

# Molecular and immunological aspects of p53 and p53-autoantibodies in head and neck squamous cell carcinoma

C. CORDES, J. VON LINGEN, T. GÖRÖGH, P. AMBROSCH, S. GOTTSCHLICH and M. HOFFMANN

Department of Otorhinolaryngology, Head and Neck Surgery, Christian-Albrechts-University Kiel, Germany

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**Abstract.** The tumour suppressor protein p53 (wild-type = wt-p53) is of major importance in the genetic integrity of the cell. Mutations of the p53-gene (mt-p53) are the most frequent genetic aberrations identified in different tumour entities. As analyzed in a wide variety of human malignomas, mt-p53 evokes a specific immune response. Yet, the possible occurrence of p53-autoantibodies in patients with head and neck squamous cell carcinomas (HNSCC) correlated to p53-mutations, p53 in sera and p53-overexpression in tissue has not been previously investigated. For the first time, the p53 status in 24 HNSCC patients was analyzed in the present study. The following parameters were investigated: analysis of mutation frequency of the p53-gene by direct sequencing of the exons 5-9, immunohistochemical detection of p53, measurement of the wt- and mt-p53-protein in sera by ELISA and p53-autoantibodies in sera by ELISA. Mutations of the p53-gene were detected in four (17%) patients. Overexpression of wt-p53 was detected by immunohistochemistry in 18 out of 24 (75%) tumours. In 8 (33%) patients the p53-protein was also detectable in sera, whereas in just one of these eight patients p53-autoantibodies were detectable simultaneously. Overall 6 out of 24 (25%) patients were found to be positive for serum p53-autoantibodies. Of these 6 cases, 5 could be assigned to tumours with immunohistochemically measurable wt-p53-overexpression. There was no correlation between p53-overexpression in tissue and p53-protein levels in sera or between p53-autoantibody levels in sera, nor in mutation frequency of the p53-gene and p53-overexpression in tissue. The results presented herein support the hypothesis that strong accumulation of p53 in the tissue is an important prerequisite for development of p53-autoantibodies. However, there must be further, yet unknown factors that

influence the p53-autoantibody production because p53-autoantibodies were not identified in sera in each case of p53-accumulation in the tissue.

## Introduction

Every year 500,000 new cases of cancer in the head and neck region occur worldwide. More than 90% of these represent squamous cell carcinomas (HNSCC) (1). The main risk factors for developing HNSCC are the carcinogenic substances of tobacco smoke and alcohol. A correlation of elevated p53 expression in HNSCC with heavy smoking (2) and heavy smoking and drinking has been shown by Field *et al* (3). The p53-gene is the most frequently mutated gene in all human malignomas (4). More than 50% of human cancers show mutations of the p53-gene. p53-mutations in HNSCC are described in 40-60% of cases, thus they are one of the most frequent mutations in these tumours (5).

The p53-gene is located at the short arm of chromosome 17 band 17p13, consisting of 11 exons. Its product, the wild-type p53-protein (wt-p53), is a phosphoprotein localised in the cell nucleus. Wt-p53 contains a DNA-binding site, an oligomerization domain and a transcription activation domain. The molecular mass is 53 kD and it consists of 393 amino acids (6).

In regular function, wt-p53 upregulates growth arrest and apoptosis-related genes in response to stress signals, thereby influencing programmed cell death, cell differentiation and cell cycle control mechanisms. wt-p53 yet can be chaperoned to the cytoplasm by the negative regulator MDM2, an E3 ubiquitin ligase. MDM2 is upregulated in the presence of active p53 and MDM2 polyubiquitinates p53 for proteasome targeting. p53 fluctuates between latent and active (DNA-binding) conformations and is differentially activated through post-translational modifications including phosphorylation and acetylation.

Especially mutations of exon 5-9 encoding the central binding site of the protein lead to severe misregulations. Mutations of this region are considered to cause malignant transformation of the cell. Moreover, the mutated p53-protein (mt-p53) inactivates wt-p53 by producing complex formation. It is implied that mutation of only one allele of p53 produces complete inactivation of p53 which is in contrast to other tumour suppressor genes (7). p53 alterations may result in release of the protein from the core of the cell into the serum

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*Correspondence to:* Dr Christian Cordes, Department of Otorhinolaryngology, Head and Neck Surgery, Christian-Albrechts-University Kiel, Arnold-Heller-Strasse 14, D-24105 Kiel, Germany  
E-mail: ccordes@hno.uni-kiel.de

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Table I. Patient characteristics and overview of the results.<sup>a</sup>

Patient	Gender	Age	Anatomical tumour site	TNM	Grading	Mutation	p53 AAB	p53 Protein	IHC
1	Male	52	Base of tongue	T4N2cM0	G3	Exon 7			+++
2	Male	54	Larynx	T3N0M0	G2	Exon 7		+	+++
3	Female	43	Larynx	T1N0M0	G2			+	-
4	Male	84	Oropharynx	T2N2bM0	G3				+++
5	Male	60	Hypopharynx	T3N2bM0	G2			+	-
6	Male	42	Hypopharynx	T4N3M1	G3	Exon 6			++
7	Male	52	Oropharynx	T2N1M0	G2				+
8	Male	60	Larynx	T1N0M0	G2			+	+++
9	Male	48	Base of tongue	T1N0M0	G2				++
10	Male	53	Larynx	T4N2cM0	G2		+	+	+++
11	Female	59	Oropharynx	T2N1M0	G3			+	+++
12	Male	75	Hypopharynx	T4N2bM0	G2				+++
13	Female	39	Oropharynx	T4N2cM0	G3		+		+++
14	Male	46	Hypopharynx	T3N2aM0	G3	Exon 5			-
15	Male	62	Hypopharynx	T4N3M1	G1			+	+++
16	Male	49	Larynx	T2N0M0	G2		+		+++
17	Male	63	Base of tongue	T2N1M0	G2		+		++
18	Male	59	Base of tongue	T4N1M0	G2				++
19	Male	70	Larynx	T3N0M0	G2				+++
20	Female	60	Oropharynx	T1N0M0	G2				-
21	Male	64	Hypopharynx	T3N2bM0	G2				-
22	Male	46	Base of tongue	T1N2bM0	G3			+	+++
23	Male	62	Oropharynx	T4N1M0	G2		+		-
24	Male	45	Larynx	T2N1M0	G2		+		+++

<sup>a</sup>p53 AAB, p53-autoantibodies; IHC, immunohistochemistry.

and this may lead to p53-autoantibody production. Crawford and colleague (8) first described this phenomenon in breast carcinoma which is reported for all cancers in 5-40% of the cases.

The role and the clinical impact of p53-autoantibodies are discussed controversially. The sensitivity of p53-autoantibodies as tumour marker in cancer patients is low, whereas the specificity as a tumour marker is high, since these autoantibodies only appear in cancer patients and in chronic inflammatory disease (9). As established for breast and bronchial carcinomas, the occurrence of p53-autoantibodies seems to be associated with poor prognosis of the patients (10). Furthermore, for patients with bladder and colon cancer serum anti-p53 antibody positive tumours had a worse prognosis than those with negative serum levels, regardless of the p53 status of the tumour (11). For HNSCC, the correlation of autoantibody occurrence and poor prognosis seems to be true as well, yet without statistical significance (12). The induction and formation process of p53-autoantibodies is poorly understood. It is hypothesized that p53-overexpression in the tissue might be capable of activating the production of p53-autoantibodies in sera (13). Other reasons for p53-autoantibody production might be structural alterations of p53-protein due to mutation of the protein. The p53-gene-mutations may lead to a modified protein formation and newly

expressed epitopes of the p53-protein subsequently leading to modification of the central binding site of the protein and to develop antigenic character (14). However, Schlichtholz *et al* (15) and Maass *et al* (16) suggest that tumour cell necrosis might release p53 from the cell subsequently accumulating in the serum thus being presented to the immune system. If this was true, not only p53-autoantibodies but also p53 itself should be detectable in sera.

To contribute to this issue, we examined p53 genetic mutation, p53 protein-expression in tissue, wt-p53 and mt-p53 expression in sera, and p53-autoantibodies in sera. As to our knowledge, this is the first study comparing the results of these parameters.

## Materials and methods

**Patients.** Serum and tissue specimens were obtained from 24 patients with HNSCC. Five tumours were localised in the base of tongue, 6 in the oropharynx, 6 in the pharynx and 7 in the larynx. All patients were treated in the Department of Otorhinolaryngology, Head and Neck Surgery, Christian-Albrechts-University Kiel, Germany. The age of the patients was from 39 to 84 years (56.1±10.9). Of the 24 patients, 20 were male and 4 female. Tumours were classified according to the TNM classification of the Union Internationale Contre

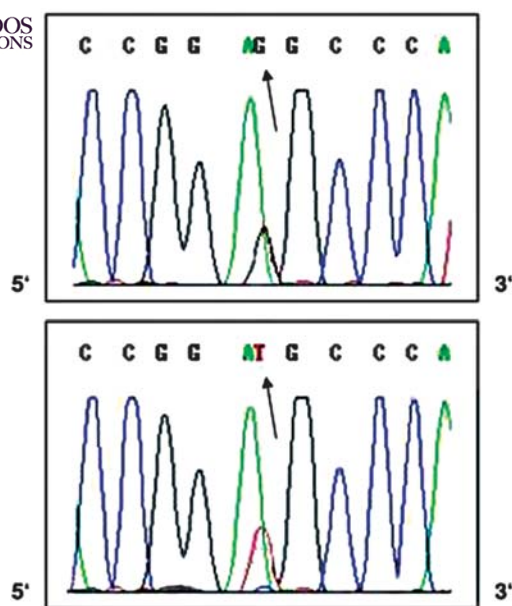


Figure 1. Part of a 5'-3' sequence profile of exon 7. Arrows indicate heterozygotic point mutation in codon 249. The upper image shows the wild-type signal (G), the lower marks the mutated gene locus (T).

Cancer (UICC) from 1987 and by grade of differentiation, age and gender (Table I). Pretherapeutic serum probes were obtained and stored in liquid nitrogen. All samples were retrieved following informed consent approved by the local ethics committee (AZ: D413/03).

**Sequencing of exons 5-9.** Prior to sequencing, DNA was extracted using the DNAeasy kit (Qiagen, Germany) and amplified by PCR using oligonucleotides specific for exon 5-9. PCR was carried out using a thermocycler with following conditions for 100  $\mu$ l reaction mixture: 10  $\mu$ l 10-fold PCR buffer, 2.5  $\mu$ l of 50 mM  $MgCl_2$ , 0.5  $\mu$ l of 5 U/ $\mu$ l Taq DNA polymerase, 2  $\mu$ l of 10 mM dNTP, 1  $\mu$ l of 100  $\mu$ M sense-anti-sense primer each and 100 ng template DNA in 83.1  $\mu$ l  $H_2O$  (all PCR reagents from Boehringer, Germany). PCR was run

for 35 cycles at 96°C for 10 sec denaturizing, 59°C for 5 sec annealing and 60°C for 4 min elongation.

After PCR the amplicons were purified using a GFX purification kit (Pharmacia, Germany), then upstream and downstream sequenced according to the method previously described by Sanger *et al* (17) with a computer assisted genetic analyzer (ABI Prism 310 genetic analyzer, Applied Bio-systems, Germany). The resulting sequence data was compared with p53 wild-type sequences (Fig. 1).

**Immunohistochemistry.** Routine staining of each tissue probe was done by haematoxylin and eosin (H&E). Immunohistochemical staining was performed using a primary monoclonal antibody (DO-7) against the protein p53 (Dako, Denmark). Negative controls were obtained by omission of the primary antibody and by incubating the primary antibody with specific blocking peptide in 5- to 10-fold molar excess before staining. Then immune histochemical core staining was evaluated from 0 to 3 fold positive (Fig. 2).

**Quantitative measurement of p53.** Sandwich-ELISA kit (Oncogene Research Products, USA) was used to measure p53-protein and p53-autoantibodies in the sera. Photometric measurement was carried out at 490 and 630 nm, respectively, using the ELISA-Reader MR500 (Dynatech, Germany). Samples with an average p53-protein concentration of  $\geq 300$  pg/ml were assessed as positive.

**Statistical analysis.** Fischer's exact test with SPSS statistical software (version 13.0) was used to assess significant differences. The data were considered statistically significant at  $p \leq 0.05$ .

## Results

Of the 24 tumour samples used, p53-gene sequencing revealed mutations in 4 out of 24 cases (17%). In all cases missense mutations occurred i.e. mutations in which nucleotides were changed, resulting in a codon encoding for a different amino acid (Table II).

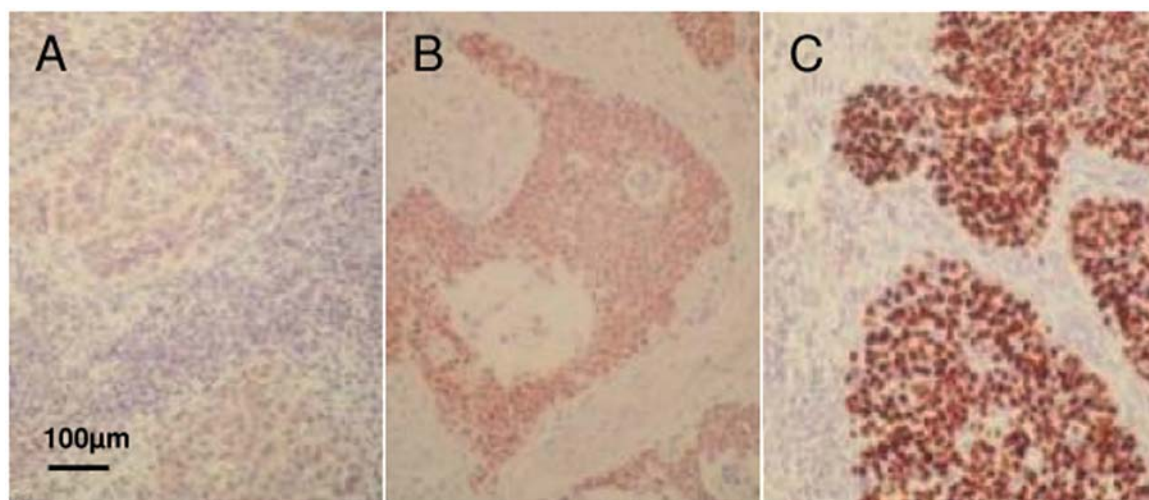


Figure 2. Immunohistochemical staining showing weak (A), moderate (B), and strong (C) nuclear accumulation of p53 (brown) in invasive growing squamous cell carcinomas.



Table II. Gene locus, change in nucleotide sequence and resulting amino acid change in the detected mutations of p53 gene.

Mutated regions	Codon	Change in nucleotide sequence	Amino acid change
Exon 5	173	Transversion GTG→TTG	Valine → leucine
Exon 6	214	Transversion CAT→GAT	Histidine → aspartic acid
Exon 7	242	Transversion TGC→GGC	Cysteine → glycine
Exon 7	249	Transversion AGG→ATG	Arginine → methionine

H&E staining confirmed the presence of HNSCC in the tissue samples which were evaluated semi-quantitative by means of immunohistochemistry. p53-overexpression was featured in 18 out of 24 cases (75%). Weak expression was detected in only 1 case, moderate expression in 4 cases and strong p53-expression in 13 cases. In the remaining 6 cases no p53-expression was detectable.

Wt-p53 and mt-p53 protein was quantified by ELISA in serum samples taken from 24 tumour patients. Overall, p53 concentrations were between 66 and 1040 pg/ml. Eight out of the 24 serum samples (33%) contained >300 pg/ml and were considered as positive for p53 in sera.

Considering the total number of samples analysed, there was no significant correlation between p53-autoantibodies in sera and p53-overexpression in the tissue ( $p=0.5$ ). p53-autoantibodies were detected in sera of only 6 out of 24 patients (25%). However, in 5 out of these 6 patients a significant correlation between p53-autoantibody production in sera and p53-overexpression in the tissue could be found ( $p=0.03$ ), suggesting that p53-overexpression is a necessary precondition for p53-autoantibody production. It remains unknown why so many patients with p53-overexpression do not produce p53-autoantibodies in the sera.

Comparing the clinical and histopathological parameters (TNM, grading, anatomical tumour site, gender and age) with the rate of p53-mutations, p53-overexpression, p53 in sera and p53-autoantibody production showed no statistically significant correlation.

## Discussion

Mutations of p53 in HNSCC are described in 40-60% of cases, thus they are one of the most frequent mutations in these tumours (5). The results presented herein, show a p53-mutation rate of 17% which is rather low compared to other studies of SCC in the head and neck region. The low incidence of mutations presented herein might be due to the applied detection method for mutations. Direct sequencing of the exons 5-9 was performed following amplification by PCR, representing a technique which is known to be very sensitive. Yet, the method is risky to overlook mutations in case wt-p53 sequences of contaminating regular cells disturb the signal of the mutated cells. In studies reporting higher mutation rates single-strand conformation polymorphism (SSCP) was applied, a technique which has shown rather frequent false positive results (1).

In 8 out of 24 patients (33%) investigated, p53 itself was detectable in serum. Regularly p53 can not be detected in patients sera since p53 is located in the nucleus with a short

biological half-life (6-20 min). Presumably, p53-accumulation occurs in tumour cells and consecutively p53 is released into sera due to tumour cell necrosis. The described process of protein release might be the prerequisite for the immunological response and the development of p53-autoantibodies. However, in this study there was no significant correlation between p53-accumulation in the tissue and the occurrence of p53-autoantibodies.

The first immunological response against p53 was described in 1979 (18). It was discovered rather by accident while researching the popular tumour virus SV40. p53 is a complex-builder with the T antigen of the virus. p53-autoantibodies were first shown by Crawford *et al* in 1982 (8). Later on, measurable p53-autoantibody levels in sera were demonstrated in various malignancies (10-14).

Depending on tumour type and method of analysis the rate of p53-autoantibodies varied between 5 and 40% (19,20). In the present study, 25% of patients autoantibodies against p53 could be shown by ELISA in pre-therapeutic serum. This quantity is consistent with previously studies on p53 autoantibodies in HNSCCs (12,16,21). Rates higher than ~40% are rarely described (22).

Two dominating hypothesis for the development of p53-autoantibodies are subject of controversy in literature. Lubin and colleagues (13) hypothesized that mutations of the p53-gene produce a modified protein formation and thus newly expressed epitopes of the p53-protein subsequently develop antigenic character. Winter *et al* (14) detected p53-autoantibodies only in patients with a point mutation in exon 7, whereas no mutation in exon 8 was detected. The significant correlation between mutation and p53-autoantibodies has been supported by other studies (23,24). Yet, there are studies (25,26) showing no significant correlation between p53-mutation and p53-autoantibody development. One of several reasons arguing against p53-mutation as single cause for autoantibody production is that p53-autoantibodies bind to epitopes that are expressed in both, mt-p53 and wt-p53 (27). Moreover, these epitopes are rather located on the C- and N-terminus of the protein, while mutations mainly occur in the central binding site of the protein (28).

No correlation between p53-mutation and occurrence of p53-autoantibodies was observed. In four out of 24 cases p53-mutations were detected. However, none of these cases showed serological evidence of p53-autoantibodies. Thus, it can be concluded that mutations of the p53-gene leading to a modified protein formation and newly expressed epitopes of the p53-protein which subsequently develop antigenic characteristics cannot be supported by the data presented herein. Furthermore, there was no correlation between



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e of p53 in the serum and to TNM stage, grading,  
ur site.

Another hypothesis favoured by different authors (16,29,30) implies an overexpression of p53 in the tissue with subsequent production of p53-autoantibodies. p53 accumulation in the tissue might be a result of tumour cell necrosis with release of p53 out of its natural compartment (15). An internalisation of antigen presenting cells might finally give a B-cell reaction (16).

In concordance with this hypothesis the results presented herein demonstrate a 75% p53-overexpression in tumour tissue. This result is at the upper limit of previously described results with a p53-overexpression rate between 35% (31) and 84% (32). Altogether, the results of this analysis do not show a significant correlation between p53-mutation and p53-overexpression in the tissue. However, in almost all cases of circulating p53-autoantibodies also an overexpression of p53 in the tissue could be seen, indicating that p53-overexpression in the tissue is a necessary precondition for p53-autoantibody production in the tested cases.

In conclusion, the results presented herein support the hypothesis that strong accumulation of p53 in the tissue is an important prerequisite for development of p53-autoantibodies. However, there must be further, still unknown factors that influence the p53-autoantibody production because p53-autoantibodies were not identified in sera in each case of p53-accumulation in the tissue. Neither the initial proposition of p53-overexpression in the tissue with consecutive p53 release into the tissue and subsequent production of p53-autoantibodies nor the assumption that mutations in the p53 genome lead to the production of p53-autoantibodies can be confirmed by the data demonstrated in this study.

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