Molecular-cytogenetic characterization of head and neck cancer: Identification of novel prognostic factors and gene targets for therapy [double dissertation 2]
Wreesmann, V.B.; Singh, B.

Citation for published version (APA):
Chapter 3

Human Papillomavirus DNA and p53 Polymorphisms in Squamous Cell Carcinomas from Fanconi Anemia Patients


Journal of the National Cancer Institute 2003; 95(22):1718-1721
Fanconi Anemia

Abstract

Fanconi anemia is an autosomal recessive disorder characterized by congenital malformations, bone marrow failure, and the development of squamous cell carcinomas (SCCs) and other cancers. Recent clinicopathologic evidence has raised the possibility that an environmental factor such as human papillomavirus (HPV) may be involved in the pathogenesis of SCCs in Fanconi anemia patients. Given the high prevalence of p53 mutations in SCCs among the general population and the lack of p53 mutations in HPV-related carcinogenesis, we evaluated the role of HPV and p53 mutations and polymorphisms in SCC from Fanconi anemia patients. We used polymerase chain reaction (PCR) screening and real-time PCR to detect and quantify HPV DNA in DNA extracted from microdissected SCCs obtained from 24 Fanconi anemia patients (n=25 SCCs; case subjects) and 50 age-, sex-, and tumor site-matched SCC patients without Fanconi anemia (n=50 SCCs; control subjects). We PCR-amplified and sequenced exons 4-9 of the p53 gene from SCC DNA. We detected HPV DNA in 84% of the SCC specimens from the case subjects and in 36% of the SCC specimens from the control subjects (P<.001). The prevalence of p53 mutations in SCCs from the case subjects (0%, 0/25) was statistically significantly lower than that of SCCs from the control subjects (36%, 12/33; P<.001). A greater proportion of patients with Fanconi anemia and SCC were homozygous for Arg72, a p53 polymorphism that may be associated with increased risk for HPV associated human malignancies, than an ethnically-matched cohort of Fanconi anemia patients without SCC (75% versus 51%; P=.05). These data suggest that Fanconi anemia is associated with increased susceptibility to HPV-induced carcinogenesis.

Fanconi anemia is a rare autosomal recessive disorder that is characterized by chromosome instability and a predisposition for cancer development [1]. It has recently been shown that Fanconi anemia patients have a high incidence of squamous cell carcinomas (SCCs), especially SCCs of the head and neck and anogenital regions.[2,3] Fanconi anemia patients have a 500- to 700-fold higher incidence of head and neck SCC than the general population and a 14% cumulative incidence of head and neck SCC by the age of 40 years.[4,5] Whereas 80%-90% of SCC patients in the general population report a history of tobacco and/or alcohol use [6,7], only 16% of the Fanconi anemia patients with SCC report a history of tobacco and/or alcohol use. Therefore, the etiology of SCC in Fanconi anemia patients may differ from that in the general population.

The high incidence of head and neck and anogenital SCC among Fanconi anemia patients, combined with the high rates of second primary tumors in these patients [4], led us to speculate that an environmental factor may play a role in squamous cell carcinogenesis in these patients. Moreover, the observed distribution of tumors, which involves regions of the body that are at high risk of human papillomavirus (HPV)-associated carcinogenesis, suggests a possible association between SCC and HPV infection in Fanconi anemia patients.[8-12] Because HPV E6 and E7 oncoproteins are expressed in HPV-associated cancers and have been found to inactivate several important tumor suppressor genes including p53 and Rb, respectively, understanding the relationship
between HPV and p53 may elucidate the underlying cellular mechanisms responsible for tumor development in the Fanconi anemia population.[13-16]

We examined the role of HPV infection (as defined by HPV DNA positivity of tumor tissue) in the pathogenesis of SCC among patients with Fanconi anemia by performing a case-control study in which the case subjects were Fanconi anemia patients diagnosed with SCC of the anus, vulva, or head and neck and the control subjects were SCC patients who did not have Fanconi anemia and who were matched to the case subjects in a 2:1 ratio for age, sex, and site of primary tumor. Matching for tobacco and/or alcohol exposure status was not possible because of the small number of appropriate cases in our tissue bank. The cohort of Fanconi anemia patients consisted of 754 diagnosis-confirmed Fanconi anemia patients registered between 1982 and 2001 by the International Fanconi Anemia Registry (IFAR).[2,4] We procured 25 tissue and blood samples from 24 Fanconi anemia patients who had pathologically confirmed SCCs and were treated at one of 24 medical institutions across North America. Tumor tissues (and blood samples, when available) for the control subjects were obtained from the Memorial Sloan-Kettering Cancer Center tumor bank. All tissue specimens were assigned random specimen numbers. To maintain subject anonymity, we collected clinical data independently from the laboratory data and integrated both types of data only after completion of the project. All investigators were blinded to the clinical information until the completion of the study. This study was approved by the Institutional Review Board of The Rockefeller University. We obtained written informed consent from the patients or their next of kin to use clinical data and tissue specimens.

Two experienced pathologists (A. G. Huvos and D. Carlson) reviewed the hematoxylin-eosin-stained sections of the surgical resection specimens to confirm the diagnoses and to identify regions of interest. All specimens were found to contain SCC. All of the vulvar cancers were warty (condylomatous; three cases) or basaloid (usual type; three cases) types, which are associated with HPV infection in the general population, and no cases were of the typical keratinizing type, which in the general population is not associated with HPV.[17] Invasive tumor tissue, adjacent non-tumor tissue, and dysplastic tissue (when available) were isolated from the tissue specimens with the use of a laser capture microdissection apparatus (PALM, Bernried, Germany). DNA was extracted from the microdissected tissue [18-20] and subjected to HPV DNA detection by polymerase chain reaction (PCR) as previously described.[21] HPV DNA positivity was defined as the presence of a 188-base-pair (bp) PCR product on an ethidium bromide-stained agarose gel. The PCR product was purified and sequenced to determine the type of HPV, as previously described.[22] We used real-time PCR to confirm HPV DNA detection and to quantify the number of HPV DNA copies per cell, as previously described.[22] Only tissue samples that were positive for HPV16 and HPV18 were used for real-time PCR, and tissue samples were considered positive only if they had more than one copy of HPV DNA per 10 cells.[22] DNA extracted from microdissected tissue was also analyzed for p53 mutations by PCR amplification of exons 4-9 of the p53 gene;
Table 1. Summary of the squamous cell carcinomas (SCCs) analyzed for the presence of human papilloma virus (HPV) DNA and the frequencies of HPV DNA positivity

<table>
<thead>
<tr>
<th>Patient Groups</th>
<th>No. of tumors</th>
<th>Subsite of tumor (No. of tumors)</th>
<th>No. of HPV DNA-positive tumors/total No. of tumors (%)</th>
<th>Median No. of HPV DNA copies per cell (range)</th>
<th>No. of HPV 16 DNA-positive tumors/No. of HPV DNA-positive tumors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fanconi anemia patients with SCC (case subjects)</td>
<td>25</td>
<td>21/25 (84)§</td>
<td>2.6 (0.16-249.3)</td>
<td>19/21 (90)</td>
<td></td>
</tr>
<tr>
<td>Fanconi anemia patients with anogenital SCC</td>
<td>7</td>
<td>Vulva (6) Anus (1)</td>
<td>68.6 (0.54-249.3)</td>
<td>4/6 (67)†</td>
<td></td>
</tr>
<tr>
<td>Fanconi anemia patients with head and neck SCC</td>
<td>18</td>
<td>Oral cavity (15) Larynx (1) Oropharynx (1) Hypopharynx (1)</td>
<td>15/18 (83)</td>
<td>15/15 (100)</td>
<td></td>
</tr>
<tr>
<td>SCC patients without Fanconi anemia (control subjects)</td>
<td>50</td>
<td>Anogenital (14) Head and neck (36)</td>
<td>18/50 (36)‡</td>
<td>16/18 (89)</td>
<td></td>
</tr>
</tbody>
</table>

*Tumors were considered HPV DNA-positive if they had more than one copy of HPV DNA per 10 cells detectable by a quantitative real-time polymerase chain reaction assay. Only HPV types 16 and 18 were evaluated by real-time polymerase chain reaction methods.

†One patient with vulvar carcinoma had HPV type 18 DNA and one patient with vulvar carcinoma had HPV type 52 DNA.
‡Statistical analysis using Fisher's exact test revealed a statistically significantly higher frequency of HPV DNA in the Fanconi anemia patients with SCC (case subjects) than in the SCC patients without Fanconi anemia (control subjects) (P<.001).
§One patient with head and neck cancer had HPV type 33 and one patient with head and neck cancer had HPV type 67.

each amplified exon was sequenced in duplicate using a previously described protocol.[23] To determine p53 codon 72 genotypes, we directly sequenced exon 4 of the DNA extracted from blood samples (DNAeasy; Qiagen, Valencia, CA) of the previously described 24 Fanconi anemia patients with SCC and 72 Fanconi anemia patients without cancer. Because specific ethnic groups vary in the distribution of p53 codon 72 genotypes, the groups of subjects were matched by ethnicity. Statistical analyses were performed using Fisher's exact test and the Wilcoxon rank sum test. A two-tailed P value less than or equal to .05 was considered statistically significant for all analyses.

We detected HPV DNA in 21 (84%) of the 25 SCC specimens from the Fanconi anemia case subjects and in 18 (36%) of the 50 SCC specimens from the control subjects (P<.001, Fisher's exact test) (Table 1). HPV DNA was not detected in any of the adjacent nontumor (n=21) or dysplastic (n=17) tissues from the Fanconi anemia patients. (We were unable to perform a similar analysis for the control group because of the small size of those tissue specimens and the lack of non-tumor and dysplastic tissue.) A subgroup analysis revealed that 15 (83%) of the 18 head and neck SCCs from the Fanconi anemia case subjects were positive for HPV DNA compared with only 13 (36%) of the 36 head and neck SCCs from the control subjects (P=.002, Fisher's exact test). Direct sequencing of PCR products amplified from SCC DNA showed that HPV16 was the
most common HPV type detected among the Fanconi anemia-associated SCCs and the control SCCs (19/21 SCCs [90%] and 16/18 SCCs [89%], respectively; \( P = .55 \), Fisher's exact test). Results of real-time quantitative PCR confirmed the presence of HPV16 or HPV18 in all samples that were positive for HPV DNA by PCR screening with consensus oligonucleotide primers. The median number of HPV DNA copies per genome was 2.6 (range=0.16-249.3 copies/genome) and 3.2 (range=0.15-190.85 copies/genome) in SCCs from Fanconi anemia patients and from the control subjects, respectively (\( P = .88 \), Wilcoxon rank sum test; data not shown).

Given the high prevalence of \( p53 \) mutations in SCCs among the general population and the lack of \( p53 \) mutations in HPV-related cervical carcinogenesis[24], we PCR-amplified and directly sequenced exons 4-9 of the \( p53 \) genes from SCC DNA to examine

<table>
<thead>
<tr>
<th>Table 2. Genotype frequencies for ( p53 ) codon 72 in Fanconi anemia patients with squamous cell carcinoma (SCC) and Fanconi anemia patients without SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Groups</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Fanconi anemia patients with SCC (case subjects), ( n = 24 )</td>
</tr>
<tr>
<td>Fanconi anemia patients without SCC (control subjects, group 1), ( n = 65 )</td>
</tr>
<tr>
<td>Age-matched Fanconi anemia patients without SCC (control subjects, group 2), ( n = 20 ) (control subjects)</td>
</tr>
</tbody>
</table>

*Values refer to the number of individuals corresponding to each of three possible genotypes for the codon 72 polymorphism of \( p53 \) (relative frequency of each genotype). NA=not applicable.
The relative frequency of Arg/Arg homozygosity of the case subjects was individually compared with the frequency of the Arg/Arg homozygosity of the control subjects in group 1 or group 2 listed above. For each group, Fisher's exact test was used to compare Arg/Arg homozygosity to the Arg/Pro heterozygosity and Pro/Pro homozygosity combined, and a two-sided \( P \) value was determined for each comparison, as listed in the table above.

\( p53 \) mutation status. None (0%) of the Fanconi anemia-associated SCCs had a \( p53 \) mutation in these exons, whereas 36% of control SCCs had a \( p53 \) mutation (\( P < .001 \), Fisher's exact test; data not shown). Results of a subgroup analysis showed that none (0%) of 18 Fanconi anemia-associated head and neck SCCs had a \( p53 \) mutation compared with 12 (39%) of 31 control head and neck SCCs (\( P = .004 \), Fisher's exact test; data not shown). These results suggest that \( p53 \) inactivation in SCCs from Fanconi anemia patients may occur through an HPV-associated mechanism rather than as a result of \( p53 \) mutations.

Storey et al. [25] reported experimental evidence that polymorphic variants of \( p53 \) differ in their susceptibility to degradation mediated by HPV-derived E6 oncoprotein. Specifically, they demonstrated that homozygosity for arginine at codon 72 (Arg72) of \( p53 \) is associated with an increased susceptibility to E6-induced degradation of \( p53 \) in vivo and, in their case series, a sevenfold increased risk for HPV-associated malignancies.
in humans.[26] However, substantial controversy exists about the role of this polymorphism in the susceptibility to HPV-associated carcinogenesis, mainly because large differences in the frequency of Arg72 homozygosity in different populations have made it difficult to define suitable control groups for cancer susceptibility studies.[27]

We examined the association between the p53 Arg72 polymorphism and SCC among the cohort of Fanconi anemia patients registered in the IFAR because they are a well-defined population at risk for SCC. We compared the p53 codon 72 genotypes of the 24 Fanconi anemia patients with SCC used in the above analyses with those of 72 Fanconi anemia patients without cancer who were registered in the IFAR and matched to the patients with SCC for ethnicity in a 3:1 ratio. We were unable to amplify exon 4 of the p53 gene of seven control patients, leaving us a total control group of 65 Fanconi anemia patients without SCC. There were no statistically significant differences between the two groups of patients with regard to sex or bone marrow transplant status (25% transplant rate in the case subjects versus 33% transplant rate in the control subjects). A statistically significantly higher proportion of Fanconi anemia patients with SCC was homozygous for the Arg72 polymorphism than Fanconi anemia patients without SCC (75% versus 51%; \( P = .05 \), Fisher's exact test) (Table 2). Notably, the Fanconi anemia patients with SCC (median age=30 years; range=15-49 years) were statistically significantly older than the Fanconi anemia patients without SCC (median age=10.2 years; range=1-48 years; \( P < .001 \), Wilcoxon rank sum test). To address differences in the at-risk period for cancer development, we compared the genotype frequencies of the 20 non-cancer Fanconi anemia patients older than 15 years with those of the 24 Fanconi anemia patients with SCC (the youngest age for developing Fanconi anemia-associated SCC was 15 years). This subgroup analysis revealed a larger difference in the frequency of p53 Arg72 homozygosity between Fanconi anemia patients with and without SCC (Table 2; \( P = .015 \)). The odds ratio of SCC associated with Arg72 homozygosity in this subgroup analysis was 5.6 (95% confidence interval=1.5 to 20.5; \( P = .01 \)).

Our study has several limitations. First, even though SCC is common among Fanconi anemia patients, the absolute number of Fanconi anemia-associated SCC cases in our analysis was relatively small because of the rarity of Fanconi anemia. Second, we did not match the SCC patients without Fanconi anemia to those with Fanconi anemia for tobacco and alcohol use status because of the lack of availability of appropriate cases in our tissue bank. This difference in exposure status represents a potential confounding factor because the frequency of p53 mutations in head and neck SCC is associated with tobacco and alcohol use.[27] Third, we could not control for sexual behavior patterns or HPV16 L1 serology status when analyzing the cancer risks associated with p53 Arg72 homozygosity. Finally, even though PCR detection of HPV DNA is an excellent screening tool, further analysis using hybrid capture HPV DNA assays and animal studies is required to confirm our findings and to establish a causal relationship between HPV infection and SCC in patients with Fanconi anemia.
Our results suggest that the high rates of head and neck and anogenital SCCs among Fanconi anemia patients may be associated with the high frequency with which onco-
genic HPV DNA is detected in their tumor tissues. Whether this high rate of HPV-assoc-
ated SCC is caused by an underlying immune dysfunction in Fanconi anemia patients or directly involves the pathway(s) defective in Fanconi anemia is currently not known. Fanconi anemia may be the second inherited syndrome identified, after epidermodys-
plasia verruciformis, that is associated with an increased susceptibility to HPV-induced carcinogenesis. However, Fanconi anemia-associated SCCs differ from epidermodysplasia verruciformis-associated SCCs in that the former are mucosal and are associated with HPV16, whereas the latter are cutaneous and are associated with HPV5 or HPV8. Moreover, compared with Fanconi anemia patients, epidermodysplasia verruciformis patients appear to have a higher risk for HPV-associated tumorigenesis, which develops in approximately 50% of cases.[28] Although mutations in two genes, EVER1 and EVER2, have been identified in epidermodysplasia verruciformis patients by linkage analysis, the precise mechanisms underlying the increased sensitivity to HPV-associated carcinogenesis in these patients are yet to be defined.[29]

As with cervical cancer attributable to HPV in the general population, SCC in Fanconi anemia patients is probably also associated with the inactivation of p53 by HPV-associated oncoproteins rather than by direct mutagenesis. In addition, Fanconi anemia patients with homozygosity for Arg72 in p53 have a 5.6-fold increased risk of developing HPV-associated cancers compared with Fanconi anemia patients who do not have Arg72 homozygosity. Thus, Fanconi anemia may represent an excellent model for studying HPV-induced carcinogenesis and associated prevention approaches.

References
7. Maier H, Sennewald E, Heller GF, Weidauer H. Chronic alcohol consumption-the


**Acknowledgments**

Funded in part by Public Health Services grants R37-HL32987 (to A. D. Auerbach) from the National Heart, Lung, and Blood Institute, RO1- CA82678 (to A. D. Auerbach) and 5T32- CA09685 (to D. I. Kutler) from the National Cancer Institute, and Research Center grants M01- RR00102 and M01-RR06020 (to The Rockefeller University Hospital General Clinical Research Center and The New York Presbyterian Hospital Children's Clinical Research Center), National Institutes of Health, Department of Health and Human Services; and the 2002 American Society of Clinical Oncology Young Investigator Award (to D. I. Kutler).

B. Singh is a recipient of the George H. A. Clowes, Jr., MD, Memorial Research Career Development Award from the American College of Surgeons.

We thank Nancy Bennett for her excellent editorial assistance.