Pathway Analysis using Gene-expression Profiles of HPV-positive and HPV-negative Oropharyngeal Cancer Patients in a Hispanic Population: Methodological Procedures

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Objective: The incidence of oral cavity and pharyngeal cancer in Puerto Rican men is higher than it is in the men of any other ethnic/racial group in the United States of America (US). The information regarding the effect of the human papilloma virus (HPV) in the gene-expression profile among patients with this cancer is limited in Hispanic community. We aim to describe the methodology for future studies to identify the molecular networks for determining overrepresented signaling and metabolic canonical pathways, based on the differential gene-expression profiles of HPV+ and HPV- samples from patients with oropharyngeal squamous cell carcinoma in Puerto Rico.

Methods: We analyzed the RNA expression of 5 tissue samples from subjects diagnosed with oropharyngeal squamous cell carcinoma, 2 HPV+ and 3 HPV-, using Affymetrix GeneChips. The relative difference between the average gene expressions of the HPV+ and HPV- samples was assessed, based on the fold change (log2-scale).

Results: Our analysis revealed 10 up regulated molecules (Mup1, LRP1, P14KA, ALYREF, and BHMT) and 5 down regulated ones (PSME4, KEAP1, ELK3, FAM186B, and PRELID1), at a cutoff of 1.5-fold change. Ingenuity Pathway Analysis showed the following biological functions to be affected in the HPV+ samples: cancer, hematological disease, and RNA post-transcriptional modification. QRT-PCR analysis confirmed only the differential regulation of ALYREF, KEAP1, and FAM186B genes.

Conclusion: The relevant methodological procedures described are sufficient to detect the most significant biological functions and pathways according to the HPV status in patients with oropharyngeal cancer in Puerto Rico. [*P R Health Sci J 2016;35:3-8*]

Key words: Network analysis, HPV, Oropharyngeal cancer, Puerto Rico, Gene expression, Microarrays

Oral cavity and pharyngeal cancer (OCPC) is the 4th and 12th most common cancer type in Puerto Rico (PR) among men and women, respectively (1). However, PR shows higher incidence of OCPC than any other racial/ethnic group in the US (3). The high incidence of OCPC represents a significant economic burden for PR. In 2004, the economic impact of OCPC in PR was approximately 6% (more than $3.6 million) of the total cancer costs (4).

Although a substantial percentage of OCPC is attributed to alcohol or tobacco (or both) exposure, approximately one quarter of the malignant cases in men and half of those in women in PR cannot be so attributed to these exposures (5). For example, previous studies indicate that the human papilloma virus (HPV) is a major risk factor for oropharyngeal squamous cell carcinoma (6,7). According to Kreimer et al.'s meta-analysis of the relevant data published in 2005 the prevalence in the

US of oral HPV infection in men and women aged from 14 to 69 years was 6.9% (95% CI: 5.7%–8.3%); for HPV type

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16 (HPV16), the prevalence was 1.0% (95% CI: 0.7%–1.3%) (8). It is estimated that the prevalence of HPV in patients with oropharyngeal cancer is about 36%, with HPV16 being the commonest type (~86%) in all HPV-positive (HPV+) tumors (8). This infection has been associated with age, sex, number of sexual partners, and current number of cigarettes smoked per day (9). However, clinically relevant HPV infection involves the oncoproteins coded by early viral genes E6 and E7. The E6/E7 mRNA method confirms the presence of transcriptionally active HPV-related head and neck squamous cell carcinoma, which has a strong predilection for the oropharynx and is strongly associated with high levels of p16 expression (10).

Most tumors with active HPV16 involvement have high p16 (INK4a) levels, low pRb levels, low cyclin D1 levels, and normal p53 levels; this pattern is significantly different from the pattern observed in HPV-negative (HPV-) tumors (11). Some HPV16+ tumors have been found to lack viral RNA expression patterns (11). In addition, HPV+ OCPC patients tend to have a better overall prognosis than do their HPV- counterparts, with HPV status appearing to affect therapeutic response and, consequently, the risk of progression is reduced and the survival rate is improved (12).

Molecular and epidemiological profiles have suggested that oropharyngeal squamous cell carcinoma (OSCC) comorbid with HPV+ is a distinctive disease entity from that which is comorbid with HPV-, differing both in terms of genome-wide expression profiles as well as in clinical outcomes (13). The full understanding of these differences in diverse populations represents an important step in the development of effective personalized treatments for this malignancy. However, molecular information regarding HPV infection and its effect on oropharyngeal cancer as pertains to Hispanics in PR and those the US is very limited, despite the fact that the incidence of this malignancy is rather high in PR (3). The aim of this study was to describe a methodology that could be used to identify molecular networks (algorithmically generated pathways with a complex interaction between genes) that can themselves be used to identify overrepresented signaling and metabolic canonical pathways, based on the differential gene-expression profiles between HPV+ and HPV- samples from OSCC patients in PR.

Methods

All the study procedures have been detailed in order to identify the molecular networks associated with the gene expression profiles between HPV+ and HPV- tumors of OSCC patients in PR. These networks identify particular genes (called *focus genes*) as representing significant biological function. The specific steps recommended to identify these types of genes are as follows:

i) Participant recruitment

The participants in this study were individuals with an initial diagnosis of OSCC who visited the HIMA San Pablo Hospital in Caguas, PR, from February through July 2012. The inclusion criteria were 1) being at least 21 years of age and 2) having undergone surgery as treatment for the diagnosed OSCC or having had a biopsy to confirm the original OSCC diagnosis. Individuals were excluded from the study if 1) they could not voluntarily consent to their participation in the study, 2) the period of time after their having been diagnosed with OSCC was greater than 12 weeks, or 3) they had a previous cancer diagnosis and/or had received treatment (radiotherapy and/or chemotherapy) before ascertainment and recruitment. Only those cases with a confirmed OSCC diagnosis were evaluated in this study (n = 7).

The sources of data were personal interviews (socio-demographic and risk-factor data), a medical record review (OSCC subsite and stages), and fresh tissue samples (HPV status and gene-expression profiling). Fresh tissue was collected during each participant's surgery or by performing a biopsy on him or her. Upon performing sample quality control, we ascertained that the samples from 2 of the 7 participants contained degraded RNA; thus, only 5 samples were analyzed.

All the participants consented to participate in the study, which was approved by the Institutional Review Board of the University of Puerto Rico Medical Sciences Campus (UPR-MSC) (protocol number: A9240112).

ii) HPV status

Human HPV DNA detection was performed via a nested-PCR strategy using PGMY09/PGMY11 as outer primers and GP5+/GP6+ as inner primers (14–16). Each reaction was carried out in a total volume of 25μl, containing 200 ng of gDNA, 12.5 μl of Bullseye HS-Taq 2X Master Mix (MIDSCI, St. Louis, MO), and 100 nmol/L of pooled PGMY09/11 primers. PCR was carried out by the activation of Bullseye polymerase (MIDSCI, St. Louis, MO) for 15 minutes at 95°C, followed by 35 cycles of 60 seconds each at 94°C, 60°C, and 72°C, with a final extension of 10 minutes at 72°C. Two microliters of the first-run PCR reaction were used as a template for the nested PCR. The conditions for the nested PCR (40 cycles) were identical to the first-run PCR, with an annealing step at 52°C for 60 seconds. The amplified products with the expected size (141 bp) were electrophoresed in 2% agarose gels and analyzed using a ChemiDoc imaging system (Bio-Rad, Hercules, CA).

iii) Gene-expression profiling and Validation by qRT-PCR

We analyzed the RNA expression profiles of 5 tissue samples from subjects diagnosed with OSCC, 2 HPV+ and 3 HPV-, using Human Gene 2.0 ST Affymetrix GeneChips, at the UPR-MSC Research Centers for Minority Institutions Program Center for Genomics in Health Disparities and Rare Diseases, using 100 ng of total RNA, as recommended by the manufacturer. RNA quality and integrity were assessed by UV absorption analysis (A260/A280) in a Nanodrop 1000 Spectrophotometer, and by microfluidic analysis, using the Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip Kit. Only RNAs that had acceptable A260/A280 ratios (from 1.7 to 2.1) and RNA

integrity numbers (RIN values of ~10) (17) were used for microarray analysis and validation by quantitative reverse transcription PCR (qRT-PCR). For qRT-PCR, 10 ng of total RNA were analyzed in duplicate with primer sets obtained from the Qiagen QuantiTect Primer Assay collection and the QuantiFast SYBR Green RT-PCR Kit (Valencia, CA) for amplification in a StepOne Plus Real-Time PCR System and the StepOne Software (Thermo Fisher Scientific Inc., Grand Island, NY) for the analysis of the qRT-PCR results. In order to obtain the fold changes (FCs) for all the genes evaluated for differential expression from HPV+ vs. HPV- tumor RNA samples, we used the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a housekeeping gene. The relative quantification values were calculated by the $\Delta\Delta C_T$ method (18).

From a total of 133,318 probesets, each gene's expression levels were obtained for each tissue sample using the Affymetrix GeneChip® Human Gene 2.0 ST Arrays. Then, the statistical method of principal component analysis (PCA) was used to identify potential clusters of subjects based on the HPV status (19). The cumulative variance of the first 2 components was 93.6%.

To assess the gene-expression profiling for every probeset, we initially assessed (with the information of the 5 tissue samples) the variability of this expression based on the coefficient of variation (CV) computed for the i-th probeset as follows:

$$CV_i = \frac{\text{standard deviation of the intensity}_i}{\text{mean of intensity}_i}$$

After discarding probesets with a CV greater than 50%, we explored the magnitude of the association between gene expression and HPV status among sample tissues of OSCC patients. The FC was computed for every probeset as follows:

$$Log_2 FC = Log_2 \left(\frac{\bar{Y}_{HPV+}}{\bar{Y}_{HPV-}} \right)$$

iv) Candidate genes

Once the FC was computed for every probeset, we identified a group of probesets for exploring the biological functions affected by the HPV status. The criterion for the selection of probesets was based on the FC; probesets with a log2FC greater than 1.5 or a log2FC less than -1.5 were included in the list of candidate genes.

v) Ingenuity pathway analysis

We used the IPA software to examine the potential biological functions affected by HPV status, based on the list of candidate genes, in OSCC. IPA transforms a list of genes into a set of relevant molecular networks (containing approximately 35 genes) that have been abstracted into a large network, called the Global Molecular Network, composed of thousands of genes and gene products that interact with each other. These networks optimize the interconnectivity between a set of user-specified genes (focus genes) that are also in the Global Molecular Network and likely represent significant biological function.

The IPA software provides as many interactions as possible between genes, to be most informative about how the genes in a given dataset appear to work together at the molecular level (www.ingenuity.com). Canonical pathways are generated prior to data input, based on the scientific literature, and do not change upon that input. IPA calculates significance based on the number of genes/molecules that map to a biological function, pathway, or network. The statistical significance assessment is performed based on the Fisher's exact test (20), where the null hypothesis is the proportion of genes mapping to the functions or pathways of the list of candidates genes equal to the proportion that map to the same functions or pathways in the entire population (total number of genes on the chip). The following 2x2 contingency table was prepared for this purpose:

Genes	Specific function	Other functions	Total
Candidate genes	a	(n_1-a)	n_1
Others	(m_1-a)	(n_2-m_1+a)	n_2
Total	m_1	m_2	N

$$p - value_{Fisher\ exact\ test} = \frac{(n1)!(n2)!(m1)!(m2)!}{n!a!(n1-a)!(m1-a)!(n2-m1+a)!}$$

where "a" indicates the number of candidate genes with specific function, "n_i," the marginal total by type of genes, "m_i," the marginal total of genes with specific functions, and "n," the total number of genes. A Fisher's exact test provided the probability of finding "a" genes with specific functions in the candidate genes from a set of n genes randomly selected from the Global Molecular Network. If the null hypothesis is rejected with some significance level (i.e., p-value<0.05), there will be evidence of biological effects in the HPV+ group different from what would be seen in the HPV- group.

vi) Ranking the networks

In order to rank the networks, a p-score was calculated based on the pFisher exact test-value, as follows:

$$p\text{-score} = -\log_{10}(p_{Fisher\ exact\ test}\text{-value})$$

The lower the p-value the higher will be the p-score. A p-score of 3 (p-value = 0.001) indicates that there is a 1 in 1,000 probability of getting a network containing at least the same number of network-eligible molecules that can be in networks from the Ingenuity Knowledge Base.

Results

Most of the participants were male (4 of the 5 individuals). The median age of the group was 65 years old. The tumors of

2 of the 5 participants were HPV+. The most frequent tumor site (60%) was the base of the tongue. Stage IV was commonly observed (4 of the 5 samples) in the tumors evaluated.

Based on the intensities of mRNA hybridization of the 133,318 probesets, the results of the PCA showed a high concentration of HPV+ tumors when the first 2 components were used; however, the HPV- tumors showed more dispersion (Figure 1). In addition, the FC used to explore the effect of HPV status showed that approximately 5% of the probesets indicated that the intensities of the HPV+ tissue samples were at least double (<-1 or >1 in $\log_2$ scale) those of the HPV- tissue samples (Table 1). Based on this result, 1758 probesets satisfied the criterion of selection for the network analysis of this study (<-1.5 or >1.5 in $\log_2$ scale).
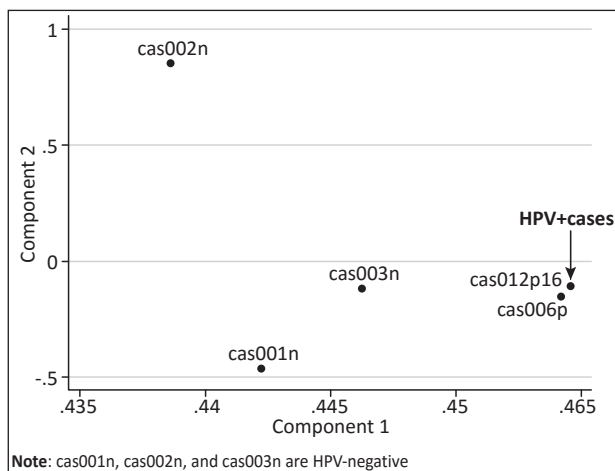

Figure 1. Categorization of gene expression in OSCC patients by HPV status, using the method of principal components

Shown in Figure 2 are the 3 most significant HPV-associated networks that were detected (using IPA), for OSSC. The results of the IPA indicated that the 5 most significant (p<0.001) HPV-associated diseases and disorders in the OSSC-containing samples were infectious and dermatological diseases, immunological and inflammatory diseases, and inflammatory response. In addition, according to the IPA results, the 5 most significant molecular and cellular functions in this group were cell death and survival, free-radical scavenging, cellular growth and proliferation, protein synthesis, and cellular development. The top up regulated and down regulated molecules that were reported by IPA, based on the FC in parentheses, are listed in Table 2.

Even though we attempted to validate all 10 genes listed in Table 2, only 9 of them could be adequately and efficiently amplified by qRT-PCR. Hence, results for relative quantification were obtained for 9 of the genes tested (Figure 3). The results of the quantitative reverse transcription PCR (qRT-PCR) validation procedure confirmed only the following 3 of those 9 genes: ALYREF, KEAP1, and FAM186B. The other 6 genes analyzed by qRT-PCR showed the differential regulation, but in the opposite sense of the expected difference.

Table 1. Number of probe sets according to the Fold change to compare mRNA expression between HPV-positive and HPV- negative individuals

Fold Change (Log 2 scale)	Probe sets	%
<-1.5	877	0.7
-1.5 to -1.0	4,706	3.5
-1.0 to 1.0	123,466	92.6
1.0 to 1.5	3,388	2.5
>1.5	881	0.7
Total	133,318	100.0

Discussion

Our results showed that differential expression is observed in HPV+ and HPV- samples of OSCC. According to the FC results, 877 and 881 probesets were downregulated or up regulated ($\log_2$FC<-1.5 and $\log_2$FC>1.5, respectively), so the ratio was close to 1. The relationship between up regulated and down regulated probesets was different from that described in the results of Lohavanichbutr et al. (13), where the ratio of up regulated probesets to down regulated probesets was 222/77 (2.88). However, our sample size for this preliminary study was much smaller than that of the Lohavanichbutr study (119 patients with OSCC) (13).

The most relevant pathway network showed that the biological functions affected in the HPV+ samples were cancer, hematological disease, and RNA post-transcriptional modification. The second most relevant pathway network showed that the biological functions affected in the HPV+ OSCC samples were RNA post-transcriptional modification, infectious disease, and cancer. The most prominent functions of the affected genes, according to Lohavanichbutr et al. (13), were DNA replication, DNA repair, and cell cycling, which are key cellular processes frequently dysregulated in cancer.

Jin and collaborators (21) showed that TNF-α played an important role in inflammation, immunity, and defense against infection and clearance of HPV. Our results, based on our IPA results, suggest that inflammatory disease was one of the 5 most significant HPV-related conditions in the OSCC samples.

A recent study of OSCC tumors from German OSCC patients suggests that ALH1A2, OSR2, GATA4, GRIA4, and IRX4 gene transcription is regulated by gene-promoter hypermethylation, which in turn correlates with HPV status in OSCC tumors (22). However, our results did not capture any of these genes in the network analysis based on our candidates list. Therefore, since our study had a small number of samples from OSCC patients with a Hispanic background, further studies are warranted to describe the biological functions affected by the HPV-infection status of OSCC patients and establish the methodological steps to investigate these functions in a Hispanic community.

Even though our study was performed with a small sample, it allowed us to describe a methodology that can be used to identify molecular networks that can themselves be used to identify overrepresented signaling and metabolic canonical
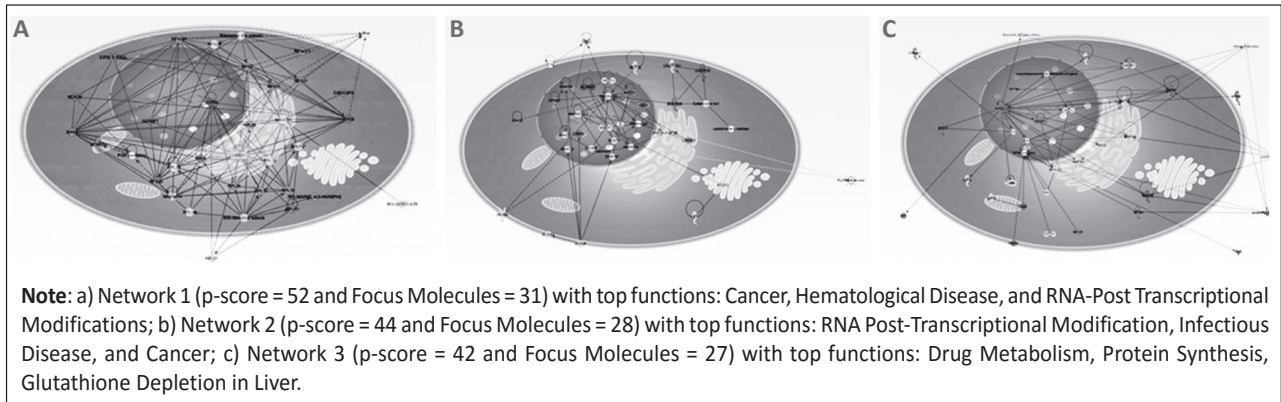
Note: a) Network 1 (p-score = 52 and Focus Molecules = 31) with top functions: Cancer, Hematological Disease, and RNA-Post Transcriptional Modifications; b) Network 2 (p-score = 44 and Focus Molecules = 28) with top functions: RNA Post-Transcriptional Modification, Infectious Disease, and Cancer; c) Network 3 (p-score = 42 and Focus Molecules = 27) with top functions: Drug Metabolism, Protein Synthesis, Glutathione Depletion in Liver.

Figure 2. Principal molecular networks detected

Table 2. Relative quantification among selected molecules

Characteristic	Molecules	Microarray FC	qRT-PCR RQ
Upregulated	Mup1	2.97	ND
	LRP1	2.97	0.82
	P14KA	2.96	0.61
	ALYREF	2.95	1.70
	BHMT	2.90	0.32
Downregulated	PSME4	-2.86	1.29
	KEAP1	-2.84	0.82
	ELK3	-2.78	0.96
	FAM186B	-2.69	0.33
	PRELID1	-2.65	2.36

RQ: relative quantification; FC: fold change; ND: not determined

Figure 3. Relative quantification among selected molecules

pathways, based on the differential gene-expression profiles between HPV+ and HPV- samples from OSCC patients in PR. We herein described, in a step-wise fashion, this methodology for those investigators who are doing specific genetic analyses of OCPC, but this methodology could be applied to other types of comparisons between tumor samples. Hence, we expect to expand this project by using a greater number of patients, as we aim to contribute to the improvement of oral health in Puerto Rico and in other regions having large Hispanic communities, any or all of which have high incidences of this malignancy.

Resumen

Objetivo: La incidencia de cáncer de la cavidad bucal y faringe entre los hombres puertorriqueños es más alta que la de cualquier otro grupo étnico/racial en los EE.UU. La información sobre el efecto del virus del papiloma humano (VPH) en la expresión genética de los pacientes con este cáncer es limitada en la comunidad hispana . En este manuscrito pretendemos describir la metodología para futuros estudios que permitan identificar las redes moleculares para determinar la señalización excesivamente y las vías canónicas metabólicas, con base en los perfiles de expresión genética diferencial entre las muestras VPH+ y VPH- de pacientes con cáncer de faringe en Puerto Rico. Métodos: Se analizó la e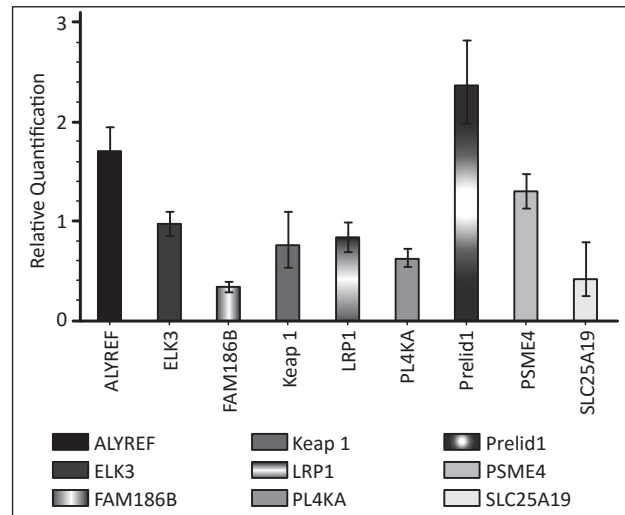xpresión de ARN de cinco muestras de tejido de pacientes con diagnóstico de OSCC, 2 VPH+ y 3 VPH-, usando GeneChips Affymetrix. Se evaluó la diferencia relativa del promedio de la expresión genética entre muestras con VPH+ y VPH-, basado en el *fold-change* (escala $\log_2$). Resultados: El análisis reveló 10 moléculas sobreexpresadas (Mup1, LRP1, P14KA, ALYREF y BHMT) y 5 subexpresadas (PSME4, KEAP1, ELK3, FAM186B y PRELID1) en un punto de corte de 1.5-*fold-change*. A través del software *Ingenuity Pathway Analysis* se demostró que las funciones biológicas afectadas por VPH+ fueron: cáncer, enfermedades hematológicas y ARN post-transcripcional modificación. El análisis QRT-PCR solo confirmó la regulación diferencial de los genes ALYREF, Keap1 y FAM186B. Conclusión: Los aspectos metodológicos más importantes son descritos para detectar las funciones y los procesos biológicos más significativos de acuerdo al estado de la infección de HPV en los pacientes con cáncer orofaringe en Puerto Rico.

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