Prognostic significance of Cyclin A in epidermoid anal cancer

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Abstract. Ultimately aiming at a more individualized therapeutic approach in epidermoid anal cancer, this study explored the prognostic and predictive impact of a set of tumour markers. From a population-based cohort of 276 patients with epidermoid anal cancer, treated according to prospective protocols, 215 pre-treatment biopsies were investigated using immunohistochemistry. The expression of p53, p21, Cyclin A and CD31 was measured semi-quantitatively. The expression rate was classified as high when immunostaining was seen in >5% of the tumour cells for p53 and p21, >20% in Cyclin A and, above median vessel count for CD31. Marker expression was correlated to survival and treatment response. A high Cyclin A expression correlated significantly with improved overall (77% vs 59%, p=0.005) and tumour-specific (81% vs 64 %, p=0.009) survival at 5 years. Also, the locoregional failure rate was significantly lower in patients with a high Cyclin A expression (12% vs 24%, p<0.05). In a multivariate Cox analysis Cyclin A was an independent prognostic factor. A low p21 expression correlated with a reduced rate of locoregional failure (14% vs 27%, p<0.05) but no impact on survival was found. For p53 and CD31 no significant correlations were obtained. Cyclin A may be an indicator of radiosensitivity and a valuable prognostic marker in epidermoid anal cancer.

Introduction

In the search for prognostic and predictive factors in epidermoid anal cancer, several markers have been investigated (1). Although prognosis after combined modality therapy is generally good, the access of reliable prognostic markers would facilitate individual treatment planning to achieve optimal results while minimizing treatment-related morbidity. For example, if the patients who ultimately require radical surgery, either because of primary or secondary failure after (chemo)radiotherapy, would be identified at an early stage, surgery could be an integrated part of the primary treatment after a lower pre-operative radiation dose, thus reducing the risk of wound healing problems following surgery after a curative radiation dose (2).

As the primary therapeutic option in epidermoid anal cancer is (chemo)radiation, prognosis is largely dependent on the sensitivity of the tumour *vis-á-vis* radiotherapy and chemotherapy. The sensitivity of a tumour may be related to the different properties of tumour cells, such as cell cycle control, apoptosis, DNA damage repair, and angiogenesis. As a means of studing these properties, immunohistochemical analyses of markers reflecting intracellular events can be used (3).

The tumour suppressor gene p53 plays an important role in cell cycle inhibition and initiation of apoptosis but has also been linked to DNA damage repair and angiogenesis (4). p21 is a down-stream effector of p53 and inhibits cell cycle progression, generally at the G_1 - S transition. However, there is also evidence that p21 expression can be induced by the p53 independent pathways (5). Cyclins are a group of proteins also related to proliferation. Cyclins interact with cyclin-dependent kinases (CDK) to sequentially regulate the cell cycle via different cell cycle transition points (6). Cyclin A is a member of the cyclin family which may be of particular interest as it is involved in two steps in the cell cycle, both in the entry of G_1 cells into S phase and in the G_2-M transition (7). Angiogenesis, often expressed as micro-vessel density (MVD), can be assessed using different markers. CD31, a platelet endothelial adhesion molecule, is a marker of angiogenesis in different tumours, including squamous cell carcinomas (8).

The aims of the present study were to explore the possible prognostic and predictive importance of p53, p21, Cyclin A and CD31 in a large, population-based cohort of patients with epidermoid anal cancer treated according to prospective protocols.

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Table I. Pre-treatment data on all the patients treated with curative intent between 1985-2000 and on the investigated cohort.

	All the patients treated with curative intent	%	Investigated cohort	%
n	276		215	78
Age (years) median (range)	69 (28-92)		69 (28-92)	
Gender				
Female	218	79	168	78
Male	58	21	47	22
Tumour location				
Anal margin	40	14	29	13
Anal canal	236	86	186	87
Histology				
Squamous	200	72	154	72
Basaloid	76	28	61	28
T-classification (UICC)				
T 1-2	190	69	145	67
T 3-4	86	31	70	33
N-classification (UICC)				
N-	211	76	158	73
N+	65	24	57	27
Treatment				
RT±bleomycin	150	54	108	50
RT±bleomycin+APR	16	6	13	6
Neoadjuvant CRT	74	27	64	30
Neoadjuvant CRT+APR	17	6	16	7
Primary APR	6	2	4	2
Local excision	7	3	7	3
Outside guidelines	6	2	3	1

UICC, International Union Against Cancer; RT, radiotherapy; APR, abdominoperineal resection; CRT, chemoradiotherapy.

Patients and methods

Patients and treatment. During the years 1985 to 2000, 308 patients were diagnosed with an invasive epidermoid anal carcinoma in the Stockholm Health Care Region (population 1.9 million). Details on this cohort of patients have been reported (9). Treatment with curative intent was delivered to 276 (90%) patients according to prospective protocols. External beam radiotherapy (RT) alone or with concomitant bleomycin was used in the early part of the series. Starting in 1989, neoadjuvant platinum-based chemotherapy was given to patients with locally advanced tumours (T≥4 cm or N^+) while less advanced cases received RT alone. During the entire period RT was delivered in 1.8-2 Gy fractions in a split course to a radical dose at 60-64 Gy. Abdominoperineal resection was reserved for poor responders after the first course to 40-46 Gy, or for recurrences after full-dose (chemo)radiotherapy. In this study, the rate of poor response to (chemo)radiotherapy was estimated by adding the number of patients who were assessed as having a poor response and underwent surgery after the first course of RT to the number of patients with residual disease after full-dose RT, thereby including all patients that, hypothetically, were candidates for abdominoperineal resection after a pre-operative dose of 40-46 Gy as part of the primary therapy. Pre-treatment data and details on therapeutic approach are presented in Table I. The study was approved by the Regional Ethics Committee.

Immunohistochemistry. Pre-treatment diagnostic biopsies from 230 (83%) of the 276 patients were available for study. Fifteen of the formalin-fixed, paraffin wax-embedded blocks were of inadequate quality and, hence, were not used. From the remaining 215 blocks, 4 μm sections were cut and immunohistochemical staining was performed applying a standard avidin-biotin complex (ABC) technique using the following antibodies: p53 (DO-1, Santa Cruz Biotechnology Inc., Santa Cruz, USA, 1:100), $p21^{WAF1}$ (Oncogene Research Products, Boston, USA, 1:50), Cyclin A (Novocastra Laboratories, Newcastle, UK, 1:100) and CD31 (Dako, Glostrup, Denmark 1:100). In all cases microwave heat-induced antigen retrieval was used and the primary antibodies were incubated for 30 min at room temperature. This was followed by incubation with the avidin-biotin-peroxidase complex for an additional 30 min. The peroxidase

Table II. Staining patterns and the relation to clinicopathological data.

	p 53		p 21		Cyclin A		CD31	
	high	low	high	low	high	low	high	low
n	108	106	148	67	109	106	105	104
Age (years) median (range)	68 (28-92)	70 (30-90)	68 (28-92)	73 (30-90)	70 (34-92)	69 (28-90)	68 (28-92)	69 (30-90)
Gender (%)								
Female	79	76	80	75	83	73	77	78
Male	21	23	20	25	17	27	23	22
Histology (%)								
Squamous	73	70	74	67	66	77	74	70
Basaloid	27	30	26	33	34	23	26	30
Location (%)								
Anal canal	89	84	89	81	89	84	82	91
Anal margin	11	16	11	19	11	16	18	9
T-stage (%)								
1-2	70	64	66	70	69	66	76	58
3-4	30	36	34	30	31	34	24	42
N-stage (%)								
Negative	74	73	75	70	73	74	72	75
Positive	26	27	25	30	27	26	28	25
Locally advanced (%)								
No	41	43	43	42	46	39	45	38
Yes	59	57	57	58	54	61	55	62

p53 and p21: high, >5% positive tumour cells; Cyclin A: high, >20% positive tumour cells; CD31: high, vessel count above median; locally advanced, T >4 cm or N^+.

reaction was visualized using diamino-benzidine tetrahydrochloride. Finally, counterstaining in Mayer's hematoxylin was performed prior to mounting with a xylene-soluble mounting medium. As negative controls, the primary antibodies were replaced by bovine serum albumin.

Sections from all the blocks were evaluated by a senior pathologist (C.R.) to confirm diagnosis. Assessment of immunohistochemical staining was performed jointly by two investigators (C.L. and P.J.N.) using a double objective microscope. For p53, p21 and Cyclin A, semiquantitative analysis based on the percentage of positive cells was used. Based on microscopic evaluation, expression of the markers was classified as high or low. In agreement with earlier reports (10-13) both p53 and p21 protein expressions were classified as high when >5% of the tumour cells showed positive staining. For Cyclin A, where no previous reports on epidermoid anal cancer are available, staining of >20% of the tumour cells was considered as high, based on the findings in squamous cell laryngeal cancer (14). When analysing CD31, the entire section was viewed in low power to identify 'hot spots'. Then, in a high-power field (x400), a graticule was used and all stained vessels were counted (8). In CD31 the median vessel count was used as a cut-off between high and low expression.

Statistical analyses. For comparison between proportions the Chi-square test and Mann-Whitney U test were used where appropriate. For assessment of the correlations between different markers, Fisher's exact test (two-tailed) was used. Survival curves were plotted using the Kaplan-Meier method and the comparison of survival between groups was by the log-rank test. For the graphical presentation, the curves were truncated at 120 months. P-values <0.05 were considered statistically significant. Cox proportional hazards model was used to perform uni- and multivariate analyses on the parameters related to survival. In the Cox analyses, patients treated with primary surgery (n=11) or outside guidelines (n=3) were excluded. T-stage, N-stage, treatment and Cyclin A expression were included in the Cox analyses.

Results

A total of 214, 215, 215 and 209 slides were available for the evaluation of p53, p21, Cyclin A and CD31, respectively. Distribution of staining patterns and the relation to clinicopathological data are presented in Table II. High expression was found in 50% for p53, 69% for p21, 51% for Cyclin A and 50% for CD31 (Fig. 1). Besides a decreased CD31 expression in T3-4 tumours, no significant correlations

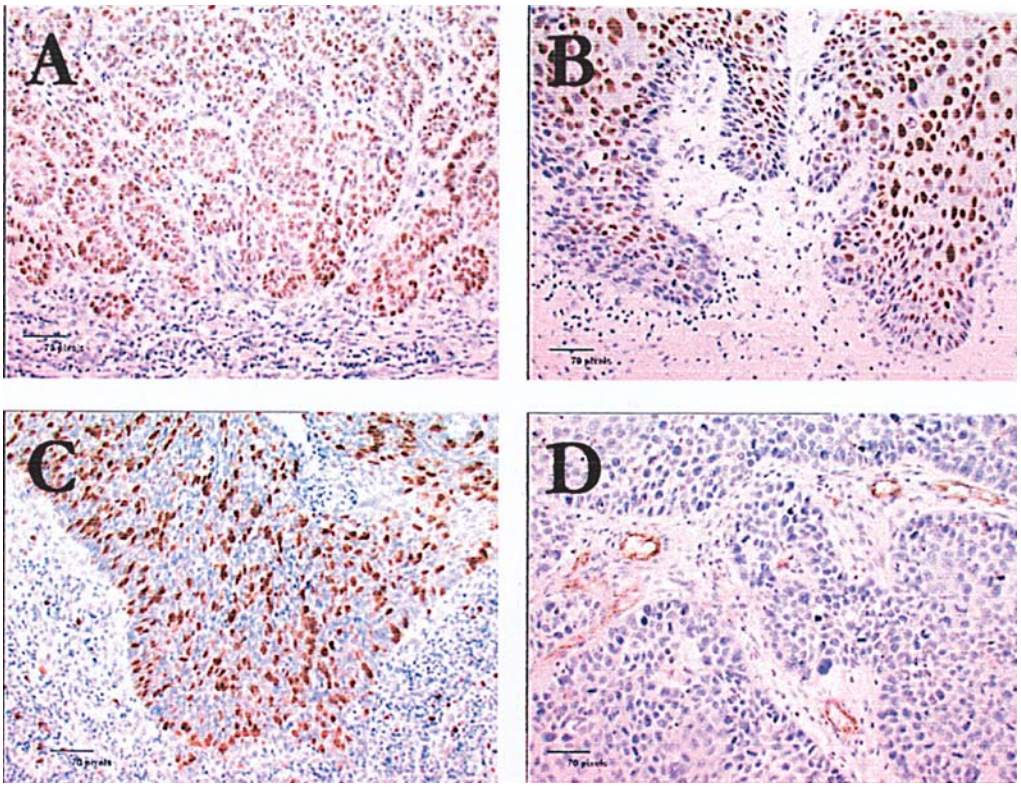


Figure 1. Invasive epidermoid anal carcinoma. Immunohistochemical staining showing tumours with a high expression of p53 (A), p21 (B), Cyclin A (C) and CD31 (D). Original magnification x20.

between immunostaining and the clinicopathological data were observed. No statistically significant differences between treatment modalities used and marker expression were found. Significant associations were found between Cyclin A expression and both p21 ($p<0.01$) and CD31 ($p<0.01$). Furthermore, a highly statistically significant correlation ($p<0.001$) was found between p53 and p21 expression.

Tumours with a high Cyclin A expression showed a statistically significant better overall (77% vs 59%, $p=0.005$) and tumour-specific (81% vs 64%, $p=0.009$) 5-year survival rate compared to patients with a low Cyclin A expression (Fig. 2A and B). Also, in patients with tumours high in Cyclin A, the rate of isolated locoregional failures was significantly lower than in those with a low expression (12% vs 24%, $p<0.05$) and with high Cyclin A there was a trend towards a decreased failure rate after an intitial complete response (14% vs 25%, $p=0.06$).

Reduced expression of p21 showed a trend, although not statistically significant, towards inferior 5-year survival (62% vs 71%, $p=0.08$) (Fig. 3). However, the rate of isolated locoregional failures was significantly increased among patients with p21-negative tumours (27% vs 14%, $p<0.05$) compared to those with a high p21 expression.

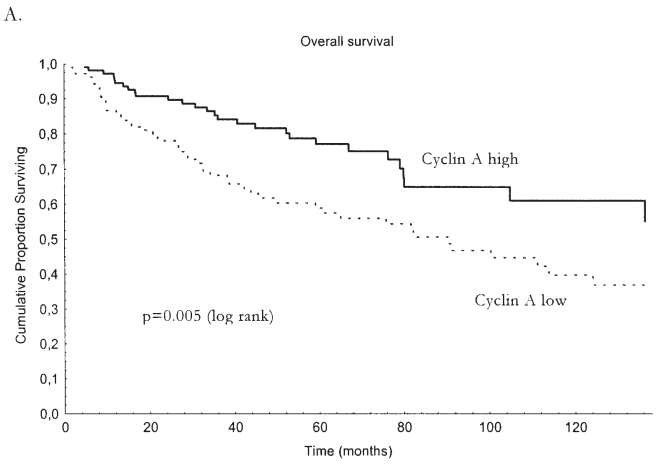
Analysis of p53 and CD31 revealed no significant differences in survival between tumours with high and low expression (data not illustrated). In addition, these two markers showed no significant impact on other outcome variables such as rate of complete response, failure after initial complete response, isolated locoregional failures and rate of distant metastasis.

Uni- and multivariate Cox analyses on the overall survival and tumour-specific survival revealed that a high Cyclin A expression was an independent prognostic factor associated with a reduction of the relative hazard to 0.54 (0.34-0.87, 95% confidence interval) of not surviving (Table III).

In both the entire cohort of 276 patients treated with curative intent and among the 215 patients analysed for marker expression the rate of poor response to (chemo)radiotherapy was 22%. Although no statistically significant differences could be found, some trends were noted. In the patients whose tumours had a high Cyclin A expression, the risk of poor response tended to be decreased compared to those with a low Cyclin A expression (18% vs 27%, $p=0.17$). In the patients with tumours with a low p21 expression the rate of poor response tended to be increased (30% vs 19%, $p=0.09$). Also, among the patients with tumours overexpressing p53 the poor response rate was 19% compared to 10% among those with a low p53 expression ($p=0.09$). Irrespective of marker expression, patients with T3-4 tumours had a much higher rate of poor response compared to patients with T1-2 tumours (38% vs 14%, $p<0.001$).

Discussion

The results of this study indicate that Cyclin A may be a significant prognostic marker for epidermoid anal cancer. Since the proliferation marker Cyclin A has not previously been investigated in conjunction with anal cancer, we used data from immunohistochemistry in another type of



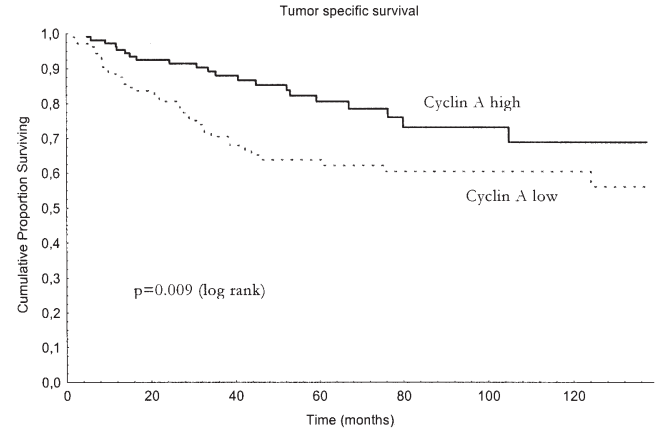


Figure 2. Survival among patients with high or low Cyclin A expression (n=215).

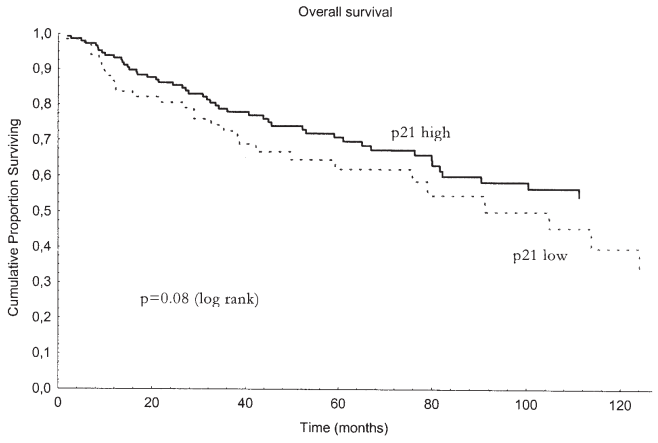


Figure 3. Overall survival among patients with high or low p21 expression (n=215).

squamous cell carcinoma to define high expression (14). The main novel finding is that a high Cyclin A expression was associated with a statistically significantly increased overall and tumour-specific survival. Furthermore, this study, being much larger than any previous study, could not confirm results of earlier reports on prognostic impact of p21 and p53 in epidermoid anal cancer (10,12,15). The marker expression rate used in this study to discriminate between high and low p53 and p21 expression was the same as previously used.

A few reports on the proliferation markers other than Cyclin A, in epidermoid anal cancer, have been published (13,16-18). A high proliferation index, measured with Ki-67, was significantly associated with better colostomy-free survival in 29 patients reported by Grabenbauer *et al* (16). In another study on Ki-67, including 55 patients, a high expression showed no statistically significant differences in overall survival, locoregional control or disease-free survival although the outcome tended to be superior regarding the two latter endpoints (77% vs 69% and 73% vs 66%, respectively) (17). These observations from small series suggest that the proliferation marker Ki-67 may be a positive prognostic marker in epidermoid anal cancer. In contrast, however, in a series of 98 anal carcinomas, Allal *et al* found no prognostic

Table III. Cox proportional hazards analyses on overall and tumour specific survival.

	Univariate		Multivariate	
	RH	95% CI	RH	95% CI
Overall survival				
T3-4/T1-2	2.03	1.26-3.28	2.34	1.37-3.98
N^+/N^-	1.43	0.88-2.32	1.55	0.89-2.70
Neoadj. CRT/RT±bleomycin	1.09	0.68-1.75	0.57	0.32-1.01
Cyclin A high/low	0.55	0.34-0.88	0.54	0.34-0.87
Tumour specific survival				
T3-4/T1-2	2.07	1.17-3.65	2.37	1.26-4.45
N^+/N^-	1.44	0.83-2.53	1.61	0.84-3.07
Neoadj. CRT/RT+/-bleomycin	1.07	0.62-1.84	0.54	0.28-1.05
Cyclin A high/low	0.52	0.30-0.90	0.51	0.29-0.88

RH, relative hazard; CRT, chemoradiotherapy; RT, radiotherapy.

value of Cyclin D1 or Cyclin E, both reflecting proliferation rates (13).

Earlier studies on Cyclin A as a prognostic marker in squamous cell carcinomas, other than anal cancer, have shown either a lack of impact, or indications that an increased expression may be an indicator of poor prognosis (14,19). In contrast to the present study, the results of these earlier studies were based on patients who received primary surgical therapy and RT was used only post-operatively. However, in squamous cell head and neck carcinomas treated with primary RT, several studies have shown Ki-67, another marker of proliferation, to be a positive prognostic marker (20-23). The results of the present study, where Cyclin A was used as a marker of proliferation and where RT was the primary therapeutic modality, are in agreement with those reports. Also, it is well known that hypoxia is associated with decreased radiosensitivity. Immunohistochemical assays, using a double staining technique, have shown an inverse association between proliferation and hypoxia in, for example, cervical and bladder carcinoma (24,25). These findings corroborate well with the results of this study and the studies on head and neck cancers where tumours with a high proliferation have a superior prognosis when RT is the primary therapeutic modality.

Previously, two reports have indicated the prognostic value of p53 in epidermoid anal cancer (10,12). The present report could not confirm these findings. In a study on 49 anal cancer patients by Wong *et al*, a significant association was found only when p53 was used as a continuous variable for disease-free survival (10). Allal *et al* investigated a cohort of 98 anal cancer patients and found p53 expression to be significantly associated with disease-free survival in a multivariate analysis (12). Our results are in agreement with several other reports on p53 in epidermoid anal cancer, showing no statistical correlation between p53 and prognosis (1). Tumour suppressor genes, such as p53, are believed to be both radiation sensitivity and chemosensitivity determinant factors (26-28), which may have influenced the differences in results, as patients in the studies by Wong *et al* (10) and Allal *et al* (12) were mainly treated with concomitant 5-fluorouracil and mitomycin C and radiotherapy whereas the patients in this study received either no chemotherapy, bleomycin, or neoadjuvant platinum-based chemotherapy with radiotherapy.

Holm *et al* reported on the reduced expression of p21 being a marker of poor prognosis in epidermoid anal cancer (15). In another study on a patient series of similar size these results could not be confirmed (13). The results on p21 of our study are in accord with those of Allal *et al* (13) and fail to show the prognostic impact of p21 in anal epidermoid cancer.

Abdominoperineal resection in patients having received high and curative doses of RT is associated with high complication rates (2,29). Prediction of poor response, i.e. a response not completely eradicating all tumour cells, to (chemo)radiotherapy on an individual level would make it possible to offer surgery to these patients after only a pre-operative (chemo)radiotherapy course, thus reducing the risk of post-operative complications. When the markers included in this study were assessed with respect to the prediction of poor response, no significant predictive potential could, however, be obtained. The clinical parameter T-stage remained the strongest risk factor for need of surgery. Future search for predictors of poor response to (chemo)radiotherapy may include more sophisticated techniques such as DNA sequencing, measurements of RNA expression and proteomic methods (3).

In conclusion, this study indicates that Cyclin A may be a valuable prognostic marker in epidermoid anal cancer. Future studies to confirm the results on Cyclin A as a prognostic marker in epidermoid anal cancer and to search for predictors of poor response to (chemo)radiotherapy are needed.

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