Distinctive microRNA expression in early stage nasopharyngeal carcinoma patients

Shuna Li a, *, Lihua Hang b, Yongming Ma a, Chaoyang Wu c

a Department of Otolaryngology and Head-Neck Surgery, Zhenjiang First People’s Hospital, The Affiliated People’s Hospital of Jiangsu University, Zhenjiang, Jiangsu, China
b Department of Anesthesia, Zhenjiang First People’s Hospital, The Affiliated People’s Hospital of Jiangsu University, Zhenjiang, Jiangsu, China
c Department of Radiation Oncology, Zhenjiang First People’s Hospital, The Affiliated People’s Hospital of Jiangsu University, Zhenjiang, Jiangsu, China

Received: March 16, 2016; Accepted: May 15, 2016

Abstract

The goal of this study was to investigate microRNAs (miRs) expression at different stages of nasopharyngeal carcinoma (NPC). MiR expression profiling at various stages of NPC was performed by miR array and further verified using quantitative real-time RT-PCR. Pathway enrichment analysis was carried out to identify the functional pathways regulated by the miRs. The expression of a selected group of identified miRs was verified in stage I NPC by in situ hybridization (ISH). A total of 449 miRs were identified with significantly different expressions between NPC tissues and normal pharyngeal tissues. Eighty-four miRs were dysregulated only in stage I NPC, among which 45 miRs were up-regulated and the other 39 were down-regulated. Pathway enrichment assay revealed that three significantly down-regulated and three significantly up-regulated miRs involved in 12 pathways associated with tumour formation and progression. Quantitative RT-PCR confirmed the miR array result. In addition, the low expression levels of hsa-miR-4324, hsa-miR-203a and hsa-miR-199b-5p were further validated in stage I NPC by ISH. This present study identified the miR signature in stage I NPC, providing the basis for early detection and treatment of NPC.

Keywords: microRNA • nasopharyngeal carcinomas • microarrays • early stage

Introduction

Nasopharyngeal carcinoma (NPC) is a common type of cancer in Southeastern Asia and Africa. It is closely related to many viral, dietary and genetic factors [1–3]. Intensity-modulated radiation therapy and active anticancer agents are standard treatment options for NPC [4]. In recent years, cancer stem cells and gene therapy are new concepts and promising strategies for NPCs, but these new technologies have yet to be applied in the clinic [5, 6]. Similar to other types of malignant tumour, TNM stages of NPC are significantly correlated with the treatment efficacy and the prognosis of the disease. Stage I NPC is easier to treat while prognosis is very poor in late stage NPC [7–9]. Therefore, it is important to understand the biomarkers and signatures of early stage NPC so that treatment can start right away.

MicroRNAs (or miRNAs) are short non-coding RNAs involved in post-transcriptional regulation of gene expression [10]. They can be found in various organisms including animals, plants and viruses, and they play a key role in diverse biological processes, such as embryogenesis, differentiation and proliferation of cells, production of cytokines or apoptosis [10, 11]. Since the initial observation, more and more miRNAs have been identified in mammalian cells and up to one-third of all protein-encoding genes are estimated to be regulated by these small molecules [12]. Based on current literature, miRNA dysregulation plays a major role in head and neck/oral cancer [13]. Identification of the dysregulated miRNAs in cancer (especially at early stages) offers great potential for early diagnosis and new therapeutic targets [14, 15]. To that end, it is crucial to study the dysregulated miRNAs in NPC. Previous studies suggest the importance to study the relationship between miRNAs and NPC [16–21]. Also, some researches have been carried out to reveal with the relationship between miRNA expression and NPC radioresistance and recurrence [22–25]. To date, little has been known regarding the dysregulated miRNAs in early stage NPC.

In this study, we employed the Agilent Microarray platform to analyse miR expression in different stages of NPC. Interestingly, 84 miRs were found dysregulated only in stage I NPC. Pathway
enrichment analysis and *in situ* hybridization (ISH) further revealed the cancerous pathways regulated by the identified miRs. We expect our results provide possible targets for the development of new gene therapies to treat NPC at early stages [26].

**Material and methods**

**Tissue samples**

All samples were obtained with approval of the Ethics Committee of the Affiliated People’s Hospital of Jiangsu University. Nasopharyngeal carcinoma tissue samples were taken from poorly differentiated squamous NPC patients at different TNM stages before treatment at the Cancer Center of the Affiliated People’s Hospital of Jiangsu University. Normal nasopharyngeal tissue samples were collected in the same hospital. Eight samples were obtained from eight NPC patients at different stages and two samples from normal nasopharyngeal tissues. Samples we used are listed in Table 1. Those 10 samples were further divided into five groups: Normal, stage I, II, III and IV for microarray analysis. According previous results, sample pooling does not significantly improve inferences. One can decrease the number of arrays required in an experiment without a loss of precision [27, 28]. All tissues were fixed in 10% neutralized formalin and embedded in paraffin. Pathological types were confirmed by haematoxylin and eosin staining and immunohistochemically staining. TNM stages were judged according to the UICC/AJCC staging system for NPC, seventh edition (2009).

**RNA isolation and microRNA microarray hybridization**

Total RNA was extracted and purified using RecoverAll™ Total Nucleic Acid Isolation Reagent (Ambion, Austin, TX, USA) following the manufacturer’s instructions. RNA concentration and integration were examined by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The MiRs in total RNA were labelled using the miRNA Complete Labeling and Hyb Kit (Agilent Technologies) following the manufacturer’s instructions. Each slide was hybridized with 100 ng Cy3-labelled RNA using miRNA Complete Labeling and Hyb Kit (Agilent Technologies) in hybridization Oven (Agilent Technologies) at 55°C, 20 r.p.m. for 20 hrs according to the manufacturer’s instructions. After hybridization, slides were washed in staining dishes (Thermo Shandon, Waltham, MA, USA) with Gene Expression Wash Buffer Kit (Agilent Technologies). Slides were scanned by the Agilent Microarray Scanner (Agilent Technologies) powered by the Feature Extraction software 10.7 (Agilent Technologies) with default settings. Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent Technologies). After normalization, differentially expressed miRs were identified through Fold Change filtering.

**Real-time quantitative PCR**

To ascertain the microarray results, miR-203a, miR-199b-5p, miR-2117, miR-4494, miR-4502 and miR-4324 were selected for quantitative real-time RT-PCR analysis. FAM-labelled Taqman ABI probe-based real-time PCR assays for miR-4324 (context sequence: CCCUAGAGCCUACC UUAA), miR-203a (context sequence: AGUGGUUCUUAACAGUUCAACAG UU), miR-199b-5p (context sequence: CCGAGUGUUAGACUAUCUGU UC), miR-2117(context sequence: UGUGUCUCUGUUGCCAAGGACAG), miR-4494 (context sequence: CCAGACUUGGGCUAGACCCAGG) and miR-4502(context sequence: GCUAGUGAUAGGUGUCUGAAG) were carried out on: ABI 7900 HT Sequence Detection System according to the ABI Taqman microRNA assay protocol. U6 small nuclear RNA was used as the internal standard for determining the relative miRNA expression level. The reactions were incubated at 50°C for 2 min., 95°C for 10 min., followed by 40 cycles at 95°C for 15 sec., 60°C for 1 min. All PCR reactions were performed in triplicate. The 2^(-ΔΔCT) method was used as relative quantification measure of differential expression.

**MicroRNAs *in situ* hybridization**

Locked nucleic acid (LNA) ISH on paraffin tissue sections was performed with a double 5'-digoxigenin (DIG)-labelled LNA probe specific for human miR-4324, miR-203a and miR-199b-5p (Exiqon, Woburn, MA, USA). 20 paraffin-embedded sections came from 20 NPC patients (five in each NPC stage) were used for ISH analysis. First, paraffin-embedded sections were deparaffinized in xylenes and then rehydrated through an ethanol dilution series. Slides were then treated with Proteinase K at 15 μg/ml for 10 min. at 37°C. Hybridization was performed at 54°C for the following: DIG labelled (U6) and double DIG (scrambled and miR-4324, miR-203a, miR-199b-5p), LNA-modified oligonucleotide ISH probes. Positive probe labelling was blue/purple. Nuclei were visualized using Nuclear Fast Red counterstain (Vector Laboratories Inc., Burlingame, CA, USA).

**Results**

**Distinctive microRNA expressions in NPC at different stages and nasopharyngitis tissues**

A total of 2006 human miRs were detected with the Agilent’s microarray platform driven by Sanger miRBase (Release 19.0). The original data were analysed using Gene Spring Software (Agilent Technologies). The information of NPC samples

| Sample | Gender | Age | TNM stage | Cancer stage |
|--------|--------|-----|-----------|--------------|
| 1      | Male   | 68  | T1N0M0    | I            |
| 2      | Male   | 56  | T1N0M0    | I            |
| 3      | Male   | 49  | T1N1M0    | II           |
| 4      | Female | 59  | T1N1M0    | II           |
| 5      | Male   | 73  | T3N0M0    | III          |
| 6      | Female | 67  | T2N2M0    | III          |
| 7      | Male   | 73  | T3N3M1    | IV           |
| 8      | Male   | 52  | T2N3M0    | IV           |

Table 1 The information of NPC samples
Technologies) after normalization for fold change to identify differentially expressed genes, based on the following selection criteria: fold change (linear) ≤ 0.5 or fold change (linear) ≥ 2. After the initial screening, 449 miRs were kept for the subsequent distinctive analysis. As shown in the Venn diagram (Fig. 1), some dysregulated miRs only appeared in certain stage, while others appeared in more than one stage of NPC.

**Distinctive microRNA expression in stage I NPC**

We are interested in distinctive miR expression at various stages of NPC, especially in stage I NPC. Stage I is crucial for the formation of NPC and an important time-point to intervene [11]. In this study, we found total 84 miRs only dysregulated in stage I NPC (Fig. 1). Among those 84 miRs, 45 were up-regulated and 39 were down-regulated (Table 2). One miR can target hundreds of genes and one gene can be targeted by multiple miRs. To that end, we selected the most dysregulated miRs for the further analysis, according to highest or lowest FC values (i.e. high FC values obtained by up-regulated miRs and lower FC values obtained by down-regulated miRs). To evaluate the biological consequence of abnormal miR expressions, we employed the miRDB software [29, 30] to analyse targeted genes and functions. We also employed the Cytoscape software (The Cytoscape v1.1 Core runs on all major operating systems and is freely available for download from http://www.cytoscape.org/as an open source Java application,) to generate miRs function network. Using DAVID tools (The Database for Annotation, Visualization and Integrated Discovery v6.7) [31, 32] we acquired pathway enrichment from gene ontology. Through the KEGG pathway databases, we examined the pathway targets enrichment (P < 0.05) of down-regulated miRNAs (hsa-miR-4324, hsa-miR-203a, hsa-miR-199b-5p) (Table 3) and up-regulated miRNAs (hsa-miR-2117, hsa-miR-4494, hsa-miR-4502) (Table 4).

Pathways in cancer, ErbB signalling, insulin signalling, adipokine signalling pathway, focal adhesion, renal cell carcinoma, aldosterone-regulated sodium reabsorption, neurotrophin signalling, Fc gamma R-mediated phagocytosis and transforming growth factor (TGF)-beta signalling were co-regulated by down-regulated miRNAs (hsa-miR-4324, hsa-miR-203a, hsa-miR-199b-5p) (Table 3). Although, three up-regulated miRs (hsa-miR-2117, hsa-miR-4494, hsa-miR-4502) modulated NPC genesis by axon guidance and apoptosis (Table 4). As the results of pathway enrichment analysis, three down-regulated miRs were involved in malignant tumour pathways. To verify the reliability of miR array result, quantitative RT-PCR was carried out to investigate the expressions of hsa-miR-4324, hsa-miR-203a, hsa-miR-199b-5p, hsa-miR-2117, hsa-miR-4494 and hsa-miR-4502 in stage I, II, III, IV NPC tissues or in normal nasopharyngeal tissues. Consistent with the array data, hsa-miR-2117, hsa-miR-4494 and hsa-miR-4502 were significantly up-regulated in stage I NPC; whereas hsa-miR-4324, hsa-miR-203a and hsa-miR-199b-5p were less expressed in stage I NPC (Fig. 2). For further investigation, ISH were performed on another 20 samples at various NPC stages (different from the samples used in microarray). In situ hybridization results confirmed low expressions of hsa-miR-4324, hsa-miR-203a and hsa-miR-199b-5p in stage I NPC (Fig. 3).

**Hierarchical clustering analysis of dysregulated miRNA expression in all NPC stages**

Hierarchical clustering analysis of the 49 miRNAs dysregulated in all stages of NPC (The “O” category in Fig. 1) was performed with R software. By Hierarchical clustering analysis, expression diversity of those 49 miRNAs was observed in NPC and normal pharyngeal tissues with visual representation. As shown in Figure 4, comparing with nasopharyngitis tissue, tumour tissues were classified into two groups: stage I and II NPC in one group, and stage III and IV NPC in the other group (x-axis).

**Let-7 family expression in the microarray data**

In the microarray analysis, nine members of let-7 family were dysregulated according the FC criteria. Five members of let-7 family

---

**Fig. 1** Venn diagram of differentially expressed miRNAs in stages of NPC. Number 1, 2, 3, 4: stage I, II, III, IV and a-o: miRNA number with diverse expression in different stage.

---

© 2016 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.
Table 2: MicroRNAs only dysregulated in stage I NPC. 45 miRNAs were up-regulated (left, FC ≥ 2) and 39 miRNAs were down-regulated (right, FC ≤ 0.5)

| Up-regulated miRNAs | Fold change (NPC/Normal pharyngeal tissue) | Down-regulated miRNAs | Fold change (NPC/Normal pharyngeal tissue) |
|----------------------|-------------------------------------------|-----------------------|-------------------------------------------|
| hsa-miR-2117         | 35.19640078                               | hsa-miR-203a          | 0.00438528                                |
| hsa-miR-4502         | 28.42966709                               | hsa-miR-4324          | 0.013114843                                |
| hsa-miR-4494         | 24.85078121                               | hsa-miR-199b-5p       | 0.015106744                                |
| hsa-miR-5686         | 6.234625357                               | hsa-miR-152           | 0.015655062                                |
| hsa-miR-3163         | 5.981554649                               | hsa-miR-532-5p        | 0.021356314                                |
| hsa-miR-139-5p       | 5.897478688                               | hsa-miR-214-3p        | 0.02341181                                 |
| hsa-miR-4436a        | 5.782638599                               | hsa-miR-132-3p        | 0.02550163                                 |
| hsa-miR-4674         | 5.673874972                               | hsa-miR-98-5p         | 0.076613182                                |
| hsa-miR-4717-3p      | 5.63948609                                | hsa-miR-199a-5p       | 0.080598807                                |
| hsa-miR-4748         | 5.538848968                               | hsa-miR-10b-5p        | 0.085482191                                |
| hsa-miR-518a-5p      | 5.429137528                               | hsa-miR-148b-3p       | 0.095244995                                |
| hsa-miR-3680-3p      | 5.411772528                               | hsa-miR-6073          | 0.114204104                                |
| hsa-miR-30b-3p       | 5.311856969                               | hsa-miR-199a-3p       | 0.125620428                                |
| hsa-miR-4519         | 5.270859003                               | hsa-miR-193a-3p       | 0.12792638                                 |
| hsa-miR-1254         | 4.898350333                               | hsa-miR-151a-3p       | 0.128389409                                |
| hsa-miR-4660         | 4.861456796                               | hsa-miR-487b          | 0.144085394                                |
| hsa-miR-4694-3p      | 4.692768851                               | hsa-miR-128           | 0.145525102                                |
| hsa-miR-4707-3p      | 4.658102545                               | hsa-miR-125a-5p       | 0.1829238                                  |
| hsa-miR-4697-3p      | 4.632601224                               | hsa-let-7e-5p         | 0.191501673                                |
| hsa-miR-4314         | 2.62042406                                | hsa-miR-99b-5p        | 0.194760485                                |
| hsa-miR-339-3p       | 2.60592703                                | hsa-miR-200b-3p       | 0.277032588                                |
| hsa-miR-4526         | 2.599055786                               | hsa-let-7d-5p         | 0.288889485                                |
| hsa-miR-1323         | 2.596089822                               | hsa-miR-22-3p         | 0.325533856                                |
| hsa-miR-1469         | 2.504560567                               | hsa-miR-324-5p        | 0.338455696                                |
| hsa-miR-6129         | 2.488412744                               | hsa-miR-365a-3p       | 0.34912837                                 |
| hsa-miR-3682-3p      | 2.43621794                                | hsa-miR-374b-5p       | 0.355823031                                |
| hsa-miR-1273c        | 2.435106719                               | hsa-miR-146b-5p       | 0.370538563                                |
| hsa-miR-4673         | 2.377707584                               | hsa-miR-146a-5p       | 0.371381254                                |
| hsa-miR-652-5p       | 2.373619362                               | hsa-miR-664a-3p       | 0.37268886                                 |
| hsa-miR-4476         | 2.274244399                               | hsa-miR-23b-3p        | 0.378322373                                |
| hsa-miR-424-3p       | 2.249633838                               | hsa-miR-361-5p        | 0.378351875                                |
**Discussion**

MicroRNAs can be dysregulated in cancer, in which they function as a group to mark differentiation states or individually as bona fide oncogenes or tumour suppressors [34]. We selected formalin-fixed, paraffin-embedded (FFPE) tissues for analysis because: (i) many studies have demonstrated that miRs are minimally affected by FFPE treatment [35–37]; (ii) FFPE NPC tissues have been effectively used for diagnosis with haematoxylin and eosin and immunostaining as these samples can be easily collected from clinical tissue banks. Stage I NPC samples (T1N0M0) are rare since enlargement of the lymph nodes occurs as the primary symptom in more than 50% NPC patients [38]. Multiple studies have been performed for miR expression profile in NPC, but specific expressions in different stages of NPC remain unrevealed [24,25]. In our microarray platform, 2006 known miRs were detected. As shown in Figure 1, 449 miRNAs were expressed in NPC at various stages. In 84 miRNAs only dysregulated in stage I NPC, three most down-regulated miRs, namely miR-203, miR-199b-5p, miR-4324, were selected for further analysis. In previous studies, those three miRs were found to be down-regulated in some forms of cancers. MiR-203 suppresses cancer cell proliferation through the inhibition of SRC in lung cancer [39]. ZNF217 and CASK were proved as other targets of miR-203 and knockdown of ZNF217 and repressing CASK expression attenuated cell proliferation, invasion and migration in colorectal cancer [40, 41]. In addition, MiR-203 can enhances 5-FU chemosensitivity via the down-regulation of TYMS in colorectal cancer [42] and drive progression of prostate cancer by suppressing LASP1 [43]. Moreover, miR-203 is regulated by C/EBPb-LIP, E2F, Jun N-terminal protein kinase and NF-jB in cancer [18, 44, 45]. The latter implies that EBV promotes malignancy by down-regulating cellular miR-203 in NPC [18]. MiR-199b-5p was deemed to be a regulator of the Notch pathway and Sonic hedgehog (SHH) pathway through its targeting of the transcription factor Hairy and enhancer of split 1 (HES1) [46], involved in transcription and post-transcription regulation in erythroid differentiation [47]. In the highly aggressive osteosarcoma cell lines and in the follicular thyroid carcinoma, miR-199b-5p was down-regulated [48, 49]. Stable nucleic acid lipid particles that encapsulate miR-199b-5p has been used as a tool to impairment of cell proliferation with no signs of apoptosis, which will be the

| Up-regulated miRNAs | Fold change (NPC/Normal pharyngeal tissue) | Down-regulated miRNAs | Fold change (NPC/Normal pharyngeal tissue) |
|---------------------|-------------------------------------------|-----------------------|-------------------------------------------|
| hsa-miR-4758-5p     | 2.49353332                                 | hsa-let-7f-5p         | 0.40459317                                 |
| hsa-miR-4257        | 2.203649232                                | hsa-let-7g-5p         | 0.40904785                                 |
| hsa-miR-4507        | 2.183758018                                | hsa-miR-425-5p        | 0.409401672                                |
| hsa-miR-4470        | 2.169207555                                | hsa-miR-29a-3p        | 0.420675499                                |
| hsa-miR-5088        | 2.168038536                                | hsa-miR-27b-3p        | 0.440837238                                |
| hsa-miR-564         | 2.153669691                                | hsa-miR-3676-3p       | 0.462569599                                |
| hsa-miR-4745-5p     | 2.134746854                                | hsa-miR-1260b         | 0.46793252                                 |
| hsa-miR-3605-5p     | 2.12263866                                 | hsa-let-7i-5p         | 0.468495144                                |
| hsa-miR-3654        | 2.120827526                                |                        |                                           |
| hsa-miR-1273e       | 2.093393465                                |                        |                                           |
| hsa-miR-4481        | 2.076230617                                |                        |                                           |
| hsa-miR-550a-3-5p   | 2.033664087                                |                        |                                           |
| hsa-miR-4294        | 2.019948765                                |                        |                                           |
| hsa-miR-3945        | 2.013610577                                |                        |                                           |

(Atlantic Journal of Cellular and Molecular Medicine, 2016)
basis for future preclinical studies [50]. Hsa-miR-4324 was down-regulated in cutaneous malignant melanoma [51]. Employing the DAVID tools, the targets of down-regulated miRNAs (hsa-miR-203a, hsa-miR-199b-5p, and hsa-miR-4324) were examined. Pathways in cancer, ErbB signalling pathway, insulin signalling pathway, adipokine signalling pathway, focal adhesion, renal cell carcinoma, aldosterone-regulated sodium reabsorption, neurotrophin signalling pathway, Fc gamma R-mediated phagocytosis and TGF-beta signalling were co-regulated by hsa-miR-4324, hsa-miR-203a, hsa-miR-199b-5p in stage I of NPC (Table 3). All these pathways were proved involved in tumour formation and progression [52–58]. Those three down-regulated miRNAs may promote the formation of NPC through these pathways. To avoid variety of pooled samples in microarray, we evaluated the expression of miR-203, miR-199b-5p and miR-4324 by ISH on FFPE sections from another group of NPC patients. The results of ISH also showed down-expression of those three miRs. Although previous researches reveal the cancer relevance of miR-203, miR-199b-5p and miR-4324, we are the first to report those three miRNAs were specifically down-regulated in stage I NPC. Future studies will focus on the mechanisms underlying how miR-203, miR-199b-5p and miR-4324 regulates NPC formation.

On the other hand, we analysed three up-regulated miRs (hsa-miR-2117, hsa-miR-4494, hsa-miR-4502) in stage I NPC. MiR-2117 has been suggested as a potential bona fide miR in ovarian cancer [59].

### Table 3 Pathways enrichment and related genes of hsa-miR-4324, hsa-miR-203a and hsa-miR-199b-5p (three down-regulated miRs in stage I NPC)

| KEGG_PATHWAY                  | Count | %     | P-value | Genes                                                                 |
|-------------------------------|-------|-------|---------|-----------------------------------------------------------------------|
| Pathways in cancer            | 40    | 2.8531| 0.001283| KITLG, GLI3, TPM3, TGFB2, LAMB4, PTK2, PAK8, PIK3CA, NKK3-1, TPR, COL4A4, PRKCA, BMP2, CTBP1, PLD1, TGF7, COL4A1, CTBP2, ENSL1, IL8, PIK3CD, STAT1, APP1, STK4, FZD4, PRKCB, RAD51, MAPK1, SM0, CDC6, CDKN1B, HIF1A, ETS1, GSK3B, JUN, MAPK9, PTCH1, LAMC1, ABL1, CRK |
| ErbB signalling pathway       | 16    | 1.1412| 0.001324| PRKCA, NRG4, ERBB3, PIK3CD, PRKCB, MAPK1, PTK2, CDKN1B, JUN, GSK3B, GAB1, PIK3CA, MAPK9, ABL1, CRK, ABL2 |
| Insulin signalling pathway    | 21    | 1.4979| 0.001621| SOCS3, PRKAG2, PHKA1, PIK3CD, HK2, PRKCI, PRKAB1, RHQ, PPP1CB, PPARGC1A, IRS1, PCK1, G6PC2, MAPK1, GSK3B, PIK3CA, MAPK9, PRKAA2, PTPN1, CRK, RAPGEF1 |
| Adipokine signalling pathway  | 13    | 0.9272| 0.002844| PPARA, SOCS3, PRKAG2, PRKAB1, IRS1, PPARGC1A, G6PC2, PCK1, ACSL1, CD63, MAPK9, PRKAA2, ACSL6 |
| Focal adhesion                | 25    | 1.7832| 0.010295| CAV2, CAV1, TNC, LAMB4, PTK2, PPP1R12A, PIK3CA, TNN, PDGFD, THBS2, RAPGEF1, COL4A4, PRKCA, COL4A1, PIK3CD, PPP1CB, FLNB, PRKCB, MAPK1, GSK3B, JUN, MAPK9, RAP1A, LAMC1, CRK |
| Renal cell carcinoma          | 12    | 0.8559| 0.01145  | MAPK1, HIF1A, EPAS1, ETS1, JUN, PIK3CD, GAB1, RAP1A, PIK3CA, CRK, RAPGEF1, TGF2B |
| Aldosterone-regulated sodium reabsorption | 8     | 0.5706| 0.026626| PRKCA, MAPK1, PIK3CD, ATP1B4, PIK3CA, NEDD4L, IRS1, PRKCB |
| Neurotrophin signalling pathway| 16    | 1.1412| 0.034362| IRAK2, PIK3CD, IRS1, RPS6KA6, MAPK1, PSEN1, MAP3K1, JUN, GSK3B, GAB1, PIK3CA, RAP1A, MAPK9, ABL1, CRK, RAPGEF1 |
| Fc gamma R-mediated phagocytosis| 13   | 0.9272| 0.041454| PRKCA, PLD2, DNM3, PLD1, WASF1, PIK3CD, ARF6, ARPC5, PRKCB, MAPK1, PIK3CA, PPAP2B, CRK |
| TGF-beta signalling pathway   | 12    | 0.8559| 0.04935  | ACVR2A, MAPK1, ACVR2B, BMP2, SMAD9, ID4, SMURF2, SMAD1, BMPR1B, THBS2, CUL1, TGF2B |

### Table 4 Pathways enrichment and related genes of hsa-miR-2117, hsa-miR-4494, hsa-miR-4502 (three up-regulated miRNAs)

| KEGG_PATHWAY                  | Count | %     | P-value | Genes                                                                 |
|-------------------------------|-------|-------|---------|-----------------------------------------------------------------------|
| Apoptosis                     | 9     | 1.1111 11 | 0.029075| BID, IRAK3, CASP7, IL1RAP, CHP2, PPP3CC, ENDOD1, PPP3CA, BIRC3 |
| Axon guidance                  | 11    | 1.358025| 0.044223| SEMA5A, PAK7, NCK2, PAK2, CHP2, PPP3CC, SEMA3A, PPP3CA, UNC5C, SRGAP1, RASA1 |
The predictive targets of miR-2117 were involved in two important pathways, apoptosis and axon guidance (Table 4). Axon guidance (i.e. axon path finding) is a process by which neurons send out axons to reach the correct targets. SEMA3F, an important molecule in axon guidance, is involved in cell adhesion, migration, invasion, and proliferation and inhibits the growth and metastasis in cancer [60–62]. The up-regulated miRs were also found to suppress apoptosis pathways. An impaired apoptosis often results in formation of tumours [63]. It has been reported that the Let-7 family associated with the growth and invasion of malignant tumours including NPC [17, 64, 65]. Interestingly, we found eight Let-7 members (namely hsa-let-7a-5p, hsa-let-7b-5p, hsa-let-7c, hsa-let-7d-5p, hsa-let-7e-5p, hsa-let-7f-
Fig. 4 Hierarchical clustering of 49 miRNAs expressed differentially between stages I, II, III, IV of NPC.

Table 5 The sample-signal value of let-7 family in different stages of NPC detected by microarray

| Systematic name | Stage I_NS | Stage II_NS | Stage III_NS | Stage IV_NS | Nasopharyngitis_NS |
|-----------------|------------|------------|--------------|-------------|--------------------|
| hsa-let-7a-5p   | 8.102136   | 9.300925   |              |             | 10.331304          |
| hsa-let-7b-5p   | 7.544303   | 8.989673   |              |             | 10.410756          |
| hsa-let-7c      | 6.1174273  | 7.374718   |              |             | 8.511084           |
| hsa-let-7d-3p   | -3.321953  | -3.308076  | -3.1660423   | -3.301246   | 0.945101           |
| hsa-let-7d-5p   | 5.027936   | 6.8193464  |              |             | 6.8193464          |
| hsa-let-7e-5p   | 3.8237662  | 6.2083373  |              |             | 9.0592785          |
| hsa-let-7f-5p   | 7.753345   | 8.574723   |              |             | 8.574723           |
| hsa-let-7g-5p   | 7.285065   | 8.342013   |              |             |                    |

Sample-signal values of let-7 family screened by FC ≥2 and FC ≤0.5 were listed in this table only.

© 2016 The Authors.
Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.
5p, hasa-let-7g-5p, hasa-let-7i-5p) were down-regulated in early stage of NPC and 1 member (hasa-let-7d-3p) was down-regulated in all stages of NPC. We speculated that since most Let-7 family members were dysregulated in early stage of NPC they may be involved in the early formation of NPC.

In summary, we have identified stage-specific miRs in NPC patients. In this study, miRs specifically dysregulated in stage I NPC. Several biological pathways were identified to be associated with the identified miRNAs. In situ hybridization further confirmed the low expressions of miR-203, miR-199b-5p and miR-4324 in stage I NPC. Although it has been reported that miRs play an important role in NPC carcinogenesis [19–21], our research advanced the field by identifying stage-specific miRs. Those miRs are important regulators of NPC formation and can serve as potential therapeutic targets or as biomarkers for early diagnosis. We also found 49 miRNAs dysregulated in every stage of NPC as compared to normal nasopharyngeal tissues. It is likely that those miRs overarch the whole progression of NPC, not just formation.

**Conflict of interest**

None.
40. Wang N, Liang H, Zhou Y, et al. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. *RNA*. 2007; 13: 1668–74.

36. Li J, Smyth P, Flavin R, et al. Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells. *BMC Biotechnol*. 2007; 7: 36.

37. Masuda N, Ohnishi T, Kawamoto S, et al. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res*. 1999; 27: 4436–43.

38. Prasad U, Pua KC. Nasopharyngeal carcinoma: a delay in diagnosis. *Med J Malaysia*. 2000; 55: 230–5.

39. Wang N, Liang H, Zhou Y, et al. miR-203 suppresses the proliferation and migration and promotes the apoptosis of lung cancer cells by targeting SRC. *PLoS ONE*. 2014; 9: e105570.

40. Li Z, Du L, Dong Z, et al. MiR-203 suppresses ZNF217 upregulation in colorectal cancer and its oncogenicity. *PLoS ONE*. 2015; 10: e0116170.

41. Zhou X, Xu G, Yin C, et al. Down-regulation of miR-203 induced by Helicobacter pylori infection promotes the proliferation and invasion of gastric cancer by targeting CASK. *Oncotarget*. 2014; 5: 11631–40.

42. Li T, Gao F, Zhang XP. MiR-203 enhances chemosensitivity to 5-fluouracil by targeting thymidylate synthase in colorectal cancer. *Oncol Rep*. 2015; 33: 607–14.

43. Hailer A, Grunewald TG, Orth M, et al. Loss of tumor suppressor miR-203 mediates overexpression of LIM and SH3 Protein 1 (LASP1) in high-risk prostate cancer thereby increasing cell proliferation and migration. *Oncotarget*. 2014; 5: 4144–53.

44. Li J, Shan F, Xiong G, et al. SGF-induced C/EBPbeta participates in EMT by decreasing the expression of miR-203 in esophageal squamous cell carcinoma cells. *J Cell Sci*. 2014; 127: 3735–44.

45. Zhang K, Dai L, Zhang B, et al. MiR-203 is a direct transcriptional target of E2F1 and causes G1 arrest in esophageal cancer cells. *J Cell Physiol*. 2015; 230: 903–10.

46. Won KY, Kim YW, Kim HS, et al. MicroRNA-199b-5p is involved in the Notch signaling pathway in osteosarcoma. *Hum Pathol*. 2013; 44: 1648–55.

47. Li Y, Bai H, Zhang Z, et al. The up-regulation of miR-199b-5p in thyroid differentiation is associated with GATA-1 and NF-E2. *Mol Cells*. 2014; 37: 213–9.

48. Laurvak SU, Munthe E, Kresse SH, et al. Functional characterisation of osteosarcoma cell lines and identification of mRNAs and miRNAs associated with aggressive cancer phenotypes. *Br J Cancer*. 2013; 109: 2228–36.

49. Rossing M, Borup R, Henao R, et al. Down-regulation of microRNAs controlling tumorigenic factors in follicular thyroid carcinoma. *J Mol Endocrinol*. 2012; 48: 11–23.

50. de Antonellis P, Liguori L, Falanga A, et al. MicroRNA 199b-5p delivery through stable mRNA-199b-5p is involved in the Notch signaling pathway in osteosarcoma. *BMC Biotechnol*. 2014; 13: 1668.

51. Arteaga CL, Engelman JA. ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics. *Cancer Cell*. 2014; 25: 282–303.

52. Singh P, Alex JM, Bast F. Insulin receptor (IR) and insulin-like growth factor receptor 1 (IGF-1R) signaling systems: novel treatment strategies for cancer. *Med Oncol*. 2014; 31: 805.

53. Vona-Davis L, Rose DP. Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression. *Endocr Relat Cancer*. 2007; 14: 189–206.

54. Duperret EK, Ridky TW. Focal adhesion complex proteins in epidermis and squamous cell carcinoma. *Cell Cycle*. 2013; 12: 3272–85.

55. Grange C, Collino F, Tapparo M, et al. Oncogenic micro-RNAs and renal cell carcinoma. *Front Oncol*. 2014; 4: 49.

56. Tomellini E, Lagadec C, Polakowska R, et al. Role of p75 neurotrophin receptor in stem cell biology: more than just a marker. *Cell Mol Life Sci*. 2014; 71: 2457–81.

57. Ramamoorthi G, Sivalingam N. Molecular mechanism of TGF-beta signaling pathway in colon carcinogenesis and status of c-myc as chemopreventive strategy. *Tumour Biol*. 2014; 35: 7295–305.

58. Wyman SK, Parkin RK, Mitchell PS, et al. Repertoire of microRNAs in epithelial ovarian cancer as determined by next generation sequencing of small RNA cDNA libraries. *PLoS ONE*. 2009; 4: e5311.

59. Secq V, Leca J, Bressy C, et al. Stromal SLIT2 impacts on pancreatic cancer-associated neural remodeling. *Cell Death Dis*. 2015; 6: e1592.

60. Rao J, Zhou ZH, Yang J, et al. Semaphorin-3F suppresses the stemness of colorectal cancer cells by inactivating Rac1. *Cancer Lett*. 2014; 358: 76–84.

61. Nasarre P, Gemmell RM, Drabkin HA. The emerging role of class-3 semaphorins and their neuropilin receptors in oncology. *Onco Targets Ther*. 2014; 7: 1663–83.

62. Indran IR, Tufo G, Pervaiz S, et al. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. *Biochim Bio-phys Acta*. 2011; 1807: 735–45.

63. Tsai CH, Lin LT, Wang CY, et al. Overexpression of cofilin-1 suppressed growth and invasion of cancer cells is associated with up-regulation of let-7 microRNA. *Biochim Biophys Acta*. 2015; 1852: 851–61.

64. Wagner S, Ngezahayo A, Mursu Escobar H, et al. Role of miRNA let-7 and its major targets in prostate cancer. *Biomed Res Int*. 2014; 2014: 376326.