Transforming Growth Factor beta-1 in cervical cancer

Hazelbag, S.

Overexpression of the αvβ6 integrin in cervical squamous cell carcinoma is a prognostic factor for decreased survival

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Abstract

Cervical squamous cell carcinomas histologically are composed of tumor cell islands surrounded by varying amounts of tumor stroma, whereof the amount and composition are influenced by local TGF-β_1. TGF-β_1 is secreted in an inactive complex with latency-associated peptide (LAP). Both LAP as well as the extra cellular matrix (ECM) protein fibronectin are important ligands for the integrin receptor $\alpha v\beta 6$. While $\alpha v\beta 6$ is only weakly expressed by normal epithelia, it is up regulated in different carcinomas where it generally reflects a more aggressive phenotype. In cervical cancer, the expression of $\alpha v\beta 6$ thus far has not been investigated. Given the ability of $\alpha v\beta 6$ to both activate TGF-β_1 and to interact with fibronectin, we studied correlations between the expression of these components and disease parameters in a large cohort of cervical cancer specimens.

Methods. We analysed $\alpha v\beta 6$ expression in primary cervical squamous carcinomas of FIGO stage IA to IIB patients and correlated the findings with formerly investigated fibronectin and TGF-β_1 expression and clinicopathological parameters. $\alpha v\beta 6$ expression was also examined in cervical intra-epithelial neoplasia (CIN) and lymph node metastases.

Results. $\alpha v\beta 6$ was only weakly expressed in normal epithelium but clearly up regulated in CIN lesions. In carcinomas, strong expression of $\alpha v\beta 6$ in tumor cells correlated with different clinicopathological parameters and with worse overall and disease free survival. Furthermore, $\alpha v\beta 6$ expression positively correlated with TGF-β_1 mRNA expression as well as with fibronectin expression.

Discussion. Overexpression of $\alpha v\beta 6$ in cervical squamous carcinomas is an unfavourable prognostic factor. This might reflect an increased capacity of $\alpha v\beta 6$ expressing tumor cells to migrate in a fibronectin rich ECM and/or to activate TGF-β_1 at the tumor/stroma interface, both of which processes may contribute to cervical cancer progression.

Introduction

With 400,000-500,000 newly affected women yearly cervical cancer is the second leading cause of cancer death among women worldwide.[1] Persistent infection of the cervical keratinocytes with high risk-Human Papillomavirus (HPV) is an established risk factor with respect to cervical carcinogenesis.[2] The hosts cellular immune response is thought to be essential in controlling both HPV infections and HPV-related neoplasms.[3,4] The invasive stage of disease is preceded by cervical intra epithelial neoplasia (CIN), where dysplastic epithelial cells take up an increasing part of the height of the epithelium from only the lower one (CIN 1) or two thirds (CIN 2) towards (nearly) the full thickness of the epithelium, but still with an intact basement membrane (CIN 3). Invasive cervical carcinomas characteristically are composed of infiltrating epithelial tumor cell nests surrounded by widely varying amounts of tumor stroma.[5-7] This specialized stroma, composed of extra cellular matrix (ECM) proteins such as fibronectin, laminin, tenascin and vitronectin, (activated) fibroblasts, inflammatory cells and capillaries, is thought to be indispensable for the tumor to grow.[8,9] The process of tumor invasion and metastasis requires complex changes in the normal epithelial cell-cell and epithelial cell-stroma interactions, in which besides extra cellular glycoproteins, the integrin family of adhesion molecules may play an important role.

Integrins are a family of cell surface receptors that mediate cell adhesion to each other or to extracellular matrix substrata.[10,11] These molecules are composed of one α- and one β subunit, both transmembrane glycoproteins consisting of large extra cellular domains and short cytoplasmic domains, which form a structural and functional bridge between the ECM outside the cell and the cellular cytoskeletal proteins. The αv integrins form a subfamily of five members (αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8) that recognize a group of overlapping ligands which generally contain the canonical tripeptide recognition sequence, arginine-glycine-aspartic acid (RGD).[12] The αvβ6 integrin binds to RGD sites in its ligand proteins fibronectin (and to a lesser degree tenascin and vitronectin), and the latency associated peptide (LAP), the latent precursor form of TGF-β_1.[13-15] αvβ6 is down regulated in differentiated epithelia, while in injured or inflamed epithelia as well as in some types of epithelial derived tumors this integrin is up regulated.[15-18]

In addition to providing anchorage for stationary cells and traction during cell movement the αvβ6 integrin may have multiple regulatory functions in oncogenesis. Enhanced or *de novo* expression of αvβ6 expression has been observed in several epithelial malignancies such as oral squamous carcinoma, breast carcinoma, colon carcinoma, gastric and ovarian carcinoma.[19-26] Recent studies have demonstrated that the extracellular and cytoplasmic domains of the αvβ6

heterodimer mediate different cellular activities. Whereas the extracellular and transmembrane domains mediate TGF-β activation, adhesion and epithelial mesenchymal transition (EMT),[27,28,23,15] the cytoplasmic domain (contains an 11-amino acid sequence that) affects proliferation, matrix metallo proteinase (MMP) production, migration and survival through cell signaling pathways.[29,27,30,22,21]
αvβ6 binding to LAP leads to activation of the latent precursor to active TGF-β_1, probably as a result of a conformational change in the latent complex, allowing mature TGF-β_1 to bind to its receptor. Up regulated expression of αvβ6 can lead to local activation of TGF-β_1, which in turn can activate a cascade of events downstream of TGF-β_1. On the other hand, TGF-β_1 has been demonstrated to up regulate the expression of αvβ6 on the surface of keratinocytes and to stimulate migration of epithelial cells on fibronectin, both in wound healing and in malignant invasive growth.[31,16,32,23]
In our previous studies we demonstrated cervical carcinoma cells *in vitro* and *ex vivo* to express TGF-β_1, which affected both the amount and composition of the intratumoral stroma.[33,34] We observed that in many cervical carcinomas especially the ECM protein fibronectin was abundantly present in the tumor stroma,[34] a phenomenon described by Goldberg *et al.* to possibly reflect the ability of these tumors to modify the peritumoral stroma and facilitate tumor invasion of stroma and vessels.[5-7] Normal squamous cervical epithelium has been described to weakly express αvβ6,[35] but no data are available on the expression in cervical carcinomas. Because of the pivotal role of the αvβ6 integrin reported for various epithelial malignancies and the possible link with TGF-β_1 and fibronectin in cervical carcinomas in particular, we studied in detail the expression of αvβ6 in normal cervical epithelium, CIN, invasive carcinomas and lymph node metastases. Furthermore, we complemented our previous findings of TGF-β_1 mRNA and fibronectin in cervical carcinomas, by investigating the relationship of these components with αvβ6 expression in the tumors, in order to better understand the mechanisms of growth and invasion of cervical cancer. Finally, the relationship with relevant clinico-pathological parameters and the prognostic meaning with respect to cervical cancer were examined.

Material and Methods

Tissue samples

From 86 patients with cervical squamous carcinoma who underwent radical hysterectomy with pelvic lymph adenectomy between 1985 and 1995, formalin fixed paraffin-embedded tissue blocks were retrieved from the archives of the Department of Pathology, Leiden University Medical Center. Paraffin blocks containing representative tumor tissue were used. From 16 of these patients paraffin blocks of lymph node metastases were present and used in this study. None of the patients had received radio-or chemotherapy prior to surgery.
Paraffin blocks of 9 (other) patients with a CIN 1, 2 or 3 lesion were included.

Clinical and histopathologic characteristics of the carcinoma group

The clinical records of the women, all treated with a radical hysterectomy type III for carcinoma of the uterine cervix between 1985 and 1995 in our hospital, were reviewed. The surgical procedure was performed by three gynaecologic oncologists of the Department of Gynaecology, Leiden University Medical Centre. The mean age of the patients was 45.4 years, with the youngest 26 years and the oldest 80 at the time of surgery. The following data were collected for analysis from the patients' records: FIGO stage, tumor size, presence of distant metastases and whether or not post-surgical radiotherapy was performed. Follow-up of patients until 2001 gave information concerning recurrence state and performance state at last follow-up.
The slides of all tumors were reviewed using conventional histological sections stained with hematoxylin and eosin by a trained pathologist. Periodic acid-Schiff staining with diastase pre-treatment and Alcian-blue staining was used to assign tumors with mucin production and squamous morphology to the adenosquamous category. By reviewing the slides, the following data were obtained: tumor size, tumor type, presence of vasoinvasion, depth of infiltration expressed in millimetres of tumor at a right angle to the basement membrane, presence of tumor-positive resection margins, parametrial involvement, lymph node involvement and number of tumor positive lymph nodes. The size of the primary tumor was subdivided into categories of < 40 mm and ≥ 40 mm. The depth of tumor infiltration was classified as < 15 mm and ≥ 15 mm.

Immunohistochemistry

Immunohistochemistry on the whole series was performed on 4 µm sections using aminopropylethoxysilane (APES) slides. Paraffin slides were deparaffinized and rehydrated, and endogenous peroxidase was blocked with MeOH/ H_2O_2 0,3

% for 20 minutes. Antigen retrieval was performed with 0.01 M Citrate buffer. The primary mouse monoclonal antibody (mAb) (2G2) against αvβ6 (1:2000) was obtained from Biogen Idec, Cambridge, Massachusetts, as recently described.[36] 2G2 was characterized for its ability to bind to denatured human beta6 (HPLC purified) and was subsequently used in developing the staining protocol for immunohistochemistry on paraffin-embedded tissue sections. The primary mAb against fibronectin was retrieved from Sigma, St. Louis, MO (1:1000). Phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) was used as a diluent for the antibodies. Incubations were performed at room temperature. Incubation with mAbs against αvβ6 and fibronectin was preceded by pre-treatment with 0.4% pepsin in 0.01 M HCl for 20 minutes at 37^{0}C. Washing in between incubations was performed 3 times for 4 minutes with PBS. After washing in PBS, slides were incubated overnight at 4^0 Celsius with the primary antibodies. The next day after washing in PBS, biotinylated secondary rabbit antimouse antibodies (1:200, Dako, Glostrup, Denmark) and subsequently a biotinylated horseradish peroxidase (HRP)-streptavidin complex (1:100, Dako) were applied for 30 minutes each. To visualize immune complexes, a 0.05% solution of diaminobenzidine (kit 5001, Dako) containing 0.0018% H2O2 in a 0.05 M Tris-HCl buffer (pH 7.6) was applied.

Brown staining of cytoplasma indicated positivity for αvβ6 in tumor cells. Brown staining of ECM components in adjacent tumor slides indicated positivity for fibronectin in stroma. Paraffin embedded sections of the sw-480/B6 cell line[37] pellets were used as positive control for αvβ6. Mouse IgG1 Ab (1:2000) (MOPC21) on serial slides was used as a negative control. For fibronectin, normal kidney tissue served as positive control and rabbit IgG on serial slides as negative control. Sections were stained simultaneously. Mayer's hematoxylin was used for counterstaining of the fibronectin staining.

For the CIN lesions a supplementary staining with P-16 monoclonal mouse IgG1 Ab (Neomarkers, clone INK4A/MTS1, 1:500) was performed as a control for recognition of the dysplastic cells.[38]

RNA in situ hybridization

RNA *in situ* hybridisation to detect TGF-β_1 mRNA was performed before on paraffin-embedded sections of the cervical squamous carcinomas and carried out as previously described.[39,40]

In short, we used a *Sma*I-*Bam*HI fragment of TGF-β_1 complementary DNA (cDNA) cloned into pBluescript KS (Stratagene, La Jolla, CA). The specific copy RNA (cRNA) probes were labelled with digoxigenin following the manufacturer's protocol (Boehringer, Mannheim, Germany). After pre treatment the tumor sections

were hybridised with 10 ng TGF-β_1 antisense riboprobe per slide for 16 h at 62 ^{0}C. Subsequently, sections were washed in 2x standard saline solution citrate (SSC) with 50% formamide at 50 ^{0}C, then in 0.1x SSC with 20 mM β-mercaptoethanol at 62 ^{0}C, and finally treated with 2 U/ml ribonuclease (RNAse) T1 (Roche, Basel, Switzerland) in 2x SSC plus 1 mM ethylenediaminetetraacetic acid (EDTA) at 37 ^{0}C. Immunodetection of digoxigenin-labeled hybrids was done using nitro blue tetrazolium (NBT) as chromogen and bicholylindolyl phosphate (BCIP) as coupling agent (Roche). Blue staining of the cytoplasm indicated positivity for TGF-β_1 mRNA. To determine the level of nonspecific binding, adjacent tumor slides were hybridised with TGF-β_1 sense riboprobes. These were included as negative controls and did not show staining. Normal kidney tissue served as a positive control. The specificity of the TGF-β_1 probe has been thoroughly tested in our lab. The probe was sequenced to verify its sequence and by Northern blotting one specific band of the appropriate size was demonstrated. Using these probes they detected high expression of TGF-β_1 mRNA in the distal tubuli of the kidney, which was confirmed by quantitative real time PCR.[40]

Immunohistochemical evaluation

The slides were scored by two of the authors. When slides were scored differently, which occurred in few cases, they were evaluated again by the two observers simultaneously until consensus was reached. Staining for αvβ6 protein and TGF-β_1 mRNA in tumor cells was scored semi quantitatively by a quality control system proposed by Ruiter *et al.*[41] The tumor slides were scored on two items: the percentage of tumor cells staining positive and the intensity of the staining for the two specific markers. Scores representing the percentage of tumor cells stained positive were as follows: 0% (absent); 1-5% (sporadic); 6-25% (local); 26-50% (occasional); 51-75% (majority); and 76-100% (large majority). Intensity of tumor cell staining was scored as 0 (no staining); 1+ (weak); 2+ (clear); and 3+ (bright). To perform statistical evaluation, the following subdivision was made: staining intensity was considered negative/weak (0-1+) versus clear/bright (2+-3+), while percentage of positive tumor cell staining was considered minority (≤ 50%) versus majority (> 50%) positive. The extent of fibronectin staining in the tumor stroma was scored at the tumor-stromal border as described by Havenith *et al*[42]: limited (< 25% immunoreactivity), moderate (25-75% immunoreactivity) and extensive (> 75% immunoreactivity).

Statistics

Statistical analysis was performed using the SPSS 11.0 software package. Associations between αvβ6 protein, TGF-β_1 mRNA expression and clinicopathological

parameters were evaluated using the Fisher Exact test, and where appropriate the Chi-square test. 5-year survival rates were calculated according to the Kaplan-Meier method using the log rank test, while univariate and multivariate analysis of overall and disease free survival was performed according to Cox proportional hazard models. All tests were two-sided and the significance level was set to 5 %, corresponding to 95 % confidence intervals (CI).

Results

Assessment of αvβ6 expression

Expression of αvβ6 integrin protein by normal cervical squamous epithelium, CIN, cervical squamous carcinomas and lymph node metastases was examined. Normal epithelium generally displayed weak staining for αvβ6 in the basal layer of cells; more differentiated cells higher in the epithelial layer in general did not show staining (Fig 1A). In especially the CIN 2 and 3 lesions αvβ6 staining also was notably present in the upper epithelial layers (Fig 1B). This staining in general was also more intense than in normal epithelium. In both CIN and carcinomas the only cells showing positive staining for αvβ6 were those of epithelial origin; inflammatory cells or fibroblasts did not display expression. In general, in carcinomas a characteristic staining pattern was observed with a more intense staining at the peripheral borders of the tumor islands, where the carcinoma cells contact the stroma, and a weaker staining intensity centrally within the tumor nests and cords, which often include the more differentiated cells (Fig 1C). Furthermore, a more intense staining was observed in small nests (which contain a higher percentage of tumor cells bordering the stroma) or individually infiltrating tumor cells, compared with a weaker staining in large, massive nests and cords of tumor cells (Fig 1D).

As described in the materials and methods section, the tumors were scored on two items: the percentage of tumor cells staining positively and the intensity of staining. Of the 86 tumors one case was excluded because of too few tumor cells in the tissue slides. In the remaining 85 tumors the score was as follows: in 1 cases no positively stained tumor cells, in 4 cases 1-5% of tumor cells positive, in 8 cases 6-25 % positive, in 13 cases 26-50% positive, in 17 cases 51-75% positive and in 42 cases 76-100% of tumor cells stained positive for αvβ6 integrin. With respect to the intensity of staining, 1 case showed no staining, 34 cases displayed weak staining (1+), 34 cases clear staining (2+) and 16 cases bright staining (3+).

Lymph node metastases of 16 of these patients were also stained for αvβ6 expression. Three cases were excluded because of too few tumor cells in the lymph

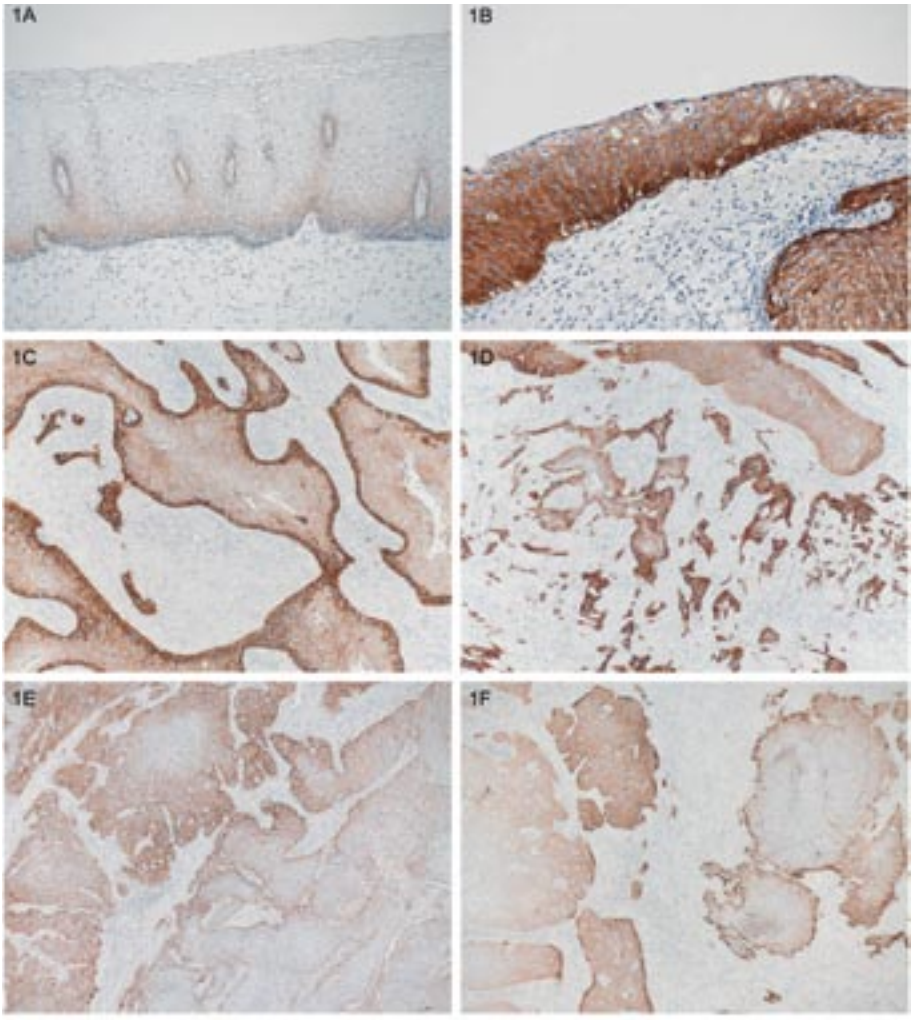

Figure 1 - αvβ6 protein expression in normal squamous cervical epithelium (1A), CIN 3 (1B), two different squamous cervical carcinomas (1C-1D) and one primary carcinoma with a lymph node metastasis of the same patient (1E-1F). αvβ6 was detected by IHC as described in the M&M section and visualized by a brown color. Note that in both CIN and carcinomas the only cells displaying positive staining for αvβ6 are those of epithelial origin; inflammatory cells or fibroblasts did not display expression.
(1A) αvβ6 protein is only weakly expressed in the cytoplasm of the basal cell layers of normal epithelium and not in the more differentiated cells higher in the epithelial layers. (1B) In CIN 3, αvβ6 is present into the upper epithelial cell layers, while the intensity of expression is also more intense, reflecting a higher amount of undifferentiated (dysplastic) cells (original magnification x 45). (1C) Surrounded by stroma (bluish) lie large tumor cell nests, which show a characteristic staining pattern of more intense staining at the peripheral borders and a weaker intensity centrally within the nests, which often include the more differentiated cells. (1D) Small tumor cell nests or individually infiltrating tumor cells show more intense staining than large, massive nests of tumor cells (original magnification x 25). (1E) The major part of the primary tumor shows a heterogeneous, weak to clear staining intensity pattern with the lymph node metastasis (1F) displaying a similar expression pattern (original magnification x 31).

nodes. The remaining 13 cases all demonstrated αvβ6 expression. Compared to the expression pattern observed in the primary tumor, 8 of 13 cases showed a similar expression pattern, 4 out of 13 cases a slightly weaker and 1 out of 13 cases a stronger expression pattern (Fig 1E-F). Lymphocytes and histiocytes in the lymph nodes did not show positive staining for the integrin.

Assessment of TGF-β1 mRNA expression and fibronectin immunoreactivity.
TGF-β_1 mRNA expression was previously examined in the cytoplasm of the carcinoma cells.[39] Inflammatory cells, known to express TGF-β_1 mRNA, served as an internal control for the quality of the mRNA served; furthermore RNA in situ hybridisation with the household gene β-actin was performed on the whole series. Expression was scored in the same way as described for αvβ6 expression. This resulted in 2 cases showing 1-5% of tumor cells expressing TGF-β_1 mRNA, 4 cases 6-25%, 6 cases 26-50%, 16 cases 51-75% and 58 cases > 75%. Intensity of expression was weak in 39 cases, clear in 36 cases and bright in 11 cases. Normal epithelial cells, as well as inflammatory cells, demonstrated clear staining.
Fibronectin immunoreactivity in the tumor stroma was limited in 18 cases, moderate in 24 cases and extensive in 40 cases. 4 cases were excluded because of poor morphology. The extent of fibronectin immunoreactivity was independent of the amount of intra tumoral stroma (scored previously[34]; Anova linear regression model, p = 0.23, data not shown).

Patients
Of the total group of 86 patients, 3 patients were diagnosed as FIGO stage IA2, 48 as stage IB1, 12 as IB2, and 23 as stage II and all underwent radical hysterectomy combined with pelvic lymph adenectomy. 26 of these patients had lymph node metastasis. 42 patients, equally balanced in the different integrin groups, received postoperative radiotherapy because of either tumor positive lymph nodes or a combination of 2 of the following unfavourable prognostic parameters: depth of infiltration ≥ 15 mm, tumor size ≥ 40 mm and presence of vasoinvasion. 26 patients suffered recurrent disease. By 2001, the authors' cut off date of follow up, 20 patients had died of disease, 60 were alive, 6 suffered from a recurrence and 6 died for causes unrelated to the primary disease, but showed no evidence of disease as concluded from the clinical record.
The median time of disease free survival was 66 months, with a minimum of 0 and a maximum of 184 months. The median time of follow up was 77 months, ranging from 0 to 185 months. Twelve patients were lost for follow up.

Association of αvβ6 staining results with clinicopathological parameters and survival analysis

Clear/bright (2+/3+) intensity of αvβ6 staining (n=50) was significantly correlated with more advanced stage of disease (p=0.05), larger tumor size (p=0.02), TGF-β_1 staining intensity (p=0.05) and recurrent disease (p=0.02) (Table 1).

The majority (> 50%) of tumor cells staining positive for αvβ6 (n=59) was significantly related to a higher FIGO stage (p=0.02), extensive fibronectin in the ECM (p = 0.002) and a more intense staining for TGF-β_1 (p = 0.04).

Furthermore, the intensity of αvβ6 staining was prognostic for worse overall and disease free survival. The stronger the staining intensity in the tumor cells the shorter the 5 year overall survival rate calculated according to the Kaplan-Meier method was (91% negative/weak vs. 76% clear vs. 54% bright; p-value survival rates: 0.01) and 5 year disease free survival rate (91% vs. 72% vs. 54% respectively; p-value survival rates: 0.004), which is illustrated in the survival curves (Fig 2). As expected and described before, well known prognostic parameters such as the presence of lymph node metastases, depth of infiltration ≥ 15 mm, vasoinvasion and FIGO stage ≥ IB2 were significant predictors for a shorter overall and disease free (5-year) survival (Table 2). Subsequently, multivariate analysis was performed using Cox's regression model on αvβ6 intensity and the strongest prognostic factors according to the univariate analysis (data not shown but in

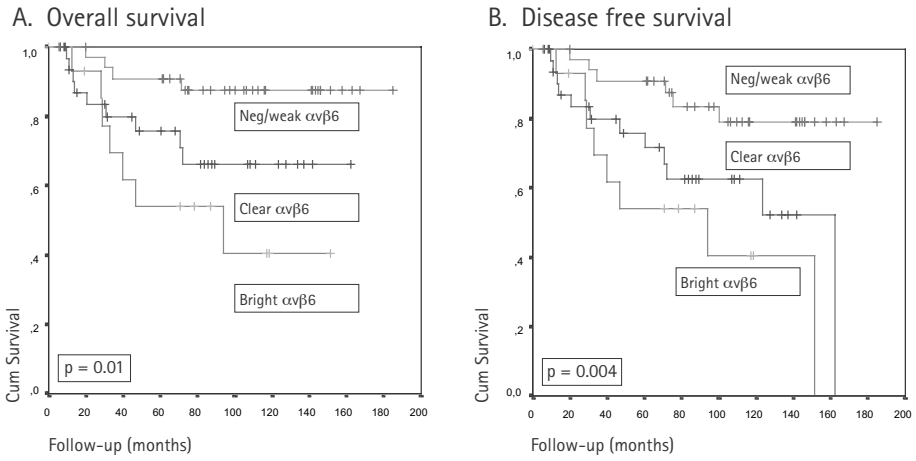

Figure 2 - Kaplan-Meier curves for (A) overall survival (p = 0.01) and (B) disease free survival (p = 0.004) in patients with negative/weak αvβ6 protein staining intensity (n = 35), clear αvβ6 staining intensity (n = 34) and bright αvβ6 intensity staining patterns (n = 16) in the primary cervical squamous carcinomas (n=85).

Table 1 - Relationship of αvβ6 with clinico-pathological parameters.

		αvβ6 **intensity** neg/weak (0-1+)		αvβ6 **intensity** clear/bright (2+/3+)			αvβ6 **percentage positive cells** <50%		αvβ6 **percentage positive cells** >50%		
	n	n (%)	*(%)*	n (%)	*(%)*	p value	n (%)	*(%)*	n (%)	*(%)*	p value
# patients	85	35		50			26		59		
FIGO stage											
≤ IB1	50	25 (50)	*(71)*	25 (50)	*(50)*		20 (40	*(77)*	30 (60)	*(51)*	
≥ IB2	35	10 (29)	*(29)*	25 (71)	*(50)*	**0.05**	6 (17)	*(23)*	29 (83)	*(49)*	**0.02**
Lymph node											
positive	26	10 (39)	*(29)*	16 (62)	*(32)*		7 (27)	*(27)*	19 (73)	*(32)*	
negative	59	25 (42)	*(71)*	34 (58)	*(68)*	0.74	19 (32)	*(73)*	40 (68)	*(68)*	0.63
Recurrent disease											
yes	26*	6 (23)	*(17)*	20 (77)	*(41)*		10 (39)	*(39)*	16 (62)	*(28)*	
no	58	29 (50)	*(83)*	29 (50)	*(59)*	**0.02**	16 (28)	*(61)*	42 (72)	*(72)*	0.32
Tumorsize											
< 40 mm	55*	29 (53)	*(83)*	26 (47)	*(58)*		17 (31)	*(71)*	38 (69)	*(68)*	
≥ 40 mm	25	6 (24)	*(17)*	19 (76)	*(42)*	**0.02**	7 (28)	*(29)*	18 (72)	(32)	0.79
Vasoinvasivion											
yes	48*	19 (40)	*(54)*	29 (60)	*(60)*		16 (33)	*(62)*	32 (67)	*(56)*	
no	35	16 (46)	*(46)*	19 (54)	*(40)*	0.58	10 (29)	*(39)*	25 (71)	*(44)*	0.64
Depth of infiltration											
< 15 mm	55*	26 (47)	*(77)*	29 (53)	*(64)*		16 (29)	*(67)*	39 (71)	*(71)*	
≥ 15 mm	24	8 (33)	*(24)*	16 (67)	*(36)*	0.25	8 (33)	*(33)*	16 (67)	*(29)*	0.71
HPV status											
negative	8*	6 (75)	*(18)*	2 (25)	*(4)*		3 (38)	*(13)*	5 (63)	*(9)*	
16	55	20 (36)	*(59)*	35 (64)	*(73)*		18 (33)	*(75)*	37 (67)	*(64)*	
other	19	8 (42)	*(24)*	11 (58)	*(23)*	0.12	3 (16)	*(12)*	16 (84)	*(27)*	0.33
TGF-β_1 **intensity**											
weak	38	20 (53)	*(57)*	18 (47)	*(36)*		16 (42)	*(62)*	22 (58)	*(37)*	
clear/bright	47	15 (32)	*(43)*	32 (68)	*(64)*	**0.05**	10 (21)	*(38)*	37 (79)	*(63)*	**0.04**
TGF-β_1 **positive tumor cells**											
≤ 50%	11	7 (64)	*(20)*	4 (36)	*(8)*		2 (18)	*(8)*	9 (82)	*(15)*	
> 50%	74	28 (38)	*(80)*	46 (62)	*(92)*	0.11	24 (32)	*(92)*	50 (68)	*(85)*	0.34
Fibronectin ECM											
< 25%	18*	11 (61)	*(32)*	7 (39)	*(15)*		10 (56)	*(40)*	8 (44)	*(14)*	
25-75%	24	11 (46)	*(32)*	13 (54)	*(27)*		10 (42)	*(40)*	14 (58)	*(25)*	
> 75%	40	12 (30)	*(35)*	28 (70)	*(88)*	0.07	5 (13)	*(20)*	35 (88)	*(61)*	**0.002**

Statistical analysis of the staining results of αvβ6 with c.p. Parameters and with the RISH results of TGF-β_1 mRNA, evaluated using chi-square and Fisher Exact tests. Statistical significant p values are bold. Incidentally missing cases are marked with *. The first row of numbers between ( ) reflect the percentage of αvβ6 within the specific c.p. parameter; the second row between ( ) in italics reflect the percentage of the c.p. parameter within αvβ6.

general corresponding results with the Kaplan-Meier survival rates). Clear/bright αvβ6 staining intensity turned out to be an independent prognostic parameter for overall (Hazard ratio 3.21; p = 0.05) and disease free (Hazard ratio 2.89; p = 0.04) survival (Table 3).

The intensity of αvβ6 staining was also evaluated in a subgroup of patients without tumor positive lymph nodes (n=60). Although the difference between weak or

Table 2 - Association of αvβ6 protein expression and clinicopathological parameters with overall and desease free 5-years survival

	n	Overall survival 5 yrs (%)	p value	Disease-free survival 5 yrs (%)	p value
	86				
αvβ6 intensity specified					
neg/weak (0-1+)	35*	91		91	
clear (2+)	34	76		72	
bright (3+)	16	54	**0.01**	54	**0.004**
αvβ6 intensity					
neg/weak	35	91		91	
clear/bright	50	69	**0.008**	66	**0.003**
αvβ6 percentage positive cells					
≤ 50%	26*	80		80	
> 50%	59	78	0.61	76	0.48
Depth of infiltration					
< 15 mm	56*	88		86	
≥ 15 mm	24	60	**< 0.001**	60	**0.001**
Tumor size					
< 40 mm	56*	83		83	
≥ 40 mm	25	69	0.19	64	0.09
Lymph node					
positive	26	57		57	
negative	60	87	**< 0.001**	85	**< 0.001**
FIGO stage					
≤ Ib1	51	88		88	
≥ Ib2	35	64	**0.04**	60	**0.04**
Vaso invasion					
present	48	74		71	
not present	36	88	0.04	88	0.06

5 year overall and disease-free survival analysis of αvβ6 and well-known prognostic parameters calculated according to the Kaplan-Meier method. * marks accidental missing cases. Statistical significant associations are in bold; p-values are calculated over the total survival time.

Table 3 - Multivariate analysis including αvβ6 intensity, lymph node metastasis and tumor infiltration depth

	Overall survival HR (95 % c.i.)	p-value	disease-free survival HR (95 % c.i.)	p-value
αvβ6 intensity clear/bright	3.21 (0.99-10.41)	**0.05**	2.89 (1.06-7.91)	**0.04**
Lymph nodes tumor positive	3.70 (1.29-10.61)	**0.02**	2.94 (1.15-7.56)	**0.03**
Infiltration > 15 mm	3.19 (1.09-9.35)	**0.04**	2.25 (0.89-5.67)	0.09

Hazard ratio (HR) with 95% confidence interval for αvβ6 intensity with the strongest prognostic parameters for overall and disease free survival by Cox proportional hazard model. Statistical significant HRs are bold.

clear staining intensity did not affect survival, bright αvβ6 staining added extra prognostic information, since after 5 years, the survival rate for negative/weak vs. clear vs. bright was 92% vs. 95% vs. 56% (p-value survival rates: 0.02). For disease-free survival this was 92% vs. 90% vs. 56% respectively (p-value survival rates: 0.06).

Discussion

In the present study we observed increased expression of αvβ6 protein staining in cervical carcinoma cells to be significantly related to a more advanced stage of disease, recurrent disease, a larger tumor size, extensive fibronectin immunoreactivity in the ECM and enhanced TGF-β_1 mRNA expression and to be predictive for a shorter overall and disease free 5 year survival rate. In both primary carcinomas and lymph node metastases a characteristic staining pattern was observed. Often a more intense staining was notified in the less differentiated tumor cells at the leading edge of (large) tumor cell nests. Also small nests or individually infiltrating tumor cells in the tumor stroma displayed stronger staining compared to the massively growing fields of tumor cells. This typical staining pattern was also described in invasive colon carcinomas and oral sqamous carcinomas and might reflect an interaction between the tumor cells and the ECM components.[23,43] As described previously, the ECM of cervical carcinomas is rich in especially the expression of the fibronectin protein, probably deposited there by peritumoral stroma cells and perhaps by tumor cells themselves.[5,34] One of the important receptors for fibronectin is the αvβ6 integrin. In the present study we found a significant semi

quantitative relationship between enhanced αvβ6-expression by tumor cells and a more abundant presence of fibronectin in the ECM. Post-EMT colon carcinoma cells (characterized by up regulation of αvβ6) as well as squamous carcinoma cells have been demonstrated to be significantly more chemotactic on transwells coated with fibronectin compared to controls (not coated or laminin-coated).[23,19] This effect could be blocked by administration of function-blocking β6 antibodies. Also in wound healing studies cell spreading and haptotactic motility was demonstrated to be in part an interaction between keratinocytes expressing αvβ6 and fibronectin in the matrix.[31,16] In cervical squamous cell carcinomas up regulation of this integrin in the tumor cells most closely neighboring the fibronectin-rich stroma, might facilitate migration and invasion of those cells too. The observed strong intensity in individually infiltrating cells possibly reflects (some degree of) EMT as described in colon carcinoma cells.[23,32] The similarity in staining pattern observed in the lymph node metastases and primary carcinomas of 13 patients suggests that the up regulation of αvβ6 integrin by cervical carcinoma cells is of importance both during the process of invasion and metastasis.

A factor which may play a role in the process of αvβ6 up regulation is TGF-β_1. This multifunctional cytokine has been demonstrated to up regulate the expression and surface exposure of especially the αvβ6 integrin not only in keratinocytes but also in colon carcinoma cells.[31,16,23] Previously we have shown that cervical carcinoma cells in vitro as well in vivo express mRNA for TGF-β_1, which influences the formation of tumor stroma and tumor infiltrate. In the present study a more intense staining for TGF-β_1 mRNA in the tumor cells correlates with enhanced αvβ6 integrin expression. This is suggestive for an auto-regulatory mechanism of the tumor, in which autocrine and paracrine TGF-β_1 results in an up-regulation of αvβ6 integrin expression by the tumor cells. Whether the vice versa mechanism of activation of latent TGF-β_1 by αvβ6 described in colon carcinoma cells and lung epithelial cells[23,15] occurs in cervical carcinomas in vivo too, can not be deduced from the present study. All together our findings are suggestive for a collaboration between αvβ6 integrin and TGF-β_1 in cervical squamous cell carcinomas, as is proposed in wound healing too. In the latter process this may lead to either healing or fibrosis, while in carcinomas it may result in increased invasive growth. Of note, alterations reported in components of the TGF-β signaling pathway, such as those affecting SMAD 2 and 4, point to a role of this pathway contributing to cervical carcinogenesis[44,45]

Normal cervical squamous epithelium displayed in most cases only (weak) staining in the basal layer of keratinocytes attached to the basement membrane and none in upper layers, which is in agreement with the observations by others that αvβ6 expression generally is down regulated in differentiated epithelial cells.[18] In

the nine premalignant specimen we examined, an up regulation of αvβ6 protein expression was observed in almost all CIN, and especially in the higher grades, compared to normal epithelium. In contrast to our findings, a study on αvβ6 expression in premalignant oral lesions demonstrated staining positivity in only 40% of the specimen, while absence of αvβ6 staining in the lesions appeared to be prognostic for non-invasiveness.[43] Since we observed αvβ6 expression in 8 out of 9 CIN, while only approximately 12 percent of CIN will precede to invasive tumors,[46] the (increased) presence of αvβ6 probably reflects the higher amount of undifferentiated epithelial cells in these lesions rather than that it predicts invasiveness.

The unfavourable prognostic significance of increased αvβ6 expression in cervical squamous carcinomas we detected is in agreement with recent observations in colon carcinoma and gastric carcinoma.[23,25] Overall survival analysis in the subgroup of patients without lymph node metastases illustrated bright αvβ6 intensity to add independent prognostic information. In contrast, Kawahima *et al.* showed increased αvβ6 expression to be associated with lymph node metastases.[25] Inhibition of cytoplasmic β6-ERK2 binding, and concomitant MAP kinase activation, has been shown to suppress growth of colon cancer in vivo.[22] The enhanced aggressive phenotype of αvβ6 expressing colon, squamous cell and ovarian carcinomas has been partly ascribed to an observed co-expression with proteases such as MMP-9 and uPA, which results in increased matrix degradation and facilitation of tumor cell invasion.[26,47,24] In oral cancer cells however the opposite has been reported too: a decrease in uPA-receptor expression, necessary for binding of uPA and plasmin generation, was correlated with enhanced αvβ6 expression, suggesting preservation of a certain amount of ECM to be essential for invasion.[48]
Besides, the relationship between increased αvβ6 expression and a larger tumor size we observed, is suggestive for an enhanced potential to proliferate of αvβ6 expressing cells, which is also demonstrated in colon cancer cells lacking endogenous αvβ6; transfection of wild-type β6 has been shown to result in enhanced tumor growth, conferred by an 11 amino acid region of the β6 cytoplasmic tail. Inoculation of carcinoma cells transfected with β6 lacking the cytoplasmic sequence in athymic mice demonstrated tumors 2-3 fold smaller when compared to wild-type.[37,22] Similar results in oral cancer were shown in which neo-expressed wild-type β6 binding to fibronectin induced fyn and subsequent MAP kinase signaling and promoted oral cancer growth and metastases in mice, which was abrogated by transfection of β6 with a kinase-dead fyn domain.[20,29]

In conclusion, the results of our immunohistochemical study are suggestive for up regulation of the αvβ6 integrin in primary cervical squamous cell carcinomas as well as in the lymph node metastases of those patients. Increased expression is related to clinical unfavourable prognostic factors and is an independent prognostic parameter for worse overall and disease free 5 year survival. This might be partly explained by an increased capacity of the tumor cells for migration and invasion due to enhanced interaction with fibronectin in the ECM, facilitating proliferation and/or protease production and concomitant matrix degradation, or by contributing to TGF-β_1 activation and its protumorigenic effects. These aspects of αvβ6 remain to be investigated in cervical carcinoma. Experiments with αvβ6 function-blocking antibodies may be promising for a future therapeutic strategy in αvβ6 expressing squamous carcinomas and metastases.

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Chapter 6