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ORIGINAL ARTICLE

# High prevalence of oral human papillomavirus infection in Fanconi's anemia patients

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**BACKGROUND: Fanconi's anemia (FA) is a rare recessive genetic disorder characterized by bone marrow failure, developmental and congenital abnormalities, which frequently evolves to aplastic anemia and neoplasias, primarily acute leukemia and head-neck carcinomas. Risk of malignancies increases after hematopoietic stem cell transplantation (HSCT), and the role of human papillomavirus (HPV) in FA carcinogenesis have been proposed.**

**OBJECTIVE: To investigate prevalence of oral HPV in FA patients without oral malignant lesions.**

**MATERIALS AND METHODS: After oral examination, 76 subjects without detectable oral malignant lesions were included and classified in four groups: 20 FA submitted to HSCT (I), 22 FA not submitted to HSCT (II), 18 severe aplastic anemia (SAA) submitted to HSCT (III) and 16 healthy subjects (IV). Liquid-based cytology sampling, HPV screening by polymerase chain reaction and genotyping by reverse hybridization were performed.**

**RESULTS: The HPV detection rates were: group I 35%, group II 27.3%, group III 38% and group IV 6.25%. Prevalence of high risk HPV types, mainly HPV16, was detected. Compared with control group, suggestions for increased likelihood of being HPV infected in SAA (OR = 9.55, 95% CI: 1.01–125.41) and FA patients submitted to HSCT (OR = 8.08, 0.83–72.29) emerged.**

**CONCLUSION: Patients without oral malignant lesions submitted to HSCT, have high prevalence of oral HPV. HPV screening and close follow up should be considered in these patients.**

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**Keywords:** Fanconi anemia; oral; human papillomavirus; mouth; hematopoietic stem cell transplantation

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## Introduction

Fanconi's anemia (FA) is a genetically and phenotypically rare disorder usually inherited as an autosomal recessive trait. A small subset of cases with an X-linked inheritance was also described. FA patients show marked clinical heterogeneity, congenital malformations, a progressive bone marrow failure and an increased predisposition to malignancies, especially anogenital and head and neck squamous cell carcinomas (HNSCC) (Meetei *et al*, 2004; Alter, 2007; Dokal, 2008). FA patients show a 500-fold higher risk of HNSCC and the cumulative risk by age of 40 years is 14% (Kutler *et al*, 2003a). The most common site for tumor development is the oral cavity, mainly the tongue. Subjects with increased susceptibility of oral cavity and genital region to local risk factors, such as human papillomavirus (HPV) may be at higher risk for SCC (Kutler *et al*, 2003a; Spardy *et al*, 2007; Hoskins *et al*, 2009). It has recently been described that FA patients have increased susceptibility to HPV-associated cancer, since they are carriers of deficiency in the DNA damage response pathway which normally attenuates the oncogenic potential of HPV16 E7 (Park *et al*, 2010). Frequency of HPV infection in FA with oral neoplasia has been reported (Kutler *et al*, 2003c; Van Zeeburg *et al*, 2008; Han *et al*, 2009), but no information is currently available for FA patients without clinically detectable oral lesions. The aim of this study was to investigate the prevalence of oral HPV infection in FA patients free of oral malignant lesions.

## Materials and methods

*Study population*

Seventy-six subjects who resulted negative after anamnesis and an oral examination for clinically detectable malignant oral lesions were the study population. They were classified in four groups: group I: 20 FA patients submitted to hematopoietic stem cell transplantation (HSCT), group II: 22 FA patients in clinical follow up

and not submitted to HSCT, group III: 18 severe aplastic anemia (SAA) patients submitted to HSCT and group IV: 16 healthy control subjects never submitted to HSCT and in good health status. Patients of groups I, II and III were recruited at the Bone Marrow Transplantation Service of Hospital de Clínicas, Federal University of Paraná, Curitiba, Brazil. Group IV subjects' were recruited at Bauru School of Dentistry, University of São Paulo, where they were attending for dentistry treatment. All subjects signed an informed consent term, which was approved by the Ethics Committee of Hospital de Clínicas, Federal University of Paraná. Age of patients refers to age at time of sample collection.

*Sample collection*

Oral squamous cells were obtained by a liquid-based cytology using the DNA-citoliq® kit (Universal Collection Medium; Digene, São Paulo, Brazil). The DNA-citoliq cytobrush was rotated five times on each of the following regions of the mouth: hard and soft palate, superior and inferior gums, cheek oral mucosa, floor of mouth and tongue.

*DNA extraction*

The DNA was extracted using the InstaGene Matrix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) extraction method according to Woo *et al* (1997) and Sakai *et al* (2007). To verify DNA extraction method and adequacy, all samples were amplified in real-time polymerase chain reaction (PCR) with primers specific for the beta globin gene following the protocol proposed by Van Duin *et al* (2002).

*HPV-DNA detection*

The PCR detection of HPV-DNA was performed by nested PCR employing MY09/11 and GP5+/6+ consensus primers. These pairs of primers may detect a broad spectrum of HPV types (De Roda Husman *et al*, 1995; Jacobs *et al*, 1995; Atalay *et al*, 2007) targeting the L1 open reading frame of the virus. The MY09/MY11 primer pair amplifies a fragment of approximately 450 bp, whereas the GP5+/6+ primer pair a fragment of approximately 150 bp. DNAs extracted from SiHa and CaSki cells (ATCC, Manassas, WA, USA) and a DNA-free sample were included as positive and negative controls, respectively, in each test run.

All PCR reactions were performed in a GeneAmp 9700 PCR System thermal cycler (Applied Biosystems, Foster City, CA, USA). The outer PCR amplifications were carried out in 25 $\mu$l containing: 1X PCR buffer [10 mM Tris (pH 8.0), 50 mM KCl, and 3.5 mM $MgCl_2$], deoxyribonucleotide triphosphates (0.2 mM each), 1 U of AmpliTaq Gold DNA polymerase enzyme (Applied Biosystems), 100 pmol of each primer (MY09/MY11) and 5 $\mu$l of purified DNA. Amplification conditions were as follows: initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 45 s and extension at 72°C for 1 min; final extension at 72°C for 7 min.

The inner PCR amplifications were carried out in 25 $\mu$l containing: 1X PCR buffer [10 mM Tris (pH 8.0), 50 mM KCl, and 3.5 mM $MgCl_2$], deoxyribonucleotide triphosphates (0.2 mM each), 1 U of AmpliTaq Gold DNA polymerase enzyme (Applied Biosystems), 50 pmol of each primer (GP5+/6+), and a 2-$\mu$l aliquot of the outer reaction. Amplification conditions were as follows: initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 2 min and extension at 72°C for 90 s; final extension at 72°C for 5 min.

Amplicons were analyzed on 2% agarose gels stained with ethidium bromide and visualized by ultraviolet transillumination.

To avoid risk of false negatives, a subset of HPV negative samples was double checked also with the INNOLiPA hybridization method (see below), and confirmed as HPV negative.

*HPV genotyping*

The HPV-DNA positive samples were genotyped by reverse hybridization using the commercial kit INNO LiPA HPV Genotyping Extra Amp (Innogenetics, Gent, Belgium). The INNO-LiPA HPV genotyping assay is based on amplification of at least 43 HPV genotypes with biotinylated SPF10 primers targeting a 65-bp fragment in the viral L1 region (Kleter *et al*, 1998, 1999).

The PCR reactions were performed according to the manufacturer's instructions in the GeneAmp 9700 PCR System thermal cycler (Applied Biosystems). An HPV-6 positive control provided by the manufacturer and a negative PCR control were included in each run. To enhance the sensitivity, a second step of PCR was performed in addition to the recommended procedure by using 2 $\mu$l of the first SPF10 PCR product. The resulted amplification products were genotyped using the reverse hybridization nitrocellulose membrane strips following the manufacturer's instructions.

*Statistical analysis*

Univariate and multivariate logistic regression models were applied to estimate crude and adjusted odds ratios (OR), respectively. Crude OR and ORs adjusted for age (treated as continuous) were calculated through logistic regression models. The logistic regression model applied to patients submitted to HSCT (groups I and III) was adjusted for age and year since HSCT.

The approximate 95% confidence intervals (CIs) of the ORs were computed using Wald standard errors. Statistical analyses were performed with StataCorp (Stata Statistical Software Release 10, College Station, TX, USA: StataCorp LP, 2007).

## Results

In Table 1 the characteristics of the study population are reported. A detailed oral examination was performed in all subjects and no oral mucosal lesions were detected, except for a leukoplakia. A tongue leukoplakia was detected in one patient: an 18-year-old young man in clinical follow-up after 10 years and 4 months of

**Table 1** Distribution and characteristics of the study population

| *Groups* | *Group I* | | *Group II* | | *Group III* | | *Group IV* | |
|---|---|---|---|---|---|---|---|---|
| *Gender* | *F* | *M* | *F* | *M* | *F* | *M* | *F* | *M* |
| Number of subjects | 8 | 12 | 13 | 9 | 8 | 10 | 7 | 9 |
| Patients' age (years) | 10–31 | | 4–18 | | 21–58 | | 4–37 | |
| Mean patients' age (years) | 17.50 | | 9.86 | | 34.77 | | 16.12 | |
| Years since HSCT | 3.4–13.5 | | – | | 5–23 | | – | |
| Years since HSCT mean | 8.3 | | – | | 12.4 | | – | |

F, Female; M, male; HSCT, hematopoietic stem cell transplantation; FA, Fanconi's anemia; SAA, severe aplastic anemia.
Group I: FA patients submitted to HSCT; group II: FA patients not submitted to HSCT; group III: SAA patients submitted to HSCT; group IV: healthy controls.

HSCT for FA (group I) presented a regular and homogenous white plaque measuring 1.5 cm on the right margin of the tongue. He noticed the lesion 1 year before the oral examination. He denied any symptoms. An excisional biopsy was performed: the histological aspect revealed an infiltrate of inflammatory cell in the subjacent lamina propria. Oral epithelial cells mouth brushing and the specific brushing performed directly on patient's leukoplakia were submitted to HPV investigation, however, they were both negative for HPV infection as well as no dysplasia was observed on histological analysis.

All DNA samples obtained from the 76 subjects included in the study were positive for beta-globin gene in SYBR Green® real-time PCR, thus proving adequate for HPV analysis.

The HPV prevalence was higher in the test groups than in the control group: group I – 35% (7/20), group II – 27.3% (6/22), group III – 38% (7/18) group IV – 6.25% (1/16). Table 2 describes HPV positive samples distribution according to groups and HPV types. The high risk HPV-16 was the most frequent genotype found in this study. In group I, HPV-16 represented 71.4% of the HPVs, in group II it was 33.3% and in group III 28.6%.

Crude ORs for HPV infection in oral epithelial cells suggested that FA patients and SAA patients submitted to HSCT had an increased likelihood of being HPV infected (OR = 8.08, 95% CI: 0.88–74.59, and OR = 9.55, 95% CI: 1.02–89.22, respectively). The crude effect, although not significant, was similar to that adjusted for age (Table 3). Higher risk of HPV infection is suggested when comparing groups I and III adjusting for patients' age at sample collection time and years since HSCT to sample collection time (OR = 4.91, 95% CI: 0.44–54.51) (Table 4). Mean age (all groups) was 17 years, and the mean time since HSCT was 10 years (groups I and III) both of which were considered in the analyses.

**Table 2** HPV positivity and typing in the study population

| *Groups*<br>*HPV positivity/total samples in each group* | *Group I*<br>*7/20* | *Group II*<br>*6/22* | *Group III*<br>*7/18* | *Group IV*<br>*1/16* |
|---|---|---|---|---|
| High risk | 5 | 3 | 4 | – |
| HPV-16 | 4 | 2 | 2 | – |
| HPV-18 | – | 1 | 1 | – |
| HPV-58 | – | – | 1 | – |
| HPV-16, 18 | 1 | – | – | – |
| Low risk | 1 | 1 | 1 | – |
| HPV-6 | 1 | 1 | 1 | – |
| Not typed | 1 | 2 | 2 | 1 |

## Discussion

This study showed that HPV can be detected with high prevalence in oral exfoliated cells of anemia patients (FA and SAA) with no detectable oral malignant lesions. Successful brushing for HPV detection in oral epithelial cells had been previously demonstrated (Giovannelli *et al*, 2002, 2006) and it has been shown as a sensitive method for detection of oncogenic HPV types in oral mucosa (Smith *et al*, 2004).

We identified high risk HPVs, mainly HPV-16, in the patients groups I, II and III, according to previous studies which demonstrated that HPV-16 was the most common HPV type detected in FA patients' tumor tissues (Miller and Johnstone, 2001; Kutler *et al*, 2003b). A double HPV-16, 18 infection case was detected in a FA patient that had been submitted to HSCT 10 years earlier, and who is now supposed to receive a close follow-up. In terms of cancer risk, detection of multiple infections can be relevant: in cervical cancer multiple HPV infections by high risk genotypes have been shown to increase the severity of the disease (Harnish *et al*, 1999; Burd, 2003). Indeed, oral infection by multiple HPV types was described by Zhang *et al* (2002) in an

**Table 3** Risk of HPV infection in the study population

| *Group* | *OR* | *95% CI* | | *OR*[a] | *95% CI*[a] | |
|---|---|---|---|---|---|---|
| Group I | 8.08 | 0.88 | 74.59 | 7.76 | 0.83 | 72.29 |
| Group II | 5.63 | 0.60 | 52.37 | 5.00 | 0.50 | 49.88 |
| Group III | 9.55 | 1.02 | 89.22 | 11.27 | 1.01 | 125.41 |
| Group IV | 1.00 | – | | 1.00 | – | |

CI, confidence interval; OR, crude odds ratio. OR[a], OR adjusted for age.

**Table 4** Risk of HPV infection in patients submitted to HSCT

| *Groups* | *OR* | *95% CI* | | $OR^a$ | *95% CI*[a] | | $OR^b$ | *95% CI*[b] | | $OR^c$ | *95% CI*[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group I | 1.00 | – | | 1.00 | – | | 1.00 | – | | 1.00 | – | |
| Group III | 1.18 | 0.32 | 4.42 | 4.91 | 0.44 | 54.51 | 1.82 | 0.41 | 8.11 | 3.82 | 0.38 | 38.82 |

OR, odds ratio; HSCT, hematopoietic stem cell transplantation; OR[a], OR adjusted for age and years since HSCT; OR[b], OR adjusted for years since HSCT; OR[c], OR adjusted for age; CI, confidence intervals.

oral carcinoma *in situ* developed after bone marrow transplantation for acute myeloid leukemia.

Although it was suggested that oral dysplasia usually precedes HPV infection in FA (Kutler *et al*, 2003c), the FA patient submitted to HSCT who presented a tongue leukoplakia resulted negative for HPV infection. This result was, however, in line with Kutler *et al* (2003c) which found an oral dysplastic lesion unexpectedly negative for HPV-DNA in a FA patient with an HPV-DNA positive tumor.

In the present study, we found increased ORs for oral HPV infection in FA and SAA in absence of malignant oral lesions. Although not statistically significant, given the small sample size, ORs may be regarded as suggestive for an association. Because of the rarity of FA, it would be difficult to increase the sample size of our study. Even if the small number of controls is a limitation of this study, published prevalences of oral HPV infection in healthy subjects of similar age are consistent with our findings (6.25%). A recent systematic review (Kreimer *et al*, 2010) reported in 4070 subjects an overall oral prevalence (any HPV) of 4.5%. Specifically in Brazilian studies, a prevalence of 4.3% in the oral cavity of 70 subjects free of lesions was reported by Giraldo *et al* (2006). A second study found a 14% oral HPV prevalence in 50 subjects (do Sacramento *et al*, 2006) when subjects ($n = 4$) with HPV positive specimens collected from tonsils and oropharynx, sites – not considered in our study – were excluded, the oral HPV prevalence was comparable with our results.

Elderly patients and patients with more than 10 years since transplantation had higher risks of HPV infection. This result is consistent with the time when patients are most prone to SCC. Deeg *et al* (1996) stated that the hazard of developing a post-transplant solid tumor in patients with FA increases at 8–9 years since transplantation. A cumulative risk of an SCC at 10 and 15 years after transplantation of 12% and 24%, was descripted, respectively (Rosenberg *et al*, 2005).

As time of onset of FA and SAA is first and second-third decades of life, respectively, and age may influence the cumulative risk of HPV infection, we controlled for age in our analyses. In our study there was a wide variation in the age distribution among different groups. Therefore, it is possible that some residual confounding by age is still present.

High prevalence of oral HPV infection in patients who already have increased susceptibility to squamous cell neoplasias may be of impact on patients' management. HPV positivity in oral epithelial cells provides information that a potential oncogenic virus is present in a mucosal area, and would suggest a close follow-up of these patients. The association between virus and squamous cell carcinogenesis in FA patients was pointed out before (Kutler *et al*, 2003b; Spardy *et al*, 2007; Hoskins *et al*, 2009): the immunosuppression associated with persistent bone marrow failure and the genetic instability may predispose these patients to viral infections and consequent cellular changes involved in carcinogenesis (Kutler *et al*, 2003a; Lowy and Gillison, 2003). Furthermore, described FA deficiency in the DNA damage repair pathway, which normally attenuates the carcinogenic potential of HPV16 E7 oncogenic protein, may allow malignant transformation of oral infected epithelial cells (Park *et al*, 2010).

This is the first study focused on HPV infection in FA patients free of clinically detectable oral malignant lesions. The present results, due to the small sample size, can only be regarded as suggestive for an association between FA, SAA patients and HPV infection. However, the high prevalence of oral HPV infection found in these patients both submitted and not submitted to HSCT indicates that HPV screening of these subjects with a close follow-up should be considered. Further investigation about persistence or clearance of HPV infection in FA is necessary in order to propose mechanisms of infection prevention.

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## Author contributions

All the authors equally contributed to this work.

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