CLINICAL REPORT

HPV-related Cancer Susceptibility and p53 Codon 72 Polymorphism

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Conflicting results regarding the association of a polymorphism at codon 72 of the p53 tumour suppressor gene and susceptibility to develop human papilloma virus (HPV)-associated cervical cancer have been published over the last year, implicating differences in ethnic background, sample origin, sample size and/or detection assay. The material for this study was collected in the identical geographical region as for 2 previous reports with contradictory results regarding the association of codon 72 genotype with squamous cell cancer (SCC). We have used an alternative detection assay, based on pyrosequencing technology, that interrogates the variable position by the accuracy of DNA polymerase. In addition to cervical clinical specimens from SCC, HPV16- and HPV18-infected adenocarcinoma cases as well as cervical intraepithelial neoplasia (CIN) were investigated. No significant association was found between p53 codon 72 genotype and the risk to develop adenocarcinoma, SCC or CIN in the Swedish population. *Key words: human papilloma virus; cervical intraepithelial neoplasia.*

Adenocarcinoma and squamous cell carcinoma (SCC) of the cervix are both associated with high-risk human papilloma virus (HPV) infection (1, 2). The E6 oncoprotein from high-risk HPV binds to and induces ubiquitin-mediated degradation of the p53 protein (3, 4) and thereby contributes to the development of cervical cancer. Thus, this E6-mediated inactivation of p53 can be functionally equivalent to inactivating p53 mutations. Mutant p53 variants have been shown to be resistant to E6-mediated degradation (5) and specific E6 variants have been proposed to be more prevalent in invasive cervical tumours than the prototype (6). These findings indicate that the interaction between p53 and E6 can be modulated and may have implications for the development of cervical carcinoma.

The single nucleotide polymorphism (SNP) in p53 exon 4 codon 72, either CGC (arginine) or CCC (proline), was first suggested to play a role in the development of HPV-associated cancers by Storey and coworkers in 1998 (7). They showed that high-risk E6 protein has a selective affinity for the p53 arginine variant and that arginine homozygous individuals were 7 times more susceptible to develop HPV-associated cancer. The association to cervical SCC was confirmed in a recent study (8). However, all other reports have not been able to confirm the association of p53 codon 72 arginine homozygotes with increased risk for cervical cancer (9–23). Intriguingly, 2 separate investigations (8, 23) based on the analysis of cervical SCCs from the identical geographical region have produced conflicting results. Our study include both SCC and adenocarcinoma cases collected from Swedish women in the same region as in the 2 reports with conflicting data mentioned above. In total we screened the codon 72 genotypes of 141 clinical samples, including adenocarcinoma, SCC, cervical intraepithelial neoplasia (CIN) and non-neoplastic specimens. An alternative DNA sequencing assay, pyrosequencing (24), that accurately determines the SNP and its adjacent positions was used.

MATERIALS AND METHODS

Samples

A total of 141 clinical specimens from cervical punch biopsies or surgical extirpation specimens were selected from a Swedish population (Uppsala County). Routine histological classification of the cases was confirmed by 2 experienced pathologist before inclusion in the study. Cases histologically defined as normal, cervicitis, hyperplasia, metaplasia and condyloma comprise the non-neoplastic control group. We preferentially selected high-risk HPV-infected cases from the cervical non-neoplastic control, CIN and cancer patient material available. However, HPV-negative or HPV low-risk infected control cases were included to increase the case number. Tissues from formalin fixed, paraffin-embedded specimens of 39 cervical adenocarcinoma, 20 SCC, 46 CIN (CIN1, 2 and 3) and 36 non-neoplastic cases were included in this study.

DNA preparation

One to three $10 \mu m$ sections were made from each case according to size of tissues available for testing. All tumour samples contained a mixture of tumour cells and normal tissue (stroma, adjacent normal epithelia and infiltrating inflammatory cells). Microdissection was not performed. Tissue sections were deparaffinized by routine procedure. DNA samples were prepared from tissue sections by digesting with proteinase K treatment, $1.0 \mu g/\mu l$ (Boehringer Mannheim, GmbH, Germany) at 56°C overnight. After incubation at 95°C for 10 min, samples were stored at −20°C for subsequent analysis.

HPV detection and typing

The HPV status was determined either as HPV negative, HPV low risk or HPV high risk positive. In the case of adenocarcinoma, classification was made to differentiate the high risk HPV types 16 and 18. The detection of high and low risk HPV types were accomplished with PCR and the ELISA-based SHARP Signal System (25). In brief, low- or high-risk specific RNA probes were hybridized to the biotinylated PCR product. Following solid-phase capture the

DNA/RNA hybrids were allowed to bind to an antihybrid/alkaline phosphatase antibody, which was detected by addition of the colorimetric substrate para-nitrophenylphenol. The detection and typing of HPV types 16 and 18 was performed by PCR with general primers and single-stranded conformation polymorphism (SSCP) (26).

PCR and template preparation

The p53 exon 4 was amplified by nested PCR as outlined in Ref. 27. The outer PCR (initial denaturation 94°C for 5 min followed by 94°C 30s, 50°C 1 min and 72°C 1 min for 30 cycles, followed by a final 10 min extension at 72°C) was followed by a nested inner PCR (initial denaturation 94°C for 5 min followed by 94°C 1 min, 50°C 40s and 72°C 1 min for 35 cycles, followed by a 10 min extension at 72°C), generated a 79 bp PCR product for pyrosequencing. Five μl (approximately 500–1000 cells/μl) was used as template for the outer PCR. The outer 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′-AGAGGAATCCCAAAGTTCCA-3′; 5′-CTGGGACCTGGAGGGCTGG-3′) and 1.5 units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Connecticut, USA) in a total volume of 50 μl. For inner nested PCR, 2.5 μl outer PCR product was used as template. The inner amplification was performed as above but with 10 pmol of each primer (5′-TCCAGATGAAGCTCCCAG-3′; 5′-AGGGGCCGCCGGTGTA-3′) and 1 U of AmpliTaq DNA polymerase in a total volume of 100 μl. The upstream inner primer was biotinylated to allow for immobilization.

Pyrosequencing

Pyrosequencing is a sequencing-by-synthesis technique that specifically analyses the sequence context of the polymorphism and the adjacent nucleotide positions (24). The principle relies on an iterative addition of the individual nucleotides to a substrate consisting of single strand template and a sequencing primer in the presence of a DNA polymerase. Successful incorporation, based on Watson–Crick base pairing, results in a quantitative release of light through an enzyme cascade which is monitored in real time.

The 79 bp biotinylated inner PCR product (50 μl) was immobilized onto 150 μg streptavidin-coated super paramagnetic beads (Dynabeads

M280; Dynal, Oslo, Norway) in 30 μl BW buffer (10 mM Tris-HCl (pH 7.6), 2.7 M NaCl, 1 mM EDTA, 0.1% Tween 20) for 30 min at 43°C. Single stranded DNA was obtained by incubating the immobilized PCR product in 20 μl 0.1 M NaOH for 5 min. The immobilized strand was resuspended in annealing buffer (10 mM Tris-OOCH$_3$ pH 7.75, 2 mM MgOOCH$_3$) containing 1 pmol sequencing primer (5′-GCTGCTGGTGCAGGGGCCA-3′) in a total volume of 10 μl. All steps were automated and performed in 96-well format using in-house robotics (A. Holmberg, unpublished). Primer hybridization was performed by incubation at 94°C for 20 s, 65°C for 2 min and subsequent cooling to room temperature. Thirty μl of 10 mM Tris-OOCH$_3$ pH 7.6 buffer was added to the single stranded DNA template before sequencing. Thereafter, real-time pyrosequencing at 28°C in a total volume of 50 μl was performed on an automated 96-well PSQ 96 instrument using enzymes and substrates from the PSQ 96 SNP reagent kit (Pyrosequencing AB, Uppsala, Sweden).

Statistical analysis

The prevalence of different genotypes among adenocarcinoma, SCC, dysplastic and non-neoplastic cases were statistically assessed by the χ^2 test.

RESULTS

Distribution of HPV types

HPV status of the selected cervical cases (see Materials and Methods for details) is summarized below. High-risk HPV was present in all 20 SCC and 46 CIN samples. The adenocarcinoma samples were positive for high-risk HPV, type 16 (17 cases) and type 18 (22 cases). The non-neoplastic samples comprised 15 HPV-negative cases, 5 low-risk HPV-positive cases and 16 high-risk HPV-positive cases.

Pyrosequencing of the codon 72 polymorphism—frequency of alleles

The single nucleotide polymorphism at codon 72 involves either a G or C residue corresponding to the amino acid
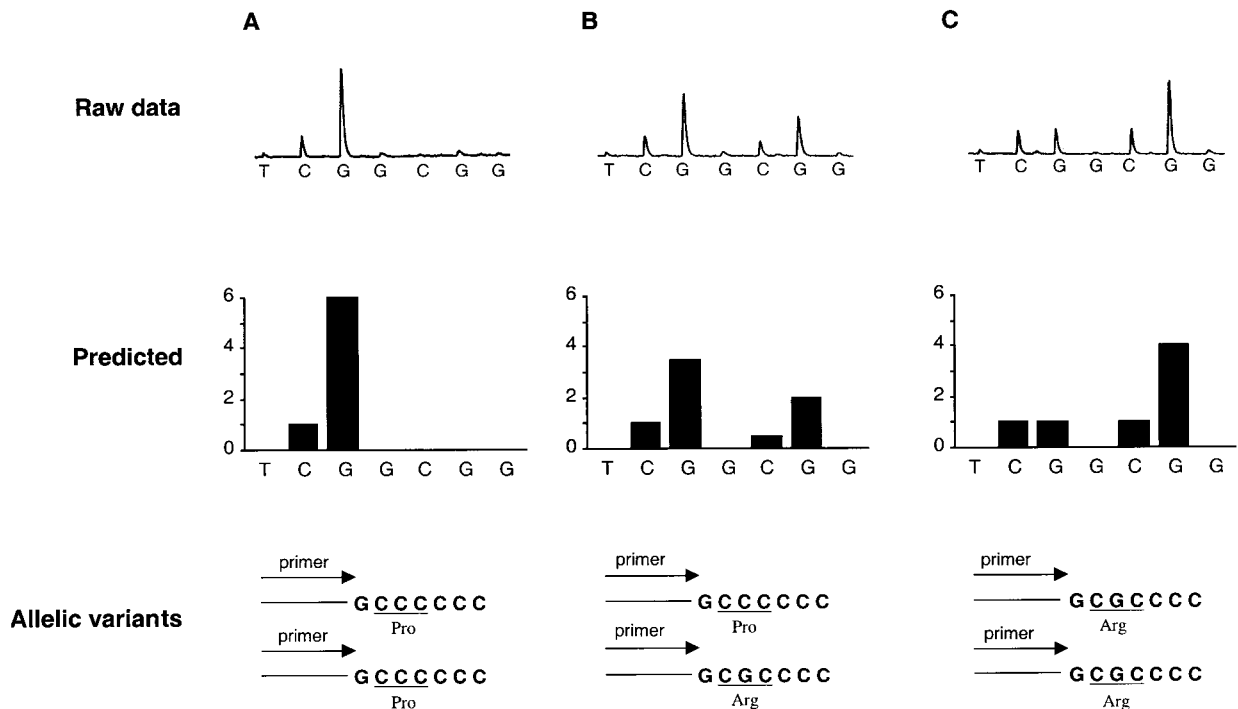

Fig. 1. The obtained raw and predicted pyrosequencing data for the allelic variants of p53 codon 72. (A) proline homozygote, (B) proline/arginine heterozygote and (C) arginine homozygote.

proline (CCC) or arginine (CGC). An example of pyro-sequencing raw data generated by sequential dispensing of nucleotides TCGGCGG is shown in Fig. 1 for the 3 possible genotypes. The sequencing primer was positioned penultimate to the SNP. The first introduced nucleotide, T, was deliberately chosen not to be complementary in order to yield a background control signal. The subsequently added nucleotide, C, was incorporated and identical for all allelic variants and set to correspond to 1 peak equivalent (extension on both alleles). The third nucleotide addition, G, interrogates the variable position by yielding 6, 3.5 or 1 peak equivalent base incorporation for the homozygous C (proline), heterozygous C/G (proline/arginine) and homozygous G (arginine), respectively. Another addition of G nucleotides was made to ensure complete incorporation. Thereafter, nucleotides C and G were added sequentially to extend the lagging arginine allele. The predicted patterns for the polymorphism is shown below (Fig. 1) and the similarities between raw-data and the three predicted patterns allowed for direct and reliable scoring of the codon 72 polymorphism.

The distribution of the p53 codon 72 polymorphism among the investigated Swedish cases using the pyrosequencing detection assay are summarized in Table I. All samples were confirmed by a repeated analysis starting from the lysed sample. The frequency of codon 72 genotypes in the non-neoplastic population was determined to be: 2.8% proline homozygous cases, 50% arginine homozygous and 47.2% proline/arginine heterozygous cases with no difference in distribution of genotypes observed between the HPV positive and HPV negative controls ($p = 0.6852$). The codon 72 genotype frequency among patients with adenocarcinoma was similar to non-neoplastic controls ($p = 0.2064$). No distinction was observed between HPV 16 and HPV 18 positive adenocarcinoma cases ($p = 0.2663$). Moreover, we did not find any significant over-representation of homozygotes for the arginine allele among either the SCC ($p = 0.6486$) or CIN patients ($p = 0.7154$) as compared to non-neoplastic controls.

Table I. *Distribution of p53 codon 72 genotypes*

Histopathological diagnosis	Number of cases (%)		
	Pro/Pro	Pro/Arg	Arg/Arg
Adenocarcinoma[a]	1 (2.6)	17 (43.6)	21 (53.8)
Adenocarcinoma (HPV 16-positive cases)	0 (0)	8 (36.3)	14 (63.6)
Adenocarcinoma (HPV 18-positive cases)	1 (5.9)	9 (52.9)	7 (41.2)
Squamous cell carcinoma	1 (5)	7 (35)	12 (60)
Cervical intraepithelial neoplasia[b]	2 (4.4)	18 (39.1)	26 (56.5)
Non-neoplastic[c,d]	1 (2.8)	17 (47.2)	18 (50)
Non-neoplastic (HPV-positive cases)	0 (0)	10 (47.6)	11 (52.4)
Non-neoplastic (HPV-negative cases)	1 (6.6)	7 (46.7)	7 (46.7)

[a]All adenocarcinoma cases; [b]Includes cervical intraepithelial neoplasia CIN 1, CIN 2 and CIN 3; [c]All non-neoplastic cases; [d]Non-neoplastic cases include samples histologically defined as: normal, cervicitis, hyperplasia, metaplasia and condyloma.

DISCUSSION

The genotype distribution of codon 72 variants in the normal Swedish population have previously been determined to be 11–12% proline homozygotes, 50–53% arginine homozygotes and 35–39% proline/arginine heterozygotes (23, 28). Comparable frequencies were found in this study, although proline homozygotes were slightly less represented (3-7%) and heterozygotes were slightly more represented (47-48%). An overestimation of homozygotes due to loss of heterozygosity (LOH) is also not likely since most previous investigations have reported none or low frequencies of LOH involving 17p in cervical cancer (0–22%) (7, 29–31). In addition, LOH in our samples would probably be masked by signals from the significant amounts of normal cells.

This study show that the codon 72 genotype distribution among high-risk HPV-infected patients with cervical adenocarcinoma is similar to controls in the Swedish population, indicating that no variant of the polymorphism at p53 codon 72 elevate the risk of developing adenocarcinoma of the cervix. Additional χ^2 tests and multivariate logit-regression analysis using the normal population (23, 28) as control confirmed the results (data not shown). These findings are in concordance with the single previous study of the association of this polymorphism with cervical adenocarcinoma based on an eastern American population (22). Considering the possible differential impact of HPV type specific E6 sequences, we investigated both HPV 16 and HPV 18-infected adenocarcinoma cases. In order to better compare non-neoplastic and neoplastic groups, we have also selected controls comprising an increased proportion of high-risk HPV-infected cases. However, no differences were found in the distribution of codon 72 frequencies either between HPV 16 and 18-infected adenocarcinomas or HPV-negative and HPV-positive controls.

Our data suggest that the p53 codon 72 polymorphism is not associated with increased probability of high-risk HPV-infected Swedish patients to develop CIN or cervical SCC. Multivariate logit-regression analysis and χ^2 tests using the normal population (23, 28) as control also confirmed these results (data not shown). This report represents the third study investigating the association between SCC of the cervix and codon 72 arginine based on cases collected in the same geographical region. The two previous reports have yielded conflicting results. Because differences in the ethnic background can be ruled out, the contradictory results may reflect differences associated with the source of DNA or with the screening methodology. We have used the same source of DNA (formalin fixed tissue sections) as the study that showed a correlation between the arginine allele and cervical cancer (8), but we here used an alternative screening technique (pyrosequencing instead of SSCP) that may have affected the outcome.

An inherent advantage of DNA sequencing, performed here in the format of pyrosequencing, as compared to SSCP, is the direct investigation of the variable position. An additional, specific advantage with pyrosequencing in typing codon 72 is that each allele combination (homozygous C, homozygous G or heterozygous C/G) will give a unique sequence pattern, which makes typing extremely robust. Simple manual comparison of predicted codon 72 patterns and the raw data from the pyrosequencer can score the variable position in a reliable manner. To further validate typing, all pyrosequencing data

(duplicates) was also confirmed by RFLP analysis (A.C. Gustafsson, unpublished).

We have recently shown that an analysis of formalin fixed tissue sections, as used here, requires great caution when limited numbers of target cells are used (32). In that comparative study we were able to demonstrate that artefact mutations appear in formalin material when the target cells are fewer than 300. These artefact mutations appear in a semi-random manner and require that potential mutation patterns obtained from an indirect screening procedure, such as SSCP (8), is confirmed with an alternative strategy (DNA sequencing). Formalin-mediated artefacts could therefore contribute to the observed differences when SSCP or DNA pyrosequencing is used to score the codon 72 polymorphism.

In conclusion, using an alternative detection assay we found that the frequency of the two different p53 codon 72 alleles were not different in high-risk HPV-associated adenocarcinoma, SCC or CIN as compared to non-neoplastic controls in a Swedish population. This report emphasizes the importance of using accurate and robust detection assays for genotyping p53 codon 72.

ACKNOWLEDGEMENTS

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