The Human Papillomavirus Type 16 E7 Oncoprotein Activates the Fanconi Anemia (FA) Pathway and Causes Accelerated Chromosomal Instability in FA Cells[▽]

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Fanconi anemia (FA) patients have an increased risk for squamous cell carcinomas (SCCs) at sites of predilection for infection with high-risk human papillomavirus (HPV) types, including the oral cavity and the anogenital tract. We show here that activation of the FA pathway is a frequent event in cervical SCCs. We found that FA pathway activation is triggered mainly by the HPV type 16 (HPV-16) E7 oncoprotein and is associated with an enhanced formation of large FANCD2 foci and recruitment of FANCD2 as well as FANCD1/BRCA2 to chromatin. Episomal expression of HPV-16 oncoproteins was sufficient to activate the FA pathway. Importantly, the expression of HPV-16 E7 in FA-deficient cells led to accelerated chromosomal instability. Taken together, our findings establish the FA pathway as an early host cell response to high-risk HPV infection and may help to explain the greatly enhanced susceptibility of FA patients to squamous cell carcinogenesis at anatomic sites that are frequently infected by high-risk HPVs.

Fanconi anemia (FA) is a rare autosomal recessive or X-linked cancer susceptibility syndrome characterized by congenital abnormalities, progressive bone marrow failure, and cellular sensitivity to DNA cross-linking agents (10). FA patients have a greatly increased susceptibility to squamous cell carcinomas (SCCs) of the anogenital tract and the oral cavity (24), sites with a predilection for infection with high-risk human papillomaviruses (HPVs) (33). An analysis of head and neck SCCs from FA patients showed that over 80% of the tumors contained high-risk HPV DNA, in particular that of HPV type 16 (HPV-16) (13). However, these results have been challenged by a study in which HPVs were undetectable in four cell lines derived from head and neck SCCs that developed in FA patients (31). Nonetheless, there is evidence that FA genes can become epigenetically inactivated during cervical carcinogenesis in the general population (22). The reason for the increased susceptibility of FA patients to SCCs that 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 (16).

High-risk HPV types, such as HPV-16, encode two oncoproteins, E6 and E7, which have important functions during the viral life cycle by reinitiating DNA replication in terminally growth-arrested host keratinocytes (1, 15, 20). The efficiency of

HPV-16 oncoproteins to subvert cell cycle checkpoints in order to create an S-phase-like milieu supportive of viral DNA replication is likely to be critical for their oncogenic potential. Unscheduled entry into S phase and the deregulation of cyclin expression, characteristics of HPV-16 E7-expressing cells (17, 18, 25), have previously been suggested to result in perturbations of DNA replication, increased stalling of replication forks, and chromosomal instability (4, 26).

Previous studies have shown that the FA pathway can become activated by stalled replication forks (9). Upon activation, the core complex of FA proteins mediates the monoubiquitination of another FA protein, FANCD2 (6). This modification is required for the translocation of FANCD2 to sites of altered DNA and the formation of nuclear foci (6). Following chromatin loading, monoubiquitinated FANCD2 interacts with proteins involved in replication fork stabilization and restart, including FANCD1/BRCA2 (14, 32).

In the present report, we asked whether high-risk HPV-encoded oncoproteins can cause an activation of the FA pathway and whether cells with a compromised FA pathway are prone to genomic instability, a hallmark of carcinogenic progression.

We first determined the presence of nuclear FANCD2 foci as a surrogate marker for FA pathway activation in 82 cervical carcinomas and 39 normal control tissue specimens. Two different cervical SCC tissue arrays (Cybrdi, Bethesda, MD, and Biomax USA, Rockville, MD) were analyzed by immunofluorescence microscopy. Briefly, slides were deparaffinized in xylene and dehydrated in 100% ethanol. After rehydration in a graded ethanol series, slides were washed in distilled H₂O and microwave treated in 0.01 M sodium citrate for 30 min. After

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FIG. 1. FANCD2 foci in normal cervical tissue and cervical SCCs. Immunofluorescence analysis of normal cervical tissue and a cervical SCC for FANCD2 demonstrates a small punctate nuclear staining in the control (top panels) in contrast to large nuclear FANCD2 foci in cervical cancer cells (bottom panels). The white line in the top right panel denotes the basement membrane. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI). Scale bar, 10 μ m.

being washed, the slides were digested using pepsin solution (Digest-all 3; Zymed Laboratories, San Francisco, CA) for 30 s at 37°C, washed in phosphate-buffered saline, blocked in 10% donkey serum, and incubated with a polyclonal anti-FANCD2 antibody (Genetex, San Antonio, TX) followed by a fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). In control tissue specimens, a small punctate FANCD2 staining was detected in 17 out of 39 samples (43.6%) (Fig. 1, top panels). Cells with this staining pattern were found almost exclusively in the supra-basal/basal strata of the epithelium and are hence likely to represent S-phase-associated FANCD2 foci (27). In striking contrast, cervical carcinomas frequently contained cells with significantly enlarged nuclear FANCD2 foci (Fig. 1, bottom panels). A proportion of these large cancer-associated FANCD2 foci showed a more complex morphology that included a ring-like shape. Out of a total of 82 cervical SCCs, 50 tumors (61%) contained cells with large nuclear FANCD2 foci. The number of tumor cells with large FANCD2-containing foci ranged from a few scattered cells (1 or fewer per high-power field [HPF]; 27 tumors) to a moderate number of positive tumor cells (1 to 5 per HPF; 15 tumors) or a high number of positive tumor cells (>5 per HPF; 8 tumors). The formation of large FANCD2 nuclear foci was not specific to the high-risk HPV-infected epithelium of the uterine cervix and was also detected in high-risk HPV-associated anal SCCs (data not shown).

The vast majority of high-risk HPV-associated carcinomas contain integrated viral DNA. We therefore analyzed next whether episomal expression, which is typically found in HPV-associated premalignant lesions, is sufficient to activate the FA pathway. Three independently generated organotypical raft cultures from immortalized human keratinocytes that stably maintained the full HPV-16 genome as episomes (2, 5) were analyzed for large nuclear FANCD2 foci by immunofluorescence microscopy as described above (Fig. 2A). A significant increase in keratinocytes displaying large FANCD2 foci was detected for HPV-16-containing raft cultures (1.7%) in comparison to a control raft culture, in which such foci were absent (0%; $P \leq 0.05$) (Fig. 2A and B). Inactivation of HPV-16 E7 by

a translational termination linker (TTL) in two independently generated raft cultures was found to significantly reduce the presence of cells with large FANCD2 foci to 0.08% ($P \leq 0.05$) (Fig. 2B). A decrease of FANCD2 focus formation was also detected in a raft culture containing HPV-16 episomes with a deletion of the core pRB-binding motif of HPV E7 (HPV-16 E7 Δ 21-24; 0.8%). Since the decrease was not as pronounced as in HPV-16 E7-TTL raft cultures, the latter results may suggest that pRB-dependent as well as pRB-independent functions of HPV-16 E7 (7) contribute to FA pathway activation. Although the frequencies of large FANCD2 foci in primary tumor samples and organotypical raft cultures were in a similar range, it is still possible that viral integration affects FA pathway activation. This notion, however, requires further testing using matched cell populations with integrated or nonintegrated viral genomes.

To corroborate these results and to explore the roles of the HPV-16 E7 and E6 oncoproteins in more detail, we generated stable populations of human telomerase reverse transcriptase (hTERT)-immortalized primary human keratinocytes (kindly provided by Karl Munger, The Channing Laboratory, Brigham & Women's Hospital, Boston, MA [23]) expressing high-risk HPV-16 E6 and/or E7 or low-risk HPV-6 E6 or E7 (Fig. 2C to F). The stable expression of HPV-16 E7 led to a significant, 5.6-fold increase in the proportion of cells with large FANCD2 foci, from 1.5% in controls to 8.4% ($P \leq 0.0005$) (Fig. 2C and D). Ectopic expression of HPV-16 E6 did not significantly increase the proportion of cells with large FANCD2 foci compared to that of the corresponding controls (4.6% versus 5.3%; $P > 0.05$) (Fig. 2D). Coexpression of HPV-16 E6 and E7 led to a moderate further increase of cells with large FANCD2 foci, from 12.1% in cells expressing HPV-16 E7 individually (HPV-16 E7/neo) to 14.9% in cells expressing both HPV-16 E6 and E7 (Fig. 2E). No significant changes in the proportion of cells with large FANCD2 foci were detected for human keratinocytes expressing low-risk HPV-6 E6 or E7 (3.5% and 5.5%, respectively) compared to controls (4.8%; $P > 0.05$) (Fig. 2F). The different baseline levels in the control keratinocyte populations are most likely related to the fact that cell populations used in Fig. 2E and F as well as HPV-16 E6-expressing cells and the corresponding controls (Fig. 2D) underwent two rounds of antibiotic selection, whereas HPV-16 E7-expressing cells and the corresponding controls (Fig. 2D) were selected only once. Nevertheless, HPV-16 E7 was able to stimulate a significant increase of cells with large FANCD2 foci regardless of the background levels in the respective controls.

To prove that the FA pathway is activated in HPV-16 oncoprotein-expressing cells, we performed a series of biochemical analyses. Chromatin fractions (P3) and soluble nuclear fractions (S3) from human keratinocytes expressing HPV-16 E7 and/or E6 or low-risk HPV-6 E6 or E7 were prepared and analyzed by immunoblotting (19) (Fig. 3A). As expected, chromatin fractions contained predominantly the monoubiquitinated form of FANCD2 (also referred to as the "long form") (6). An increased recruitment of the long form of FANCD2 to chromatin was detected in HPV-16 E7-expressing keratinocytes compared to controls (2.3-fold; quantifications of band intensities were performed using NIH ImageJ software, <http://rsb.info.nih.gov/ij/>) (Fig. 3A). Importantly, we found that FANCD1/BRCA2 is also recruited to chromatin in HPV-16

FIG. 2. Induction of large nuclear FANCD2 foci involves HPV-16 E7 and occurs independently of viral integration. (A) Immunofluorescence microscopic analysis of organotypical cultures of primary human keratinocytes that were either untransfected (control) or stably transfected with full-length HPV-16 episomes. Note the small punctate staining in a control cell in comparison to the large FANCD2 focus in a cell containing HPV-16. Nuclei were stained with DAPI. Scale bar, 10 μ m. (B) Quantification of the percentage of cells showing large nuclear FANCD2 foci in organotypical raft cultures that either were untransfected (control) or contained HPV-16 episomes. In addition, raft cultures obtained from keratinocyte populations transduced with E7-deficient HPV-16 episomes with a TTL inserted at nucleotide 711 of the E7 gene were analyzed. Each bar indicates the mean and standard error for at least two independent immunofluorescence experiments, with at least 100 cells counted per experiment. Statistical significance was calculated using Student's *t* test for independent samples. (C) Immunofluorescence microscopic analysis of hTERT-immortalized primary human keratinocytes either stably expressing pCMVneo-based empty vector (control) or HPV-16 E7. Note the small punctate staining in a control cell in comparison to the large nuclear FANCD2 foci in a cell expressing HPV-16 E7. Nuclei stained with DAPI. Scale bar, 10 μ m. (D to F) Quantification of the proportion of cells with large FANCD2 foci in immortalized human keratinocyte populations stably expressing high-risk HPV-16 E6 or E7 (D), coexpressing HPV-16 E6 and E7 (E), or of low-risk HPV-6 E6 or E7 (F). In all experiments, the expression of HPV genes was confirmed by PCR. Each bar represents the mean and standard error for triplicate quantifications of at least 100 cells. Asterisks indicate statistically significant differences in comparison to controls (Student's *t* test for independent samples).

E7-expressing cells (Fig. 3A). In addition, increases in the short form of FANCD2 and in FANCD1/BRCA2 protein levels were detected in S3 fractions from HPV-16 E7-expressing cells compared to controls (Fig. 3A).

No changes in the chromatin association of FANCD2 or FANCD1/BRCA2 were detected in keratinocytes expressing HPV-16 E6 (Fig. 3B). In fact, we noted a moderate decrease of FANCD1/BRCA2 in S3 and P3 fractions of HPV-16 E6-expressing cell populations (Fig. 3B).

The coexpression of HPV-16 E7 and E6 in immortalized human keratinocytes led to only a minor increase in chromatin-bound FANCD2 compared to controls (2.4-fold in comparison to the 2.2-fold increase in cells expressing HPV-16 E7 alone) (Fig. 3C). No further increase of FANCD1/BRCA2 in P3 fractions was detected in cells coexpressing HPV-16 E6 and E7 compared to cells expressing HPV-16 E7 alone (Fig. 3C). As expected from experiments shown in Fig. 3A, HPV-16 E7

caused an increase of both FANCD2 and FANCD1/BRCA2 in soluble S3 fractions.

A minor increase in the chromatin-associated long form of FANCD2 was detected in P3 fractions from keratinocytes expressing low-risk HPV-6 E7 (1.3-fold) (Fig. 3D). Remarkably, low-risk HPV-6 E7 also caused an increase in the short form of FANCD2 in S3 fractions, along with an increase of FANCD1/BRCA2 (Fig. 3D).

Taken together, these results corroborate our finding that the HPV-16 E7 oncoprotein causes an activation of the FA pathway.

Based on these results, we speculated that a compromised FA pathway would render cells prone to accelerated high-risk HPV-associated chromosomal instability. To test this hypothesis, immortalized human fibroblasts of FA complementation group D2 (GM16633; Coriell Cell Repositories, Camden, NJ) were stably transfected with high-risk HPV-16 E6 and/or E7,

FIG. 3. HPV-16 E7 stimulates recruitment of monoubiquitinated FANCD2 to chromatin. (A to D) Immunoblot analyses of subcellular fractions S3 (soluble nuclear proteins) and P3 (chromatin-enriched) (19) obtained from hTERT-immortalized primary human keratinocytes stably expressing empty vector (control) or HPV-16 E7 (A), HPV-16 E6 (B), HPV-16 E6 and E7 (C), or low-risk HPV-6 E6 or E7 (D) (same cell populations as used in Fig. 2C to F). Antibodies used for immunoblotting were directed against FANCD2 (Genetex) or FANCD1/BRCA2 (Calbiochem, San Diego, CA). Note the differences in the monoubiquitinated form of FANCD2 (long form, L) in comparison to the nonubiquitinated form (short form, S). Immunoblot for ORC2 (BD Biosciences, San Diego, CA) is shown to demonstrate chromatin enrichment of P3 fractions. Ponceau S (Pon S) staining is shown to visualize total protein loading.

low-risk HPV-6 E6 or E7, or empty vector (Fig. 4). hTERT-immortalized primary human keratinocytes manipulated to stably express HPV-16 E7 were used as FA-proficient control cells. As expected from previous studies (3, 11), the chromosomal aberrations detected in metaphase spreads obtained from HPV-16 E7-expressing keratinocyte populations increased from 1.6% in controls to 6.7% in HPV-16 E7-expressing cells, albeit without reaching statistical significance (Fig. 4B). In FANCD2-deficient fibroblasts, HPV-16 E7 caused a statistically significant 2.9-fold increase of metaphases with chromosomal aberrations, from 3.3% in controls to 9.7% ($P \leq 0.001$) (Fig. 4A and C). These abnormalities included chromatid breaks and chromosome fusions (Fig. 4A). Coexpression of HPV-16 E6 and E7 in FANCD2-deficient cells led to only a minor further increase in metaphases, with chromosomal aberrations increasing from 9.8% in double-transfected HPV-16 E7/neo cells to 12.3% (1.3-fold). Expression of HPV-16 E6 alone or low-risk HPV-6 E6 or E7 did not cause a statistically significant increase of chromosomal alterations in comparison to empty vector controls (Fig. 4C). The finding that the ex-

pression of HPV-16 E7 in FANCD2-deficient cells did not lead to a more pronounced increase in chromosomal aberrations compared to the level seen in keratinocytes was somewhat unexpected. To determine what the cause of these results may be, we asked whether FANCD2-deficient cells expressing HPV-16 E7 undergo enhanced apoptosis. We detected increased poly(ADP-ribose) polymerase and caspase-3 cleavage in FANCD2-deficient cells expressing HPV-16 E7 but not in FA cells expressing empty vector or in keratinocyte populations (data not shown). These results suggest that HPV-16 E7 stimulates enhanced apoptosis in FANCD2-deficient cells and that the frequency of metaphase chromosome aberrations in HPV-16 E7-expressing FA cells is hence likely to be an underrepresentation. The fact the coexpression of HPV-16 E7 and E6 in FA cells does not significantly accelerate chromosomal instability could be related to p53-independent proapoptotic mechanisms that have previously been reported for FA cells (12) or to cell type-specific effects, since immortalized fibroblasts were used.

Collectively, our results show that the HPV-16 E7 oncopro-

FIG. 4. Expression of HPV-16 E7 in FANCD2 fibroblasts accelerates chromosomal instability. (A) Metaphase spreads from stably transfected empty vector (control) or HPV-16 E7-expressing immortalized FANCD2 fibroblasts. Chromosomes were stained with DAPI. Inserts depict chromatid breaks (top right and bottom right) or chromosome fusion (bottom left). (B and C) Quantification of the percentage of metaphase spreads with chromosome aberrations from stably transfected empty vector (control) or HPV-16 E7-expressing immortalized human keratinocytes (B) or immortalized FANCD2-deficient fibroblasts stably expressing low-risk HPV-6 E6 or E7 or high-risk HPV-16 E6 and/or E7 (C). Populations expressing HPV-16 E6 and E7 and the corresponding control (HPV-16 E7/neo) underwent two rounds of transfection and antibiotic selection. Each bar represents the mean and standard error for at least triplicate quantifications of a minimum of 25 metaphases. Asterisks indicate statistically significant differences in comparison to controls (Student's *t* test for independent samples).

tein can activate the FA pathway and that HPV-16 E7 causes accelerated chromosomal breakage in the absence of a functional FA pathway. Since the FA pathway responds to replication stress, it is possible that HPV-16 E7-induced FA pathway activation is caused by replication fork perturbations. Deregulation of cyclin expression and unscheduled S phase entry, important activities of HPV-16 E7 (17), have previously been shown to stimulate replication stress (17, 18, 25). Nonetheless, the precise molecular mechanism of FA pathway activation by HPV-16 E7 and whether HPV-16 E7 can interfere directly with FA protein expression and/or function remains to be determined. In addition, the mechanisms leading to increased chromosomal breakage in FANCD2-deficient cells warrant further investigation. Stalled forks are substrates for homologous recombination, and DNA double-strand breaks that occur as repair intermediates (28) may remain unrepaired

in FA-deficient cells (21), thereby leading to increased chromosomal instability.

The FA pathway has been implicated in the regulation of common fragile site stability (9). These chromosomal regions have been suggested to represent preferred regions for HPV genome integration (29). Whether the FA pathway plays a role in viral genome integration or in other aspects of high-risk HPV-associated cellular immortalization and transformation remains to be determined.

Our findings are in agreement with a previous study reporting an enhanced susceptibility of FA fibroblasts to transformation by viral oncogenes (30). It needs to be emphasized, however, that the precise role of HPV infection in squamous cell carcinogenesis in FA patients is currently unclear, in particular since some reports failed to detect high-risk HPVs (31). An alternative explanation would be that an impaired FA pathway

promotes chromosomal instability at these anatomic sites in the absence of an HPV infection but in a manner that resembles the insults imposed by high-risk HPV infection in the general population. Although our studies suggest that the HPV-16 E7 oncoprotein may be critical for accelerating chromosomal instability in FA-deficient cells, it is very likely that the E6 oncoprotein contributes to tumor formation in FA patients by inactivating the p53 tumor suppressor. The finding that *Fancd2* knock-out mice deficient in p53 show increased tumor formation and chromosomal instability lends support to this notion (8).

Taken together, our results establish the FA pathway as an early host cell response to high-risk HPV infection and may help to explain the increased risk of FA patients for SCCs at sites of predilection for HPV infection.

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