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Serum antibody responses against human papillomavirus in relation to tumor characteristics, response to treatment, and survival in carcinoma of the uterine cervix

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Abstract To investigate whether the serum antibody responses to human papillomavirus (HPV) in cervical carcinoma were related to the clinical and histopathological features of the tumors and how the antibody responses were affected by treatment, pretreatment serum samples from 66 patients with carcinoma of the cervix were studied for the presence of IgA or IgG responses against six defined HPV epitopes. Posttreatment serum samples were drawn from the same patients 2–24 months after initiation of treatment. There was no significant correlation between pretreatment level of any of the investigated antibodies and clinical stage or differentiation of tumor. For the IgA responses to the epitopes 245:16 and 245:18 in the E2 protein there was a significant correlation between an increased pretreatment antibody level and a shortened survival. A high pretreatment value of IgA against 245:16 was also associated with the absence of any complete response after therapy. The antibody levels declined dramatically after therapy for most of the antigens studied. However, this decline was seen both among the 53 patients with complete remission and among the 13 patients with remaining or progressive disease. Thus, the investigated serological responses were not useful as tumor markers, since patients with progressive, late-stage disease may fail to mount an antibody response to these proteins. However, pretreatment levels of the serological responses to the HPV epitopes 245:16 and 245:18 were associated with prognosis in cervical cancer.

Key words Cervical cancer · Human papillomavirus · Serology · Tumor markers · Prognostic markers

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Introduction

The oncogenic types of human papillomaviruses (HPV), especially types 16 and 18, are established as major etiological factors for development of carcinoma of the uterine cervix, as demonstrated in numerous studies on the molecular biology and epidemiology of HPV [5, 10, 13]. An explanatory model for the transforming ability of HPV has been provided by the demonstration of the role of the early antigens E6 and E7 for inactivation of the tumor-suppressor proteins p53 and Rb respectively [4, 12]. Antibody responses evoked by HPV have also been studied epidemiologically in relation to the risk of cervical cancer. Serological responses to various proteins and defined epitopes of HPV 16 and 18 have been associated with an up to tenfold relative risk of cervical cancer [3, 6–8, 9].

E6 and E7 antibody responses have been observed in connection with invasive cervical carcinoma but not in relation to cervical intraepithelial neoplasia, suggesting that these responses are mounted when invasiveness has been established and may be dependent on tumor load [9] or, alternatively, that the E6 and E7 responses reflect a pathogenic mechanism involved in the progression of disease into invasive carcinoma [4, 12]. We have previously found non-significant tendencies of association between the serological response to some antigens, particularly E7, and tumor stage [3]. If these antibody responses were dependent on clinical stage, they could be useful as tumor markers. On the other hand, if they were not dependent on tumor load, it

would seem more plausible that the observed associations between antibody responses and cervical cancer were reflections of the oncogenic infection and, thus, conveying an etiological or pathogenic significance.

These questions are of relevance for several reasons. Apart from the obvious interest from a strictly scientific, tumor-biological standpoint, one reason is the possibility of utilizing HPV antibodies as tumor markers in clinical practice. Another reason is that serological methodology may be potentially useful for screening purposes, and for cancer prediction and intervention.

In order to elucidate these issues further we studied the connection between antibody responses to a set of HPV epitopes in relation to some important clinical features in patients with cervical carcinoma, including stage at diagnosis, tumor differentiation, response to treatment and prognosis.

Materials and methods

Patients

All patients were referred to and treated at the Department of Gynecological Oncology at the University Hospital in Umeå, Sweden, during 1984–1991. The mean age of the patients was 53 years (range 20–82 years). All biopsy specimens were reexamined at the Department of Pathology at the same hospital. Of the 66 cases, 52 patients had cervical squamous cell carcinoma and 11 patients had cervical adenocarcinoma. Two patients had adenosquamous carcinoma, and in 1 case the tumor was anaplastic, not permitting further classification. Four patients had well-differentiated, 42 moderately, 18 poorly differentiated tumors, and 2 biopsy specimens could not be graded histologically.

Staging was performed according to the International Federation of Gynecology and Obstetrics (FIGO). Treatment was given according to the following general principles. After preoperative brachytherapy, patients with stages I and II underwent radical hysterectomy, followed by postoperative pelvic irradiation usually to 45 Gy in selected cases. Patients with stage III disease received external irradiation, in some cases followed by brachytherapy. For patients with stage IV disease external irradiation alone or in combination with chemotherapy was usually given. After treatment, 55 patients attained complete remission, 5 partial remission, 2 had stationary disease and 4 progressed during therapy. Of those with primary complete remission, 2 patients had a recurrence of the disease before the posttreatment serum sample was drawn. For stage distribution at the time of pre- and posttreatment sampling see Table 1. The range of follow-up time was 5–108 months. Of the 66 patients, 27 died during follow-up. The cause of death was cervical cancer in 22 cases and intercurrent disease in 5 patients.

Blood sampling

In addition to initial blood sampling at diagnosis, a second blood sample was drawn during the follow-up time in 66 of the 95 cases of cervical cancer that were treated during 1984–1991 [3]. The mean time between pre- and posttreatment samples was 7 months (range 2–24 months). All blood specimens were stored at − 80° C.

Table 1 Clinical stage of the patients at the time of pretreatment ($n = 66$) and posttreatment ($n = 13$) sampling

	No. patients with stage:					
Sample	IA	IB	IIA	IIB	III	IV
Pretreatment	4	35	14	5	7	1
Posttreatment	0	0	1	4	2	6

Serological techniques

Enzyme-linked immunosorbent (ELISA) assays were performed as previously described [3]. Briefly, synthetic peptides or purified bovine papillomavirus were coated onto microtiter plates (Costar, Cambridge, Mass.), which were subsequently blocked with 10% horse serum in phosphate-buffered saline. Sera were diluted in horse serum in phosphate-buffered saline containing 1 mM EDTA at 1:30, 1:50, or 1:100, depending on the antigen to be tested, and incubated on the plates for 23 h at 37° C. Bound antibodies were detected with a monoclonal antibody to human IgA or IgG (Eurodiagnostics, Aapeldorn, The Netherlands), followed by a horseradish-peroxidase-conjugated antibody to mouse IgG (Southern Biotechnology). Absorbances in the linear interval of the absorbance curve, i.e. in the 0.2–1.0 absorbance interval, were used to calculate single-dilution absorbance titers. Prior to calculation of absorbance titers, the absorbance of the same sample reacted with uncoated wells was subtracted.

The sequences of the HPV peptides selected for testing in this study are listed elsewhere [3]. These antigens have been extensively characterized and reviewed [2] previously. Briefly, antibody responses to the epitopes E7:5 (derived from HPV 16 E7), 245:16 (derived from HPV 16 E2), 245:18 (derived from HPV 18 E2) and L1:13 (derived from HPV 16 L1) are elevated among cervical cancer patients [2, 3]. The response to epitope L2:49 (derived from HPV 16 L2) is not associated with cervical cancer [3], but increased L2:49 and L1:13 responses are found among patients with asymptomatic HPV 16 infection [11]. The HPV 16 E7-derived epitope E7:2/Dillner [1] overlaps the previously studied E701/Muller peptide, which has a well-studied association with cervical cancer [3, 9], and has similar reactivity in ELISA.

Statistical methods

Statistical analyses were carried out, first after division of antibody levels for each protein into two categories with the median as cut-off point and, second, with three groups after division into quartiles with the two lowest quartiles taken together. Tumor differentiation was divided into highly or moderately differentiated tumors, and poorly differentiated lesions respectively. Clinical stage was divided into stages IA or IB, and stages IIA, IIB, IIIA, IIIB or IV respectively. Response to treatment was also divided into complete remission (no clinically detectable tumor on standard examinations) and remaining tumor after completed therapy, irrespective of stage.

Associations between pretreatment antibody levels and clinical features (pretreatment tumor differentiation, tumor stage and response to treatment) were studied in logistic regression models. Exact confidence levels for odds ratios were calculated using the permutational distribution of the sufficient statistics.

Prognosis in relation to pretreatment antibody levels was studied in Cox proportional-hazards models with antibody levels in serum as categorical covariates. As failure, death from cervical cancer was used. Deaths due to intercurrent diseases were treated as censored observations. Adjustment to age of patient at time for pretreatment sample was made by dividing the material into two groups with 50 years of age as cut-off point.

Results

Pretreatment antibody levels in relation to tumor characteristics

There was no statistically significant association between antibody level and clinical stage (Table 2) or histopathological differentiation (not shown) for any of the 12 serological responses examined with two groups of antibody levels. The results were essentially the same when dividing the serological levels into three categories (not shown). Nor did separate analysis of the 52 cases of squamous cell carcinoma reveal any significant association between antibody responses and the clinical characteristics (not shown).

Pretreatment antibody levels in relation to response to treatment

After therapy 55 patients attained a complete response, whereas disease remained in 11 cases. Analysis of pretreatment antibody level, categorized into two groups, showed no association with the clinical outcome, in terms of complete response or not, for any one of the antibodies examined, except for IgA against 245:16, for which a high pretreatment value was associated with remaining disease after therapy (odds ratio 5.67, 95% confidence interval 1.04–58.65, $P = 0.04$).

Table 2 Relative risk of advanced stage in cervical cancer in relation to antibody levels of pretreatment serum sample. Conditional logistic regression analysis of IgG and IgA antibody levels against a panel of 12 antigens among 66 cases of incident cervical cancer. The cut-off levels used to determine positivity were (in the order the antigens listed): 0.209, 0.103, 0.197, 0.160, 0.100, 0.100, 0.203, 0.135, 0.309, 0.209, 0.537 and 0.100 difference in absorbance. Response variable is stage, categorized as 0 and 1 for stage IA or IB, and IIA, IIB, III or IV, respectively. (*OR* odds ratio, *CI* confidence interval)

Antigen	$10^3 \times$ Mean value, ΔA (range)		OR	95% CI
	Low stage	High stage		
E7:5 IgG	281 (64–1160)	253 (88–858)	0.62	0.23–1.86
E7:5 IgA	197 (0–960)	124 (0–834)	0.54	0.17–1.60
245:16 IgG	229 (0–640)	246 (55–711)	0.88	0.30–2.63
245:16 IgA	200 (0–1020)	198 (0–766)	1.45	0.49–4.40
E7:2 IgG	97 (0–509)	178 (0–1110)	2.01	0.64–6.47
E7:2 IgA	42 (0–383)	32 (0–274)	0.85	0.12–4.87
L2:49 IgG	381 (0–1616)	427 (0–1541)	0.69	0.23–2.05
L2:49 IgA	230 (0–1490)	361 (0–1600)	0.69	0.23–2.05
245:18 IgG	338 (0–966)	357 (93–1110)	1.13	0.38–3.39
245:18 IgA	340 (0–2430)	261 (0–1084)	0.88	0.30–2.63
L1:13 IgG	507 (0–824)	537 (274–844)	0.88	0.30–2.63
L1:13 IgA	112 (0–796)	123 (0–687)	0.89	0.29–2.68

Pretreatment antibody levels in relation to survival

An increased pretreatment antibody level was found to be associated with a shortened survival for IgA responses against the proteins 245:16 and 245:18 (Table 3). When cases with squamous cell carcinoma were analyzed separately, the results did not significantly differ from those obtained for the whole material (not shown).

Antibody levels during follow-up

For most antibodies examined the antibody levels declined dramatically between samples 1 and 2 (Tables 4, 5; Fig. 1). However, contrary to expectation, the decline

Table 3 Survival of cervical cancer patients in relation to antibody levels of pretreatment serum sample. Cox regression analysis of IgG and IgA antibody levels against a panel of 12 antigens among 66 cases of incident cervical cancer. The cut-off levels used to determine positivity were (in the order the antigens listed): 0.209, 0.103, 0.197, 0.160, 0.100, 0.100, 0.203, 0.135, 0.309, 0.209, 0.537 and 0.100 difference in absorbance. Age adjustment via a dichotomous covariate with cut-off level equal to 50 years $Exp(\beta)$ relative risk of death from cervical cancer for positive versus negative individuals

Antigen	Individuals pos/neg	Deaths pos/neg	Exp(β)	95% CI
E7:5 IgG	34/32	12/10	2.15	0.88–5.29
E7:5 IgA	33/33	9/13	0.93	0.38–2.27
245:16 IgG	33/33	11/11	1.03	0.45–2.38
245:16 IgA	33/33	14/8	2.81	1.16–6.81
E7:2 IgG	23/43	11/11	1.83	0.78–4.28
E7:2 IgA	8/58	1/21	0.29	0.04–2.16
L2:49 IgG	33/33	10/12	0.70	0.30–1.63
L2:49 IgA	33/33	10/12	0.82	0.35–1.90
245:18 IgG	33/33	12/10	1.23	0.53–2.84
245:18 IgA	33/33	16/6	2.69	1.04–6.96
L1:13 IgG	33/33	14/8	1.80	0.75–4.30
L1:13 IgA	28/38	7/15	0.58	0.24–1.42

Table 4 Comparison of pre- and posttreatment sera of 53 patients who were in complete remission at the time of posttreatment sampling (number of seropositive patients and mean antibody levels measured as difference in absorbance at 405 nm × 1000)

Antibody	Pretreatment		Posttreatment		*P* (Wilcoxon)
	No.	$10^3 \times$ mean antibody level (ΔA)	No.	$10^3 \times$ mean antibody level (ΔA)	
E7:5 IgG	9	256	1	94	< 0.001
245:16 IgG	46	222	23	120	< 0.001
E7:2 IgG	30	192	12	55	< 0.001
L2:49 IgG	12	382	12	342	0.003
245:18 IgG	43	330	7	107	< 0.001
L1:13 IgG	44	506	8	169	< 0.001
245:16 IgA	42	197	23	110	< 0.001
E7:5 IgA	10	170	5	131	< 0.001
E7:2 IgA	20	44	14	27	0.737
L2:49 IgA	15	286	12	235	< 0.001
245:18 IgA	36	270	18	216	< 0.001
L1:13 IgA	7	112	7	105	0.001

Table 5 Comparison of pre- and posttreatment sera of 13 patients who had clinical disease at the time of posttreatment sampling

Antibody	Pretreatment		Posttreatment		P (Wilcoxon)
	No.	$10^3 \times$ mean antibody level (ΔA)	No.	$10^3 \times$ mean antibody level (ΔA)	
E7:5 IgG	3	326	0	145	< 0.001
245:16 IgG	11	295	4	91	< 0.001
E7:2 IgG	8	246	4	101	0.005
L2:49 IgG	4	473	4	359	0.016
245:18 IgG	12	410	3	127	< 0.001
L1:13 IgG	8	573	1	165	0.001
245:16 IgA	12	208	7	92	0.001
E7:5 IgA	2	153	0	87	0.133
E7:2 IgA	5	14	7	36	0.039
L2:49 IgA	4	274	3	221	0.100
245:18 IgA	11	460	4	340	0.048
L1:13 IgA	3	133	2	126	0.778

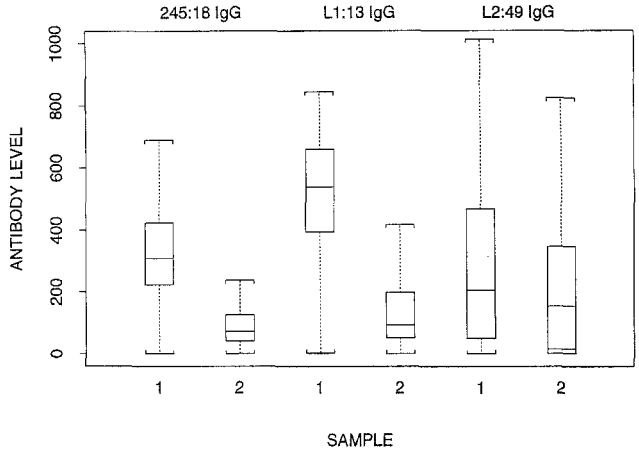

Fig. 1 Box plots exemplifying the difference in distribution of antibody levels between pre- and posttreatment samples (samples 1 and 2) for two seriological variables that showed a regular decline (245: 18 IgG and L1: 13 IgG) and one variable that only changed slightly (L2: 49 IgG). Antibody levels are measured as $\Delta A_{405} \times 10^3$

was of a similar magnitude for patients who achieved a durable complete response and for patients who had progressive or recurrent disease. Among patients with complete remission a decline of antibody levels was found that was statistically significant for all epitopes except for IgA to E7:2 (Table 4). This antibody was the only one showing an increase among patients without complete remission, all others were decreasing (Table 5). Cases with the most pronounced decline between sample 1 and sample 2 were those having the highest levels in sample 1. Thus, for most antibodies, a clear correlation was found between the difference between levels at sample 1 and sample 2, and the antibody level at sample 1 (not shown). In line with these observations, there was also a correlation between the magnitude of decline from sample 1 to sample 2, and a shortened survival, i.e. for those antibodies that showed an association between a high level in sample 1 and a shortened survival, namely IgA against 245:16 and 245:18 as described previously.

Discussion

The concept that antibodies to HPV may behave as tumor markers in cervical cancer has attracted considerable interest [9]. In a Finnish study [6] of antibody responses to the HPV16 E2 peptide 245:16 in 27 women with cervical carcinoma, increasing IgA antibody levels indicated significantly worse 2-year disease-free survival than did stable or decreasing antibody levels, whereas no effect was detected for IgG. In the present study, the association of IgA anti-245:16 with survival was confirmed. The presence of pretreatment IgA both to 245:16 and to 245:18 showed a clear correlation with a shortened survival. Although not significantly associated with tumor stage or grade at diagnosis, the level of these antibodies thus appeared to be of relevance for survival prediction.

Antibodies to E7 have been reported to be associated with tumor stage [3, 9], although the associations have not been statistically significant. The E7:2 IgG response, which has previously been reported to be associated with tumor stage [9], was not significantly associated with stage in this study, although a tendency was seen.

The decline of antibody responses after treatment was most pronounced for those responses that were strongly associated with untreated cervical cancer, such as L1:13 and 245:18 IgG, whereas the responses that had no association with cervical cancer, namely E7:2 IgA and IgA and IgG to L2:49, were much less or not significantly affected. These findings could be expected if the tumor-associated antibody responses were dependent on the presence of tumor. The decline of antibody responses also among the 13 patients with progressive/recurrent disease is puzzling. The most plausible explanation is that the radiotherapy and/or the disease process itself caused an immunosuppression, which resulted in failure to maintain these antibody responses. In any case, it is clear that these responses are not useful as tumor markers to monitor the course of disease. Rather they could be viewed as markers of cervical cancer risk. The question of when these antibodies are induced during development of cervical neoplasia could not be answered in this study. It will need to be elucidated in prospective studies.

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Note added in proof A recent study by Baay et al (Baay MFD, Duk JM, Burger MPM, de Bruijn HWA, Stolz E and Herbrink P (1995) Follow-up of antibody responses to HPV-16 E7 in patients treated for cervical carcinoma. J Med Virol, in press) analysed IgG antibodies against an E7 peptide (corresponding to our E7:2 peptide) after treatment of cervical cancer. In accordance with our results, most patients (12 out of 15) showed a decline of antibodies during treatment. However, in the patient group studied by Baay et al, 5 patients who initially were in remission after treatment had a recurrence during follow-up. In 4 of the 5 patients the E7 antibodies increased again before or concomitantly with recurrence. Our study contained only 2 patients with relapse after complete remission. The results of Baay et al thus suggest that further studies are needed on the usefulness of HPV antibodies in detecting relapses after clinical remission.