REAL TIME POLYMERASE CHAIN REACTION AND FLUORESCENT IN SITU

HYBRIDIZATION IN THE DETECTION OF THE PHYSICAL STATE OF HUMAN

PAPILLOMAVIRUS 16 AND 18 IN PARAFFIN EMBEDDED CERVICAL TISSUE

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DEDICATION

I dedicate this work to my dearest sister, Sharifa Davis-Anthony. Thank you for always believing in me. Thank you for being my biggest cheerleader. Many thanks for your words of encouragement. Your unconditional love and support has been the glue that has held me together!

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BPC04	Control primer for β-globin
BGH20	Control primer for β-blobin
DAPI	4',6-diamidino-2-phenylindole
dH20	distilled water
DNA	deoxyribonucleic acid
E	Early genes
FFPE	Formalin Fixed Parraffin Embedded
FISH	Fluorescent In Situ Hybridization
HCL	Hydrochloride
H & E	Hematoxylin & Eosin
HPV	Human Papillomavirus
HR	High risk
HRP	Horseradish peroxidase
L	Late genes
LCR	Long Control Region
LR	Low risk
M	Molarity
mL	milliter
mM	millimolar
mm	millimeter
N	Normality
NaCl	Sodium Chloride

p53	Tumor suppressor protein
p97	HPV genome early promoter
PBS	Phosphate Buffered Saline
PGMY09	Biotinylated primer for HPV 16 and 18
PGMY11	Biotinylated primer for HPV 16 and 18
Rb	Retinoblastoma
RT-PCR	Real Time Polymerase Chain Reaction
SSC	Saline Sodium Citrate
TNT	**T**ris-HCL,**N**aCl and **T**ween 20 wash buffer
Tris-HCL	Trisaminomethane hydrochloride
TSA	Tyramide Signal Amplification
μg	microgram
μL	microliter
μM	micromole

INTRODUCTION

Human Papillomavirus Background Information

Human papillomaviruses (HPV) are non-enveloped DNA viruses that are

approximately 50-55 nm in diameter. The virus has an icosahedral capsid that consists of

72 pentamers of the L1 major capsid protein. The viral genome is a circular, double

stranded DNA with 8 open reading frames (genes) all contained on one strand. There are

well over 100 different HPV types that have been characterized based on genomic

similarities [1]. These types are further characterized and classified according to their

neoplastic ability into low risk (LR) and high risk (HR) types. LR types cause condyloma

acuminata; while HR types are associated with dysplasias of the cervix and cervical

cancer. HR types are the etiologic agent of 100% of cervical cancer, 60% of

oropharyngeal cancer, 95% of anal cancer, 50% of vulvar, 65% of vaginal and 35% of

penile cancer [2].

HPV Epidemiology

Based on cross-sectional studies there are over 70 million Americans that are

infected with HPV. There are more than 10 million new cases each year in the United

States [2]. Nearly all sexually active individuals will become infected with an HPV type

sometime during their lifetime. The American Cancer Society statistics predicts that there

will be well over 12,000 new cases of invasive cervical cancer and approximately 4,100

women will die from cervical cancer in the United States just within the year 2015 alone.

The aforementioned statistics are applicable to the United States. However, most cases of

cervical cancer occur in women living in poor countries. The highest incidence of

cervical cancer occurs in women living in certain parts of Central and South America,

Asia and in sub-Saharan Africa. Indeed, HPV is responsible for a considerable health burden.

Physical State of HPV

The most common HR HPV types in cervical cancer are HPV 16 and 18 [3,4]. High risk HPV DNA is found in human epithelial cells in one of two physical states: episomal (extrachromosomal) or integrated into the human genome (Figure 1). Integration most often disrupts the E2 gene, an 1100 base pair gene with numerous functions; one function of importance involves transcriptional regulation of E6 and E7. Disruption of E2 leads to unregulated, over-expression of E6 and E7, resulting in a selective growth advantage for the virus. The E6 and E7 viral oncoproteins target the p53 and retinoblastoma (Rb) tumor suppressor genes, respectively [5,6]. As the E2 gene is disrupted, E2 protein is not produced, and thus there is no suppression of the p97 promoter resulting in un-controlled expression of the aforementioned viral oncoproteins [6].

Disruption of the E2 gene during viral integration is of clinical significance. Disruption of this 1100 base pair gene can be assessed using molecular tools. One such molecular tool is real time polymerase chain reaction (RT-PCR). RT-PCR is a molecular technique that quantifies the amount of a targeted DNA fragment or gene. The method depends on a fluorescent taqman probe, a primer pair (reverse and forward primers) and the presence of the targeted gene or DNA fragment of interest. In the case of viral integration and resultant E2 disruption, no amplification of the E2 gene can occur.

Figure 1: Schematic depiction of the physical state of HPV. HR HPV is found in human epithelia either as episomal (extrachromosomal) and/or integrated into the host/cellular genome. Also shown in the figure is disruption of the E2 gene upon integration of the viral DNA into the human genome (bottom diagram). *LCR-Long Control Region. *p97-HPV genome promoter for E6 and E7. Courtesy of Dr. Helena Spartz.
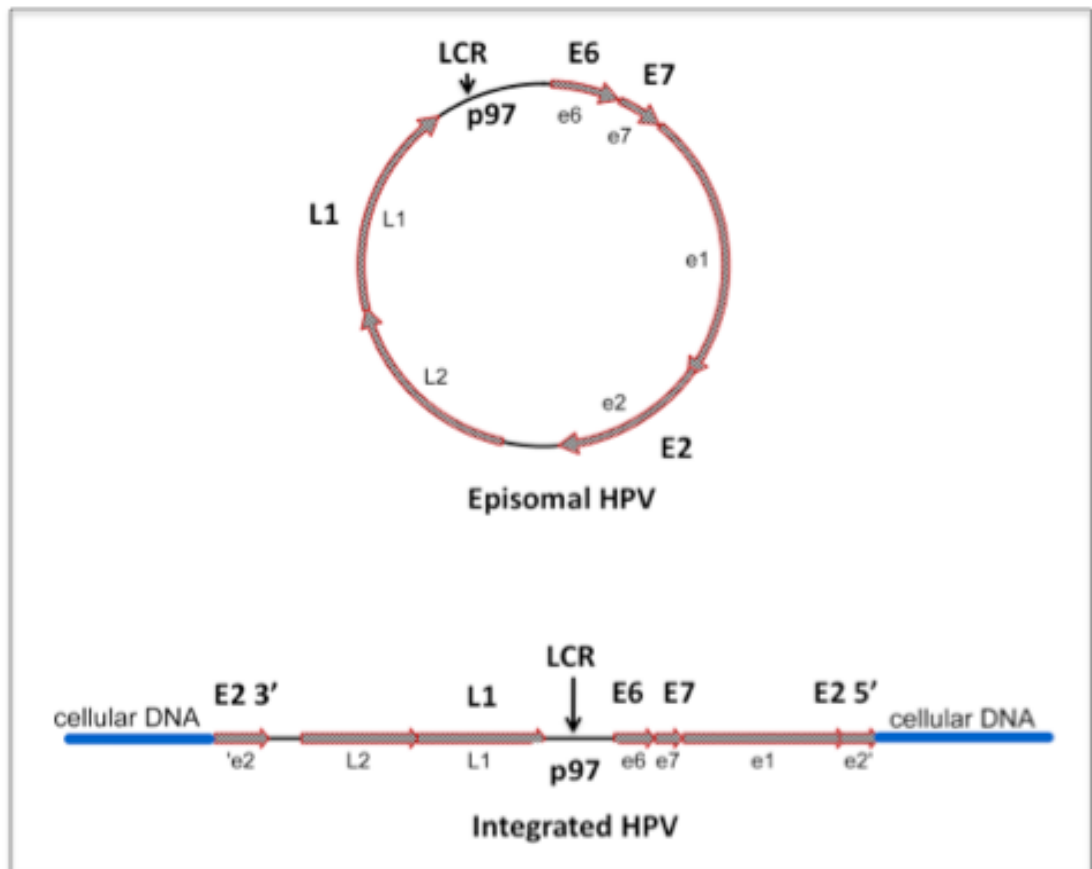
Study Rationale

The ability to use molecular tools such as RT-PCR in evaluation of HPV physical state is of significant importance for many reasons: 1) Integration of HR HPV DNA into the human genome is considered to be a critical event in the progression to invasive cervical cancer [5]; 2) Evaluating the physical state of HPV will provide greater understanding into how pre-neoplastic lesions cause cancer years later 3) Evaluating the physical state of HPV could guide early intervention and therapies. We therefore sought to describe the physical state of HPV DNA in formalin-fixed paraffin embedded (FFPE) cervical tissue using two assays: tyramide amplified fluorescent DNA in situ hybridization (FISH) and RT-PCR. We are specifically interested in determining the feasibility of RT-PCR in the detection of HPV 16 and 18 integration.

MATERIALS AND METHODS

Materials

Our study was performed using FFPE cervical cancer tissue obtained from obstetrics and gynecology clinics in Botswana and Jamaica and the pathology laboratory of Indiana University and Wishard Hospitals. Cervical cancer tissues were examined to verify the presence of invasive cancer. Those invasive cancer samples shown to contain HPV 16 or 18 in a previous study were chosen [16].

DNA Extraction

Formalin-fixed paraffin embedded cervical samples were removed from microscope glass slides with a new razor blade and transferred into a labeled micro-centrifuge tube. Each cervical sample was de-paraffinzed by washing in 1 mL of octane for 10 minutes, followed by 10 minutes of centrifugation at highest speed. This process was repeated to ensure complete removal of residual paraffin. One milliliter of absolute ethanol was added and tissues were centrifuged for 10 minutes at maximum speed to remove residual octane. Samples were allowed to dry for 20 minutes in a tabletop speed vacuum. Tissues were treated with 200 μg of proteinase K buffer (QIamp MinElute Media Kit) suspended in 300 uL of Specimen Transport Medium (QIAGEN) and digested overnight at 55° C. After overnight digestion, DNA was extracted from all samples using the QIAamp MinElute Media Kit (QIAGEN) according the manufacturer's instructions. The quality and purity of extracted DNA was evaluated using spectrophotometry (Courtesy of Brahim Qadadri).

Determination of HPV Types

HPV types in cervical cancers were determined using the Roche Linear Array HPV Genotyping Assay (Roche, Indianapolis, IN USA). This assay amplifies the polymorphic L1 region of 37 HPV types (including the oncogenic HPV types 16 and 18) using biotinylated primers, PGMY09 and PGMY11. Polymerase chain reaction system control primers, BPCO4 and BGH20, were used to amplify the human β-globin gene to determine specimen adequacy.

Real Time PCR (RT-PCR)

RT-PCR was used to assess the HPV viral genome status based on the E2 to E6 gene copy number (E2/E6 ratio). In the case of viral integration there is disruption of the E2 gene and hence loss of amplification during RT-PCR; but the E6 gene is left intact resulting in expression of this gene, which can be detected during the RT-PCR reaction. For this reason, the viral genome was regarded as integrated when the E2/E6 ratio was between 0.00 and 0.33. While, the viral genome was regarded as mixed (containing both episomal and integrated HPV DNA) when the E2/E6 ratio was between 0.34 and 0.66, and episomal when the E2/E6 ratio was between 0.67 and 1.00. Amplification and quantitative measurements of HPV 16/18 E2 and E6 genes were carried out in a total reaction volume of 20 μL. The E2 and E6 primers were at a concentration of 0.5 uM per reaction for the 3' and 5' primers. The E2 and E6 fluorophore-labeled probes for HPV 16 and 18 were used at a final concentration of 0.25 uM. Most HPV 16/18 integration occurs within the hinge region of the E2 gene. Therefore, the E2 primers and probes were directed at the hinge region (Figure 2).

Figure 2: Schematic diagram of the E2 gene. The E2 gene is approximately 1100 base pairs long and consists of three regions: an amino region, a hinge region, and a carboxy region. Integration of HPV into the human genome can disrupt anyone of these regions of the E2 gene. However, the majority of disruption occurs within the hinge region of the gene.
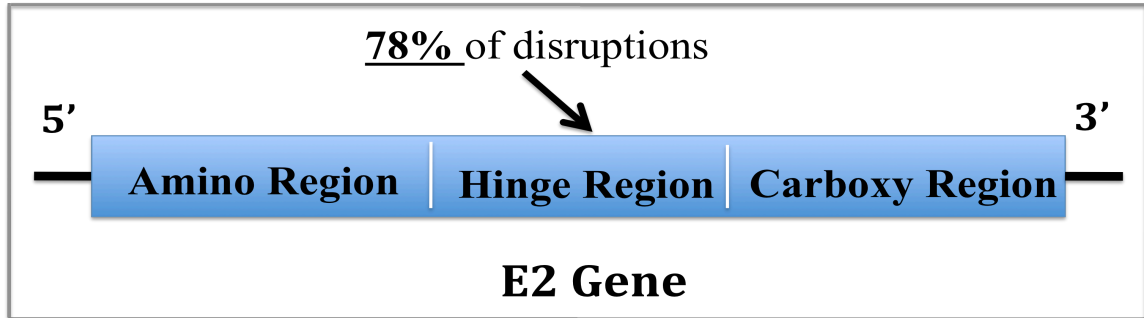
The conditions for PCR consisted of: 12 minutes at 95°C for one cycle, followed by denaturation for 10 seconds at 95°C, 30 seconds at an annealing temperature of 55°C, 1 second at 72°C (x45 cycles) in single signal acquisition mode for DNA amplification and detection. Reactions were allowed to cool to 40°C for 30 seconds. Six point standard curves for E2 and E6 of HPV 16/18 where obtained using 10-fold serial dilutions (10^1 to 10^6 copies/uL) of whole plasmids. To check for accuracy and reproducibility of standard curve calculations, experiments were repeated twice. HeLa cells, which are known to harbor the integrated form of HPV 18 [14] served as a positive HPV 18 integrated control. While, SiHa cells, known to harbor the integrated form of HPV16 [15] served as a positive HPV 16 integrated control. HPV 16/18 plasmids served as non-integrated controls (Courtesy of Dr. Aaron Ermel).

Fluorescent DNA In Situ Hybridization (FISH)

Formalin-fixed, paraffin embedded cervical sections were fixed to glass slides by incubating at 80°C for 15 minutes and de-paraffinized by soaking in Xylene. Slides were hydrated in a graded ethanol series prior to being rinsed in hydrogen peroxide. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide diluted in methanol for 30 minutes. Next, slides were treated with 0.2 N HCL for 20 minutes followed by washing in dH2O for 3 minutes. Subsequently, sections were dried at 37°C for 10 minutes. Cervical specimens were digested with 25 μg/mL Proteinase K in 10 mM Tris-HCL pH 7.4 for 35 minutes at 37°C. Slides were washed with PBS, dehydrated and dried. During the hybridization process, one drop (approximately 15 uL) of Enzo HPV16/18 biotinylated genomic probe (Enzo Diagnostics, Inc., Farmingdale, NY) was added to each individual slide, followed by the application of a Corning 22 x 22 mm glass cover slip to each slide. The probe and target DNA were denatured by incubating slides at 95°C for 5 minutes. Slides were then incubated at 37°C overnight in a humidity chamber to allow for maximum hybridization between probe and target DNA.

The following day, coverslips were removed by washing slides in 2x SSC/0.05% Tween 20 for 5 minutes. Subsequently, slides were washed for 40 minutes in 2x SSC/0.05% Tween 20 in a shaker at 50°C. The Tyramide Signal Amplification (TSA) Cyanine 3 System (NEN Life Science Products, Boston, MA) was used to detect hybridized DNA. All sections were initially blocked with Tris-NaCl blocking buffer (1 M Tris HCL, pH 7.5, 5 M NaCl and blocking reagent -PerkinElmer) for 10 minutes. Subsequently, sections were incubated with streptavidin-HRP (1:100 dilution). All sections were washed three times using TNT wash buffer (0.1 M Tris-HCL, pH 7.5, 0.15

M NaCl, 0.05% Tween 20) for 15 minutes. Two drops (approximately 30 μl) of TSA-

direct Cy3 fluorophore reagent (1:50 dilution; NEN Life Science Products, Boston, MA)

were applied to each slide and incubated for 10 minutes to increase signal enhancement.

Sections were washed using TNT buffer, dehydrated and allowed to air dry before

mounting with Vectashield (Vector Laboratories, Burlingame, CA). All sections were

analyzed using fluorescent microscopy at a wavelength of 550/570-nm for Cy3 and

340/488 nm for DAPI. Cervical cancers known to contain HPV type 16 or 18 were used

as positive controls. Slides treated with PBS instead of Enzo probes served as

corresponding negative controls. Two distinct fluorescent patterns were observed: a

diffuse nuclear pattern suggestive of episomal HPV DNA, while punctate dots suggested

integrated HPV DNA [7,8,9] (Figure 3). A combination of both a diffuse nuclear pattern

and punctate dots suggested both episomal and integrated (mixed) HPV DNA.

Figure 3. Cartoon depiction of the FISH assay. Cartoon depiction of the steps involved in Fluorescent In Situ Hybridization (FISH) including an illustration of the physical states of HPV DNA. High risk HPV DNA is found in human epithelia in two physical states either as episomal (extrachromosomal) particles or integrated into the human genome. The episomal state consists of multiple copies of the virus; while the integrated form of HPV DNA exist as a single copy integrated into the human genome. The resulting two distinct forms can be seen under fluorescent microscopy as either a diffuse nuclear pattern suggestive of episomal HPV DNA or punctate dots suggestive of integrated HPV DNA. Courtesy of Dr. Helena Spartz.
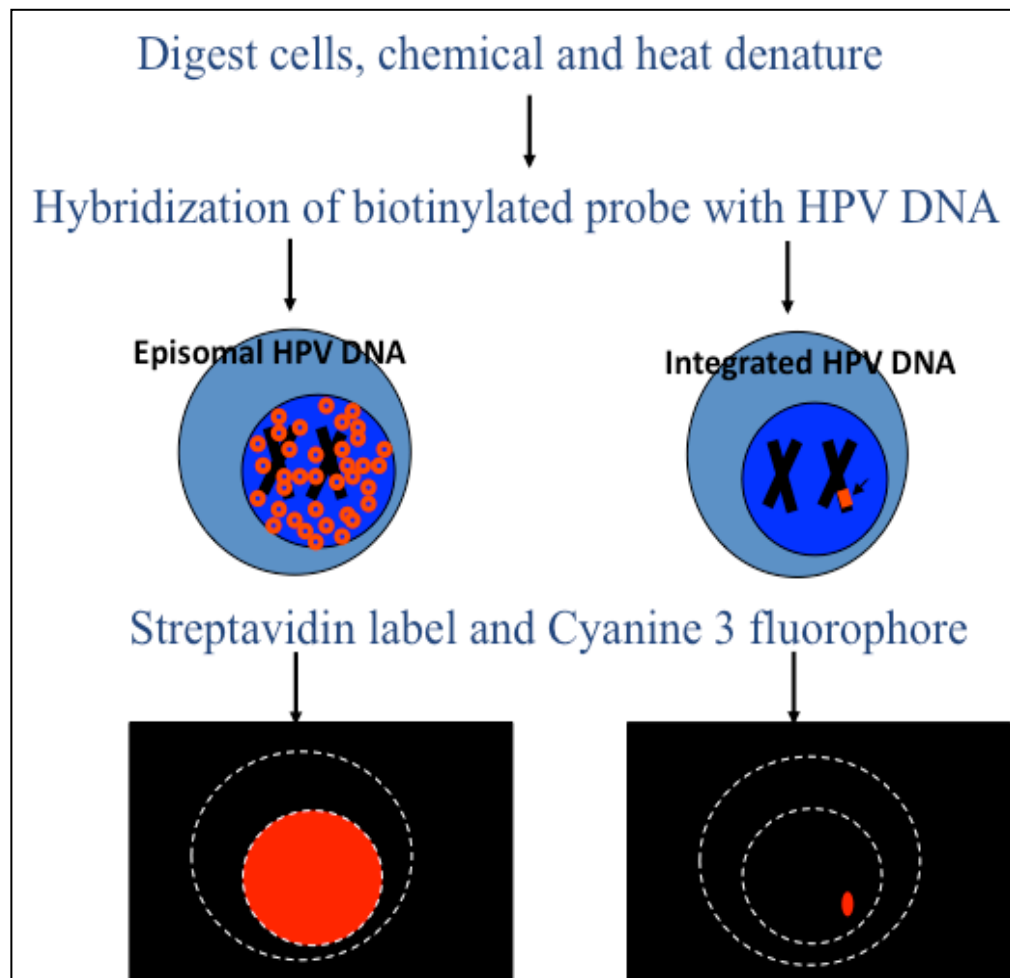
RESULTS

The study was performed on cervical cancer specimens obtained from 35 women. Thirty cases were from women living in Indiana; three cases were from women living in Botswana; and 2 cases were from women living in Jamaica. Based on prior studies, these cancers were known to contain HPV 16 (n = 27), HPV 18 (n = 7) or both HPV16 and HPV 18 (n = 1) (Table I).

Table 1. HPV types in cervical cancer. Included in the table is the exact number of cases that were infected with either human papillomavirus 16 (HPV 16) or human papillomavirus 18 (HPV 18).

Study Site:	Indiana	Botswana	Jamaica	Total
Cases	30	3	2	35
HPV 16 alone	26	1	0	27
HPV 18 alone	3	2	2	7
HPV 16 and 18	1	0	0	1

Detection of the Physical State of HPV 16 and 18 by RT-PCR

The physical state of HPV DNA was analyzed using RT-PCR via amplification of E2 and E6, then calculating the ratio of E2 and E6. All 35 cancers contained amplifiable HPV 16 or 18 signals by RT-PCR. From the 35 cervical cancers, 16 cases (45.7%) had an E2/E6 ratio between 0.67 and 1.00 indicating episomal HPV; 2 cases (5.7%) had an

E2/E6 ratio between 0.34 and 0.66 indicating mixed (episomal and integrated) HPV; while, 17 cases (48.6%) had an E2/E6 ratio between 0.00 and 0.33 indicating integration (Table II and III).

Detection of the Physical State of HPV 16 and 18 by FISH

From the 35 cervical cancers, FISH was positive in detecting HPV 16 or 18 in 26 cases (74.3%). FISH was used to assess the physical state of HPV based on fluorescent patterns observed under fluorescent microscopy (Figure 3). In the FISH assay, 6 cases (23.1%) had a diffuse nuclear pattern observed under fluorescent microscopy indicating episomal HPV; 8 cases (30.8%) had a combination of both a diffuse nuclear pattern and punctate dots indicating mixed (episomal and integrated) HPV; while, 12 cases (46.2%) had only punctate dots indicating HPV integration (Table II and III).

Table 2. RT-PCR and FISH analysis of the physical state of HPV 16 and 18 in 35 carcinomas. RT-PCR assessed the physical state based on the E2/E6 ratio; while, FISH assessed the physical state based on fluorescent patterns observed under fluorescent microscopy. "Mixed"= both episomal and integrated fluorescent pattern.

HPV 16 or 18 Detection	RT PCR Results	FISH Results
Total	35	35
Negative	0	9
Positive	35 (100%)	26 (74.3%)
Episomal	16 (45.7%)	6 (23.1%)
Mixed	2 (5.7%)	8 (30.8%)
Integrated	17 (48.6%)	12 (46.2%)

Table 3. Comparison of RT-PCR and FISH. The E2/E6 ratios were compared to FISH. A diffuse nuclear fluorescent pattern indicated the presence of episomal HPV, while punctate dots indicated integrated HPV DNA. An E2/E6 ratio between 0.00 and 0.33 indicated integrated HPV; an E2/E6 ratio between 0.34 and 0.66 indicated mixed HPV; and an E2/E6 ratio between 0.67 and 1.00 indicated episomal HPV.

Specimen Number	HPV type	E2/E6 ratio	HPV 16/18 FISH pattern	[a]β- globin
1	16	0.770 (Episomal)	Episomal	Positive
2	16	1.1384 (Episomal)	Episomal	Positive
3	16	1.200 (Episomal)	Episomal	Positive
4	16	0.955 (Episomal)	Episomal	Positive
5	16	1.378 (Episomal)	Episomal	Positive
6	16	0.00469 (Integrated)	Integrated	Positive
7	16	0.00648 (Integrated)	Integrated	Positive
8	16	0.14286 (Integrated)	Integrated	Positive
9	16	0.21573 (Integrated)	Integrated	Positive
10	16	0.08547 (Integrated)	Integrated	Positive
11	16 ,18	0.2021 (Integrated)	Mixed	Positive
12	16	0.18814 (Integrated)	Mixed	Positive
13	16	0.1800 (Integrated)	Mixed	Positive
14	16	0.0003 (Integrated)	Mixed	Positive
15	16	0.0425 (Integrated)	Episomal	Positive
16	16	0.7942 (Episomal)	Integrated	Positive
17	16	1.000 (Episomal)	Integrated	Positive
18	16	0.6568 (Mixed)	Integrated	Positive
19	16	1.054 (Episomal)	Mixed	Positive
20	16	0.9198 (Episomal)	Mixed	Positive
21	16	1.4988 (Episomal)	Mixed	Positive
22	16	1.00 (Episomal)	Mixed	Positive
23	16	1.333 (Episomal)	Negative	Positive
24	16	0.01923 (Integrated)	Negative	Positive
25	16	0.330 (Integrated)	Negative	Positive
26	16	0.8403 (Episomal)	Negative	Positive
27	16	0.2036 (Integrated)	Negative	Positive
28	16	0.010 (Integrated)	Negative	Positive
29	18	0.000 (Integrated)	Integrated	Positive
30	18	1.089 (Episomal)	Integrated	Positive
31	18	0.4296 (Mixed)	Negative	Positive
32	18	0.0461 (Integrated)	Integrated	Positive
33	18	0.3000 (Integrated)	Integrated	Positive
34	18	1.2310 (Episomal)	Negative	Positive
35	18	0.8045 (Episomal)	Negative	Positive
HeLa	18	0.000 (Integrated)	Integrated	Not applicable
Normal Cervix	Negative	Not applicable	Negative	Not applicable

[a]Samples were deemed as either being β-globin positive or negative.

Comparison of RT-PCR and FISH Results

Although RT-PCR and FISH were conducted on 35 cervical cancers, 25 cases were used in the direct comparison of both assays. Nine cases were excluded for the comparison because of negative FISH results (Table III). One case was excluded because of co-infection with both HPV 16 and 18. The combined HPV16/18 probe was used in fluorescent in situ hybridization, and it was therefore impossible to determine which HPV type (16 or 18) contributed to the pattern detected under fluorescent microscopy.

Overall, of the 25 samples that yielded positive results in both assays, 8 samples had both punctate dots when observed under fluorescent microscopy and a corresponding E2/E6 ratio between 0.00 and 0.33 (Fig 4, Panel B). Therefore, both assays indicated HPV integration for these 8 cancers (Table IV).

Table 4. Comparison of FISH analysis and RT-PCR results. Comparison of FISH analysis and RT-PCR results in the assessment of the physical state of human papillomavirus 16 (HPV 16) and human papillomavirus 18 (HPV 18).

RT PCR	FISH			
	Integrated	Mix	Episomal	Total
0.00 − 0.33 Integrated	8	3	1	12
0.34 − 0.66 Mixed	1	0	0	1
0.67 − 1.00 Episomal	3	4	5	12
	12	7	6	25

Moreover, of the 25 samples that yielded positive results in both assays, no samples exhibited a mixed fluorescent pattern under fluorescent microscopy and had an E2/E6 ratio between 0.34 and 0.66 (Table IV). However, 7 samples had a mixed fluorescent pattern with FISH. Of these, 3 had an E2/E6 ratio between 0.00 and 0.33 suggestive of the integrated form of HPV DNA (Fig. 4, Panel C). Four cases had an E2/E6 ratio between 0.67 and1.00 suggestive of the episomal form of HPV DNA.

Lastly, of the 25 samples that yielded positive results in both assays, 5 samples exhibited both a diffuse fluorescent pattern and had an E2/E6 ratio between 0.67 and 1.00 indicating the episomal form of HPV (Fig. 4, Panel A). Therefore, both assays indicated that these 5 samples contained the episomal form of HPV DNA.

Figure 4. FISH and RT-PCR analysis of three cervical carcinomas, HeLa cells, and normal cervix. Panel A. Case #S11-7498 depicting episomal HPV (diffuse nuclear fluorescent pattern). The E2/E6 ratio of this same sample was 0.77 also indicating the presence of episomal HPV DNA. Panel B. Case #2101-115 depicting integrated HPV (punctate dots fluorescent pattern). The E2/E6 ratio of this cancer was 0.00 indicating HPV integration. Panel C. Case #S09 3020 depicting mixed HPV (both a diffuse nuclear and punctate dots fluorescent pattern). However, the E2/E6 ratio of this cancer was 0.180, indicating integrated HPV. Panel D. HeLa cells (a known integrated HPV 18 positive cervical cancer) showing punctate dots fluorescent pattern and an E2/E6 ratio of 0.00 both indicating HPV integration. Panel E. Normal cervix (non-infected HPV cervical tissue based on PCR); FISH depicting absence of fluorescent signal after application of HPV16/18 probe. Original magnification 500X. H&E: Hematoxylin and Eosin.
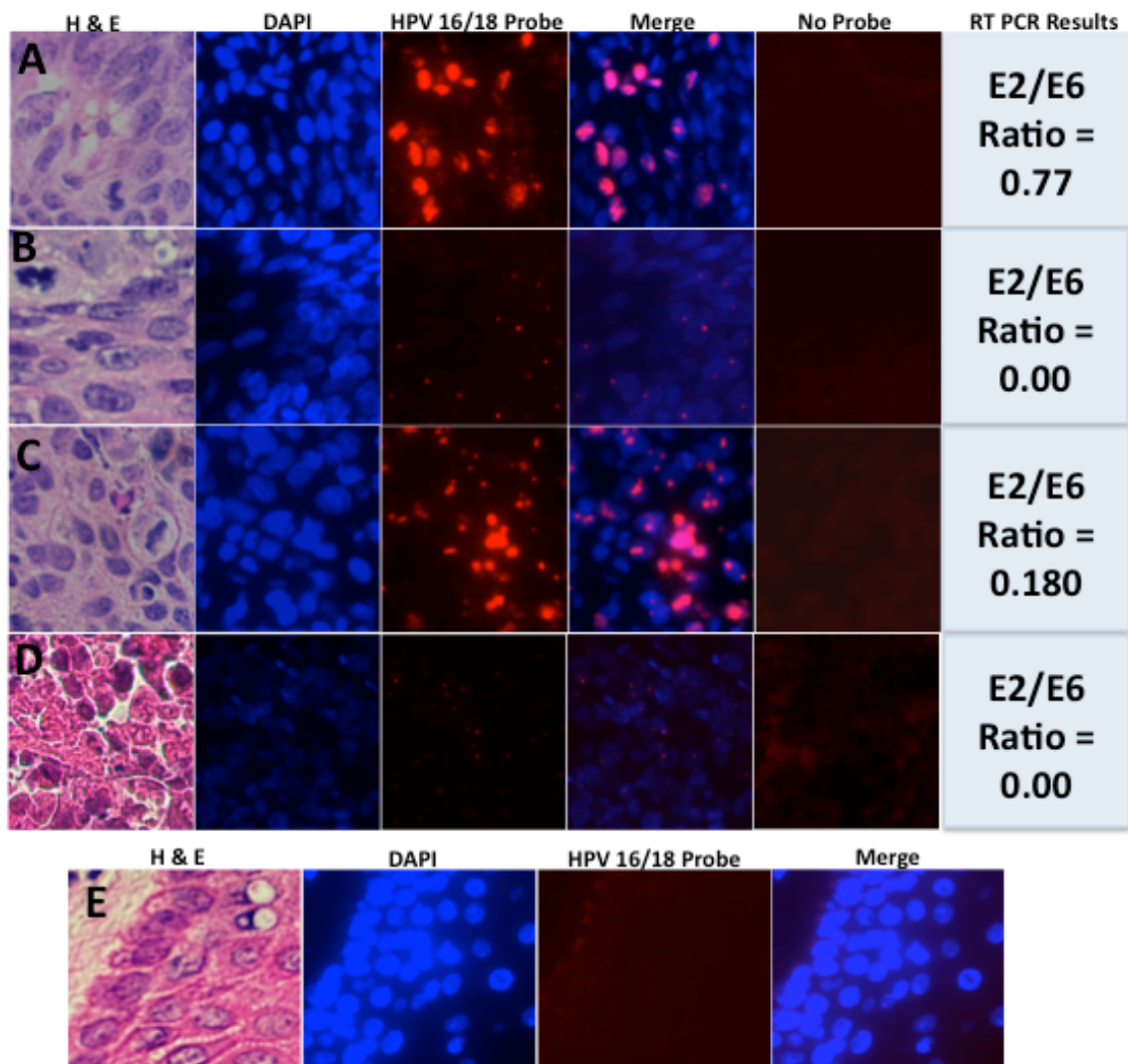
DISCUSSION

Integration of HR HPV into the human genome is a critical event in the progression from cervical dysplasia to invasive cervical cancer [5, 10, 11]. The ability to detect and evaluate HPV integration, and hence the physical state of HR HPV, is pivotal. Assessing the physical state of HPV can provide greater understanding into how pre-neoplastic lesions can cause cancer years later. Equally important, evaluating the viral genome status of HPV is necessary in informing clinical models for early intervention and therapies. Therefore, it is critical that assays are developed that can accurately detect integration of high risk HPV.

The ability to use molecular tools such as RT-PCR in the detection and evaluation of HPV integration is thus becoming increasingly important. While a variety of diagnostic methods have been developed to detect HPV in cervical biopsy specimens, RT-PCR confers high sensitivity and the assay can be performed on a variety of cervical samples including both cervical smears and paraffin-embedded cervical tissue. In this study, we evaluated the physical state of HPV using RT-PCR, while tyramide amplified fluorescent in situ hybridization (FISH) was used for comparison. We found a fair degree of correlation between RT-PCR and FISH in determining the physical state of HPV 16 and 18 DNA in FFPE cervical tissue.

We showed that in 13 of 25 cervical samples there was an agreement of the HPV physical status as determined by both assays. Specifically, of the 25 samples that yielded positive results in both assays, we observed that amongst 8 of these samples (32%) there was agreement of the assays in determining the presence of HPV integration. Furthermore, we observed that 5 of the cervical samples (20%) showed an agreement

amongst both assays in determining the presence of the episomal form of HPV DNA. Thus, RT-PCR was able to demonstrate that in the integrated forms of the virus the E2 gene is disrupted resulting in loss of amplifcation, while the E6 gene is left intact resulting in gene expression. Therefore, a very low E2/E6 ratio is observed with viral integration, as compared to the episomal viral state.

The highest degree of agreement occurred amongst the cervical cancers that contained either the integrated or episomal form of HPV DNA. There was no agreement amongst the two assays in cervical specimens that harbored the "mixed" (combination of both episomal and integrated pattern) form of HPV DNA. RT-PCR deemed one cervical sample as harboring the mixed form of HPV DNA based on the E2/E6 ratio. However, FISH conducted on this same sample yielded negative results. Similarly, the cervical samples deemed as having a mixed physical state based on fluorescent patterns observed by FISH, were either deemed as being episomal or integrated based on the E2/E6 ratio. The significance of this observation is not entirely known. However, it is thought in cases where there are discrepancies this could be due to integration that disrupted an area other than the hinge region of the E2 gene. Recall that the E2 gene is an 1100 base pair gene consisting of three regions: an amino region, a hinge region, and a carboxy region. Upon integration of the gene into the host genome any one of these regions of the E2 gene can be disrupted. In our present study, the E2 primers and probes were only directed at the hinge region of the E2 gene due to the large percentage of disruption (78%) occurring at this region during viral integration. However, a small percentage (22%) of E2 gene disruption via viral integration can take place in either the amino or carboxy region of the gene, and our RT-PCR would not detect these.

Furthermore, there are limitations that exist within the FISH method that may certainly contribute to disagreement amongst the two assays that were observed in our study. Namely, previous studies also performed on cervical specimens using in situ hybridization, found that small quantities of integrated HPV DNA can be concealed if a high quantity of episomal DNA exists within the same sample giving rise to discrepancies in determining the physical state of HPV DNA using in situ hybridization assays [8,12]. Additionally, Wierdon et al. [13] compared in situ hybridization, in situ PCR and tyramide signal amplification in determining HPV physical state and believed that the episomal form of HPV DNA can exist in other forms such as concatamers, which consists of multiple HPV copies. The authors stated that it is possible that these episomal concatamers can appear as punctate dots under fluorescent microscopy suggesting the integrated form of HPV rather than portraying the existence of the true episomal form of HPV DNA.

To date, there has been only a few studies that have been performed using formalin fixed paraffin-embedded cervical specimens comparing RT-PCR and FISH in determining the physical state of HPV 16 and/or 18 DNA. Biesaga et al. [5] analyzed formalin-fixed paraffin embedded sections of 85 cervical specimens using RT-PCR as the reference method and in situ-hybridization with tyramide signal amplification in the detection of HPV 16 and 18 integration. They found a significant correlation between the two methods in determining the viral genome status of HPV 16 and 18. In regards to RT-PCR, the viral genome was regarded as integrated when the E2/E6 ratio was 0, as episomal when the ratio was 1 or more, and mixed when the E2/E6 ratio was between 0 and 1. According to the authors, the sensitivity and specificity of in situ hybridization

with tyramide signal amplification (ISH-TSA) in detecting the integrated form of HPV 16/18 was 70.0% and 60.9% respectively, as compared to RT-PCR. They found no statistically significant difference between RT-PCR and ISH TSA in the detection of HPV viral genome status.

Similarly, Fujii et al. [10] performed in situ hybridization on formalin fixed paraffin embedded cervical sections and analyzed exfoliated cervical cells using RT-PCR. They found significant correlation between the two methods in determining the physical state of HPV 16. The authors found a concordance rate of 86% (37 of 43 cases were positive for HPV 16 integration) between the two methods for the detection of integrated HPV 16. In regards to the E2/E6 ratio, the authors established a cut off value of 0.79 to distinguish the mixed episomal and integrated physical state from the pure episomal form. The cut off value was based on a 99% confidence interval of 0.79 to 1.61. Thus, if the E2/E6 ratio were less than 0.79, the chance of the physical state of HPV 16 being in the pure episomal form would be less than 1%. Likewise, the integrated form of HPV 16 DNA was considered to be present when the E2/E6 ratio was below 0.79.

Still, other studies have been performed using only RT-PCR to detect the physical state of HPV with differing E2/E6 cut off values. Saunier et al. [11] analyzed 151 cervical smears and 22 paraffin-embedded cervical cancer biopsy specimens with an E2/E6 cut off value of 0.8. According to the authors, an E2/E6 ratio of 0.8 or higher represented the episomal form of HPV 16; E2/E6 values equaling 0 represented purely integrated HPV 16 DNA, while an E2/E6 ratio between 0 and 0.8 represented the mixed form of HPV 16 DNA. It is certainly evident that discrepancies exist in determining a universal cut off value when using the E2/E6 ratio as a surrogate for detecting the

physical state of HPV. Thus, while RT-PCR may represent an acceptable assay suitable in screening women for pre –cancerous lesions, involving integration of high risk HPV DNA, universal E2/E6 cut off values must be established.

In conclusion, our research extends the observations of the aforementioned studies, which compared RT-PCR and in situ hybridization in the detection of HPV physical state. While a limitation in our study included the small sample size, we were still able to observe a fair correlation between RT-PCR and tyramide amplified fluorescent DNA in situ hybridization (FISH) among cervical cancers that were positive for HPV 16 or 18 in both assays. Thus, our study is still in accordance with previous studies that have been conducted showing concordance amongst RT-PCR and FISH in detecting the viral genome status of high risk HPV. In regards to our study, conducting RT-PCR and FISH on a larger sample size and designing over-lapping E2 primers for RT-PCR and probes targeting all three regions (the amino, hinge and carboxy regions) of the E2 gene can increase the utility of RT-PCR as an assay in detecting HPV integration. Nonetheless, large-scale clinical studies will be indispensable in validating RT-PCR as a molecular tool in detecting the viral genome status of HPV. Even so, the use of RT-PCR and the E2/E6 ratio as a surrogate for HPV integration appears to be promising and may prove to be an essential clinical method in the future for determining the physical state of oncogenic HPV.

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15. Evans MF, Aliesky HA, and Cooper K. Optimization of biotinyl-tyramide-based in situ hybridization for sensitive background-free applications on formalin-fixed, paraffin-embedded tissue specimens. BMC Clin Pathol. 2003; 3:1-17.

16. Ermel A, Ramogola-Masire D et al. Invasive cervical cancers from women living in the United States or Botswana: differences in human papillomavirus type distribution. Infect Agent Cancer. 2014; 9: 1-8.

CURRICULUM VITAE

AISHA N. DAVIS

EDUCATION:

2012-2015: **M.S. Translational Science**
Indiana University

2010-2015: **M.D. Doctor of Medicine**
Indiana University School of Medicine

2007-2010: **M.S. Medical Science**
Indiana University School of Medicine

2001-2005: **B.S. Biology**
Spelman College

RESEARCH AND TRAINING EXPERIENCE:

Master of Science in Translational Research
Indiana University
Primary Investigator: Dr. Darron R. Brown, Department of Infectious Disease
MD/MS Fellowship, June 2012- June 2013
- Research aim: To describe the physical state of Human Papillomavirus (HPV) DNA in formalin-fixed paraffin embedded cervical tissue using two assays: DNA in situ hybridization and real time PCR. Evaluation of HPV physical state can provide greater understanding into pre-neoplastic lesions causing cancer years later; knowledge can guide early intervention and therapies.
- Performed DNA in situ hybridization on archival formalin-fixed paraffin embedded cervical tissue obtained from Africa (Botswana), the Caribbean (Jamaica) and Indiana University and Wishard Hospital in Indianapolis.
- Utilized fluorescent imaging and confocal microscopy to evaluate the physical state (episomal vs integrated) of HPV 16 and 18 in archival formalin-fixed paraffin embedded cervical tissue.

Cancer in the Under-Privileged, Indigent or Disadvantaged (CUPID) Research Program
Johns Hopkins School of Medicine
Primary Investigator: Dr. Sandra B. Gabelli, Department of Biophysics and Biophysical Chemistry
Summer Fellowship, June 2011 – August 2011
- Research Project included purification and attempted crystalization of Lactate Dehydrogenase A (LDH-A). LDH-A expression is up-regulated in metatstatic cancers and is overexpressed in invasive & hypoxic carcinoma. LDH-A is a potential target for anticancer therapeutics. Purpose of study was to purify and crystalize LDH-A in

the presence of competitive inhibitors.
- •Cloned and transformed LDH-A into BL21 (DE3) competent E.Coli cells . Purfied LDH-A via immobolized metal ion affinity chromatography and gel filtration chromatography.
- •Attempted crystalization of LDH-A using X-ray crystallography.

Master of Science in Medical Science
Indiana University School of Medicine
Primary Investigator: Dr. Jian-Ting Zhang, Department of Pharmacology and Toxicology
Senior Research Project, January 2010- May 2010
- •Research aim: To study the effects of protein expression, plasma membrane trafficking and efflux function of ABCG2, a multidrug resistant, ATP-binding cassette plasma membrane transporter.
- •Conducted cell culture experiments including the transfection of Human Embryonic Kidney (HEK293) cells with various subdomain swapping/ mutated ABCG2 constructs in order to study the oligomerization activity of ABCG2.
- •Utilized immunofluorescence staining and confocal imaging to determine the extraacellular location of the various mutated ABCG2 constructs.
- •Employed flow cytometry to study drug efflux activity of the various subdomain swapping/ mutated ABCG2 constructs in the presence of a chemotherapeutic substrate.

University of Mississippi Medical Center
Primary Investigator: Dr. Drazen Raucher, Department of Biochemistry
Research Assistant, June 2005 – May 2007
- •Research aim: To design thermally responsive polypeptides that only target tumorous cells as an alternative anticancer therapeutic.
- •Synthesized, expressed and purified thermally responsive Elastin-like polypeptide (ELP)- doxorubicin(dox) conjugate proteins. Conducted cell culture and ELP-dox conjugate polypeptide treatment in vitro experiments using human uterine, breast and ovarian carcinoma cell lines. Conducted drug efflux studies via flow cytometry using human uterine, breast and ovarian carcinoma cell lines. Conducted in vivo drug testing and toxicology tests in mice and rats.
- •Trained research technicians, graduate students and summer lab technicians in the use of laboratory equipment and experimental protocol.

PUBLICATIONS AND PRESENTATIONS:

Massodi I, Bidwell GL 3rd, Davis A, Tausend A, Credit K, Flessner M, Raucher D (2009) Inhibition of ovarian cancer cell metastasis by a fusion polypeptide Tat-ELP. Clinical Exp Metastasis 26 (3): 251-60

Bidwell GL 3rd, Davis AN, Raucher D (2008) Targeting a c-Myc inhibitory polypeptide to specific intracellular compartments using cell penetrating peptides. J Control Release 135 (1): 2-10

Bidwell GL 3rd, Davis AN, Fokt I, Priebe W, Raucher D (2007) A thermally targeted elastin-like polypeptide doxorubicin conjugate overcomes drug resistance. Invest New Drugs 25 (4): 313-326

Oral Presentation: "HPV Integration and its Role in Cervical Cancer," *Infectious Disease Research Conference*. Indianapolis, IN, April 2013.

Oral Presentation: "Toward the Structural Based Design of Inhibitors of *Homo sapiens* Lactate Dehydrogenase-A," *Johns Hopkins CUPID Research Symposium.* Baltimore, MD, August 2011.

HONORS, AWARDS, SCHOLARSHIPS:

Graduate/Medical School

Gold Humanism Honor Society Member   2014-Current
This national honor recognizes senior medical students,
residents, role-model physician teachers, and other
exemplars who demonstrate excellence in clinical care,
leadership, compassion, and dedication to service.

IUSM Medical Student Scholar   2010-2015
$10,000 merit-based scholarship awarded to incoming
 freshmen.

CTSI Research Fellowship Award   2012- 2013
$24,500 award funded by Indiana Clinical and Translational
Science Institute (CTSI) given to one or two highly motivated
2nd or 3rd year medical school students to complete 12months
of uninterrupted research while taking graduate school
courses to attain a Master's Degree in Translational Science.

CTSI ANU-IU Translational Science & Medicine Interchange   August 2012
Conference Award
All expense paid trip to Canberra, Australia funded by CTSI.
Award allowed CTSI MD/MS fellows an opportunity to attend
Australia National University (ANU) 3rd Annual Bootes course
in Translational Medicine. Conference granted MD/MS fellows
an opportunity to learn about translational research from a global
perspective.

Dr. George and Lula Rawls Scholarship Award   2010-2011
One of three minority students to receive a $1,000 scholarship
as an award of excellence for academic achievement.

Confucius Institute China Summer Abroad Scholar   May 2011
Awarded a merit-based scholarship to study in China for one
month.Visited hospitals and interacted with Chinese patients
and medical students. Also enrolled in Chinese language
courses at Sun-Yat-sen University in Guangzhou, China.

Undergraduate/High School
Beta Kappa Chi National Scientific Honor Society   2003-2005
Spelman College, Atlanta, GA

Alpha Epsilon Delta Pre-Medical Honor Society   2002-2005
Spelman College, Atlanta, GA

Dean's List   2002-2005
Spelman College, Atlanta, GA

Who's who Among College Students Award   2001-2002
Spelman College, Atlanta, GA

Certificate of Superior Achievement: Salutatorian Award   June 2001
Charlotte Amalie High School, St.Thomas, U.S. Virgin Islands
Graduated #2 out of a class of 325 students

U.S House of Representative: Certificate of Special Congressional June 2001
Recognition
Charlotte Amalie High School, St.Thomas, U.S. Virgin Islands
Salutatorian Congressional Recognition Award

VOLUNTEER EXPERIENCES:

IU Student Outreach Clinic (IU-SOC)   2012-2014
The IU-SOC is a non-profit student-run clinic dedicated to
providing free medical care and other services to the underserved
and uninsured of Indiana. As a medical student volunteer, I conducted
history and physical exams and performed phlebotomy.

Westside Community Health Fair   2010-2012
Annual health fair organized by Eskenazi Health and the IU
School of Medicine's Internal Medicine Student Interest Group,
provides free health screenings and information to underserved
members of Indianapolis. At the annual fair I have assisted in diabetes
and blood pressure screening, as well as DM type II and Hypertension
education.

Wheeler Mission   2010-2012
As a student volunteer at a non-denominational, Christian, social services

organization, which provides critically needed goods and services to the homeless, poor, and needy of central Indiana, I assisted in the preparation and serving of food.

Honduras Alternate Spring Break   March 2011
A week-long community service program in the small town of Rigores, Honduras. Part of a group of eight students and seven physicians that treated and cared for roughly 1,000 impoverished patients with limited access to healthcare.

Julian Center   2010-2011
Volunteered at Indiana's largest domestic violence emergency shelter and family justice center, which support victims of domestic violence and sexual assault. As a volunteer, I organized a health and nutrition arts and crafts day for the children. I also participated in monthly bible study and prayer.

LEADERSHIP:

Global Health Student Interest Group Hunger Banquet   2012-2013
Outreach and Recruitment Chair

Student National Medical Association   2010-2011
Community Service co-chair

PROFESSIONAL AFFLIATIONS:

National Health Care for Homeless Council   2014 – Current

Student National Medical Association   2011 – 2015