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The involvement of E6, p53, p16, MDM2 and Gal-3 in the clinical outcome of patients with cervical cancer

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Abstract. High-risk human papilloma virus (HPV) is the leading cause of cervical cancer. HPV oncogenes are responsible for the development of malignancy, and the E6 oncoprotein that HPV expresses induces the degradation of tumour suppressor protein p53 (p53). This degradation leads to the upregulation of p16; however, unidentified proteins may also serve a role in the development and progression of cervical cancer. Therefore, the aim of the present study was to analyse the expression levels of E6, p53, p16, MDM2 proto-oncogene (MDM2) and galectin-3 (gal-3) in cervical cancer specimens. A total of 250 cervical cancer tissue slides were used. The expression of E6, p53, p16, MDM2 and gal-3 was analysed with immunohistochemical methods and a semi-quantitative scoring. SPSS software was used for the statistical evaluation of staining results and survival analysis of patients with cervical cancer. Cervical cancer specimens demonstrated significantly increased E6 staining with advanced T-status and increased International Federation of Gynecology and Obstetrics classification. E6, p53 and p16 demonstrated significantly different expression levels in squamous epithelial tissue compared with adenocarcinomas. MDM2 and gal-3 demonstrated positively correlated expression levels in cervical cancer. In addition, gal-3 expression was correlated with poor prognosis in p16-negative cases. A negative correlation between the expression of E6 and a mutated form of p53 was also identified in cervical cancer. p53 mutation was demonstrated to be common in cervical cancer, and gal-3 and MDM2 appeared to act in a combined manner in this type of tumour. As gal-3 is overexpressed in the cervical cancer tissue of patients with poor prognosis, the use of gal-3 inhibitors should be investigated in future studies.

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Introduction

Cervical cancer is the fourth most frequent cancer in women globally with ~530,000 new cases in 2012, accounting for 7.5% of all female cancer-associated mortalities (1). A major cause of cervical cancer is persistent infection with high-risk human papillomavirus (HR-HPV) (1). HPV subtypes 16 and 18 cause ~70% of all cases of HPV (1,2). At present, >170 HPV types have been identified (3). Infection with 15 subtypes of HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) may lead to cancer, which is why these 15 types are known as carcinogenic or high-risk types (4). The genome of human papillomaviruses consists of ~8,000 base pairs and contains six early genes (E6, E7, E1, E2, E4 and E5) and two late genes (L1 and L2) (5). Upon replication of the viral gene E6, E6 oncoprotein is expressed, which alters the cell cycle (6). E6 oncoprotein and E6-associated protein (E6-AP) form a complex that binds to p53 and causes its proteolytic degradation (7).

The tumour suppressor protein p53 (p53) signalling pathways leads to cell cycle arrest or apoptosis in case of DNA damage (8). As E6 oncoprotein induces the degradation of p53, the function of this important cell cycle protein is disturbed following HPV infection (9). In addition, the cell cycle regulation protein p16 is expressed at high levels in HPV-infected epithelial cells, and thus acts as a marker for the diagnosis of HPV-associated carcinoma (9,10). In non-carcinoma tissues p53 is regulated by MDM2 proto-oncogene (MDM2) through a negative feedback mechanism. MDM2 promotes the ubiquitination and proteasome-dependent degradation of p53 (11). There is also an association between MDM2, p53 polymorphism and the progression of cervical carcinoma (12).

A protein previously demonstrated to be associated with cervical cancer is galectin-3 (gal-3) (5). Galectins are defined as lectins with a galactose-binding ability and a characteristic amino-acid sequence (13). Galectin is a name proposed by Hirabayashi and Kasai (14) for a family of animal lectins. Galectins are typically soluble and metal-independent in

their activity (15). They have similar features to cytoplasmic proteins, including no disulfide bridges, no sugar chains, no signal sequences and, in most cases, their N-terminal amino acids are acetylated (16). It is possible to classify galectins into the following three types on the basis of their structural architecture: Proto, chimera and tandem-repeat types. Gal-3 is a chimera-type galectin (17).

Gal-3 may increase the invasiveness of cervical cancer by activating vascular endothelial growth factor receptor-3 (5). Therefore, the aim of the present study was to systematically analyse the expression and interactions of E6, p53, p16, MDM2 and gal-3 in cervical cancer specimens.

Materials and methods

Ethical approval. The present study was approved by the local Ethics Committee of the Ludwig-Maximilians-University of Munich (approval no. 259-16; Munich, Germany), and was performed in compliance with the guidelines of the Helsinki Declaration. Patient data were fully anonymised.

Specimens. Archived formalin-fixed paraffin-embedded (FFPE) sections from 250 cases of cervical cancer were used in the present study; it was possible to analyse 248 cases as there was no tumour tissue present on two sections (Table I). Cervical dysplasia [cervical intra-epithelial neoplasia (CIN) stage III] (18) and non-dysplastic cervical tissue (3 sections of each) was used for the E6 immunohistochemical staining, and breast cancer tissue was used for the mutated p53 immunohistochemistry. Specimens were obtained from the Department of Obstetrics and Gynecology of Ludwig-Maximilians-University of Munich, and were obtained from patients undergoing surgery there between 1993 and 2002. Follow-up data were received from the Munich Cancer Registry (Munich Tumour Centre, Munich, Germany).

Table I. Clinical parameters of the patients included in the present study.

Clinical parameter	No./total no.	%
Age (years)		
≤50	143/248	58
>50	105/248	42
No. of metastasis positive lymph nodes		
0	149/248	60
1-4	97/248	39
NA	2/248	1
Tumour size (cm)		
<2	111/248	45
2-4	128/248	52
>4	9/248	3
Tumour grade		
I	20/248	8
II	141/248	57
III	78/248	31
NA	9/248	4
Tumour subtype		
Squamous	199/248	80
Adenocarcinoma	49/248	20
Progression (over 236 months)		
None	190/248	77
≥1	58/248	23
Survival (over 236 months)		
Right censured	210/248	85
Succumbed	38/248	15

NA, not applicable as data not available.

Immunohistochemistry. The FFPE sections (3-μm-thick) were dewaxed in xylol, endogenous peroxidase was inhibited with 3% methanol/H_2O_2 and sections were rehydrated in a descending ethanol gradient. To stain for mutated p53, wild-type p53, E6, gal-3 and MDM2, the slides were pre-treated in citrate buffer (100˚C; pH 6.0) for antigen retrieval. Following this, non-specific binding of the primary antibodies was blocked, and incubation with the primary antibodies followed (Tables II and III). Incubation with the secondary antibodies and the following steps of the detection system and colour development are illustrated in Tables II and III. For p16 detection, the specimens were automatically stained using the Ventana BenchMark XT Stainer (Ventana Medical Systems, Inc., Oro Valley, AZ, USA) and the CINtec Histology kit (cat. no. 9517; Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions, while all other antibodies were stained for manually. For wild-type p53, the slides were washed in PBS/0.05% Tween-20. All other slides were washed in PBS only. Finally, the slides were counterstained with hemalaun (Waldeck GmbH, Münster, Germany) for 2 min at room temperature, dehydrated in an ascending series of ethanol and stored.

Slides were examined with a Zeiss Axiophot light photomicroscope (Zeiss GmbH, Jena, Germany). Digital images were obtained with a digital-camera system (CF20DXC; KAPPA Messtechnik, Gleichen, Germany). All specimens were evaluated by a pathologist. The intensity and distribution patterns of the staining reaction was evaluated by two blinded, independent observers, including the gynecological pathologist, using the semi-quantitative immunoreactive (IRS)-score, as previously described (19), to asses steroid receptors (20) and cathepsin D (21) expression. The IRS score was calculated by multiplication of optical staining intensity (graded as 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) and the percentage of positive stained cells (0, no staining; 1, ≤10% of the cells, 2, 11-50% of the cells, 3=51-80% of the cells and 4, ≥81% of the cells) and without knowing the pathological evaluation, the diagnosis or the standard performed hematoxylin reaction for 2 min at room temperature for each specimen.

Statistical analysis. Data were analysed using SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA) for Microsoft Windows and visualised using Microsoft Office 7 (Microsoft Corporation, Redmond, WA, USA). Spearman coefficients were calculated to assess correlations, while the

Table II. Procedures for gal-3 and MDM2 staining.

Protocol	Gal-3	MDM2
Blocking method	Horse serum[a], 20 min, RT	Goat serum[a], 20 min, RT
Primary antibody, dilution, incubation duration, incubation temperature, cat. no.	Anti-galectin-3, 1:1,000 in PBS, 16 h, 4˚C; NCL-GAL3[b]	Anti-MDM2, 1:100 in PBS, 16 h, 4˚C, NCL-MDM2[b]
Secondary antibody, dilution, incubation duration, incubation temperature, cat. no.	Biotynilated anti-mouse IgG[a], 30 min, RT; PK-6100	Biotinylated goat anti-mouse IgM, 30 min, RT ZMB2020[c]
Detection of secondary antibody	ABC-complex[a], 30 min	ABC-complex[a], 30 min
Chromogen	1 mg/ml DAB[d], 5 min	1 mg/ml DAB[d], 1 min

[a]Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA. [b]Novocastra; Leica Microsystems GmbH, Wetzlar, Germany. [c]Linaris GmbH, Dossenheim, Germany. [d]Dako, Glostrup, Denmark. Gal-3, galectin-3; MDM2, MDM2 proto-oncogene; Ig, immunoglobulin; DAB, 3,3'-diaminobenzidine; RT, room temperature.

Table III. Procedures for mutated p53, wild-type p53 and E6 staining.

Protocol	Mutated p53	p53 wild-type	E6
Blocking method	Reagent 1[a]; 5 min, RT	Reagent 1[a]; 5 min, RT	Reagent 1[a]; 5 min, RT
Primary antibody, dilution, incubation duration, incubation temperature, cat. no.	Anti-p53, 1:100 in PBS, 16 h, 4˚C, ab32049[b]	Anti-p53, 1:200 in PBS, 16 h, 4˚C, ab26[b]	Anti-E6, 1:150 in PBS, 1 h, RT, ab70[b]
Post blocking method	Reagent 2[a]; 20 min, RT	Reagent 2[a]; 20 min, RT	Reagent 2[a]; 20 min, RT
Secondary antibody, dilution, incubation duration, incubation temperature, cat. no.	HRP-Polymer Reagent 3[a], 30 min, RT POLHRP-100	HRP-Polymer Reagent 3[a], 30 min, RT POLHRP-100	HRP-Polymer Reagent 3[a]; 30 min, RT POLHRP-100
Chromogen	1 mg/ml DAB[c], 1 min	1 mg/ml DAB[c], 1 min	1 mg/ml DAB[c], 1 min

[a]From the ZytoChem-Plus HRP Polymer-kit; Zytomed Systems GmbH, Berlin, Germany. [b]Abcam, Cambridge, UK. [c]Dako, Glostrup, Denmark. RT, room temperature; HRP, horseradish peroxidase; DAB, 3,3'-diaminobenzidine.

Mann-Whitney U test was applied to examine differences between groups. Differences in survival were assessed using the log-rank test and survival curves were plotted in accordance with Kaplan-Meier estimator. $P<0.05$ was considered to indicate a statistically significant difference and data were expressed as the mean ± standard error. Cox regression analysis was used to compare the risk of mortality in patients with and without gal-3 expression when the effects of further factors were accounted for. Independent variables included in the Cox regression model were gal-3 expression, age at the time of surgery, histological subtype, tumour size, lymph node status (pN), metastasis, tumour grade, International Federation of Gynecology and Obstetrics (FIGO) stage (22,23), and E6, mutated p53 and MDM2 expression status.

Results

Evaluation of E6 oncoprotein immunohistochemistry and the detection of mutated p53 on control slides. CIN III tissue slides were used for the evaluation of E6 oncoprotein staining. Moderate expression levels of E6 were observed in the CIN III sections (Fig. 1A). There was no expression of the E6 oncoprotein, and therefore no staining observed, in the non-dysplastic cervical tissue (Fig. 1B). Breast cancer tissue was used to evaluate the staining of mutated p53 (Fig. 1C), which exhibited nuclear and cytoplasmic staining.

E6 oncoprotein staining. A total of 81% of all cervical cancer tissue examined expressed E6 oncoprotein (data not shown). Cervical cancer specimens demonstrated significantly increased staining with a higher T stage (according to the Tumor-Node-Metastasis classification system) (24). T1 stage carcinomas (Fig. 2A) demonstrated E6 staining with a median IRS of 2, while T2 (Fig. 2B) and T3 (Fig. 2C) stage carcinoma tissues had a significantly higher median E6 expression of IRS 3 ($P=0.017$; Fig. 2D).

FIGO 1 carcinoma tissues had a median E6 expression of IRS 2 (Fig. 2E). FIGO 2 (Fig. 2F) and FIGO 3 (Fig. 2G) carcinoma tissues had a median IRS of 4. FIGO 4-classified cervical cancer tissue had a median E6 IRS score of 6 (Fig. 2H). E6 demonstrated a significant positive correlation with the FIGO classification ($R=0.277$, $P<0.001$; Fig. 2I).

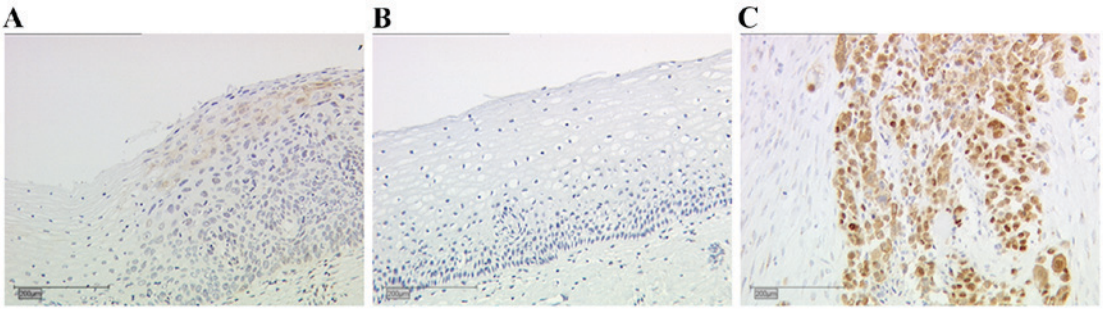

Figure 1. Representative images of the staining for E6 in dysplastic and non-dysplastic cervical tissue samples, and mutated tumour protein p53 in breast cancer samples. (A) E6 staining in cervical dysplasia. (B) No expression of E6 was detected in non-dysplastic cervix samples. (C) Breast cancer tissue demonstrated expression of mutated p53. Scale bar, 200 μm.

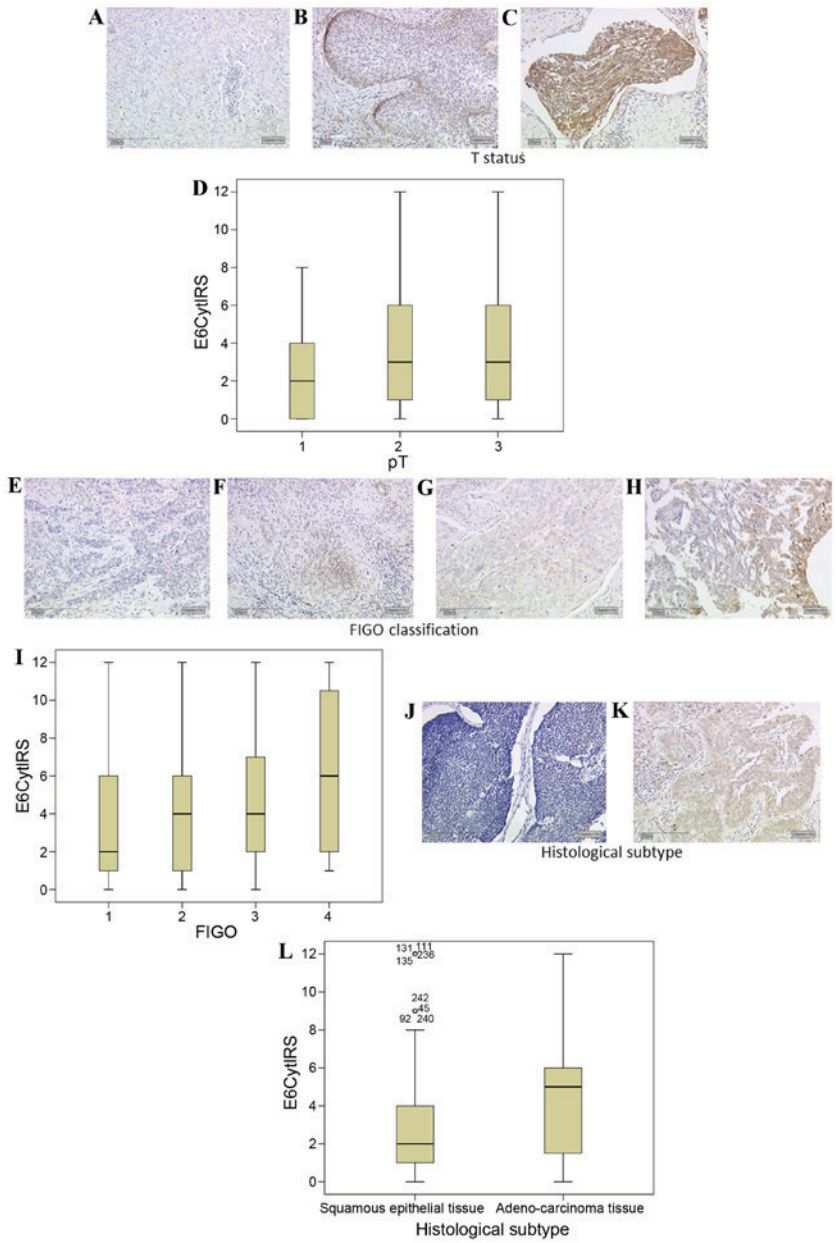

Figure 2. E6 expression is enhanced with cervical cancer tumour staging. (A) Low intensity E6 expression was observed in T1 tumours, whereas (B) T2 and (C) T3 staged tumours demonstrated increased expression of E6. (D) Box plot summary of the IRS for each tumour stage (P=0.017, FIGO 1 vs. 3). E6 expression was positively correlated with FIGO classification, with (E) FIGO 1 classified tissue demonstrating low expression of E6 while (F) FIGO 2 and (G) FIGO 3 classified tissue demonstrated increased expression levels, and (H) FIGO 4 tissue further increased expression levels. (I) Box plot summary of the IRS for each FIGO stage (P<0.001, FIGO 1 vs. 4). (J) Squamous epithelial tissue demonstrated lower levels of E6 staining than (K) adenocarcinoma tissue. (L) Box plot summary of the IRS for each histological subtype. Scale bar, 200 μm. FIGO, International Federation of Gynecology and Obstetrics; IRS, immunoreactive score; E6Cyt, E6 cytoplasmic; pT, pathological tumour stage.

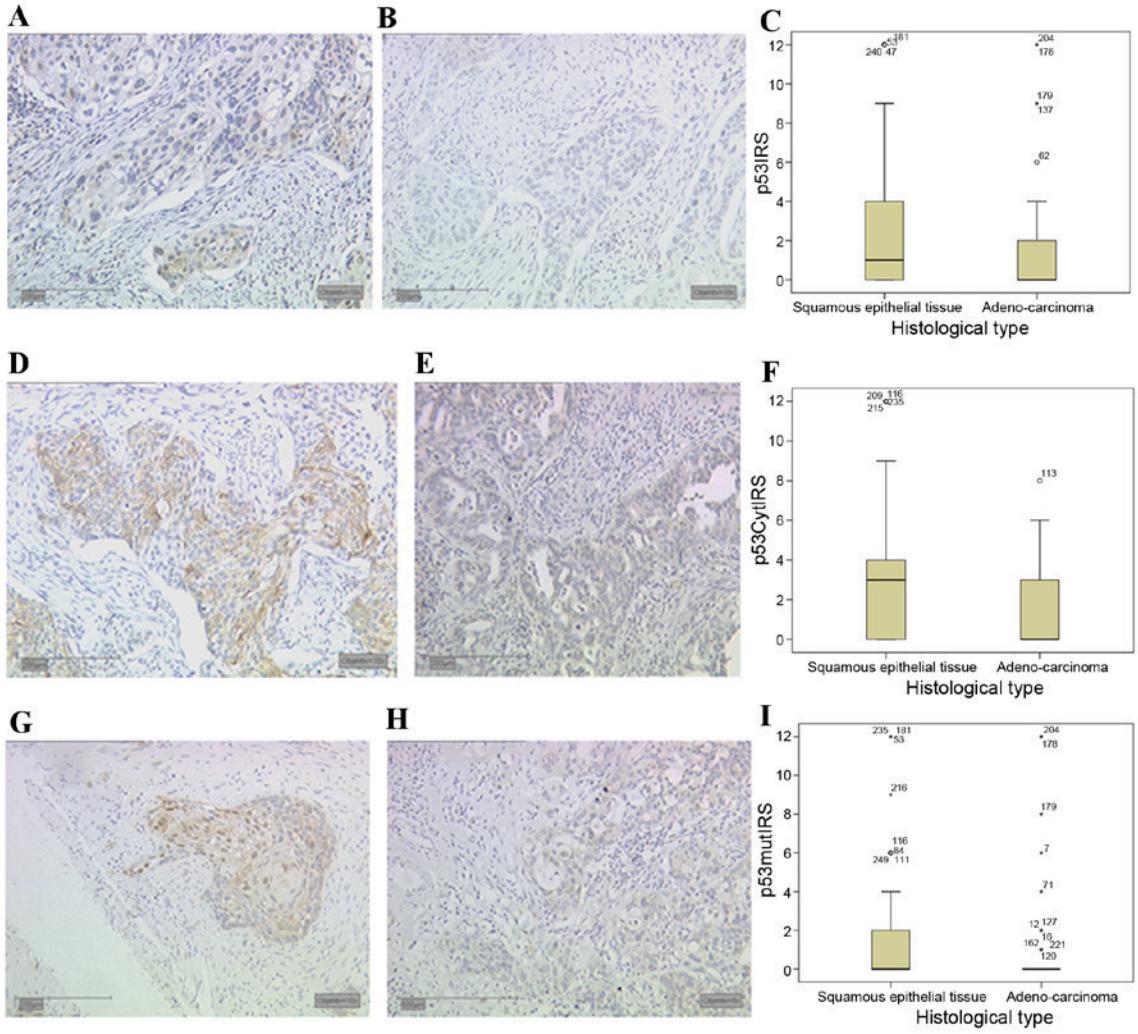

Figure 3. Tumour protein p53 expression in cervical cancer tissues. (A) Squamous epithelial tissue demonstrated a nuclear expression of wild-type p53, whereas (B) adenocarcinoma tissue had lower levels of p53 nuclear staining. (C) Box plot summary of the nuclear p53 IRS for each histological subtype. (D) Squamous epithelial tissue demonstrated higher expression of wild-type p53 in the cytoplasm compared with (E) adenocarcinoma tissue. (F) Box plot of the cytoplasmic p53 IRS for each histological subtype. (G) Squamous epithelial tissue demonstrated expression of mutated p53, while (H) adenocarcinoma tissue demonstrated almost no staining. (I) Box plot summary of the mutated p53 IRS for each histological subtype. Scale bar, 200 μm. IRS, immunoreactive score; p53Cyt, cytoplasmic wild-type p53; p53mut, mutant p53.

E6 demonstrated significantly different expression levels in cervical cancer tissue dependent on the histological subtype. Squamous epithelial carcinomas (Fig. 2J) had a median expression of IRS 2. Adenocarcinoma tissue (Fig. 2K) had significantly increased staining with a median of IRS 5 (P=0.015 vs. squamous epithelial carcinoma; Fig. 2L).

Wild-type and mutated p53 expression. Expression of wild-type p53 was observed in the nucleus and cytoplasm of 60 and 66% of all cervical cancer specimens, respectively. Significantly different expression levels in cervical cancer tissue in different histological subtypes were also observed for p53 expression. Wild-type p53 demonstrated a median nuclear expression (Fig. 3A) of IRS 1 in squamous epithelial tissue, whereas in adenocarcinoma tissue (Fig. 3B) the median nuclear expression was significantly decreased in comparison (IRS 0, P=0.024; Fig. 3C).

In addition to nuclear expression, wild-type cytosolic p53 expression also demonstrated significant differences associated with the histological subtype. In squamous epithelial tissue (Fig. 3D) a median expression of IRS 3 was observed, whereas in comparison the median cytosolic expression of p53 was significantly decreased in adenocarcinoma tissue (Fig. 3E) to IRS 0 (P<0.001; Fig. 3F).

The monoclonal antibody that recognises a previously described mutated form of p53, (25) also revealed significant staining differences associated with the histological subtype of cervical cancer. In addition, 42% of all cervical cancer tissue slides demonstrated nuclear expression of mutated p53, and 67% of all cases demonstrated mutated p53 expression in the cytoplasm. Although the median expression of mutated p53 in squamous epithelial tissue (Fig. 3G) and adenocarcinoma tissue (Fig. 3H) was 0, differences between the subtypes were significant (P=0.011; Fig. 3I).

Expression of p16 oncoprotein in cervical cancer tissue. p16 overexpression is routinely used in the Pathology Department of the Ludwig-Maximilians-University of Munich as a marker for HPV-associated head and neck squamous carcinoma (11). A total of 94% of cervical carcinoma cases tested

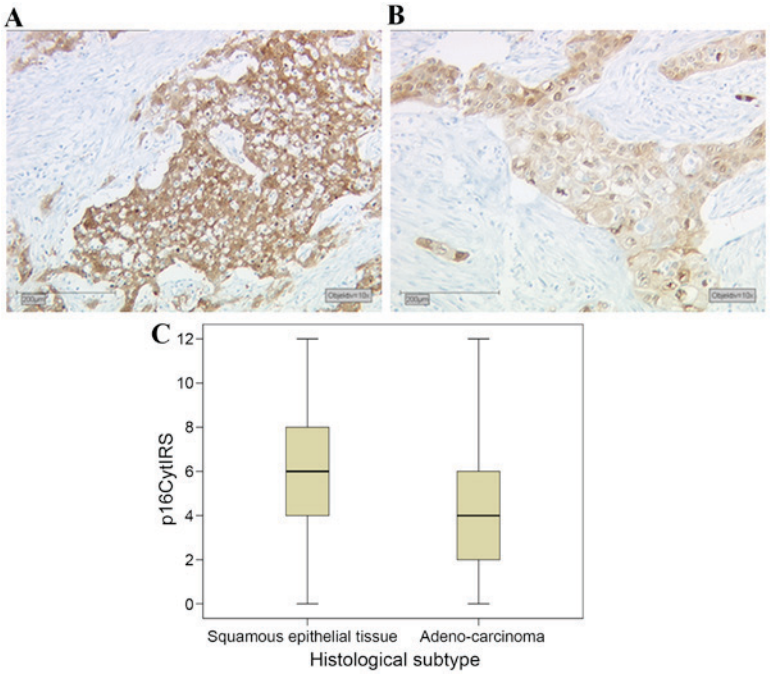

Figure 4. Expression of p16 in cervical cancer tissues (A) Squamous epithelial tissue demonstrated higher p16 expression levels compared with (B) adenocarcinoma tissue. (C) Box plot summary of the p16 IRS for each histological subtype. Scale bar, 200 μm. IRS, immunoreactive score; p16Cyt, cytoplasmic p16.

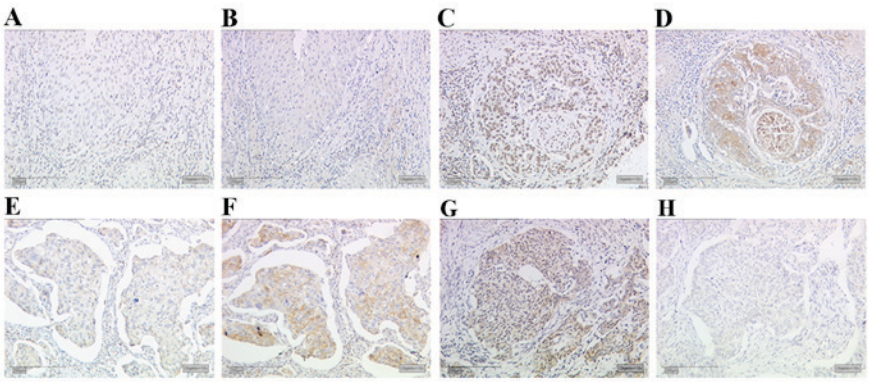

Figure 5. Expression of MDM2 and gal-3 in cervical cancer. Low expression of (A) MDM2 and (B) gal-3 was identified in the same area of cervical cancer. In another case, high expression of (C) MDM2 and (D) gal-3 were observed in the same are. Samples with (E) low E6 expression demonstrated high expression levels of (F) mutated p53. Another serial slide series with high expression of (G) E6 demonstrated low expression of (H) mutated p53. Scale bar, 200 μm. MDM2, MDM2 proto-oncogene; gal-3, galectin-3.

demonstrated p16 expression, and 61% of these cases were p16-overexpressing according to pathological evaluation. The cell cycle protein p16 demonstrated significant differences in expression between different histological subtypes of cervical cancer. Squamous epithelial tissue (Fig. 4A) had a median expression of IRS 6, while adenocarcinoma tissue (Fig. 4B) had a significantly lower expression in comparison, with an IRS of 4 (P<0.001; Fig. 4C). Notably, p16 demonstrated no significant correlation with E6 oncoprotein expression (data not shown).

Correlation analysis. A significant correlation was identified between MDM2 and gal-3 expression in cervical cancer tissue (R=0.181, P=0.005; data not shown). Cases of cervical cancer with low MDM2 expression (Fig. 5A) also demonstrated low gal-3 expression (Fig. 5B). Likewise, a case with high MDM2 expression (Fig. 5C) demonstrated high gal-3 expression (Fig. 5D). Cases of cervical cancer with low E6 oncoprotein expression (Fig. 5E) demonstrated enhanced staining of the mutated form of p53 in the same area of the tumour (Fig. 5F). However, cases with high expression of E6 (Fig. 5G) revealed low expression of mutated p53 (Fig. 5H). The statistical evaluation confirmed these results of serial section staining (R=-0.140, P=0.028; Table IV). A significant correlation was also identified between the expression of MDM2 and mutated p53 in cervical cancer tissue (R=0.144, P=0.025; Table IV). The correlation analyses and clinical parameters are summarised in Table IV.

Gal-3 is a negative prognosticator in p16-negative patients with cervical cancer. In patients with cervical cancer with no or very low p16 expression, gal-3 expression was correlated with a

Table IV. Correlation analyses of clinical parameters and immunohistochemical staining parameters.

Clinical parameter	Statistic	Age	Histology	pT	pN	pM	Grade	FIGO	E6	p53-mutated	MDM2	Galectin-3
Age (years)	Correlation coefficient	-.019	.004	-.115	-.065	-.140*	.354[b]	.133[a]	.169[b]	.107	.026	.050
	Sig. (2-tailed)	.766	.945	.070	.307	.028	.000	.037	.008	.095	.686	.438
	N	250	250	250	250	246	250	245	244	246	241	240
Histology	Correlation coefficient	.028	1.000	.000	-.073	-.108	-.084	.045	.156[a]	-.162[a]	.075	.029
	Sig. (2-tailed)	.664	.	.994	.249	.089	.185	.477	.015	.010	.242	.652
	N	245	249	249	249	249	249	249	245	249	244	243
pT	Correlation coefficient	.276[b]	.000	1.000	.359[b]	-.202[b]	.182[b]	.380[b]	.174[b]	.055	-.019	-.039
	Sig. (2-tailed)	.000	.994	.	.000	.001	.004	.000	.006	.384	.762	.540
	N	246	249	250	250	250	250	250	246	250	245	244
pN	Correlation coefficient	.037	-.073	.359[b]	1.000	-.172[b]	.212[b]	.240[b]	.033	-.050	-.058	-.033
	Sig. (2-tailed)	.559	.249	.000	.	.007	.001	.000	.610	.435	.370	.608
	N	246	249	250	250	250	250	250	246	250	245	244
pM	Correlation coefficient	-.081	-.108	-.202[b]	-.172[b]	1.000	-.146*	-.150[a]	-.037	.004	.032	-.074
	Sig. (2-tailed)	.206	.089	.001	.007	.	.021	.018	.560	.951	.619	.252
	N	246	249	250	250	250	250	250	246	250	245	244
Grade	Correlation coefficient	-.081	-.084	.182[b]	.212[b]	-.146[a]	1.000	.093	.084	-.065	-.175[b]	-.047
	Sig. (2-tailed)	.203	.185	.004	.001	.021	.	.142	.191	.302	.006	.461
	N	246	249	250	250	250	250	250	246	250	245	244
FIGO	Correlation coefficient	.076	.045	.380[b]	.240[b]	-.150[a]	.093	1.000	.227[b]	-.099	.021	.067
	Sig. (2-tailed)	.233	.477	.000	.000	.018	.142	.	.000	.120	.748	.296
	N	246	249	250	250	250	250	250	246	250	245	244
E6	Correlation coefficient	.088	.156[a]	.174[b]	.033	-.037	.084	.227[b]	1.000	.093	.001	.009
	Sig. (2-tailed)	.173	.015	.006	.610	.560	.191	.000	.	.147	.986	.893
	N	242	245	246	246	246	246	246	246	246	244	243
p53-mutated	Correlation coefficient	.092	-.290[b]	.016	-.019	.004	-.115	-.065	-.140[a]	1.000	.133[a]	.169[b]
	Sig. (2-tailed)	.148	.000	.796	.766	.945	.070	.307	.028	.	.037	.008
	N	246	249	250	250	250	250	250	246	250	245	244
MDM2	Correlation coefficient	.026	.075	-.019	-.058	.032	-.175[b]	.021	.001	-.042	1.000	.181[b]
	Sig. (2-tailed)	.686	.242	.762	.370	.619	.006	.748	.986	.511	.	.005
	N	241	244	245	245	245	245	245	244	245	245	243
Galectin-3	Correlation coefficient	.050	.029	-.039	-.033	-.074	-.047	.067	.009	-.020	.181[b]	1.000
	Sig. (2-tailed)	.438	.652	.540	.608	.252	.461	.296	.893	.760	.005	.
	N	240	243	244	244	244	244	244	243	244	243	244

[a]P<0.05; [b]P<0.01. pT, pathological tumour stage; pN, pathological node stage; pM, pathological metastasis stage; FIGO, International Federation of Gynecology and Obstetrics stage; MDM2, MDM2 proto-oncogene.

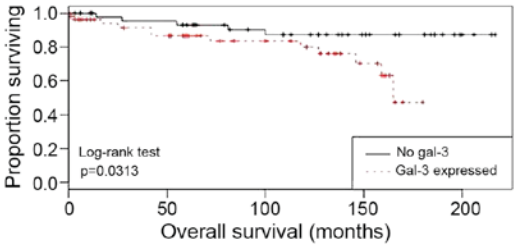

Figure 6. Kaplan-Meier overall survival analyses for patients with cervical cancer with gal-3 expression (red) compared with those with no gal-3 expression (black). Gal-3, galectin-3.

poor prognosis in overall survival analyses (P=0.0313; Fig. 6). Multivariate Cox regression analysis was performed to test which histopathological variables were independent prognosticators for survival rate in the tested breast cancer collective. It was demonstrated that the histological subtype (P=0.02), tumour size (P=0.011) and pN (P=0.045) were independent prognosticators for overall survival (Table V). No significant effect was demonstrated for the other histopathological variables.

Table V. Cox regression of overall survival on cervical cancer variables.

Parameters	P-value	Hazard ratio	95.0% CI	
			Lower	Upper
Age (years)	0.071	1.029	0.997	1.062
Histology	0.002	3.576	1.586	8.063
pT	0.011	1.270	1.057	1.525
pN	0.045	2.113	1.016	4.395
pM	0.702	1.305	0.335	5.085
Tumour grade	0.065	1.717	0.968	3.048
FIGO	0.875	0.994	0.926	1.068
E6CytIRS	0.475	0.961	0.863	1.071
p16CytIRS	0.696	1.026	0.903	1.165
p53IRS	0.267	0.892	0.729	1.092
p53mutIRS	0.975	0.996	0.765	1.296
MDM2IRS	0.460	1.050	0.922	1.196
Gal-3 IRS score	0.452	0.937	0.792	1.109

CI, confidence interval; pT, pathological tumour stage; pN, pathological node stage; pM, pathological metastasis stage; FIGO, International Federation of Gynecology and Obstetrics stage; IRS, immunoreactive score; Cyt, cytoplasmic; mut, mutated; MDM2, MDM2 proto-oncogene; Gal-3, galectin-3.

Discussion

Within the present study, immunohistochemical evaluation of E6 oncoprotein expression was conducted. In addition, E6 expression levels were demonstrated to be associated with the histological subtype. The expression of wild-type p53 and a mutated form of p53 were identified in the cervical cancer specimens tested. Finally, correlation analyses revealed a combined positive expression pattern for galectin-3 and MDM2, and a negative correlation between E6 and mutated p53 expression.

Although the early era of HPV research identified that ≤99.5% of cervical cancer cases are HPV-associated (26), it remains controversial in the literature whether viral load and disease severity are positively correlated (12). Therefore, the present study investigated a number of markers that are associated with HPV-driven changes in cell cycle proteins. Using these markers permitted a comparative analysis of the influence of HPV on the progression of cervical cancer for >10 years following surgery.

The replication of the viral genes E6 and E7 results in the cellular expression of E6 and E7 oncoproteins, which interfere with the cell cycle (6). E6 oncoprotein binds to E6-AP, forming a complex that selectively binds to p53 and leads to its ubiquitin-dependent proteolytic degradation (7). The present study demonstrated that E6 immunohistochemistry was a fast and simple method for the detection of the HPV-associated oncoprotein E6 in cervical cancer tissues. As a routine practice, E6 and E7 are detected using either *in situ* hybridisation (11) or polymerase chain reaction (27) methodology due to the non-specific immunohistochemical staining results of antibodies used in former studies (28).

In the present study a well-tested antibody, and specific antigen retrieval and staining protocol was used, resulting in the establishment of a useful immunohistochemical evaluation protocol for the detection of the HPV E6 oncoprotein. The optimal results were obtained with the E6 antibody supplied by Abcam (Cambridge, UK). The advantage of immunohistochemical evaluation is that it is easier to apply and less expensive compared with mRNA *in situ* hybridisation. mRNA *in situ* hybridisation may be the optimal way to detect HPV; however, this method is more complicated for routine detection compared with immunohistochemistry (26). Evaluation of E6 immunohistochemical staining in cervical cancer tissue has previously revealed positive correlations with advanced T staging and FIGO classification (22). Although specific studies have indicated correlations between E6/E7 gene expression and the clinicopathological parameters of cervical cancer (26), such a correlation was not demonstrated in the present study.

An additional finding of the present study is the negative correlation between E6 and mutated p53 expression. Mutations of the gene encoding p53 (*TP53*) are the most frequent alterations in multiple human malignancies (29-31). In total, >50% of human tumours contain a mutation/deletion of *TP53*, ranging from 5-80% depending on the type, stage and etiology of the tumours (32). A number of previous studies have investigated a potential genetic link between these variations and cancer susceptibility, but the results have been controversial. A previous meta-analysis study from 49 pooled studies failed to demonstrate a link between a common *TP53* mutation (25) and cervical cancer susceptibility (33). Later on, the same mutation was revealed to be associated with higher pancreatic cancer risk among males; however, results also indicated that it may protect Arab women against the development of breast cancer (34,35). Multiple other mutations of *TP53* have since been described. Mutations that deactivate p53 in cancer are primarily located in the central DNA binding domain. These mutations typically ablate the ability of the protein to bind to

its target DNA sequences, prevent the transcriptional activation of p53 target genes. In total, ~80% of the most common p53 mutants demonstrate the capacity to exert dominant-negative effects over wild-type p53 and thus prevent the activation of transcription. In contrast, only 45% of the less frequent mutants studied have this capacity (36).

The mutation detected by the antibody used in the present study is an mutation at position 20 (serine to aspartic acid), which abolishes the phosphorylation site on p53. The phosphorylation of this serine when DNA damage is detected weakens the interaction between p53 and MDM2, thereby stabilising p53 (37-39). Thus, this mutation maintains increased protein levels of p53 following DNA damage. The analysis of the immunohistochemical detection of mutated p53 revealed that cervical cancer specimens derived from squamous epithelial tissue demonstrated significantly higher expression levels compared with adenocarcinoma tissue. In addition, a positive correlation between the expression of mutated p53 and MDM2, and a negative correlation between the expression of mutated p53 and E6, were identified. Therefore, it is possible to speculate that E6 also degrades the mutated form of p53. In a previously published study, this mutation was demonstrated to be associated with the improved survival of patients with cervical cancer (25).

Finally, a positive correlation between MDM2 and gal-3 expression was demonstrated in cervical cancer tissue. Little information concerning the involvement of galectins in cervical cancer exists at present. Research has primarily focused on gal-1 (40,41), gal-7 (42,43) and gal-9 (44). A previous publication described the influence of gal-3 on vascular endothelial growth factor C expression and its influence on the enhancement of cervical cancer cell invasiveness (5). The present study demonstrated that gal-3 was a negative independent prognosticator for the overall survival of patients with p16-negative cervical cancer. In this group of patients, gal-3 may be responsible for the aggressiveness of cervical cancer, whereas in p16-positive carcinomas different factors/signal transduction pathways may be responsible.

In the present study, a total of 250 cervical cancer cases were systematically analysed for the expression and interaction of E6, p53, p16, MDM2 and gal-3 in FFPE tumour tissue. Significantly increased levels of E6 staining were correlated with an advanced T stage and FIGO classification. Furthermore, MDM2 and gal-3 expression levels were positively correlated in cervical cancer. In addition, gal-3 expression levels were negatively correlated with prognosis in p16-negative cases. As gal-3 is overexpressed in the cervical cancer tissue of patients with a worse prognosis, the investigation of gal-3 inhibiting compounds is an additional task for the development of alternative treatments for this tumour type. In addition, a negative correlation between E6 and a mutated form of p53 in cervical cancer was identified. In conclusion, the results of the present study indicate that immunohistochemical staining may be a useful method for the detection the HPV E6 oncoprotein.

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