p53 Codon 72 Polymorphism in Squamous Cell Carcinoma of the Head and Neck Region

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Abstract. *Background: The impact of codon 72 polymorphism of the human tumour suppressor gene p53 on the risk of developing squamous cell carcinomas of the head and neck (HNSCC) remains unclear because of contradictory results found by several studies. Patients and Methods: We genotyped a group of 77 patients with advanced HNSCC by using a direct sequencing method. Results: There were no significant differences in the age of the patients at the time of the first diagnosis nor in the 5-year survival rates. There was no additive effect between different risk factors (alcohol, nicotine) and codon 72 polymorphism. Compared to the frequency of homozygosity encoding for Arg/Arg in the Eurasian population given in literature, the present study has shown a significantly higher frequency of homozygosity for Arg/Arg at codon 72 than commonly detected. Conclusion: These findings may indicate codon 72 polymorphism as a risk factor for HNSCC or point to a high variability of codon 72 polymorphism among ethnic groups.*

A large body of data exists concerning the environmental component of head and neck cancer risk, namely, tobacco and alcohol exposure. However, our current knowledge is sparse in regard to other host or genetic factors that may contribute to head and neck carcinogenesis. One of the host genetic factors that has been discussed as a susceptibility factor towards developing cancer is the codon 72 polymorphism in exon 4 of the *p53* tumor suppressor gene. This polymorphism at amino acid 72 results in either a proline residue (*Pro*) or an arginine residue (*Arg*). These two variants of wild-type *p53* appear to be different, both biochemically and biologically (1). For cervical and lung cancer, codon 72 polymorphism has been implicated as a risk marker (2-4). The association between this polymorphism and human papillomavirus (HPV)-related cancer has received considerable attention following experimental research demonstrating that the *Arg* form of the p53 protein was more vulnerable than the *Pro* form to binding and degradation by the HPV-E6 oncoprotein. However research on this topic has produced controversial results (5) and few data are available for head and neck cancer. Recently, it was demonstrated that the codon 72 allelic status has a major impact on the p73-dependent apoptosis in *p53* mutants. The clinical response following cisplatin-based chemo-radiotherapy for advanced head and neck cancer may be influenced by this polymorphism. Cancers expressing the *Arg* variants have lower response rates than those expressing *Pro* mutants (6). Furthermore, apoptosis seems to be correlated with the codon 72 allelic status in HNSCC. Homozygous *Pro* appears to be an important regulator of apoptosis *via* the Fas/FasL pathway in HNSCC (7). Recent studies on codon 72 polymorphism may indicate an oncogenic potential of the exon 4/codon 72 genotype, but other groups have not confirmed these findings (8, 9). Therefore in this study a group of 77 advanced squamous cell carcinomas of the head and neck was investigated for correlation between the codon 72 allele constitution of the *p53* gene and the development of head and neck cancer.

Patients and Methods

Patients. The study group consisted of 77 patients with histologically proven squamous cell carcinoma of the head and neck region (HNSCC). Cancer sites included the epipharynx (n=3), oropharynx (n=24), hypopharynx (n=17), larynx (n=31) and cancer of unknown primary (n=2). All the patients underwent primary surgery or a combined therapy with subsequent irradiation. Their age ranged from 36 to 77 years, with a median age of 56.8 years. All the tumors were classified

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Key Words: p53, tumour suppressor gene, codon 72, head and neck cancer.

according to the TNM-classification of the UICC, 1987 (10). All patients signed a written consent for the scientific use of the tumor material according to the criteria approved by the ethical committee of the University of Kiel.

PCR and DNA sequencing. Fresh tumor biopsies were taken during surgical resection of the tumor and immediately shock frozen after removal of the tissue. Frozen sections were used to define nearly pure tumor tissue and the DNA was extracted using a DNA extraction kit (Qiagen, Hilden, Germany) or a trizol-extraction method, and amplified by PCR using primers specific for exon 4 (11). PCR was carried out using a thermo cycler (T3, Biometra, Göttingen, Germany) with the following conditions for a 100 μl reaction mixture: 10 μl 10-fold PCR buffer, 2.4 μl of 50 mM $MgCl_2$, 2 μl of 10 mM dNTP, 1 μl of 100 μM sense and anti-sense primer each, 0.5 μl of 5 U/μl Taq DNA polymerase and 900 ng template DNA in 83.1 μl H_2O_2 (all PCR reagents from Gibco BRL, Karlsruhe, Germany). High stringency PCR was carried out for 35 cycles at 94˚C (60 s), 65˚C (30 s) and 72˚C (75 s), finished by a final extension-step at 72˚C (5 min). The PCR product was purified using a GFX purification kit (Pharmacia, Freiburg, Germany) and then sequenced by the dideoxy-chain-termination method using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Weiterstadt, Germany). All amplified exons were up- and downstream sequenced using the DNA sequencer ABI Prism 310 (Applied Biosystems, Darmstadt, Germany).

Statictical analysis. Statistical analyses were performed with the SPSS 10.0 software package (Munich, Germany). For the overall group and subgroups, not only the median and arithmetic mean but also the quartile and standard deviation for the time of the first diagnosis and the duration of treatment were determined. Other characteristics were represented in the tables for frequency. To evaluate survival time, a Kaplan-Meier survival analysis was carried out and the log-rank test was used with a 95% confidence interval as significance test.

Results

Codon 72 polymorphism. In 54 out of 77 tumor DNA samples (70.1%), the sequencing for codon 72 revealed a CGC-base triplet encoding the amino acid *Arg*. These patients were classified as homozygous for *Arg*/*Arg*. In 2 patients (2.6%), we detected a signal (12-14) giving the sequence CCC at codon 72 encoding for *Pro*. These patients were defined as homozygous for *Pro*/*Pro*. The remaining 21 patients (27.3%) showed two signals (CGC and CCC) after sequencing. Consequently these patients were categorized as heterozygous genotype for codon 72 (*Arg*/*Pro*).

Age at the time of first diagnosis. The age at the time of diagnosis ranged from 36 to 77 years. The median age of the group homozygous for *Arg* was 57.0 years, for *Pro* 65.5 years and of the group with the heterozygous genotype, 54.0 years. The entire median age at the time of diagnosis was 54.0 years. Using the *t*-test, no significant association between the genotypes for homozygous *Arg* and heterozygous genotypes and the age at the time of the first diagnosis ($p=0.154$) was observed. The group homozygous for *Pro* included only two cases, hence statistical analysis was not performed.

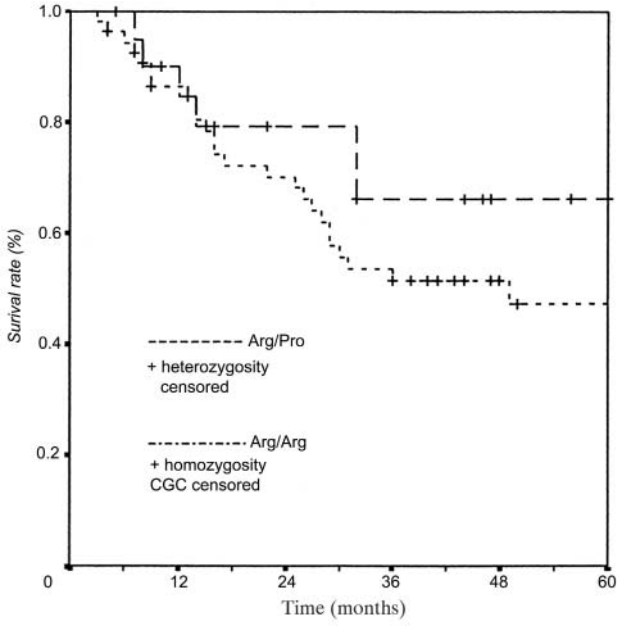

Figure 1. *Kaplan-Meier survival analysis for codon 72 variants.*

Median survival time. A Kaplan-Meier survival analysis was performed to evaluate the survival time. These data are shown in Figure 1. The median survival for patients with the homozygous *Arg*/*Arg* genotype was 49 months and the 5-year survival rate 47%. The group with the heterozygous genotype showed a median survival of 90 months and a 5-year survival rate of 66%. As mentioned above, analysis of the group *Pro*/*Pro* was not carried out. There was no statistically significant difference in the median survival between the *Arg*/*Arg* and the *Arg*/*Pro* genotype using the log-rank test ($p=0.49$).

Tobacco and alcohol exposure. Data for tobacco and alcohol consumption according to median age and genotype are listed in Table I. There was no significant difference in genotype frequency between smokers and non-smokers using the *t*-test.

Discussion

When summarizing the work on the codon 72 polymorphism in exon 4 of the *p53* gene for squamous cell cancer in head and neck, a few characteristics are obvious. The clinical significance of the codon 72 polymorphism in

Table I. *p53 polymorphism genotype according to median age at diagnosis and tobacco exposure.*

	Codon 72	N	Age (years)		
			median	arithmetic mean	standard deviation
Non-smoker/no alcohol abuse	Arg	4/16	57/61	61/62.1	10.1/8.3
	Arg/Pro	5/10	56/58	56.2/57.4	6.5/9.2
	Pro	–/1	–/–	–/66	–/–
	Total	9/59	57/60.6	58.3/60.6	8.1/88.8
Smoker/alcohol abuse	Arg	50/38	56.5/55.5	57.3/55.6	9.5/9.3
	Arg/Pro	16/11	53.5/52	53.3/50.9	10.8/9.8
	Pro	2/1	65.5/–	65.5/65	0.7/–
	Total	68/50	56.5/54	56.6/54.8	9.9/9.6

head and neck cancer is still unclear. Many studies dealing with head and neck cancer present very heterogeneous data concerning codon 72 polymorphism frequencies in squamous cell cancer. A few authors described an association between codon 72 polymorphism and susceptibility to head and neck cancer (7), while others doubt such an association (8, 9). In our case, a group of 77 patients with advanced squamous cell carcinomas of the head and neck was investigated for a correlation between the codon 72 alleles of the *p53* gene and the existence of head and neck cancer. In the present study homozygosity for *Arg*/*Arg* at the codon 72 was more commonly detected in comparison to that in Eurasian populations given in the literature. But comparing the current studies, some facts should be noted. The ethnic group seems to have a strong impact on the frequency of different genotypes (15, 16). There is not only a difference between Eurasians and African-Americans in codon 72 genotypes, as dectected in some Anglican-American studies (12-14), but there is also a different frequency among Eurasians themselves (16, 17). A Swedish group postulated an increasing *Pro* allele frequency from North to South (15). A meta-analysis in cervical and lung neoplasia demonstrated that the use of convenient control subjects when looking for associations with disease status may lead to spurious findings as a result of confounding due to population stratification (*e.g.* if cases and controls come from different racial/ethnic backgrounds) (18, 19). There are intercessional facts for case-only studies (20, 21).

Furthermore, there is a great variety not only of study designs but also of methods used. A substantial interlaboratory variation in genotype analysis has been demonstrated (22). Most groups dealing with codon 72 polymorphism applied methods such as restriction length polymorphism (RFLP) and single-strand conformational polymorphism (SSCP). Here a direct sequencing method, a more laborious but also more accurate and reliable technique, was employed. Furthermore, most of the studies used white blood cells as their DNA source. In contrast, our study used tumor tissue for genotyping, like other recent studies (11, 22, 23). Storey *et al.* (4) used tumor material for their codon 72 study on cervical cancer and showed that the rate of loss of heterozygosity (LOH) at exon 4 was low and therefore the DNA source is not an influential factor for defining the codon 72 genotype. However, recent analysis of the frequencies for the *Arg*/*Arg* genotype in the healthy European population revealed data between 36.6% (4) and 65% (24) in contrast to 70.1% in the present study. Consequently, for the *Pro*/*Pro* and *Arg*/*Pro* genotype, ethnical variation has been shown (8, 14). Summarizing the available data for codon 72 polymorphism in healthy Europeans and squamous cell cancer patients, all stated frequencies for the homozygous genotype *Arg*/*Arg* are lower than that observed in the present study. However, rating the different study designs and methods, or different kinds of statistical analysis, the current findings may point to a correlation between the *Arg*/*Arg* genotype in exon 4 of codon 72 and the development of HNSCC.

The epidemiology of HNSCC is similar to the epidemiology of lung and bladder cancer in that it is strongly related to tobacco exposure. In most case control studies, the risk of HNSCC is also strongly influenced by exposure to alcohol (25). Tobacco exposure and alcohol appear to act synergistically to increase the risk of mucosal surfaces in the mouth and pharynx developing cancer. The codon 72 polymorphism with the *Arg*/*Arg* genotype was suspected to have oncogenic potential in lung cancer and it was postulated that this polymorphism affects the risk of lung cancer unrelated to smoking (26). Jin *et al.* (13) and Kawajiri *et al.* (2) suggested both a potential susceptibility of the *Pro*/*Pro* genotype for smoking-induced lung cancer. Jin *et al.* (13) stated that patients with the susceptible genotype appeared to be younger at the time of diagnosis and have a lower mean cigarette consumption than the other genotypes of codon 72. In contrast, the present study has shown no relationship between the median age at the onset of the tumor nor the survival time and the codon 72 genotype. Furthermore, there was no significant difference in median age at the time of onset between *Arg*/*Arg* and *Pro*/*Pro* allele carriers within the groups of smoker/non smoker and alcohol abuse/no alcohol abuse. These findings indicate that there is no relationship between tobacco and alcohol exposure and codon 72 polymorphism as an additional risk factor. This confirms the findings of Mc Williams *et al.* (9). Differences in HPV positivity by head and neck cancer site could hide a genotypic bias in

HPV-infected patients, since about 18-36% of cases are expected to be positive for HPV, depending on the techniques used (27). We do not have HPV typing information for our study. Schwartz *et al.* (28) found that the oropharynx and the tonsils were the head and neck sites most likely to harbour HPV, which concurs with other data (27). Both a positive (28) and an inverse correlation (29) between smoking and the presence of HPV in host DNA has been reported. Interestingly enough, we observed the major frequency of homozygosity for *Arg*/*Arg* in cases with cancer sites in the oropharynx (79.2%), although due to the small group size there was no significant association.

In summary, to address the effect that codon 72 polymorphism may have in altering the risk of head and neck cancer developing, we conducted a case-only study of the codon 72 polymorphism in 77 cases of HNSCC and its association with different risk factors, age at the time of first diagnosis, survival and tumor localization. Neither the time of the first diagnosis nor the 5-year survival rates demonstrated any significant differences. Furthermore, no additive effect between different risk factors (alcohol, nicotine) and codon 72 polymorphism was recognized. In point of fact, the present study showed that homozygosity for *Arg*/*Arg* at codon 72 in HNSCC is more commonly detected than would be inferred for the Eurasian population as given in the literature. These findings might assume codon 72 polymorphism as a risk factor for HNSCC or reveal a high variability of codon 72 polymorphism among different ethnic groups.

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Received March 5, 2007
Revised June 26, 2007
Accepted July 9, 2007