Expression of Human MicroRNAs and Oncogenes in HPV-16 and HPV-18 Transfected Keratinocyte Cell Line

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ABSTRACT

Background: Cervical Cancer is the second most common cancer next to breast cancer among women in the United States of America and is caused by the Human Papilloma virus (HPV). Genital HPVs are categorized into high-risk (HR) and low-risk (LR) types. Cervical cancer is largely attributable to persistent infection with HR-HPV types. HR-HPV types -16 and -18 are predominantly associated with 70% of cases of cervical cancers, whereas the two LR-HPV types -6 and -11 cause 90% of anogenital wart cases. **Objective of the study:** The aim of this study was to study the expression of microRNAs (miRNAs) and two oncogenes, E6 and E7, in keratinocytes transfected with HPV-16 and HPV-18. **Materials and Methods:** In this study, primary keratinocyte cell lines derived from the foreskin were transfected with the plasmids carrying HPV-16, HPV-18, or HPV-16 and HPV-18 together. Total RNA was extracted from transfected and un-transfected keratinocyte cells using miRNeasy Mini Kit. We then performed microarray profile of 84 human miRNAs using Human Cancer PathwayFinder PCR Array, and also studied the expression of E6 and E7 using qRT-PCR in transfected and un-transfected keratinocyte cells. **Results:** Cell culture revealed dysplastic cells, and increase in cell aggregation was more pronounced in keratinocytes transfected together with HPV-16 and HPV-18 than with either alone. Microarray profiling demonstrated that out of 84 miRNAs, a total of 50 miRNAs were up-regulated or down-regulated in keratinocyte transfected with HPV-16, HPV-18, or together. Out of the 50 miRNAs, hsa-miR-215-5p was highly up-regulated showing a 12 fold increase in expression in keratinocytes transfected with HPV-16 and HPV-18 together. This study also showed that E6 and E7 viral oncogenes were highly expressed in HPV transfected keratinocyte cells. The log10 relative mRNA abundance of E6 was 3.7 and 5.2 in HPV-16 and HPV-18 transfected keratinocytes, respectively. The relative mRNA abundance of E7 was 4.2 and 4.0 in HPV-16 and HPV-18 transfected keratinocytes, respectively. Interestingly, the relative mRNA abundance of E6 and E7 in keratinocytes transfected with HPV-16 and HPV-18 together was 5.0 and 3.6, respectively. **Conclusion:** In conclusion, our study suggests that expression of miRNAs can be used as a potential diagnostic tool for HPV infection.

INTRODUCTION

❖ The American Cancer Society estimates about 12,990 new cases of invasive cervical cancer will be diagnosed and about 4,120 women will die from cervical cancer in 2016. Cervical cancer is one of the leading causes of death among women in the United States (American Cancer Society 2016).

❖ Human Papillomavirus (HPV) is a small deoxyribonucleic acid (DNA) virus that infects the skin or mucosal cells. The genome is circular, double-stranded, and approximately 8-kB in length. The genome encodes for six early proteins (E1, E2, E4, E5, E6, and E7) that are responsible for virus replication and two late proteins (L1 and L2) that are viral structural proteins. Papillomaviruses infect keratinocytes in the basal layer of epithelium tissues and replicate in nucleus of infected keratinocytes (Zheng and Baker 2006).

❖ At least 13 or more than 100 known HPV genotypes can cause cervical cancer and are associated with other anogenital cancers and cancers of the head and neck. The two HR-HPVs (HPV-16 and HPV-18) cause approximately 70% of all cervical cancers (World Health Organization 2016).

❖ Each year, out 33, 000 new cases of cancer, HPV is known to cause about 26, 900 of these cases. HPV is also associated with genital warts and respiratory papillomatosis (Center for Disease Control and Prevention 2014).

❖ Groundbreaking evidence shows miRNA profiling exhibits great potential and promise in understanding the progression of HPV oncogenesis (Zheng and Wang 2011).

❖ Wang *et al.* in 2014 reported elevated expression of miR-16, miR-25, miR-92a, and miR-378 and decreased expression of miR-22, miR-27a, miR29a, and miR-100 due to viral oncoproteins E6 and E7.

RESEARCH OBJECTIVE

The objective of this research was to study the expression of cellular microRNAs (miRNAs) and two oncogenes, E6 and E7 in response to HPV-16 and HPV-18 transfection in a keratinocyte cell line.

MATERIALS AND METHODS

1. Preparation of Complete Keratinocyte Growth Media: Keratinocyte growth kit (Cat# PCS-200-040), 10 µg/mL Gentamicin + 0.25 µg/mL Amphotericin (Cat# ATCC-PCS-999-025), 10 Units/mL Penicillin + 10 µg/mL Streptomycin + 25 ng/mL Amphotericin B (Cat# PCS-999-002) and 33 µM of Phenol Red (Cat# PCS-999-001, ATCC, Manassas, VA).

2. Preparation of Primary Epidermal Keratinocyte Cells: The Primary Epidermal Keratinocyte Cells (Cat# PCS-200-010, ATCC, Manassas, VA) was added to complete Keratinocyte Growth Media.

3. Counting Primary Epidermal Keratinocyte Cells: Neubauer hemocytometer.

4. Cell Culture: Keratinocyte cells were cultured in complete Keratinocyte Growth Media and incubated at 37°C in 5% CO2.

5. Transfection: Keratinocyte cells were transfected with Plasmids (HPV-16, HPV-18, and HPV-16 & HPV-18 together, using Lipofectamine 2000 Reagent (Cat# 11668030, Invitrogen, Carlsbad, CA).

6. Dysplastic Cell and Cell Aggregation Formation: Transfected and un-transfected keratinocyte cells were monitored on regular basis and observed for dysplastic cells and cell aggregation formation for two weeks.

7. RNA Extraction from Transfected and Un-transfected Keratinocyte Cells: Total RNA was extracted using miRNeasy Mini Kit (Cat# 217004, Qiagen GmbH, Hilden, Germany).

8. Optical Density Readings: Total RNA quantitation for transfected and un-transfected was achieved by measuring the optical density at 260/280 nm using NanoDrop Lite (Thermo Scientific, Wilmington, DE).

9.cDNA Synthesis: cDNA synthesis was carried out using miScript® II RT Kit (Cat# 218160, Qiagen GmbH, Hilden, Germany), using template RNA from both transfected and un-transfected keratinocytes cells.

10. Mature MicroRNA Profiling: Mature miRNA quantification was carried out using miScript SYBR® Green PCR Kit (Cat# 218073, Invitrogen, Qiagen GmbH, Hilden, Germany).

11. Data Analysis: Mature miRNA expression data was carried using QIAEGN gene globe, generating fold expression.

12. E6 and E7 mRNA Expression: Expression of E6 and E7 in both transfected and un-transfected keratinocytes cells was carried out using iTaq™ Univeesal SYBR® Green One-Step Kit (Cat# 172-5151, Bio-Rad Laboratories, Inc., Hercules, CA).

RESULTS

Un-transfected and Transfected Primary Keratinocyte Cell Lines with HPV-16, HPV-18, and HPV-16 and HPV-18 Together

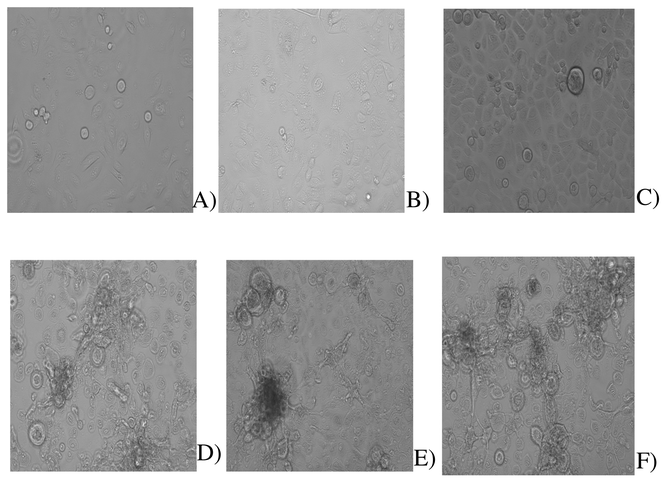

Figure 1. Un-transfected keratinocyte cells (A, B and C) show no change in morphology of the cells. Whereas, the keratinocyte cells transfected with HPV-16 (D), HPV-18 (E), and HPV-16 and HPV-18 together (F) show dysplastic cells and cell aggregation.

Table 1. MicroRNAs Up-regulated and Down-regulated in Keratinocytes Transfected with HPV-16, HPV-18 and Together

No.	MicroRNAs	Up-regulated and Down-regulated (Folds)		
		HPV-16	HPV-18	HPV-16+HPV-18
1	hsa-let-7a-5p	-2.1179	-1.0072	-1.2269
2	hsa-miR-133b	1.557	3.128	7.31
3	hsa-miR-122-5p	1.557	3.128	7.31
4	hsa-miR-20b-5p	-2.2621	-2.2241	-2.1396
5	hsa-miR-335-5p	1.2354	-1.0214	2.9327
6	hsa-miR-142-5p	2.3718	3.0411	7.1069
7	hsa-miR-96-5p	-1.1451	-1.8497	-2.2271
8	hsa-miR-184	1.6165	3.1282	7.3105
9	hsa-miR-15a-5p	-1.2836	-2.341	-1.9898
10	hsa-miR-205-5p	-2.2328	-2.2701	-3.4922
11	hsa-miR-181a-5p	-2.5729	-2.4209	-4.4663
12	hsa-miR-140-5p	-2.1128	-2.0487	-1.1044
13	hsa-miR-146b-5p	-2.6159	-1.462	-1.096
14	hsa-miR-193b-3p	-1.4721	-1.2185	-2.2323
15	hsa-miR-183-5p	-2.7444	-2.5484	-1.3626
16	hsa-miR-34c-5p	-3.789	-3.712	-1.5884
17	hsa-miR-138-5p	-1.8508	-3.1931	-5.3407
18	hsa-miR-373-3p	1.8199	3.5455	6.4179
19	hsa-miR-218-5p	-1.1289	1.1648	2.0048
20	hsa-miR-29b-3p	1.3262	-1.1071	-2.1402
21	hsa-miR-146a-5p	1.5569	3.1282	7.3105
22	hsa-miR-206	1.5569	3.1282	7.3105
23	hsa-miR-124-3p	-2.2177	-16.6537	-5.021
24	hsa-miR-21-5p	-2.5046	-1.3302	1.034
25	hsa-miR-100-5p	-2.4061	-1.026	-1.1746
26	hsa-miR-10b-5p	-2.3283	-1.7955	2.1762
27	hsa-miR-1-3p	1.5569	3.1282	7.3105
28	hsa-miR-150-5p	-2.0376	-1.0585	3.5813
29	hsa-miR-7-5p	1.6412	2.1858	1.5003
30	hsa-miR-191-5p	-2.0061	-1.0002	-1.1021
31	hsa-miR-9-5p	1.5569	3.1282	7.3105
32	hsa-let-7f-5p	-2.8238	-1.4172	-1.4992
33	hsa-miR-10a-5p	1.2872	1.6324	2.9117
34	hsa-miR-15b-5p	-2.143	-1.1423	-1.1495
35	hsa-miR-210-3p	1.6696	1.2317	-2.054
36	hsa-miR-17-5p	-1.8316	-2.321	-2.8256
37	hsa-miR-98-5p	-2.0734	-1.0733	1.0008
38	hsa-miR-34a-5p	-1.086	-1.0417	-2.4802
39	hsa-miR-144-3p	1.5569	3.1282	7.3105
40	hsa-miR-128-3p	1.1796	1.0763	2.092
41	hsa-miR-143-3p	1.1725	2.3559	5.5056
42	hsa-miR-215-5p	1.3538	2.7201	12.2071
43	hsa-miR-19a-3p	1.502	1.1425	-2.5882
44	hsa-miR-193a-5p	1.0281	-1.2577	-2.3183
45	hsa-miR-126-3p	1.2059	2.2345	-1.2903
46	hsa-miR-372-3p	-1.357	1.4807	3.4604
47	hsa-miR-149-5p	2.0047	2.1652	1.3673
48	hsa-miR-203a-3p	1.289	2.1404	2.0593
49	hsa-miR-32-5p	1.7932	3.1282	7.3105
50	hsa-miR-181c-5p	-3.0118	-2.8521	-5.1895

MicroRNA expression in HPV transfected keratinocytes was normalized with un-transfected keratinocytes and miRNA Transcription Control (miRTC)

E6 mRNA Level in Untransfected and HPV-Transfected Keratinocytes

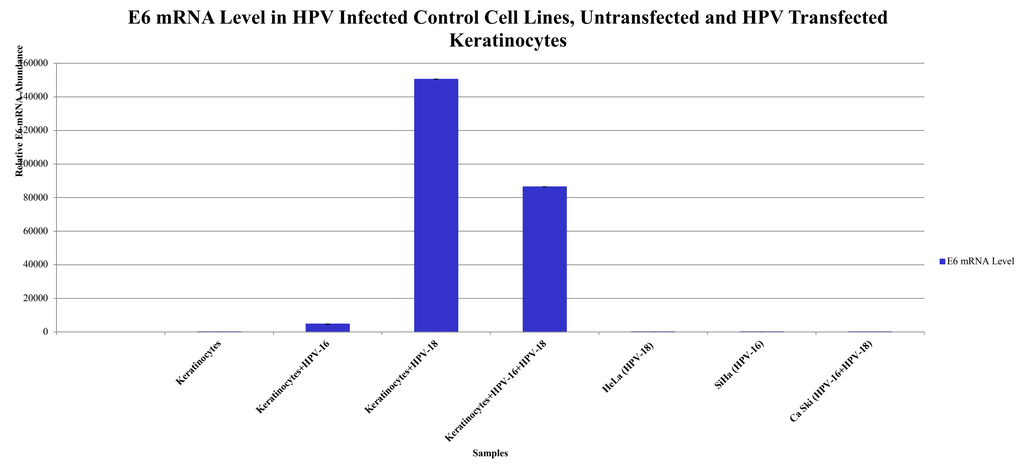

Figure 2. Expression of E6 in Un-transfected and HPV-Transfected Keratinocytes. Keratinocytes Transfected with HPV-18 show higher relative mRNA abundance for E6.

E7 mRNA Level in Untransfected and HPV-Transfected Keratinocytes

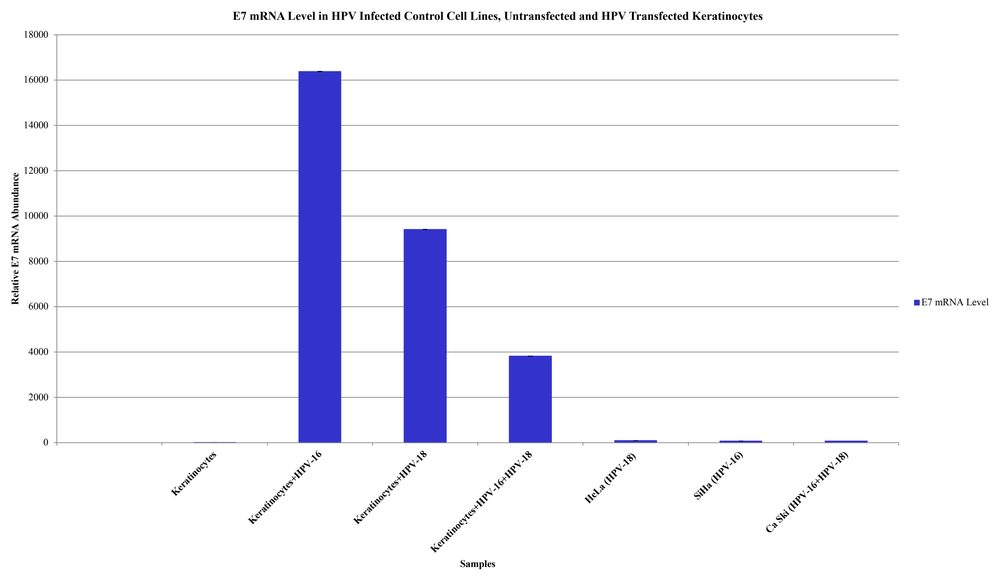

Figure 3. Expression of E7 in Un-transfected and HPV-Transfected Keratinocytes. Keratinocytes Transfected with HPV-16 show higher level of relative mRNA abundance for E7.

DISCUSSION

- In this project, miRNAs expression is regulated by oncogenic HPV16 andHPV18 infection in keratinocyte cultures and were comprehensively investigated by miRNA array analysis.
- We identified 50 miRNAs responsive to HPV-16, HPV-18, and HPV-16 &HPV-18 infection, including two RNAs that were significantly up-regulated and down-regulated.
- The expression of miR-215 displayed significant fluctuation over the course of transfection with HPV-16 and HPV-18.
- The expression of miR-124 displayed decreased expression over the course of transfection with HPV-18.
- The level of expression of E6 oncogene was higher with HPV-18 infection and HPV-16 and HPV-18 together.

CONCLUSION

- Over a 10 day period, keratinocyte cells showed dysplasia and cell aggregation was observed after transfection.
- The abundance of oncogenes, E6 and E7 in HPV transfected keratinocytes showed that these oncogenes are up-regulated contributing to the formation of dysplastic cells and aggregation of cells.
- New miRNAs were identified showing that they play a important role and could be used as a diagnositic tool for HPV infection.

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