INVESTIGATIVE REPORT

The Role of p53 Codon 72 and Human Papilloma Virus Status of Cutaneous Squamous Cell Carcinoma in the Swedish Population

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The arginine variant of the p53 codon 72 polymorphism as well as anogenital and epidermodysplasia verruciformis (EV) types of human papilloma virus (HPV) are suggested to confer increased risk for developing cutaneous squamous cell carcinoma (SCC). In this pilot study, we analysed the p53 codon 72 genotype distribution in 106 microdissected samples from normal and tumour tissues of 53 cases of cutaneous SCC and 96 controls from Sweden. Both normal and tumour samples from cases of SCC were screened for anogenital and EV HPV. The $p53_{Arg}$ allele was not associated with the development of cutaneous SCC. Anogenital HPV (44%) was more prevalent than EV HPV (12%). Data also indicate that anogenital HPV is more common in tumour samples, but HPV infection was not identified as a significant risk factor for developing SCC. The presence of anogenital HPV, but not EV HPV might be a risk factor for development of cutaneous SCC. *Key words: single nucleotide polymorphism; loss of heterozygozity (LOH); epidermodysplasia verruciformis (EV); anogenital.*

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A nucleotide substitution from G to C gives rise to an amino acid change from arginine to proline at the polymorphic site in p53 exon 4, codon 72. These $p53_{Arg}$ and $p53_{Pro}$ variants have different structural conformations and are not functionally equivalent. $p53_{Arg}$ differs from $p53_{Pro}$ in transcriptional activity and has a reduced ability to induce apoptosis and suppress transformed cell growth, potentially increasing the risk of developing cancer (1). The $p53_{Arg}$ variant has also been shown to act as an intragenic modifier of mutant p53 behaviour by enhancing binding and inactivation of the p73 gene product, leading to inhibition of p73-induced apoptosis (2). The clinical relevance has been demonstrated in different forms of human epithelial squamous cell cancers (2, 3), and $p53_{Arg}$ has been demonstrated to represent a significant

risk factor in the development of human papilloma virus (HPV)-associated cancers (4).

Epidemiological and experimental studies have clearly shown that HPV infection is the main aetiologic factor for cervical cancer. The HPV E6 gene products play a critical role in cervical carcinogenesis by interfering with p53 function, consequently deregulating the cell cycle. E6 from both high and low risk anogenital HPV types binds to the $p53_{Arg}$ allele with enhanced affinity as compared with the $p53_{Pro}$ variant, resulting in increased ubiquination and degradation of the p53 protein (4). As a consequence, $p53_{Arg}$ in combination with HPV may be hypothesized to provide an augmented risk for persistent DNA damage making not only cervical, but also skin epithelial cells, more cancer-prone. Anogenital HPV types have in fact been found to be associated with an increased risk for developing cutaneous squamous cell carcinoma (SCC) in immunosuppressed renal transplant recipients (4), and in SCC of distal digits and periungual skin (5). Recently, anogenital HPV was also found in immunocompetent individuals with cutaneous SCC (6).

Particular cutaneous HPV subtypes are involved in the development of non-melanoma skin cancer (NMSC) associated with the inherited disorder epidermodysplasia verruciformis (EV) (7). In analogy with anogenital HPV types, EV HPV subtypes have the ability to transform rodent fibroblasts (8–11). However, none of the three investigated cutaneous HPV types have been shown to transform human keratinocytes (8, 11). Multiple pathways for EV HPV transforming activity have been suggested, including both partly p53-dependent and p53-independent mechanisms (12). An important example is the E6-stimulated degradation of Bak via the ubiquitin-mediated pathway (13, 14). There appears to be a crucial additional requirement for UV in the oncogenic mechanism of the EV HPV subtypes (15). This may be inferred to the p53 as well as to the Bak-targeted binding and degradation, which both have the consequence of inhibiting the apoptotic response to UV. None of the experiments investigating the EV HPV E6 protein interaction with TP53 have been able to confirm any binding (8, 16). However, these studies have limitations such as evaluation of limited HPV types (only HPV 1, 8 and 47 tested), species-specific

differences (16), as well as the use of systems which might be devoid of cofactors necessary for EV HPV E6 protein to bind and degrade TP53. This raises the possibility of an inability to disclose weak, but yet biologically significant, protein interactions. Consequently a biologically significant EV HPV E6/TP53 interaction may not be completely excluded. Considering the weaker transforming activity of the EV HPV subtypes (9–11), synergistic or additive effects, such as p53 polymorphism, may provide a significant contribution to the development of cutaneous SCC.

In this pilot study we aimed to elucidate the potential association of $p53_{Arg}$ and HPV status, individually or together, with increased risk of developing cutaneous SCC in a Swedish population. We investigated the p53 codon 72 genotypes of 106 microdissected samples from 53 cases of cutaneous SCC and 96 unselected healthy Swedish blood donors in a case-control study. In addition, analysis of preferential loss of heterozygosity (LOH) in tumour samples of heterozygous cases was performed. Lastly, tumour and normal samples from cases of cutaneous SCC were screened for anogenital and EV types (HPV 5, 8, 15, 20, 24, 38) of HPV.

MATERIALS AND METHODS

Samples

Normal and tumour tissues were microdissected from 53 cases of formalin-fixed, paraffin-embedded specimens of cutaneous SCC collected from the pathology archives comprising specimens from a Swedish population (Uppsala County). Routine histological classification was confirmed by a pathologist prior to inclusion into this study. Tissue sections were deparaffinized, cut and stained with H&E prior to microdissection. Microdissected samples were digested with proteinase K, 1.0 μg/μl (Boehringer Mannheim, GmbH, Germany) at 56°C overnight to facilitate amplification of genomic DNA. Following inactivating incubation at 95°C for 10 min, samples were stored at −20°C for subsequent analysis. Peripheral blood from a total of 96 unselected healthy blood donors from the same region in Sweden constituted the control group.

p53 c72 status determination

A nested PCR approach was used to amplify exon 4 from the p53 gene. Genomic DNA was used as template for the outer PCR (initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 1 min and a final extension at 72°C for 10 min) in a total volume of 50 μl. The amplification mixture comprised 10 mM Tris-HCl (pH 8.3), 2 mM MgCl$_2$, 50 mM KCl, 0.1% Tween 20, 0.2 mM dNTPs, 5 pmol of each primer (5′-AGAGGAATCC-CAAAGTTCCA-3′ vs 5′-CTGGGACCTGGAGGGCTGG-3′) and 1.5 units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA).

To generate a template for pyrosequencing, outer PCR product was used for nested inner PCR (initial denaturation

94°C for 5 min followed by 35 cycles of 94°C for 1 min, 50°C for 40 s and 72°C for 1 min and a 10-min extension at 72°C). The amplification mixture comprised 10 mM Tris-HCl (pH 8.3), 2 mM MgCl$_2$, 50 mM KCl, 0.1% Tween 20, 0.2 mM dNTPs, 5 pmol of each primer (5′-biotin-TCC-AGATGAAGCTCCCAG-3′ vs 5′-AGGGGCCGCCGGT-GTA-3′) and 1 U of AmpliTaq DNA polymerase in a total volume of 50 μl. The quality of the PCR product was subsequently analysed using agarose gel electrophoresis.

The template for pyrosequencing was prepared using MBS robotics and software (Magnetic BioSolutions, Stockholm, Sweden). Briefly: the biotinylated inner PCR product was immobilized onto streptavidin-coated super paramagnetic beads (Dynabeads M280; Dynal, Oslo, Norway) in B/W-buffer (10 mM Tris-HCl (pH 7.6), 2.7 M NaCl, 1 mM EDTA, 0.1% Tween 20). Single-stranded DNA was obtained by incubating the immobilized PCR product in 0.2 M NaOH melting away the non-biotinylated strand. The immobilized strand was washed and subsequently resuspended in annealing buffer (20 mM Tris-acetate, 2 mM Mg-acetate, pH 7.6) containing 1 pmol/μl sequencing primer (5′-GCTGCTGGTGCAGGGGCCA-3′). Real-time pyro-sequencing was subsequently performed on an automated 96-well PyroSequencer using the LucKit SNP 96 (Genotage, Uppsala, Sweden) enzymes and substrates.

Status determination of anogenital HPV types

The genital HPV status was determined by single-step PCR and DNA sequencing using the universal degenerated primers GP5+(5′-TTTGTTACTGTGGTAGATACTAC-3′) and GP6 +(5′-GAAAAATAAACTGTAAATCATATTC-3′) designed to detect multiple cervical HPV subtypes (17). Microdissected material was used as template for PCR (40°C for 10 min followed by initial denaturation at 95°C for 10 min and thereafter 94°C for 1 min, 48°C for 2 min and 72°C for 90 s for 40 cycles and a final extension at 72°C for 7 min) in a total volume of 25 μl. The amplification mixture comprised 10 mM Tris-HCl (pH 8.3), 3 mM MgCl$_2$, 50 mM KCl, 0.1% Tween 20, 0.2 mM dNTPs, 0.5 μM of each primer, 0.2 units of uracil N-glycosylase (UNG) (Applied Biosystems, Foster City, USA) and 0.8 units of platinum Taq DNA polymerase (Invitrogen Life Technologies, USA). The quality of PCR products was inspected by agarose gel electrophoresis.

PCR detection of β-globin DNA was used as DNA template quality control. Microdissected material was used as template for PCR (initial denaturation at 95°C for 10 min and thereafter 40 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min and a final extension at 72°C for 7 min) in a total volume of 20 μl. The amplification mixture comprised 10 mM Tris-HCl (pH 8.3), 4 mM MgCl$_2$, 50 mM KCl, 0.1% Tween 20, 0.16 mM dNTPs, 0.5 μM of each primer (5′-GAAGAGCCAAGGACAGGTAC-3′ and 5′-CAACTTCATCCACGTTCACC-3′) and 0.8 units of platinum Taq DNA polymerase (Invitrogen Life Technologies). The quality of the PCR products was inspected by agarose gel electrophoresis. Note that PCR success rates for β-globin amplification and HPV are less than the success rate for p53 due to the decreased sensitivity of single PCRs as compared with nested PCR. In six cases where anogenital HPV was detected, the β-globin control PCR tested negative for that sample, indicating that DNA-positive (i.e. informative) samples might be underestimated. These HPV-infected samples were included in the data analysis.

Status determination of EV HPV types

The HPV status of EV-associated HPV subtypes 5, 8, 15, 20, 24 and 38 was determined by type-specific PCRs according to Struijk and co-workers with some modifications (18). Single-step PCR with EV HPV type-specific primers was performed using microdissected material (initial denaturation at 95°C for 10 min, 45 cycles at 95°C for 20 s, 55°C (HPV 15, 20, 38), 57°C (HPV 24) or 60°C (HPV 5, 8) for 40 s and 72°C for 1 min, before a final extension at 72°C for 10 min) in a total volume of 50 μl. The amplification mixture comprised 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl$_2$, 50 mM KCl, 0.2 mM dNTPs, 10 pmol of each primer and 1 unit of AmpliTaqGold DNA polymerase (Applied Biosystems). The quality of the PCR product was subsequently analysed using agarose gel electrophoresis. Negative controls were introduced between every seventh sample.

The sensitivity of the different PCRs was determined for the different EV HPV types by testing serial-fold dilutions of EV HPV plasmids for the following types HPV 5, HPV 8, HPV 15, HPV 20, HPV 24 and HPV 38 in the absence and presence of spiked genomic DNA. The sensitivity of the PCR was demonstrated to be in the range of 1–100 copies for all types. The identity of EV HPV types was validated by analysis of fragment length on an Agilent 2100 bioanalyser (Agilent Technologies) and subsequent DNA sequencing (see below). PCR detection of genomic DNA (20p11.21) was used as DNA template quality control. Microdissected material was used as template for PCR (initial denaturation at 95°C for 10 min and thereafter 33 cycles of 95°C for 20 s, 55°C for 40 s and 72°C for 1 min and a subsequent final extension at 72°C for 10 min) in a total volume of 50 μl. The amplification mixture comprised 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl$_2$, 50 mM KCl, 0.2 mM dNTPs, 10 pmol of each primer (5′-CCTCAATGACAAGAGTGTGC-3′ and 5′-CAGTCATGGTACAGACACAG-3′) and 1 unit of Ampli-TaqGold DNA polymerase (Applied Biosystems). The sensitivity of genomic PCR was approximately 100 copies. In one case EV HPV was detected in a sample that tested negative for genomic DNA. This sample was included in the data analysis.

Sequencing of HPV products

Purification of PCR products were performed before running the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) according to the manufacturer's instructions with temperature cycling at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min repeated for 25 cycles using one of the PCR primers. After ethanol purification automatic sequencing was performed with the ABI 377/ABI3700 (PE Applied Biosystems). The subtype of HPV was determined (anogenital) or validated (EV HPVs) by BLAST homology search against NCBI Standard nucleotide-nucleotide BLAST. A nucleotide sequence was regarded as a distinct HPV type if it shared >90% homology with a known type. Eighteen samples with anogenital HPVs contained overlapping sequence information hindering identification of individual virus genotypes.

Statistics

The distribution of p53 codon 72 alleles in SCC cases as compared to healthy controls was tested for homogeneity using the χ^2 test, presuming Hardy Weinberg equilibrium since the control samples showed almost perfect equilibrium. For the cases of SCC the association of anogenital and EV

HPV types with cancer tumours, regardless of p53 codon 72 status or specifically in combination with the p53$_{Arg}$ allele, was tested using the χ^2 test. With paired observations from both normal and tumour tissue from the same patient a one-sided McNemar's test was performed to analyse the risk for increased HPV prevalence in tumour samples.

RESULTS

To investigate the implicated potential association of p53$_{Arg}$ allele with development of cutaneous SCC in the Swedish population we performed a pilot case-control study of the allelic distribution of p53 codon 72 genotypes in 53 cases (106 samples) of SCC (normal and tumour) in addition to 96 normal control samples. We could not establish a statistically significant different allelic distribution for normal samples from SCC cases, as compared to the control group ($p = 0.09$; Table I). In particular there was no over-representation of the p53$_{Arg}$ allele in the SCC group. Heterozygous cases of SCC were also investigated for preferential loss of the p53$_{Pro}$ allele. The proportion of LOH was determined by inspection of chromatograms, with all cases of LOH demonstrating a complete loss of one allele. An overall p53 LOH frequency of 32% (6 of 19) was detected in tumour samples (data not shown). There was no indication of preferential loss of the p53$_{Pro}$ allele in tumours (3 of 6). No LOH was observed overall for the normal samples (0 of 19).

Conflicting data have been reported on the involvement of HPV in cutaneous SCC development. Here we analysed the presence of anogenital and EV types of HPV in normal and tumour samples microdissected from cases of SCC to identify any indication of association between HPV infections with increased risk for developing cancer either in combination with, or regardless of, the p53 codon 72 genotype.

Presence of anogenital HPV infection was determined by a single degenerate PCR, coupled with sequence analysis. In addition, the presence of genomic DNA in each sample was determined by a single-step β-globin PCR. Samples that showed successful amplification of genomic DNA were considered informative and the remaining samples were excluded from this part of the analysis. In total, 27 samples were found to be infected by anogenital HPV in 20 of 45 informative cases, corresponding to an overall prevalence of 44%. In histologically normal tissues, 30% of the samples (8/27) contained anogenital HPV, while tumour tissues

Table I. *Allelic distribution of p53 codon 72 genotype in microdissected normal tissue from Swedish cutaneous squamous cell carcinoma (SCC) cases and healthy Swedish controls*

	p53$_{Arg}$/$_{Arg}$	p53$_{Arg}$/$_{Pro}$	p53$_{Pro}$/$_{Pro}$	$\Sigma_{samples}$
Normal$_{SCC}$	30 (55%)	19 (36%)	5 (9%)	53
Control	62 (65%)	31 (32%)	3 (4%)	96

showed an increased frequency of infection affecting 53% of the samples (19/36) (Table II). The data from informative paired normal and tumour samples coming from the same SCC patient demonstrated that none of the anogenital HPV infections was restricted to merely normal tissues. However, in five cases both normal and tumour tissues were infected, and in three cases anogenital HPV was detected only in the tumour sample. Albeit not statistically significant, these data are indicative of an increased prevalence of anogenital HPV in tumour as compared with normal tissues (one-sided $p = 0.125$). The number of potential multiple anogenital HPV infections as determined by DNA sequencing is significant, 67%, while the remaining 33% were single infections of HPV type 16, 18 and 45. The distribution of samples infected with anogenital HPV types did not show any significant deviation from the genotype distribution in the control group, either when grouped according to p53 genotype or when considered regardless of the p53 genotype (see Tables I and II).

The presence of EV HPV was determined by single-step PCRs using type-specific primer sets (HPV 5, 8, 15, 20, 24, 38). Additional screening for the presence of genomic DNA was performed by single-step PCR. Samples that showed successful amplification were considered informative, while the remaining samples were excluded from this part of the analysis. In total six samples were found to be infected by EV HPV in 5 of 41 informative cases, corresponding to an overall prevalence of 12%. In histologically normal tissues, 17% (5/30 samples) contained EV HPV, while infection was detected in 3% (1/31) of tumour samples (Table II). All of the EV HPV infected samples were p53$_{Arg/Arg}$ homozygous. In four cases the EV HPV infections were restricted to normal samples (i.e. HPV 15, HPV 20 and two HPV 38 infections). In a single case, paired normal and tumour samples from the same patient were infected, but with different EV HPV subtypes (HPV 8 in normal tissue and HPV 38 in tumour tissue). Co-infection of anogenital and EV types of HPV was demonstrated in a single tumour sample.

In conclusion, the p53 codon 72 genotype was not found to be associated with increased risk of developing SCC. Cases of cutaneous SCC were found to have a higher prevalence of anogenital than EV types of HPV infections. Data also indicate an increased prevalence of

anogenital HPV infections in tumour as compared with normal tissues. However, no statistically significant association could be established between development of cutaneous SCC and the presence of anogenital HPV, EV HPV or when considering all HPV types as one group — either in combination with the p53$_{Arg}$ allele or regardless of the p53 genotype.

DISCUSSION

So far evidence for the role of p53$_{Arg}$ as a risk factor for cutaneous SCC appears contradictory. Some reports confirm an association of p53$_{Arg}$ with increased risk for developing cutaneous SCC in immunosuppressed cases (19, 20), while another report shows conflicting data (21). In the presence of HPV, an association between the p53$_{Arg}$ variant and increased risk for developing cutaneous SCC has been shown for both immuno-compromised (4) and immunocompetent cases (22). However, other groups found no correlation of SCC with p53$_{Arg}$, HPV or a combination thereof in either immunocompetent or immunosuppressed cases (23, 24). Similarly, there are also several conflicting reports on the association of p53 status and risk of developing cervical SCC in different ethnic groups (4, 25–28). In particular, when studying the Swedish population we (29) and another group (30) did not identify this correlation, while a third study claimed to confirm the association (31). For example, differences in genotyping methodologies and inter-laboratory variation have been suggested as causes of discerning results (29, 32). There is also a possibility that the p53 c72 is in linkage disequilibrium with alternative cancer-promoting genes or polymorphisms in diverse ethnic groups, thus functioning as a marker, or that the p53 c72 polymorphism represents a greater risk factor in particular genetic backgrounds.

In concordance with our previous report on p53$_{Arg}$ and the risk for acquiring cervical SCC in a Swedish population (29), this allele was also not found to be associated with the development of cutaneous SSC in this study. Likewise, preferential loss of the p53$_{Pro}$ allele in tumours was not evident. However, the importance of the p53 gene in the development of cutaneous SCC is not ruled out. Approximately 32% of the heterozygous tumours had lost one p53 allele. This was in an

Table II. *Human papilloma virus (HPV) status in normal and tumour samples from cases of cutaneous squamous cell carcinoma according to p53 codon 72 genotype*

Location/type of HPV	p53$_{Arg/Arg}$		p53$_{Arg/Pro}$		p53$_{Pro/Pro}$		$\Sigma_{samples}$	
	Normal	Tumour	Normal	Tumour	Normal	Tumour	Normal	Tumour
Anogenital	4/18 (22%)	12/23 (52%)	3/7 (42%)	3/8 (38%)	1/2 (50%)	4/5 (80%)	8/27 (30%)	19/36 (53%)
EV	5/19 (26%)	1/20 (5%)	0/8 (0%)	0/8 (0%)	0/3 (0%)	0/3 (0%)	5/30 (17%)	1/31 (3%)

EV: epidermodysplasia verruciformis.

expected range, as previously published data on LOH of the p53 gene in cutaneous SCC present frequencies varying from 18% ($n=49$) (20) to 45% ($n=11$) (33) and 59% ($n=36$) (34). This relatively high frequency of LOH in tumour cells rather underscores a link between p53 and risk for developing SCC, as alternative p53 polymorphisms or mutations may be selected through the deletion of one of the p53 alleles.

Skin type and susceptibility to sunburn, which are known risk factors for SCC, have been found to be associated with the p53$_{Arg}$ genotype (20). In this study, the effect of this possible confounder is considered negligible since all investigated cases and controls were collected in the same region and may be assumed to be of the same skin type. According to the literature, UV radiation is the main requirement for induction of cutaneous SCC (35). In theory, UV may also cause an increase in HPV viral load (36). However, anamnesis of UV exposure is a confounding parameter that is almost impossible to retrieve and evaluate, even in tumours from the same location from individuals living in the same region. Hence, it seems reasonable to assume only minor gains when controlling for UV exposure in this study and consequently this confounder was not considered.

In all, 44% of the SCC cases were found to be infected by anogenital HPV and 12% by EV-associated HPV, which is in agreement with previous data on the prevalence of HPV in immunocompetent individuals (6, 37–40). The lower prevalence of anogenital HPV in normal tissues (30%), as compared with the prevalence in tumour tissues (51%), indicates anogenital HPV as a risk factor for cutaneous SCC. The increase in anogenital HPV frequency in tumours, regardless of p53 codon 72 genotype could be hypothesized to be attributable to the alternative, p53-independent, cancer-promoting mechanism of the HPV E6 protein causing degradation of Bak (41, 42). Interestingly, HPV single infections containing anogenital HPV types 16, 18 or 45 were not identified as restricted to normal tissues, although present in three tumour tissues as well as in one normal sample, but with a parallel tumour infection. Our findings are supported by a recent report (6) demonstrating that high-risk anogenital HPV subtypes are risk factors for the development of cutaneous SCC. Furthermore, both studies demonstrate the occurrence of a large proportion of multiple infections of anogenital HPVs in SCC, which was not observed in basal cell cancer samples (6).

This study has involved a microdissection strategy to isolate and analyse defined areas of tumour growth. The low level of EV-associated HPV in these tumour samples was also observed by Iftner and co-workers (6), and indicates a minor role of the EV-associated HPV subtypes (HPV 5, 8, 15, 20, 24, 38) in the pathogenesis of SCC, although the effect of other subtypes cannot be ruled out. The observation that normal skin has a higher proportion of EV-associated HPV infection as compared with tumour samples could be explained by the fact that normal microdissected skin material is more heterogeneous than a sample corresponding to a clonally expanded tumour material. Furthermore, we could also demonstrate that two adjacent samples (normal/tumour) could harbour two different EV-associated HPVs, indicating a demarked and localized spreading of the virus.

In summary, our results indicate that the p53$_{Arg}$ allele is not associated with the development of cutaneous SSC, and that the presence of anogenital, but not EV-associated, HPV might be a risk factor for development of cutaneous SCC. A larger study is required to clearly distinguish the true association of implicated risk factors.

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