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HPV status is associated with altered PIWI-interacting RNA expression pattern in head and neck cancer

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Introduction

Head and neck squamous cell carcinoma (HNSCC) affects over 550,000 patients worldwide each year ¹. Tobacco and alcohol consumption, together with human papillomavirus (HPV) infection are the main etiological factors ²⁻⁴. Many studies have found that patients with HPV-positive HNSCC have a better prognosis, with increased survival and less chance of recurrence ⁵⁻⁷. However, there have been contradictory reports showing either no or poor associations with outcome. Nevertheless, the growing proportion of HPV-positive cases poses an urgent need for prognostic biomarkers that guide patient management according to HPV status ^{3,6,8,9}.

Small RNAs, particularly microRNAs (miRNAs), have proven to be promising prognostic tools in HNSCC patients ^{10,11}. Piwi-interacting RNAs (piRNAs), an abundant class of small non-coding RNA that regulate genome stability ¹²⁻¹⁴, are emerging as a valuable addition to the category of small RNA biomarkers. Recently, piRNA expression patterns have been shown to be deregulated in a variety of cancer types, including head and neck tumors ¹⁵⁻²²; however, the relationship between piRNA expression and clinicopathological features in HNSCCs has yet to be elucidated. Interestingly, we have reported the observation of an association between piRNA expression and nodal metastasis in HNSCC ¹⁶.

Here, we describe the expression pattern of piRNAs in HNSCC using a custom pipeline to generate piRNA transcriptomes for more than 20,000 human piRNAs from 498 non-malignant and tumor tissues from HNSCC patients, and investigate their association with HPV infection status and patient survival. Our results reveal that specific piRNA expression patterns are defined by HPV status and virus type. Importantly, a piRNA expression signature was able to stratify patients based on overall survival, suggesting the potential utility of piRNA in assessing HNSCC patient prognosis.

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The authors declare no conflicts of interest.

Materials and methods

Sample Acquisition

A total of 498 HNSCC small RNA sequencing libraries (455 tumors and 43 matched non-malignant tissue) generated by The Cancer Genome Atlas (TCGA) consortium were acquired from the Cancer Genomics Hub (cgHUB) Data Repository (dbgap Project ID: 6208). The demographics and clinical data of these cases were obtained from the TCGA data portal and the USCS Cancer browser (Supplementary Table S1) .

Assembly of piRNA Transcriptome

A custom sequence analysis pipeline was developed to deduce piRNA expression from raw sequencing data (FASTQ files) ¹⁶. Briefly, our pipeline includes sample accrual, quality control and data quantification. Raw sequence BAM files received from the cgHUB were converted to FASTQ files using the Picard analysis package (<http://picard.sourceforge.net>) and quality trimmed. Poor quality bases (Phred quality score ≤ 20) as well as potential miRNA-associated sequences (read length < 24 bp) were removed. The remaining reads were re-mapped to human genome hg19 using STAR aligner ²³, with specific parameters for short reads mapping. piRNA were annotated using a custom piRNA reference transcriptome which was generated using the information from the functional RNA database (fRNAdb v 3.4) with corresponding NCBI accession IDs corresponding to piRNA sequences. Transcript abundance was determined using PartekFlow™ platform in conjunction with the Partek Genome Suite (Partek Inc., MO, USA). Expression values were represented using reads per kilobase of exon model per million mapped reads (RPKM) ²⁴. piRNA with RPKM values ≥ 1 in at least 10% of samples were considered as robustly expressed and included in further analyses.

Global Analysis of piRNA Expression Patterns

Expression matrices were aligned to clinicopathological features in order to perform comparisons between piRNA expressions levels. Statistical analysis between groups were carried out using the non-parametric Mann-Whitney U test followed by false discovery rate (FDR) correction through the Benjamini-Hochberg method ²⁵. Box plots were generated using Gene-E software (<http://www.broadinstitute.org/cancer/software/GENE-E/>). In all comparisons, a p-value ≤ 0.05 was considered significant. Circular representation of genome-wide piRNA expression in non-malignant and malignant HNSCC tissue was generated using the CircosPlot software ²⁶.

Survival Analysis

Overall and recurrence-free survival information for the 455 HNSCC cases was collected from the UCSC cancer genome browser (<https://genome-cancer.ucsc.edu>). The association of multiple piRNA with patient survival was first evaluated using a Cox proportional hazard model. Significantly associated piRNAs were used to calculate sample was multiplied by its corresponding Cox coefficient, to obtain an individual piRNA weight ²⁷. The probability of death from disease was calculated by summing all the individual piRNA weights for a given patient, and then the patients were ranked based on these probabilities. Kaplan-Meier

survival curves of patients belonging to the upper and lower tertiles were compared using the log rank test.

Results

piRNAs are Differentially Expressed in Head and Neck Non-Malignant and Tumor Tissue

To identify piRNAs that might delineate tumor and non-malignant tissues, we analyzed piRNA expression in 43 non-malignant and 455 HNSCC samples. While the number of piRNAs expressed in either types of somatic tissue is small relative to the total number of piRNAs in the genome, we found intriguing patterns of differential expression between HNSCC tumors and their non-malignant counterparts. A total of 305 piRNAs were robustly expressed in both non-malignant and tumor tissue (Figure 1, Supplementary Table S2). Globally, we observed that piRNA expression was considerably more abundant in tumors relative to non-malignant tissue. A total of 247 were significantly differentially expressed between non-malignant and tumor tissues (Mann-Whitney U-test, FDR-BH p-value = 0.05). Interestingly, 25 were exclusively expressed in non-malignant tissue, while 87 were only expressed in tumors.

piRNAs Are Differentially Expressed in HPV Positive and Negative Cases

We next focused our attention on piRNA expression patterns in malignant tissues. Since HPV infection is an important driving event in HNSCC tumorigenesis²⁸⁻³⁰, we investigated whether HPV status might be associated with a unique piRNA expression pattern. To this end, we compared the piRNA expression patterns between HPV-negative (HPV-, N=370) and HPV-positive (HPV+, N=83) tumors. Forty-one piRNAs were differentially expressed (Figure 2, Supplementary Table S3), with 30 being overexpressed in HPV+ cases and 11 overexpressed in HPV- samples.

Since HPV16 and HPV18 have the highest oncogenic potential, we assessed whether expression of any of these piRNAs might specifically associate with these HPV types. We found that 11 out of 41 piRNAs (26.8%) were differentially expressed between HPV16 or 18 positive patients (N=67) compared to cases harboring other HPV types 33, 35, and 56 (N=22 combined) (Mann-Whitney U-test, FDR-BH p-value = 0.05, Table 1). Remarkably, all these piRNAs were expressed at lower levels in HPV16/18 HNSCC samples (Supplementary Figure 1).

A piRNA Signature is Associated with Patient Outcome in HPV-Positive HNSCC

According to the literature, HPV+ patients have better overall survival compared to HPV- patients. Since we observed distinct piRNA expression patterns within the HPV+ cohort, we next evaluated the association of the 11 differentially expressed piRNAs with outcome. While we did not find any association with recurrence free survival, we found a five piRNA signature that was significantly associated with overall survival in these patients (p-value χ^2 test=0.002, Table 2 and Figure 3). Patients with high probability scores had significantly worse survival (log rank p-value=0.00092, Figure 4). We tested this signature in HPV- patients as well as all patients regardless of HPV status, and found no significant

association with overall survival in these groups, indicating this signature is specific to HPV + patients (Supplementary Table S4).

Discussion

Considerable advances have been made in the detection and chemoprevention of head and neck cancer^{4,31}; however, major challenges still remain regarding the improvement of patient management once the disease is diagnosed. Additionally, the interaction between biological risk agents, such as HPV, and cellular mechanisms are still not completely understood. In this proof of principle study, we show that another important component of the small RNA transcriptome, piRNA, is likely to play a role in patient outcome.

We assessed piRNA expression of 498 non-malignant and tumor tissues from TCGA consortium using a custom pipeline to assemble a piRNA transcriptome. Our findings revealed that, 1) piRNAs are differentially expressed between malignant HNSCC and non-malignant tissues, 2) piRNA expression patterns are specific to the HPV status of the tumor, and 3) a five piRNA expression signature can be used to predict HPV+ HNSCC overall survival.

It has been widely established that HPV+ HNSCC is a distinct disease and develops through different mechanisms than carcinomas with other etiologies^{3,32,33}. miRNA dysregulation has been previously implicated in the development and progression of HPV+ HNSCC tumors. For example, members of the mir106b~25 cluster were considerably up-regulated in HPV associated HNSCC; while mir-199-1 was highly downregulated in HPV+ patient samples³⁴. These HPV-associated miRNAs may play a mechanistic role in the distinct clinical behavior of HPV-infected tumors, and suggest potential utility of miRNAs as biomarkers^{34,35}. While initial studies have shown that HPV infection affects the expression of some small non-coding RNAs, such as miRNAs, its effect on piRNA expression is unknown. The association of piRNA with patient outcome has been previously demonstrated in other cancer types, such as breast and gastric, among others^{16,17,19}. Thus, it is of great relevance to characterize changes in piRNA expression patterns in the context of HPV status, as these piRNAs may represent novel predictors of clinical outcome in HPV+ HNSCC.

A deeper investigation of piRNA expression within malignant tissues revealed a group of piRNAs whose expression was significantly associated with the presence of oncogenic HPV types HPV16/18. One of the most highly differentially expressed piRNAs is FR140858 (DQ598918) (Table 1). The 31 nucleotides of the piRNA sequence displays 100% complementarity with positions 99,691,656 to 99,691,686 in chromosome 7, which encompasses the sequences of different transcripts of the minichromosome maintenance complex component 7 (MCM7) gene. Interestingly, the expression of MCM7 is induced through the activity of the HPV viral oncogene E7³⁶ and significantly correlates with HPV infection in cervical carcinoma³⁷. Although the mechanisms of piRNA-mediated silencing of mRNA targets are still emerging³⁸, the loss of the piRNA FR140858 in HPV16/18-associated HNSCC could potentially promote MCM7 expression, a possibility that may be assessed in future mechanistic studies. Although more validation studies are needed,

piRNAs hold great promise as potential prognostic biomarkers, owing to their small non-coding RNA features such as small size, stability in biofluids and archival materials, and variety of detection methods³⁹. Also, considering there are 10–25 times more piRNA species (20,000–50,000) than microRNAs, the impact of their deregulation is likely at least as relevant.

In summary, we have identified loci that actively express piRNA in non-malignant and malignant head and neck tissues. Although it is generally accepted that HPV+ HNSCC patients have a better prognosis compared to HPV– cases⁵⁻⁷, our results indicate this cohort can be further stratified based on piRNA expression, suggesting a potential clinical utility of piRNAs in head and neck cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights of results from “HPV status alters PIWI-interacting RNA expression pattern in head and neck cancer”

- piRNAs are differentially expressed between HNSC and non-malignant tissues
- piRNA expression patterns are specific to the HPV status of the tumor
- A piRNA expression signature can predict overall survival in HPV+ HNSC patients

Figure 1. Genome-wide expression of piRNAs in head and neck non-malignant and tumor tissue
The expression of 305 piRNAs in non-malignant (blue) and tumors (red) are presented in a circos plot, which relates piRNA genes to the chromosomal location.

Figure 2. HPV status and piRNA expression patterns

Comparison of piRNA expression patterns between HPV negative (average of 370 HPV–HNSCC tumor piRNA transcriptomes, purple bars) and HPV positive cases (average of 83 HPV+ HNSCC tumor piRNA transcriptomes, yellow bars).

Figure 3. Expression boxplots of the five survival-associated piRNAs (RPKM) in HPV+ tumors
On each boxplot, the average is denoted by a black diamond, the line within each boxplot indicates the median value, the border of the box indicates the 25th and 75th percentiles, and the dashed line represents the range.

Figure 4. A five piRNA signature delineates the outcome of patients with HPV+ HNSCC

The expression values of the piRNAs in the model were transformed into a score representing the probability of death by disease by multiplying the expression values of each piRNA by their respective Cox proportional hazard coefficient (Table 2), and then summing their values. The Kaplan-Meier curve shows patients with a high score (top 30th percentile) had worse survival than those with a low score (bottom 30th percentile).

Table 1

piRNAs differentially expressed in HPV positive cases with and without HPV16/18

Tag	NCBI Accession	Mean HPV16/ 18	Median HPV16/ 18	Mean other HPVs	Median other HPVs	p Value	Over- lapping gene
FR004819	DQ596390	103.47	40.41	204.02	136.97	0.00904	
FR018916	DQ597887	3.48	2.00	2.89	1.00	0.00936	RPL13A
FR136216	DQ598104, DQ598103	244.52	198.75	362.82	297.57	0.00417	PRRC2B
FR140858	DQ598918	2316.20	2366.00	3039.78	2636.00	0.04866	MCM7
FR197104	DQ601526	1.78	1.00	3.61	2.50	0.00266	RBM33
FR213383	DQ598648	99.87	53.59	176.97	143.06	0.00045	RPL3
FR222326	DQ597945	37.69	3.00	96.94	10.50	0.03806	
FR237180	DQ598649	40.15	10.81	151.98	40.15	0.00507	RPL3
FR245310	DQ596225	15.28	7.00	27.33	16.00	0.04054	
FR279668	DQ571955	8.69	6.00	10.78	8.00	0.01283	
FR298757	DQ570394	1.38	0.00	2.24	0.83	0.04258	

Table 2

Five piRNAs significantly associated with patient overall survival

Variable	Cox coefficient*	Standard error	Wald Chi- Square	Pr > Chi ²	Hazard ratio	Hazard ratio Lower bound (95%)	Hazard ratio Upper bound (95%)
FR018916	−0.125	0.058	4.636	0.031	0.882	0.787	0.989
FR140858	−0.001	0.000	8.241	0.004	0.999	0.999	1.000
FR197104	0.135	0.080	2.848	0.091	1.145	0.978	1.340
FR237180	0.003	0.001	5.580	0.018	1.003	1.001	1.006
FR298757	0.103	0.075	1.878	0.171	1.109	0.957	1.285

* Regression coefficient for piRNA association with patient overall survival. 11 piRNAs from Table 1 were initially tested for correlation with survival using a Cox regression model. A combination of the five piRNAs shown was significantly associated with patient overall survival.