Potentially Prognostic Micro-RNAs in HPV-Associated Oropharyngeal Carcinoma

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Running Head: HPV associated miRNAs in OPC

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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 favourable outcome, compared to HPV-negative OPCs. The biological basis behind this differential outcome is currently un-elucidated. Deregulation of micro-RNAs (miRNAs) is associated with oncogenesis of various malignancies, suggesting that miRNA expression profiling have the potential to unravel the complex biology of human tumours. In this study, we evaluated the miRNA profiles of archival formalin fixed and paraffin embedded diagnostic biopsy specimens from 88 non-metastatic 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. Further examination of these candidate miRNAs will inform biological insights into the mechanisms underlying the differences between HPV-positive and HPV-negative OPC.
ABSTRACT

PURPOSE

Deregulation of micro-RNAs (miRNAs) is associated with almost all human malignancies. Human papillomavirus (HPV)-associated oropharyngeal carcinoma (OPC) has a significantly more favourable outcome compared to 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 RT-PCR. Seven FFPE specimens of histologically normal tonsils were utilized as controls.

RESULTS

Overall, 224 miRNAs were expressed in >80% of the investigated samples, with 128 (57%) being significantly differentially-expressed between tumor vs. normal tissues (p<0.05). Up-regulated miR-20b, miR-9 and miR-9* 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 biological 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 appear to correlate with clinical outcome, independent of p16 status. Further evaluations will offer biological insights into the mechanisms underlying the differences between HPV-positive vs. HPV-negative OPC.
INTRODUCTION

Over the past two decades, the incidence of Human Papilloma Virus (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 to HPV-negative OPC patients (1, 2). Intriguingly, HPV-positive patients experience a significantly superior clinical outcome when treated with either radiation therapy (RT) alone, or combined chemo-RT, despite presenting with higher grade and stage of disease (3). We have previously reported that ~60% of OPC patients seen at the Princess Margaret Cancer Center were HPV-positive, with 3-year overall survival (OS) rates of 88% vs. 67% in favour of HPV-positive vs. negative patients (4). However, the biological mechanisms behind this unique clinical entity of HPV-associated OPC remain to be elucidated.

Deregulation of micro-RNAs (miRNAs) is clearly associated with 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 disease (6). Additional miRNA studies in HNSCC have reported potentially predictive sets of miR-205 and let-7d, or miR-210 as a hypoxia marker (7-10). MiRNAs associated with OPC have been reported previously; however, overlap in these candidate prognostic miRNAs has remained limited, as well as lack of concordance in HPV-associated miRNAs (11-14). Hence, to better understand the role of miRNAs in HPV-associated OPC, we conducted a comprehensive miRNA profiling
focused solely on OPC, using the same samples that have been previously analyzed for HPV status (4).

MATERIALS & 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 demonstrated that HPV status as defined by p16 immunohistochemistry (IHC), HPV16 in-situ hybridization (ISH), or HPV16 E6 transcript levels using qRT-PCR were strongly concordant (4). Given that these were small diagnostic biopsies, only 88 samples had sufficient remaining tumour tissues to perform global miRNA profiling.

RNA Purification from FFPE Samples

A representative section from each sample was stained with H&E, and reviewed by a head & neck cancer (HNC) pathologist (B P-O or I W) to identify regions containing >70% malignant epithelial cells for macro-dissection. Seven normal tonsillar epithelial FFPE tissues from individuals who underwent a tonsillectomy were included as controls. University Health Network (UHN) Institutional Research Ethics Board (REB) 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, Austin, TX), 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 performed using the TaqMan Low Density Array (TLDA) Human Micro-RNA Panel v1.0. (Applied Biosystems, CA, USA), which enabled the simultaneous quantification of 365 human miRNAs plus 3 endogenous controls (RNU6B, 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 Ct 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 >80% of the tumors, and >6 “normals” were eliminated from analysis. Undetermined Ct values, or Ct values >36, were imputed to 40. All samples were normalized by the mean of the endogenous controls, and converted into a ratio of abundance compared to the geometric mean of the abundance of the seven 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, performed in the R statistical environment (v2.6.1) (16). Two-way ANOVA and the Benjamini-Hochberg correction for FDR of multiple testing were performed 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-value <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 performed 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 two 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 hazard ratios (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 performed; 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 1). The 5-year OS for HPV-positive vs. negative patients were 65% vs. 30%; the DFS rates were 70% vs. 20% respectively.

Amongst the 365 interrogated miRNAs, 224 were expressed in >80% of the samples, with 128 being significantly differentially-expressed between the 88 OPC vs. 7 normal tonsillar epithelial tissues (p<0.05). Ninety-two of these 128 miRNAs were >2-fold differentially-expressed (Supplementary Fig 2), with the majority (120 of 128; 94%) being up-regulated in OPC (Supplementary Table 1). A detailed comparison of the top six most significantly differentially-expressed miRNAs (miR-21, let-7g, miR-25, let-7f, miR-130b, and miR-151) are provided in Figure 1. These dysregulated miRNAs appeared to be randomly distributed amongst different chromosomal regions, with the exception of 1p34.2 and 7q22.1, within which 6 of the top 40 aberrantly-expressed miRNAs are located (bolded in Supplementary Table 1).

MiRNAs Associated With HPV/p16 status

The miRNAs associated with either HPV16 ISH, or p16 IHC was next investigated. Comparison of the two sets of miRNAs demonstrated that the fold change in miRNA expression defined by either HPV ISH, or p16 IHC, were highly correlated (R²=0.78; Supplementary Fig 3). Figure 2 compared the miRNAs that were significantly associated with p16 IHC or HPV16 ISH as defined by the –log₁₀ (p-values), again demonstrating a strong correlation (R²=0.8). Using a cutoff of p<0.01, nine miRNAs were significantly associated with both p16 IHC and HPV16...
ISH-positive OPCs, which included up-regulated miR-20b, miR-9, miR-9*, miR-492, miR-545, miR-591, miR-422a, and down-regulated miR-193b and miR-107. Since 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 2) was conducted using the samples from an independent HNSCC cohort that we had previously profiled, which contained eleven p16 positive and eight 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 Figure 3, miR-9 and miR-9* retained statistical significance (p=0.04); the remaining five miRNAs were also 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 cut-off, and treated as binary predictors, three potential miRNA signature sets were identified that associated with OS, DFS, and DM (Fig 4). The candidate prognostic panel for OS was defined by up-regulated miR-107 and miR-151, and down-regulated miR-492 (Fig 4B; p<0.0001). For the DFS set, this comprised of up-regulated miR-107, miR-151, miR-182, and miR-361, with down-regulated miR-20b (Fig 4C; p≤0.0001). The DM set was defined by up-regulated miR-151, miR-361 and miR-324-5p, as well as down-regulated 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 biological functions of these aforementioned miRNAs using *in silico* analysis (Fig 5). For miRNAs differentially-expressed in OPC (Supplementary Table 2), miR-30c, miR-30e-3p, and miR-30e-5p all resided within the 5th 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) (19). As described in our previous publication (5), over-expression 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 (phosphatase and tensin homolog deleted on chromosome 10) (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) (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 (LPS) inflammatory stimuli mediated by the pro-inflammatory 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'UTR of HIF-1α and VEGF (27).

Over-expression of miR-151 with its host gene protein tyrosine kinase 2 (PTK2/FAK) has been reported to induce tumour invasion and metastasis in hepatocellular carcinoma (HCC) (Fig 5C) (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 up-regulated in multi-drug resistant cell lines (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 2/3 being HPV-positive; 1/3 being HPV-negative. A panel of miRNAs significantly differentially-expressed between tumor vs. normal tissues (p<0.05) was identified, with miR-21 being the most significantly up-regulated miRNA. MiRNA-21 is one of the most consistently reported aberrant miRNAs in HNSCC, known to target multiple tumour suppressors such as PTEN, and Bcl-2 (5, 7, 10, 12). Up-regulated miR-20b, miR-9 and miR-9* were also significantly associated with HPV/p16-status. Furthermore, there were three candidate miRNA sets that associated significantly with OS, DFS, and DM, even after adjusting for HPV status.

Similar to our previous HNSCC miRNA study (5), up-regulation of the miR-106b-25 cluster was also observed in these OPC samples. These included the non-random over-expression of miR-25, miR-93 and miR-106b, located on chromosome 7q22.1, within the intronic region of MCM7. Over-expression of MCM7, as well as regulation of the miR-106b-25 cluster and MCM7 by E2F1, with subsequent interference of TGF-ß signaling in HNSCC has been previously noted (5). Interestingly, TGF-β polymorphisms have been reported to be a susceptibility marker for HPV16 status amongst OPC patients (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-ß 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 2) (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 non-random region that harbored five (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 ~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) (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). Amongst 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 2 and Fig 5B) (38). Up-regulation of miR-9 and its passenger strand miR-9* were amongst the most significantly associated miRNAs with both OPC, and specifically p16-positive OPC. Over-expression 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 down-regulation was associated with poor DFS (Fig 4 & 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 down-regulated 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 over-expressed 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) (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 immuno-expression, particularly in association with p16 (4).

Finally, in relation to the potential miRNA signature associated with clinical outcome, up-regulation of miR-151 was the only miRNA associated with all of OS, DFS and DM (Fig 4A), as well as being amongst the top 10 significantly deregulated miRNAs in OPC (Supplementary Table 1). Its chromosomal location on 8q24.3 is frequently amplified in human cancers, including
HNSCCs (44). Over-expression 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). Down-regulation of miR-152 is another well-characterized tumour-suppressor miRNA, commonly inactivated by promoter hypermethylation (30, 45), targeting several known important mediators of tumour progression (Fig 5C) (30). It resides in the intron of the host gene Coatomer protein complex ζ2 (COPZ2), which is also a putative tumor suppressor (46).

Oncogenic miR-182 (Fig 4 & 5) belongs to the miR-183-96-182 cluster which was reported to regulate zinc homeostasis in prostate cancer, and consistently up-regulated in multi-drug resistant cell lines (31). It has been implicated in metastasis and poor survival in cancers (32, 33). Over-expression of miR-107 was observed to be associated with worse outcome (Fig 4), and inversely related to HPV-positivity in OPC (Supplementary Table 2). 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 (34, 47). It resides within the introns of the host gene PANK1, involved in catalyzing the formation of Coenzyme A (CoA) during the Krebs Cycle (48). Down-regulation of miR-107 has been noted in HNSCC (49), but in contrast, for this study, we report a contradictory observation in that miR-107 over-expression was associated with poor survival. These data indicate that dysregulation of miR-107 in OPC might well be quite complex; although its oncogenic function has been previously reported (34, 47, 50).

A six-miRNA signature set that associated with survival in OPC was recently reported (11). None of these six miRNAs overlapped with our three potential miRNA signature sets. Among the five miRNAs that were suggested to be associated with HPV by Gao et al, up-
regulated miR-9 and miR-155 were also among the significantly HPV associated miRNAs in our current study (Supplementary Table 2). However, no overlap was observed in HPV-associated miRNAs between those 5 miRNAs Gao et al with the 21 HPV-associated miRNAs reported by Lajer et al (11, 13). Down-regulation of miR-145 was found to be associated with HPV in both our current study and that conducted by Lajer et al (13). MiR-381 and miR-101 were also identified in both studies, but with the fold changes in opposite directions (13). One possible reason for the limited overlap amongst these various studies (Lajer et al vs. current vs. Gao et al) could be due to the different number of miRNAs being included at the outset (847 vs. 365 vs. 96). Moreover, each study included different proportions of HPV-positive patients: 42% vs. 64% vs. 82% for Lajer et al vs. current vs. Gao et al, respectively. The methods used to define HPV-positivity were also distinct (combination of two positives of p16 IHC, RT-PCR, or HPV ISH vs. p16 IHC vs. RT-PCR). Controversy still remains regarding the best method of HPV detection, with discrepancies ranged from 60% to 92% amongst these aforementioned methods (4, 13). These variations hopefully will be resolved by the development of miRNA sequencing of FFPE samples, which will enable the interrogation of all known human miRNAs, and the integration of efforts currently being undertaken by the TCGA head and neck cancer group.

**Conclusion**

We have conducted a comprehensive miRNA profiling study focused strictly on OPC, which identified three 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 tumour progression, located within FRA and HPV integration sites. Further examination of these candidate miRNAs will inform
biological insights into the mechanisms underlying the differences between HPV-positive and HPV-negative OPC.

Acknowledgements

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LEGEND

**Figure 1**  Expression levels of the top 6 miRNAs associated with OPC (miR-21, let-7g, miR-25, let-7f, miR-130b, and miR-151), comparing the 88 tumours to that of the 7 normal tonsillar epithelial tissues. The data are presented as fold change in log2 space, normalized to that of RNU44, 48, and 6B.

**Figure 2**  Comparison of the association between miRNAs with p16 IHC, and association between miRNAs with HPV16 ISH, as defined by p-values. The x and y axes refer to -log_{10} (p-values) of HPV16 ISH associated miRNAs, and -log_{10} (p-values) of p16 IHC associated miRNAs, respectively. These two sets of p-values are highly correlated, with R^2=0.8. MiRNAs with significant associations (p<0.01) for both HPV16 ISH and p16 IHC are specifically identified; green denotes down-regulation; red denotes up-regulation.

**Figure 3**  Validation of the most significant p16-associated miRNAs was performed by utilizing the OPC samples from an independent HNSCC cohort (N=19). The top seven 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 to the average Ct values of the endogenous controls (RNU44, 48, and 6B).

**Figure 4**  Potential miRNA signature sets that associated with OS, DFS, and DM. A) Venn diagram of the candidate miRNAs as a function of clinical outcome (OS, DFS, and DM). B) Kaplan-Meier survival curves for OS as a function of the median expression level of the three miRNAs (miR-107, miR-151, and miR-492). C) DFS actuarial plot as a function of the median expression level of the 5 miRNAs (miR-107, miR-151, miR-182, miR-20b, and miR-361). D) DM actuarial plot as a function of the median expression levels of the 5 miRNAs (miR-151, miR-492, miR-361, miR-152, and miR-324-5p). The p-values indicated in all 3 graphs have already
been adjusted for p16 status. Green denotes under-expression; red denotes over-expression of each candidate miRNA.

**Figure 5**  *In silico* analysis of selected key OPC-associated miRNAs. A) Selected miRNAs significantly differentially-expressed between OPC vs. normal tissues: miR-30c, miR-30e-5p, miR-25, miR-93, and miR-106b, along with their host genes, and putative mRNA targets. B) Selected miRNAs significantly associated with p16 status: miR-381, miR-412, miR-380-5p, miR-487b, miR-382, miR-9/9*, miR-34a, and miR-20b. C) Selected miRNAs significantly associated with clinical outcome: miR-151, miR-152, miR-182, and miR-107. Green denotes under-expression; red denotes over-expression of candidate miRNAs.

**References**

Figure 1
MiRNAs with significant differential expression between OPC vs. normal

- **miR-21**
P<0.0001

- **let-7g**
P<0.0001

- **miR-25**
P<0.0001

- **let-7f**
P<0.0001

- **miR-130b**
P<0.0001

- **miR-151**
P<0.0001
**Figure 2.** Comparison of the association between miRNAs and p16 IHC and the association between miRNAs and HPV16 ISH, as defined by p-values. The x and y axis refer to $-\log_{10}(p$-values) of HPV16 ISH associated miRNAs and $-\log_{10}(p$-values) of p16 IHC associated miRNAs, respectively. These two sets of p-values are highly correlated, with $R^2=0.8$. MiRNAs with significant association (p<0.01) with both HPV and p16 are specifically identified; green denotes down-regulation; red denotes up-regulation.
Expression in OPC miRNA profile

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<td>miR-142-3p</td>
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Expression in HNSCC miRNA profile

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Figure 3. Validation of the most significant p16+ve OPC associated miRNAs with OPC samples from previous HNSCC miRNA profiling (Hui et al, 2009). Higher (– delta Ct) value indicates higher expression of miRNA level. neg = HPV-ve; pos = HPV+ve. FC = fold change of miRNA expression between HPV+ve vs. HPV-ve OPCs.
Figure 4: Potential OPC miRNA prognostic sets.

A) Venn diagram showing the overlap of miRNAs associated with OS, DFS, and DM:
- miR_107 (up)
- miR_182 (up)
- miR_20b (down)
- miR_151 (up)
- miR_492 (down)
- miR_361 (up)
- miR_152 (down)
- miR_324-5p (up)

B) OS probability (%)
- Low Risk: Black line
- High Risk: Red line
- p=0.002

C) DFS probability (%)
- Low Risk: Black line
- High Risk: Red line
- p=0.0001

D) DM probability (%)
- Low Risk: Black line
- High Risk: Red line
- p=0.0087
Figure 5. *In silico* analysis of OPC associated miRNAs

A) Differentially expressed in OPCS

- miR-30c/ miR-30e-3p/ miR-30e-5p
  1p34.2- NFYC

- miR-25/ miR-93/ miR-106b
  7q22.1 – MCM7

- miR-34a
  1q22.1/5q14.3/15q26.1

- miR-20b
  Xq26.2

- FRA/HPV integration

- TGF-β
- PTEN

- DNMT1/E2F3/ MET

B) Associated with p16

- miR-381/ miR-412/ miR-380-5p/ miR-487b/ miR-382
  14q32.31

- miR-9/miR-9*
  1q22.1/5q14.3/15q26.1

- miR-34a
  1p36.22

- miR-20b
  Xq26.2

- p53
- HIF-1α
- VEGF

C) Associated with clinical outcome

- miR-151
  8q24.3-PTK2

- miR-152
  17q21.32-COPZ2

- miR-182
  7q32.2

- miR-107
  10q23.31-PANK1

- miR-152

- Of Metastasis

- Drug resistance

- Cellular Metabolism
Table 1
Clinical Description of the 88 OPC Patients

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>HPV+ve (n=56; 64%)</th>
<th>HPV-ve (n=32; 36%)</th>
<th>p-values</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 years</td>
<td>Mean: 67+11 years</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>(range: 27-80 years)</td>
<td>(range: 46-93 years)</td>
<td></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>0.003</td>
</tr>
<tr>
<td>II</td>
<td>2 (5%)</td>
<td>7 (22%)</td>
<td></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>RT</td>
<td>CRT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26 (46%)</td>
<td>26 (81%)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>30 (54%)</td>
<td>6 (19%)</td>
<td></td>
</tr>
</tbody>
</table>

RT: radiation therapy  CRT: chemo-RT
Potentially Prognostic Micro-RNAs in HPV-Associated Oropharyngeal Carcinoma

Angela B.Y. Hui, Alice Lin, Wei Xu, et al.

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