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Human papillomavirus and p53 polymorphism in codon 72 in head and neck squamous cell carcinoma

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Abstract. The impact of a polymorphism of the wild-type human tumour suppressor gene p53(wt) on carcinogenesis is subject of controversy ever since a higher susceptibility of p53 to HPV-E6 mediated degradation when encoding for Arginine at codon 72 ($p53_{Arg}$) was first reported. The issue remained unclear because various studies investigating this question for different tumour entities and different geographical regions demonstrated diverging results. In the present study, the HPV status and p53 genotype frequency of 42 head and neck cancers was analysed and compared to results reported in the recent literature. Applying PCR and cycle sequencing techniques, HPV DNA was demonstrated in 12/42 (29%) of the cases and the overall distribution of the p53 allele was: 64, 31 and 5% for $p53_{Arg}$, $p53_{Arg/Pro}$ and $p53_{Pro}$, respectively. There was no statistically significant association between HPV status and p53 genotype distribution. The results of our study and of the reviewed literature do not support a relevant role of the p53 polymorphism in head and neck carcinogenesis, either taken alone or in association with the HPV status.

Introduction

Epidemiology oriented studies strongly confirm the conclusion of the molecular biology based observations, pointing to an etiological involvement of human papillomaviruses (HPV) in a subset of head and neck carcinomas (1,2). Infections with high risk HPV types, most frequently HPV type 16 (HPV16), were shown to be regularly detectable in ~20-30% for oro-, hypopharyngeal as well as laryngeal squamous cell carcinomas and ~60% for squamous cell carcinomas of the Waldeyer's tonsillar ring (1-5). From a mode of action perspective, expression of the viral oncogenes E6 and E7 dysregulates the control of crucial cell mechanisms, particularly the apoptotic pathway and the cell cycle. The E6 gene product binds wild-type p53, triggering its ubiquitin-mediated degradation, thus preventing a cell cycle block and induction of apoptosis of DNA-damaged cells, whereas the E7 protein binds pRb. The corresponding disruption of cell cycle regulation, with down-regulation of pRb and cyclin D1 expression as well as up-regulation of $p16^{INK4a}$ has been observed in patient derived material (4,6-9). In HNSCC, tumours with lower retinoblastoma gene product (pRb) levels, overexpression of $p16^{INK4a}$ as well as lack of p53 mutations, viral activity in form of E6/E7 mRNA expression finally leading to carcinogenesis can, most likely, be expected.

In wild-type p53 gene, a common *Acc*II restriction fragment length polymorphism in codon 72 of exon 4 (Arg72Pro) has been demonstrated. It arises from a single base pair substitution, and results in either a proline (CCC, $p53_{Pro}$) or an arginine (CGC, $p53_{Arg}$) residue (10).

In 1998, Storey *et al* reported that the $p53_{Arg}$ protein is more susceptible to E6-mediated proteolytic degradation than the $p53_{Pro}$ protein and that the $p53_{Arg}$ allele is over-represented in cervical cancer patients (11). The conclusion that the $p53_{Arg}$ allele confers a higher risk for cervical cancer development than the $p53_{Pro}$ allele has been supported by some subsequent studies. However, in a majority of cervical cancer studies the published results of Storey and co-workers could not be confirmed (12-16). Analysis on p53 codon 72 polymorphism and HPV has also been carried out in other human cancers such as oesophageal, skin, lung, breast and head and neck carcinomas, with just as diverging results as reported for cervical cancers (17).

In the present study, we investigated the genotypic frequency of p53 codon 72 polymorphism in correlation with the presence or absence of HPV to determine whether or not specific p53 genotypes facilitate HPV-associated carcinogenesis in our local HNSCC cases.

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Materials and methods

Tissue specimens and DNA extraction. Tissue samples of histopathologically confirmed HNSCC were obtained during surgery from 42 patients (31 male; 11 female; 38-82 years, mean: 61.2±10.1 years) receiving treatment at the Department of Otorhinolaryngology, Head and Neck Surgery at the Christian-Albrechts-University of Kiel, Germany. Written informed consent from all patients was obtained prior to surgery. The specimens were immediately stored at -80˚C for subsequent molecular analysis. The anatomical location of the primary tumours of the investigated tissue samples was: hypopharynx (n=14), larynx (n=13), palatine tonsil (n=6), oral cavity (n=4), oropharynx (n=2), lower lip (n=1), nasal cavity (n=1), and paranasal sinus (n=1). For further clinico-pathological features and results see Tables I and II. DNA was extracted from 25 mg frozen tissue sample as described previously (18,19).

HPV PCR amplification and Southern blot hybridization of the PCR results. The presence of amplifiable DNA in every individual sample was confirmed by PCR applying ß-globin primers. For detection of HPV DNA, PCR was performed using type specific primer PCR for HPV16, a combination of type specific primers for HPV6, 11, and 18, and consensus primer PCRs and subsequent Southern blot hybridization of the PCR products with corresponding probes as described previously (18,19).

DNA sequencing of p53 exon 4. PCR was carried out applying exon 4 specific primer pairs (p53 E4-1 5'-CTGGTCCTC TGACTGCTCTTTT-3'; p53 E4-2 5'-GAAGTCTCATGGA AGCCAGC-3') and using a thermocycler (T3, Biometra, Göttingen, Germany) with the following reagents for a 100 μl reaction mixture: 10 μl 10-fold PCR buffer, 2.4 μl of 50 mM $MgCl_2$, 2 μl of 10 mM dNTP, 1 μl of 100 μM sense and anti-sense primer each, 0.5 μl of 5 U/μl Taq DNA polymerase and 900 ng template DNA in 83.1 μl H_2O (all PCR reagents from Gibco BRL, Karlsruhe, Germany). High stringency PCR was carried out for 35 cycles at 94˚C (60 sec), 65˚C (30 sec) and 72˚C (75 sec), finished by a final extension-step at 72˚C (5 min). The PCR product was purified using a GFX purification kit (Pharmacia, Freiburg, Germany) and then sequenced by the dideoxy-chain termination method (20) using the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Weiterstadt, Germany). All amplified exons were sequenced up- and downstream using the ABI Prism 310 (Applied Biosystems, Darmstadt, Germany) DNA sequencer.

Statistical analysis. The impact of HPV infections and/or p53 polymorphisms on survival applying the Kaplan-Meier method could not be analysed due to the relatively small number of patients per group. Chi-square and Fisher's exact test were used to test for differences between the groups.

Results

HPV analysis. All 42 tissue specimens obtained from 42 patients were studied by type-specific PCR and subsequent Southern blot hybridization with type-specific internal probes for the detection of HPV6, 11, 16, and 18. All negative samples were subsequently subjected to consensus-primer PCR and Southern hybridization.

Out of 42 tissue specimens (29%) 12 were HPV positive. HPV16 was identified in eight cases (67%). Additionally, four cases were positive for HPV DNA in the consensus primer PCR, but not in type-specific PCR (Table I). HPV6, 11, and 18 were not identified in any of the investigated tissue specimens.

Concerning the anatomical location of the primary tumours, the distribution of the HPV positive cases was: Waldeyer's tonsillar ring [2/6 (33%)], hypopharynx [4/14 (29%)], and larynx [4/13 (31%)]. Additionally, HPV was detected in SCC of the lower lip (n=1) and the paranasal sinuses (n=1), respectively. HPV was not detected in any case derived from the oral cavity (n=4), the oropharynx (n=2), and the nasal cavity (n=1).

p53 codon 72 polymorphism. In 27 out of 42 tumour DNA samples (64%), the sequencing for codon 72 revealed a CGC-base triplet encoding the amino acid Arg. These patients were classified as homozygous for Arg/Arg ($p53_{Arg}$). In 2 patients (5%) we detected a signal giving the sequence CCC at codon 72 encoding for *Pro*. These patients were defined as homozygous for *Pro*/*Pro* ($p53_{Pro}$). The remaining 13 patients (31%) showed two signals (CGC and CCC) after sequencing. Consequently these patients were categorized as heterozygous for codon 72 (*Arg*/*Pro*, $p53_{Arg/Pro}$). For details see Table I. Comparing the p53 polymorphism and HPV status of the analyzed patient population with the data published for patient groups from different geographical locations, a complex picture arises (Table II). For instance, the overall presence of the $p53_{Arg}$ phenotype varies from 81.8 to 28.8%, the $p53_{Arg/Pro}$ genotype is present in between 52.7 and 10.4% of analyzed patients, whereas the p53 Pro genotype with a distribution between 19 and 5% seems slightly more homogeneous (Table II). Due to the relative small number of patients investigated these cannot be considered representative of their respective regional populations.

Regarding the comparative analysis of HPV positivity and specific p53 codon 72 polymorphisms, the variety is huge. For instance, the HPV positivity in $p53_{Arg}$ patients varies between 92 and 32% of the analyzed cases and between 5 and 0% in the $p53_{Arg/Pro}$ group (Table II).

HPV status and p53 codon 72 polymorphism. Out of the 12 patients identified as HPV positive, 11 showed $p53_{Arg}$ and one patient showed $p53_{Pro}$ in exon 4 at codon 72. Yet, no HPV positive patient showed a $p53_{Arg/Pro}$ genotype (see Tables I and II). Out of the 30 HPV negative patients 16 (53%) showed $p53_{Arg}$, 13 patients (43%) $p53_{Arg/Pro}$, and one patient (3%) showed $p53_{Pro}$ in exon 4 at codon 72.

Statistical analyses. The analysis of the HPV status with the genotype of exon 4 at codon 72 of wild-type p53 by Chi-square test was statistically not significant (p=0.124). However, this might be due to the small number of tumour samples included in this study. Due to the small number of cases containing the proline homozygous genotype ($p53_{Pro}$) we combined this group

Clinicopathologic features and HPV- and p53-polymorphism results of investigated HNSCC.

Case	Sex	Age	Tumour site	TNM	HPV	p53-polymorphism
1	F[a]	82	Tonsil	T4N2cM0	HPV16	Arg
2	F	51	Tonsil	T2N2aM0		Arg
3	M	61	Tonsil	T3N0M0	HPV16	Arg
4	M	61	Tonsil	T2N0M0		Pro/Arg
5	M	52	Tonsil	T2N0M0		Pro/Arg
6	M	53	Tonsil	T2N2cM0		Arg
7	F	68	Oral cavitiy	T3N1M0		Pro/Arg
8	M	50	Oral cavitiy	T4N2bM0		Arg
9	M	61	Oral cavitiy	T3N0M0		Arg
10	F	78	Oral cavitiy	T2N2bM0		Arg
11	M	77	Oropharynx	T3N0M0		Arg
12	M	60	Oropharynx	T3N2aM0		Arg
13	F	53	Hypopharynx	T2N2bM0		Pro/Arg
14	M	47	Hypopharynx	T3N2bM0		Pro/Arg
15	M	66	Hypopharynx	T4N0M0		Pro/Arg
16	M	63	Hypopharynx	T4N2bM0		Arg
17	M	67	Hypopharynx	T3N2bM1	HPV-cons.[b]	Arg
18	M	62	Hypopharynx	T3N2bM0		Arg
19	F	57	Hypopharynx	T4N0M0	HPV-cons.	Arg
20	M	60	Hypopharynx	T4N1M0		Arg
21	M	67	Hypopharynx	T4N1M0		Arg
22	M	38	Hypopharynx	T4N2cM0		Pro
23	M	69	Hypopharynx	T3N2bM0	HPV-cons.	Arg
24	F	46	Hypopharynx	T4N2aM0		Arg
25	M	60	Hypopharynx	T2N3M0	HPV16	Arg
26	M	52	Hypopharynx	T4N2M0		Pro/Arg
27	M	62	Larynx	T3N0M0		Pro/Arg
28	F	70	Larynx	T4N2cM0		Pro/Arg
29	F	67	Larynx	T3N2bM0		Arg
30	M	58	Larynx	T3N0M0	HPV16	Arg
31	M	71	Larynx	T3N0M0	HPV-cons.	Arg
32	M	62	Larynx	T4N1M0		Arg
33	M	67	Larynx	T4N0M0	HPV16	Arg
34	F	60	Larynx	T1aN0M0		Pro/Arg
35	M	70	Larynx	T2N0M0		Arg
36	M	67	Larynx	T4N2bM0		Pro/Arg
37	M	45	Larynx	T4N0M0		Arg
38	M	74	Larynx	T2N0M0		Pro/Arg
39	M	42	Larynx	T2N2bM0	HPV16	Pro
40	F	75	Nasal cavity	T4N0M0		Pro/Arg
41	M	50	Paranasal sinus	T4N0M0	HPV16	Arg
42	M	68	Lower lip	T2N0M0	HPV16	Arg

[a]F/M, male/female; [b]HPV-cons., HPV-positive after L1/L2 consensus primer PCR.

with the group of cases containing the $p53_{Pro/Arg}$ genotype and correlated these cases with the HPV status applying Fischer's exact test, again without statistical significance ($p=0.099 > 0.05$).

Table II. HPV- and p53-polymorphism results of this study in comparison to results of recent studies performed in different geographical parts of the world.

	Hof[a]			Sum[b]			Nag[c]			Cor[d]			Sche[e]			Per[f]		
HPV	Pos.[h]	Neg.	Total	Pos.	Neg.	Total	Pos.	Neg.	Total	Pos.	Neg.	Total	Pos.	Neg.	Total	Pos.	Neg.	Total
Cases																		
Arg[g]	11	16	27	26	76	112	14	17	31			26	17	49	66	11	52	63
(%)	(92)	(53.3)	(64)	(56.5)	(52.8)	(53.7)	(34)	(24.6)	(28.8)	(52)	(48)	(52)	(46)	(57)	(54)	(68.8)	(85)	(82)
Arg/Pro	0	13	13	15	55	70	20	38	58			16	20	35	55	1	7	8
(%)		(43.3)	(31)	(32.6)	(38.2)	(36.8)	(49)	(55.1)	(52.7)	(47)	(37)	(32)	(54)	(41)	(45)	(6.2)	(12)	(10)
Pro	1	1	2	5	13	18	7	14	21			8		1	1	4	2	6
(%)	(8.0)	(3.3)	(5)	(10.9)	(9.0)	(9.5)	(17)	(20.3)	(19.1)	(0)	(9)	(16)	(0)	(1.1)	(1)	(25.0)	(3.3)	(7.8)
Controls																		
Arg				19	149				13			71			114			84
(%)	/	/	/	(51.4)	(55)	/	/	/	(50)	/	/	(50)	/	/	(59)	/	/	(60)
Arg/Pro				14	98				11			61			66			47
(%)	/	/	/	(37.8)	(36.2)	/	/	/	(42.3)	/	/	(43)	/	/	(34)	/	/	(33)
Pro				4	24				2			10			7			10
(%)	/	/	/	(10.8)	(8.8)	/	/	/	(7.6)	/	/	(7)	/	/	(13)	/	/	(7.1)

[a]Hoffmann *et al*, present study with percentage in parenthesis. [b]Summersgill *et al* (12), controls derived from oral rinses. [c]Nagpal *et al* (13), controls derived from oral scraps, of which 27% tested positive for HPV. [d]Cortezzi *et al* (14), controls derived from oral mucosal swabs, of which 10.6% tested positive for HPV. [e]Scheckenbach *et al* (16), controls derived from blood lymphocytes, on which no HPV analysis was performed. [f]Perrone *et al* (15), controls derived from blood lymphocytes, on which no HPV analysis was performed. [g]p53, p53 polymorphism, either coding homozygous for Arginin, heterozygous for Arginin and Prolin or homozygous for Prolin. [h]Pos./Neg., HPV positives and HPV negatives after HPV analysis.

In this study we investigated the HPV status and genotypic frequencies of p53 at codon 72 in 42 carcinomas of the head and neck (HNSCC) searching for reproducible patterns in the p53 allelic distribution and compared the results to the presently available literature. The underlying hypothesis is the same as postulated for cervical carcinomas: a higher susceptibility of the wild-type p53 to HPV-E6 mediated proteolytic degradation when encoding homozygously for *Arg* in exon 4 at codon 72 (11).

Analysing fresh frozen tissue specimens of HNSCC taken from 42 patients, the detected HPV prevalence of 29% is within the range that should be expected when a comparable study population employing identical methodological standards is investigated (1-4,18). The rising incidence of especially HPV associated tonsillar carcinomas with shown HPV prevalences of up to 60% (1-5) could not be demonstrated due to the small number of cases in each tumour group investigated in this study population.

Focussing on the distribution of the analysed p53 allele at codon 72, the homozygous allele for $p53_{Arg}$ was most commonly detected in tumour specimens (64%). These data are comparable with the data of the majority of previously published studies on the topic derived from different geographical regions (Germany, Italy, Brazil, and USA, see Table II for details), all of them reporting homozygosity for $p53_{Arg}$ in 52%-81% of the tumour cases (12-16). However, Nagpal *et al* (13) demonstrated only 28.8% of cases to be homozygous for $p53_{Arg}$. This might be explained by ethnic differences of the investigated Indian population compared to the other study groups discussed or because of differing risk factors besides HPV, like betel nut use habits. With 31% and 5% for $p53_{Arg/Pro}$ heterozygosity and $p53_{Pro}$ homozygosity, respectively, the demonstrated frequencies in our study are in the range of the published data for HNSCC, as well ($p53_{Arg/Pro}$: 10.4-52.7%; $p53_{Pro}$: 1-19.9%). Again only with exception of data published by Nagpal *et al* (13), all studies cited (including ours) show the genotype prevalence of $p53_{Arg}$, $p53_{Arg/Pro}$ and $p53_{Pro}$ in descending order for tumour cases. Taking into account the geographical differences, the comparison of the genotype frequencies of the p53 polymorphism among the control groups, where available, show striking similarities in the different study groups ($p53_{Arg}$ 50-59.6%, $p53_{Arg/Pro}$ 33.3-43%, $p53_{Pro}$ 7-10.8%), as well (12-16).

Although there were slight differences in the allele distribution when the HPV positive group was compared to the HPV negative group, these differences did not reach statistical significance in the present study. Of the HPV positive patients, 92% presented the $p53_{Arg}$ allele, which would be in agreement with the hypothesis of an enhancement between this allele and head and neck carcinogenesis of HPV positive tumours. $p53_{Arg/Pro}$ heterozygosity was not seen in any of the HPV positive cases and there was only one case of $p53_{Pro}$ homozygosity in the HPV negative and HPV positive HNSCC group, respectively. Cortezzi *et al* (14) and Scheckenbach *et al* (15) did not find any example for homozygosity for $p53_{Pro}$ in the HPV-positive group, at all. In contrast, Perrone *et al* (16) described just the greater frequency of $p53_{Pro}$ homozygosity in their HPV-positive tumour group as the only result with statistical significance and postulated the presence of the $p53_{Pro}$ allele as being a risk factor for the development of HPV-associated oropharyngeal SCC. The incidence of the $p53_{Pro}$ allele in the study of Nagpal *et al* (13) is described with 19.09% in the tumour group (7.6% in the control group). Those authors argue that there is a strong association of the $p53_{Pro}$ genotype with ethnicity, revealed by a significant decrease in the frequency of the $p53_{Pro}$ allele with increasing latitude. The studies from India (13) and Brazil (14) discussed herein show higher frequencies for the homozygous $p53_{Pro}$ allele when focusing on the tumour group (19.09 and 16%) compared to the results from Germany (15), Italy (16) and the USA (12) (Table II). However, when testing the control groups comparable frequencies of $p53_{Pro}$ were demonstrated in all studies (7-10.8%). Since ethnicity based influences should be detected in both, tumour and a control group, the association of latitude with p53 genotype distribution seems at least doubtful.

Due to the lack of a control group consisting of healthy individuals in this case-only study, we compared our allelic distribution data to the results of Scheckenbach *et al* (15). This study group additionally analysed the genotypic frequency of p53 codon 72 polymorphism in 193 healthy controls from Germany using blood samples. At present, further data on the normal distribution of p53 allelic variants in the German population are not available. Scheckenbach and co-workers reported a genotype distribution of 59, 35, and 7% for $p53_{Arg}$, $p53_{Arg/Pro}$ and $p53_{Pro}$, respectively. It was pointed out that these results are almost identical to those reported for the Netherlands, a neighbouring country to Germany, comparable in ethnicity and latitude. Interestingly, with an allelic distribution of 64, 31, and 5% for $p53_{Arg}$, $p53_{Arg/Pro}$ and $p53_{Pro}$, respectively, the results demonstrated for the tumour group in our study are more comparable to the data of the control group described by Scheckenbach and co-workers than to the tumour group therein. Comparing the tumour with the control group, Scheckenbach *et al* demonstrated a significant deviation between the groups with over-representation of $p53_{Arg/Pro}$ heterozygotes (45 vs. 35%) and under-representation of $p53_{Pro}$ homozygotes (7 vs. 1%) in the tumour group. However, considering all cited studies one needs to question the comparability of the presented data, in general, since there are numerous and various facts obviously influencing the data (Table II). Therefore, it is not surprising that there is no obvious tendency in distribution of the different p53 codon 72 exon 4 allele, taken alone or in association to the HPV status of the material. In conclusion, reliable interpretation of data on p53 polymorphism without inclusion of a large representative control group and many cases into the study design, apparently the weakest point in the present study, turns out to be very difficult.

In summary, the data of the present study and of the cited literature reviewed show a similar distribution of the p53 codon 72 polymorphism in control groups investigated, independent of ethnicity, latitude as well as tobacco, alcohol and betel nut consumption habits. Furthermore, there are noticeable deviations of the allele frequencies when tumour specimens are analysed (Table II), yet without reproducible

statistical significance. The latter is true for the p53 codon 72 polymorphism taken alone and, with only few exceptions, in association to the HPV status. Thus, it can be concluded that there is no codon 72 genotype obviously protecting or enhancing the p53 susceptibility to HPV-E6 mediated proteolytic degradation. Further attempts to prove such an association should be based on a large scale study, most likely in a multi-centre setting employing identical methodological standards and, moreover, applying matched controls and cases.

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