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Advances in Brief

Is the p53 Inactivation Frequency in Squamous Cell Carcinomas of the Head and Neck Underestimated? Analysis of p53 Exons 2–11 and Human Papillomavirus 16/18 E6 Transcripts in 123 Unselected Tumor Specimens

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Abstract

Mutations and interaction with high-risk human papillomavirus (HPV) E6 oncoprotein are well-established mechanisms of p53 inactivation. In a series of 123 unselected squamous cell carcinomas of the head and neck (SCCHN), we performed sequence analysis of the entire coding region of p53 transcript and determined the presence of the E6 transcripts of HPV 16 and 18. Aberrant p53 transcripts were identified in 97 (79%) SCCHN. HPV 16 and/or 18 E6 transcripts were detected in 37 (30%) tumor specimens, including 20 (77%) of the 26 p53 wild-type tumors. The likely inactivation of p53 in 117 (95%) of the 123 SCCHN suggests that this event could be obligatory in the multistep process of carcinogenesis.

Introduction

Abrogation of normal p53 function appears to be the most prevalent molecular alteration in human cancers, which allows tumor cells to survive, proliferate, and continue to progress despite the accumulation of mutations. Currently, p53 mutations are thought to contribute to the manifestation of ~40% of SCCHN[3] (1, 2). There have been many attempts to establish p53 as a marker of progression, prognosis, and response to antineoplastic treatment. However, a considerable range of mutation frequencies has been reported based on different methods of assessment of the p53 status, such as immunohistochemical detection of accumulated protein or partial sequence analysis of genomic DNA (3). As yet, the predictive value of p53 mutations remains unresolved because of contradictory results (4).

To establish a firm basis for the assessment of the p53 status in a large series of SCCHN, we sequenced the entire coding region of p53 transcript, *i.e.*, exons 2–11. In addition, we determined the frequency of E6 transcripts of HPV 16 and 18 in these tumors. Inactivation of p53 can occur through its enhanced degradation via complex formation with the E6 oncoprotein of the high-risk HPV 16 and 18 (5), and this mechanism is considered to be particularly relevant in SCCHN (2, 6).

Materials and Methods

Sample Collection. Specimens of 123 unselected and histologically confirmed SCCHN were investigated, including all sites (14 p.o. cavity, 33 oropharynx, 30 hypopharynx, 44 larynx, 1 ear, and 1 nose) and stages (T_{1-4}, N_{0-3}, $M_{0/1}$) of disease.

Analysis of p53 Transcript. In a previous study, we demonstrated that RNA for p53 transcript analysis can be readily isolated from frozen sections of SCCHN (7). Ample levels of mutated p53 transcript were present in the tumor cells, and wild-type p53 expression in adjacent normal cells did not interfere with the detection of aberrant transcripts. The same protocol was used in the present study. Briefly, tumor RNA was isolated from a 25-μm section of snap-frozen tumor specimens with the QIAshredder and RNeasy-Kit (Qiagen, Hilden, Germany) and reverse transcribed using random hexanucleotides and Superscript (Life Technologies, Inc., Eggenstein, Germany), according to the manufacturer's instruction. Aliquots of the conversion mixture were amplified by PCR in a thermal cycler (Biometra, Göttingen, Germany) with specific primers (Table 1) and Qiagen Master Mix (Qiagen), subdividing the p53 transcript into three overlapping amplification products (7, 8). The PCR comprised 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 64°C, and extension for 1 min at 72°C. The amplification reaction was preceded by 3 min at 95°C and followed by 7 min at 72°C. The quality of RNA preparations was controlled by RT-PCR of glyceraldehyde-3-phosphate dehydrogenase. Amplificates were checked on an ethidium bromide-stained agarose gel. The samples were purified with spin columns (Millipore, Eschborn, Germany) and mixed with specific oligonucleotide primers and PRISM Big-Dye Terminator Cycle Sequencing Ready Reaction kit (ABI, Weiterstadt, Germany). The cycle sequencing reaction consisted of 25 cycles of denaturation for 15 s at 96°C and annealing and extension for 4 min at 60°C. The sequencing products were purified using gel filtration columns (DyeEx Spin Kit; Qiagen) and analyzed with a semiautomated sequencer (ABI 310).

Detection of HPV 16/18 E6 Transcript. The E6 transcripts of HPV 16 and 18 were amplified by RT-PCR from tumor and positive control cDNAs (CasKi: HPV 16; HeLa: HPV 18) using specific primers (Table 2). The reaction included 35 cycles of denaturation for 45 s at 95°C, annealing for 45 s at 56°C (HPV 16) or 67°C (HPV 18), and extension for 40 s at 70°C. The PCR was preceded by 2 min at 95°C and followed by 7 min at 72°C. Amplification products with sizes of 209 and 400 bp for HPV 16 and 18, respectively, were separated on agarose gels, denaturated in 0.5 M NaOH and 1.5 M NaCl for 10 min, neutralized in 0.5 M Tris-HCl (pH 7.5) and 1.5 M NaCl, and blotted overnight onto a nylon membrane (ABI) using a standard upward capillary transfer protocol. The membrane was dried at room temperature and cross-linked with UV light (312 nm) for 5 min. Using HPV cDNA as templates, specific probes were amplified by RT-PCR, including 23 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C. The reaction was preceded by 3 min at 95°C and followed by 5 min at 72°C. Amplification products were checked on an ethidium bromide-stained agarose gel (HPV 16: 123 bp; HPV 18: 267 bp), purified with spin columns (Millipore), and labeled with alkaline phosphatase (AlkPhos labeling kit; Amersham, Freiburg, Gemany). Briefly, probes (100 ng/10 μl) were boiled for 5 min and subsequently cooled for 5 min on ice. Ten μl of reaction buffer, 2 μl of labeling reagent, and 10 μl of cross-linker (1:10 cum aqua dest.) were added, and the mixture was incubated for 1 h at 37°C. The membrane was incubated in hybridization buffer (AlkPhos labeling kit) for 1 h at 55°C, probes were added, and hybridization proceeded overnight at 55°C. After washing with primary [2 M urea, 0.1% SDS, 50 mM $NaPO_4$ (pH 7), 150 mM NaCl, 1 mM $MgCl_2$, and 0.2% blocking reagent of AlkPhos labeling kit; 2 × 10 min; 55°C] and secondary [50 mM Tris (pH 10), 100 mM NaCl, and 2 mM $MgCl_2$; 2 × 10 min; room temperature] buffers, the membrane was covered with CDP-Star (New England Nuclear, Cologne, Germany), incubated for 5 min in the dark, and exposed to X-ray film. Detection of HPV 16/18 E6 transcript was confirmed in independent hybridization experiments.

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[3] The abbreviations used are: SCCHN, squamous cell carcinomas of the head and neck; RT-PCR, reverse transcription-PCR; HPV, human papillomavirus.

Table 1 *Primers for p53 cDNA amplification and sequencing*

Primers containing additional sites: *Bam*HI, CCGGAATTCC; *Eco*RI, CGGATCC.

Fragment	Tag	Position	Sequence
p53-1	1-1[a–c]	146–164	*Bam*HI-GCG TGC TTT CCA CGA CG
	1-2[a,d]	810–790	*Eco*RI-CCT TCC ACT CGG ATA AGA TG
	1-3[b,c]	281–302	*Bam*HI-ACT TCC TGA AAA CAA CGT TCT G
p53-2	2-1[a,c]	554–573	*Bam*HI-TTG CAT TCT GGG ACA GCC AA
	2-2[a,d]	1273–1253	*Eco*RI-GGC ATC CTT GAG TTC CAA GG
	2-3[b,c]	747–766	*Bam*HI-ACC ATG AGC GCT GCT CAG AT
	2-4[b,d]	1026–1007	*Eco*RI-TCA AAG CTG TTC CGT CCC AG
p53-3	3-1[a–c]	970–989	*Bam*HI-CAC CAT CAT CAC ACT GGA AG
	3-2[a,d]	1506–1487	*Eco*RI-CTG ACG CAC ACC TAT TGC AA

[a] Primer for cDNA amplification.
[b] Primer for sequencing analysis.
[c] Sense primer.
[d] Antisense primer.

Results

Transcript analysis of the entire coding region of p53 led to the identification of 103 aberrations in 97 (79%) of the 123 SCCHN, comprising 91 tumors with one and 6 with two alterations (Table 3; Fig. 1). We detected 79 point mutations (70 missense, 6 nonsense, and 3 silent mutations), 18 deletions, and 6 insertions. Ten deletions and 1 insertion involved exon borders and were, therefore, considered to be the consequence of erroneous splicing. Only four of these splice site mutations resulted in the loss of an entire exon or insertion of a complete intron in the respective transcript, whereas the remainder showed a partial loss of exons. All tumors with silent point mutations harbored a second aberration: deletions in two cases and a missense point mutation in one case. Hot spots were identified at codons 155 ($n = 6$), 245, 248, and 282 ($n = 5$ each). Of the 103 aberrations, 23 (22%) were located outside the most commonly analyzed region of exons 5–9, including 13 point mutations, 8 deletions, and 2 insertions. Hence, deletions and insertions accounted for only 23% (24 of 103) of all aberrations, whereas 43% (10 of 23) clustered outside exons 5–9. In addition, the present transcript analysis allowed the determination of five p53 polymorphisms described previously. Expression of the rare p53 alleles for codons 21, 36, 47, and 213 were found in 0, 2 (1.6%), 0, and 5 (4%) SCCHN, respectively. On the other hand, codon 72 polymorphism was frequently observed; 83 (67%) tumors encoded arginine, 11 (9%) proline, and 29 (24%) both amino acids at this position.

HPV 16 and/or 18 E6 transcripts were detected by RT-PCR and gel electrophoresis in 20 (16%) tumor specimens (16 × HPV 16 E6, 4 × HPV 18 E6). To enhance both sensitivity and specificity of detection, we performed subsequent Southern blot analysis and hybridization with HPV E6-specific probes. Seventeen additional tumors were found to harbor HPV 16 and/or 18 E6 transcript, and two tumors initially identified as HPV 18 E6 positive also expressed HPV 16 E6. HPV 16 and/or 18 E6 expression thus occurred in 37 (30%) of the 123 SCCHN (27 × HPV 16 E6, 7 × HPV 18 E6, and 3 × HPV 16 and 18 E6; Table 4). These included 20 (77%) of the remaining 26 p53 wild-type SCCHN, whereas only 17 (18%) of the 97 p53 mutant tumors contained HPV 16 and/or 18 E6 transcripts.

Merging of the results of p53 transcript analysis and HPV 16 and/or 18 E6 transcript detection suggests that inactivation of the tumor suppressor p53 through either mutation or high-risk, HPV E6-mediated degradation occurred in 117 (95%) of the 123 SCCHN (Table 4).

Finally, characteristics of patients (age and gender) and tumors (localization, grading, and Tumor-Node-Metastasis stage) were correlated with the occurrence and type of both p53 alterations and E6 transcripts. Mutations of p53 were equally frequent in carcinomas of the hypopharynx (27 of 30; 90%), larynx (35 of 44; 80%), and p.o. cavity (11 of 14; 79%). In contrast, aberrant p53 transcripts were identified in only 15 (45%) of 33 oropharyngeal tumors. Correspondingly, the prevalence of HPV 16/18 E6 transcripts was considerably higher in carcinomas of the oropharynx (17 of 33; 52%) as compared with those of the hypopharynx (8 of 30; 27%), p.o. cavity (4 of 14; 29%), or larynx (8 of 44; 18%). None of the other parameters showed any significant correlations (data not shown).

Discussion

Here, we report the results of a detailed analysis of the p53 status in a large series of unselected SCCHN, applying both p53 transcript sequence analysis of the entire coding region and HPV 16/18 E6 transcript detection. With this approach, we found a p53 aberration frequency of ~80%. Furthermore, the majority of p53 wild-type tumors turned out to harbor the oncogenic E6 transcript. Thus, the percentage of SCCHN that presumably lacks normal p53 function amounts to an impressive 95% in this series.

In addition, it must be remembered that various other mechanisms are known to interfere with normal p53 activity in cancer cells, *e.g.*, overexpression of mdm-2 or inactivation of $p14^{ARF}$ (9, 10). Moreover, biallelic loss of the gene or transcriptional silencing of p53, both of which have been reported in SCCHN cell lines (7), results in a complete loss of transcript in tumor cells. Because of wild-type p53 transcript expression in adjacent normal cells, which is amplified in an RT-PCR-based approach, such aberrations and epigenetic mechanisms remain unidentified in the analysis of tissue samples. The present study, therefore, provides strong support for the concept of p53 inactivation being a prerequisite for the development of SCCHN.

It is well accepted that p53 regulates cell cycle progression as well as apoptotic cell death (11). Thus, the inactivation of the p53 stress response pathway would provide precancerous cells with a selective advantage for unregulated growth and the acquisition of genetic alterations on the one hand (12). On the other hand, it would supply manifest cancer cells with a pivotal mechanism to avoid apoptosis, which is otherwise promoted through the activation of oncogenes and accumulation of DNA damage in the multistep process of carcinogenesis (13, 14).

Because the *p53* gene spreads over >20 kb, and because the majority of aberrations are missense mutations within the DNA-

Table 2 *Primers for HPV 16/18 E6 transcript detection*

Fragment	Sequence
HPV 16 E6	GCT TTG AGG ATC CAA CAC GC[a]
Blot fragment	TGC AGC ACG AAT GGC ACT GG[b]
HPV 16 E6	AAT GTT TCA GGA CCC ACA GG[a]
Hybridization probe	CTC ACG TCG CAG TAA CTG TT[b]
HPV 18 E6	GCT TTG AGG ATC CAA CAC GG[a]
Blot fragment	TGC AGC ACG AAT GGC ACT GG[b]
HPV 18 E6	CGG AAC TGA GCA CTT CAC TG[a]
Hybridization probe	CAC CGC AGG CAC CTT ATT A[b]

[a] Sense primer.
[b] Antisense primer.

Table 3 *Itemization of 103 p53 aberrations identified in 97 SCCHN by examination of the entire coding region via transcript analysis in a series of 123 unselected SCCHN*

p53 Aberrations	103 (100%)	Outside exons 5–9
Point mutations	79 (77%)	13/79 (16%)
Missense mutations	70	8
Nonsense mutations	6	5
Silent mutations[a]	3	0
Deletions/presumed splice mutations	18 (17%)	8/18 (44%)
Incorrectly spliced transcripts	10	5
Insertions/presumed splice mutations	6 (6%)	2/6 (33%)
Incorrectly spliced transcripts	1	1
Located outside exons 5–9		23/103 (22%)

[a] All of these with one additional mutation.

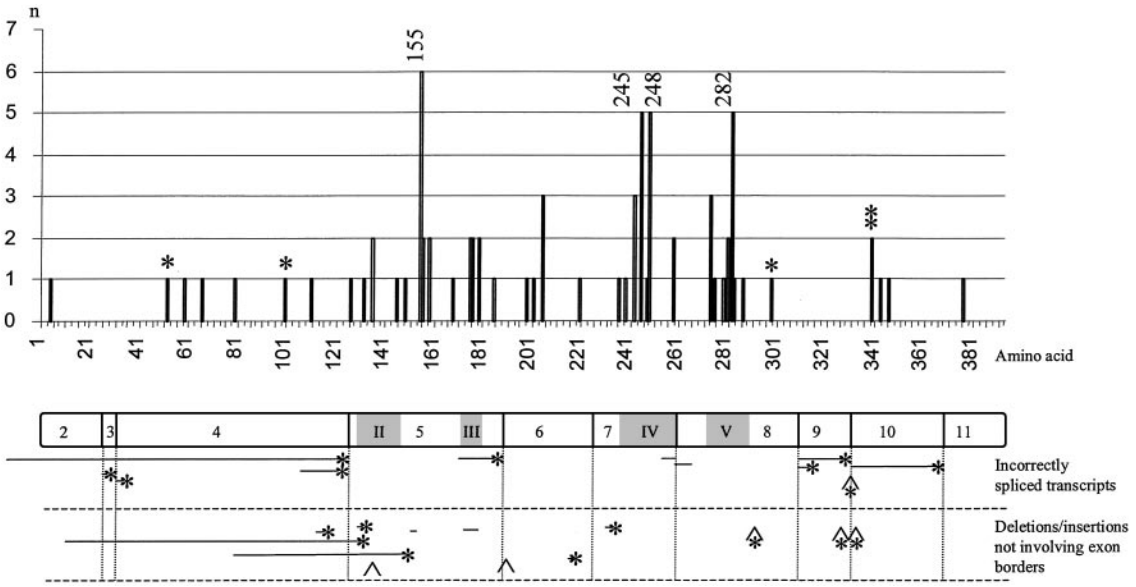

Fig. 1. Distribution of 103 p53 aberrations identified by examination of the entire coding region via transcript analysis in 97 of 123 unselected SCCHN. *n*, number of point mutations; |, point mutation; —, deletion; ^, insertion; *, truncation of translation. Exons 2–11 are depicted as *open boxes*, and conserved domains II-V are inserted as *shaded boxes*.

binding region, sequence analysis of genomic DNA is commonly restricted to exons 5–8 or 5–9. For SCCHN, such investigations have generated an average p53 mutation frequency < 50%. In a comprehensive study, Gillison *et al.* (2) performed genomic DNA sequence analysis of exons 5–9 in a subgroup of 166 of 253 SCCHN and detected mutant p53 in 65 (39%) tumors. More recently, Sisk *et al.* (15) analyzed the DNA of exons 5–8 and identified mutant p53 in only 8 (25%) of 32 SCCHN. In contrast, analysis of the entire coding region, *i.e.*, exons 2–11, also detects mutations outside the so-called hot spot region, which, as demonstrated here, may reveal an important percentage of p53 mutations. In the present series of 123 SCCHN, 23 (22%) of the 103 p53 mutations were located outside exons 5–9.

Mutations at exon/intron borders can result in incorrectly spliced transcripts, causing the complete or partial loss of exons or insertion of intronic sequences. In comparison with sequence analysis of genomic DNA, the transcript-based approach allows the accurate determination of the resulting, erroneously spliced transcript. Including our own preclinical study (7), two *in vitro* investigations and a single clinical study comprising 20 tumor specimens have used previously p53 transcript analysis of the entire coding region in SCCHN (7, 16, 17). In accordance with the present study, they also revealed high p53 mutation frequencies and noticeable numbers of aberrantly spliced transcripts. Furthermore, two studies analyzing both genomic DNA and transcript of p53 in SCCHN cell lines (7) and breast carcinoma specimens (18) have demonstrated that mutated *p53* alleles sometimes fail to produce the corresponding transcript, probably attributable to epigenetic mechanisms or deletion of promotor sequences. Again, analysis of p53 RNA provides the advantage of detecting the expressed transcripts, which are likely to be translated into protein.

Table 4 *Identification of p53 aberrations by examination of the entire coding region via transcript analysis and detection of HPV 16/18 E6 transcripts in a series of 123 unselected SCCHN*

SCCHN	123	(100%)
Aberrant p53 transcript	97	(79%)
Single aberrations	91	
Double aberrations[a]	6	
HPV 16/18 E6 transcript	37	(30%)
HPV 16 E6 transcript	27	
HPV 18 E6 transcript	7	
HPV 16 and 18 E6 transcripts	3	
HPV 16/18 E6 transcripts in p53 wild-type tumors	20	(16%)
Inactivation of p53	117	(95%)

[a] Three of these with one silent mutation.

In the present series, p53 transcripts derived from the rare alleles of polymorphisms described previously at codons 21, 36, 47, and 213 were identified in only 7 (6%) SCCHN, which is well within the range reported for normal populations.[4] Codon 72 polymorphism, on the other hand, was a frequent event; in the majority of cases, we detected transcripts coding for the arginine variant, followed by arginine-proline-coding transcripts, and solely proline-coding transcripts. This amino acid exchange is suggested to affect certain biochemical and functional properties of p53, including the interaction with viral E6 oncoprotein and induction of apoptosis (19, 20). However, because allele frequencies of the codon 72 polymorphism are known to show considerable differences in distinct populations (21), it remains to be determined whether the prevalence of transcripts coding for arginine reflects the allele distribution in the German population or may indeed confer an enhanced susceptibility to SCCHN.

Increase in the turnover of p53 protein is believed to be the most significant oncogenic property of the multifunctional HPV 16 and 18 E6 proteins (5). Because we have observed the respective E6 transcripts in 37 SCCHN, their presence in these tumors is likely to be of biological relevance (6). In this regard, the high frequency of E6 transcript detection in p53 wild type (77%) as compared with p53 mutant (18%) SCCHN is particularly remarkable. According to previous reports and confirmed by us, the majority of E6-positive carcinomas was localized in the oropharynx (2, 15). Prospective studies should further examine the potential clinical relevance of p53 mutations and/or high-risk HPV E6 expression in this particular disease.

The pivotal role of p53 in cancer development and progression, as well as tumor response to both radiotherapy and antineoplastic drugs, has tempted many researchers to establish the p53 status as a prognostic factor for survival or predictive marker for treatment outcome. Thus far, however, such studies have not succeeded in clearly demonstrating the anticipated correlations but instead resulted in contradictory observations (10). There may be several reasons for this: (*a*) in most instances, the p53 status was assessed with inadequate methods, such as immunohistochemistry, single-strand conformation polymorphism analysis, or partial sequence analysis (7, 10, 17); (*b*) diverse types and localizations of p53 mutations as well as other mechanisms

[4] Internet address: www.iarc.fr/p53/Polymorphism.html.

of p53 inactivation are supposed to exert differential effects on cancer behavior and, consequently, on the course of disease (2, 22, 23). As shown here, 95% of the SSCHN lack normal p53 function; therefore, inactivation of p53 *per se* is not a suitable choice for a prognostic or predictive marker; and (*c*) elementary requirements for planning, conduction, and analysis of clinical studies on prognostic and predictive factors are frequently disregarded. In particular, this applies to the utilization of historical data, sample size, heterogeneity of patients with respect to disease and treatment, and biometric considerations (24, 25).

In the past, the incidence of p53 mutations in SCCHN, and potentially other malignancies, has been considerably underestimated. It is quite obvious that a significant number of p53 aberrations is missed when the analysis is confined to DNA of exons 5–8 or 5–9. Furthermore, the majority of p53 wild-type SCCHN expresses high-risk HPV E6 transcript. The likely inactivation of p53 in almost all SCCHN has a major impact on the interpretation of earlier reports and the design of future investigations on p53 in this disease. Finally, this result emphasizes the potential of novel treatment strategies that either restore p53 function or target p53 dysfunction (26, 27).

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