

# Detection of High Risk Human Papillomavirus DNA Sequences in Head and Neck Squamous Cell Carcinoma in Iranian Fanconi Anemia Patients

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## Abstract

**Objective:** Fanconi anemia (FA) is an autosomal recessive disorder characterized by congenital malformations, bone marrow failure, development of squamous cell carcinoma (SCC), and other cancers. Human papillomavirus (HPV) in the oral cavity or oropharynx has been associated with an increased risk of laryngeal papillomatosis, invasive squamous cell carcinoma of the head and neck (HNSCC) and cervical and other genital cancers. The prevalence of HPV DNA in the oral cavity/oropharynx in FA patients and controls was compared.

**Materials and Methods:** A risk factor questionnaire and oral exfoliated cells were collected from FA patients. The study group consisted of 22 FA patients with HNSCC (case subjects) and 24 patients with HNSCC without FA (control subjects). HPV DNA was detected using polymerase chain reaction (PCR) and specific primers that covered high risk types of HPV. Moreover, special serological assays were used for the detection of specific antibodies against HPV in patient's sera.

**Results:** HPV DNA was detected in 82% of the SCC specimens from the case subjects which was statistically higher ( $p < 0.05$ ) than the SCC specimens from the control subjects (62.5%). In all cases, the presence of HPV antibodies in patient's sera has been shown. Among the case subject specimens, 14 cases, and in the control subjects, 11 cases were infected by high risk HPV (hr-HPV).

**Conclusion:** These data confirm that HPV infection, especially with high risk types (16,18), could be one of several risk factors for HNSCC particularly in FA patients.

**Keywords:** Human Papillomavirus, Fanconi Anemia, Squamous Cell Carcinoma, Head and Neck Carcinoma

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## Introduction

Fanconi anemia (FA) is a rare autosomal recessive cancer predisposing syndrome characterized by congenital abnormalities, progressive bone marrow failure, and cellular sensitivity to DNA cross-linking agents (1). Each of the FA genes is located in a cell cycle checkpoint and DNA repair pathway. The FA gene defects are associated with cellular hypersensitivity to DNA cross-linking agents and oxidative stress, increased double strand DNA breaks and chromosomal instability, loss of telomere integrity, and cell cycle prolongation. Systematic reviews of the FA literature, surveys of FA

patients, and data from the International Fanconi Anemia Registry (IFAR) have identified an unusual preponderance of head and neck squamous cell carcinomas (HNSCC) and anogenital tract malignancies among FA patients (2-6). The risk of HNSCC among FA patients enrolled in the IFAR is approximately 500-fold higher than seen among the general population. The majority of FA patients do not have a history of alcohol or tobacco use (the strongest risk factors for HNSCC in the general population) (7). Whereas 80%-90% of SCC patients in the general population report a history of tobacco and/or alcohol use (8, 9) only

16% of the FA patients with SCC report a history of tobacco and/or alcohol use. Therefore, the etiology of SCC in FA patients may differ from that in the general population. In 2000, head and neck cancer was ranked as the eighth leading cause of cancer death worldwide (10). However, among the general population, a subset of HNSCC and anogenital tumors share a common etiology: human papilloma viruses (HPV) (11, 12). HPVs are a family of small DNA viruses that infect epithelial cells of the skin and mucosa. They are highly specific to their respective host. The involvement of HPV in oral and oropharyngeal carcinogenesis was first proposed by Syrjanen et al. (13). Although numerous studies have reported HPV DNA in normal and pre-neoplastic oral mucosa, as well as oral and oropharyngeal carcinomas, many of these studies were small hospital-based cross-sectional studies. More recently, larger studies of HPV DNA prevalence in the head and neck mucosa have shown that HPV may be an additional independent risk factor for a subset of HNSCC (14-19). Several high-risk HPV types have been detected in head and neck cancers, even though there is a predominance of HPV type 16 (17, 20-23). The pooled probability of detection of any high-risk HPV is 2.8 times more likely than for a low-risk subtype. HPV16 and 18 have been detected in 30% of oral squamous cell carcinomas (OSCC), while other high-risk types have been detected in less than 1% of these tumors. Although the likelihood of high-risk HPV being detected may be higher in samples of SCC, there has been substantial heterogeneity in detection rates between studies (10). On the basis of these observations, Kutler et al. (24) hypothesized that HPV may also play a role in the pathogenesis of HNSCC and anogenital tumors in FA patients. Nonetheless, there is evidence that FA genes can become epigenetically inactivated during cervical carcinogenesis in the general population (25). The reason for the increased susceptibility of FA patients to SCCs which frequently arise at sites of HPV infection, and why they develop these tumors at a significantly younger age than the general population, is currently unknown (26). This study aims to evaluate the prevalence of HPV DNA in the oral cavity/oropharynx of FA patients in comparison to non-FA patients and provide evidence that FA patients may have an inherited susceptibility to HPV associated malignancies.

## Materials and Methods

### *HPV detection in Iranian FA patients*

The study consisted of two groups: 22 FA patients with HNSCC (case subjects) and 24 patients with

HNSCC without FA (control subjects).

Tumor tissue samples from 22 FA patients were collected from archived, paraffin-embedded specimens (micro dissected oral cavity/oropharynx and head and neck) from the Pathology Laboratory of Ali Asghar Hospital (Tehran, Iran). Similar samples from 24 non-FA patients (control group), from the Pathology Laboratory of Rasool Akram Hospital (Tehran, Iran) were also examined. Tumors consisted of 30 fresh, frozen samples and 16 that were obtained from paraffin-embedded tissue blocks. Meanwhile, patients were asked to give blood samples for further serum analysis. The study was approved by the Ethical Committee of Iran University of Medical Science. All donors gave their informed written consent and completed a written questionnaire to obtain information related to their lifestyles. A total of 22 FA patients, nine girls and thirteen boys from Iranian families with a mean age of  $9.9 \pm 3.9$  years were selected. The average age of non-FA patients was  $44.37 \pm 13.7$ , with 12 males and 12 females. More clinical data about these patients have been summarized in table 1. H&E-stained slides were reviewed and evaluated by a pathologist for the presence and grade of SCC. Special serological assays were used for detection of specific antibodies against HPV in the patient's sera.

### *DNA extraction*

About 10- $\mu$ m sections were cut from paraffin blocks or fresh tissue and micro dissected using microsurgical forceps. Uninvolved non-mucosal tissue was micro dissected as a representative normal. Paraffin-embedded samples were placed in xylene for 12 hours and centrifuged at 13,500 rpm. The pellets were then digested in 20  $\mu$ l of proteinase K (20  $\mu$ g/ml; Fluka, Buchs, Switzerland) and incubated at 55 °C for 12 hours. Subsequently, 100  $\mu$ l of aqueous NaCl solution (5 M) and 90  $\mu$ l of CTAB (cetyltrimethyl-ammonium-bromide)/NaCl (10 g/0.7 M; Fluka) were added and incubated at 65 °C for 10 minutes. The sample was mixed with 800  $\mu$ l of chloroform/isoamylalcohol (24:1; Fluka) and centrifuged for 5 minutes at 13,000 rpm. The resulting water phase was mixed with 600  $\mu$ l of fresh phenol/chloroform/isoamylalcohol (24: 24: 1; Fluka) for another 5 minutes at 13,000 rpm followed by washing with 100% isopropanol at room temperature for 5 minutes and centrifugation for 10 minutes at 13,000 rpm. The pellet was washed twice with 70% ethanol and stored at -20°C. Fresh tissue was treated in a similar fashion, although these samples did not require xylene treatment. DNA isolation and PCR were performed in two different rooms.

**Table 1: Univariate analysis of patient/tumor characteristics and HPV presence stratified by oropharyngeal and non oropharyngeal tumor site**

Number of Patients	Oropharynx (n=6)		Non oropharynx (n=18)	
	HPV Status		HPV Status	
Characteristics	No. Positive (n=4)	No. Negative (n=2)	No. Negative (n=11)	No. Positive (n=7)
<i>Tobacco exposure</i>	2	1	2	0
Non Smokers	2	1	9	7
Smoker (Current+ Former)	-	-	-	-
Unknown				
<i>Alcohol intake**</i>				
<10	2	-	2	1
>10	-	-	1	0
Unknown	2	2	8	6
<i>Lymph node status</i>				
Negative	3	1	10	5
Positive	1	1	1	2
<i>Tumor Grade</i>				
Well/Moderate	1	2	4	3
Poor	3	-	7	4
Unknown	-	-	-	-
<i>Basaloid Morphology</i>				
Not Present	2	1	5	1
Present	2	1	5	2
Not Available	-	-	1	4

\* HPV: Human papilloma virus. \*\* 10 cc of alcohol per week.

### PCR

The quality of DNA extracted from all specimens described above was controlled by amplification of a portion of the  $\beta$ -globin gene. Five  $\mu$ l purified total cellular DNA was used for  $\beta$ -globin PCR to assess DNA quality. The primers used were: 5-CAACTTCATC-CACGTTCCACC-3' and 5-GAAGAGCCAAG-GACAGGTAC-3' spanning 268 bp. Amplified DNA was analyzed by agarose gel electrophoresis.

### Consensus L1-PCR

General consensus primers GP5+/GP6+ (27) and MY09/MY11 (28) were used to amplify the corresponding part of the HPV L1 gene. GP5+/GP6+ primers amplify a region of about 140 bp within

the L1 open-reading frame of more than 37 different HPV types. More details have been shown in table 2. The PCR was performed in a final reaction volume of 50  $\mu$ l, containing 10 ng of the DNA sample, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 2.5mM MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP (deoxynucleoside triphosphate), 100 pmol each of forward and reverse primers (synthesized by Microsynth, Balgach, Switzerland), and 0.25 U of Taq-DNA-Polymerase (Roche Applied Science, Basel, Switzerland). PCR conditions were as follows: preheating at 95 °C for 5 minutes followed by 30 cycles at 95 °C for 30 seconds, 30 seconds at 52 °C, and 1 minute at 72 °C, and a final extension for 7 minutes at 72 °C.

**Table 2: Sequences of primers used in this study, their location in the HPV genome and corresponding PCR product length**

Name	Oligonucleotide sequence (5'-3')	Use	Gene bp	Gene bank location
MY11a	GCMCAGGGWCATAAYAATGG	Forward	L1	4506583-6602b
MY09a	CGTCCMARRGGAWACTGATC	Reverse	L1	7015-7034b
GP5+	TTTGTACTGTGGTAGATACTAC	Forward	L1	150 6624-6649b
GP6+	GAAAAATAAACTGTAAATCATATTC	Reverse	L1	6719-6746b

Each PCR experiment was performed with negative (water) and positive controls. Purified DNA was extracted from the HPV 16-positive Caski cell line.

To determine the HPV type, the PCR product was subjected to an enzyme immunoassay with different HPV type specific oligonucleotides, as described by Jacobs et al. (27). Briefly, a streptavidin-coated microtiter plate (Roche, Vienna, Austria) was loaded with 5 µl of the amplified reaction mixture in 50 µl freshly prepared 1× SSC/0.5% Tween-20 solution (Merck, Darmstadt, Germany) and incubated at 37°C for 60 minutes to bind the biotinylated components. After three washing steps to remove unbound biotinylated PCR products, the DNA was denatured into single strands. Type specific oligonucleotides (30-mers) were selected on the basis of sequence information derived from the EMBL database. The oligonucleotides were provided from Pharmacia Corp. which were synthesized by the methoxy-phosphoramidite method. The sequences of oligonucleotides have been shown in table 3.

**Table 3: Sequences of HPV type-specific oligonucleotides**

HPV probe	Sequence (5'-3')
Probe 16	GTCATTATGTGCTGCCATATCTACTTCAGA
Probe 18	TGCTTCTACACAGTCTCCTGTACTCTGGGCA
Probe 31	TGTTTGTGCTGCAATTGCAAACAGTGATAC

A cocktail of digoxigenin-labeled oligonucleotides specific for a number of high-risk HPV types was then added, followed by hybridization for 1 hour at 37°C. Unbound oligonucleotides were removed by washing. Detection of a specifically bound digoxigenin-labeled probe was achieved by immunochemistry, and the optical density at 405 nm was subsequently measured at times points (27). HPV DNA typing demanded that the EIA be performed with each digoxigenin labeled HPV type-specific oligonucleotide.

**HPV-ELISA**

A capture ELISA was used to detect serum HPV IgA and IgG antibodies to HPV-16, HPV-18 and HPV-31. ELISAs were a modification of that described by Heim et al. (29), using polymer solutions for blocking (polyvinyl alcohol, PVA) and secondary antibody (polyvinylpyrrolidone, PVP) enhancement. PVA-50 (molecular weight, 50,000) and PVP-360 (molecular weight, 360,000) were obtained from Sigma Chemical Co. This enhanced ELISA enabled the use of serum at 1:100 dilutions. Briefly, monoclonal

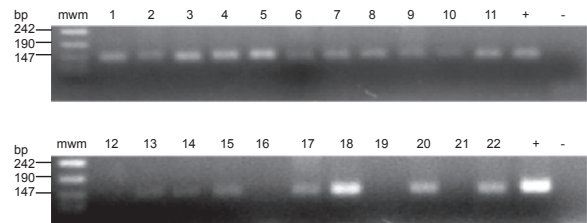
antibodies directed against the major conformational, neutralizing epitope on HPV-16, HPV-18 and HPV-31 (Sigma, USA) were used as capture antibodies. Plates were coated with 100 µl monoclonal antibody at a dilution of 1:2000 in PBS pH 7.4 overnight at 4°C, washed twice with PBS and blocked with 100 µl 0.05% PVA in PBS for 2 hours at room temperature. Plates were washed six times with PBS and wells filled with 100 µl of 0.05 µg virus-like particle (VLP) to HPV-16, HPV-18 and HPV-31 in PBS for 1 hour at 37°C. Subsequent procedures were done as with other ELISAs but using 0.8% PVP and 0.5% PVA in PBS for the dilution of the horseradish peroxidase conjugated anti-human IgA and IgG and six washes between ELISA procedures. Total IgA estimations were performed on each sample to determine the adequacy of the sample prior to HPV antibody testing as 2.3 µg/ml in children, 4.7 µg/ml in adolescents and 1.8 µg/ml in adults.

**Statistical analysis**

All statistical analyses were carried out using SPSS (SPSS Inc., Chicago, Illinois, USA) software version 15. To analyze the results of the presence of HPV between groups, the  $\chi^2$  test of independent proportions was used to determine 95% limits of confidence intervals (95% CI). P-value of less than 0.05 was considered as a significant level.

**Results**

DNA was extracted from all samples for PCR amplification. The positive control was always positive. DNA extracted from tissue sections was positive for  $\beta$ -globin gene in all cases, indicating that quality and quantity of DNA were satisfactory. HPV DNA was detected in 18 (82%) investigated FA patients (Fig 1) and in 15 (62.5%) non FA patients ( $p < 0.05$ ).



**Fig 1: Detection of HPV by general-primer-mediated PCR (GP-PCR) in 22 FA patients. GP-PCR products after electrophoresis on a 2% agarose gel and ethidium bromide staining. Numbers indicate patients' numbers; bp, base pairs; mwm, molecular weight marker (pUCBM21-DNA cut with HpaII and DraI plus HindIII); +, positive control; -, negative control.**



HPV-16 was the predominant type in both FA and non-FA patients. There were 14 (78%) FA patients out of 18 samples infected by HPV-16. In the control group, 11 (73%) samples were also HPV-16-positive. All were HPV-16 antibody positive ( $p > 0.05$ ). Additionally in both groups, 3 (9%) patients had HPV-18 and -31 antibodies. The results of other HPV types in the case and control groups have been summarized in table 4. In all of these samples, antibodies of specified HPV types were detected. This result was confirmed when DNA extraction and PCR were repeated.

**Table 4: Number of people who were positive for different HPV types in each group**

Group HPV Type	Number of FA patients	Number of Non-FA patients
HPV 18 Only	2	1
HPV 16 and 18	5	4
HPV 31 Only	2	3
HPV 16 and 31	1	-
HPV 31 and 18	2	1

## Discussion

Although HPV genomic DNA has been detected in head and neck cancers (14, 20, 21, 30) its etiologic role in HNSCC development has remained unclear. Firstly, the role of HPV in oral and oropharyngeal carcinogenesis was reported by Syrjanen et al. (13). Over the past two decades many small hospital-based studies have been completed on HPV DNA in the mucosa of normal, oral and oropharyngeal carcinomas. Recent larger studies have proved the presence of HPV DNA in the head and neck mucosa, so it can be concluded that HPV (predominantly HPV type 16) might be an important risk factor for a group of HNSCC (14-24). According to our results HPV DNA was detected in 82% of investigated FA patients and 62.5% of non-FA patients (Fig 1). In a similar research, analysis of HNSCCs from FA patients has shown that over 80% of the tumors contained high-risk HPV DNA, in particular that of HPV type 16 (HPV-16) (24). This result is similar to what we report in this article, independent of alcohol and tobacco exposure. Koyama et al. in a 2007 experiment using *in situ* PCR (MY09/11) demonstrated a high prevalence of HPV in SCC (100%) which is in accordance with our observation (31). In conjunction with recent epidemiologic studies, our data provides evidence that HPVs may be etiologically linked to a defined subset of head and neck

cancers. Our results suggest that the high rates of HNSCCs among FA patients may be associated with the high frequency of oncogenic HPV DNA detected in their tumor tissues. Whether this high rate of HPV-associated SCC is caused by an underlying immune dysfunction in FA patients or directly involves the pathway(s) defective in FA is currently unknown. The significant difference between the frequency of HPV DNA in FA patients and non-FA patients may describe a different etiology of HNSCC in these patients. HPV genomic sequences have also been identified in HNSCC, but markedly varied estimates of viral prevalence (range, 8%-100%) have impeded clarification of the relationship between HPV presence and head and neck cancer development (32). Despite this variability, studies (20, 21) have suggested an association of HPVs with cancers in the oropharynx and, especially, with tonsillar carcinomas (33-35). Our findings suggest that HPV-positive oropharyngeal cancers can be a distinct molecular-pathologic entity etiologically linked to infection by high-risk HPVs, especially HPV16. In contrast to our data, Van Zeeburg et al. has reported that human papillomavirus was not detected in any of the FA or sporadic HNSCC cell lines. (36) It has been shown that HPV does not play a major role in squamous cell carcinogenesis in the cohort of FA patients and that FA SCCs are genetically similar to sporadic SCCs despite having a different etiology (36, 37).

In the general population, HPV-associated cancers develop as a consequence of persistent HPV infection plus a series of genetic and epigenetic changes in an infected cell that often occur in association with viral DNA integration (31, 38, 39). Although the immune impairment associated with cytopenia of FA could play a role in HPV persistence, most FA patients do not have difficulties handling infections, and the IFAR patients who have undergone bone marrow transplantation do not appear to have a lower incidence of HNSCCs or vulvar cancer than FA patients not treated with bone marrow transplantation (6). The relatively young age of FA patients with HNSCC or vulvar cancer suggests that the period between HPV infection and development of cancer may be considerably shorter in this patient population than in the general population. This situation could arise if the combination of HPV infection and a defect in the FA pathway results in a decrease in the number of additional cellular changes required for progression to cancer and/or an increase in

the rate at which these changes occur. Many hypothetical interactions are possible. For example, because FA is associated with a high rate of apoptosis, which inhibits tumorigenesis, the ability of HPV E6 to inhibit apoptosis may represent one mechanism by which HPV could cooperate with the FA defect to shorten the time to cancer development (39). The risks and timing of HPV exposure in FA, as well as a possible immune impairment in FA that may favor persistence over clearance of HPV infection, could be addressed experimentally by prospectively screening FA patients for the incidence of oral and genital HPV infections. If exposure to HPV type 16 (and/or HPV type 18) occurs primarily during adolescence or later, the prophylactic HPV virus-like particle vaccine, which targets HPV type 16 (and HPV type 18) and is currently in clinical trials, might be able to prevent most FA cancers attributed to HPV (40, 41). It may be useful to explore possible functional interactions between FA gene defects and HPV in detail. In addition to the effects on apoptosis noted above, other possible interactions include HPV E6 induction of telomerase, which would overcome the telomere shortening associated with FA; HPV E7 stimulation of the cell cycle, which would antagonize cell cycle prolongation that is characteristic of FA; and HPV E6 and E7 induction of genomic instability and double-strand DNA breaks (39, 42) which would enhance similar defects in FA. This study has several limitations. First, even though SCC is common among FA patients, the absolute number of FA-associated SCC cases in our analysis was relatively small because of the rarity of FA. Second, we did not match SCC patients without FA to those with FA for tobacco and alcohol use status because of the lack of availability of appropriate cases in our tissue bank. According to what Koch et al. has reported in 1999, this difference in exposure status represents a potential confounding factor because the frequency of some cell cycle regulatory genes such as p53 mutations in HNSCC is associated with tobacco and alcohol use (43). Mouse models of HPV and FA can help address these interactions as well as other possible interactions (44, 45).

## Conclusion

However, we need to know more about the natural history and predictive value of oral HPV infection before HPV DNA detection can be incorporated into oral cancer screening. Clinicians should also consider the possibility of FA

in young HNSCC patients who do not smoke or drink alcohol, particularly in those patients with unusually severe toxicity from chemotherapy or radiation therapy. Moreover research studies must be done on larger sample groups. It would also be worthwhile to look for HPV in other solid tumors arising in FA patients, such as skin and esophageal carcinomas, because the role of HPV infection in the development of those tumors in the general population remains unclear. HPV-associated cancer susceptibility syndromes can provide surprising new directions to guide this research.

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