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Deficiencies in the Fanconi Anemia DNA damage Response Pathway Increase Sensitivity to HPV-Associated Head and Neck Cancer

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Abstract

Patients with the rare genetic disease, Fanconi Anemia (FA), are highly susceptible to squamous cell carcinomas arising at multiple anatomical sites including the head and neck region. Human papillomaviruses (HPVs), particularly HPV16, are associated with ~20% of head and neck squamous cell carcinomas (HNSCCs) in the general population. Some but not other investigators have reported that HNSCCs in FA patients are much more frequently positive for HPV. In addition, studies have demonstrated an interaction between the HPV16 E7 oncoprotein and the FA pathway, a DNA damage response pathway deficient in FA patients. Based on these studies, it was hypothesized that the FA pathway contributes to repair of DNA damage induced by HPV16 E7, providing one explanation for why FA patients are predisposed to HPV-associated HNSCCs. To determine the importance of the FA pathway in modulating E7's oncogenic abilities, we crossed *K14E7* transgenic (*K14E7*) and *fancD2*-knockout mice (*FancD2*$^{-/-}$) to establish *K14E7*/*FancD2*$^{-/-}$ and *K14E7*/*FancD2*$^{+/+}$ mice and monitored their susceptibility to HNSCC when treated with a chemical carcinogen. *K14E7*/*FancD2*$^{-/-}$ mice had a significantly higher incidence of HNSCC compared with *K14E7*/*FancD2*$^{+/+}$ mice. This difference correlated with an increased proliferative index and the increase in expression of biomarkers that are used to assess levels of DNA damage. These animal studies support the hypotheses that FA patients have increased susceptibility to HPV-associated cancer and that the FA DNA damage response pathway normally attenuates the oncogenic potential of HPV16 E7.

Keywords

HPV16 E7; FancD2; Fanconi Anemia (FA); Head and Neck Cancer

Introduction

Fanconi Anemia (FA) is a heterogeneous, recessive genetic disease characterized by congenital abnormalities, progressive bone marrow failure, and a predisposition to leukemia

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and solid tumors, particularly squamous cell carcinomas (SCCs) including head and neck SCCs (HNSCCs), at remarkably young ages. The frequency of HNSCCs in FA patients is highly elevated compared to that in the general population (1-4). So far, hematopoietic stem cell transplantation (HSCT) is the only way to treat bone marrow failure, which is the main cause to death in FA patients. Even after HSCT, FA patients suffer from a high incidence of HNSCCs (5-7) most likely due to defects in DNA damage repair, and consequent increases in genomic instability, in epithelial cells of the head and neck region.

FA disease is caused by biallelic mutations in one of at least thirteen different genes (*FA* genes). FA proteins (Fanc proteins; FancA, B, C, D1, D2, E, F, G, I, J, L, M, and N) encoded by these genes act together to protect genomic integrity, though the mechanism by which they do this remains poorly understood. The FA pathway is known to be activated by DNA interstrand cross-links (ICLs) induced by chemicals and/or found at sites of stalled DNA replication forks (8). Eight of the *FA* gene products interact in a holo-complex that localizes to sites of damage or stalled replication forks. This in turn leads to the mono-ubiquitination of another *FA* gene product, FancD2 (9). DNA damage induced by UV, IR, and chemical compounds can also activate the FA pathway through phosphorylation of FancD2 by the ATM kinase; however, sensitivity of the FA pathway to these forms of damage is less than that to ICLs (10-12).

High-risk human papillomaviruses (HPVs) are etiologic agents in approximately 20% of HNSCCs in the general population, primarily amongst tumors of the oropharynx. HPV type 16 (HPV16) is present in over 90% of the HPV-positive HNSCCs (13-14). HPV16 encodes three oncogenes, *E5*, *E6*, and *E7*. A mouse model for HPV-associated HNSCC has been developed in which HPV16 transgenic mice, when treated with a chemical carcinogen that induces DNA adducts akin to those caused by tobacco-associated carcinogens synergizes with HPV16 oncogenes to induce HNSCC. The cancers that arise in these mice share many histopathological properties with HPV positive HNSCC in humans. The site at which tumors arise is less restrictive than in humans, presumably reflective of the pattern of HPV transgene expression throughout the mouse oral/upper GI epithelia. Between E6 and E7, HPV16 E7 has greater potential to cause head and neck cancer in animal models (15) with E6 contributing to increased incidence in combination with E7 (Jabbar et al., in press). The contribution of HPV16 E5 to HNSCC in this mouse model has not been fully evaluated but appears to be much less than that of E7 (Strati and Lambert, unpublished results)

In FA patients from North America a high percentage (84%) of HNSCC arising at various sites in the head and neck region (i.e. not restricted to the oropharynx) were found to be positive for high-risk HPV DNA. Consistent with a role of HPV in these cancers, mutations in p53, a tumor suppressor that is inactivated by HPV16 E6, were not found in HNSCCs from these patients. However, in patients from European countries, HNSCCs were HPV DNA negative and over 50% of these tumors had p53 mutations (16-17). Given these conflicting clinical data, it remains unclear whether HPVs play a major role in HNSCCs arising in FA patients.

Several studies point to a contribution of FA deficiency in HPV-associated disease at the molecular level. In tissue culture, expression of the HPV16 *E7* oncogene was shown to stimulate transcription of *fancD2* (18) and activate the FA pathway (19). In another study making use of organotypic cultures of human keratinocytes to recapitulate the HPV life cycle, HPV16 and HPV18-induced epithelial hyperplasia was increased in *fancA*-deficient cells or in cells knocked down in its expression; whereas, restoration of *fancA* expression attenuated this effect (20). These studies indicate that there is an interplay between HPV and the FA pathway. The implications of this interplay on HPV-associated carcinogenesis remain unclear.

Given the above findings, we performed experiments using mice to ask whether deficiency in the FA pathway leads to an increase in HPV16 E7-mediated HNSCC. We crossed *K14E7* transgenic mice, which display heightened susceptibility to head and neck cancer (15,21) to *fancD2* gene knock-out mice (22) and the incidence of head and neck cancers in HPV transgenic and nontransgenic mice was scored in *fancD2*-sufficient and *fancD2*-deficient backgrounds. We found a significantly increased incidence of head and neck tumors in *K14E7/FancD2*$^{-/-}$ mice compared to in *K14E7/FancD2*$^{+/+}$ mice. No tumors arose in nontransgenic mice regardless of *fancD2* status. Increased tumor incidence in the FA-deficient *E7*-transgenic mice correlated with increases in cell proliferation and DNA damage foci. In addition, we found that two biomarkers, p16 and MCM7 (21,23), used in distinguishing between HPV-positive and HPV-negative HNSCC arising in humans are up-regulated in the tumors arising in *E7*-transgenic mice regardless of FA-status. Our findings support the hypotheses that FA patients have greater susceptibility to HPV-associated HNSCC and that E7's oncogenic properties are attenuated by a functional FA pathway. Based upon these conclusions, HPV-specific prophylactic vaccines could be useful in reducing SCC burden in FA patients.

Materials and Methods

Mice

K14E7 transgenic mice (24), on the FVB genetic background, were crossed to *fancD2* gene knockout (*FancD2*$^{-/-}$) mice (22), on the 129 genetic background, to generate F_1 mice, *K14E7/FancD2*$^{+/-}$ and *NTG/FancD2*$^{+/-}$ (FVB/129 mixed background). All experimental mice (*NTG/FancD2*$^{+/+}$, *NTG/FancD2*$^{-/-}$, *K14E7/FancD2*$^{+/+}$, and *K14E7/FancD2*$^{-/-}$ mice) were males generated by intercrossing F_1 mice. All mice were genotyped by PCR. Mice were injected i.p. with 0.3 mL bromodeoxyuridine (BrdUrd; 12.5 mg/mL) 1 hr before euthanasia to measure cell proliferation. Tongues and esophagi were harvested and processed as previously described (21). Mice were housed in the Association for Assessment of Laboratory Animal Care-approved McArdle Laboratory Animal Care Unit. All procedures were carried out in accordance with an animal protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee.

4-nitroquinoline-1-oxide–induced head and neck carcinogenesis study and histologic analysis

The treatment and guidelines for histological analysis of tumors were previously described (21). Briefly, mice were treated with 4-nitroquinoline-1-oxide (4-NQO; 10 μg/ml) in their drinking water for 8 weeks and then held off treatment for an additional 16 weeks. At the end point or when mice became moribund, they were euthanized, tumors in tongue and esophagus were scored, and tissues collected for histopathological analyses.

Immunofluorescence

Immunofluorescence was performed as described previously (25-26). Antibodies used included anti-p16 (1:50 in 5% nonfat milk/5% horse serum; M156, Santa Cruz Biotech.), anti-Mcm7 (1:200 in 5% horse serum; Neomarkers), anti-cytokeratin 14 conjugated to FITC (CK14; 1:100 in 5% horse serum; CBL 197F, Millipore), and anti-bromodeoxyuridine (BrdUrd; 1:50 in 5% horse serum; Calbiochem).

Quantifying BrdUrd positive nuclei, γH2AX foci, and 53BP1 foci positive cells

At least three mice of each genotype, *NTG/FancD2*$^{+/+}$, *NTG/FancD2*$^{-/-}$, *K14E7/FancD2*$^{+/+}$, and *K14E7/FancD2*$^{-/-}$ mice, were selected and ~8 to 10 frames (400x) of cells within the

suprabasal (CK14 negative) and basal (CK14 positive) layers of tongue and esophagus epithelia were quantified for each mouse.

Statistical analysis

The MSTAT software program[1] was used for determining statistical significance. Two-sided Fisher's exact test was used to determine the significance of differences in tumor incidence between each group of mice (Table 1). To determine the significance for BrdUrd, γH2AX foci, and 53BP1 foci positive cells between each group of mice, a one-sided Wilcoxon rank-sum test was used (Fig.2 and 3).

Results

fancD2-deficiency sensitizes mice to HPV-associated tumorigenesis

To test the hypothesis that FA-deficiency leads to increased susceptibility to head and neck cancers caused by HPV, we made use of a mouse model for HPV-associated head and neck cancer (15,21) in which HPV-transgenic mice develop head and neck cancers when exposed to a chemical carcinogen, 4-nitroquinoline-1-oxide (4-NQO). This chemical carcinogen is absorbed by the epithelia lining the oral cavity and esophagus where a metabolite of 4-NQO causes DNA mutations. Prolonged treatment (16 weeks) with 4-NQO at a high dose (10X that used in our own studies) is sufficient to efficiently induce head and neck cancers in nontransgenic mice (27). At the ten-fold lower dose used in our studies, 4-NQO synergizes with HPV16 oncogenes to induce head and neck cancers with few if any cancers arising in nontransgenic mice (21). In the current study, we treated mice at the dose used in our past studies but for a shorter duration (8 weeks treatment compared to 16 weeks). This shorter treatment regimen was chosen because it confers lower incidence of tumors in *E7*-transgenic mice (Jabbar et al., manuscript in preparation), thereby providing a better context in which to monitor any contribution of FA-deficiency in increasing the incidence of head and neck cancer. Differences have been observed in the tumor susceptibility of male vs female *fancD2*$^{-/-}$/*p53*$^{+/-}$ mice (28) with higher incidence of spontaneous tumors observed in female mice owing largely to tumors arising in the mammary glands and ovaries. In the present study only male mice were used.

As stated above, of the HPV16 oncogenes, *E7* contributes greatest to head and neck cancer in mice (15). Because of this, and the fact that tissue culture studies indicated that the FA pathway promotes repair of E7-induced damage (20), we crossed *K14E7* mice to *fancD2*-null mice (22) to generate four different genotypes: *NTG/FancD2*$^{+/+}$, *NTG/FancD2*$^{-/-}$, *K14E7/FancD2*$^{+/+}$, and *K14E7/FancD2*$^{-/-}$. Male mice were treated with 4-NQO for 8 weeks, then held for an additional 16 weeks before being sacrificed. Visible tumors on the tongues and esophagi of mice were counted, and tissues collected for histopathological analyses. No tumors were detected in either the *NTG/FancD2*$^{+/+}$ or *NTG/FancD2*$^{-/-}$ mice, indicating that FA-deficiency alone was not sufficient to induce head and neck cancer under this carcinogen-treatment regimen (Table 1). Only 4 out of 20 *K14E7/FancD2*$^{+/+}$ mice (20%) had overt tumors (Table 1) consistent with prior observations that tumors arise in only a small fraction of E7 mice treated for only 8 weeks with 4-NQO (Jabbar et al., manuscript in preparation). However, a much higher fraction (11 out of 21; 52%) of *K14E7/FancD2*$^{-/-}$ mice developed overt tumors (Table 1). This incidence of tumors in the *K14E7/FancD2*$^{-/-}$ mice was significantly higher compared to the *K14E7/FancD2*$^{+/+}$ mice (P=0.05; two-sided Fisher's exact test). These data indicate that FA-deficiency increases the susceptibility of mice to E7-induced head and neck tumors.

[1] http://www.mcardle.wisc.edu/mstat/

The majority of overt tumors arising in *K14E7/FancD2*$^{-/-}$ mice are carcinomas

Tissues from mice with overt tumors were harvested, fixed, sectioned and subjected to detailed histopathological analysis. Specifically, every tenth 5 μm section of the tongue and esophagus was stained with hematoxylin and eosin (H&E) and analyzed histopathologically to determine the worst stage of neoplastic disease in each animal (Table 2). As described previously (21), a progressive disease was observed in the *E7*-transgenic mice in this study (see Supplemental figure 1), ranging from normal epithelium, to benign papillomas to invasive cancer (i.e. carcinomas). Carcinomas were subdivided into grade I, II, and III, based on the degree of differentiation (keratinization) within the cancer. Whereas only one of the tumors arising in the *K14E7/FancD2*$^{+/+}$ mice was a carcinoma, 8 out of 11 tumors arising in the *K14E7/FancD2*$^{-/-}$ mice were carcinomas (Table 2). These histopathological findings demonstrate that FA-deficiency increases the severity of neoplastic head and neck disease in *E7*-transgenic mice.

Expression of two biomarkers in carcinomas arising in *K14E7/FancD2*$^{+/+}$ and *K14E7/FancD2*$^{-/-}$ mice

Two biomarkers, p16 and MCM7, have proven useful in distinguishing HPV-positive from HPV-negative HNSCCs both in the mouse (21) and humans (25,29-31). HPV16 E7 is thought to play a dominant role in the induction of MCM7 and p16 in both head and neck (21) and cervical (25) cancers. To determine if these proteins were also up-regulated by E7 in head and neck cancers arising on a FA-deficient background, sections from cancers arising in *K14E7/FancD2*$^{+/+}$ and *K14E7/FancD2*$^{-/-}$ mice were subjected to p16- and MCM7-specific immunofluorescence. Both proteins were highly expressed in cancers arising in *E7*-transgenic mice regardless of *fancD2* status (Fig. 1). Furthermore, the expression patterns of MCM7 and p16 in normal epithelium of the tongue and esophagus correlated with E7 expression, not *fancD2* status (Supplemental figure 2). Consistent with these findings, we previously observed MCM7 and p16 staining exclusively in HPV-positive head and neck tumors in our mouse model (21). These findings, when taken together, suggest that these biomarkers will retain their usefulness in identifying HPV-positive disease in the FA patients suffering from HNSCCs.

Deficiency in *fancD2* induces DNA synthesis in the basal epithelial compartment, but not in the suprabasal epithelial compartment, and this activity is independent of E7

A prior study demonstrated that FA deficiency can lead to hyperplasia in the context of organotypic raft cultures of HPV oncogene immortalized human keratinocytes (20). This raised the possibility to us that increases in cell proliferation could contribute to the increase in head and neck cancers in the *fancD2*-deficient *E7*-transgenic mice. We therefore monitored cell proliferation in the epithelia lining the tongue and esophagus of the different mouse genotypes used in this study. For this experiment, groups of mice (n=3) of each genotype were injected i.p. with BrdUrd 1 hr before being sacrificed, and BrdUrd-specific immunofluorescence was performed to detect its incorporation into newly synthesized DNA (Fig. 2A). The frequency of BrdUrd-positive cells was then quantified in each tissue (Fig. 2B). On the *fancD2*-sufficient background, E7 caused significant increases in the frequency of BrdUrd-positive cells in both the basal and suprabasal compartments of the tongue and esophagus epithelia (*K14E7/FancD2*$^{+/+}$ vs. *NTG/FancD2*$^{+/+}$ mice; $P<0.05$), consistent with prior observations (15). In the absence of E7, deficiency in *fancD2* alone caused a significant increase in the proliferative index in the basal but not the surpabasal compartment of the epithelia in both the tongue and esophagus (*NTG/FancD2*$^{-/-}$ vs. *NTG/FancD2*$^{+/+}$ mice; $P<0.05$ for basal only). In the presence of E7, deficiency in *fancD2* also caused a significant increase in the proliferative index in the basal but not the surpabasal compartment of the epithelia in both the tongue and esophagus (*K14E7/FancD2*$^{-/-}$ vs. *K14E7/FancD2*$^{+/+}$ mice; $P<0.05$ for basal only). Additionally, we scored cells progressing

through G2/M by using immunofluorescence of Histone 3 phosphorylated at serine 10 (H3S10) which is a well-known marker for mitosis (32). We found that *fancD2* deficiency or expression of E7 led to increased numbers of H3S10-positive cells (Supplemental figure 3). This correlated with the increases in DNA synthesis in these same mouse strains (Fig. 2). FancD2 deficiency, however, led to only subtle increases in percentage of H3S10-positive cells in the E7 transgenic tissue (Supplemental figure 3). This correlates with the observation that E7's induction of DNA synthesis is only slightly enhanced on a FancD2 deficient background (Fig. 2). Taken together these findings indicate that both HPV16 E7 oncoprotein and *fancD2*-deficiency act can independently and additively induce cell cycle progression specifically in the basal epithelia of the animal tissues.

DNA damage foci-positive cells accumulate in head and neck epithelia in E7 expressing mice: influence of FA pathway

A number of observations support the hypothesis that HPV16 E7 induces genomic instability. Expression of E7 in cells leads to an accumulation of DNA breaks based upon use of the comet assay, which scores for the presence of single and/or double strand DNA breaks, as well as DNA interstrand cross-links (ICLs) damage (33-34). Expression of E7 in cells also causes an accumulation of chromosomal abnormalities as evidenced by losses or gains of specific alleles (33,35) or gross changes scored by karyotypic analyses for chromatid breaks, chromatid fusions or polyploidy (35). That E7 causes an accumulation of damaged DNA is further supported by the observation that in the nuclei of cells expressing E7 there is an increased frequency of DNA damage foci (33), as detected using antibodies specific to histone 2AX phosphorylated at Serine-139 (γH2AX), which is a marker for activated DNA damage responses triggered by the presence of damaged DNA in the nuclei of cells (36-37).

All of the above-described studies were performed in tissue culture. We wanted to determine whether E7 is capable of inducing DNA damage in the context of our mouse model for head and neck cancer and, if so, learn whether this property of E7 is affected by the status of the FA pathway. Specifically we wanted to test the hypothesis that the FA pathway suppresses E7's capacity to induce DNA damage. It is not possible to directly quantify DNA damage *in situ* in the context of tissue samples; therefore, we monitored for the presence of DNA damage indirectly by quantifying the abundance of cells with DNA damage foci. γH2AX-specific immunofluorescence was performed on sections of tongue and esophagus from *E7*-transgenic and nontransgenic mice on the *fancD2*-sufficient and *fancD2*-deficient backgrounds (Fig. 3A). E7 led to a large and statistically significant increase in cells with γH2AX-positive nuclear foci both in the basal and suprabasal compartments of the epithelia lining the tongue and esophagus (Fig. 3B), consistent with prior findings in tissue culture (33). This E7-dependent induction in the number of cells with γH2AX-positive foci was further increased on the *fancD2*-deficient background (Fig. 3B). This latter increase was statistically significant in the basal and suprabasal compartment of the esophagus and the suprabasal compartment of the tongue, and marginally significant in the basal compartment of the tongue (Fig. 3B). The fact that there was greater frequency of cells with DNA damage foci in *E7*-transgenic, FA-deficient epithelia than in *E7*-transgenic, FA-sufficient epithelia is consistent with the hypothesis that the FA pathway suppresses E7's capacity to induce DNA damage. Similar results were observed in the epidermis lining the *K14E7* mouse torso; another stratified epithelia in which E7 is expressed. In this tissue, we again observed E7-dependent induction of γH2AX-positive nuclear foci both in the basal and suprabasal compartments, and this induction was further heightened on the *fancD2*-deficient background (Supplemental figure 4).

A recent study carried out in tissue culture also supports the hypothesis that the FA pathway promotes repair of E7-induced damage (20). Specifically, in HPV18 E6/E7 immortalized

fancA-deficient keratinocytes grown in organotypic cultures to produce stratified epithelium, there was observed an increased frequency of cells with high numbers of 53BP1-positive nuclear foci compared to a derivative cell line that had been complemented for *fancA*. 53BP1, like γH2AX, is a marker for DNA damage foci and provides an indirect measure of the level of DNA damage in a cell (38). We repeated our own analysis of mouse tissues using 53BP1 as an alternative marker for DNA damage foci (Fig. 3C). As observed with γH2AX-specific immunofluorescence (Fig. 3B), E7 caused a large and statistically significant induction in the frequency of cells with 53BP1-positive nuclear foci in both the basal and suprabasal compartment of the tongue and esophagus epithelia (Fig. 3D). In *fancD2*-deficient tissue E7 caused a further increase in the frequency of cells positive for 53BP1-positive nuclear foci in both the basal and suprabasal compartments of the tongue and esophagus. This further increase was again statistically significant in the both compartments of the esophagus and the suprabasal compartment of the tongue, and marginally significant in the basal compartment of the tongue (Fig. 3D). Thus, using two different markers for DNA damage foci, we find evidence in support of the hypothesis that the FA pathway suppresses E7-dependent induction of DNA damage.

In the absence of E7, we found no evidence for *fancD2*-deficiency leading to an increase in the frequency of cells positive for γH2AX-positive nuclear foci, but there was a modest yet significant increase in the frequency of cells positive for 53BP1-positive nuclear foci specifically in the suprabasal compartment of the tongue and esophagus epithelia (Fig. 3D). It is unclear what this modest increase reflects, as in nontransgenic mice this compartment of the epithelia is post-mitotic; therefore it is not likely to reflect DNA damage arising from normal DNA replicative processes.

Discussion

FA pathway suppresses HPV-associated carcinogenesis in tongue and esophagus

A link between HPVs and SCCs from FA patients is of interest to both the clinical and basic research fields. In prior studies of FA patients, geographical and technical differences between study groups make establishing such a link unclear (16-17). Some researchers, however, have demonstrated clear correlations between the oncogenic activity of HPVs and deficiency in the FA pathway *in vitro* (19-20). Our *in vivo* findings, that HPV-associated cancer incidence is significantly induced in the tongues and esophagi when the FA pathway is compromised by the lack of *fancD2* gene (Table 1 and 2), supports the conclusions of prior *in vitro* studies. Our findings might explain why HPV16 E7 activates the FA pathway in human cells (19). Such an activation could counteract the oncogenic activities of E7. Our results also are consistent with the observation *in vitro* that rescue of the FA pathway inhibits HPV-associated hyperplasia (20). Based upon the cumulative studies, we hypothesize that the FA pathway suppresses the oncogenic activities of HPV16 E7. Moreover, these data provide further argument for the value of early vaccination of FA patients against HPVs to prevent onset of HPV-associated neoplasia.

FancD2 controls cell proliferation in basal epithelial cells

We observed *fancD2*-deficiency increased the frequency of cells supporting DNA replication in the basal epithelium, even in the absence of HPV16 E7 expression (Fig. 2). This supports the hypothesis that increases in cell proliferation contribute to epithelial cancers in FA patients. This finding also correlates with prior findings that post-translational modification of FancD2 by the ATM and ATR pathways play a role in the S-phase checkpoint in cells *in vitro* (10,12,39). In the absence of exposure to exogenous DNA damaging agents, ubiquitinated-FancD2 protein is observed in S phase of cells (10,12,40). Furthermore, lymphoblastoid cell lines deficient in the FA pathway lack the ability to delay

S-phase progression in response to DNA damage from DNA cross-linking agents (41-42). In the latter cell type, a corresponding induction of programmed cell death is thought to contribute ultimately to bone marrow failure in FA patients (43).

De-regulation of cell proliferation by HPV16 E7 is induced by deficiency in the FA pathway

Unscheduled entry into S phase and the deregulation of cyclin expression in epithelium are the well-known properties of HPV16 E7 (44). *fancD2* gene knock-down increases the proliferation in organic raft culture system using keratinocytes immortalized by HPV16 and 18 oncogenes *in vitro* (20). Our finding that deficiency in the FA pathway increased cell proliferation in epithelium *in vivo* induced by E7 expression (Fig. 2) is consistent with the previous observation. We did not observe an effect of fancD2-deficiency on proliferation within the suprabasal compartment of epithelia in the presence of E7 expression. This would suggest that E7's ability to deregulate the cell in the suprabasal compartment is not modulated by the FA pathway. However, deficiency in the FA pathway did result in increased proliferation in the basal compartment of epithelium expressing the E7 oncoprotein (Fig. 2B). But because FA deficiency, in the absence of E7 expression, can induce basal cell proliferation (see above), it remains unclear whether the increase in basal cell proliferation in *E7*-transgenic, *fancD2*-null epithelia reflects an ability of the FA pathway to partially suppress E7-induced proliferation or an ability of FA deficiency to act independently to cause cell proliferation. Regardless, the predisposition of FA-deficient *E7*-transgenic mice to develop cancer correlated with an overall heightened rate of basal cell proliferation.

FA pathway modulates DNA damage induced by HPV16 E7

Whether the FA pathway acts directly to suppress DNA damage induced by HPV16 E7 is an important question in light of the fact that E7 is more oncogenic on a FA-deficient mouse background. E7 activates FA pathway as demonstrated by the accumulation of mono-ubiquitinated FancD2 *in vitro* (19). E7 induces DNA damage as detected by the comet assay (33) and DNA damage response, as measured by the induction of γH2AX-positive nuclear foci *in vitro* (33). Our findings that E7-dependent DNA damage responses were increased on the FA-deficient background *in vivo*, as evidenced by the increase in numbers of cells with γH2AX and 53BP1-positive nuclear foci (Fig. 3), is consistent with recent *in vitro* results demonstrating that *fancA* gene complementation significantly reduced the number of 53BP1 foci per nucleus in *fancA* gene deficient keratinocytes immortalized by HPV18 (20). Together, these results support the hypothesis that the FA pathway suppresses E7-induced DNA damage, and raise the interesting corollary that this property of the FA pathway contributes to its ability to suppress HPV-induced neoplasia.

One of the possible forms of DNA damage induced by E7 is double strand DNA breaks (DSBs), given that γH2AX and 53BP1-positive nuclear foci are commonly considered to be readouts for DSBs and DNA damage responses to IR (45-46). γH2AX/53BP1 double positive nuclear foci were observed in the epithelium expressing HPV16 E7 (data not shown). In addition, anaphase bridges, which represent chromosomal fusion caused by DSBs have been observed in cells expressing *E7* (34). These data are consistent with the hypothesis that E7 induces DSBs and that E7-induced DSBs accumulate to a greater extent on a FA-deficient background. Further studies are needed to clearly define the nature of the DNA damage arising in the context of our mouse model for HPV-associated HNSCC.

HPV and HNSCCs in FA patients

Taken together, our studies and that of others in the field are pointing to a model in which cell proliferation and DNA damage induced by the dominant HPV oncogene, *E7*, arise to a greater extent in an FA deficient cellular context. Consistent with this model, recent data

indicates a role of the FancD2 protein in preventing chromosome instability and aneuploidy (47). Accumulated, unrepaired DNA damage is likely to lead to increased genomic instability. This instability of the human genome may in turn contribute to increases in the incidence of SCCs in HPV-positive FA patients. In addition, the increased epithelial proliferation seen in FA deficient cells/tissues may increase the infectivity of HPVs, thereby increasing the chance for persistent infections arising in FA patients (20). Together, these properties conferred by FA-deficiency are likely to contribute to increased susceptibility of FA patients to HPV-associated cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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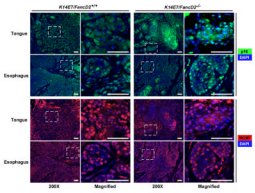

Figure 1. Examination of two biomarkers, p16 and MCM7, in carcinomas arising on tongue and esophagus in *K14E7/FancD2*$^{+/+}$ and *K14E7/FancD2*$^{-/-}$ mice treated with 4-NQO, a carcinogen

Shown are representative images from sections stained with anti-p16 (green), and, and anti-MCM7 (red) antibodies and counterstained with DAPI (4′, 6-diamidino-2-phenylindole) (blue). Magnified images of areas highlighted in the dashed boxes are shown to the right. Scale bar, 50 μm.

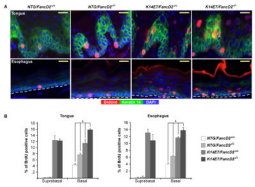

Figure 2. Examination of abilities of HPV16 E7 on DNA synthesis under the deficiency of *fancD2* gene

A, To assess the newly synthesis DNA at the suprabasal and basal epithelial layers of the tongue and esophagus tissues, mice were injected with BrdUrd before sacrifice and their tissues were stained for anti-BrdUrd (red) and anti-cytokeratin 14 (CK14) protein (green) antibodies. DAPI (blue) is used for a nuclear counter staining. Scale bar, 20 μm. ***B***, At least three mice of each genotype (n=3), *NTG/FancD2*$^{+/+}$, *NTG/FancD2*$^{-/-}$, *K14E7/FancD2*$^{+/+}$, and *K14E7/FancD2*$^{-/-}$ mice, were selected and ~8 to 10 frames of cells at the suprabasal (CK14 negative) and basal (CK14 positive) layers of tongue and esophagus epithelia were quantified for each mouse. The amount of BrdUrd positive nuclei over the number of total cells was plotted in each case (columns); bars, SD. Asterisk (*) means that in the basal epithelial layer of the tongue and esophagus tissues, both the deficiency of *fancD2* gene and the expression of HPV16 *E7* oncogene were showing significantly increased DNA synthesis between genotypes, but not in the suprabasal epithelial layer. All statistical comparisons were performed using a one-sided Wilcoxon rank sum test (* P=0.02).

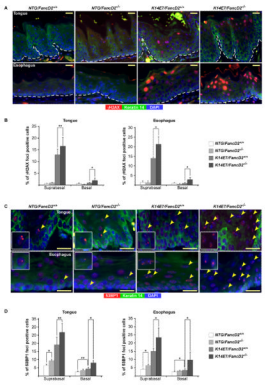

Figure 3. DNA damage response induced by HPV16 E7 via γH2AX and 53BP1 under the deficiency of *fancD2* gene

A and C, to assess induced DNA damage response by HPV16 E7 in the deficiency of *fancD2* gene, tissues from each group were stained for anti-γH2AX (red), anti-53BP1 (red), and anti-cytokeratin 14 (CK14) protein (green) antibodies. DAPI is used for a nuclear counter staining. Scale bar, 20 μm. 53BP1 foci positive cells are highlighted by yellow arrows in the images of panel ***C***. Insets in panel C provide magnified views of cells with 53BP1-positive nuclear foci. ***B and D***, At least three mice of each genotype (n=3), *NTG/FancD2*$^{+/+}$, *NTG/FancD2*$^{-/-}$, *K14E7/FancD2*$^{+/+}$, and *K14E7/FancD2*$^{-/-}$ mice, were selected and ~8 to 10 frames of cells at the basal (CK14 positive) and suprabasal (CK14 negative) layers of tongue and esophagus epithelia were quantified for each mouse. The amount of cells with γH2AX positive foci over the number of total cells was plotted in each case (columns); bars, SD. The differences in the number of cells with γH2AX foci between the groups were statistically compared (*K14E7/FancD2*$^{+/+}$ versus *K14E7/FancD2*$^{-/-}$ suprabasal P=0.06/P=0.02, basal P=0.02/P=0.02 at tongue/esophagus) (B). The differences in the number of cells with 53BP1 foci (yellow arrow) between the groups were statistically compared (*NTG/FancD2*$^{+/+}$ versus *K14E7/FancD2*$^{+/+}$ suprabasal P<0.05/P<0.05, basal P=0.06/P=0.02 at tongue/esophagus; *NTG/FancD2*$^{+/+}$ versus *NTG/FancD2*$^{-/-}$ suprabasal P=0.02/P=0.02 at tongue/esophagus; *K14E7/FancD2*$^{+/+}$ versus *K14E7/FancD2*$^{-/-}$ suprabasal P=0.06/P=0.02, basal P=0.02/P=0.02 at tongue/esophagus) (D). All statistical comparisons were performed using a one-sided Wilcoxon rank sum test (* P=0.02 and ** P=0.06). Note: Exposure times used were the same for all samples analyzed. Some cells in tissues of *K14E7/FancD2*$^{+/+}$ and *K14E7/FancD2*$^{-/-}$ mice had very bright γH2AX-specific nuclear staining at the exposure used. Shorter exposure confirmed that these cells retained punctuate nuclear staining reflective of DNA damage foci.

Table 1

Comparison of 4-NQO induced over tumor incidences in animal tissues (tongue and esophagus)

Genotype	Group size, n	Animal tissues w/overt tumor, n (%)		
		Tongue & Esophagus	Tongue	Esophagus
NTG/FancD2$^{+/+}$	24	0 (0%)	0 (0%)	0 (0%)
NTG/FancD2$^{-/-}$	26	0 (0%)	0 (0%)	0 (0%)
K14E7/FancD2$^{+/+}$	20	4 (20%)	2* (10%)	3* (15%)
K14E7/FancD2$^{-/-}$	21	11 (52%)	5 (24%)	6 (29%)

Note : The difference in tumor incidence between *K14E7/FancD2*$^{+/+}$ and *K14E7/FancD2*$^{-/-}$ groups was statistically significant (P=0.05). Also, the differences in the tumor incidence between *K14E7/FancD2*$^{+/+}$ and *NTG/FancD2*$^{+/+}$ (P=0.04), and *NTG/FancD2*$^{-/-}$ groups (P=0.03) are statistical significant. All statistical comparisons were performed using a two-sided Fisher's exact test.

*One *K14E7/FancD2*$^{+/+}$ mouse had tumors in both the tongue and esophagus.

Table 2

Histopathological analysis of sample with overt lesions

Genotype	Grade of overt lesions, n (%)			
	Papilloma	Carcinoma		
		Grade I	Grade II	Grade III
K14E7/FancD2$^{+/+}$	3 (75%)			1 (25%)
K14E7/FancD2$^{-/-}$	3 (27%)	3 (27%)	2 (18%)	3 (27%)