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Original Article

Expression of HPV-induced DNA Damage Repair Factors Correlates With CIN Progression

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Summary: Human papillomaviruses (HPVs) are DNA viruses with epithelial tropism. High-risk types of HPV are the causative agents of the majority of cervical cancers and are responsible for a number of other anogenital as well as oropharyngeal cancers. The life cycle of HPV is closely linked to the differentiation state of its host cell and is dependent on the activation of specific pathways of the DNA damage response. Several proteins from the ataxia telangiectasia mutated and the ataxia telangiectasia mutated and Rad3-related DNA repair pathways, which are essential for maintaining genomic stability in cells, are upregulated in HPV-positive cells and are required for viral replication. Our studies examine the expression of 5 such DNA repair factors—pCHK2, pCHK1, FANCD2, BRCA1, and H2AX—in cervical specimens from patients diagnosed with low-grade, intermediate-grade, or high-grade lesions. The percentage of cells expressing pCHK2, pCHK1, FANCD2, and BRCA1 is significantly higher in high-grade squamous intraepithelial lesions compared with that of either low-grade squamous intraepithelial lesions or normal tissue, particularly in differentiated cell layers. In addition, the distribution of this staining throughout the epithelium is altered with increasing lesion grade. This study characterizes the expression of pCHK2, pCHK1, FANCD2, H2AX and BRCA1 during cervical cancer progression and provides additional insight into the role of these DNA damage response proteins in viral transformation. **Key Words:** Papillomavirus—Epithelial—Differentiation—Cervical cancer.

Human papillomaviruses (HPVs), members of the Papillomaviridae family of viruses, are small, nonenveloped viruses with double-stranded DNA genomes. HPVs infect undifferentiated cells in the basal layers of stratified epithelia where they establish their 8 kb genomes as extrachromosomal elements, or episomes. Infections by high-risk types of HPV produce asymptomatic lesions, or dysplasias, that are typically cleared by the host's immune system within 2 yr (1). Failure to clear this initial infection leads to a persistent infection, which can last for up to several decades and this, along with the integration of viral DNA into host chromosomes, greatly increases the risk of cancer (2). High-risk HPVs have been linked to the development of many oropharyngeal and anogenital cancers including cervical cancer, for which it is the etiological agent in 99.9% of cases (3). Although HPV infection is often necessary for the malignant progression to cervical cancer, it is not sufficient, as transformation requires the accumulation of additional cellular mutations.

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HPV-related squamous intraepithelial lesions have traditionally been classified using the 3-tiered Richart classification system for cervical intraepithelial neoplasia (CIN), where CIN1 refers to koilocytic change/mild dysplasia, whereas CIN2 and CIN3 define moderate and severe dysplasia, respectively (4). More recent guidelines recommend a 2-tiered system of low-grade squamous intraepithelial lesion (LSIL), comprised of CIN1, and high-grade squamous intraepithelial lesion (HSIL), comprised of both CIN2 and CIN3. This system has been demonstrated to have better diagnostic reproducibility and also correlates better with the dichotomous interaction of HPV with squamous epithelial cells (productive transient infection vs. persistent infection with progression to precancer) (5). Using either system, lesions are graded according to morphologic changes observed in the differentiation pattern of the affected epithelium. Grading is assigned based on the thickness of the epithelium affected by neoplastic cells, with expansion of the basal cell layer in the lower third of the epithelium observed in CIN1 and expansion into the upper third of the epithelium in CIN3 (6). CIN3 may also refer to a carcinoma *in situ*, which is a precursor to invasive cancer (7). Each of these lesions may regress to normal over time, though the likelihood of this occurring decreases with increasing grade (8). Despite the effectiveness of cervical cancer prevention by screening and vaccination, it is estimated that there will be 12,820 new cases of cervical cancer and 4210 cervical cancer deaths in the United States in 2017 (9). Worldwide, it was estimated that there were 527,600 new cases of cervical cancer and 265,700 cervical cancer deaths in 2012 (10). Identifying reliable biomarkers or predictors of disease progression may lead to more accurate classification of cervical neoplasia, which in turn will allow for appropriate treatment of the lesions most likely to progress without overtreatment of those most likely to regress (6,11).

The host DNA damage response is a network of cellular pathways that prevents the propagation of damaged DNA to maintain genomic integrity (12). Mutations in components of these pathways or their loss of function is a step in carcinogenesis (13). Central to this response are the ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases and their downstream signaling pathways. The ATM pathway is activated in response to DNA double-strand breaks and initiates its response by phosphorylating several downstream effector proteins, including CHK2 and BRCA1 (14,15). ATR responds to replication stress and the presence of single-stranded DNA at stalled replication forks. It also signals by phosphorylating specific effector proteins, such as CHK1 and proteins of the Fanconi anemia (FA) pathway (16,17).

DNA viruses often modulate components of these pathways to promote completion of their viral life cycles. Studies from our laboratory, and others, have shown that DNA damage response proteins are activated in HPV-positive cells compared with normal cells and are required for completion of the viral life cycle. HPV activates both the ATM and ATR pathways, indicated by the presence of pCHK2 and pCHK1, respectively, and this is required for viral replication in undifferentiated and differentiated cells (18,19). Similarly, HPV replication requires activation of FANCD2, a key regulatory protein in the FA pathway, and BRCA1 and γH2AX, which coordinate in the repair of damage signaled for by these pathways (20–22). As such, the involvement of the DNA damage response in the HPV viral life cycle has become increasingly clear; however, its role in viral transformation has not yet been fully explored. High-risk HPVs increase the expression of these, and several other, DNA damage response proteins during infection, suggesting that these proteins may serve as promising biomarkers for CIN diagnosis and progression (18). This study examines the role of the DNA damage response in HPV-related cervical carcinogenesis by investigating the correlation between the levels of DNA repair proteins and cervical intraepithelial lesion grade.

MATERIALS AND METHODS

Samples

Deidentified samples from cervical excision specimens with histologically confirmed cervical intraepithelial lesions that were received in the Northwestern Memorial Hospital Department of Pathology between June 1, 2014 and June 1, 2015. Hematoxylin and eosin–stained slides were reviewed for confirmation of the diagnosis by a gynecologic pathologist (K.P.M.). A second gynecologic pathologist (L.Z.B.) subsequently reviewed the slides, blinded to the original diagnoses, and any discrepant diagnoses were reviewed by both pathologists concurrently at a 2-headed scope to achieve a consensus interpretation.

p16 Staining

Immunohistochemistry for p16 (clone G175-405; Biocare, Concord, CA) was performed by the Pathology Core Facility at the Northwestern University

Lurie Cancer Center on all 10 cases of morphologic CIN2. Positive staining was defined as diffuse, band-like nuclear, and cytoplasmic reactivity in at least the lower third of the epithelium, with all other staining patterns considered negative. Immunohistochemistry for p16 was performed on all cases of morphologic CIN2.

Immunohistochemistry Detection by Immunofluorescence

Slides were baked overnight at 55°C. The following day, slides were deparaffinized using a series of washes —xylene, 100% ethanol, 95% ethanol, 70% ethanol, and double-distilled water. Slides were then incubated in a glass chamber containing antigen retrieval buffer (10 mM citrate buffer pH 6.0–2.42 g sodium citrate dihydrate, 0.378 g citrate acid in 1 L double-distilled water) for 30 min in a 99°C water bath. The chamber was removed from the bath and allowed to cool to room temperature. Slides were rinsed in double-distilled water for 1 min before incubation in wash buffer (0.05 M tris hydrochloric acid, 0.15 M sodium chloride, 0.05% Tween 20). The slides were blocked with Normal Goat Serum with 0.1% Triton X-100 for 1 hr at 37°C and incubated with primary antibody in Normal Goat Serum with 0.1% Triton X-100 overnight at 4°C. The next day, slides were washed in wash buffer before incubation with Alexa Fluor secondary antibody for 1 hr at room temperature. Following washes, the slides were incubated in 4',6-diamidino-2-phenylindole solution for 5 min and mounted in gelvatol.

Antibodies

pCHK1 (S317) (catalog no.12302; Cell Signaling, Danver, MA), pCHK2 (T68) (catalog no. 2661; Cell Signaling), FANCD2 (catalog no. ab108928; Abcam, Cambridge, MA), BRCA1 (catalog no. OP92; Oncogene, St Louis, MI), γH2AX (S139) (catalog no. 05-636; Millipore, St Louis, MI).

Image Analysis

Images were taken on a Zeiss Axioscope and imported into ImageJ for analysis. For each antibody, the percentage of positive-staining cells was evaluated by comparing the number of cells with nuclear, focal staining to the total number of cells as determined by

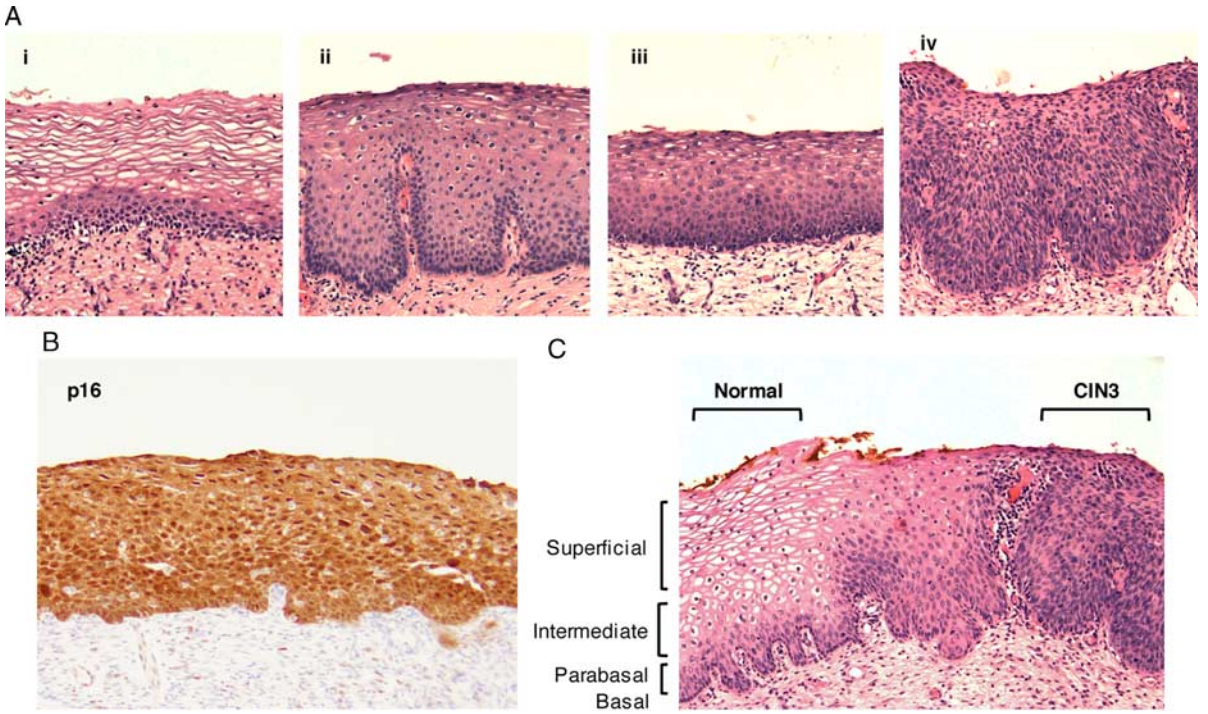

FIG. 1. (A) Hematoxylin and eosin (H&E) staining of normal (i), cervical intraepithelial neoplasia 1 (CIN1) (ii), CIN2 (iii), and CIN3 (iv) samples. (B) p16 immunohistochemical staining showing diffuse block staining was used to confirm CIN2 samples as high-grade squamous intraepithelial lesions. (C) H&E staining of a CIN3 lesion with adjacent normal tissue. Samples were divided into basal, parabasal, intermediate, and superficial layers for analysis.

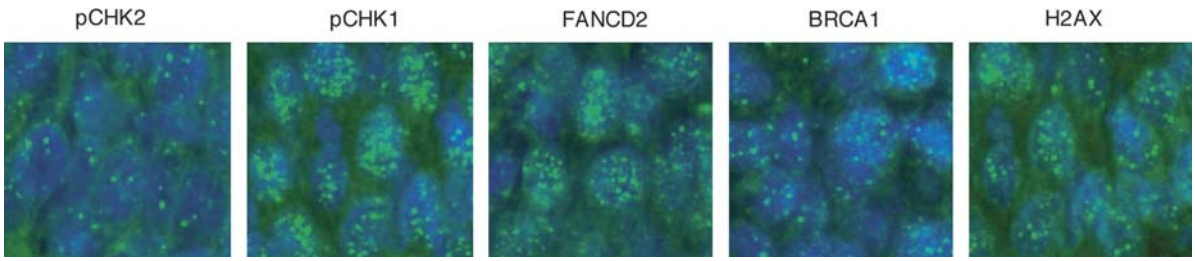

FIG. 2. Immunofluorescence staining of pCHK2, pCHK1, FANCD2, BRCA1, and γH2AX in cervical intraepithelial neoplasia 3 lesions (green). Cells were counterstained with 4',6-diamidino-2-phenylindole (blue).

4',6-diamidino-2-phenylindole staining. Percentages were then correlated with epithelial cell layer (basal, parabasal, intermediate, superficial).

RESULTS

As the role of DNA damage response pathways in cervical cancer progression has not been well studied, we conducted a retrospective review of cervical specimens in which we aimed to determine initially whether DNA repair protein levels are altered with CIN progression. To investigate the correlation between DNA damage response protein expression and cervical intraepithelial lesion grade, we obtained a total of 30 patient samples—10 of which were classified as CIN1, 10 as CIN2, and 10 as CIN3 based on consensus diagnosis (Fig. 1A). All CIN2-diagnosed lesions were confirmed as HSIL by p16 immunohistochemical staining (Fig. 1B) (23). Patient samples with normal tissue adjacent to the intraepithelial lesion were obtained when possible for use as a negative control (Fig. 1C). For the purpose of this study, epithelial cell layers were divided into 4 categories for assessment: basal, parabasal, intermediate, and superficial (Fig. 1C).

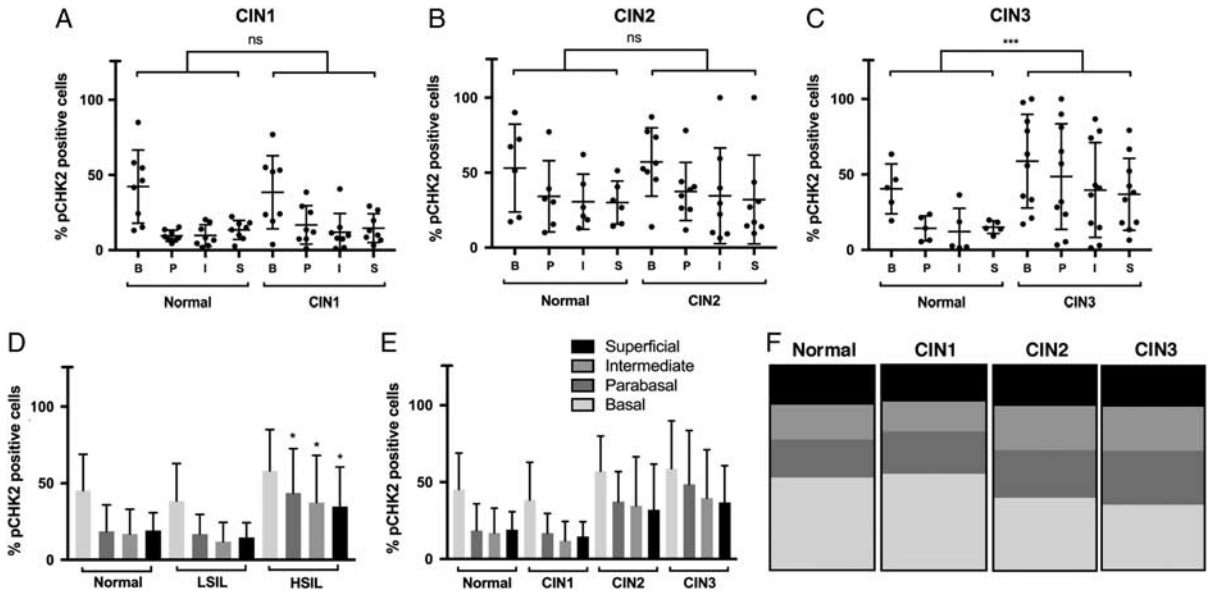

FIG. 3. (A–C) Graph shows the percentage of pCHK2-positive cells in cervical intraepithelial neoplasia 1 (CIN1), CIN2, or CIN3 lesions compared with normal tissue from the same patient. Each point represents the percentage of pCHK2 expressing cells from an individual patient. Percentages were calculated for each epithelial cell layer where B, basal; P, parabasal; I, intermediate; and S, superficial. Error bars represent the SD between samples. An analysis of variance test was used to determine statistical significance between normal and CIN tissue. $***P \leq 0.001$; ns, not significant. (D) Graph represents a 2-tier analysis of the percentage of pCHK2-positive cells in normal, low-grade squamous intraepithelial lesions (LSILs), and high-grade squamous intraepithelial lesions (HSILs) by epithelial cell layer. (E) Graph demonstrates a 3-tier analysis of the percentage of pCHK2-positive cells in CIN1, CIN2, and CIN3 lesions by epithelial cell layer. Error bars represent the SD between samples. A standard Student *t* test was used to determine statistical significance. $*P \leq 0.05$ between HSIL and LSIL samples. (F) The percentage of cells with pCHK2 staining in basal, parabasal, intermediate, and superficial cell layers represented as a parts of whole table to show the relative distribution of pCHK2 staining in normal and CIN tissue.

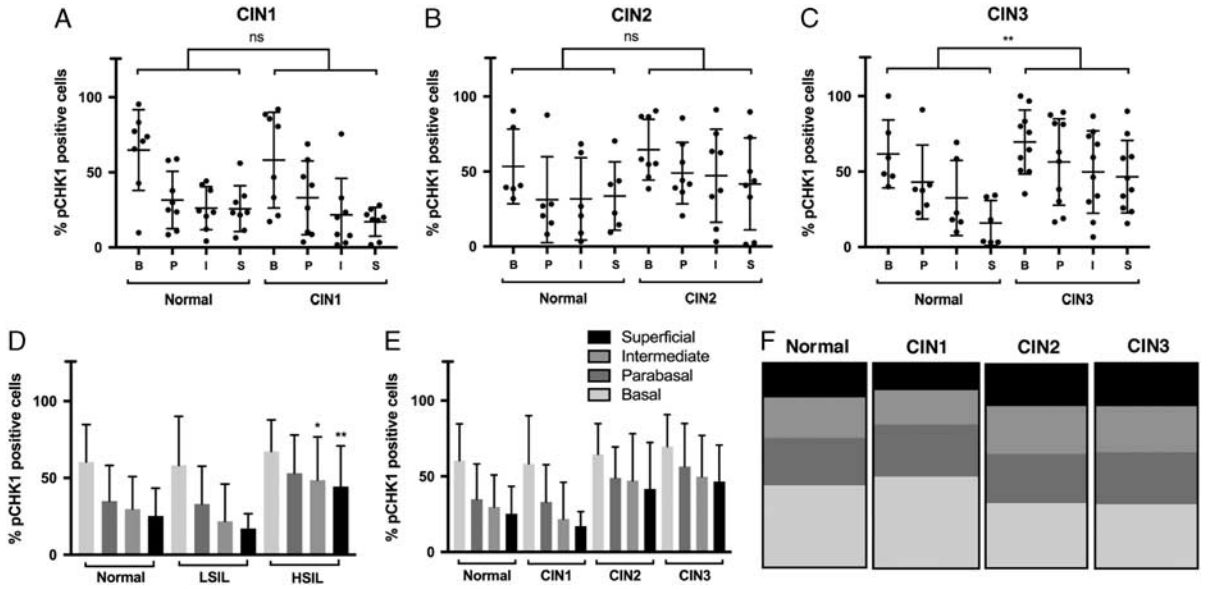

FIG. 4. (A–C) Graph shows the percentage of pCHK1-positive cells in cervical intraepithelial neoplasia 1 (CIN1), CIN2, or CIN3 lesions compared with normal tissue from the same patient. Each point represents the percentage of pCHK1 expressing cells from an individual patient. Percentages were calculated for each epithelial cell layer where B, basal; P, parabasal; I, intermediate; and S, superficial. Error bars represent the SD between samples. An analysis of variance test was used to determine statistical significance between normal and CIN tissue. $^{**}P \leq 0.01$; ns, not significant. (D) Graph represents a 2-tier analysis of the percentage of pCHK1-positive cells in normal, low-grade squamous intraepithelial lesions (LSILs), and high-grade squamous intraepithelial lesions (HSILs) by epithelial cell layer. (E) Graph demonstrates a 3-tier analysis of the percentage of pCHK1-positive cells in CIN1, CIN2, and CIN3 lesions by epithelial cell layer. Error bars represent the SD between samples. A standard Student *t* test was used to determine statistical significance. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ between HSIL and LSIL samples. (F) The percentage of cells with pCHK1 staining in basal, parabasal, intermediate, and superficial cell layers represented as a parts of whole table to show the relative distribution of pCHK1 staining in normal and CIN tissue.

DNA Damage Response Protein Expression is Altered During CIN Progression

We began by evaluating the expression of pCHK2 in cervical neoplasia (18). Discrete focal staining was observed in the nuclei of cells stained for pCHK2, as well as for pCHK1, FANCD2, BRCA1, and γH2AX, which is indicative of DNA repair pathway activation and similar to what is seen in *in vitro* studies of these proteins during HPV infection (Fig. 2) (20,22,24). The percentage of cells expressing pCHK2 was similar between CIN1 lesions and adjacent normal tissue (Fig. 3A), as well as between normal tissue and CIN2 lesions from the same patient (Fig. 3B). When comparing CIN3 lesions to normal tissue, we observed a significant increase in the percentage of pCHK2-positive cells (Fig. 3C). Interestingly, our analysis found that there was a significant increase in the percentage of cells expressing pCHK2 between HSIL and LSIL samples, particularly in differentiated cell layers (Fig. 3D). This correlates with reports that HPV activates an ATM-dependent DNA damage response, that is, maintained at high levels through differentiation in HPV-positive cells compared with normal, uninfected cells (18). A comparison of pCHK2 expression using the 3-tier system, however, revealed no significant change in expression between CIN2 and CIN3 lesions suggesting that pCHK2 expression may distinguish between HSIL and LSIL, but not further between CIN2 and CIN3 (Fig. 3E).

Of particular interest was the shift in the distribution of pCHK2 expression that was observed across epithelial cell layers between different lesion grades. In CIN1, pCHK2 was mainly expressed in the basal layer of cells, similar to as seen in normal tissue; however, in CIN2 and CIN3 lesions, pCHK2 is expressed at near equal proportion in all cell layers, becoming most evenly distributed in CIN3 tissue (Fig. 3F).

Next, we evaluated the relationship between pCHK1 expression and CIN progression. As seen with pCHK2, the percentage of pCHK1 positive–staining cells was similar between normal tissue and CIN1 lesions from the same patient. A slight, but not significant, increase was seen in the percentage of pCHK1-positive cells in CIN2 lesions and this increase became significant in CIN3 tissue (Figs. 4A–C). Again, a significant increase was seen in pCHK1 expression between HSIL and LSIL samples, predominately in upper differentiated cell layers (Fig. 4D). When pCHK1 expression was

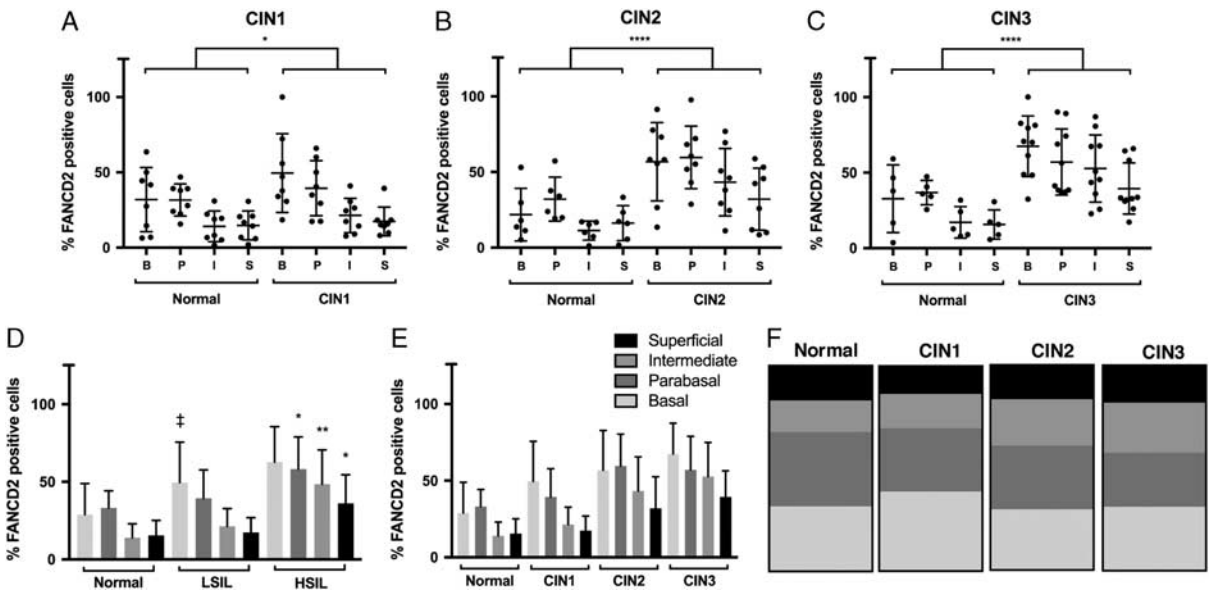

FIG. 5. (A–C) Graph shows the percentage of FANCD2-positive cells in cervical intraepithelial neoplasia 1 (CIN1), CIN2, or CIN3 lesions compared with normal tissue from the same patient. Each point represents the percentage of FANCD2-expressing cells from an individual patient. Percentages were calculated for each epithelial cell layer where B, basal, P, parabasal; I, intermediate; and S, superficial. Error bars represent the SD between samples. An analysis of variance test was used to determine statistical significance between normal and CIN tissue. $*P \leq 0.05$; $****P \leq 0.0001$; ns, not significant. (D) Graph represents a 2-tier analysis of the percentage of FANCD2-positive cells in normal, low-grade squamous intraepithelial lesions (LSILs), and high-grade squamous intraepithelial lesions (HSILs) by epithelial cell layer. (E) Graph demonstrates a 3-tier analysis of the percentage of FANCD2-positive cells in CIN1, CIN2, and CIN3 lesions by epithelial cell layer. Error bars represent the SD between samples. A standard Student *t* test was used to determine statistical significance. $*P \leq 0.05$; $**P \leq 0.05$ between HSIL and LSIL samples; $\ddagger P \leq 0.05$ between LSIL and normal samples. (F) The percentage of cells with FANCD2 staining in basal, parabasal, intermediate, and superficial cell layers represented as a parts of whole table to show the relative distribution of FANCD2 staining in normal and CIN tissue.

compared using the 3-tiered system, we observed an increase in the percentage of pCHK1-positive cells in intermediate and superficial layers with increasing lesion grade, but the levels between CIN2 and CIN3 layers were comparable (Fig. 4E). This correlated with the distribution of pCHK1 between epithelial cell layers where expression shifted from mostly basal to a more even distribution in HSIL, with CIN2 and CIN3 showing a very similar pattern (Fig. 4F).

Immunofluorescence staining of FANCD2 revealed a significant increase in FANCD2-expressing cells in CIN epithelia compared with normal tissue and the percentage of cells expressing FANCD2 increased with lesion grade (Figs. 5A–C). In addition, a 2-tier analysis of FANCD2 expression found that there was a significant increase in the percentage of differentiated cells expressing FANCD2 in HSIL compared with LSIL (Fig. 5D). Interestingly, there was also a significant increase in FANCD2 expression in basal cells from LSIL tissue when compared with normal tissue (Fig. 5D). This is in line with previous findings that FANCD2 is an important regulator of HPV replication in undifferentiated cells during viral infection and suggests that basal cells from LSIL lesions may be actively replicating viral DNA (20). As with pCHK2 and pCHK1, a 3-tiered analysis of FANCD2 expression showed an increase in the percentage of FANCD2-positive cells in nearly all epithelial cell layers, though no significant change was detected between CIN2 and CIN3 (Fig. 5E). Analysis of FANCD2 distribution between lesions found that in normal epithelia, FANCD2 was mainly expressed in the parabasal layer of cells, but this shifted to predominately basal layer expression in CIN1 lesions. As lesions progressed to CIN2 and CIN3, FANCD2 expression moved toward a more even distribution and was almost equally expressed between cell layers in CIN3 lesions (Fig. 5F).

Immunofluorescence analysis of BRCA1 expression revealed that a similar percentage of cells expressed BRCA1 in CIN1 lesions, compared with normal tissue (Fig. 6A). There was an increased percentage of BRCA1-expressing cells in both CIN2 and CIN3 lesions; however, this increase was significant only between CIN3 cells and normal cells from the same

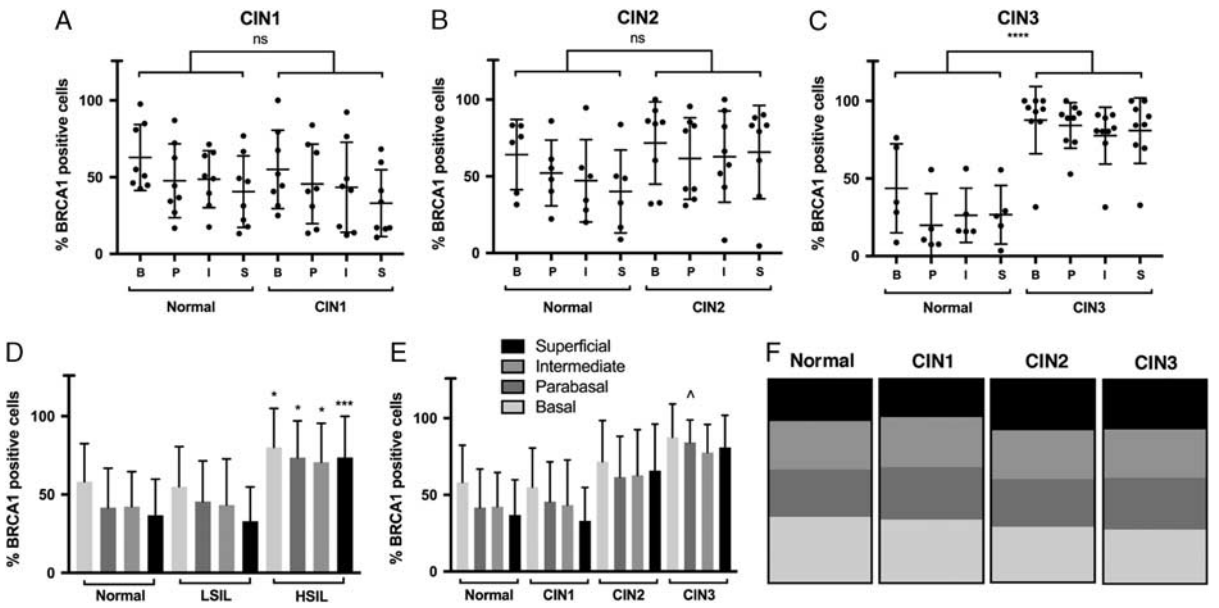

FIG. 6. (A–C) Graph shows the percentage of BRCA1-positive cells in cervical intraepithelial neoplasia 1 (CIN1), CIN2, or CIN3 lesions compared with normal tissue from the same patient. Each point represents the percentage of BRCA1-expressing cells from an individual patient. Percentages were calculated for each epithelial cell layer where B, basal; *P*, parabasal; I, intermediate; and S, superficial. Error bars represent the SD between samples. An analysis of variance test was used to determine statistical significance between normal and CIN tissue. $****P \leq 0.0001$; ns, not significant. (D) Graph represents a 2-tier analysis of the percentage of BRCA1-positive cells in normal, low-grade squamous intraepithelial lesions (LSILs), and high-grade squamous intraepithelial lesions (HSILs) by epithelial cell layer. (E) Graph demonstrates a 3-tier analysis of the percentage of BRCA1-positive cells in CIN1, CIN2, and CIN3 lesions by epithelial cell layer. Error bars represent the SD between samples. A standard Student *t* test was used to determine statistical significance. $*P \leq 0.05$; $***P \leq 0.001$ between HSIL and LSIL; $\wedge P \leq 0.05$ between CIN2 and CIN3. (F) The percentage of cells with BRCA1 staining in basal, parabasal, intermediate, and superficial cell layers represented as a part of whole table to show the relative distribution of BRCA1 staining in normal and CIN tissue.

patient (Figs. 6B, C). These increases were also reflected in our 2-tier analysis, which found that the percentage of cells expressing BRCA1 was significantly higher in HSIL cells than in LSIL in all epithelial cell layers (Fig. 6D). In addition, there was a significant increase in BRCA1-positive cells in the parabasal cell layer of CIN3 tissue compared with CIN2 (Fig. 6E). Distribution analysis revealed that BRCA1 was expressed evenly throughout all epithelial cell layers in both normal and CIN tissue; although normal and CIN1 may have a slightly higher proportion of BRCA1-positve cells in the basal layer of cells (Fig. 6F).

Lastly, to determine whether the expression of γH2AX correlates with CIN progression, immunofluorescence was performed against γH2AX on tissue from CIN lesions (25). Despite variability in the percentage of cells expressing γH2AX in both normal and CIN tissue (Figs. 7A–C), an upward trend could be observed between CIN progression and the percentage of γH2AX-positive cells in differentiated cell layers (Fig. 7E). This was significant between CIN1 and CIN3 in superficial cell layers, but not CIN2 and CIN3. Further, we found no significant change between the percentage of cells expressing γH2AX in HSIL and LSIL tissue (Fig. 7D). We did find, however, that the distribution of γH2AX expression was altered with CIN progression. In CIN1, γH2AX was expressed more so in the basal layer of cells, most similar to in normal tissue, but as seen with other DNA damage response proteins, it was distributed more evenly through the epithelium in CIN2 and CIN3 (Fig. 7F).

DISCUSSION

High-risk HPVs are the causative agents of 99.9% of cervical cancers, which is the fourth most common cause of cancer-related death in women worldwide (26). With no cure for HPV infection and significant mortality from advanced stage cervical cancers, the early and accurate diagnosis of CIN lesion progression may improve both treatment and survival. DNA damage response pathways are responsible for maintaining genomic integrity, and mutations in genes of these pathways lead to congenital defects and cancer

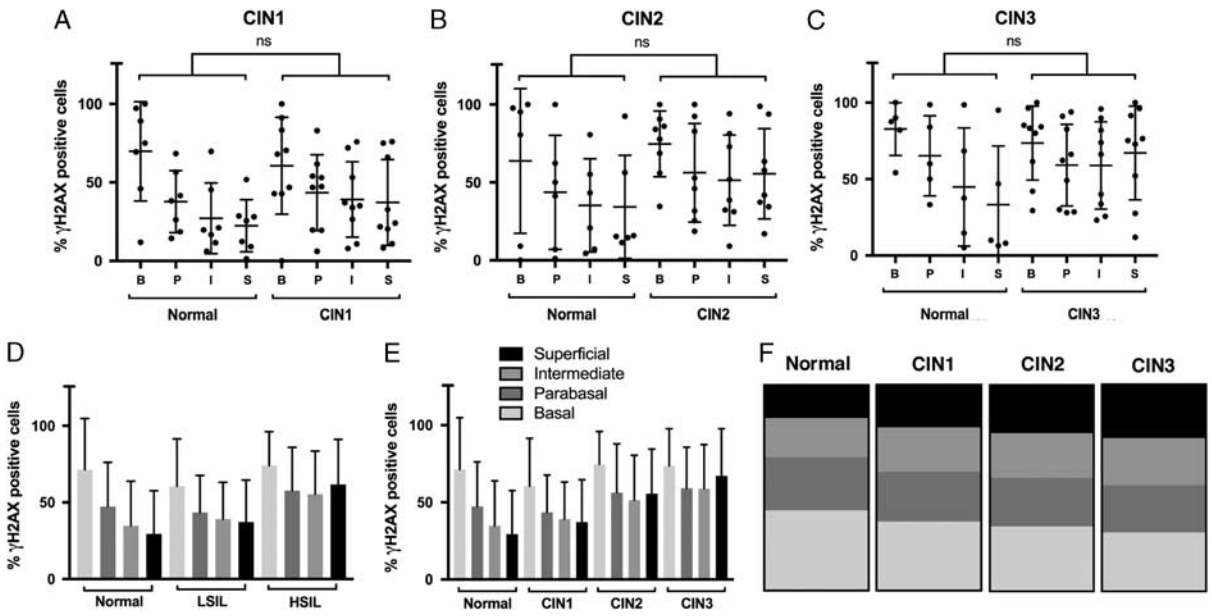

FIG. 7. (A–C) Graph shows the percentage of γH2AX-positive cells in cervical intraepithelial neoplasia 1 (CIN1), CIN2, or CIN3 lesions compared with normal tissue from the same patient. Each point represents the percentage of γH2AX expressing cells from an individual patient. Percentages were calculated for each epithelial cell layer where B, basal; *P*, parabasal; I, intermediate; and S, superficial. Error bars represent the SD between samples. An analysis of variance test was used to determine statistical significance between normal and CIN tissue. ns, not significant. (D) Graph represents a 2-tier analysis of the percentage of γH2AX-positive cells in normal, low-grade squamous intraepithelial lesions, and high-grade squamous intraepithelial lesions (HSILs) by epithelial cell layer. (E) Graph demonstrates a 3-tier analysis of the percentage of γH2AX-positive cells in CIN1, CIN2, and CIN3 lesions by epithelial cell layer. Error bars represent the SD between samples. A standard Student *t* test was used to determine statistical significance. (F) The percentage of cells with γH2AX staining in basal, parabasal, intermediate, and superficial cell layers represented as a parts of whole table to show the relative distribution of γH2AX staining in normal and CIN tissue.

predisposition (14). This host response is intricately involved in the HPV life cycle, as HPV activates the ATM and ATR pathways as well as the FA pathway for viral replication (18–20). Given this association and its role in carcinogenesis, we investigated whether these DNA repair proteins might correlate to the progression from viral infection to cervical cancer.

Our studies examined the expression of 5 DNA repair proteins in cervical intraepithelial lesions, that were previously shown to be involved in the HPV viral life cycle, pCHK2, pCHK1, FANCD2, BRCA1, and γH2AX (18–22). For this study, immunofluorescence was used to evaluate protein expression as opposed to traditional chromogenic immunohistochemistry because of its ability to generate higher-resolution images that allow for the detection of nuclear foci, which are characteristic of DNA repair pathway activation. Although staining intensity is often used as a measure of protein levels, we found that the signal intensity was often too inconsistent between samples to serve as a reliable measure in this project. This may be due to either differences in overall protein levels between patients or variability in our staining procedure. We therefore evaluated the percentage of cells with nuclear, focal staining along with the distribution of this staining through the epithelium. We would expect, however, that a more technically involved analysis of protein levels would reveal increased levels of these proteins in high-grade lesions, given the results of a number of *in vitro* studies (18–20).

We identified 4 proteins, pCHK1, pCHK2, FANCD2, and BRCA1, as having altered expression during CIN progression. Each of these proteins was expressed in a higher percentage of cells in HSIL lesions compared with LSIL suggesting that activation of these proteins may correlate with lesion grade. Moreover, pCHK1, pCHK2, FANCD2, and γH2AX showed different distribution patterns in higher grade lesions compared with normal and low-grade tissue further supporting a relationship between DNA damage repair protein expression and lesion grade. These results suggest that activation of the DNA damage response may not only be important for viral replication, but for viral transformation as well.

Interestingly, for all 5 proteins, we found that CIN3 lesions showed a more even distribution in expression

between basal, parabasal, intermediate, and superficial cell layers than seen in low-grade and intermediate-grade lesions. CIN3 is characterized as an expansion of basal-like cells through the majority of the epithelial thickness. As the basal cell layers of normal tissue and low-grade lesions often had the highest percentage of positive staining cells, an expansion of this population into suprabasal layers would likely display a similar expression pattern.

Our intention was to assess whether any of these 5 DNA repair proteins could serve as potential biomarkers for disease progression. Although we did identify significant changes in protein expression patterns, there was no clean cut discrimination in the expression of these proteins between lesion grade. Given the overlap observed in the percentage of cells expressing each of these proteins of interest, further studies would be needed to draw any conclusions about their potential as biomarkers for CIN progression.

While our data does not show a significant change between the percentage of cells expressing γH2AX in low-grade and high-grade lesions despite the presence of novel staining and distribution patterns, a recent study suggested γH2AX as a potential biomarker for differentiating between these types of lesions (27). Using chromogenic immunohistochemical staining, this study observed a gradual increase in basal and surface γH2AX expression in high-grade cervical intraepithelial lesions when compared with normal and low-grade tissues. Although this study only divided the epithelium into lower (basal) and higher (surface) segments, it is possible that with the analysis of a larger number of samples, we would see similar results with γH2AX, as well as with the other DNA damage response proteins tested.

Before this investigation, few studies had examined the correlation between DNA damage response protein expression and CIN progression; although there is some evidence that these proteins may be linked to HPV persistence and cancer progression. First, gene profiling identified the differential expression of DNA damage response genes in low-risk and high-risk HPV infections of the vulva (28). In a separate study, genetic variants in DNA repair genes, including the FA core component FANCA, had been linked to an increased risk for viral persistence and progression to cervical cancer (29). Overall, our studies provide additional insight into the relationship between DNA repair protein expression and cervical intraepithelial lesion grade and we have identified 4 proteins, pCHK1, pCHK2, FANCD2, and BRCA1, as having altered expression during CIN progression that, with further analysis, may ultimately prove useful in the diagnosis and treatment of cervical cancer.

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