Retention of the Arginine Allele in Codon 72 of the p53 Gene Correlates with Poor Apoptosis in Head and Neck Cancer

Regine Schneider-Stock,* Christian Mawrin,[†]
Christiane Motsch,[‡] Carsten Boltze,*
Brigitte Peters,[§] Roland Hartig,[¶] Peter Buhtz,*
Anja Giers,[‡] Astrid Rohrbeck,* Bernd Freigang,[‡]
and Albert Roessner*

From the Departments of Pathology,* Neuropathology,[†] Head and Neck Surgery,[‡] Biometrics,[§] and Immunology,[¶] Otto-von-Guericke University, Magdeburg, Germany

The allele constitution at codon 72 of the p53 gene (CGC-arginine or CCC-proline) plays a major role in inducing apoptosis in p53 mutant cells. To verify this, we determined GC-status, p53-mutations, and p53-loss of heterozygosity (LOH) in a group of 54 squamous cell carcinomas of the head and neck (SCCHN). A novel approach, using a one-step real-time PCR analysis with fluorescent hybridization probes, was applied to detect the GC status in tumors and corresponding blood samples. p53 mutations in exons 4 to 8 were detected by PCR-SSCP-sequencing analysis. Apoptosis was determined immunohistochemically using antibodies against Fas, FasL, p53, Bcl2, and terminal deoxy-transferase-mediated dUTP nick end labeling (TUNEL) staining. The overall frequency of p53-LOH in SCCHN was 45.2%. In cases of LOH, there was a preferential loss of the proline allele, which was associated with an up-regulation of Bcl2 and lack of co-expression of Fas/FasL and, thus, impaired apoptosis ($P < 0.001$). Apoptosis was not observed in tumors carrying the arginine allele. p53 mutations were detected in 29.6% of SCCHN and preferentially occurred at the arginine allele ($P = 0.01$). p53 alterations were more frequently observed in tumors of the oral cavity, oropharynx and hypopharynx, whereas they were rare in larynx carcinomas ($P = 0.07$). The p53-LOH status was not found to be significantly correlated with sex, age, TNM-status, or tumor grading. We conclude that apoptosis is correlated with the allelic status of codon 72 in SCCHN. Homozygous proline 72 appears to be an important regulator of apoptosis via the Fas/FasL pathway in SCCHN. (*Am J Pathol* 2004, 164:1233–1241)

There is an important restriction fragment length polymorphism (RFLP) in codon 72 of exon 4 of the p53 gene

coding for proline (CCC) or arginine (CGC). Thus, each individual inherits a p53 genotype that can be heterozygous (Arg/Pro) or homozygous for either arginine (Arg/Arg) or proline (Pro/Pro) at codon 72. These two variants of wild-type p53 appear to be different both biochemically and biologically.¹ The correlation between arginine constitution and human papilloma virus (HPV)-related cancer might be due to increased susceptibility of p53 to degradation by E6 protein of HPV16/18.^{2–4} There are discrepancies regarding the association between codon 72 polymorphism and the risk of oral cancer. A few authors described an association between codon 72 polymorphism and susceptibility to tobacco-related cancers,⁵ while others doubted such a correlation.⁶ Furthermore, McGregor et al⁷ reported that Arg72 constitution is related to an increased susceptibility to sunburn. Recently, it has been demonstrated by Bergamaschi et al⁸ that the codon 72 allelic status has a major impact on the p53-dependent apoptosis in p53-mutant advanced head and neck cancers. Furthermore, this study clearly demonstrated that SCCHN tumors expressing Pro72 mutants show a better clinical response following cis-platin-based chemo-radiotherapy than those expressing Arg72 mutants.

The role of membrane death receptor-mediated apoptosis is well-established both *in vitro* and *in vivo*.^{9,10} When cell-surface Fas engages with Fas ligand (FasL), several proteins are recruited to the intracellular “death domain” of Fas to form a death-inducing signaling complex. Disturbances in apoptosis mediated via the Fas/FasL system play a key role in the pathogenesis of cancer.¹¹ A p53-responsive element was found in both human and mouse Fas death receptor genes.^{12,13} Mutant p53 overexpression can also trigger continuous expression of Bax and Bcl2, proteins that regulate apoptosis via the mitochondrial signal cascade.^{14,15} Recently, it was reported that the allele constitution at codon 72 of the p53 gene (CGC-arginine or CCC-proline) contributes to the

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Address reprint requests to Regine Schneider-Stock, Ph.D., Molecular Tumor Genetics Lab, Department of Pathology, Otto-von-Guericke University, Leipziger Str. 44, 39120 Magdeburg, Germany. E-mail: Regine.Schneider-Stock@medizin.uni-magdeburg.de.

apoptosis induction of p53 mutant cells by influencing the interaction of p53 with p73.^{6,16} Furthermore, Arg72 seems to be preferentially mutated, whereas Pro72 is preferentially lost in various squamous cell cancers.^{6,7} The relationship between codon 72 status and apoptotic regulation in human SCCHN is still unclear. Since codon 72 is localized in a proline-rich region of the p53 gene that is essential for p53-mediated apoptosis, we investigated the association between p53-LOH, p53 mutations and p53 protein expression, and the expression of three apoptosis-related proteins (Fas, FasL, and Bcl2) in a group of 54 SCCHN.

Materials and Methods

Tissues

We analyzed 54 patients suffering from advanced head and neck squamous cell cancers, treated in the Department for Head and Neck Surgery of the Otto-von-Guericke University of Magdeburg. Twelve (22.2%) tumors were located in the oral cavity, 22 (40.7%) in the oropharynx and 13 (24.1%) in the hypopharynx. Five (9.3%) tumors were supraglottic, and two (3.7%) were glottic cancers. There were 47 male and seven female patients; the average age was 57 years. Twenty-seven patients had stage pT4-cancer, 13 had a pT3-tumor, and 14 had a pT2-tumor, but none of them was affected by a small stage pT1 tumor. In 21 patients, cervical lymph node metastases did not occur (pN0). Ten patients were diagnosed at stage pN1, and 22 patients at stage pN2. One patient had cervical metastasis with a size of more than 6 cm (pN3). Only two of the carcinomas were well differentiated (GI), 40 carcinomas were moderately (GII), and 12 were poorly differentiated (GIII). In the analyzed group were 45 patients with a history of alcohol and nicotine abuse. Seven patients had neither alcohol nor nicotine abuse in their history, and two were smokers only. All patients were treated by radical surgery, and 45 patients underwent postoperative radiotherapy.

DNA Extraction

Tumor DNA and blood DNA were extracted using a standard proteinase K-phenol-chloroform extraction protocol.

Exon 4-LOH (Codon 72 of the p53 Gene)

The restriction fragment length polymorphism (RFLP) in codon 72 of exon 4 of the p53 gene was determined using the LightCycler (Roche Diagnostics, Mannheim, Germany). Primers and oligonucleotide hybridization probes were chosen using the TIB MOLBIOL computer program (Berlin, Germany: www.TIBMOLBIOL.de/oligo_ag.html) to ensure their total gene specificity. Primer sequences (GenBank Accession No. X54156) were sense 5'-GATGCTGTCCCCGACGA-3' (position 12053–12070) and antisense 5'-AGGGGCCGCCGTG-TAG-3' (position 12181–12163) amplifying a 128 bp fragment. The sequence of the 5'-fluorescein-labeled probe

Figure 1. A and B: SSCP-analysis of exon 5 (A, tumor Tu15) and of exon 7 (B, tumor Tu2); arrows show the aberrant migrating bands of PCR products with p53 mutations. C: Control gel electrophoresis of p53 exon 4 PCR products; lane 1 (tumor Tu29) and lane 3 (tumor Tu30) show a typical heteroduplex structure in addition to the double-stranded (ds) PCR product. This is an indication of larger rearrangements.

was 5'-GATGAAGCTCCCAGAATGCCAGAGGCT-3', position 12105–12131. The LCRed 640 probe was designed to detect the C-variant (5'-TCCCCCGGTIICCCCTG-CACCA-3', position 12134–12155). If G is present, the resulting mismatch destabilizes the sample probe-hybrid, and this effect is further enhanced by inserting two additional inositol bases into the probe, yielding a melting temperature that is lower in mismatch ($T_m = 62.5^\circ\text{C}$) than in normal-match cases ($T_m = 69^\circ\text{C}$). The different melting curve profiles (Figure 1) allow for the exact genotyping without post-PCR handling. Fluorescence melting peaks were obtained by plotting the

negative derivative of fluorescence over temperature (-dF/dT) versus temperature (T) showing melting temperatures (T_m).

PCR

For PCR of exon 4 of the p53-gene, we used the Light-Cycler-DNA Master Hybridization Probes Kit (Roche Diagnostics). The 20 μ l PCR reaction contained the exon 4 primer set (10pmol each), two hybridization probes (4pmol of the fluoresceine-labeled probe and 2pmol of the LCRed 640 probe), 3 mmol/L $MgCl_2$, 1 $\times$ LC-DNA Master Hybridization Probe Mix, and 100 ng DNA.

PCR conditions were as follows: a first denaturation step (95°C, 30 seconds) followed by 45 cycles of denaturation (95°C, 10 seconds), annealing (60°C, ramping 0.1, for 10 seconds), and extension (72°C, 5 seconds), one melting cycle (45°C to 80°C, 20 seconds), and a final cooling step (40°C, 30 seconds).

p53 Intron-1 Polymorphism

The intron-1 polymorphic region was amplified by using the following touch-down PCR: 95°C for 5 minutes, 20 cycles of 95°C for 10 seconds, 66°C for 10 seconds and 72°C for 20 seconds, with a stepwise decrease of 2°C in each fifth cycle, and finally 30 cycles at 95°C for 45 seconds, 58°C for 1 minute, and 72°C for 1 minute. An aliquot of the PCR product was electrophoresed on native 8% polyacrylamide gels, cross-linked with piperazine diacrylamide, and visualized by silver staining. An allelic loss was considered in cases when, in comparison with non-tumor DNA, the signal of a tumor band disappeared or signal intensity was reduced by more than 50%. Evaluation was done twice, visually, or by densitometry (VDS, Pharmacia Biotech, Uppsala, Sweden) in unambiguous cases.

Immunohistochemistry

For Fas, FasL and Bcl-2 immunohistochemistry, we used monoclonal mouse antibodies and antigen retrieval by microwave heating of Fas (clone DX3, 1:10 dilution, EDTA (pH 8.0), Dako, Hamburg, Germany), FasL (clone 5D1, 1:50 dilution, Glycubuffer (pH 3.0), Novocastra, Hamburg, Germany) and Bcl-2 (clone 124, 1:10 dilution, EDTA (pH 8.0), Dako) after inhibition of endogeneous peroxidase activity. The primary antibodies were incubated for 1 hour at 37°C. Slides were subsequently incubated with a 1:10 dilution of normal swine serum (Vector, Burlingame, CA). After washing in PBS (pH 7.4), the samples were incubated with a 1:200 dilution of biotinylated anti-mouse secondary antibody (Vector) for 30 minutes at room temperature. The detection of bound antibody was accomplished using the avidin-biotin complex method (Dianova UniTect A.B.C. System XHC1, Hamburg, Germany). A 0.1% solution of 3,3'-diaminobenzidine (5 minutes) was used as a chromogen. Specificity for immunostaining was checked by omitting single steps in the immunohistochemical protocol and by replacing primary antibody with non-immune serum. Tissue from a xenotransplant of a dedifferentiated thyroid carcinoma was used as a positive control. Estimating 10 high-power fields, we found a very low staining intensity and an inhomogeneous staining pattern if less than 20% of cells showed positivity. Therefore we considered a section immunohistochemically positive if cytoplasm was strongly positively stained (with pronounced membrane staining for Fas and Fas-L) in more than 20% of tumor cells.

Apoptosis Detection by the Terminal Deoxy-Transferase-Mediated dUTP-Biotin Nick End Labeling Method

Apoptosis was detected using the terminal deoxy-transferase-mediated dUTP nick end labeling (TUNEL) method

Table 1. LOH and Protein Expression Status of SCCHN with p53 Mutations

Tumor number	Exon/Codon	Base substitution	Amino acid exchange	p53-LOH	p53 protein expression (%)
1	7/248	CGG to CAG	Arg to Glu	LOH	60
2	7/248	CGG to CAG	Arg to Glu	LOH	80
4	8/273	CGT to CAT	Arg to His	LOH	70
7	5/175	CGC to CAC	Arg to His	LOH	80
8	4/83	76bp-insertion	frameshift	LOH	0
10	5/177	4bp-deletion	frameshift	WT	0
14	intron 5, 9bp 5' end of exon 6 splice origin	C to T	splicing?	WT	90
15	5/175	CGC to CAC	Arg to His	WT	90
16	Intron 7, 11bp 3'-end of exon 7 splice origin	A to T	splicing?	WT	0
41	5/168	CAC to CCC	His to Pro	n.i./ Pro	55
24	5/179	CAT to AAT	His to AsN	n.i./Arg	80
29	4/41	5bp-insertion/inversion	frameshift	n.i./Arg	0
30	4/75	deletion	frameshift	n.i./Arg	0
31	intron 4, 7 bp 5'-end of exon 5 splice origin	A-insertion	splicing?	n.i./Arg	70
38	4/53	TGG to TGA	stop	n.a./Arg	0
49	8/279-281	10bp-deletion	frameshift	?/Arg*	0

*Blood status was not known.

LOH, loss of heterozygosity; WT, wild type; n.i., not informative; n.a., no analysis.

Table 2. Genetic and Immunohistochemical Data of Patients with Germline Arg/Pro Status

Tumor number	Age	Sex	Localization*	pT	pN	Clinical stage	Tumor grade	Codon 72 status	Exon 4 LOH	Intron 1 LOH	p53 Mutation type	p53-IH (%)	Bcl2-IH	Fas-IH	FasL-IH
1	60	m	3	2	0	2	2	Arg	LOH	LOH	missense	60	+	+	—
2	58	m	2	4	2	4	3	Arg	LOH	LOH	missense	80	+	—	—
3	54	m	4	4	2	4	2	Arg	LOH	LOH	WT	10	+	—	—
4	57	m	2	4	2	4	2	Arg	LOH	LOH	missense	70	+	+	—
5	41	m	1	4	0	4	2	Arg	LOH	LOH	WT	0	+	—	+
6	42	f	3	4	1	4	1	Arg	LOH	LOH	WT	80	+	—	—
7	57	m	4	3	1	4	2	Arg	LOH	LOH	missense	80	+	—	—
8	57	m	4	1	1	4	2	Arg	LOH	LOH	frame	0	+	—	—
9	65	m	3	2	2	4	2	Pro	LOH	LOH	WT	0	—	+	+
10	53	f	1	3	2	4	2	Arg/Pro	het	het	frame	0	—	—	—
11	49	m	4	4	0	4	3	Arg/Pro	het	LOH	WT	0	—	—	—
12	44	f	1	4	2	4	2	Arg/Pro	het	het	WT	15	+	—	—
13	54	m	3	2	0	2	2	Arg/Pro	het	LOH	WT	10	—	—	+
14	57	m	1	3	0	3	2	Arg/Pro	het	het	splicing	90	—	—	+
15	50	m	2	4	1	4	2	Arg/Pro	het	het	missense	55	—	+	—
16	48	m	3	4	0	4	3	Arg/Pro	het	het	splicing	0	—	—	+
17	49	m	1	4	1	4	3	Arg/Pro	het	het	WT	0	—	—	—
18	70	m	1	2	2	4	2	Arg/Pro	het	het	WT	0	—	—	—
19	43	f	1	4	0	4	2	Arg/Pro	het	het	WT	0	—	+	—
20	50	m	4	4	2	4	2	Arg/Pro	het	het	WT	0	—	—	+
21	70	m	2	3	2	4	2	Arg/Pro	het	het	WT	0	—	—	—
22	68	m	3	4	0	4	3	Arg/Pro	het	het	WT	0	—	+	—
23	60	m	1	3	2	4	2	Arg/Pro	het	het	WT	15	—	+	—

*Carcinomas of the 1, oropharynx; 2, hypopharynx; 3, oral cavity; 4, supraglottis; 5, glottis.

m, male; f, female; Arg, arginine; Pro, proline; LOH, loss of heterozygosity; het, heterozygous; WT, wild type; IH, immunohistochemistry; —, immunonegative; +, immunopositive; pT, pathological tumor stage; pN, nodal stage.

in situ apoptosis detection kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, five paraffin sections of each Arg, Arg/Pro, and Pro status were mounted on glass slides and treated with xylene for 5 minutes and then in 100%, 95%, or 75% ethanol. The deparaffinized tissue samples were incubated with proteinase K (2 mg/ml) at room temperature for 15 minutes. After phosphate-buffered saline (PBS) washing, endogenous peroxidase was blocked by the addition of 3% H₂O₂. Tissues were then treated with terminal deoxynucleotidyl transferase and bio-

tinylated dUTP. After stopping the reaction with TB buffer (30 mmol/L sodium chloride, 30 mmol/L sodium citrate), the samples were investigated by fluorescence microscopy (Axiovert 200, Zeiss, Intermedic, Germany).

Statistics

Statistical analyses were carried out using the χ^2 -test or Fisher's exact test in cross tables, and one-way analysis of variance (for comparison of group means). *P* values

Table 3. Genetic and Immunohistochemical Data of Patients with Germline Arg/Arg Status

Tumor number	Age	Sex	Localization*	pT	pN	Clinical stage	Grade	Codon 72 status	Exon 4 LOH	Intron 1 LOH	p53 Mutation type	p53-IH (%)	Bcl2-IH	Fas-IH	FasL-IH
24	35	f	3	4	2	4	3	Arg	n.a.	LOH	missense	80	+	—	—
25	61	f	1	2	1	3	2	Arg	n.a.	het	WT	10	+	+	—
26	60	m	2	4	1	4	2	Arg	n.a.	LOH	WT	0	+	—	—
27	62	m	2	2	2	4	2	Arg	n.a.	het	WT	0	+	—	+
28	47	m	2	4	2	4	2	Arg	n.a.	het	WT	80	+	—	—
29	43	m	1	3	1	3	2	Arg	n.a.	LOH	frame	0	—	+	—
30	43	m	1	3	2	4	2	Arg	n.a.	LOH	frame	0	+	—	+
31	59	m	1	3	1	3	2	Arg	n.a.	LOH	splicing	70	+	—	—
32	74	m	2	3	0	3	3	Arg	n.a.	LOH	WT	0	+	+	—
33	59	m	1	2	0	2	3	Arg	n.a.	het	WT	0	+	—	—
34	69	m	4	2	0	2	2	Arg	n.a.	LOH	WT	20	+	—	—
35	68	m	1	2	1	3	2	Arg	n.a.	LOH	WT	0	+	+	—
36	51	m	1	2	3	4	2	Arg	n.a.	het	WT	20	+	—	—
37	51	m	2	4	2	4	2	Arg	n.a.	het	WT	70	+	—	+
38	74	m	1	4	2	4	2	Arg	n.a.	het	stop	0	+	—	—
39	64	m	1	4	0	4	3	Arg	n.a.	het	WT	50	+	—	—
40	73	m	5	4	0	4	2	Arg	n.a.	het	WT	90	+	—	+

*Carcinomas of the 1, oropharynx; 2, hypopharynx; 3, oral cavity; 4, supraglottis; 5, glottis.

m, male; f, female; Arg, arginine; Pro, proline; LOH, loss of heterozygosity; het, heterozygous; WT, wild type; IH, immunohistochemistry; —, immunonegative; +, immunopositive; n.a., not available; pT, pathological tumor stage; pN, nodal stage.

Table 4. Genetic and Immunohistochemical Data of Patients with Germline Pro/Pro Status

Tumor number	Age	Sex	Localization*	pT	pN	Clinical stage	Grade	Codon 72 status	Exon Intron		p53 Mutation type	p53-IH (%)	Bcl2-IH	Fas-IH	FasL-IH
									4	1					
									LOH	LOH					
41	81	m	2	4	0	4	3	Pro	n.a.	het	missense	90	—	+	+
42	64	m	2	4	1	4	2	Pro	n.a.	het	WT	0	—	+	+

*Carcinomas of the 1, oropharynx; 2, hypopharynx; 3, oral cavity; 4, supraglottis; 5, glottis.

m, male; f, female; Arg, arginine; Pro, proline; LOH, loss of heterozygosity; het, heterozygous; WT, wild type; IH, immunohistochemistry; —, immunonegative; +, immunopositive; n.a., not available; pT, pathological tumor stage; pN nodal stage.

less than 0.05 were considered statistically significant, and $P < 0.10$ was regarded as a statistical trend. All statistical tests were two-sided. Calculations were carried out by SPSS-9.0 software (SPSS, Chicago, IL).

Results

Mutation Frequency and Mutation Spectrum

P53 mutations were identified in 16 of 54 (29.7%) tumors (Table 1). Representative PCR and SSCP gels are shown in Figure 1. We found seven missense (43.8%), five deletion/insertions (frame-shifts) (33.3%), three splicing mutations (18.7%), and one stop-mutation (6.2%). Seven mutations corresponded to the transition type (five G:A at CpG islands, one C:T and one G:A outside of CpG islands) and only three were transversions (A:C, A:T, and C:A). Five mutations occurred at known hot-spot codons (Arg175, Arg248, and Arg273). To date, four exon mutations have not been described in the Hainaut p53 database for head and neck cancer (tumors 29, 30, 8, and 41).¹⁷

p53-LOH Frequency

p53-LOH was detected in 19 of 42 (45.2%) advanced head and neck carcinomas (Tables 2, 3, 4, and 5). The intron 1-VNTR-marker showed higher informativity (42 of 42 = 100%) versus exon 4-RFLP marker (23 of 42 = 54.7%). Exon 4-LOH was found in 9 of 23 informative cases (39.1%), (Figure 2). Intron 1-LOH was detected in 19 of 42 informative cases (45.2%). There were six cases with partial deletions; in five cases, the deletion involved

both markers; in eight cases only the intron 1-marker showed allelic loss.

Correlation of p53 Mutations with p53 Protein Expression

Nuclear accumulation of p53 protein was seen in 21 of 54 (38.9%) tumors (5 cases $\leq 30\%$ and 16 cases $> 30\%$ immunopositive nuclei, respectively). All tumors with missense mutations at hot-spot codons (tumors 1, 2, 4, 7, 15, 24, and 41) showed nuclear p53 protein accumulation, whereas the five tumors with frame-shift mutations (tumors 8, 10, 29, 30, and 49), the single tumor with a stop-mutation (tumor 38), and one of three tumors with splice mutations (tumor 16) showed clear null-staining, confirming that such mutations result in a truncated p53 protein molecule (Tables 1 to 5). In addition, 8 of 28 (28.6%) wild-type p53 tumors identified by PCR-SSCP-sequencing analysis also expressed p53 protein. To reevaluate these discordant results, separate PCR reactions were performed and the PCR products were re-analyzed. In none of these p53 immunopositive tumors was a p53 mutation identified.

Arg/Pro Status and p53 Alterations (Mutations + LOH)

The Arg/Pro status of patients blood was Arg/Arg [17 of 42 (40.5%)], Arg/Pro [23 of 42 (54.8%)], and Pro/Pro [2 of 42 (4.8%)]. Codon 72 status of these 42 cases was Arg/Arg [25 of 42 (59.5%)], Arg/Pro [14 of 42 (33.3%)], and Pro/Pro [3 of 42 (7.1%)] (Tables 2 and 6). Nine of 23

Table 5. Genetic and Immunohistochemical Data of Patients with Undetermined Germline Status

Tumor number	Age	Sex	Localization*	pT	pN	Clinical stage	Grade	Codon 72 status	Exon Intron		p53 Mutation type	p53-IH (%)	Bcl2-IH	Fas-IH	FasL-IH
									4	1					
									LOH	LOH					
43	59	f	3	4	0	4	2	Pro	n.a.	het	WT	0	—	+	+
44	59	m	2	3	0	3	2	Pro	n.a.	het	WT	0	—	+	+
45	75	m	5	4	0	4	3	Arg	n.a.	het	WT	90	+	—	+
46	78	m	1	2	2	4	2	Arg	n.a.	het	WT	10	+	+	—
47	65	m	1	4	2	4	2	Arg	n.a.	het	WT	90	+	—	+
48	53	m	1	2	2	4	3	Arg	n.a.	het	WT	0	+	—	+
49	39	m	1	2	2	4	2	Arg	n.a.	het	frame	0	+	—	+
50	36	m	3	4	0	4	1	Arg	n.a.	het	WT	0	+	—	—
51	49	f	1	2	0	2	2	Arg	n.a.	het	WT	0	+	—	—
52	86	f	3	4	0	4	2	Arg	n.a.	het	WT	10	+	—	+
53	47	m	2	3	2	4	2	Arg/Pro	het	het	WT	10	—	+	—
54	35	m	3	3	2	4	2	Arg/Pro	n.a.	het	WT	70	—	—	+

*Carcinomas of the 1, oropharynx; 2, hypopharynx; 3, oral cavity; 4, supraglottis; 5, glottis.

m, male; f, female; Arg, arginine; Pro, proline; LOH, loss of heterozygosity; het, heterozygous; WT, wild type; IH, immunohistochemistry; —, immunonegative; +, immunopositive; n.a., not available; pT, pathological tumor stage; pN nodal stage.

Figure 2. Visualization of the melting temperature to identify the genotype variant at codon 72 in exon 4 of the p53 gene. **A:** If G (Arg) is present, the resulting sample probe-mismatch yields a lower melting temperature (62.5°C) than the normal-match cases (C: Pro, 69°C). **B:** Two peaks showing the heterozygous status Arg/Pro without LOH in the blood, whereas the proline signal is lost in the corresponding tumor tissue (LOH ↓). Tu, tumor; NT, non-tumorous tissue

informative cases (39.1%) showed allelic loss for the exon 4-marker (Figure 2). There was a preferential loss of the proline allele [8 of 9 cases (88.9%), Table 2]. Intron 1-LOH was mostly found in tumors bearing the Arg status [17 of 19 (89.5%)], (Tables 2 to 5).

P53 mutations tended to occur more frequently in the Arg allele; allelic losses (exon 4 and exon 1) were found significantly more often in tumors bearing at least one Arg allele ($P = 0.01$). Interestingly, tumors having both alterations were only of the Arg type ($P = 0.021$), (Table 6).

Expression Status of Apoptosis-Related Proteins Fas, FasL, and Bcl2

Representative immunostains are given in Figure 3. Fas protein expression was detected in 17 of 54 (31.5%) tumors, FasL in 20 of 54 (37%) tumors, whereas Fas/FasL protein expression simultaneously occurred in only 5 of 54 (9.2%) tumors, (Table 7). Bcl2 protein expression was found in 33 of 54 (61.1%) tumors (Table 7). Fas/FasL and Bcl2 expression were inversely correlated ($P = 0.024$),

Fas/FasL did not correlate with p53 protein expression ($P = 0.89$). Sixteen of 21 (76.2%) p53 immunopositive tumors expressed Bcl2. In contrast, no expression of the anti-apoptotic protein Bcl2 was detected in five p53 immunopositive tumors. Sixteen of 33 (48.5%) p53 immunonegative tumors were Bcl2-positive ($P = 0.09$).

Arg/Pro Status and Expression of Fas, FasL, and Bcl2

Pro status of the tumor significantly correlated with high Fas/FasL expression ($P < 0.01$). Simultaneous Fas/FasL expression never occurred in heterozygous Arg/Pro and in Arg tumors (Table 7, Figure 3). The apoptosis-inducing feature of tumors carrying the Pro allele was underlined by the frequent demonstration of DNA strand breaks in the TUNEL assay (Figure 4). Expression of the anti-apoptotic protein Bcl2 was never observed in tumors having the Pro status; this protein was expressed in only 1 of 16 (6.3%) heterozygous Arg/Pro tumors. p53 immunopositive tumors tended to show the Arg status.

Clinicopathologic Data and Genetic and Immunohistochemical Findings

There was a tendency for p53 alterations to occur more frequently in the proximal tumors ($P = 0.07$). We did not find a significant correlation between, sex, age, tumor grade, pT-status, nodal status, and Arg/Pro status of the tumor, p53 mutation status, or overexpression of any of the apoptosis-related immunohistochemical markers examined. Interestingly, non-alcoholics and non-smokers never showed p53-LOH ($P = 0.055$). p53 alterations were more frequently observed in tumors of the oral cavity, oropharynx, and hypopharynx, whereas they were rare in larynx carcinomas ($P = 0.07$).

Discussion

A group of 54 advanced squamous cell carcinomas of the head and neck was investigated for a correlation between codon 72 allele constitution of the p53 gene and apoptosis induction. We found evidence for a preferential loss of the codon 72 proline allele in SCCHN and demonstrated a positive association between arginine status and reduction of apoptotic tumor cell death.

SCCHN develop in a multi-step process, acquiring different molecular alterations during carcinogenesis. So far, mutations of the p53 tumor suppressor gene have

Table 6. Correlation between Tumor Arg/Pro Status and p53 Mutations and p53-LOH

	N	Total N (%)	Tumor status			P
			Pro/Pro N (%)	Arg/Pro N (%)	Arg/Arg N (%)	
p53 mutation	54	16 (29.6)	1/5 (20)	4/12 (25)	11/33 (33.3)	n.s.
p53-LOH	42	19 (45.2)	1/3 (33.3)	2/12 (14.3)	16/25 (64)	0.010
p53 alterations*	43	9 (20.9)	0/3 (0)	0/14 (0)	9/26 (34.6)	0.021

*p53-LOH and/or p53 mutation.

Arg, arginine; Pro, proline; N, number of cases; n.s., not significant; LOH, loss of heterozygosity.

Figure 3. Serial sections of representative immunostains: a section was considered immunohistochemically positive if cytoplasm was strongly positively stained, with pronounced membrane staining for Fas and Fas-L, in more than 20% of tumor cells (**insets**). Pro72 allele status correlated with high Fas and FasL expression. Simultaneous Fas/FasL expression never occurred in heterozygous Arg/Pro. Fas/FasL and *Bcl2* expression were inversely correlated: Bcl-2 was never observed in tumors having the Pro72 status and was found in only one of the heterozygous Arg/Pro tumors.

been demonstrated to play a major role in the development of SCCHN.¹⁸ Regarding the frequency of hot-spot mutations, type of base changes (transition *versus* transversion) and the distribution of mutations, our spectrum of p53 mutations was essentially identical to that of the IARC database.¹⁷ When excluding chain-terminating mutations (premature stop codon, splicing, and frame-shift mutations), we found transdominant mutations preferentially at Arg72. There was one recessive mutation (codon 168: CAC

to CCC) at the Pro72 allele. Codon 72 in the mutant alleles was Arg in 11 of 33 (33.3%) tumors and Pro in 1 of 5 (20%) cases. Mutant Arg72 allele thus tended to be over-represented in the tumors. This is consistent with the observation of Marin et al⁶ who found that mutant alleles containing Arg72 are preferentially selected during tumorigenesis.

There is an expanding body of literature suggesting that host factors, including genetic polymorphisms, may explain some of the individual differences in cancer oc-

Table 7. Correlation between Tumor Arg/Pro Status and Expression of Apoptotic Proteins

Status	N	Positive protein expression				
		Bcl2-IH N (%)	Fas-IH N (%)	FasL-IH N (%)	Fas/FasL N (%)	p53>49% N (%)
Pro or Pro/Pro	5	0 (0)*	5 (100)*	5 (100)*	5 (100)*	1 (20)
Arg/Pro	16	1 (6.3)	5 (31.3)	5 (31.3)	0 (0)	3 (18.8)
Arg or Arg/Arg	33	32 (96.6)	7 (21.2)	10 (30.3)	0 (0)	13 (39.4)
Total	54	33 (61.1)	17 (31.5)	20 (37)	5 (9.2)	17 (31.5)

*Significant for $p < 0.01$.

Arg, arginine; Pro, proline; N, number of cases; IH, immunohistochemistry.

currence.¹⁹ These studies correlate allelic status at codon 72 in the blood of patients with manifestation of specific tumor types. In a recent work, Bergamaschi et al⁸ reported that patients with p53-mutant head and neck cancer carrying codon 72 Arg allele have a worse response to chemotherapy than those having the Pro allele. However, in contrast to our study these authors focused on p73-dependent apoptosis, but our results add evidence that the codon 72 allele status is important for the induction of apoptosis in SCCHN.

The allelic status at codon 72 of the p53 gene has been already suggested to influence the induction of apoptosis in human cancer.⁶ As codon 72 is located in a proline-rich region of the p53 gene that is essential for p53-mediated apoptosis, the frequent occurrence of p53 mutations in SCCHN may affect the regulation of apoptotic tumor cell death, thus influencing the biological properties and prognosis of these tumors. That p53 is involved in the regulation of apoptosis in SCCHN has been demonstrated by the observation that the introduction of wild-type p53 decreases telomerase activity level, suppresses cell growth in SCCHN cell lines,²⁰ and induces apoptosis.^{3,20} Furthermore, Marin et al have shown that the p53 mutation status of tumor cells influences oxygenic stress-induced apoptosis.⁶ In the latter study, all p53 mutants bound to p73 contained the Arg allele at codon 72, suggesting that the presence of Arg allele in the p53 mutants prevents tumor cells from apoptotic cell death.⁶ We found that allelic loss at the p53 gene in SCCHN occurs generally at the Pro allele while the Arg allele is

retained. This imbalance resulted in markedly reduced apoptosis in tumors bearing the Arg allele, which, in turn, could influence the clinical outcome of these patients. In many cancers, such as bladder and gastric carcinomas, special codon 72 variants may serve as risk factors for neoplasia and may play a role in modulating environmental risk factors (smoking, alcohol, chemical toxins) for cancer development.^{21,22}

The Fas/FasL system is involved in the induction of apoptosis in a variety of fetal and adult tissues.²³ FasL (Fas ligand) is a key molecule in normal immune development, homeostasis, modulation, and cell function, that induces apoptosis by binding to its receptor Fas. There is evidence that the Fas receptor can be regulated at the transcriptional level by the tumor suppressor p53.²⁴ Drug-induced up-regulation of the Fas receptor is dependent on the p53 status of tumor cell lines.¹² The up-regulation of Fas receptor has been found to be reconstituted in p53 null cells by exogenous wild-type p53 transfection.¹² Our findings are in agreement with the literature where we show that Fas receptor protein expression is absent in most tumors of high immunopositivity for 53. In contrast, the regulation of FasL clearly involves p53-independent mechanisms.²⁵ Indeed, FasL was overexpressed in approximately two-thirds of tumors showing high p53 immunopositivity.

Despite the multiple levels at which p53 seems to control Fas-induced apoptosis, few studies have addressed this relationship *in vivo*. In the esophagus, Fas expression can be used as a marker for differentiating between normal gastric mucosa and gastric metaplasia.²⁶ Interestingly, si-

Figure 4. Representative TUNEL assay in two head and neck tumors bearing only the Pro (A) and the Arg (B) allele, respectively, at p53 codon 72 position. The extent of DNA fragmentation was determined using fluorescence microscopy showing a high apoptotic rate in the Pro tumor only.

multaneous expression of Fas and FasL does not necessarily lead to apoptotic cell death. Various anti-apoptotic mechanisms may exist in Fas/FasL co-expressing cells that protect these cells against apoptosis.²⁷ In our study, we found that Fas or FasL was expressed only in about one third of the tumors, and only a minority of tumors expressed Fas and FasL simultaneously. Expression of Fas and FasL in SCCHN *in situ* was heterogeneous; there were FasL-positive and FasL-negative regions of the tumor. Similar to Bennett et al²⁸ and Gastman et al,²⁹ we observed that the numbers of infiltrating lymphocytes were higher in SCCHN regions with higher FasL expression, but lower in tumor areas with low FasL expression. It is known that in contrast to its receptor, Fas-L is expressed on cytotoxic T-lymphocytes where it contributes to their cytotoxic function and mediates the elimination of peripheral T-cells following immune response.³⁰

Tumors having the Arg72 constitution showed lack of co-expression of Fas and FasL and high expression of *Bcl2* proteins, which was associated with impairment or lack of apoptosis. The expression of these proteins was reversed in tumors having the Pro72 constitution and this was associated with improvement of apoptosis induction. In heterozygous Arg/Pro tumors, we found neither Fas/FasL co-expression nor lack of *Bcl2* protein expression except for only one tumor. The Arg allele seems to prevent apoptosis. Thus, there was a significant correlation between codon 72 genotype and apoptosis induction.

We conclude that apoptosis is correlated with the codon 72 allelic status in SCCHN. Homozygous Pro72 appears to be an important regulator of apoptosis via the Fas/FasL pathway in SCCHN.

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