

Short Communication

p53 Codon 72 Polymorphic Variants, Loss of Allele-Specific Transcription, and Human Papilloma Virus 16 and/or 18 E6 Messenger RNA Expression in Squamous Cell Carcinomas of the Head and Neck

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Abstract

A polymorphism at codon 72 of the human tumor suppressor p53 determines translation into either arginine or proline. Yet, the impact of this amino acid variability on the risk to develop malignant tumors, particularly carcinomas associated with human papilloma virus (HPV) infections, remains unresolved because of contradictory results. To address a potential correlation between the different genotypes and the manifestation of squamous cell carcinomas of the head and neck (SCCHN), we determined the p53 codon 72 in 193 healthy subjects and 122 unselected SCCHN with known HPV status. Furthermore, loss of allele-specific transcription was analyzed in p53 codon 72 heterozygous (Arg/Pro) SCCHN and correlated with HPV

16 and/or 18 E6 transcript expression. We found a moderately increased risk (odds ratio, 1.86; 95% confidence interval, 1.0-3.3) for individuals with germ line heterozygosity to develop SCC of the pharynx. On the other hand, p53 codon 72 polymorphic variants, most notably the Arg/Arg genotype, showed no association with the presence of HPV 16 and/or 18 E6 transcript. Moreover, there was no evidence for HPV-driven selection in SCCHN with allele-specific loss of transcription. Our data suggest that the p53 codon 72 polymorphism has a minor impact on the development of SCCHN. (Cancer Epidemiol Biomarkers Prev 2004;13(11):1805-9)

Introduction

The tumor suppressor phosphoprotein p53 is a sequence-specific DNA-binding transcription factor comprising four domains: a highly charged acidic region (transactivation domain), a hydrophobic proline-rich region (protein interaction domain), a central region (DNA-binding domain), and a highly basic COOH-terminal region (oligomerization domain). Cellular stress results in the activation and post-translational stabilization of p53 (1). This up-regulation of p53 is considered to be decisive for the triggering of two major stress response mechanisms: (a) cell cycle arrest through repression of cyclin-dependent kinases and (b) programmed cell death via induction of promoters of apoptosis as well as transcription-independent pathways (2-4).

Within the proline-rich region of p53, a common sequence polymorphism at codon 72 arises from a single

base pair substitution encoding either proline or arginine. This nonconservative amino acid change is associated with altered electrophoretic mobility of the two polymorphic variants, thus suggesting structural modification of the p53 protein (5-7). Several reports have described differences for functional properties of codon 72 polymorphic wild-type p53, including (a) susceptibility to malignant transformation (8, 9), (b) ubiquitin-mediated degradation by "high-risk" human papilloma virus (HPV) E6 protein (10), (c) induction of programmed cell death (11), (d) transcriptional activity (12), (e) binding to the p53 homologue p73 (13, 14), and (f) cell cycle progression after cell damage (15).

The possible impact of p53 codon 72 genotypes on the development of tobacco-induced and HPV-related malignancies, such as lung cancer, cervical cancer, and squamous cell carcinomas of the head and neck (SCCHN), is an ongoing issue of debate. We reported recently circumstantial evidence for the inactivation of p53 in almost all SCCHN, suggesting that this event could be mandatory in the multistep process of carcinogenesis. In a series of unselected SCCHN, we identified aberrant p53 transcripts in 80% of the tumor specimens and detected HPV 16 and/or 18 E6 transcript in most of the remaining p53 wild-type tumors (16). In continuation

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of these investigations, we now analyzed the p53 codon 72 genotype in tumor specimens and a population-based control group. In addition, we determined the expressed p53 alleles in the SCCHN of Arg/Pro heterozygous individuals, thus not only loss of the p53 gene by DNA deletion but also loss of p53 expression by other mechanisms, such as mutation of the promoter or hypermethylation. Hence, in contrast to existing reports, the present study benefits from the identification of biologically relevant HPV E6 transcript (17) and considers the phenomenon of loss of transcription (18, 19).

Materials and Methods

Patients and Control Group. The study consists of 122 consecutive patients with histologically confirmed SCCHN, including all sites (13 oral cavity, 33 oropharynx, 30 hypopharynx, 44 larynx, 1 ear, and 1 nose) and stages (T_{1-4} , N_{0-3} , and $M_{0/1}$) of disease. Tumor samples were immediately snap frozen and 5 μ m sections stained with hemalaun and eosin were done to identify regions exclusively made up by tumor cells. The control group comprised 193 healthy individuals (blood donors). Patients and controls match with regard to ethnicity (white Caucasians) and residence (Germany); these are the two major determinants for variations in the p53 codon 72 polymorphism (20). Patient and tumor characteristics are summarized in Table 1. As for the SCCHN, we published recently the results of p53 exons 2 to 11 transcript analysis and HPV 16 and/or 18 E6 transcript detection (16).

p53 Genotype at Codon 72. DNA was isolated from 25 μ m sections of snap-frozen tumors and peripheral blood lymphocytes (Genomic DNA purification kit, Genra Biosystems, Minneapolis, MN). A 341-bp exon 4 fragment was amplified in a standard PCR reaction using Qiagen Mastermix (Qiagen, Hilden, Germany) and primers PU4 (5'-CACCCATCTACAGTCCCCCTTGC-3') and PD4 (5'-ACGGCCAGGCATTGAAGTCTCATG-3'). PCR amplification comprised 35 cycles of denaturing for 30 seconds at 94°C, annealing for 30 seconds at 66°C,

and extension for 30 seconds at 72°C. The PCR was preceded by 3 minutes at 94°C and followed by 7 minutes at 72°C. The samples were purified with spin columns (Millipore, Eschborn, Germany) and digested with 10 units *Bst*UI per 10 μ L for 3 hours at 60°C (New England Biolabs, Frankfurt, Germany). *Bst*UI cuts the Arg-coded allele into two fragments of 210 and 131 bp, whereas the Pro-coded allele remains intact. The digested PCR products were separated on precasted polyacrylamide gels (CleanGel, Amersham Biosciences, Freiburg, Germany) according to the manufacturer's protocol and then silver stained (DNA Silver Staining Kit, Amersham Biosciences). The established SCCHN cell lines UD-SCC 2 and UD-SCC 3 served as controls, which are Arg and Pro coded, respectively (21).

Transcript of the p53 Genotype at Codon 72. To determine loss of allele-specific transcription in tumors that developed in patients with germ line heterozygosity, a partial p53 transcript analysis was done as described previously (16). In brief, after RNA isolation from snap-frozen tumor specimens, cDNA synthesis was carried out using random hexanucleotide primers. A p53 transcript fragment including codon 72 was amplified by reverse transcription-PCR with primers 1-1 (5'-CCGGATCCGCGTGCTTTCCACGACG-3') and 1-2 (5'-CGGAATTCCTTCCACTCGGATAAGATG-3'). Amplificates were checked and purified and cycle sequencing was done using the PRISM AmpliTaq BigDye Ready Reaction Dye Terminator sequencing kit (Applied Biosystems, Weiterstadt, Germany) with primer 1-3 (5'-CCGGATCCACTTCTGAAAACAACGTTCTG-3'). The reaction products were purified and analyzed with a semiautomated sequencer (ABI 310, Applied Biosystems). To identify the threshold that is indicative for loss of transcription, we analyzed serial cDNA blends prepared from the cell lines UD-SCC 2 (Arg) and UD-SCC 3 (Pro). Discriminable peak amplitudes were reliably obtained with a mixing ratio of 1:1.5 (data not shown). Accordingly, loss of transcription was defined if the amplitude of the guanine peak was reduced to <40% of the cytosine allele (this indicates loss of transcription of the Arg-coded allele) or vice versa (this indicates loss of transcription of the Pro-coded allele).

Statistical Analysis. For statistical analysis, tumors were grouped into SCC of the pharynx (oropharynx and hypopharynx) and nonpharynx (larynx, oral cavity, ear, and nose). The Hardy-Weinberg equilibrium assumption was assessed by the standard method of comparing the observed numbers of individuals in the different genotype categories with those expected under the Hardy-Weinberg equilibrium for the estimated allele frequency. Genotype distributions in the tumor and control groups were compared by means of contingency table analysis (χ^2 test for independence). Furthermore, odds ratios (OR) and the respective 95% confidence intervals (95% CI) were determined. Fisher's exact test was done to analyze the relationship between HPV 16 and/or 18 E6 transcript expression and (a) p53 codon 72 genotype, (b) loss of p53 transcription per se, and (c) loss of allele-specific transcription of p53 codon 72. All statistical analyses were done with the Instat 3.00 Software (GraphPad, San Diego, CA) and statistical significance was taken as a nominal $P < 0.05$.

Table 1. Tumor and patient characteristics

| | Cases |
|-----------------|-------|
| Age (y) | |
| ≤ 45 | 16 |
| 46-55 | 33 |
| 56-65 | 46 |
| > 66 | 27 |
| Gender | |
| Male | 99 |
| Female | 23 |
| pT | |
| 1 | 8 |
| 2 | 26 |
| 3 | 35 |
| 4 | 53 |
| pN ¹ | |
| 0 | 28 |
| 1 | 16 |
| 2 | 72 |
| 3 | 5 |
| x | 1 |

Results

In the group of 193 control subjects, the genotypes Arg/Arg, Arg/Pro, and Pro/Pro were found in 114 (59%), 66 (34%), and 13 (7%) individuals, respectively (Table 2). For the 122 patients with SCCHN, the p53 codon 72 polymorphism genotypes divided into 66 Arg/Arg (54%), 55 Arg/Pro (45%), and 1 Pro/Pro (1%), showing a significant deviation from the control group with overrepresentation of Arg/Pro heterozygotes (45% versus 34%) and underrepresentation of Pro/Pro homozygotes (1% versus 7%). With regard to different tumor localizations, this was particularly true for Arg/Pro heterozygosity in patients who developed SCC of the pharynx (49% versus 34%) compared with patients with SCC of other sites (41% versus 34%). The calculation of ORs and 95% CIs revealed a moderately increased risk for developing SCC in heterozygote individuals (OR, 1.58; 95% CI, 1.0-2.5), especially for pharynx cancer (OR, 1.86; 95% CI, 1.0-3.3). For both populations, the genotype distributions correspond to the Hardy-Weinberg equilibrium assumption with allele frequencies of 0.762 (controls) and 0.766 (SCCHN) for Arg-coded alleles and 0.238 (controls) and 0.234 (SCCHN) for Pro-coded alleles.

High-risk HPV 16 and/or 18 E6 transcript was detected in 17 (13x HPV 16, 2x HPV 18, and 2x HPV 16 and 18) of 66 (26%) Arg homozygotes and in 20 (13x HPV 16, 6x HPV 18, and 1x HPV 16 and 18) of 55 (36%) heterozygote specimens (Table 3). No E6 transcript was present in the single Pro homozygote tumor sample. When considering the entire group of SCCHN, the proportion of carcinomas positive for HPV 16 and/or 18 E6 transcript was not significantly different in Arg homozygote compared with heterozygote individuals. Accordingly, we could not identify a higher risk for persisting HPV E6 expression in Arg homozygote samples. Seventeen of the 37 (46%) SCCHN expressing HPV 16 and/or 18 E6 transcript were in the oropharynx (33 of 122 SCCHN; 27%); hereof, 11 turned out to be heterozygous, whereas only 6 were Arg homozygous (OR, 3.06; 95% CI, 0.74-12.63).

For 26 of the 55 (47%) SCCHN having occurred in heterozygote individuals, we observed loss of transcrip-

Table 2. p53 Codon 72 polymorphism genotype distribution in healthy controls (*n* = 193) and patients with SCCHN (*n* = 122), *n* (%)

| | Arg/Arg | Pro/Pro | Arg/Pro | Total |
|---------------------------|----------|---------|----------------------|-------|
| Controls | 114 (59) | 13 (7) | 66 (34) | 193 |
| SCCHN* | 66 (54) | 1 (1) | 55 (45) [†] | 122 |
| Pharynx [‡] | 31 (49) | 1 (2) | 31 (49) [§] | 63 |
| Other sites | 35 (59) | 0 | 24 (41) [¶] | 59 |

NOTE: Differences in genotype distribution were evaluated by the χ^2 test for independence. The risk for the development of SCCHN in patients harboring heterozygote versus homozygote genotypes was determined by the calculation of OR and 95% CI (genotype distribution in control group served as reference).

* $\chi^2 = 8.52$, *df* = 2, *P* = 0.01.

[†]OR, 1.58; 95% CI, 1.0-2.5.

[‡] $\chi^2 = 5.94$, *df* = 2, *P* = 0.05.

[§]OR, 1.86; 95% CI, 1.0-3.3.

^{||} $\chi^2 = 4.51$, *df* = 2, *P* = 0.10.

[¶]OR, 1.32; 95% CI, 0.7-2.4.

Table 3. p53 Codon 72 polymorphism genotype and detection of HPV 16 and/or 18 E6 transcript in SCCHN (*n* = 122)

| | HPV negative (<i>n</i> = 85) | HPV positive (<i>n</i> = 37) | OR (95% CI) |
|--------------------------|----------------------------------|----------------------------------|------------------|
| Arg/Arg (<i>n</i> = 66) | 49 | 17 | 1.0 (reference) |
| Arg/Pro (<i>n</i> = 55) | 35 | 20 | 1.17 (0.91-1.49) |
| Pro/Pro (<i>n</i> = 1) | 1 | 0 | Not done |

NOTE: The risk for persisting expression of HPV E6 transcript in heterozygote versus homozygote individuals was determined by the calculation of OR and 95% CI.

tion; 16 (62%) tumors failed to express the Pro-coded allele and 10 (38%) tumors lost expression of the Arg-coded allele (Table 4). Thus, the Arg allele seems to be preferentially retained and expressed in SCCHN.

Finally, we correlated allele-specific transcription and HPV 16 and/or 18 E6 expression (Table 4). Loss of transcription in p53 codon 72 heterozygote SCCHN was of similar frequency in HPV-negative (17 of 35; 49%) and HPV-positive (9 of 20; 45%) tumors. Furthermore, in HPV-positive SCCHN, loss of transcription of the Pro-coded (*n* = 5) or Arg-coded (*n* = 4) allele occurred at an almost identical rate. Regarding HPV-negative tumors, a preference for loss of transcription of the Pro-coded allele was observed (11 of 17; 65%), but this difference is statistically not significant.

Discussion

The common p53 single nucleotide polymorphism encoding either Pro or Arg at residue 72 has been extensively studied as a potential risk factor for the development of malignancies. In general, two questions were asked: (a) Are the genotypes Arg/Arg, Arg/Pro, and Pro/Pro per se linked with different cancer susceptibilities? (b) Is the Arg/Arg genotype associated with HPV-related cancers?

Primarily, the genotype distribution of the p53 codon 72 polymorphism depends on ethnicity and latitude (20). Subsequent studies have shown that there are no gender-specific or age-specific differences (22-24). Because no data were available on the normal distribution of p53 allelic variants in the German population, we determined

Table 4. Loss of p53 transcription and HPV 16 and/or 18 E6 expression in SCCHN patients with p53 codon 72 germ line heterozygosity (*n* = 55)

| | No loss of transcription (<i>n</i> = 29) | Loss of transcription (<i>n</i> = 26) |
|-------------------------------|--|---|
| HPV negative (<i>n</i> = 35) | 18 | 17 Pro coded 11 Arg coded 6 |
| HPV positive (<i>n</i> = 20) | 11 | 9 Pro coded 5 Arg coded 4 |

NOTE: Fisher's exact test was done to determine the influence of the presence of HPV E6 transcript on loss of p53 transcription (*P* = 0.23) and preferential allele-specific loss of p53 transcription (*P* = 0.69).

the genotypes in healthy individuals. For the control group, we observed allele frequencies of 0.762 for Arg and 0.238 for Pro with a genotype distribution of 59%, 34%, and 7% for Arg/Arg, Arg/Pro, and Pro/Pro, respectively. These figures are almost identical to those reported for Netherlands, a neighboring country that is very well comparable regarding the main criteria such as ethnicity and latitude (25).

Several studies have been conducted to investigate the association between p53 codon 72 polymorphism and risk of developing SCCHN, but none of them found a positive correlation (25-29). We also found no such correlation for the SCCHN cases as a whole. However, our data suggest that individuals with germ line heterozygosity hold a moderately increased risk to develop SCC of the oropharynx and hypopharynx (OR, 1.86; 95% CI, 1.0-3.3). This result may reflect different roles of Arg-coded and Pro-coded p53 alleles in the development of SCCHN arising from the mucosa of the upper digestive tract (pharynx) and other sites. Site-specific differences in the etiology of SCCHN have already been reported for both environmental risk factors and genetic determinants of cancer susceptibility (30-32). Because p53 peptides harboring Pro and Arg at codon 72 are supposed to adopt different conformations (5-7), heterotetramers may interact distinct from homotetramers with target molecules differentially expressed in the head and neck region.

It has been shown that p53 protein containing Arg at codon 72 is more readily eliminated by the E6 protein of oncogenic HPV 16 and 18 (10). Also included in this preclinical report was the genotype analysis of 30 cervical tumors and 12 skin carcinomas, which revealed a significant overrepresentation of the homozygous Arg/Arg genotype compared with 41 control subjects. However, neither a subsequent study nor other groups were able to confirm this association (33-35). Likewise, no correlation between p53 codon 72 polymorphism and presence of HPV-DNA was found in SCC of the oral cavity (28, 36-38).

In the present study, we determined the expression of HPV 16 and/or 18 E6 transcript, which—compared with detection of viral DNA—is considered to be a superior indicator for biologically relevant (i.e., persisting and active) viral infection (16, 17). In the entire group of 122 SCCHN, we saw no significant correlation between p53 codon 72 polymorphism and HPV 16 and/or 18 E6 expression. This was also true for SCC of the oropharynx, which is frequently associated with oncogenic HPV (16, 38).

Depending on the employed microsatellite markers, the incidence of loss of heterozygosity near the p53 locus at 17p ranges between 38% and 71% (39-44). In a previous study, we observed loss of heterozygosity in 14 of 32 (44%) informative SCCHN with the microsatellite marker TP53 (45). However, because this marker is located ~20 kb upstream of p53 exon 1, it may not exactly reflect the p53 status of the analyzed sample. In the present study, we assessed the allele-specific loss of expression by partial sequence analysis of the transcript for SCCHN arising in p53 codon 72 Arg/Pro heterozygotes. This approach uncovers not only loss of the p53 gene and consequently loss of transcription of the

involved allele but also epigenetic phenomena like transcriptional silencing, which has been shown to occur in breast cancer and in SCCHN cell lines (18, 19). We detected loss of transcription in ~50% of the 55 heterozygous SCCHN specimens, and this loss was independent from HPV 16 and/or 18 E6 expression.

Several studies have described preferential retention of the Arg-coded allele in various solid tumors (13, 14, 46). We determined the loss of allele-specific p53 expression and looked for a possible association with the presence of HPV 16 and/or 18 E6 transcript. In our study, ~50% of the HPV-positive tumors showed loss of transcription of the Arg-coded allele, suggesting that there is no selection pressure for allele-specific loss of transcription due to HPV 16 and/or 18 E6 expression. In HPV-negative tumors, on the other hand, we found a preferential loss of transcription for the Pro-coded allele. One possible explanation for this finding is that the nonrandom loss of the Pro-coded allele in HPV-negative tumors favors a selection for mutations in the Arg-coded allele. Interestingly, inactivation of the p53 family member p73 by mutant p53 protein is, at least for some mutants, more pronounced when Arg is the amino acid at codon 72 (13, 47).

In such a clinical study, a typical and difficult problem to solve is the generalization of results obtained in small, potentially nonrepresentative tumor samples. The phenomenon of tumor heterogeneity is well established and the impact of HPV 16 infection on polyploidy and chromosomal instability has been emphasized recently (48, 49). However, genetic and epigenetic alterations of p53 were described to be remarkable stable in SCCHN (19).

Microdissection is often used to control for tumor cell enrichment in DNA-based loss of heterozygosity analyses. Considering the special sensitivity of RNA to degradation, we did frozen sections of the tumor samples to identify "regions of interest." Nevertheless, loss or silencing of both p53 alleles prevents p53 expression in tumor cells, and wild-type p53 expressed in normal cells—due to the amplification by reverse transcription-PCR—will be the only present. Hence, these tumors will be misclassified (i.e., they show no loss of transcription).

In summary, p53 codon 72 seems to predict a moderate risk for the development of oropharyngeal and hypopharyngeal cancer in individuals with germ line heterozygosity. There is no evidence for the preferential infection of any genotype with high-risk HPV 16 and/or 18, and expression of HPV E6 is not involved in allele-specific loss of transcription.

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