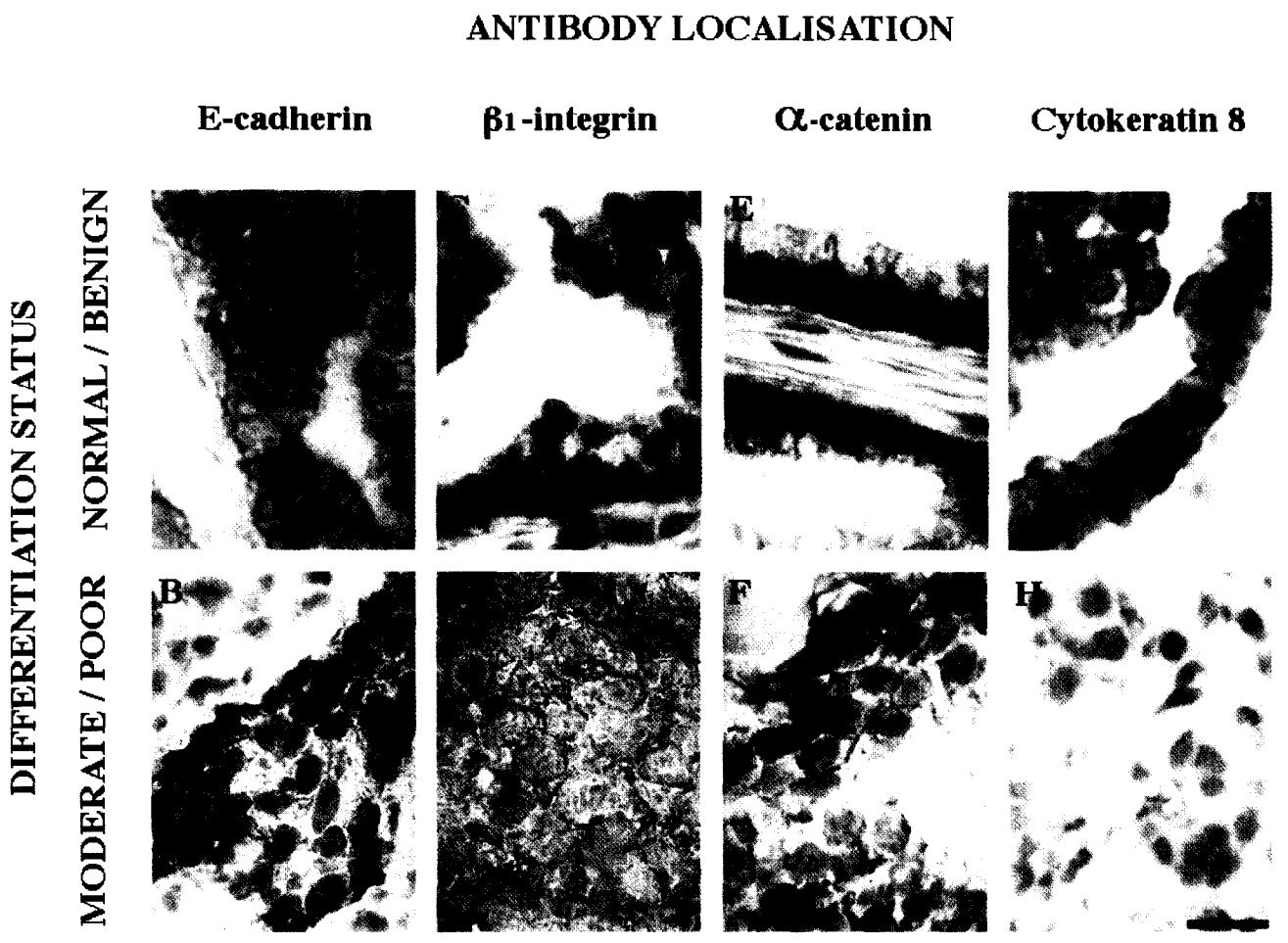

Figure 4. Intracellular localisation of cell adhesion molecules. Staining for E-cadherin, β_1 integrin, CD44 and CK8 for normal (A), (C), (E) and (G) and tumour (B), (D), (F) and (H) biopsies. The arrow in (C) indicates the strong polarised staining of the basal cells for β_1 integrin which was not present in the G5 tumour shown in (D) as the basal cells have been lost. All the sections are shown at 312.5× magnification with the scale bar representing 20 μm.

The correlation between E-cadherin and CD44 expression for moderate to poorly differentiated tumours has also never been reported before. Determination of the changes in expression of individual spice variants in human carcinomas as well as the increase in expression of total CD44 protein may help to provide a clearer understanding of any such possible mechanisms which may be controlling this event.

The loss of expression of cytokeratins 8 and 18 in the poorly differentiated tumours was totally unexpected. We are confident that this result is not artifactual because every sample was stained in duplicate and the entire data set was stained twice for both antigens. Furthermore, the normal tissue stains positively for both antigens. Several workers have measured the expression of CK8 and CK18 in cell lines and in tumour tissues and all report a continued expression. However, cytokeratin expression has not previously been investigated in detail with respect to loss of differentiation in prostate cancer, although Okada and associates described the heterogeneity of CK8 staining in more poorly differentiated tumours [31]. It is not unreasonable to find a loss of what is, after all, a differentiation marker with de-differentiation. The fact that there is a coordinated loss of both cytokeratins and E-cadherin in 50% of the tissues in this study reinforces this assumption. This finding clearly has important implications for the establishment of cell lines from prostate tumours because CK8 and 18 are used to determine whether cells are of epithelial origin.

An alternative view is that a newly described differentiated cell type, the neuroendocrine (NE) cell, becomes oncogenically transformed and forms small hormone independent foci [32]. These cells may have a different cytokeratin expression profile to the luminal cells. To our knowledge, there is no study of the cytokeratin expression changes in NE cells in tumour development, although there is one study which indicates that CK18 is expressed in normal NE cells [33].

We were consistently unable to detect expression of the HPV16 E1–E4 protein which we have confirmed in a recent study of HPV16 DNA detection using tissues from the same source as those described here [34]. This is at variance with a number of reports which suggest the presence and expression of HPV16 genes in prostatic tumours [35].

This is the first comparative study of the expression of different types of adhesion molecule in CaP with loss of differentiated phenotype. Such studies yield greater insight than studies of individual molecules. Clearly, it is the panoply of expression of surface markers along with other tumorigenic events which determines the tumorigenic or metastatic ability of the tumour cells and which ultimately determines the speed with which an individual tumour may

progress through to metastasis. It will be interesting to determine whether, for instance, the loss of E-cadherin expression at an early stage in combination with a gain of β_1 integrin expression predicts a poorer outcome than tumours which retain E-cadherin expression. An understanding of the mechanisms that control the expression of the various adhesion molecules is therefore required and may result in new treatment strategies such as toxin targeting or enhanced gene therapy. We suggest from this study that use of E-cadherin immunohistochemistry in conjunction with that for β_1 integrin and/or CD44 may be a more effective means of diagnosis and prognosis than the use of E-cadherin alone.

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