microRNA expression profiling of nasopharyngeal carcinoma

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Abstract. Nasopharyngeal carcinoma (NPC) is posing a serious health problem worldwide. The association between its pathogenesis and microRNAs (miRNA) has not been elucidated. In this study, miRNA expression profiling was performed to screen the miRNA expression changes in 8 NPC tissues and 4 normal nasopharyngeal tissues. Thirty-four miRNAs were identified to be differentially expressed; of these, one miRNA (*miR-18a*) was overexpressed and 33 miRNAs (*miR-34b, miR-34c, let-7 family*, etc.) were under-expressed in NPC tissues compared to the normal samples. Validation was performed by real-time quantitative PCR for two altered miRNAs (*miR-34b* and *let-7g*) and one non-differentially expressed miRNA (*miR-30c*). Unsupervised hierarchical clustering analysis showed that the aberrant miRNAs were correlated with the clinical stage of NPC patients. In addition to several biological pathways that are well characterised in NPC and which were significantly targeted by the underexpressed miRNAs, two novel pathways, nervous system development and sensory perception of sound, were identified to be strongly associated with NPC development. Furthermore, a *c-Myc* centered miRNA regulatory network was inferred in NPC. Our study reveals that aberrantly expressed miRNAs play important roles in NPC tumorigenesis and may serve as potential targets for novel therapeutic strategies in the future.

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Introduction

MicroRNAs (miRNAs) are a newly identified class of short (about 19-22 nt), endogenous, non-coding, single-stranded RNA molecules, which are conserved in sequence between distantly related organisms. More and more evidence has proved miRNAs play important roles in diverse biological functions, including development, differentiation, proliferation and apoptosis (1,2). MiRNAs regulate protein expression by binding to the 3' untranslated regions (3' UTR) of messenger RNAs (mRNAs) and suppressing translation or inducing degradation or deadenylation with varying degrees of sequence complementarity (3). In humans, strong links between cancer and miRNA deregulation have been suggested by recent studies. The relevance of miRNAs to cancer was first reported in 2002 when Calin *et al* observed that *miR-15a* and *miR-16-1* were down-regulated or even deleted in the majority of chronic lymphocytic leukemia patients (4). They also found that many known miRNAs are located in the regions of human chromosomes with high frequencies of copy number alterations in cancers (4). So far, distinct miRNA profiles have been reported and the potential role of miRNAs in various human haematological and solid cancers have been indicated by many studies. Lu *et al* used miRNA expressions to classify human cancers associated with the developmental lineage and differentiation state (5). Johnson *et al* reported that the down-regulation of let-7 is a significant cause of lung cancer tumorigenesis (6). This body of evidence indicates that the aberrations of miRNA expression in cancer are strongly correlated with tumor carcinogenesis and progression.

Nasopharyngeal carcinoma (NPC) is a non-lymphomatous, squamous cell malignancy arising from the epithelial cells lining of the nasopharynx (7). NPC was vastly common in Southeast Asia, particularly amongst the Cantonese population of southern China, such as Guangdong Province and Guangxi Province. The incidence is ~30-80/100,000 people per year and has remained high for decades. Because of the high risk and incidence region specificity, it is important to elucidate the mechanism of NPC carcinogenesis. Thus far, little research has been conducted to investigate the role of miRNAs in NPC carcinogenesis. Zhang *et al* proposed that the interactions between *miR-141* and tumor-related genes *c-Myc*, *Splunc1*, *Brd3*, *Ubap1*, and *Pten* contribute to the progression of NPC (8). Xia *et al* found that miR-200a targeting ZEB2 and CTNNB1 inhibits NPC cell growth,

migration and invasion (9). Wong *et al* demonstrated that miRNAs of *let-7* family suppress nasopharyngeal carcinoma cell proliferation through down-regulating *c-Myc* expression (10). Shi *et al* validated that underexpressed *miR-100* leads to *Plk1* overexpression, which in turn contributes to NPC progression (11). In addition to the investigation of individual miRNA, high-throughput techniques have been applied to identify deregulated miRNAs in NPC. Chen *et al* used a stem-loop real-time-PCR assay to examine the expression levels of 270 human miRNAs in 13 NPC samples and 9 adjacent normal tissues. They identified 35 miRNAs whose expression levels were significantly altered in NPC samples and inferred some cancer-related pathways enriched with targets of down-regulated miRNAs (12). Sengupta *et al* performed a miRNA microarray experiment to explore the expressions of 207 miRNAs between 31 NPCs and 10 normal tissues. They demonstrated the involvement of *miR-29c* in NPC metastasis by regulating mRNAs encoding extracellular matrix proteins (13). However, the relationship between miRNAs and NPC tumorigenesis is still unclear.

In this study, we recruited the Illumina miRNA microarray platform, which covers a total of 735 human miRNAs, to profile miRNA expression and used it to analyze human miRNAs in 8 NPC samples and 4 normal nasopharyngeal tissues. We identified 34 significantly differentially expressed miRNAs and explored the relationship between their expression and NPC progression. To evaluate the biological consequences of the miRNAs dysregulation, a relative stringent target prediction followed by pathway enrichment analysis was conducted to identify the functional pathways specifically regulated by the aberrantly expressed miRNAs. We expect our analysis to provide some clues to estimate the mechanism of miRNA effects on NPC carcinogenesis.

Materials and methods

Tissue samples. Nasopharyngeal carcinoma tissue samples were obtained from 8 poorly differentiated squamous NPC patients before treatment at the Institute of Nasopharyngeal Carcinoma, The People's Hospital of Guangxi Zhuang Nationality Autonomous Region, Nanning, China. In addition, normal nasopharyngeal tissues were obtained from 4 different donors in the same hospital. All samples obtained were with consent in accordance with approval granted by the Ethics Committee of the Institute of Nasopharyngeal Carcinoma, The People's Hospital of Guangxi Zhuang Nationality Autonomous Region. Fresh NPC and nasopharyngeal tissues were immediately snap-frozen in liquid nitrogen and stored at -80˚C until use. All of the samples were used to do microarray and quantitative real-time PCR (qRT-PCR). The NPC samples we used are listed in Table I. The diagnosed stages I, II, III and IV were classified by otorhinolaryngologic pathologist according to the 2008 GuangZhou Staging of NPC.

RNA extraction and miRNA array hybridization. Total RNA was extracted from 8 NPC tissues and 4 nasopharyngeal tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer's instructions. All RNA samples were examined for concentration and purity based on the agarose gel electrophoresis and absorbance ratio at 260-280 nm to make sure the RNA without any protein and DNA.

Table I. The information of NPC samples used in the study.

Sample	Gender	Age	TNM stage	Cancer stage
Tumor 1	Female	35	T2NIM0	II
Tumor 2	Male	46	T2NIM0	II
Tumor 3	Female	24	T3NIM0	III
Tumor 4	Male	46	T4N2M0	IV
Tumor 5	Female	39	T3N1M0	III
Tumor 6	Male	69	T3N1M0	III
Tumor 7	Male	65	T2N0M0	II
Tumor 8	Male	56	T4N3M0	IV

We used the Illumina human v1 miRNA panel (based on miRBase release 9.0) for miRNA analysis. RNA (100 ng) was amplified using the Illumina Total Prep RNA amplification kit (Ambion Cat. No. IL1791, Austin, TX, USA) to generate biotinylated cRNA. An aliquot (1.5 mg/30 ml) of the labeled cRNA for each sample, prepared in a probe cocktail that included GEX-HYB hybridization buffer, was hybridized to an Illumina human v1 miRNA panel at 58˚C for 16 h. After hybridization, the chips were washed, coupled with streptavadin-Cy3 and scanned in the Illumina BeadArray Reader. The expression profiles have been deposited in NCBI's Gene Expression Omnibus (GEO) with accession number GSE22587.

Microarray data analysis. Data analysis and visualization were performed using Illumina BeadStudio Gene Expression Software (Illumina, Inc., San Diego, CA, USA). With Illumina gene expression array, each probe is measured at least 30 times independently on random distributed beads. This large number of technical replicates allows robust estimation of the hybridization intensity and the measurement error for each probe. We first transformed raw data generated from BeadStudio using a variance stabilization transformation algorithm (14) and then normalized them using cubic spline algorithm and background subtraction provided by BeadStudio Software.

In general, a large fraction of miRNAs were either not expressed or non-detectable, such that these miRNAs are considered as weak signals. In this research, we adopted a two-step strategy to deal with the weak signals. First, we only chose the miRNA probes with detection p-value ≤ 0.01 in at least half of the chips for the further analysis, which means when the detection p-value was ≤ 0.01 in at least 4 NPC tissues and 2 normal nasopharyngeal tissues, the miRNA probe would be included in subsequent analysis. The detection p-value, calculated by comparing the distribution of the transcript signal to that of the negative control signal, was set at ≤ 0.01 to identify transcript that were expressed above background. Note that the detection p-values were automatically reported in BeadStudio. This procedure reduced the number of miRNA probes from 735 to 276 for final data analysis. Second, for the remaining 276 miRNA probes, when

the detection p-value >0.01 in one chip, this corresponding signal intensity was considered to be missed. To avoid losing useful information and to facilitate data analysis, we proposed an approach to impute the missing data. It can be noted that for each chip, the minimum intensity of miRNAs with detection p≤0.01 is always higher than the maximum intensity of miRNAs with detection p-value >0.01, thereby the minimum signal intensity of miRNAs with detection p≤0.01 can be considered as the threshold in this chip to separate the valid intensity from background noise. Then, for each chip, we ranked the signal intensities of all probes with detection p≤0.01. If the signal intensity of a probe was lower than the threshold, we provided the threshold as the intensity for this miRNA probe. Otherwise, we would preserve its original intensity.

To detect significantly differentially expressed miRNAs, we employed the software named SAM (significant analysis of microarray, version 3.02) (15). The cut-off was set at q-value ≤0.05 (a false discovery rate below 0.05 that the given miRNA is expressed at different levels between NPC samples and normal samples). The software Cluster and TreeView was used to perform unsupervised hierarchical clustering analysis (16).

Real-time quantitative PCR. The quantitative real-time RT-PCR was used to validate our microarray results. The quantification of miRNA was performed by using Bulge-Loop™ miRNA qPCR (RiBo Co., Cat. No. MQP-0101, Guangzhou, China) following the manufacturer's instruction in an ABI7900 Thermocycler (Applied Biosystems, Foster City, CA, USA). Reverse transcriptase reactions included purified total RNA (2 μg), 5 nmol/l stem-loop RT primer, 5X RT buffer, 0.2 mmol/l each of dNTPs, 100 U RT reverse transcriptase and 20 U RNase inhibitor. The RNA template and stem-loop RT primer were mixed to 11 μl and incubated for 10 min at 70˚C, 2 min in ice. The other components were added to 25 μl. The 25-μl reactions were incubated in the ABI7900 thermocycler plate for 60 min at 42˚C, 10 min at 70˚C, and held at 4˚C. The 20 μl PCR included 2 μl RT product, 1X SYBR-Green Mix, 500 nmol/l miRNA forward primer and 500 nmol/l miRNA reverse primer, as recommended by the manufacturer. The reactions were incubated at 95˚C for 20 sec, then followed by 40-45 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 70˚C for 10 sec. The 2-ΔCt method was used as relative quantification measure of differential expression. All reactions were run in triplicate. U6 small nuclear RNA was used as the internal control for determining the relative miRNA expression level. In relation to the expression of small nuclear U6 RNA, the expression level of specific miRNA for each RNA sample was calculated, reflecting by the value of ΔCt (Ct of miRNA – Ct of U6). Two sided Wilcoxon rank sum test was performed to analyze the expression differences of the three selected miRNAs between NPC samples and normal samples with the SPSS software (version 11.5).

Pathway analysis of the target genes of differentially expressed miRNAs. Distinct miRNA target prediction methods may result in considerably different target gene sets, herein, to rule out the possibility of bias introduced by only one miRNA target prediction method, we adopted the method introduced by Ozen *et al* (17). If a given target was identified using at least three of four different algorithms, including TargetScan (release 4.1, containing 669 human miRNAs) (18), miRanda (January 2008 release, total 475 human miRNAs) (19), PITA (version 6, total 418 human miRNAs) (20), and PicTar (May 2007 release, total 178 human miRNAs) (21), it was considered likely to be a genuine miRNA target.

In addition, we queried a published mRNA expression dataset to determine if any of the potential targets were not only significantly differentially expressed in NPC compared to normal tissues, but also expressed in an inverse manner relative to corresponding miRNAs, as would be predicted if there was deregulation due to action of the miRNA targeting them. Sengupta *et al* has reported that they conducted a mRNA microarray experiment containing 31 nasopharyngeal carcinoma (NPC) tissue samples and 10 normal nasopharyngeal tissues to identify the molecular mechanism of NPC (22). For this mRNA expression dataset, we mapped the probe or probeset IDs to NCBI Refseq IDs. When multiple probe sets are mapped to the same Refseq ID, their values are averaged to represent the expression level of this Refseq gene. We then performed two-sided t-test for each gene to compare the expressions between NPC and normal tissues. To address the multiple test issues, we employed BH correction method to adjust p-values (23). The mRNA was considered as deregulated if it satisfied the condition of adjusted p-value ≤0.05. A total of 634 genes possibly targeted by underexpressed miRNA genes were significantly increased in NPC, whereas a total of 50 target genes of the only one overexpressed miRNA, *miR-18a*, were significantly decreased in NPC.

We then enriched these target genes in curated pathways and Gene Ontology (GO) categories by the aid of GenMAPP 2.0 software, which is a widely used pathway visualization and analysis tool for biological data (24). For convenience, we uniformly denoted the identified pathway or GO term as pathway. We used the GenMAPP software to map the deregulated mRNA genes in the dataset of Sengupta *et al* in all pathways. To evaluate whether miRNA targets are more likely than expected by chance to be contained within one pathway, we test if the targets of the aberrantly expressed miRNA are enriched in this pathway. From all differentially expressed genes in the dataset of Sengupta *et al*, a rate of differential expression was computed for the entire array as (number of total miRNA targeted deregulated genes/number of total deregulated genes). For each of a pre-specified pathway, a rate of differential expression was computed as (number of miRNA targeted deregulated genes in this pathway/number of total deregulated genes contained in the pathway). When a pathway is unrelated with miRNA modulation, differential expression occurs irrespective of this pathway, and the differential expression rates for the complete array and the pathway are the same. We tested this null hypothesis against the alternative that the rates were higher in the list of a pathway by comparing the differential expression rates for gene targeted by aberrantly expressed miRNAs and entire array. P-values were estimated using one-sided hypergeometric test and the BH method was used to address the problem of multiple test. The pathways statistically enriched with by miRNA targets were identified using a threshold of adjusted p≤0.10.

Results

Differential expression of miRNAs between NPC and normal tissue. A total of 735 human miRNAs were detected with Illumina microarray platform in each sample, which include the sequences information from the Sanger Institute miRBase Database (Release 9.0) and the public, accepted miRNAs reported before. After the process of dealing with weak signals, 276 miRNAs were included in subsequent differential expression analysis. We applied the two class unpaired method provided by SAM software and identified 33 significantly underexpressed miRNAs and 1 significantly overexpressed miRNA (Table II).

MiR-18a, which belongs to *miR-17-92* cluster, was the only one overexpressed gene in our results. It was reported that *miR-18a* prevents translation of ERα, potentially blocking the protective effects of estrogen and promoting the development of hepatocellular carcinoma in women (25). *MiR-17-92* cluster (encoding *miR-17-5p*, *miR-17-3p*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b-1*, and *miR-92-1*) located in intron 3 of *C13orf25* gene. *miR-17-92* promotes tumor angiogenesis by regulating the expressions of *Tsp1* and *CTGF* (26). He *et al* reported that the levels of the primary or mature miRNAs derived from the *miR-17-92* locus are often substantially increased in B-cell lymphoma samples and cell lines. Enforced expression of the *miR-17-92* cluster acted with *c-Myc* expression to accelerate tumor development in a mouse B-cell lymphoma model.

Thirty-three underexpressed miRNAs included the *miR-34b/miR-34c* cluster, the *miR-195/miR-497* cluster and a majority of members of *let-7* family. Among these miRNAs, *miR-34b* and *miR-34c* were the top two underexpressed miRNAs in NPC. Genes encoding miRNAs in the *miR-34* family are direct transcriptional targets of p53, whose induction by DNA damage and oncogenic stress depends on p53 both *in vitro* and *in vivo*. Ectopic expression of *miR-34* induces cell cycle arrest in both primary and tumor-derived cell lines, which is consistent with the observed ability of *miR-34* to down-regulate the program of genes promoting cell cycle progression. The p53 network suppresses tumor formation through the coordinated activation of multiple transcriptional targets, and *miR-34* may act in concert with other effectors to inhibit inappropriate cell proliferation (27). Moreover, induction of *miR-34b* and *miR-34c* leads to apoptosis or cellular senescence, whereas reduced *miR-34b/c* expression attenuates p53-mediated cell death (28,29). On the other hand, *miR-34* enhances the activities of p53 by inhibiting silence information regulator I (*SIRTI*). The positive feedback regulatory network based on p53 and *miR-34* families play an important role in suppression of oncogenesis and deterioration (30).

It should be mentioned that most members of *let-7* family in our microarray data were down-regulated. The *let-7* miRNA is a founding member of the miRNA family and is conserved in invertebrates and vertebrates, including humans, where the *let-7* family consists 11 very closely related genes (31). Our microarray platform detected eight members of *let-7* family (*let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i*) and seven in them showed significantly underexpression in NPC. Johnson *et al* reported that the *let-7* family negatively regulates *let-60/RAS*. Loss of *let-60/RAS* suppresses *let-7*, and 3'UTR (untranslated region) of *let-60/RAS* contains multiple *let-7* complementary sites (LCSs), allowing *let-7* to regulate RAS expression (6). The expression level of *let-7* is lower in lung tumors than in normal lung tissues, while RAS protein is significantly higher in lung tumors. The inverse relationship between *let-7* and *RAS* suggested a possible regulation mechanism for cancer cell proliferation. In addition, Johnson *et al* showed that *let-7* overexpression caused human cancer cells to decrease cell cycle progression and *let-7* directly regulated a few key cell cycle proto-oncogenes, e.g., *RAS*, *CDC25a*, *CDK6*, and *cyclin D*, thus controlling cell proliferation by reducing flux through the pathways promoting the G1 to S transition (32).

Validation of microarray results using quantitative real-time RT-PCR. Quantitative RT-PCR method with a stem-loop primer was employed to validate the reliability of miRNA

Table II. The differentially expressed miRNAs in NPCs compared to normal tissues.

miRNA ID	q-value (%)	Fold change (log ratio NPC/Normal)
hsa-miR-18a	0	2.23
hsa-miR-34c	0	-2.17
hsa-miR-642	0	-1.89
hsa-miR-34b	0	-1.85
HS_38.1	0	-1.83
hsa-miR-449	0	-1.56
hsa-miR-10b	0	-1.43
hsa-miR-92b	0	-1.37
hsa-miR-625	0	-1.22
hsa-miR-497	0	-1.16
hsa-miR-576	0	-1.15
hsa-miR-100	0	-0.82
hsa-let-7b	0	-0.75
hsa-miR-150	0	-0.65
hsa-miR-152	0	-0.63
hsa-let-7d	0	-0.58
hsa-miR-342	0	-0.56
hsa-miR-26b	0	-0.49
hsa-let-7g	0	-0.49
hsa-miR-29a	0	-0.48
HS_204.1	0	-0.47
hsa-let-7f	0	-0.46
hsa-miR-29b	0	-0.38
hsa-let-7e	0	-0.35
hsa-let-7a	0	-0.32
hsa-miR-30d	0	-0.28
hsa-miR-375	2.47	-1.74
hsa-miR-155	2.47	-0.50
hsa-miR-195	2.47	-0.33
hsa-miR-768-3p	2.47	-0.29
HS_210	4.3	-1.15
hsa-let-7c	4.3	-0.62
hsa-miR-425-5p	4.3	-0.46
hsa-miR-221	4.3	-0.24

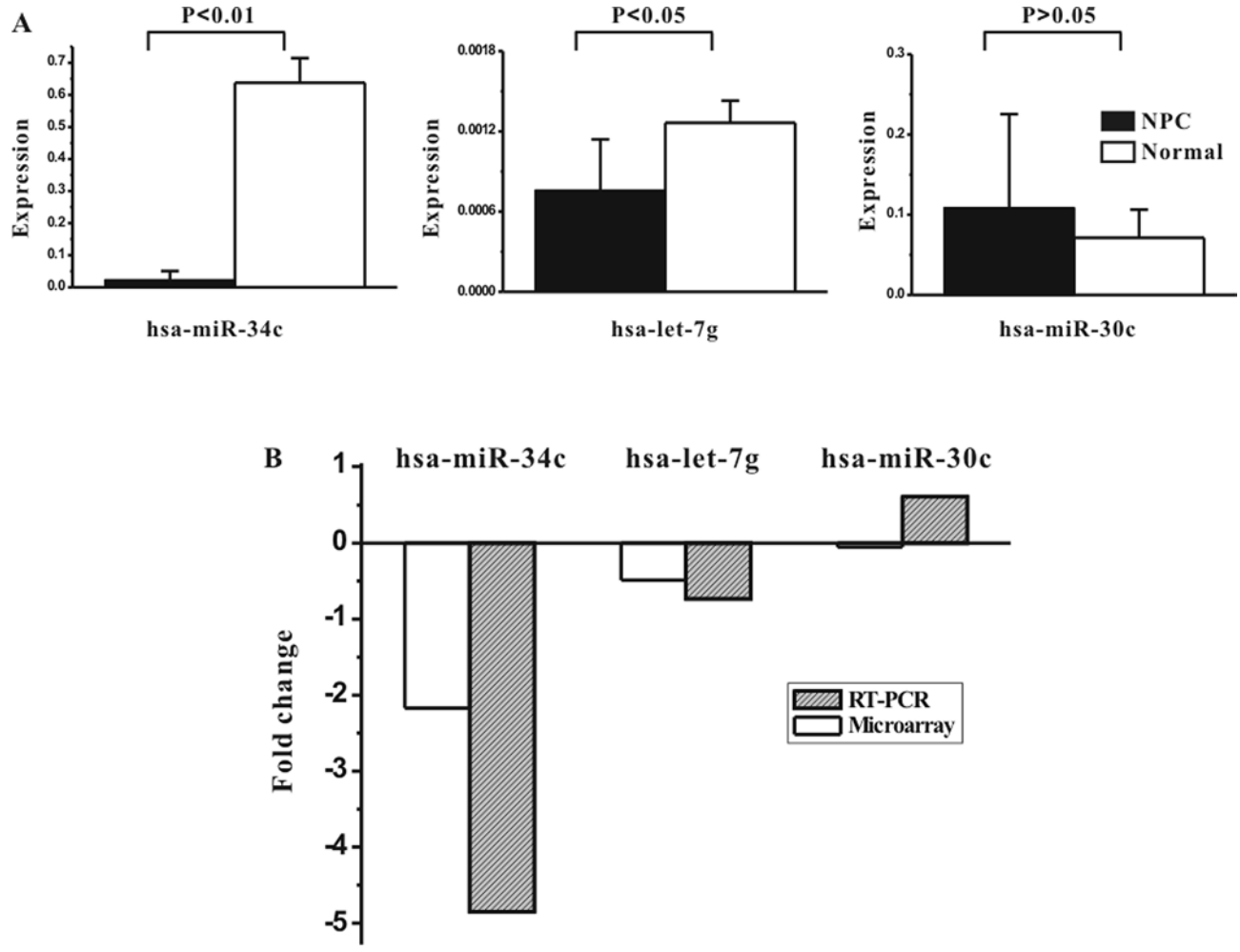

Figure 1. (A) Confirmatory studies of selected miRNAs by real-time RT-PCR. The graph shows the two aberrant miRNAs (*miR-34c* and *let-7g*) the microarray results also showed the significant alteration in real-time RT-PCR. (B) Fold change comparison between the microarray and real-time RT-PCR in three miRNAs. The *miR-34c* and *let-7g* showed the same trends in microarray and real-time RT-PCR. However, the *miR-30c* did not show the same trend, which can be explained by the expression difference of *miR-30c* between NPC and normal tissue and has no statistical significance, so it is possible that the logarithm of fold change is either positive or negative.

array result. Since mature miRNAs is very short (only 19-22 nt) without the polyA tail, the amplification could not be conducted using the Oligo(dT) methods. Here we used the stem-loop primer to extend the mature miRNA on the reverse step designed by Chen *et al* (33). Stem-loop RT primers are better than conventional ones in terms of RT efficiency and specificity.

To validate our miRNA microarray result, we carried out quantitative RT-PCR to investigate the expressions of 3 miRNAs in 8 NPC tissues and 4 normal tissues. *MiR-34c* is significantly underexpressed in NPC with a great fold change; *let-7g* is also significantly underexpressed in NPC, however, with a moderate fold change; whereas *miR-30c* shows no significantly differential expression between NPC and normal tissue. As expected in RT-PCR results, both *miR-34c* and *let-7g* reveal significant lower expression in NPC tissues with respect to normal tissues, whereas the difference of *miR-30c* expression between NPC and normal tissue is of no statistical significance (Fig. 1A). In addition, for each miRNA, we correlated the fold changes from RT-PCR and microarray (Fig. 1B). For *miR-34c*, the fold change is great in both microarray and RT-PCR. For *let-7g*, which fold change is consistently relative low in microarray experiments, the fold change of this miRNA in RT-PCR is about -1. Nevertheless, the expression of *miR-30c* is the opposite between microarray and RT-PCR. It can be explained by the fact that *miR-30c* has no differential expression between NPC and normal tissues. The logarithm of its fold change in microarray experiment is very close to zero, therefore, so long as the difference of expressions in RT-PCR experiment is not statistically significant, it is possible that the logarithm of fold change is either positive or negative. Taken together, it is indicated that RT-PCR results are consistent with our microarray data.

Cluster analysis on differentially expressed miRNAs. Unsupervised hierarchical clustering analysis was done by using total 34 differentially expressed miRNAs, and this analysis resulted in clearly segregated NPC samples from the normal samples (Fig. 2). Moreover, as shown in Fig. 2, the tumor tissues can be classified into two groups. We associated the groups with tumor progression stage listed in Table I. Surprisingly, three II stage tumors (T1, T2, T7) and

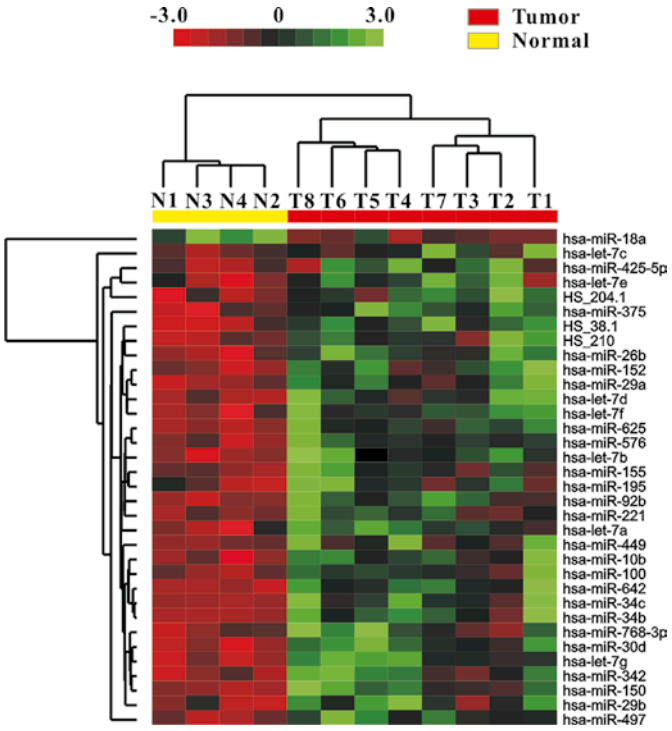

Figure 2. Unsupervised hierarchical clustering of 34 differentially expressed miRNAs in 4 normal (yellow) and 8 NPC (red) samples. Samples were clustered using Pearson correlation (uncentered) and average linkage.

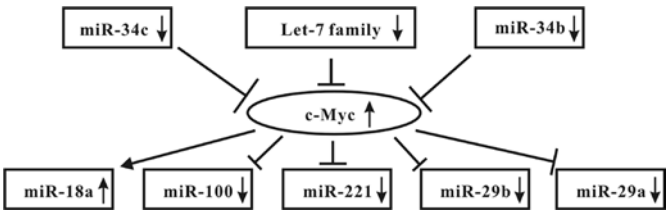

Figure 3. *c-Myc* centred miRNA regulatory network in NPC. *c-Myc* and *miR-18a* are marked with ascending arrow because their expression are increased in NPC. Reduced miRNAs are marked with descending arrow. The up-regulating and down-regulating regulatory effect are indicated by an arrow and a bar, respectively.

one III stage tumor (T3) are in one group; and the other group includes two III stage tumor (T5,T6) and two IV stage tumor (T4, T8). This demonstrates that hierarchical clustering of miRNA expression data by using multiple miRNAs can group NPCs into classes with clinical relevance, also suggesting that these differentially expressed miRNAs may be of special interest in future NPC research because they are involved in the progression of cancer.

Identification of pathways enriched with targets of differentially expressed miRNAs. To evaluate the biological consequences of the miRNA abnormal expressions, we examined the pathway enrichment of targets of down-regulated and up-regulated miRNAs, respectively. For example, focal-adhesion is an important pathway involve in tumor formation and progression, where the kinases is an important mediator of growth-factor signaling, cell proliferation, cell survival and cell migration. The genes in this pathway generally increase their expression in human tumors (34). We observed that the targets of underexpressed miRNAs are enriched in focal-adhesion pathway. The rate of total up-regulated miRNA targets to total up-regulated mRNA genes are 0.261 (584/2231). Thirty-four up-regulated mRNA genes can be mapped into Focal-adhesion pathway, among which 18 genes are regulated by underexpressed miRNAs. The rate of 0.529 (18/34) is significantly higher than the background rate of 0.261 (adjusted $p \leq 0.05$), indicating that the activation of focal-adhesion pathway has strong association with the underexpression of some miRNAs in NPC.

Six GO categories and no pathways were found to be statistically enriched with the targets of the overexpressed miRNA (Table IIIA). The possible explanation for the lack of relevant pathways is that only one miRNA was identified as overexpressed in NPC, whose targets are not enough to be enriched in some pathways. In our study, microarray expression analysis indicated that almost all miRNAs are underexpressed in NPC tissues (33,34). As miRNAs are negative regulators of protein-coding genes, underexpression of these miRNAs are expected to cause an up-regulation of their target genes and alterations of the associated cellular pathways in NPC tissues. In total 22 GO categories and 6 pathways were found to be statistically enriched with targets of the underexpressed miRNAs (Table IIIB), among which some well known cell processes linked with tumor pathogenesis, such as cell-cell signaling, cell adhesion, cell differentiation, cell mobility, and cell senescence, are recognized as strongly associated with miRNA modulation. Several published pathway analyses have reached a conclusion that WNT signaling pathway is abnormally regulated in NPC (12,35,36). In our study, 7 of 11 up-regulated genes in this pathway of 'Wnt receptor signaling pathway', are targeted by underexpressed miRNAs, consistent with their observations. Moreover, the whole genome-wide mRNA expression analysis shows that the pathway 'extracellular matrix structural constituent' is significantly enriched with up-regulated mRNA genes. Sengupta *et al* identified that most of the genes, encoding extracellular matrix proteins, can be regulated by *miR-29c*, which is underexpressed in NPC (13). Although *miR-29c* expression is not significantly decreased in our study, *miR-29a* and *miR-29b* are found to be underexpressed in NPC. *MiR-29a* and *miR-29b* share a majority of targets in the category of 'extracellular matrix structural constituent' with *miR-29c*. In fact, 9 of 13 up-regulated collagens (Table IIIB) can be mediated by *miR-29a* and *miR-29b*, as reported by Sengupta *et al* (13), indicating that the pathways of 'extracellular matrix structural constituent' and 'collagen' are associated with tumor cell invasiveness and metastatic potential of NPC.

Interestingly, two novel pathways are identified to be subject to miRNA regulation. One is sensory perception of sound, where 9 of 15 up-regulated genes are targeted by underexpressed miRNAs. Hearing impairment is frequently associated with nasopharyngeal carcinoma, nonetheless, whether the high frequency hearing loss is the cause or the effect of NPC is still unclear (37-39). It is conjectured that the up-regulation of genes involved in sound perception is a mechanism of compensation for the impairment of hearing ability. The other pathway is 'nervous system develop-

Table III. Statistically enriched pathways by targets of differentially expressed miRNAs.

A, Specifically targeted pathways by overexpressed miRNA.

Enriched GO/Pathway	Target[a]	Total[b]	P-value[c]
GO			
Metal ion binding (F)	13	192	1.20E-02
Zinc ion binding (F)	11	185	4.25E-02
Regulation of transcription\DNA-dependent (P)	8	119	4.19E-02
Nucleus (C)	14	307	7.07E-02
Transcription factor activity (F)	5	72	8.17E-02
Transcription (P)	6	102	8.71E-02

B, Specifically targeted pathways by underexpressed miRNAs.

Enriched GO/Pathway	Target[a]	Total[b]	P-value[c]
Pathway			
Neurogenesis	17	23	3.65E-04
Hs_Kit_Receptor_Signaling	9	11	1.58E-02
Hs_B_Cell_Receptor_Signaling	12	18	2.06E-02
Hs_IL-2_Signaling	10	14	2.18E-02
Hs_Focal_Adhesion	18	34	2.59E-02
Hs_Senescence_and_Autophagy	12	21	6.27E-02
GO			
Cell differentiation (P)	25	47	7.02E-03
Synapse (C)	8	9	9.14E-03
Actin binding (F)	15	24	9.17E-03
Nervous system development (P)	18	29	9.66E-03
Regulation of translation (P)	7	8	1.54E-02
Multicellular organismal development (P)	40	94	1.58E-02
Phosphate transport (P)	14	25	3.08E-02
Membrane (C)	140	429	3.19E-02
Collagen (C)	9	13	3.20E-02
Transcription factor activity (F)	44	116	4.63E-02
Protein binding (F)	260	871	4.72E-02
Sequence-specific DNA binding (F)	21	46	4.96E-02
Basement membrane (C)	9	14	4.98E-02
Extracellular matrix structural constituent (F)	13	24	5.00E-02
Receptor activity (F)	40	102	5.19E-02
Sensory perception of sound (P)	9	15	6.55E-02
Endocytosis (P)	8	13	7.98E-02
Cell-cell signaling (P)	19	43	8.35E-02
Wnt receptor signaling pathway (P)	7	11	8.72E-02
Heart development (P)	9	16	8.75E-02
Cell motility (P)	12	24	8.76E-02
Transcriptional activator activity (F)	11	21	8.85E-02

[a]The number of genes targeted by differentially expressed miRNAs in a GO/Pathway. [b]Total overexpressed/underexpressed genes in a GO/Pathway. [c]The p-value adjusted by the BH method to display the enrichment of miRNA targets in this GO/Pathway, representing the association of this pathway with miRNA modulation. P, F and C in parentheses are denoted as the corresponding term from GO biological process, molecular function and cellular component.

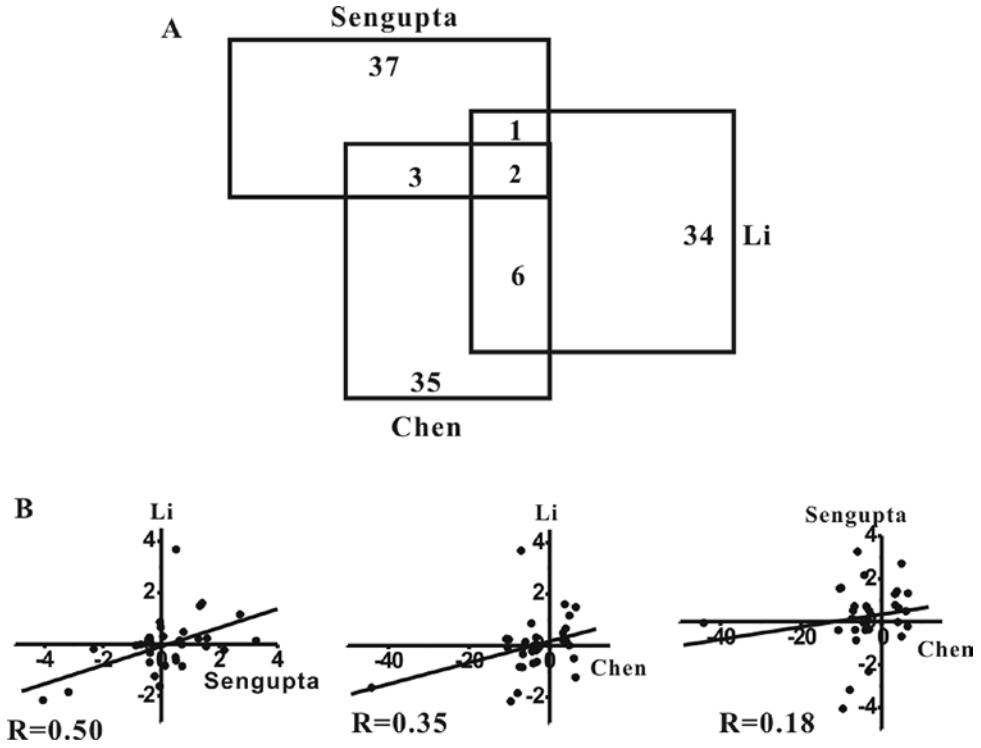

Figure 4. The comparison of three high-throughput NPC dataset. (A) Venn diagram of differentially expressed miRNAs identified in our dataset (dataset Li), dataset Chen *et al* (12) and dataset Sengupta *et al* (13) (B) the data correlation between two datasets. The x-axis and y-axis show the expressions of miRNAs (log2 transformed fold change) in the corresponding dataset. Regress linear line and the Pearson correlation coefficient (R) are displayed in each plot.

ment', including 'neurogenesis' and 'synapse'. Especially for the pathway of 'synapse', almost all up-regulated synapse genes (8/9) are regulated by underexpressed miRNAs, such as SNAP25, SV2B, and SYT1. There are three possible explanations for the relationship between nervous system development and nasopharyngeal carcinoma. First, not only the skull base, but the adjacent central nervous system, is commonly invaded by NPC, and the occurrence of central nervous system metastasis from nasopharyngeal carcinoma (NPC) have been published (40,41). Second, nasopharyngeal carcinoma patients after radiotherapy treatment are prone to central nervous system (CNS) infection (42). Third, the activation of Wnt pathway also induces neuronal circuit development, such as synaptic differentiation, mature synapse modulation and synaptic plasticity (43). These two novel pathways enriched with miRNA targets provide an intriguing clue to the biological consequences of the miRNAs abnormal expression in NPC.

Reconstruction of c-Myc centered miRNA regulatory network. *c-Myc* is an oncogene involved in cell proliferation, differentiation, or cell death (44). It also exhibits significantly elevated expression in NPCs as compared to normal tissues (22). A few of the miRNAs are directly regulated by *c-Myc* or regulate *c-Myc*, leading to the altered expressions of various mRNAs encoding tumor suppressors that block cell proliferation (45). Here, based on our identified differentially expressed miRNAs, we inferred a *c-Myc* centred miRNA regulatory network in NPC (Fig. 3), including two levels of regulation. First, the members of *let-7* family and *miR-34* family can directly down-regulate *c-Myc* expression. Ectopic expression of *let-7* family in nasopharyngeal carcinoma cells resulted in inhibition of cell proliferation through down-regulation of *c-Myc* expression (10). *MiR-34* family, including *miR-34b* and *miR-34c*, are critical regulators of the *c-Myc* expression and serve to remove *c-Myc* to prevent inappropriate replication which may otherwise lead to genomic instability (46). Second, *c-Myc*, as a transcription factor, can directly modulate an array of miRNAs. The link between *miR-18a* and *c-Myc* is well known. *miR-18a*, one of six members of *miR-17-92* cluster, is directly activated by *c-Myc* as a way to fine tune the activity of another *c-Myc* target E2F1 (47). Recently, Mott *et al* experimentally validated that *miR-29* expression can be suppressed by *c-Myc*, promoting the malignant phenotype (48). Kim *et al* proposed that *c-Myc* repressing several miRNAs, including *miR-100* and *miR-221*, plays a role in cell cycle progression (45). It is of great interest to elucidate the regulatory mechanisms of miRNAs in the *c-Myc* pathway in NPC carcinogenesis. Further studies are warranted that more and more miRNAs would be added in this regulatory network. Thus, the inferred *c-Myc* centred network proposes a relevant role of *c-Myc* in NPCs, and opens an important new facet to our understanding of epigenetic alterations in tumors.

Discussion

Two set of high-throughput data have been published to screen the abnormally expressed miRNAs between NPC and its normal counterpart. Chen *et al* employed a quantitative

RT-PCR assay to compare the expressions of 270 miRNAs and identified 35 dys-regulated miRNAs (12). Sengupta *et al* recruited a microarray spotted with 207 miRNAs to examine their expressions. Among these miRNAs, 37 miRNAs exhibit significantly differential expressions between 31 NPC samples and 10 normal samples (Wilcoxon rank sum test, at 5% false discovery rate) (13). For convenience, we call our dataset and these two published dataset as dataset Li, dataset Chen and dataset Sengupta. Eight miRNAs are shared in the list of abnormal expressions by dataset Li and dataset Chen; whereas only 3 miRNAs are differentially expressed in both dataset Li and dataset Sengupta (Fig. 4A). The inconsistency may be explained by heterogeneity of samples and use of different expression measuring platforms. Chen *et al* chose corresponding adjacent normal nasopharynx tissue from patients as normal samples. For dataset Sengupta, the normal references include nasopharyngeal tissues and tissues from other part of NPC patients, whereas our normal samples are normal nasopharyngeal tissues collected from different donors. In addition, the three datasets fail to show good inter-platform concordance. Because Chen *et al* enumerated only the expressions of their significantly differentially expressed miRNAs, there are only 30 miRNAs whose expressions are available in all three datasets. To assess the data consistency, we calculated the Pearson correlation coefficients of these 30 miRNAs between any two platforms. As illustrated in Fig. 4 , the highest correlation is 0.50, indicating that good agreement was not observed between the platforms. It is noticed that the correlation between dataset Li and dataset Sengupta is much higher, suggesting the better data reproducibility in similar techniques because Sengputa *et al* and our study adopted the microarray platform, while Chen *et al* used a stem-loop RT-PCR assay to quantify the expression levels of miRNAs in NPC tissues. Nevertheless, several miRNAs are commonly detected to be abnormally expressed. For example, *miR-18a* is overexpressed in the datasets Li and Chen, which is reported as potential oncogenes in various tumors (49). Reduced *miR-29c*, up-regulating the mRNAs encoding extracellular matrix proteins, contributes the invasion of NPC (13). In both dataset Chen and Sengupta, *miR-29c* is consistently down-regulated. In our dataset, the expression of *miR-29c* in NPCs is moderately lower than those in normal tissues, yet not meeting the statistical significance level. However, the other two members of *miR-29* family, *miR-29a* and *miR-29b* are significantly down-regulated in our dataset. In fact, the significant underexpression of *miR-29b* is also observed in dataset Sengupta. The seed sequence of *miR-29c* is identical to that of *miR-29a* and *miR-29b*, leading to heavily overlapping targets of the three members of *miR-29* family. Therefore, it is suggesting that the mechanism of decreased *miR-29c* to elevate the expression of genes related to extracellular matrix may be compensated by underexpression of *miR-29a* and *miR-29b* in our study. Two underexpressed miRNAs, *miR-34b* and *miR-34c* are overlapped in all three datasets. The reduced expression of *miR-34b* and *miR-34c* frequently occur in various cancers, which is believed to be strongly associated with p53 network (27-29,50). Interestingly, the down-modulated *let-7* family members are observed only by our study. Wong *et al* have experimentally confirmed the reduced expression level of *let-7* family in nasopharyngeal carcinoma cells (10). The main functionality of down-regulated tumor-suppressive *let-7* family is interpreted to directly induce a few key proto-oncogenes, such as *RAS* and *c-Myc* (51). The *let-7* miRNAs may be of special interest to be tumor suppressors in future nasopharyngeal carcinoma research.

In our study, a group of miRNAs were identified as significantly differentially expressed between NPC and normal tissues. The number of overexpressed miRNAs is much lower than that of down-regulated miRNAs. Only 1 miRNAs showed overexpression in NPC, whereas 33 miRNAs were underexpressed in NPC. The demonstration of the widespread down-regulation of miRNAs in NPC is consistent with the findings by Chen *et al* and Sengupta that a large number of miRNAs are down-modulated in NPC tissues (12,13). Accumulated research has reported the widespread down-regulation of miRNA expression in human cancers (5,17,52,53). Lu *et al* used a bead-based detection system to investigate the miRNA expression in multiple human cancers. Their research displayed that a majority of miRNAs are underexpressed in cancer tissues as compared to normal tissues (5). Mattie *et al* also found widespread down-regulation of miRNA on two biopsies of breast cancer versus pooled normal tissue using a microarray-based approach (53). Ozen *et al* observed that almost all of the miRNAs detected were down-regulated in the majority of the prostate cancer samples (17). Zhang *et al* speculated that DNA copy number loss may contribute to the widely down-regulation of miRNAs because miRNAs are frequently located in cancer-associated regions of the human genome (54). In addition, Thomson indicated that the widespread down-regulation of miRNAs observed in human cancers might be due to a failure in miRNA processing, especially the activity of the enzyme Drosha, which digests the primary miRNA (pri-miRNA) in the nuclease to release hairpin, precursor miRNA (pre-miRNA) (55). The decreased activity of *Drosha* leads to the deficiency of biogenesis of pre-miRNA and mature miRNA, and then the widespread down-regulation of miRNA can be observed. This provides a hint that the deregulation of genes taking part in the biogenesis of miRNAs may play critical roles in tumorigenesis (56).

In conclusion, our study identified 34 aberrant miRNAs occurred in human NPC tissues with respect to normal tissues using the high-sensitive and high-throughput microarray technology. Several well characterised biological pathways were identified to be significantly enriched with targets of the under-expressed miRNAs. Two novel pathways, nervous system development and sensory perception of sound, were identified strongly associated with NPC development. Furthermore, our study revealed that a *c-Myc* centred miRNA regulatory network may play roles in NPC tumorigenesis. Our work indicates that miRNAs are potential diagnosis biomarkers and probable factors involved in the pathogenesis of NPC. This study of differentially expressed miRNAs may lead to finding their potential for improving diagnosis, prognosis and their impact on further therapeutic strategies.

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