Five miRNAs as Novel Diagnostic Biomarker Candidates for Primary Nasopharyngeal Carcinoma

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Abstract

MicroRNAs (miRNAs) play an essential role in the development and progression of nasopharyngeal carcinomas (NPC). Despite advances in the field of cancer molecular biology and biomarker discovery, the development of clinically validated biomarkers for primary NPC has remained elusive. In this study, we investigated the expression and clinical significance of miRNAs as novel primary NPC diagnostic biomarkers. We used an array containing 2,500 miRNAs to identify 22 significant miRNAs, and these candidate miRNAs were validated using 67 fresh NPC and 25 normal control tissues via quantitative real-time PCR (qRT-PCR). Expression and correlation analyses were performed with various statistical approaches, in addition to logistic regression and receiver operating characteristic curve analyses to evaluate diagnostic efficacy. qRT-PCR revealed five differentially expressed miRNAs (miR-93-5p, miR-135b-5p, miR-205-5p and miR-183-5p) in NPC tissue samples relative to control samples \((p<0.05)\), with miR-135b-5p and miR-205-5p being of significant diagnostic value \((p<0.01)\). Moreover, comparison of NPC patient clinicopathologic data revealed a negative correlation between miR-93-5p and miR-183-5p expression levels and lymph node status \((p<0.05)\). These findings display an altered expression of many miRNAs in NPC tissues, thus providing information pertinent to pathophysiological and diagnostic research. Ultimately, miR-135b-5p and miR-205-5p may be implicated as novel NPC candidate biomarkers, while miR-93-5p, miR-650 and miR-183-5p may find application as relevant clinical pathology and diagnostic candidate biomarkers.

Keywords: miRNAs - nasopharyngeal carcinomas - qRT-PCR - biomarkers

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Introduction

Nasopharyngeal carcinoma (NPC) is highly prevalent in Southeast Asia relative to other regions, particularly in the Cantonese region around Guangdong in China (Qiu et al., 2011). Due to its unique location and a lack of specific symptoms, NPC is rarely detected during regular medical examinations and is often highly invasive in the late stages (Guo et al., 2013; Liu et al., 2013b). If these patients are diagnosed earlier or if relapses can be predicted sooner, clinical management would be greatly improved (Lin et al., 2014).

Despite key etiological factors including genetic susceptibility, environmental factors and latent infection with Epstein-Barr virus (EBV) being well established, the complex mechanisms driving NPC development and progression are not fully understood (Marquitz, 2012; Yang et al., 2013). One of the major events in NPC development is the inactivation of tumor suppressor genes, yet unlike other head and neck cancers, gene mutation or deletion is uncommon (Lee et al., 2002; Abbasi et al., 2011; Luo et al., 2012). Conversely, the down-regulation of tumor suppressor gene expression by microRNAs (miRNAs) is increasingly recognized to be an important mechanism of nasopharyngeal tumorigenesis (Li et al., 2014; Ma et al., 2014). miRNAs are endogenous, small non-coding RNA molecules that completely or partially bind to target mRNAs and lead to degradation to ultimately inhibit gene expression (Li et al., 2011; Marquitz et al., 2012; Liu et al., 2014). miRNAs serve as essential posttranscriptional regulators that can specifically influence cancer development and progression, with aberrant expression patterns observed in multiple cancer types (Marquitz, 2012).

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Clinical Characteristics of Nasopharyngeal Carcinoma Patients According to Training Set and Validation Set

| Variable                  | Samples | p-value* |
|---------------------------|---------|----------|
|                          | NPC (n=67) | Control (n=25) |
| Sex                       |          |          |
| Male                      | 56       | 16       |
| Female                    | 11       | 9        |
| Average Ages (years)      | 51.87±2.257 | 31.67±2.427 | 0.0001 |
| Histological types        |          |          |
| Squamous carcinoma        |          |          |
| T stages                  |          |          |
| T1                        | 12.50%   |          |
| T2                        | 28.10%   |          |
| T3                        | 18.8     |          |
| T4                        | 40.60%   |          |
| N stages                  |          |          |
| N0                        | 19.40%   |          |
| N1                        | 30.60%   |          |
| N2                        | 33.30%   |          |
| N3                        | 16.70%   |          |
| TNM stages                |          |          |
| I                         | 3.30%    |          |
| II                        | 12.90%   |          |
| III                       | 29.00%   |          |
| IV                        | 54.80%   |          |
| Family history            |          |          |
| yes                       | 16.10%   |          |
| no                        | 83.90%   |          |
| Smoking                   |          |          |
| Smoking                   | 19.40%   |          |
| No smoking                | 80.60%   |          |
| Region                    |          |          |
| Zhanjiang                 | 26.80%   |          |
| Other region              | 70.20%   |          |

* (NPC vs Control)
### Table 2: Information of 21 miRNAs According to the Chip

| miRNA Name | Accession No. | Human | Non-Cancer | Non-Cancer | Controls | Controls | H | P | Fold | Change |
|------------|--------------|-------|------------|------------|----------|----------|---|---|------|--------|
| hsa-miR-93-5p | HMM0000093 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-205-5p | HMM0002264 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-211-3p | HMM0003476 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-483-5p | HMM0004646 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-155-5p | HMM0000646 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-29b-3p | HMM0000100 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-96-5p | HMM0000959 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-642a-3p | HMM0010924 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-3198 | HMM0015083 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-5100 | HMM0022259 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-5739 | HMM0023888 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-486-5p | HMM0002177 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-199a-5p | HMM0000231 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-99b-5p | HMM0000689 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-650 | HMM0003320 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-183-5p | HMM0000261 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-10a-5p | HMM0000253 | P     | +           | +           | +        | +        |   |   |      |        |
| hsa-miR-30e-3p | HMM0000693 | P     | +           | +           | +        | +        |   |   |      |        |

For comparison of means between two groups, a two-tailed t-test was used. Significance was accepted at $p<0.05$.

### Bioinformatics

The selected miRNAs were further analyzed to identify their function and the pathways that they modulate using Ingenuity Pathway analysis (DAVID, http://david.abcc.ncifcrf.gov/home.jsp). This pathway analysis software identifies the putative targets of the input miRNA(s) and then develops networks and functions among the genes and targets. Before starting the analysis, the confidence was set to “highly predicted” and “experimental observed” and the species set to “human”. The miRNA targets were then predicted using an integrated database including miRecords, Tarbase and TargetScan Human. The highly predicted targets were then matched and paired with miRNA expression data using the expression pairing function of mirWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/). We assumed that the expression of a given miRNA was negatively correlated with the miRNA expression of its targets, which is a widely accepted and experimentally verified supposition (Li et al., 2011). The result identified the biological functions and canonical pathways associated with our data and were generated automatically using the core-analysis option in DAVID.

### Results

#### Histological stainings

Two pathologists independently identified the results of nasopharyngeal carcinoma or chronic inflammation of nasopharyngeal mucosa for all samples. Representation H and E staining section is as shown in Figure 1.

#### Differentially expressed miRNA profiles between NPC and non-cancer patients identified using the Agilent human miRNA microarray 19.0

miRNAs were extracted from six samples with 3 NPC and 3 NC (non-cancer controls), independently. Of the 2047 miRNAs probed, 22 miRNAs were differentially expressed in NPC tissue relative to controls samples ($p<0.05$, FDR $\geq 2$ fold or $\leq 0.5$ fold, signal value $\geq 5$) (Table 2), with 15 being up-regulated and 7 down-regulated in the NPC samples relative to the control.
Validation of differentially expressed miRNAs using qRT-PCR

We next examined the expression of the 22 miRNAs in the 67 NPC and 25 normal control samples using qRT-PCR. Following statistical analysis using a Wilcoxon test, five miRNAs (miR-93-5p (p<0.01), miR-135b-5p (p<0.001), miR-205-5p (p<0.001), miR-650 (p<0.05) and miR-183-5p (p<0.05)) were significantly overexpressed in NPC samples relative to the control samples. The expression of the other miRNAs was not significantly different between the NPC and normal control specimens (p>0.05) (Figure 2).

The relationship between miR-93-5p and miR-183-5p expression and clinical histopathological features

Variations in the expression profiles of miR-93-5p, miR-135b-5p, miR-205-5p and miR-183-5p during different TNM stages, T stages and N stages were analyzed in NPC samples using the Kruskal-Wallis test (Figure 2). Our data revealed that miR-650 was up-regulated and related to the various T stages (p<0.05), whereas miR-93-5p and miR-183-5p were up-regulated and related to various N stages (p<0.05 or p<0.01). However, the correlations between expression profiles of the five miRNAs and TNM stages were not significantly different (p>0.05). Spearman-Rho analysis was performed to compare the relative expression of miR-93-5p and miR-183-5p in NPC samples, with a significant correlation (p<0.05) noted with the N stages (miR-93-5p: r=-0.386 and miR-183-5p: r=-0.437) as shown as Figure 3.

Assessing the predictive value of miR-205-5p and miR-135b-5p expression for NPC detection

A Spearman-Rho test was performed to compare the relative expression of miR-93-5p, miR-135b-5p, miR-205-5p, miR-650, and miR-183-5p in 92 tissues samples. The results revealed a significant correlation in miR-205-5p (r=0.521, p<0.01) and miR-135b-5p (r=0.489, p<0.01). To investigate the predictive value of the five different miRNAs in NPC, we measured their expression levels in 67 NPC and 25 NC tissue samples. The results of binary logistic regression analysis revealed a significant negative correlation between miR-205-5p and miR-135b-5p expression and the risk of NPC (p<0.05). Moreover, patients with high miR-135b-5p expression levels were at a higher risk of NPC compared with those with low miRNA expression profiles (odds ratio (OR) =1.212). Similarly, patients with high miR-205-5p expression levels were at a higher risk of NPC compared with those with low expression (OR=1.673). ROC curve analyses (Figure 4) also showed that both miRNAs could be used to differentiate NPC samples from control samples, with
Discussion

NPC is a malignancy with a high occurrence in Southern China. Accumulating evidence has demonstrated that miRNAs play important roles in various physiological and pathological processes, including carcinogenesis. miRNAs can function as oncogenes or tumor suppressors, with their dysregulation involved in multiple processes including cell proliferation, apoptosis, cell-cycle regulation and invasion in various diseases. For example, it was found miR-10b is up-regulated and promotes migration and invasion in metastatic NPC cell lines and associated with the expression of E-cadherin and MMP-9 (Sun et al., 2013). It suggested that miRNAs work with adhesion molecules to influence NPC metastasis. In addition, miRNAs are potential novel biomarkers for various cancers. miRNA expression can be pathognomonic or tissue-specific, with human miRNAs easily preserved well in formalin-fixed and fresh snap frozen specimens (Lu et al., 2005; Xi et al., 2007). Therefore, miRNA expression profiles are promising for characterizing tumors and could serve as potential diagnostic and prognostic markers to enhance treatment (Iorio and Croce, 2012). By performing an initial miRNA microarray followed by two sets of individual qRT-PCR analyses, we identified a correlation between two miRNAs and the N stages of NPC, and identified two miRNAs able to discriminate between NPC and normal tissues.

Among the five tissue miRNAs identified in this study, some were previously reported to play important roles in cancer (Zheng et al., 2013; Zhi et al., 2013). While NPC miRNA expression studies have highlighted the importance and potential roles of miRNAs in disease, a deeper understanding is needed (Liu et al., 2013b). miR-183-5p was reported to be an early predictive biomarker for prostate cancer with aggressive progression characteristics (Larne et al., 2013), while conversely in lung cancer its expression was decreased (Xie et al., 2013). The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten) is known to be regulated by miR-183-5p (Sarver et al., 2010). While no previous findings have characterized miR-183-5p in NPC, our data revealed a significant correlation between miR-183-5p and the NPC N stages (p<0.05). miR-93-3p has been found to be significantly up-regulated in laryngeal squamous cell carcinoma and in cisplatin-resistant ovarian cancer cells, and may promote tumor growth and angiogenesis by targeting integrin-β8 (Fu et al., 2012; Cao et al., 2013). Additionally, a decreased miR-93 expression has been implicated as a prognostic factor for colon cancer (Xiao et al., 2013). In a colorectal cancer study, miR-93 suppressed the expression of ERBB2, p21 and the Vascular Endothelial Growth Factor VEGF, which all play roles in cell proliferation. Furthermore, miR-93 also suppressed colorectal tumor growth in tumor cell seeded Balb/c nude mice (Yang et al., 2012). miR-93 has also been reported to regulate VEGF expression in experimental models of diabetes both Asian Pac J Cancer Prev and Asian Pac J Cancer Prev (Long et al., 2010). While no previous findings have characterized miR-93-3p in NPC, our data revealed a significant correlation between miR-93-3p and the NPC N stages.

In NPC, host- and Epstein-Barr virus (EBV)-encoded miRNAs play key roles in almost all of the steps of epithelial cell carcinogenesis, including epithelial-mesenchymal to stem-like transition, cell growth, migration, invasion and tumorigenesis (Chen et al., 2009; Liu et al., 2012; Lo et al., 2012). EBV is detected in all NPC cases regardless of differentiation subtype (Wolf et al., 1975; Raab-Traub et al., 1987). Further investigation of potential EBV miRNA target genes revealed the inhibition of tumor suppressor genes, such as PTEN, and the extensive dysregulation of several pathways that are commonly involved in NPC, such as Wnt signaling. In HeLa cells transfected with miR-BART6-5p RNAs, which is EBV-encoded, the expression of miR-205-5p was increased more than 2-fold (Iizasa et al., 2010). In the current study, miR-205-5p expression was considerably higher in NPC tissues relative to control samples. This finding is consistent with the understanding that EBV is detected in all NPC tumors and further supports the potential usefulness of miRNAs as diagnostic biomarkers.

Although few previous studies have quantified the expression of miR-205 in NPC patients using qRT-PCR, its expression has been assessed in lung cancer tissues. A recent study used high-throughput microarrays to measure miRNA expression in 122 adenocarcinoma and squamous cell carcinoma (SCC) specimens and demonstrated that the expression of hsa-miR-205 in malignant tissues is an accurate marker of SCC lung cancer (Lebanony et al., 2009). Furthermore, the relative expression of miR-205-5p was significantly higher in non-small cell lung cancer tissues compared with non-cancer adjacent tissues paired specimens (Jiang et al., 2013). Consistent with these observations, our findings displayed an increased miR-205-5p expression in NPC tissues. Nevertheless, the role of miR-205 remains controversial. For example, the down-regulation of miR-205 is useful in distinguished melanoma from nevus (Kozubek et al., 2013). Moreover, the relative expression of miR-205-5p in any Gleason pattern was decreased significantly compared with normal tissues (Tsuchiyama et al., 2013), to include prostate cancer samples compared with non-prostate cancer samples (Larne et al., 2013). These results could be explained by a previous report that miRNA expression profiles are tissue-specific (Lu et al., 2005). In this study, we report for the first time that miR-135b-5p can serve as a biomarker capable of discriminate NPC from normal control patients. Although there are few studies describing the function of miR-135b-5p, a recent report showed that miR-135b-5p was upregulated in highly aggressive osteosarcoma cell lines (Lauvrak et al., 2013).

The current findings are preliminary and may be limited by the small number of patients examined. As such, further corroboration via large-scale and multicenter studies is necessary before clinically useful and reliable recommendations could be generated. Furthermore, future studies should be conducted over multiple time intervals.
to assess miRNA expression profiles during the pre- and post-operative periods, in addition to identify changes in these profiles over time.

These results are the first evidence suggesting that miR-135b-5p and miR-205-5p have the potential to be used as clinical markers for the diagnosis of NPC. Therefore, miR-135b-5p and miR-205-5p may have potential diagnostic applications in NPC patients. While additional studies are needed to confidently link these miRNA biomarkers with disease progression, survival and anti-cancer drug resistance development, they present themselves as potentially useful diagnostic tools in NPC.

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