Potentially Prognostic miRNAs in HPV-Associated Oropharyngeal Carcinoma

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Abstract

Purpose: Deregulation of miRNAs is associated with almost all human malignancies. Human papillomavirus (HPV)-associated oropharyngeal carcinoma (OPC) has a significantly more favorable outcome compared with HPV-negative OPCs; however, the underlying mechanisms are not well understood. Hence, the objectives of this study were to determine whether miRNA expression differed as a function of HPV status and to assess whether such miRNAs provide prognostic value beyond HPV status.

Methods: Global miRNA profilings were conducted on 88 formalin-fixed and paraffin-embedded (FFPE) OPC biopsies (p16-positive: 56; p16-negative: 32), wherein the expression levels of 365 miRNAs plus 3 endogenous controls were simultaneously measured using quantitative real-time (qRT)-PCR. Seven FFPE specimens of histologically normal tonsils were used as controls.

Results: Overall, 224 miRNAs were expressed in more than 80% of the investigated samples, with 128 (57%) being significantly differentially expressed between tumor versus normal tissues (P < 0.05). Upregulated miR-20b, miR-9, and miR-9/C3 were significantly associated with HPV/p16-status. Three miRNA sets were significantly associated with overall survival (miR-107, miR-151, miR-492; P = 0.0002), disease-free survival (miR-20b, miR-107, miR-151, miR-182, miR-361; P = 0.0001), and distant metastasis (miR-151, miR-152, miR-324-5p, miR-361, miR492; P = 0.0087), which retained significance even after adjusting for p16 status. The associated biologic functions of these miRNAs include immune surveillance, treatment resistance, invasion, and metastasis.

Conclusion: We have identified several miRNAs, which associate with HPV status in OPC; furthermore, three candidate prognostic sets of miRNAs seem to correlate with clinical outcome, independent of p16 status. Furthermore, evaluations will offer biologic insights into the mechanisms underlying the differences between HPV-positive versus HPV-negative OPC.

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Introduction

Over the past 2 decades, the incidence of human papillomavirus (HPV)-associated head and neck squamous cell carcinomas (HNSCC) involving the oropharynx has been increasing, nowadays comprising the majority of oropharyngeal carcinoma (OPC) cases seen in North America (1–3). There are several unique clinical characteristics of HPV-positive OPC, such as younger age at diagnosis, lower likelihood of heavy smoking or alcohol, and greater degree of sexual activity, compared with patients with HPV-negative OPC (1, 2). Intriguingly, HPV-positive patients experience a significantly superior clinical outcome when treated with either radiotherapy alone or combined chemoradiotherapy, despite presenting with higher grade and stage of disease (3). We have previously reported that approximately 60% of patients with OPC seen at the Princess Margaret Cancer Center were HPV-positive, with 3-year overall survival (OS) rates of 88% versus 67% in favor of HPV-positive versus negative patients (4). However, the biologic mechanisms behind this unique clinical entity of HPV-associated OPC remain to be elucidated.

Deregulation of miRNAs is clearly associated with the development and progression of human malignancies. We have conducted global miRNA profiling of HNSCC (5), through which the miR-375 metadherin axis was newly identified as a potentially important pathway that could partially explain the propensity for lung metastases in this
Translational Relevance

The incidence of human papilloma virus (HPV)-associated oropharyngeal carcinoma (OPC) has been increasing. HPV-positive OPCs have several unique clinical characteristics and with a significantly more favorable outcome, compared with HPV-negative OPCs. The biologic basis behind this differential outcome is currently unelucidated. Deregulation of miRNAs is associated with oncogenesis of various malignancies, suggesting that miRNA expression profiling have the potential to unravel the complex biology of human tumors. In this study, we evaluated the miRNA profiles of archival formalin-fixed and paraffin-embedded diagnostic biopsy specimens from 88 nonmetastatic OPC samples (p16+/ve:58; p16−/ve:34). We have identified a panel of p16/HPV-associated miRNAs and three potential miRNA signature sets that are associated with clinical outcome, independent of p16 status. Furthermore, examination of these candidate miRNAs will inform biologic insights into the mechanisms underlying the differences between HPV-positive and HPV-negative OPC.

Materials and Methods

Patient information

A subset of diagnostic formalin-fixed paraffin-embedded (FFPE) blocks from our previously published 111-sample OPC report was evaluated (4). That study showed that HPV status as defined by p16 immunohistochemistry (IHC), HPV16 in situ hybridization (ISH), or HPV16 E6 transcript levels using quantitative real-time PCR (qRT-PCR) were strongly concordant (4). Given that these were small diagnostic biopsies, only 88 samples had sufficient remaining tumor tissues to carry out global miRNA profiling.

RNA purification from FFPE samples

A representative section from each sample was stained with hematoxylin and eosin stain and reviewed by head and neck cancer pathologists (B. Perez-Ordonez or I. Weinreb) to identify regions containing more than 70% malignant epithelial cells for macrodissection. Seven normal tonsillar epithelial FFPE tissues from individuals who underwent a tonsillectomy were included as controls. University Health Network (UHN, Toronto, Canada) Institutional Research Ethics Board approval has been obtained for this study. All blocks were processed randomly, with clinical outcome unknown, to avoid experimental bias. Total RNA enriched for small RNA species was isolated using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE samples (Ambion), according to the manufacturer’s instructions (15).

MiRNA profiling using TaqMan low density array

Quality of RNA samples was assessed by qRT-PCR analysis of the endogenous control RNU44 using TaqMan microRNA Assay (Applied Biosystems), as previously described (6, 15). Global miRNA expression on 88 OPC and 7 normal samples was conducted using the TaqMan Low Density Array (TLDA) Human MicroRNA Panel v1.0 (Applied Biosystems), which enabled the simultaneous quantification of 365 human miRNAs plus 3 endogenous controls (RNU16B, RNU44, and RNU48). In brief, total RNA of each sample was first reverse-transcribed with the Multiplex RT pool set, then quantitated with a TLDA array using an Applied Biosystems 7900 HT Real-Time PCR system, with the C_t values determined by threshold method, according to the manufacturer’s protocol (15).

Data processing

The TLDA data were processed and analyzed as previously described (15) with some minor modifications. Any miRNAs with undetermined values in more than 80% of the tumors and more than 6 “normals” were eliminated from the analysis. Undetermined C_t values, or C_t values more than 36, were imputed to 40. All samples were normalized by the mean of the endogenous controls and converted into a ratio of abundance compared with the geometric mean of the abundance of the 7 normal samples, as we have previously described (15).

Significant differences in miRNA expression between tumor and normal samples were assessed using the Wilcoxon rank sum test, with multiple comparisons adjusted by Benjamini–Hochberg false discovery rate (FDR) correction, conducted in the R statistical environment (v2.6.1; ref. 16). Two-way ANOVA and the Benjamini–Hochberg correction for FDR of multiple testing were conducted on each miRNA to investigate the association with p16 and HPV status. The Spearman correlation coefficient was used to calculate P values of the consistency of the miRNA signals by using p16 and HPV as outcomes, for each miRNA.

To explore potential prognostic effects, we applied univariate survival analysis to detect association of single miRNA expression level with OS, disease-free survival (DFS), or distant relapse-free survival (DM) as previously described (4). After identifying potentially important miRNAs (P < 0.05), we applied multivariate models on these miRNAs. Model selection procedure was conducted using a stepwise selection algorithm, conducted separately for each outcome. Finally, after model selection, additional analyses were conducted to generate a risk score based on the significant miRNAs identified from the multivariate
analysis. The risk score was based on the weighted combination of the miRNAs with the estimated Cox proportional hazard regression model coefficient as the weight (17). For each outcome, patients were dichotomized into 2 categories of low (risk score < median), or high risk (risk score > median); following which, outcomes were compared for each risk group. Test of difference between the risk categories was assessed using the log-rank test or Cox proportional hazard regression model, with $P$ values, and HRs (including 95% confidence interval (CI)).

To validate the prognostic effect, we carried out internal validation procedures using a bootstrap algorithm (18) and constructed the bootstrap CI. The bootstrap is one of the resampling techniques, and bootstrap datasets were created by sampling with replacement. The bootstrap method has been shown to provide a valid estimation of prediction error and can correct for the bias of the parameter estimate (18). We applied bootstrap based on 500 replications; these results were obtained using the PROC SURVEYSELECT procedure in SAS version 9.3, and presented as the bootstrap HR CIs of the prognostic effect adjusted for p16 status.

### Results

**Differentially expressed miRNAs in OPC**

Genome-wide miRNA profiles of 88 OPC patient samples were conducted; the clinical characteristics are provided in Table 1. The median follow-up time for all patients has now extended to 5.9 years, with 5-year OS, and DFS rates at 55% and 52%, respectively (Supplementary Fig. S1). The 5-year OS for HPV-positive versus HPV-negative patients were 65% versus 30%; the DFS rates were 70% versus 20% respectively.

Among the 365 interrogated miRNAs, 224 were expressed in more than 80% of the samples, with 128 being significantly differentially expressed between the 88 OPC and 7 normal tonsillar epithelial tissues ($P < 0.05$). Ninety-two of these 128 miRNAs were more than 2-fold differentially expressed (Supplementary Fig. S2), with the majority (120/128; 94%) being upregulated in OPC (Supplementary Table S1). A detailed comparison of the top 6 most significantly differentially expressed miRNAs (miR-21, let-7g, miR-25, let-7f, miR-130b, and miR-151) are provided in Fig. 1. These dysregulated miRNAs seemed to be randomly distributed among different chromosomal regions, with the exception of 1p34.2 and 7q22.1, within which 6 of the top 40 aberrantly expressed miRNAs are located (bold information in Supplementary Table S1).

**miRNAs associated with HPV/p16 status**

The miRNAs associated with either HPV16 ISH or p16 IHC were next investigated. Comparison of the 2 sets of miRNAs showed that the fold change in miRNA expression defined by either HPV ISH, or p16 IHC, were highly correlated ($R^2 = 0.78$; Supplementary Fig. S3). Figure 2 compared the miRNAs that were significantly associated with p16 IHC or HPV16 ISH as defined by the $-\log_{10} (P)$ values, again showing a strong correlation ($R^2 = 0.8$). Using a cutoff of $P < 0.01$, 9 miRNAs were significantly associated with both p16 IHC and HPV16 ISH-positive OPCs, which included upregulated miR-20b, miR-9, miR-9$_3$, miR-492, miR-545, miR-591, miR-422a, and downregulated miR-193b and miR-107. Because p16 IHC is a broadly accepted surrogate marker for HPV status, shown in multiple studies to correlate with patient outcome (1, 4), subsequent analyses were based on the p16 IHC data for these patients.

Validation of the HPV/p16-associated miRNAs (Supplementary Table S2) was conducted using the samples from an independent HNSCC cohort that we had previously profiled, which contained 11 p16 positive and 8 p16 negative OPC samples (5). Seven of the top 10 most significant HPV/p16-associated miRNAs were also included in the previous list of investigated miRNAs of HNSCC profiles (5). As shown in Fig. 3, miR-9 and miR-9$_3$ retained statistical significance ($P = 0.04$); the remaining 5 miRNAs were also expressed.

### Table 1. Clinical description of the 88 patients with OPC

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>HPV positive ($n = 56 ; 64%$)</th>
<th>HPV negative ($n = 32 ; 36%$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>41 (73%)</td>
<td>24 (75%)</td>
<td>0.95</td>
</tr>
<tr>
<td>Female</td>
<td>15 (27%)</td>
<td>8 (25%)</td>
<td></td>
</tr>
<tr>
<td>Age (median; range)</td>
<td>Mean: 55 ± 11 y (range: 27–80 y)</td>
<td>Mean: 67 ± 11 y (range: 46–93 y)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>1 (3%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2 (5%)</td>
<td>7 (22%)</td>
<td>0.003</td>
</tr>
<tr>
<td>III</td>
<td>5 (12%)</td>
<td>7 (22%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>49 (83%)</td>
<td>17 (53%)</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>26 (46%)</td>
<td>26 (81%)</td>
<td>0.003</td>
</tr>
<tr>
<td>CRT</td>
<td>30 (54%)</td>
<td>6 (19%)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: C-RT, chemoradiotherapy; RT, radiotherapy.
consistently aberrantly expressed, but did not reach statistical significance, likely due to the small sample size of the validation cohort.

**Potential prognostic miRNA sets**

Using the miRNA profiles of these 88 samples, with their median value as the cutoff, and treated as binary predictors, 3 potential miRNA signature sets were identified that were associated with OS, DFS, and DM (Fig. 4). The candidate prognostic panel for OS was defined by upregulated miR-107 and miR-151, and downregulated miR-492 (Fig. 4B; \(P < 0.0001\)). For the DFS set, this composed of upregulated miR-107, miR-151, miR-182, and miR-361 with downregulated miR-20b (Fig. 4C; \(P \leq 0.0001\)). The DM set was defined by upregulated miR-151, miR-361 and miR-324-5p, as well as downregulated miR-492 and miR-152 (Fig. 4D; \(P = 0.0088\)). Even after adjusting for p16 IHC status, these \(P\) values still retained significance (adjusted \(P\) values were 0.0002, 0.0001, and 0.0087 for OS, DFS, and DM, respectively).

**In silico analysis**

We proceeded to investigate the putative biologic functions of these aforementioned miRNAs using *in silico* analysis (Fig. 5). For miRNAs differentially expressed in OPC (Supplementary Table S2), miR-30c, miR-30e-3p, and miR-30e-5p all resided within the fifth intron of NFYC on chromosome 1p34.2 (19). NFYC is the nuclear transcription factor Y-γ, which binds with CCAAT motifs in the promoter region of several genes, associated with MHC class II determinants in immune response (Fig. 5A; ref. 19). As described in our previous publication (5), overexpression of miR-25, miR-93, and miR-106b and its host gene MCM7 can be regulated by E2F1, interfering with TGF-β signaling in HNSCC. Furthermore, the miR-106b-25 cluster has been reported to target PTEN (20).

Six of the HPV/p16-associated miRNAs (miR-381, miR-412, miR-380-5p, miR-487b, and miR-382) are all located at a common fragile site (FRA) at 14q32.3 (Fig. 5B; refs. 21, 22). Aberrations of 14q32.31 miRNA cluster have been previously described in human malignancies with an emerging role in immune regulation (23, 24). MiR-9 was found to be induced by lipopolysaccharide-inflammatory stimuli mediated by the proinflammatory cytokines IL-1β and TNF-α (25). Moreover, expression of miR-34a can be induced by p53 under genotoxic stress (26), and miR-20b has been described to target the 3’ untranslated region of hypoxia-inducible factor-1α and VEGF (27).

Overexpression of miR-151 with its host gene protein tyrosine kinase 2 (PTK2/FAK) has been reported to induce tumor invasion and metastasis in hepatocellular carcinoma (HCC; Fig. 5C; ref. 28). It also acts synergistically with FAK to enhance HCC cell motility and spreading (28, 29). Several miR-152 targets have also been identified, including the DNA methyltransferase DNMT1, E2F3, MET, and Rictor (30). Moreover, miR-182 was reported to be upregulated in...
miR-20b, miR-9, and miR-9
tumour suppressors such as
aberrant miRNAs in HNSCC, known to target multiple
miRNA. MiRNA-21 is one of the most consistently reported
tified, with miR-21 being the most significantly upregulated
miRNAs, respectively. These 2 sets of
ISH-associated miRNAs, and
metastasis (34).
miR-107 has been associated with mammalian develop-
ment (31), associated with both
metastasis (32) and poor clinical outcome (33). Finally,
miR-107 has been associated with mammalian development and cellular metabolism (34).

**Discussion**

We have conducted a global miRNA profiling study focused strictly on OPC, with approximately two-third being HPV-positive and one-third being HPV-negative. A panel of miRNAs significantly differentially expressed between tumor versus normal tissues (P < 0.05) was identified, with miR-21 being the most significantly upregulated miRNA. MiRNA-21 is one of the most consistently reported aberrant miRNAs in HNSCC, known to target multiple tumour suppressors such as PTEN and TP53 and Bcl-2 (5, 7, 10, 12). Upregulated miR-20b, miR-9, and miR-9* were also significantly associated with HPV/p16-status. Furthermore, there were 3 candidate miRNA sets that were associated significantly with OS, DFS, and DM, even after adjusting for HPV status.

Similar to our previous HNSCC miRNA study (5), upregulation of the miR-106b-25 cluster was also observed in these OPC samples. These included the nonrandom over-expression of miR-25, miR-93, and miR-106b, located on chromosome 7q22.1, within the intronic region of MCM7. Overexpression of MCM7, as well as regulation of the miR-106b-25 cluster and MCM7 by E2F1, with subsequent interference of TGF-β signaling in HNSCC, has been previously noted (5). Interestingly, TGF-β polymorphisms have been reported to be a susceptibility marker for HPV16 status amongst patients with OPC (35). Furthermore, the miR-106b-25 cluster can also target PTEN (20), which in turn will activate AKT, which correlates with adverse outcome for patients with OPC (36). Hence, dysregulation of MCM7 and the miR-106b-25 cluster could lead to OPC development via aberrant TGF-β and PTEN signaling (Fig. 5A).

Many of the p16-associated miRNAs were located at common FRAs, such as 1p, 1q, 5q, 8p, 14q32.31, 16p13.12, 17p, and Xq (Supplementary Table S2; ref. 21). This is consistent with previous studies reporting that the majority (53%) of deregulated miRNAs were located in cancer-associated genomic regions or FRAs (21). Chromosome 14q32.31 is the only nonrandom region that harbored 5 (miR-381, miR-412, miR-380-5p, miR-487b, and miR-382) of the p16-associated miRNAs, which is also one of the most common HPV integration sites in cervical cancer (22). In fact, a cluster of 46 miRNAs (3.2% of 1,426 human miRNAs; http://genome.ucsc.edu/cgi-bin/hgTracks) have been mapped to this small region of approximately 45 kb. Aberrations of the 14q32.31 miRNA cluster has been documented in many human cancers and its role in immune surveillance is an emerging area of evaluation (Fig. 5B; ref. 23, 24). Hence, in OPC, frequent aberrant expression of these p16-associated miRNAs at 14q32.31 might relate to this being a preferential HPV integration site. This postulate would be supported by the recent emerging exome sequencing data from TCGA wherein 14q32.3 amplifications have been noted for HPV-positive HNSCC (https://wiki.nci.nih.gov/display/TCGAM/03-08-12+HNSCC+AWG).

Enhanced immune response induced by HPV has been suggested as one possible mechanism for the superior outcome of HPV-associated OPC (37). Among the p16-associated miRNAs, several have been previously described to be associated with immune regulation, such as miR-9, miR-9*, miR-146a, miR-34a, and miR-155 (Supplementary Table S2 and Fig. 5B; ref. 38). Upregulation of miR-9 and its passenger strand miR-9* were among the most significantly associated miRNAs with both OPC, and specifically p16-positive OPC. Overexpression of miR-9/9* has been described in other human cancers (39), with miR-9 potentially involved in immune response during inflammatory stimuli (25). In addition, miR-9 might also have an oncogenic role in mediating angiogenesis and metastasis by inducing Myc to target E-cadherin, priming cancer cells for epithelial–mesenchymal transition (39). MiRNA-20b was the most significant p16-associated miRNA in OPC, and its downregulation was associated with poor DFS (Fig. 4 and 4C). The potential tumor suppressor role of miR-20b has been investigated wherein low circulating levels of miR-20b was reported in lung cancer and mantle cell lymphoma, also associated with poor outcome (40). Inhibition of miR-20b can lead to an increase HIF-1α and VEGF under normoxia; conversely, an increase of miR-20b in hypoxic tumor cells decreased HIF-1α and VEGF (27). This negative regulatory loop between HIF-1α and miR-20b has been suggested as one of multiple mechanisms by which tumor cells can rapidly adapt to varying oxygen concentrations (27).

MiR-34a was found to have higher expression levels in HPV-positive OPC, which is distinctly different from cervix cancer whereby miR-34a is downregulated by E6 via destabilization of p53 (41). In OPC, the relationship between
p53 and HPV remains unclear (4, 42). Our previous study observed p53 to be overexpressed on IHC in 63% of cases and only borderline association with HPV positivity (4). Expression of miR-34a was shown to be induced by p53 under genotoxic stress conditions (Fig. 5B; ref. 26). Furthermore, there is a positive feedback loop wherein p53 induces miR-34a; in turn, miR-34a activates p53 by inhibiting SIRT1, leading to an increase in miR-34a expression in cells with wild-type p53, resulting in enhanced apoptosis (43). This might, in part, explain the high incidence (70%) of OPCs harboring p53 immunoexpression, particularly in association with p16 (4).

Finally, in relation to the potential miRNA signature associated with clinical outcome, upregulation of miR-151 was the only miRNA associated with all of OS, DFS, and DM (Fig. 4A), as well as being among the top 10 significantly deregulated miRNAs in OPC (Supplementary Table S1). Its chromosomal location on 8q24.3 is frequently amplified in human cancers, including HNSCCs (44). Overexpression of miR-151 has been reported to promote invasion and metastasis in HCC, by targeting the putative metastasis suppressor RhoGDIA, which in turn synergized with FAK to enhance motility (28, 29). Downregulation of miR-152 is another well-characterized tumor suppressor miRNA commonly inactivated by promoter hypermethylation (30, 45), targeting several known important mediators of tumor progression (Fig. 5C; ref. 30). It resides in the intron of the host gene Coatomer protein complex \( \zeta \) (COPZ2), which is also a putative tumor suppressor (46).

Figure 3. Validation of the most significant p16-associated miRNAs was conducted by using the OPC samples from an independent HNSCC cohort \( N = 19 \). The top 7 miRNAs were investigated (miR-20b, -9, -9*, -193b, -422a, -107, and 142-3p); both fold change and \( P \) values are presented. "\( -\Delta Ct\)" refers to the differences between the measured miRNAs compared with the average \( Ct \) values of the endogenous controls (RNU44, 48, and 6B).
multidrug-resistant cell lines (31). It has been implicated in metastasis and poor survival in cancers (32, 33). Overexpression of miR-107 was observed to be associated with worse outcome (Fig. 4) and inversely related to HPV-positivity in OPC (Supplementary Table S2). It is a member of the miR-15/107 miRNA gene group associated with mammalian development (34), as well as regulation of the miRNA processing machinery and cellular metabolism.
HPV-Associated miRNAs in Oropharyngeal Carcinoma

Conclusion
We have conducted a comprehensive miRNA profiling study focused strictly on OPC, which identified 3 potential miRNA signature sets associated with clinical outcome, independent of HPV status. The emerging picture of these dysregulated miRNAs points to a complexity of pathways involved in immune response, and tumor progression, located within FRA and HPV integration sites. Furthermore, examination of these candidate miRNAs will inform biologic insights into the mechanisms underlying the differences between HPV-positive and HPV-negative OPC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References

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