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Patterns of antibody responses to nonviral cancer antigens in head and neck squamous cell carcinoma patients differ by human papillomavirus status

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There have been hints that nonviral cancer antigens are differentially expressed in human papillomavirus (HPV)-positive and HPV-negative head and neck squamous cell carcinoma (HNSCC). Antibody responses (AR) to cancer antigens may be used to indirectly determine cancer antigen expression in the tumor using a noninvasive and tissue-saving liquid biopsy. Here, we set out to characterize AR to a panel of nonviral cancer antigens in HPV-positive and HPV-negative HNSCC patients. A fluorescent microbead multiplex serology to 29 cancer antigens (16 cancer-testis antigens, 5 cancer-retina antigens and 8 oncogenes) and 29 HPV-antigens was performed in 382 HNSCC patients from five independent cohorts (153 HPV-positive and 209 HPV-negative). AR to any of the cancer antigens were found in 272/382 patients (72%). The ten most frequent AR were CT47, cTAGE5a, c-myc, LAGE-1, MAGE-A1, -A3, -A4, NY-ESO-1, SpanX-a1 and p53. AR to MAGE-A3, MAGE-A9 and p53 were found at significantly different prevalences by HPV status. An analysis of AR mean fluorescent intensity values uncovered remarkably different AR clusters by HPV status. To identify optimal

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antigen selections covering a maximum of patients with ≤10 AR, multiobjective optimization revealed distinct antigen selections by HPV status. We identified that AR to nonviral antigens differ by HPV status indicating differential antigen expression. Multiplex serology may be used to characterize antigen expression using serum or plasma as a tissue-sparing liquid biopsy. Cancer antigen panels should address the distinct antigen repertoire of HPV-positive and HPV-negative HNSCC.

What's new?

Head and neck squamous cell carcinoma remains a deadly disease but new immunotherapeutic approaches are underexplored. Here the authors tested for antibody responses against human antigens to characterize the expression of such antigens in tumors positive or negative for human papillomavirus (HPV). Antibody responses were significantly different in prevalence and pattern based on HPV-status in a large patient cohort. The authors urge independent confirmation of their results but point out that multiplex serology of tumor antigens could be a promising strategy to identify immunotherapeutic targets based on HPV status.

Introduction

Globally, head and neck squamous cell carcinoma (HNSCC) is diagnosed in almost 900,000 cases annually resulting in approximately 450,000 cancer deaths per year.[1] Human papillomavirus (HPV)-positive oropharyngeal squamous cell carcinoma has been recognized as a distinct entity of HNSCC causally associated with HPV.[2] Clinically, a significant prognostic advantage for HPV-positive oropharyngeal squamous cell carcinoma has been determined in numerous studies for different primary treatment strategies.[3,4]

Cancer antigens are immunogenic proteins or peptides that can be recognized by the immune system. Shared cancer antigens include germline antigens such as cancer-testis antigens that are exclusively expressed in tumor tissue and germline cells[5,6] or cancer-retina antigens,[7] oncogenes or mutated tumor-suppressor genes overexpressed in cancer tissue such as p53[8] and foreign antigens such as viral antigens.[9] The expression of cancer antigens can be determined by RNA or protein detection in tissue biopsies. However, such analyses are expensive, availability of tissue may be limited and an invasive procedure is needed to obtain such biopsies. Although antibody responses (AR) to viral antigens have previously been used to identify HPV-positive patients,[10–12] AR to shared, nonviral antigens may play an important role for immunotherapy of HPV-positive and HPV-negative HNSCC.[13,14]

The analysis of serum or plasma AR could be a noninvasive way to characterize antigen expression indirectly.

The aim of our study was to analyze AR to cancer antigens in serum or plasma samples of HNSCC patients by HPV status.

Methods

This section was written with respect to the *Reporting Recommendations for Tumor Marker Prognostic Studies* (REMARK Guidelines).[15]

Patients

In our study, 382 patients with histologically diagnosed HNSCC, an available serum or plasma sample taken prior to the initiation of treatment and written informed consent according to the Helsinki Declaration II were selected (with local ethics committee approval). Patients were treated per institutional guidelines at five large head and neck cancer centers, namely University Medical Center Ulm, Heidelberg University Hospital, University Hospital Leipzig (Germany), University Hospital Padua (Italy) and University Hospital St. Gallen (Switzerland). Detailed patient characteristics are provided in Table 1 for oropharyngeal squamous cell carcinoma, cancer of unknown primary (CUP) and nonoropharyngeal squamous cell carcinoma.

HPV status

HPV status was determined at the treating center according to institutional standards. For n = 309 patients, a multiplex HPV-DNA PCR (GP5+/GP6+ primers followed by Sanger sequencing for HPV typing as previously described[16]) and p16 immunohistochemistry (n = 264) was performed. For n = 155 patients, HPV-16 E6*I mRNA status was available. Molecular HPV status was considered positive if two of the following three parameters were positive: HPV-DNA of known high-risk types, HPV-16 E6*I RNA, p16 immunohistochemistry. All other combinations were considered HPV negative. Molecular HPV status showed a significant correlation with the results of HPV serology (Pearson correlation coefficient = 0.775, $p < 0.001$). Thus, for patients lacking data for determination of the molecular HPV status, primarily nonoropharyngeal cancers, results from HPV serology to high-risk types were used as a surrogate parameter resulting in a combined marker HPV (mol/ser).

Material

A serum or plasma sample was prospectively collected prior to treatment initiation, aliquoted and stored at −20°C until use. Prospective sample collection was in accordance with local ethics committee approvals.

Table 1. Patient characteristics by primary site (oropharynx, CUP, nonoropharynx)

		Primary site							
		Oropharynx (54.9%)		CUP (11.8%)		Nonoropharynx (44.0%)		Total cohort	
		n	%	*n*	%	*n*	%	*n*	%
T	1	28	13.7	n.a.	n.a.	22	16.9	50	13.1
	2	89	43.4	n.a.	n.a.	32	24.6	121	31.7
	3	36	17.6	n.a.	n.a.	29	22.3	65	17.0
	4	52	25.4	n.a.	n.a.	47	36.2	99	25.9
	Missing	0	0.0	n.a.	n.a.	0	0.0	43	11.3
	Total	205	100	47	100	130	100	382	100
N	0	41	20.0	0	0.0	63	48.5	104	27.2
	1	26	12.7	17	36.2	20	15.4	63	16.5
	2	129	62.9	22	46.8	40	30.8	191	50.0
	3	9	4.4	7	14.9	7	5.4	23	6.0
	Missing	0	0.0	1	2.1	0	0.0	1	0.3
	Total	205	100	47	100	130	100	382	100
M	0	203	99.0	43	91.5	126	96.9	372	97.4
	1	2	1.0	4	8.5	4	3.1	10	2.6
	Missing	0	0.0	0	0.0	0	0.0	0	0.0
	Total	205	100	47	100	130	100	382	100
Stage	I	6	2.9	0	0.0	14	10.8	20	5.2
	II	17	8.3	0	0.0	17	13.1	34	8.9
	III	31	15.1	17	36.2	30	23.1	78	20.4
	IVA/B	149	72.7	25	53.2	65	50.0	239	62.6
	IVC	2	1.0	4	8.5	4	3.1	10	2.6
	Missing	0	0.0	1	2.1	0	0.0	1	0.3
	Total	205	100	47	100	130	100	382	100
HPV status (mol or ser)	HPV negative	81	39.5	33	70.2	108	83.1	222	58.1
	HPV positive	124	60.5	14	29.8	22	16.9	160	41.9
	Missing	0	0.0	0	0.0	0	0.0	0	0.0
	Total	205	100	47	100	130	100	382	100
Treatment approach	Surgical	148	72.2	38	80.9	101	77.7	287	75.1
	Nonsurgical	54	26.3	5	10.6	23	17.7	82	21.5
	Other (not curative)	2	1.0	2	4.3	4	3.1	8	2.1
	Missing	1	0.5	2	4.3	2	1.5	5	1.3
	Total	205	100	47	100	130	100	382	100
Sex	Male	156	76.1	42	89.4	106	81.5	304	79.6
	Female	49	23.9	5	10.6	24	18.5	78	20.4
	Total	205	100	47	100	130	100	382	100
Smoking	Nonsmoker	51	24.9	9	19.1	32	24.6	92	24.1
	Smoker	152	74.1	36	76.6	94	72.3	282	73.8
	Missing	2	1.0	2	4.3	4	3.1	8	2.1
	Total	205	100	47	100	130	100	382	100

Cancer-antigen serology

Among the 382 patients, a serum sample only was available for 260 patients and a plasma sample only for 86 patients. For 36 patients both, serum and plasma samples were available from the same timepoint. Serum and plasma mean fluorescence intensity (MFI) values showed a high correlation coefficient of $r = 0.960$ (95% CI 0.95–0.97, $p < 0.001$, slope 1.097; Supplementary Fig. S1). The serum sample MFI was taken whenever available and plasma sample MFI only if no serum sample was available.

Full-length proteins of selected cancer antigens were produced for multiplex serology as previously described.[17–20] The cancer antigen panel is shown in Table S1.

In brief, genes encoding for 16 cancer-testis antigens, 5 cancer-retina antigens, 8 oncogenes, 29 HPV antigens (from 8 high-risk HPV types) and 2 control antigens (JC virus protein 1 and BK virus protein 1) were cloned into the pGEX4T3 tag vector for expression in *Escherichia coli* BL21 as fusion proteins with N-terminal glutathione-S-transferase and a small C-terminal tagging epitope (tag) as previously described.[19,21] Recombinant protein expressed from the parental vector encoding the glutathione-S-transferase-tag fusion protein without insert was used to determine serological background. Anti-glutathione-S-transferase (GEHealthcare, Munich, Germany), anti-tag and anti-mouse HRP secondary antibodies (Dianova) were used to confirm full-length protein expression and protein integrity.

Multiplex serology was performed as previously described.[17–19,21] For each antigen and bead set, 2,500 glutathione-casein coated beads per sample were used and sera or plasma were measured at 1:1,000 dilutions. Reporter fluorescence of the beads was determined with the Bio-Plex analyzer (BioRad) and expressed as MFI of at least 100 beads per set per well. Antigen-specific reactivity was calculated as the difference among antigen-MFI, glutathione-S-transferase-tag-MFI and a blank. This value was used for further analyses. Cutoffs were determined graphically for nonviral antigens. For viral antigens, cutoffs were available from previous studies.[12]

Statistics

For statistical analysis, the SAMPL guidelines were respected.[22]

Prism version 7.0c (GraphPad Software, Inc, San Diego, CA) was used to graph ungrouped AR prevalences.

IBM SPSS statistics version 25.0 was used for statistical analysis unless indicated otherwise. Two-sided Pearson correlation was used to quantify correlations between MFI values of AR at baseline.

Frequencies of AR were compared between HPV-positive and HPV-negative patients using two-sided Chi-squared tests. For p53, MAGE-A antigens and NY-ESO-1 one-sided testing was performed based on available data in the literature[14,23,24] or own data from independent data sets which were indicating a significantly different prevalence by HPV status. Heatmaps of correlation coefficients between AR were created using R (version 3.5.1) and function Diana from R cluster package.

p-Values <0.05 were considered significant, but corrections for multiple testing were performed to reduce statistical errors. Corrections for multiple testing were done using Prism version 7.0c (GraphPad Software, Inc) with a false discovery rate approach for each hypothesis using the two-stage step-up method of Benjamini, Krieger and Yekutieli.[25] Given the exploratory nature of the study, a false discovery rate of up to 15% was tolerated.

The selection of antigens is formulated as a multiobjective optimization problem. Achieving a maximum coverage within a cohort of patients with a selection of antigens while at the same time minimizing the number of antigens in the respective selection is a mathematical problem that can be solved using multiobjective optimization methods. Multiobjective optimization was performed to identify antigen selections with at most ten antigens covering a maximum of patients in the whole cohort, HPV-positive or HPV-negative patients.

Instead of a single optimal solution, in multiobjective optimization a set of optimal solutions (Pareto-optimal set) needs to be found. The solutions of the Pareto-optimal set are the optimal trade-offs between the optimization objectives.[26]

We use an adaption of the multiobjective evolutionary algorithm NSGA-II[27] to our selection problem. This algorithm is a population-based metaheuristic where a set of solutions (antigen selections) is evolved iteratively by applying recombination and mutation operators to the solutions. After a specified number of iterations, the algorithm returns a set of antigen selections containing the trade-offs between the objectives patient coverage and number of antigens.

From the trade-offs, we select the antigen selection with at most ten genes that has the largest coverage.

Data availability

Results from multiplex serology are not publicly available, but may be obtained upon request to the corresponding author.

Results

Patient characteristics of the cohort of 382 patients with HNSCC are presented in Table 1. The majority of patients had an oropharyngeal tumor, explaining the high rate of HPV-positive patients (41.6%). Mean age was 61 years in the whole cohort (range: 18–91 years) as well as in HPV-positive (range: 29–85 years) and HPV-negative patients (range: 18–91 years).

Among the 382 patients, 272 (72%) were seropositive for any of the 29 autoantigens tested. These results are graphically presented in Figure 1 with the top ten AR highlighted.

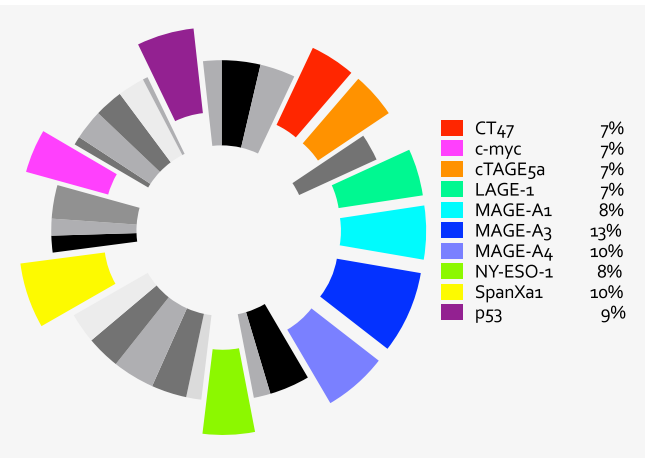

Figure 1. Diagram of antigen reactivities to the panel of 29 autoantigens. Overall 749 antigen reactivities (AR) were found in 272/382 (72%) patients. The top ten antigens (425 reactivities) with the highest prevalences in the cohort are highlighted. With these identified ten antigens, 182/272 (67%) patients with any AR and 182/382 (47.6%) of the whole cohort were covered. [Color figure can be viewed at wileyonlinelibrary.com]

AR of individual patients to antigens in the panel and the overlap between the respective AR for HPV-positive and HPV-negative were visualized in Figure 2. The overlap between the respective AR was also different ($overlap_{HPV\text{-}negative}$ = 1.09, $overlap_{HPV\text{-}positive}$ = 0.70). These differences in AR prevalences and overlap indicate that AR prevalences and patterns between HPV-positive and HPV-negative patients are distinct.

The rate of patients with 0 to 1 AR was higher in HPV-positive patients than in HPV-negative patients, but did not differ by age. A detailed overview of the AR numbers in all patients by HPV status and by age is provided in Table S2.

Thus, two-sided Chi-squared tests were performed for each of the 29 autoantigens in the panel comparing AR prevalence in the group of HPV-positive and HPV-negative patients. For those antigens for which independent data indicated a different antigen prevalence, one-sided Chi-sqaured tests were utilized (namely MAGE-A3, MAGE-A4, MAGE-A9, NY-ESO-1 and p53).

Significantly different AR prevalences were found for MAGE-A3, MAGE-A9 and p53 (Table S3). These results remained significant after correction for multiple testing with a false discovery rate of 5%. The results expressed as an odds ratio graph are shown in Figure 3.

We investigated the relationship of the sum of MFI as an indirect measure for antigen load compared to T-category, stage, HPV status and age. The distribution of the sum of MFI for all nonviral cancer antigens in the panel was significantly different by T category: The sum of MFI was higher in CUP (4,792) compared to T1/2 (1,408) or T3/T4 (2,554) (Kruskal–Wallis test: $p < 0.001$). The mean sum of MFI in Stage I/II (1259) was not significantly lower than in Stage III/IV (2,496) (Whitney–Mann *U*-test: $p = 0.326$). However, the group of Stage I/II patients was very small ($n = 54$). In HPV-negative patients (2,609), the mean sum of MFI was significantly higher than in HPV-positive patients (1,910) (Whitney–Mann *U*-test: $p = 0.009$), but did not differ by age (<65: mean sum of MFI = 1,992; ≥65 = 2,923; Whitney–Mann *U*-test: $p = 0.581$).

A correlation analysis of AR MFI values to the tested autoantigen panel revealed significant correlations between Camel, LAGE-1, NY-ESO-1 and MAGE-A9, the antigens of the MAGE-A family (MAGE-A1, -A3, -A4, -A9), the SSX-family (SSX2, SSX4) and between cTAGE5a, OY-TES-1, SpanXa1 and IMP-1. Interestingly, correlations of AR MFI for HPV-negative and HPV-positive patients revealed the same significant pairwise correlations, but the values of correlation coefficients were higher for HPV-positive patients. This becomes evident in the respective heatmaps resulting in a different hierarchical clustering (Fig. 4). A much stronger correlation between MAGE-A9, Camel, NY-ESO-1 and LAGE-1a as well as between cTAGE5a, OY-TES-1, SpanXa1 and IMP-1 can be seen in the HPV-positive patients. The respective correlation matrices with detailed values for correlation coefficients and *p*-values are provided in Tables S4 (HPV-negative) and S5 (HPV-positive). The correlation matrix for the whole cohort is presented in a heatmap in Figure S2. The correlations described above form four visible clusters in the heatmap. Detailed values for correlation coefficients and *p*-values of the whole cohort are provided in Table S6.

Given the different patterns of AR by HPV status, a multi-objective optimization was performed to identify

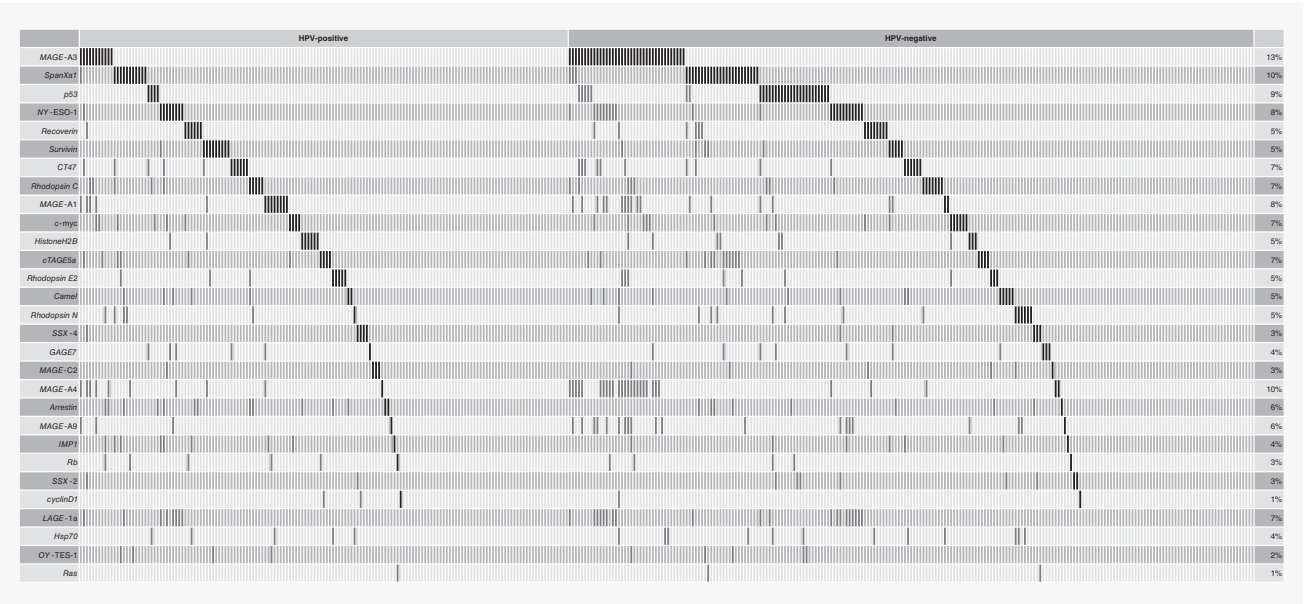

Figure 2. Individual AR to the cancer antigen panel by HPV status. Antigens are presented in rows, patients in columns. Individuals are grouped by HPV status. Each AR is symbolized by a bar in the respective antigen row. ARs overlapping with AR to other antigens are displayed in gray. In the last column, the coverage of the respective antigen within the whole cohort is indicated. The graph shows that 105/159 (66%) of HPV-positive patients and 167/223 (75%) of HPV-negative patients had any AR to the tested autoantigen panel (Chi-squared, two-sided: $p = 0.06$).

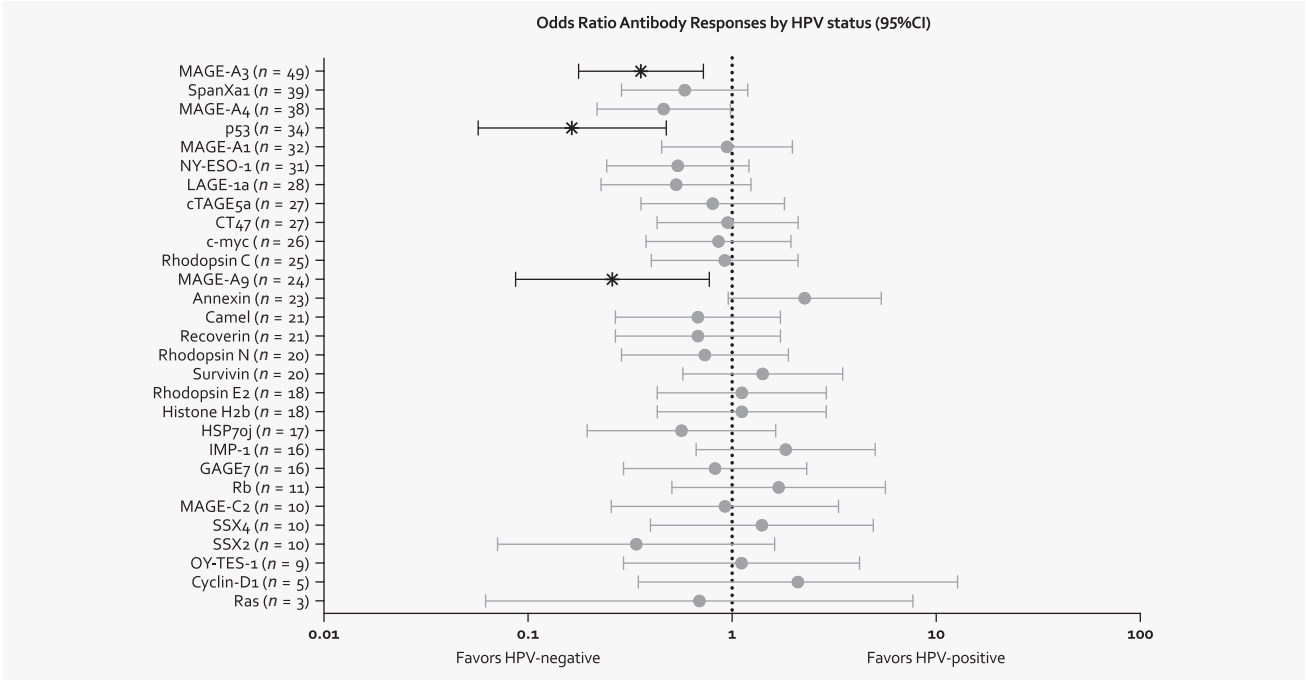

Figure 3. Odds ratio graphs for antibody responses by HPV status. The odds ratio and the 95% confidence interval (95% CI) are shown. The antibody responses are ordered by prevalence in the whole cohort (*n*). Significant odds ratios are indicated by an asterisk symbol and printed in black.

combinations of ≤10 AR resulting in maximum coverage within the respective group (all patients, HPV-negative, HPV-positive). These antigen selections were compared to the selection of the most frequent ten antigens (described in Fig. 1) with regard to maximum coverage in the respective group and in all patients (Fig. 5). Selecting the ten most frequent AR resulted in a coverage of 121/223 (54.3%) in HPV-negative patients and 61/159 (38.4%) in HPV-positive patients.

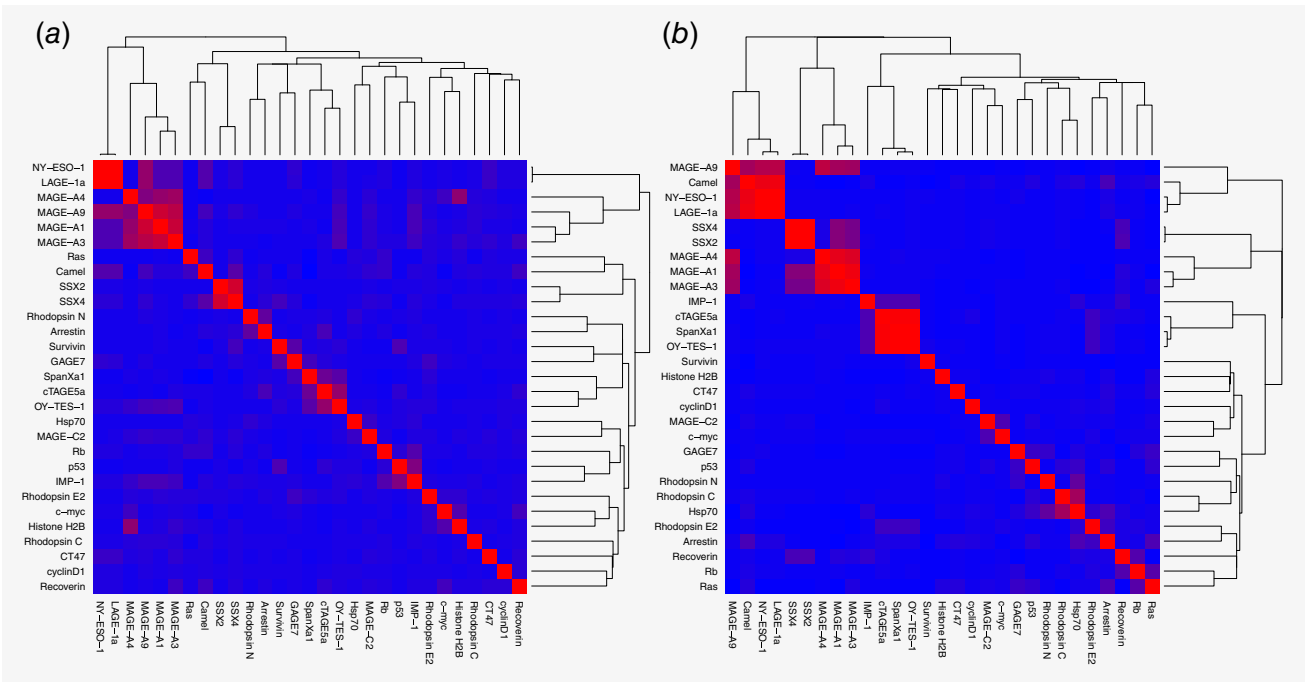

Figure 4. Heatmaps of Pearson correlation coefficients. The heatmap was created using R (version 3.5.1) and function Diana from R cluster package with a single gradient and two colors for (*a*) HPV-negative patients and (*b*) HPV-positive patients. [Color figure can be viewed at wileyonlinelibrary.com]

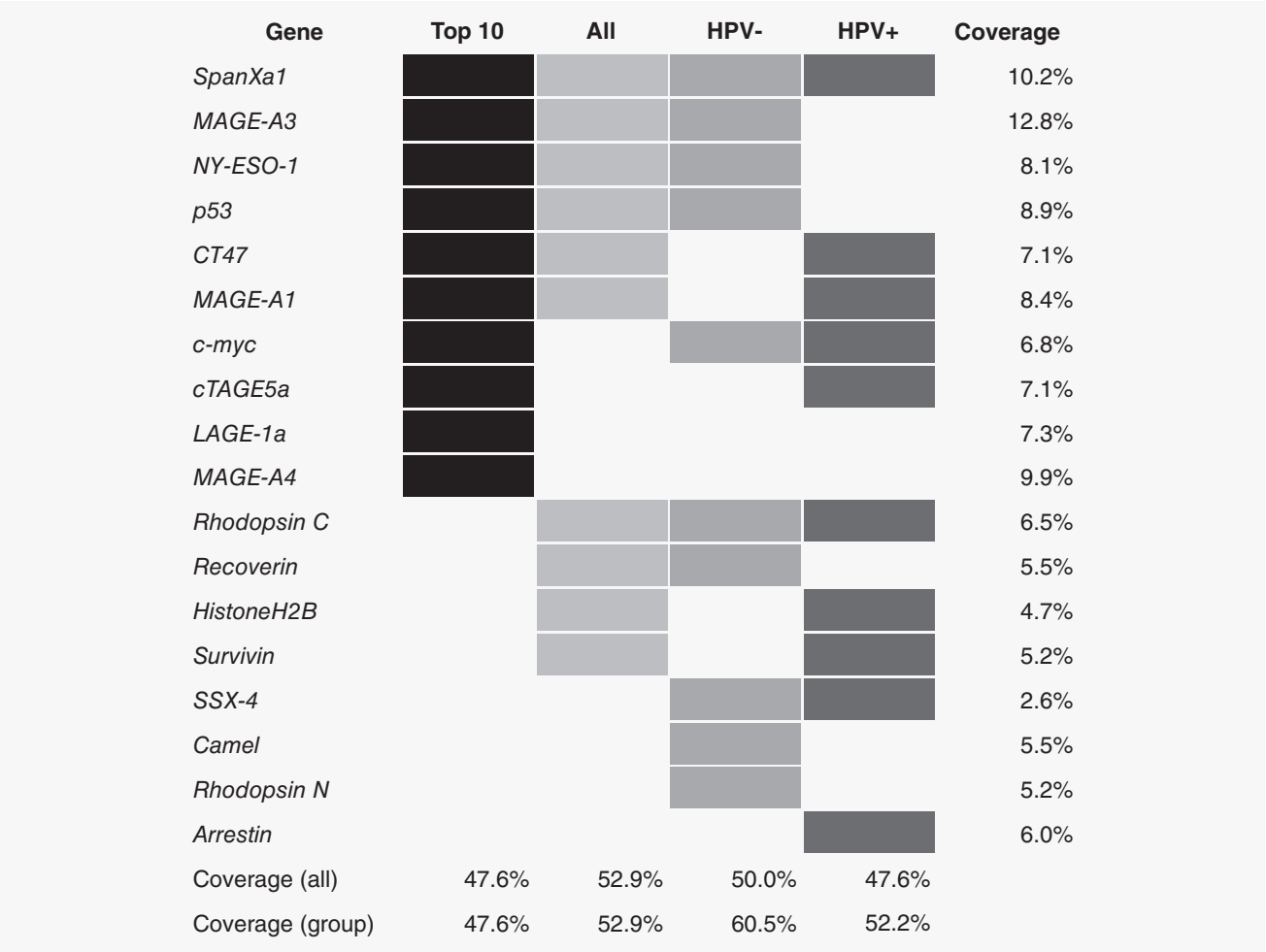

Figure 5. Comparison of coverage for ten different cancer antigen selections. Antigens are shown in rows, solutions in columns. The coverage for the whole cohort and the respective group optimized are provided in the last two rows.

After multiobjective optimization for coverage and antigen number, the coverage in the whole cohort could be increased to 202/382 (52.8%). In HPV-negative patients, 128/223 (57.4%) and in HPV-positive patients 74/159 (46.5%) could be covered with this solution. Optimization for HPV-negative patients resulted in an increased coverage of 135/223 (60.5%) within HPV-negative patients, but only 56/159 (35.2%) in HPV-positive patients. Optimization for HPV-positive patients resulted in a coverage of 83/159 (52.2%) in the HPV-positive cohort, but only 99/223 (44.3%) in the HPV-negative cohort.

These results show that antigen selections with higher coverages within the respective groups than by selecting the ten most prevalent AR can be found by multiobjective optimization.

Discussion

We were able to detect AR to a selected panel of 29 cancer antigens in serum or plasma in the majority of patients from a large HNSCC cohort. With only 10 out of the 29 antigens, approximately 66% of all AR could be detected. Significantly different prevalences for AR to MAGE-A3, MAGE-A9 and p53 by HPV status were found. AR patterns were remarkably different between HPV-negative and HPV-positive patients. Multiobjective optimization may be key to develop tailored antigen panels to improve the performance of multiplex serology to cancer antigens as a liquid biopsy of the cancer-antigen repertoire in the tumor.

MFI values are a quantitative measure and are closely correlated with antibody concentrations/antibody titers. They can be considered as a surrogate for antibody concentrations as previously shown[28,29] and may also be considered an indirect measure of antigen load.[30–32] The mean sum of MFI was higher in CUP patients and in HPV-negative patients. This may indicate that the cancer antigen panel selected for our study was tailored better for HPV-negative patients. To date, there are limited data available for nonviral cancer antigen expression in HPV-positive patients. In a recently published study, AR to 23 cancer antigens were evaluated in 36 HNSCC patients (27 HPV-negative and 9 HPV-positive HNSCC) and 15 healthy controls.[33] Due to the sample size of that study, the authors were unable to determine conclusive prevalences of AR by HPV status. Thus, this is the first study defining AR

prevalences to a large panel of cancer antigens in a large cohort of HNSCC patients.

In two previous studies in melanoma patients,[18,20] the prevalence of AR to 29 cancer antigens[20] and 43 cancer antigens,[18] respectively, was higher and AR patterns were also different. In studies aiming at establishing cancer antigen serology as a diagnostic tool to detect colorectal cancers,[17] gastric cancer[19] or prostate cancer[34] AR frequencies and patterns were distinct as well. This indicates disease-specific patterns of cancer antigen expression.

Even within HNSCC, HPV status defined different patterns of nonviral cancer antigens. Although the overall prevalence of AR was not significantly different by HPV status, a trend to reduced frequencies of AR to the selected cancer antigen panel was found in HPV-positive patients. The fact that 10/29 antigens are sufficient to detect 2/3 of all detected AR further indicates that a modification of the cancer antigen panel, preferably semipersonalized for HPV status may improve coverage within the respective cohort. A multiobjective optimization for coverage and number of antigens for all patients and by HPV status resulted in markedly improved coverage in the respective groups with the same number of antigens (Fig. 5). Thus, a modification of the cancer antigen panel may improve the performance of multiplex serology to define the individual nonviral cancer antigen repertoire.

With regard to the implications of AR to cancer antigens for cancer-specific immunity, published data are somewhat conflicting. Several publications indicate a correlation between T cell immunity and the presence of humoral immunity,[35–37] but others did not find an association between antibody and cellular responses.[38] Even if specific T cells to cancer antigens are found, these seem to be functionally impaired as indicated by increased expression of co-inhibitory immune checkpoints such as CTLA-4 or LAG-3.[30] Immune checkpoint modulation seems to reconstitute functionality of NY-ESO-1 specific immunity as previously shown.[39]

Previously published data indicate that humoral immunity to cancer antigens may not be a surrogate for active cancer immunity, but rather an indirect measure of antigen expression.[30–32,40] Several studies have associated AR to certain cancer antigens with detrimental or beneficial prognosis.[18,20,30] In most studies and for most antigens, a negative prognostic impact—consistent with our results—was found. This was also the case for some AR in the panel described here (data not shown). At the same time, such patients are candidates for antigen-specific immunotherapy which may improve the detrimental outcome. AR serology may identify patients who are at high risk of death, but also who may benefit from immunotherapy such as immune checkpoint modulation potentially in combination with vaccination based on the individual cancer-antigen repertoire defined by multiplex serology.

Unfortunately, transcriptome or protein expression data are not available from the cohort in order to verify protein expression in AR positive patients which represents a limitation to our study.

In the future, a modification of the antigen panel for multiplex serology may improve coverage of patients to identify patients who may benefit from immunotherapy. The results of this analysis are exploratory and need validation in independent cohorts, preferably from prospective clinical trials.

In conclusion, our data show that AR patterns to nonviral antigens are distinct based upon HPV status which can be utilized for the development of immunotherapy for HNSCC.

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References

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018;68:394–424.
2. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* 2000;92:709–20.
3. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med* 2010;363:24–35.
4. Licitra L, Perrone F, Bossi P, et al. High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. *J Clin Oncol* 2006;24:5630–6.
5. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991;254:1643–7.
6. von Boehmer L, Mattle M, Bode P, et al. NY-ESO-1-specific immunological pressure and escape in a patient with metastatic melanoma. *Cancer Immun* 2013;13:12.
7. Bazhin AV, Schadendorf D, Willner N, et al. Photoreceptor proteins as cancer-retina antigens. *Int J Cancer* 2007;120:1268–76.
8. Schuler PJ, Harasymczuk M, Visus C, et al. Phase I dendritic cell p53 peptide vaccine for head and neck cancer. *Clin Cancer Res* 2014;20:2433–44.
9. Welters MJP, Ma W, Santegoets S, et al. Intratumoral HPV16-specific T cells constitute a type I-oriented tumor microenvironment to improve survival in HPV16-driven oropharyngeal cancer. *Clin Cancer Res* 2018;24:634–47.
10. Schroeder L, Wichmann G, Willner M, et al. Antibodies against human papillomaviruses as diagnostic and prognostic biomarker in patients with neck squamous cell carcinoma from unknown primary tumor. *Int J Cancer* 2018;142:1361–8.
11. Holzinger D, Wichmann G, Baboci L, et al. Sensitivity and specificity of antibodies against HPV16 E6 and other early proteins for the detection of HPV16-driven oropharyngeal squamous cell carcinoma. *Int J Cancer* 2017;140:2748–57.
12. Broglie MA, Jochum W, Michel A, et al. Evaluation of type-specific antibodies to high risk-human papillomavirus (HPV) proteins in patients with oropharyngeal cancer. *Oral Oncol* 2017;70:43–50.
13. Laban S, Atanackovic D, Luetkens T, et al. Simultaneous cytoplasmic and nuclear protein expression of melanoma antigen-A family and NY-ESO-1 cancer-testis antigens represents an independent marker for poor survival in head and neck cancer. *Int J Cancer* 2014;135:1142–52.
14. Laban S, Giebel G, Klumper N, et al. MAGE expression in head and neck squamous cell carcinoma primary tumors, lymph node metastases and respective recurrences-implications for immunotherapy. *Oncotarget* 2017;8:14719–35.
15. Sauerbrei W, Taube SE, McShane LM, et al. Reporting recommendations for tumor marker prognostic studies (REMARK): an abridged explanation and elaboration. *J Natl Cancer Inst* 2018;110:803–11.
16. Jacobs MV, de Roda Husman AM, van den Brule AJ, et al. Group-specific differentiation between high- and low-risk human

papillomavirus genotypes by general primer-mediated PCR and two cocktails of oligonucleotide probes. *J Clin Microbiol* 1995;33:901–5.
17. Chen H, Werner S, Butt J, et al. Prospective evaluation of 64 serum autoantibodies as biomarkers for early detection of colorectal cancer in a true screening setting. *Oncotarget* 2016;7:16420–32.
18. Michels J, Becker N, Suciu S, et al. Multiplex bead-based measurement of humoral immune responses against tumor-associated antigens in stage II melanoma patients of the EORTC18961 trial. *Oncoimmunology* 2018;7:e1428157.
19. Werner S, Chen H, Butt J, et al. Evaluation of the diagnostic value of 64 simultaneously measured autoantibodies for early detection of gastric cancer. *Sci Rep* 2016;6:25467.
20. Zornig I, Halama N, Lorenzo Bermejo J, et al. Prognostic significance of spontaneous antibody responses against tumor-associated antigens in malignant melanoma patients. *Int J Cancer* 2015;136:138–51.
21. Waterboer T, Sehr P, Michael KM, et al. Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. *Clin Chem* 2005;51:1845–53.
22. Lang TA, Altman DG. Basic statistical reporting for articles published in clinical medical journals: the SAMPL Guidelines. *Science Editors' Handbook*. European Association of Science Editors, 2013.
23. Seiwert TY, Zuo Z, Keck MK, et al. Integrative and comparative genomic analysis of HPV-positive and HPV-negative head and neck squamous cell carcinomas. *Clin Cancer Res* 2015;21:632–41.
24. TCGA-Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature* 2015;517:576–82.
25. Benjamini Y, Krieger AM, Yekutieli D. Adaptive linear step-up procedures that control the false discovery rate. *Biometrika* 2006;93:491–507.
26. Deb K. *Multi-objective optimization using evolutionary algorithmsed*. New York, NY: John Wiley & Sons, Inc, 2001.
27. Deb K, Pratap A, Agarwal S, et al. A fast and elitist multiobjective genetic algorithm: NSGA-II. *IEEE Trans Evol Comput* 2002;6:182–97.
28. Sankaranarayanan R, Prabhu PR, Pawlita M, et al. Immunogenicity and HPV infection after one, two, and three doses of quadrivalent HPV vaccine in girls in India: a multicentre prospective cohort study. *Lancet Oncol* 2016;17:67–77.
29. Brown D, Muller M, Sehr P, et al. Concordance assessment between a multiplexed competitive Luminex immunoassay, a multiplexed IgG Luminex immunoassay, and a pseudovirion-based neutralization assay for detection of human papillomaviruse types 16 and 18. *Vaccine* 2014;32:5880–7.
30. Daudi S, Eng KH, Mhawech-Fauceglia P, et al. Expression and immune responses to MAGE antigens predict survival in epithelial ovarian cancer. *PLoS One* 2014;9:e104099.
31. Scanlan MJ, Welt S, Gordon CM, et al. Cancer-related serological recognition of human colon cancer: identification of potential diagnostic and immunotherapeutic targets. *Cancer Res* 2002;62:4041–7.
32. Stockert E, Jager E, Chen YT, et al. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med* 1998;187:1349–54.
33. Lechner A, Schlosser HA, Thelen M, et al. Tumor-associated B cells and humoral immune response in head and neck squamous cell carcinoma. *Oncoimmunology* 2019;8:1535293.
34. Adeola HA, Smith M, Kaestner L, et al. Novel potential serological prostate cancer biomarkers using CT100+ cancer antigen microarray platform in a multi-cultural South African cohort. *Oncotarget* 2016;7:13945–64.
35. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, et al. Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. *Proc Natl Acad Sci U S A* 2010;107:7875–80.
36. Gnjatic S, Atanackovic D, Jager E, et al. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. *Proc Natl Acad Sci U S A* 2003;100:8862–7.
37. Jager E, Nagata Y, Gnjatic S, et al. Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. *Proc Natl Acad Sci U S A* 2000;97:4760–5.
38. Pfirschke C, Gebhardt C, Zornig I, et al. T cell responses in early-stage melanoma patients occur frequently and are not associated with humoral response. *Cancer Immunol Immunother* 2015;64:1369–81.
39. Yuan J, Adamow M, Ginsberg BA, et al. Integrated NY-ESO-1 antibody and CD8+ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proc Natl Acad Sci U S A* 2011;108:16723–8.
40. Ademuyiwa FO, Bshara W, Attwood K, et al. NY-ESO-1 cancer testis antigen demonstrates high immunogenicity in triple negative breast cancer. *PLoS One* 2012;7:e38783.