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Evaluation of 3,3'-Diindolylmethane with Gardasil Quadrivalent HPV Vaccine in K14-HPV16 -Transgenic Mice Cervical Histology

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Abstract. *Background: The effects of 3,3'-diindolylmethane (DIM) together with the Gardasil vaccine on cervical histology were evaluated using the K14-HPV16-transgenic mouse model. The possibility that DIM could enhance the efficacy of this preventive vaccine in this model was explored. Materials and Methods: Transgenic mice were given 1000 mg/kg of DIM in the diet for 28 weeks. The mice were injected with Gardasil Quadrivalent HPV vaccine. Some mice were sacrificed at 28 weeks. Other groups were removed from the DIM diet after 28 weeks to a diet with no DIM for either 4 or 8 weeks. Results: Cervical histology indicated that a high percentage of transgenic mice fed DIM and vaccinated with Gardasil manifested normal cervical epitheliums at 4 weeks after DIM discontinuation. Conclusion: Vaccination pre-supplemented with DIM may provide with a window of protection of at least four weeks in this transgenic model. However, extrapolation to the effect in humans is beyond the limited scope of the histological data presented here.*

While increased screening has reduced the incidence of cervical cancer by over 80% in the United States, this cancer remains the second leading cause of cancer-related death in women worldwide (1). The Gardasil vaccine can prevent initial infection by two of the high risk human papilloma virus (HPV) types HPV-16 and -18 that are responsible for about 70% of all cervical cancer (2). The vaccine does not prevent cervical epithelial changes and has no effects on cervical intraepithelial neoplasia (CIN) in those individuals already infected with HPV. Not everyone infected with HPV develops cancer. Additional genetic alterations are needed for malignant progression (3). The HPV oncoproteins E5, E6 and E7 are the primary viral factors responsible for the initiation and progression of cervical cancer. These oncoproteins inhibit negative growth regulation by host cell proteins, and induce genomic instability (1). 3,3'-Diindolylmethane (DIM) alters the phase-I metabolism of estradiol (E_2) in favor of C-2 hydroxylation pathways and away from C-16 hydroxylation. 16α-Hydroxyestrone actually promotes HPV viral expression (4-7). DIM also suppresses viral infection in children with recurrent respiratory papillomatosis (8, 9) and inhibits proliferation of the viral transcripts E6 and E7 in a human cervical carcinoma CaSki cell line and inhibits viral oncogene expression (10, 11). DIM induces a G_1 cell-cycle arrest in breast cancer MCF-7 cells which is accompanied by inhibition of cyclin-dependent kinase expression (12). DIM inhibits cell adhesion, spreading and invasion associated with the up-regulation of Phosphatase and Tensin Homolog (*PTEN*), a tumor suppressor gene and *E-cadherin*, a regulator of cell-cell adhesion in T-47-D human breast cancer cells (13). Additionally, DIM prevents PTEN loss *in vivo* in the K14HPV16-transgenic mouse and in humans (14). DIM also elevates key cytokines that confer immunity (15-17). The combined effects of DIM and Gardasil Quadrivalent HPV vaccine were evaluated in a transgenic mouse model using cervical histopathology as a biomarker. The possibility that DIM may enhance the efficacy of this preventive vaccine in the K14-HPV16 transgenic mouse model was explored.

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Key Words: 3,3'-Diindolylmethane (DIM), Gardasil Quadrivalent HPV vaccine, cervical intraepithelial neoplasia (CIN), human papilloma virus (HPV).

Materials and Methods

The mouse model. This study employed a well-known transgenic mouse model commonly used in studies involving cervical dysplasia and cervical cancer (18-22). Tumorigenesis in this model is estrogen-dependent. Epithelial dysplasia and metaplasia are observed after four months of estradiol treatment, leading to

high-grade dysplasia and multi-focal carcinomas by six months or earlier (17-19, 20, 21). Upon arrival at the Hackensack University Medical Center's Department of Biological Resources the mice were quarantined for as long as deemed necessary by the veterinarian. Breeder pairs were obtained from The Mouse Models of Human Cancers Consortium (MMHCC) Repository (Frederick, MD, USA) that is funded by the National Cancer Institute (NCI). The breeder pairs were approximately forty-five days old and weighed between twenty-five and thirty grams. The hemizygote K14-HPV16 strain was bred with the FVB wild type to produce the first litter of pups. At weaning, the pups were genotyped (21, 22). Those mice exhibiting the E6-E7 exon (K14-HPV16 positive) were further bred with FVB wild-type in order to generate progeny for the experimental protocols. All females were trochar implanted with E2 pellets (0.25 mg/90 day release; Innovative Research of America, Sarasota, FL, USA) under anesthesia. E_2 pellets were replaced at 3-month intervals. The experimental research reported in this manuscript was performed with the approval of the Institutional Animal Care and Use Committee (IACUC) at Hackensack University Medical Center (Protocol Approval number 170).

Mouse Diet. Mice were fed irradiated AIN-93M diet (Purina TestDiets, Richmond, IN, USA) and were given sterile water via sterile water bottles. The mouse diet was supplemented with 1000 mg/kg of DIM (LKT Laboratories, St. Paul, MN, USA).

Vaccination. Mice were vaccinated with the Gardasil Quadrivalent HPV Vaccine (Merck, Rahway, NJ, USA). Mice received 0.4 μl doses of the vaccine at week 20, week 22, and then at week 28 by intra-muscular injection in the upper front leg region. This approximates the schedule in humans (three doses of vaccine over eight months). The dose was adjusted for body weight, life span and the interval between doses in humans relative to life span in mice. The vaccine was administered using a 0.5-μl calibrated syringe (World Precision Instruments, Sarasota, FL, USA). For tissue collection, the mice were anesthetized and euthanized at 28, 32 or 36 weeks. Fresh tissue from the reproductive tract and surrounding soft tissue was acquired from each animal at necropsy. The uterus and cervix were dissected and immediately frozen at –80˚C. Each specimen was serially-sectioned at 12 μm thickness by using a Cryostat Leica CM 1850 (Leica Microsystems Inc, Buffalo Grove, IL, USA) and 10 to 15 sections were collected at 20 μm intervals for hematoxylin and eosin staining. Examination of serial sections was carried out with a Leica CME binocular light microscope (Leica Microsystems Inc, Buffalo Grove, IL, USA). Images were obtained by a Zeiss Axioplan microscope with a Sony color digital camera DXC-S500 attached.

A grading system for transgenic mouse cervical squamous carcinogenesis developed by Riley *et al*. (22) was used to classify cervical histological samples. The grading system is based on the established criteria for classification of human cervical neoplasia or malignancy accounting for differences between the mouse model and humans and has been described previously (19, 23).

Statistics. The mice were divided into four groups: Group 1 (n=34), DIM-only; Group 2 (n=22), DIM and vaccinated at 20, 22, and 28 weeks and then immediately sacrificed; Group 3 (n=25), DIM and vaccinated then switched to a diet without DIM for four weeks and then sacrificed, and Group 4 (n=34), DIM vaccinated as described but switched to a diet with no DIM for eight weeks and then sacrificed. Since severity of CIN (Normal, CIN I, CIN II CIN III and neoplastic carcinoma *in situ* or NCIS) is ordinal, it was of interest to examine if severity increased with the time which DIM was withheld from the mice, which is also ordinal. For these ordinal scales, the Jonckheere-Terpstra test (24, 25) was performed. Furthermore, pair-wise comparisons of the groups were conducted with the Hochberg procedure (26) adjusting for multiple comparisons. All categorical data are presented as frequencies (percentages). A value of $p<0.05$ was considered statistically significant. All data analysis was conducted using SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

The cervico-uterine region was examined in the K14-HPV16 transgenic mouse model. Figure 1 represents the sequence of transformation from normal to NCIS or carcinoma *in situ* in the epithelium at the cervical transformation zone. The figure illustrates examples of cervical dysplasia or neoplasia from vaccinated mice in various groups and visually validates the biomarker. Normal epithelium (Figure 1) has a single and organized layer of basal cells. As dysplasia develops, there is mild squamous hyperplasia of the basal cells, forming two layers of epithelial cells in CIN I (Figure 1) without projections of the epithelium into the stroma of the cervix. Moderate hyperplasia and irregularities of the epithelial stromal border forming papillae are due to the hyperplasia of the squamous cells in CIN II (Figure 1). The cytological squamous cell progression is more evident in CIN III (Figure 1), where there are pronounced papillomatoses (black arrow) and the basal cells are observed at the stratum espinosum and granular stratum . Cellular dysplasia is illustrated in Figure 1 and immature hyperchromatic cells can be seen (white arrows). Additionally, some cellular anaplasia displaying noticeable pleomorphism and variable size and shaped nuclei can also be seen in Figure 1 (E, white arrow). Finally, severe irregularities as well as branching of the epithelial stromal border, and severe hyperplasia can be seen in NCIS (Figure 1) maintaining the integrity of the basement membrane. No glandular hyperplasia or keratin pearls were observed in any of the processed specimens. The Jonckheere Terpstra test reported a Z statistic of 2.50 with a two-sided p-value of 0.0124. At the 0.05 α-level, this indicated that there were significant differences among the treatment groups in their respective orderings of CIN severity (Figure 2). Pair-wise comparisons with Hochberg adjustment revealed that there was a significant increase in CIN severity in Group 4 when compared to Group 1 ($p=0.0017$). There was no significant increase in CIN severity between Group 1 and Group 2 ($p=0.8206$); or between Group 1 and Group3 ($p=0.3903$). CIN severity was significantly lower in mice in Group 2 when compared to Group 4 ($p=0.0014$). A significant

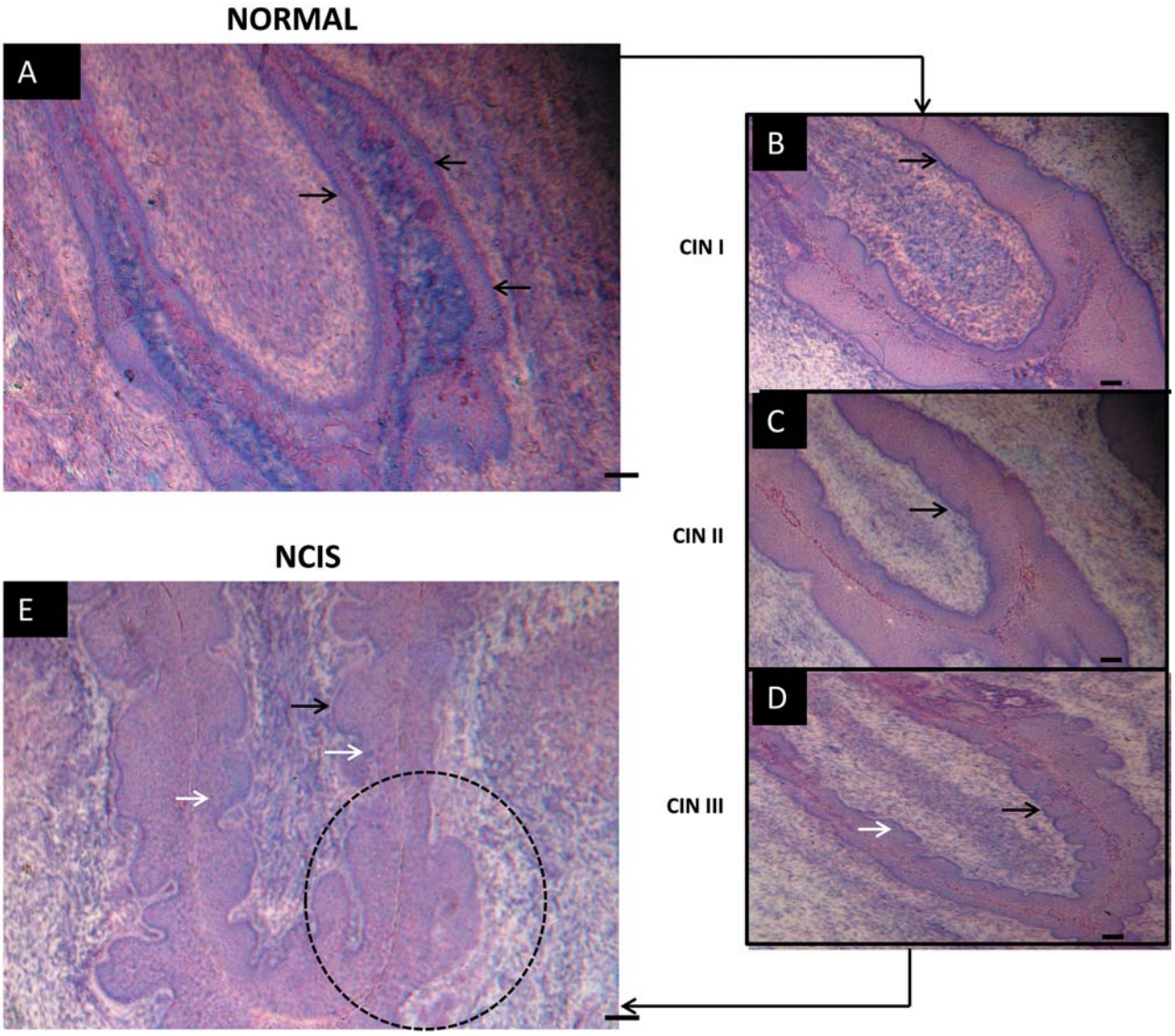

Figure 1. *Histopathology of the cervical epithelium in the K14-HPV16-transgenic mouse model showing the sequence of transformation from normal (A) to (NCIS) or carcinoma in situ (E) in the epithelium at the cervical transformation zone, at 10x object magnification. All mice received (DIM) and were vaccinated. The calibration bar represents 0.1 mm (lower right corner) and was measured by a Reichert-Jung micrometer of 2 mm, divided into units of 0.01 mm. The black arrow shows the basal cells of the cervical epithelium and indicates the changes that occur in this area as CIN increases in severity to NCIS. The white arrow shows immature hyperchromatic cells as well as cellular anaplasia. The dashed oval shows severe hyperplasia and branching of the epithelial stromal border. These changes are described in greater detail in the Results section.*

increase in CIN severity was also observed between Group 3 and Group 4 (p=0.0003). In contrast, there were no significant differences between Group 2 and Group 3 (p=0.5593). The results illustrated that more severe CIN indications were observed in Group 4. To determine if there was a significant change in mortality between the treatment groups, the Jonckheere Terpstra test was performed. The test reported a Z statistic of 2.155, with a two-sided p-value of 0.0311. At the 0.05 α-level, there is evidence that mortality was increased with increasing DIM cessation. Mortality rates of mice in Group 1 (n=10 deaths/44 mice) was 22.7% *versus* 43.3% in mice of Group 4 (n=26/60). This led to an exploratory investigation to determine if there was some clinical between-group significance. Unadjusted comparisons indicated that the difference in mortality between Group 1 and Group 4 was significant (p=0.0299).

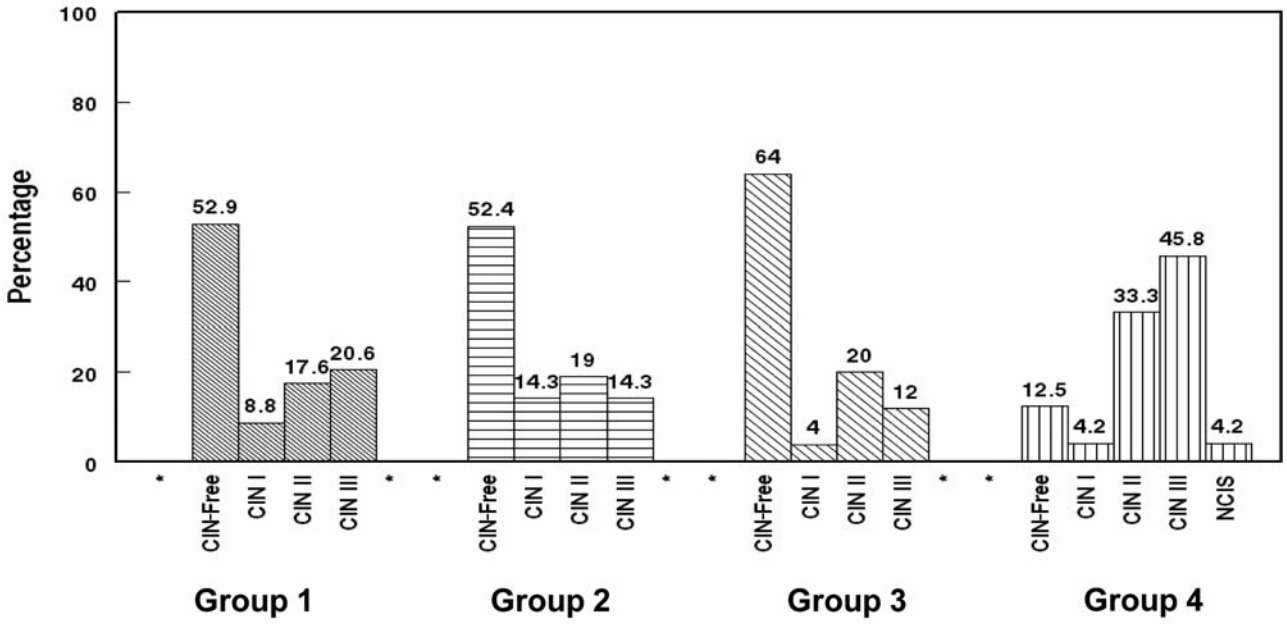

Figure 2. Transgenic mice were vaccinated after feeding with 1,000 mg/kg of (DIM) for 28 weeks then without DIM for no, four and eight weeks. Mice (except for the DIM only group, Group 1) were vaccinated at 20. 22 and 28 weeks to roughly correspond to the human vaccine administration schedule. A significant decrease in the percentage of CIN-free mice, as well as increased CIN severity was observed in the DIM group vaccinated as above but switched to a diet with no DIM for eight weeks (Group 4) (p =0.0017). The mice were divided into four groups: Group 1 (n=34), DIM-only; Group 2 (n=22), DIM and vaccinated at 20, 22, and 28 weeks and then immediately sacrificed; Group 3 (n=25), DIM and vaccinated then switched to a diet without DIM for four weeks and then sacrificed, and Group 4 (n=34), DIM vaccinated as described but switched to a diet with no DIM for eight weeks and then sacrificed. The numbers at the top of each bar represent the percentage of the number of mice in each group.

Discussion

In this study, the interactive effects of DIM and Gardasil Quadrivalent HPV vaccine on mouse cervical histology were evaluated. In the K14-HPV16 transgenic mouse model, cervical histological changes closely-mirror similar changes in humans and may be used as biomarkers of disease progression (22). Group 1, DIM-only, and Group 2, DIM with vaccination, have a high percentage of CIN-free mice and a low percentage of mice with CIN. Group 3, DIM with vaccination then four weeks without DIM, showed a similar histological pattern to Group 1 and to Group 2. In Group 4, DIM with vaccination then eight weeks without DIM, the histological profile is reversed and a low percentage of CIN-free (12.5 %) mice accompanied by high percentages of CIN II, CIN III and even NCIS are apparent (Figure 2). The mortality data indicated a significant increase in mouse mortality in Group 4 when compared to the other three Groups. DIM protects the cervical epithelium from HPV-caused lesions in several ways (10-12, 23). One mechanism of action suggests co-operation between PTEN, p53 and Rb that induce high-grade astrocytoma in the adult brain and in other types of cancer (27). DIM prevents PTEN loss *in vivo* in the K14HPV16-transgenic mouse and in humans (14). DIM inhibits cell adhesion, spreading and invasion associated with the up-regulation of PTEN and E-cadherin (13). This correlates with data from Weng *et al.* (28) indicating that DIM induces G_2/M arrest and apoptosis by modulating mitogen-activated protein kinases (MAPK) and p53 signaling. Mutations of the *PTEN* gene are a step in the development of many types of cancer (29). PTEN acts as a tumor suppressor gene through the action of its phosphatase protein product. This phosphatase is involved in the regulation of the cell cycle, preventing cells from growing and dividing too rapidly (30). When the PTEN enzyme is functioning properly, it acts as part of a chemical pathway to signal cells to stop dividing and can cause cells to undergo programmed cell death (apoptosis), when necessary. These functions prevent uncontrolled cell growth. DIM prevents PTEN loss *in vivo* in the K14-HPV16-transgenic mouse and in human samples of cervical dysplasia (14). PTEN expression decreases as cervical dysplasia increases and is absent in cervical cancer. DIM promotes the expression of PTEN in the mouse model and in humans with cervical dysplasia. DIM inhibits the development of cervical cancer by up-regulating *PTEN* to prevent cervical carcinoma. The possibility that DIM may enhance the short-term efficacy of this preventive vaccine in the K14-HPV16-transgenic mouse model may be cautiously extrapolated to humans who have been exposed to the virus and need protection. The

correlation of mouse *versus* human lifespan is an approximation. In this study, we make general comparisons that have been accepted for this purpose (31, 32). From birth to one month of life, the maturation rate of mice is 150-times that of humans. From one to six months, mice mature about 45-times faster than humans. After six months, the rate of mouse to human life span is around 25-times faster. These general comparisons are important when comparing the mouse *versus* life span. For instance, the time of successive vaccinations that we chose compare favorably to the vaccination times of adolescent humans. Mice vaccinated at 20, 22 and 28 weeks would be the equivalent of humans that are adolescent or in early maturity. The time post-vaccination (with DIM removed for four and eight weeks post vaccination respectively) therefore becomes important. Weeks of the mouse life span at this stage can be generally approximated to human years. The findings suggest that vaccination pre-supplemented with DIM administration provides a window of protection of at least four weeks in this transgenic model. Comparison of mouse to human life span suggests that DIM supplementation, coupled with vaccine administration, could be effective in humans already infected with the virus. However, extrapolation to the effect in humans is beyond the limited scope of the data presented here. Biochemical findings from serum and tissue samples obtained from this study are currently being analyzed and could confirm or refute this supposition.

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