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p53 codon 72 polymorphism, loss of heterozygosity and high-risk human papillomavirus infection in a low-incidence German esophageal squamous cell carcinoma patient cohort

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Abstract. Epidemiological studies in endemic geographic regions for esophageal squamous cell carcinoma (ESCC) suggested a number of risk factors, including modifications of the p53 tumor suppressor by codon Arg72Pro polymorphism, loss of heterozygosity (LOH) or human papillomavirus type 16 or 18 (HPV 16/18) infection. The p53 Arg72 variant has been suggested to be a high-risk factor in HPV-associated tumors. The present study analysed these associations in a low incidence geographic region in Germany. Fifty-three paraffin-embedded tumors and 53 surrounding normal squamous epithelium were investigated. For detection of p53 codon Arg72Pro polymorphism, direct sequencing for exon 4 was conducted. LOH analysis was performed using three micro-satellite markers at the p53 gene locus, and all cases were screened for high-risk HPV infection (HPV 16 and 18) with primer specific PCR and confirmed by sequencing. The p53 codon 72 genotype distribution was identical to published studies of normal Caucasian population, suggesting no general influence of this polymorphism on esophageal cancer risk in Germany. One case showed a p53 mutation in exon 4. p53 LOH was found in 13/44 (30%) informative cases without any correlation to histopathological characteristics. Of 53 (17%) samples, 9 showed HPV 16 or 18 infection. We found no association between p53 codon 72 genotypes and increasing HPV infection rates. Interestingly, 8/9 HPV-positive cases showed at least one p53 Arg72 allele. These results indicate an important role of p53 in ESCC also in low-incidence regions. In combination with the p53 Arg72 variant HPV infection could contribute to the risk of ESCC development in these cases, as has been demonstrated for high-risk regions.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the six most common malignant diseases in the world with a remarkable geographical distribution (1). Environmental exposures such as tobacco, alcohol, chronic mucosal irritation and ethnic background increase the risk of developing ESCC (2). Moreover, genetic changes affecting the p53 tumor suppressor such as different mutations, loss of heterozygosity (LOH) and high-risk human papillomavirus (HPV) infection are important for carcinogenesis in the esophagus. The p53 gene has a common sequence polymorphism resulting in either proline (CCC) or arginine (CGC) at amino-acid position 72. This polymorphism occurs in the proline-rich domain of the protein, which is necessary to induce apoptosis. The Arg72 variant induces apoptosis more effectively than does the Pro72 variant (3). In smokers, several studies have suggested an increased risk of lung cancer associated with the Pro/Pro genotype (4,5). In contrast, Arg/Arg homozygotes are frequently found in non-smoking patients with lung cancer (5,6).

Allelic deletions detected as LOH have been proved useful for mapping regions of DNA that contain tumor suppressor genes (7). Cancer lesions show a high frequency of LOH in the p53 tumor suppressor gene locus on chromosome 17p13.1 measured by repetitive DNA sequences (microsatellites), scattered widely within the genome (8). The p53 codon 72 polymorphism is also affected by LOH in tumors (9). Either arginine or proline could be lost by this mechanism. With the progression of (pre-) cancerous lesions in the esophagus, the rate of LOH at 17p13.1 raising the possibility that these changes may be one of the important mechanisms driving precancerous lesions to esophageal squamous cell carcinoma (1).

It was found previously that the p53 codon 72 arginine genotype is a high-risk factor for development of HPV associated cervical carcinoma (10). The arginine allele was found to be more susceptible to degradation by HPV E6 protein than the proline allele, resulting in a high frequency

Table I. Tumor staging and grading of the ESCC cohort.

TNM	T_{Cis}	T_1	T_2	T_3	T_4	G1	G2	G3	N_0	N_1
Cases	1	6	5	39	2	2	27	23	24	29
Percentage	(2%)	(11%)	(9%)	(74%)	(4%)	(4%)	(52%)	(44%)	(45%)	(55%)

Table II. Primer sequence and PCR conditions.

	Primer sequences (5' to 3')	PCR conditions
Microsatellite markers		
D2S123 (2p21-16.3)	Sense: AAACAGGATGCCTGCCTTTA Antisense: GGACTTTCCACCTATGGGAC	2 min at 95˚C; 1 min at 95˚C, 1 min at 60˚C, 1 min at 72˚C (35 cycles); 8 min at 72˚C; cooled to 8˚C
ß-globin	Sense: GTTGGCCAATCTACTCCCAGG Antisense: CTCACTCAGTGTGGCAAAG	2 min at 95˚C; 1 min at 95˚C, 1 min at 60˚C, 1 min at 72˚C (50 cycles); 8 min at 72˚C; cooled to 8˚C
P53 sequence analysis		
Exon 4	Sense: TTGCCGTCCCAAGCAAGT Antisense: GGGAAGGGACAGAAGATG	3 min at 95˚C; 1 min at 95˚C, 1 min at 56˚C, 1 min at 72˚C (35 cycles); 8 min at 72˚C; cooled to 8˚C
LOH-p53 analysis		
Tp53alu (17p13.1)	Sense: CGAGGAGGTTGCAGTAAGCGGA Antisense: ACAGCTCCTTTAATGGCAG	3 min at 95˚C; 1 min at 95˚C, 1 min at 55˚C, 1 min at 72˚C (40 cycles); 10 min at 72˚C; cooled to 8˚C
D17S918 (17p12)	Sense: TGTTGAGCTTTCCTGTAATC Antisense: TTCCTCACACAACCTATTGA	3 min at 95˚C; 1 min at 95˚C, 1 min at 51˚C, 1 min at 72˚C (45 cycles); 8 min at 72˚C; cooled to 8˚C
D17S786 (17p13.1)	Sense: TAGAGGGATAGGTAGCCGAG Antisense: GGATTTGGGCTCTTTTGTAA	2 min at 95˚C; 1 min at 95˚C, 1 min at 51˚C, 1 min at 72˚C (35 cycles); 8 min at 72˚C; cooled to 8˚C
Detection of HPV-DNA		
HPV 16	Sense: GCAACCAGAGACAACTGATC Antisense: TTGTAATGGGCTCTGTCCG	10 min at 94˚C; 1 min at 94˚C, 2 min at 55˚C, 2 min at 72˚C (40 cycles); 8 min at 72˚C; cooled to 8˚C
HPV 18	Sense: TCACGAGCAATTAAGCGACT Antisense: CTGAGCTTTCTACTACTAGC	10 min at 94˚C; 1 min at 94˚C, 2 min at 55˚C, 2 min at 72˚C (40 cycles); 8 min at 72˚C; cooled to 8˚C

of cervical SCC in individuals homozygous for arginine at the codon (9). This raises the possibility that homozygosity for codon Arg72 may lead to an increased susceptibility to other types of HPV related cancers as well. HPV infection occurs infrequently in association with ESCC in patients from North America (11,12) but regularly in high-incidence areas like P.R. China. The p53 codon Arg72 homozygous genotype is one of the high-risk genetic factors for HPV associated ESCC among the Chinese population (13). Especially HPV 16 and 18 encode two major oncoproteins, E6 and E7, and are implicated in the pathogenesis of squamous cell carcinoma (SCC) by deactivating the p53 tumor suppressor (14).

In the present study, we examined the relationship between the distribution of the p53 codon Arg72Pro genotype, loss of heterozygosity at the p53 gene locus, and the presence of HPV 16 and 18 DNA in a series of ESCCs and normal surrounding tissues in an ESCC patient cohort from Germany, a low incidence geographic region.

Materials and methods

Cancer and normal tissue probes. Fifty-three formalin-fixed, paraffin-embedded ESCCs (51 males and two females) with a median age of 58 years (range, 39-76 years) and 53 corresponding samples of histopathological normal squamous epithelium were included in this study. All cases were obtained from the archives of the Institute of Pathology, University of Regensburg and the Institute of Pathology, University of Bonn, Germany. The lesions were classified according to the International Union Against Cancer (UICC) guidelines (15). All patients gave informed consent for the study. The histopathological characteristics of the tumors are shown in Table I.

DNA isolation and quality control. DNA was extracted after manual microdissection of tumor and normal squamous epithelial cells. In brief, after deparaffinization and methylene

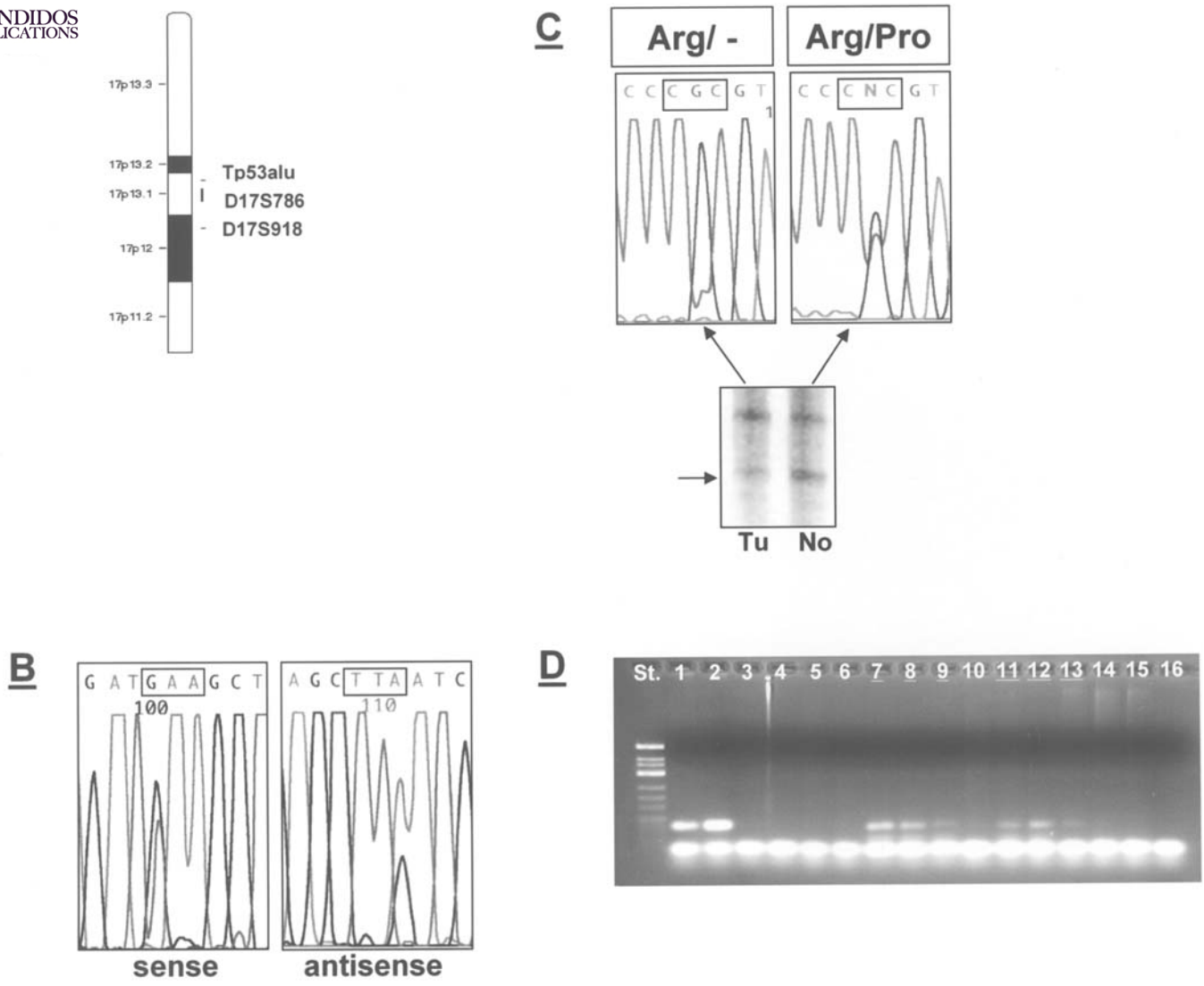

Figure 1. (A) Location of the microsatellite markers for p53 LOH analysis. (B) p53 exon 4 mutation, codon 62 (GAA to TAA) detected in one ESCC (sex, male; age, 76 years; diagnosis, pT3G2N1MX; no HPV 16/18 infection, LOH in the marker Tp53alu). Sense and antisense direction is shown. (C) p53 codon 72 sequencing and LOH of the proline allele in the tumor compared with the normal tissue. Note loss of the proline allele in the tumor. (D) Detection of HPV 16 DNA in ESCC. Lane 1 and 2, positive controls; lanes 3-15, ESCC samples; lane 16, negative control. ESCC with positive HPV 16 signals are marked (underlined numbers).

blue staining, microdissection was performed under an inverted microscope. A purity of at least 80% of tumor cells was obtained. DNA was extracted using the High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. DNA quality was assured by performing a PCR amplification of the polymorphic microsatellite marker D2S123 and a 540-bp ß-globin fragment (for primer sequences and PCR conditions see Table II). PCR products were separated using 2% agarose gels and visualized under ultraviolet light by using 0.05% ethidium bromide. Only DNA samples showing at least one specific PCR product were used for further analysis.

Genomic DNA sequencing of p53. Exon 4 of the p53 gene containing the polymorphic sequence variant at codon 72 was analysed using direct genomic sequencing. Primer sequences and PCR conditions are shown in Table II. PCR products containing exon 4 were purified by polyethylene glycol precipitation (equal volume of PCR product and PEG-Mix containing 26% PEG8000, 0.6 mol/l Na acetate, pH 5.5, 6.6 mmol/l $MgCl_2$). Purified DNA was sequenced in both directions using the PRISM Ready Dye Terminator cycle sequencing kit (Applied Biosystems GmbH, Weiterstadt, Germany) and an Applied Biosystems 373 sequencer. Sequencing reaction was as follows: initial incubation for 2 min at 96˚C, 25 cycles of denaturation for 15 sec at 96˚C, 15-sec annealing at 56˚C and 4-min elongation at 60˚C.

p53 deletion analysis. LOH analysis for the p53 gene locus on chromosome 17p13.1 was performed using three polymorphic microsatellite markers (Tp53alu, D17S918, D17S786, see Table II and Fig. 1A). Location of the markers and primer sequences were taken from the Genome Database (www.gdb.org). All primers were obtained from Proligo France SAS (Paris, France). Matched normal/tumor DNA samples were amplified by PCR in a 25-μl volume containing 0.2 mmol/l dNTP, 0.3 μmol/l primers, 1.5 mmol/l $MgCl_2$, 0.5 U Taq polymerase (Roche Diagnostics GmbH, Mannheim, Germany),

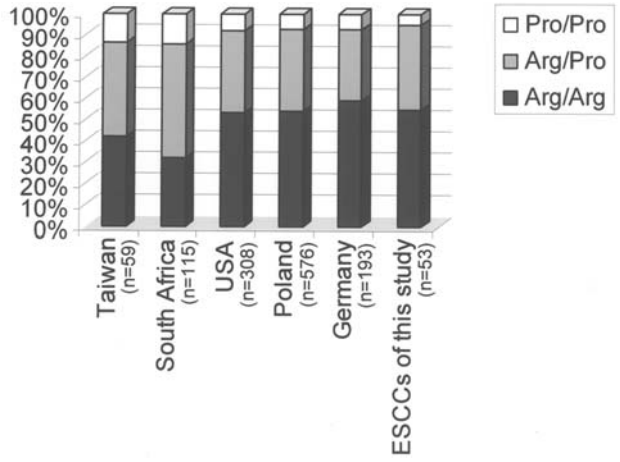

Figure 2. Comparison between the p53 codon 72 polymorphism distribution in normal control groups from various case/control studies (30-34) and ESCC cases from this study. Analysed ESCC cases showed a very similar distribution to the control groups of other studies on Caucasians (USA, Poland, Germany). Studies from South Africa and Taiwan clearly showed a different allele distribution with higher frequency of the proline allele in comparison to Caucasians. These regions are endemic for ESCC and are geographical high-risk populations for HPV-associated tumors. n, number of analysed cases.

using 100 ng isolated DNA as a template. PCR conditions (Table II) were optimized by gradient PCR and were carried out in an MJ research thermocycler (PTC 100). The amplification products were detected by polyacrylamide gel electrophoresis and silver staining as described previously (16). The silver-stained gels were assessed visually by two independent observers (AP and RS), and informative cases were scored as allelic loss when intensity of the signal for the tumor allele was decreased to at least 50% relative to the matched normal allele. To avoid errors due to preferential amplification of one allele during the PCR reaction, all detected allelic losses were verified by a second independent PCR reaction.

Detection of HPV 16/18 DNA. HPV type 16 and 18 specific PCR was performed as previously described (17). Matched normal and tumor tissues were screened for virus DNA using the primers and PCR conditions shown in Table II. Amplification was carried out in a 50-μl reaction volume containing 0.1% Triton-X-100, 0.2 mmol/l dNTPs, 2 μmol/l primers, 1.5 mmol/l $MgCl_2$, 0.5 U Taq polymerase and 100 ng DNA as template. As positive controls HPV-16 positive CaSki- and HPV-18 positive HeLa cell lines were used. The amplification products were detected by agarose gel electrophoresis (3%) and ethidium bromide staining. PCR products, indicating HPV infection of a patient, were sequenced as described above using sense PCR primers to verify the result. The received nucleotide sequence was used for standard nucleotide-nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) to show concordance of the sequence with the database entry for HPV 16/18.

Results

p53 codon 72 genotype distribution. The p53 codon 72 polymorphism (C/G) was examined by DNA sequencing for exon 4. The allelic distribution in the germline DNA of the patients was as follows: Arg/Arg, 29/53 (54.7%); Arg/Pro, 21/53 (39.6%); Pro/Pro, 3/53 (5.7%). In the corresponding ESCCs the following distribution was found: Arg/Arg, 31/53 (58.5%); Arg/Pro, 16/53 (30.2%); Pro/Pro, 6/53 (11.3%). The differences in the allelic distribution could be explained by the results of the LOH analysis (see below). Lacking a healthy control group the distribution of the p53 codon 72 polymorphism in the germline DNA of our patient cohort was compared to data of healthy controls in previously published studies (30-34) (Fig. 2). Our patient cohort showed a very similar allelic distribution to the control groups of three studies investigating Caucasian populations. Studies from endemic regions for ESCC (Taiwan, South Africa) showed a clearly different distribution of the polymorphism. This indicates that the p53 codon 72 polymorphism is not correlated with an increased risk for ESCC in our cohort. There was also no correlation between any allelic variant and histopathological characteristics of the tumors. The sequence analysis of exon 4 revealed a nonsense mutation (G to T; GAA to TAA; Glu to STOP; codon 62) in 1/53 tumors (Fig. 1B). This patient also showed LOH of the remaining allele.

LOH analysis of the p53 locus. LOH analysis for the p53 gene locus on chromosome 17p13.1 was performed using three polymorphic microsatellite markers. Of 53 patients, 44 showed heterozygosity in at least one microsatellite marker analysed, eight patients were homozygous for all three markers, and DNA from one tumor showed no amplification products at all. Overall, 13/44 (30%) of the informative cases showed LOH in the analysed p53 areas without any correlation to histopathological characteristics. LOH appears in early tumor stages such as T1 but also in T4 tumors. Of 13 patients with LOH, 5 were heterozygous for the codon 72 polymorphism and a loss of the Arg or Pro allele was analysed. Of 5 (40%) tumors, 2 showed loss of the Pro allele, and 3 (60%) showed loss of the Arg allele (Fig. 1C). Therefore the allelic distribution varied between the germline DNA of the patients and the corresponding ESCCs.

HPV 16 and 18 detection. Matched normal and tumor tissues were screened for HPV 16 and 18 DNA with virus-specific primers. Of 53 (17%) of the samples, 9 showed HPV 16 or 18 infection (Fig. 1D). All cases were confirmed by sequencing. We found the HPV DNA both in the tumor and in the surrounding squamous epithelium. Six samples contained the HPV 16 virus, and three contained the HPV 18 virus type. We did not find an infection with both virus types. Of 6 (17%) of the HPV 16-positive samples, 1 was homozygous for the arginine allele, and 5 (83%) were heterozygous. Of 3 (67%) of the HPV 18-positive specimens, 2 showed the Arg/Arg, and 1 (33%) showed the Pro/Pro genotype. Therefore 8/9 (89%) HPV-infected probes are associated with the arginine allele. In 3/8 (38%) HPV-positive Arg-associated specimens LOH at the p53 gene locus was found.

Discussion

p53 gene alterations are initiating or very early events in SCC regardless of anatomic site and already evident in premalignant

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18). These alterations include p53 mutations and mal deletions at the p53 gene locus. In addition, allelic variations in the coding region of p53 were also identified recently (3). The p53 codon Arg72 homozygous genotype is one of the high-risk genetic factors for HPV-associated ESCC among the Chinese population (13). In our study p53 codon 72 genotype distribution in ESCC patients was identical to healthy Caucasian control groups. This indicates that the p53 codon 72 polymorphism does not influence the general risk for ESCC in Germany. To our knowledge, this is the first study analysing the p53 Arg72Pro polymorphism in ESCC patients in a low endemic region.

Sequence analysis of exon 4 of the p53 gene revealed 1 case (2%) with a nonsense mutation. This low frequency of mutations in exon 4 is in accordance with previous studies showing that p53 mutations are most frequently found in exons 5-9 in ESCC (20,21).

Deletion of 17p is very common in most tumor types and is the most frequent way to inactivate the remaining wildtype allele in a tumor with p53 mutation. We found that 30% of the tumors in our patient cohort showed LOH at chromosome region 17p13.1. There are several studies dealing with p53 deletion in ESCC. The detected rates of LOH vary from 55% to 80% (22-24). All of these studies analysed tumors from high-risk populations. This might explain the discrepancy to our findings of a lower rate of p53 LOH. Another reason could be that 9 of 44 tumors with interpretable results were informative for the microsatellite markers D17S918 and D17S786 but not for the Tp53alu marker. These markers are located downstream of the p53 gene locus, and therefore smaller deletions might have been missed in our study. Nevertheless our data indicate an important and early role of p53 alterations in esophageal carcinogenesis in a low-risk population. LOH also affected the polymorphic codon 72 region in exon 4. Sixty percent of the ESCCs with LOH and both allelic variants in the corresponding germline DNA showed a deletion of the arginine allele and retention of the Pro72 variant. The Arg72 variant induces apoptosis more effectively than does the Pro72 variant (3). Therefore it might be speculated that preferred deletion of the Arg72 allele could generate cells which are more resistant to apoptotic stimuli. Experimental data for this hypothesis are missing. Large studies in different populations should be performed to clarify the importance of a possible preferential deletion of Arg72 in the esophageal carcinogenesis.

HPV may be a causative factor in carcinoma of the esophagus (17). The HPV infection rates range from 21% to 75% (13,19). E6 from HPV 16 and 18 is more effective in degradation of p53 Arg72 than p53 Pro72 *in vitro*. p53 Arg72 may therefore represent a risk factor for HPV-associated tumorigenesis (10). In the present study we detected a relatively low rate of HPV high-risk type 16 or 18 infection in the ESCCs from Germany. Only 9/53 (17%) cases showed an infection with HPV 16 or 18. Our results are in contrast to a previously published study by Awerkiew and co-workers (25), who found no HPV-positive ESCC in 23 patients from Germany but a high incidence (35%) of Epstein-Barr-virus (EBV) in ESCC. Recently these authors showed that the presence of EBV in esophageal cancer is restricted to tumor infiltrating lymphocytes (26). Only one other study also detected EBV in ESCC but with a much lower frequency (8%) (27). In addition, two studies from Greece and Thailand investigated EBV infection in ESCC and did not detect any positive tumors (28,29) suggesting a minor role of EBV in ESCC.

Nevertheless, the important role of the p53 codon 72 polymorphism in HPV-associated cancer had already been demonstrated for ESCC in endemic regions (9). Interestingly, in our tumor cohort 8/9 (89%) HPV-positive ESCC cases showed at least one p53 Arg72 allele. Because of the small overall number of HPV-positive tumors this difference did not reach statistical significance (data not shown). Of 8 (37.5%) cases, 3 were homozygous for the Arg72 variant. In these cases fast degradation of the p53 protein by the E6 oncoprotein could have driven genomic instability and tumor initiation. Of 8 (62.5%) cases, 5 displayed both allelic variants of the p53 gene. A supposed degradation of the Arg72 variant by E6 in these cases could lead to functional activity of only the Pro72 variant and to a decreased apoptotic sensitivity of the cells. This condition might also increase the risk for malignant transformation of the cells, but additional experimental data are needed to validate this hypothesis.

In summary, we showed that alterations of p53 play a considerable role in ESCC from a low-incidence German patient cohort. The distribution of the p53 codon 72 polymorphism seems not to be a general risk factor for ESCC in Caucasians. Even in a low-risk population, infection with HPV 16 and 18 is detectable in ESCC patients and might increase the risk for ESCC. In these cases, the p53 Arg72 allele could contribute to a higher risk for ESCC development by quick degradation of p53 via the viral E6 oncoprotein, as is known to occur in patients from high-incidence geographical regions.

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