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Original Article

Functional synergy between microRNAs in nasopharyngeal carcinoma

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Abstract: This study aimed to further understand the functional synergy between microRNAs in the pathogenesis of nasopharyngeal carcinoma (NPC). The microarray dataset GSE46172 was downloaded from Gene Expression Omnibus. After missing values imputation using the Impute package (default K value of 10) and across-array normalization by the quantile normalization method using the PreprocessCore package, the differential expression analysis was performed using the LIMMA (Linear Models for Microarray Data) package of R. MiRNAs with $|\log_2 FC$ (Fold change)$| > 1$ and adjusted *P*-value < 0.05 were considered as differentially expressed miRNAs (DEMs). Totally, 47 DEMs were screened, including 30 up-regulated and 17 down-regulated ones. And 33 miRNAs formed 515 miRNA-miRNA pairs, sharing with at least one common target gene, and miR-203 and miR-526b shared the most common target genes (1169). 365 miRNA pairs have functional synergism, and miR-139-5p and miR-141 shared the most common GO terms (703). In conclusions, MiR-141 and miRNA-138 may have critical roles in NPC pathogenesis, mainly working via functional synergy with many other miRNAs to regulate genes that are involved in cell cycle regulation and apoptosis in NPC pathogenesis.

Keywords: Nasopharyngeal carcinoma, differentially expressed microRNAs, GO term, miRNA-miRNA functional synergistic network

Introduction

Nasopharyngeal carcinoma (NPC) is a prevalent cancer in Southern China and Southeast Asia, which is characterized by high invasiveness and metastasis. Theoretically, 70% patients can be cured, whereas approx. 30%-40% of the patients finally developed distant metastases because of poor early diagnosis, which is attributable to the deep location of tumor cells in the lymphatic-rich nasopharynx and their propensity for lymphatic spread. Genetic and environmental factors involved in NPC pathogenesis are complicated, such as Epstein Barr Virus (EBV) infection. Actually, nearly all the NPC cases are EBV positive [1]. Hence, effective biomarkers for NPC early diagnosis are urgently demanded.

MicroRNAs (miRNAs) are small non-coding RNA molecules of about 22-25 nucleotides in length, which post-transcriptionally control gene expression by inhibiting translation or degrading target mRNA via binding to the complementary sequences in the 3'-untranslated regions. Some miRNAs can act as oncogenes (thus termed "oncomiRs"), such as miR-155, miR-21 and miR-17-92 cluster (miR-17-5p, miR-17-3p, miR-19a, miR-20a, miR-92-1) [2, 3], and some as the tumor suppressors, such as miR-16-1, let-7 family, miR-145 and miR-34a [4]. Recent studies have demonstrated the involvement of miRNAs in NPC, such as miR-218, miR-141, and miR-200a [5-7]. And *EBV*-encoding miRNAs, such as BART1-5p, miR-BART16, and miR-BART17-5p are closely involved in NPC progression [8]. Choy et al. have demonstrated that miR-BART5 targets the host gene, *PUMA* (p53-upregulated modulator of apoptosis), the down-regulation of which will inhibit the apoptosis of virus infected host cells [9].

Previously, Plieskatt et al. have identified the miRNA signatures associated with NPC by both the microarray analysis and RNA-Seq methods, which produced similar results [10]. In the pres-

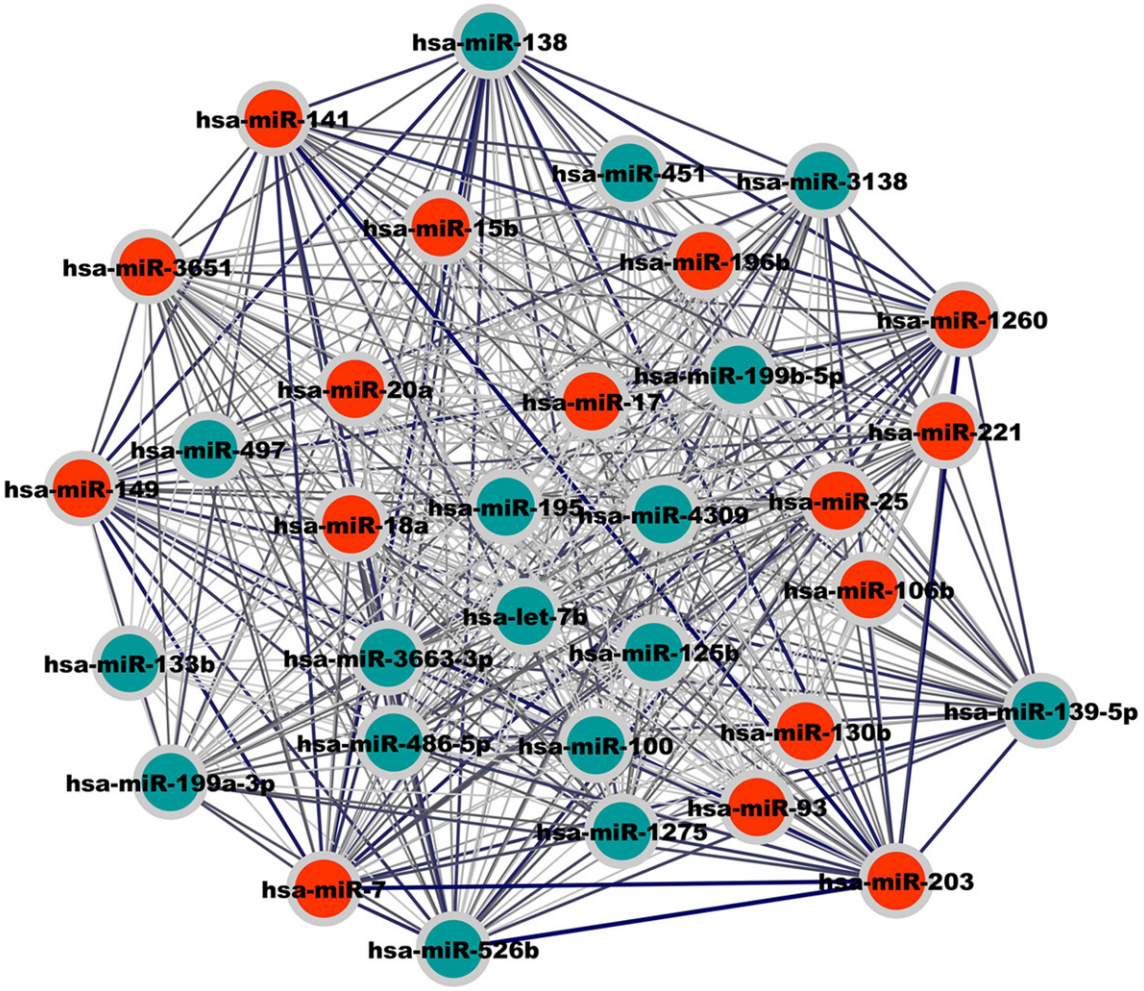

Figure 1. The miRNA-miRNA interaction network. Red, up-regulated miRNAs; green, down-regulated miRNAs. An edge connecting two genes indicating the presence of common target gene between them, and the width is proportional to the number of the common target genes.

Table 1. The top ten miRNA pairs with most co-regulated genes

microRNA	microRNA	Number of co-regulated genes
hsa-miR-203	hsa-miR-526b	1169
hsa-miR-203	hsa-miR-7	1040
hsa-miR-1260	hsa-miR-203	1025
hsa-miR-141	hsa-miR-203	1021
hsa-miR-149	hsa-miR-203	901
hsa-miR-138	hsa-miR-7	898
hsa-miR-149	hsa-miR-7	892
hsa-miR-138	hsa-miR-203	843
hsa-miR-1260	hsa-miR-7	828
hsa-miR-138	hsa-miR-149	804

ent study, using the miRNA microarray data contributed by them, we attempted to investigate the roles of different miRNAs in NPC pathogenesis and to unveil whether there is functional synergy between them, in order to further understand the mechanisms of NPC occurrence.

Materials and methods

Microarray data

The miRNA dataset GSE46172 containing eight unique samples were downloaded from Gene Expression Omnibus. Of the eight samples, four are from non-keratinizing NPC tissues, and the other four control samples were normal non-NPC tissues from four patients with NPC. The miRNA annotation platform is GPL7824 (Agilent-031181 Unrestricted_Human_microRNA_V16.0_Microarray).

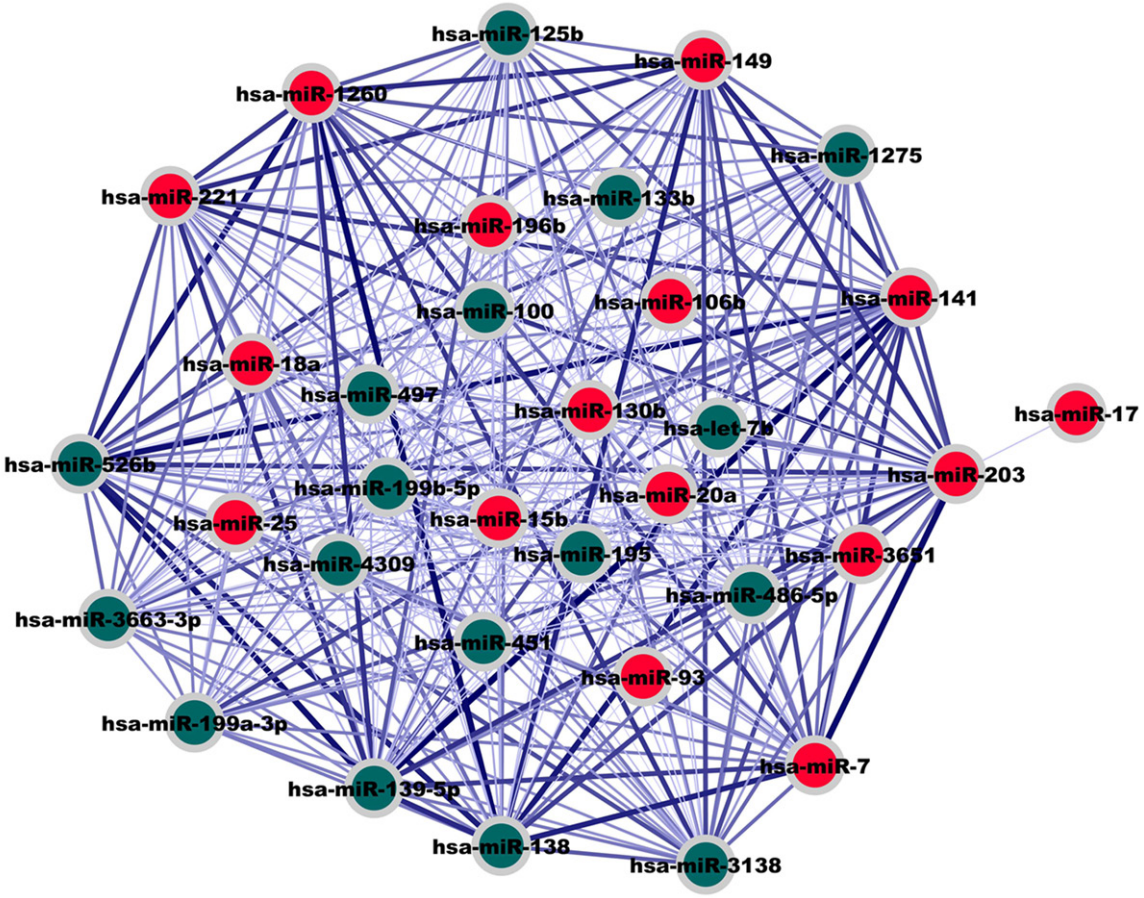

Figure 2. MiRNA-miRNA functionally synergistic network. Red, up-regulated miRNAs; green, down-regulated miRNAs. An edge connecting two genes indicating the presence of common target gene between them, and the width is proportional to the number of the common target genes.

Microarray data preprocessing

Probes were transformed into corresponding miRNAs using the GPL file. The average expression value of multiple probes corresponding to the same miRNA was taken as the final expression value of this miRNA. K-Nearest neighbor algorithm was used toimpute the missing expression values using the Impute package (default K value of 10) [11]. The across-array normalization was performed by the quantile normalization method using the PreprocessCore package of R [12].

Screening of DEMs

The differential expression values of miRNAs between the NPC lesion tissues and the controlnon-NPC tissues were calculated using the LIMMA (Linear Models for Microarray Data) package of R [13]. MiRNAs with $|\log_2 FC$ (Fold change)| > 1 and adjusted *P*-value < 0.05 were considered as DEMs.

Integration of miRNA-gene interaction data and construction of miRNA-miRNA network

miRNA-gene regulation data were downloaded-from miRecords [4], TarBase [5], HMDD [6], TargetScan [7], PicTar [8] and DIANA-microT [9]. miRNAs with common target genes were integrated to construct a regulation network.

Construction of miRNA-miRNA functional synergistic network (MFSN)

Symbols of genes commonly targeted by at least two miRNAs were converted into UniProtKB identifiers. ClusterProfiler package was used to perform the functional annotation of commonly target genes based on GO (gene ontology) database (*P*-value < 0.05, q-value < 0.05) [14]. If two miRNA significantly regulate the same GO biological process term, they are considered to be functionally synergistic. The resulting network was visualized using cytoscape [15].

Table 2. The top ten miRNA-miRNA pairs with functional synergism

microRNA	microRNA	Number of enrichment GO BP terms of co-regulated genes
hsa-miR-139-5p	hsa-miR-141	703
hsa-miR-1260	hsa-miR-138	669
hsa-miR-203	hsa-miR-7	660
hsa-miR-141	hsa-miR-526b	645
hsa-miR-139-5p	hsa-miR-526b	642
hsa-miR-1260	hsa-miR-526b	636
hsa-miR-138	hsa-miR-141	614
hsa-miR-138	hsa-miR-526b	598
hsa-miR-138	hsa-miR-7	586
hsa-miR-138	hsa-miR-149	585

GO, gene ontology; BP, biological process term.

Results

Screening of DEMs

Totally, 47 DEMs were screened, of which 30 ones up-regulated and 17 were down-regulated.

Integration of miRNA-gene interaction data and construction of miRNA-miRNA network

Among the 47 DEMs screened, 33 miRNAs including 16 up-regulated ones and 17 down-regulated ones formed 515 miRNA-miRNA pairs, each pair sharing at least one common target gene (**Figure 1**).

Among them, hsa-miR-203 and hsa-miR-526b shared the most common target genes (up to 1169 target genes), followed by miR-203 and miR-7, miR-1260 and miR-203, miR-141 and miR-203, miR-149 and miR-203, respectively (**Table 1**). Furthermore, each of the former four miRNAs pairs regulated more than 1000 common target genes.

Construction of miRNA-miRNA functionally synergistic network

For the 33 miRNAs with 515 interaction pairs, 365 miRNA pairs shared common GO terms, which are functionally synergistic (the functional synergistic network is shown in **Figure 2**). Among them, miR-139-5p and miR-141 shared the most common GO terms (up to 703 terms), followed by miR-1260 and miR-138, miR-203 and miR-7, miR-141 and miR-526b, miR-139-5p and miR-526b, successively (**Table 2**).

Among the top ten miRNA pairs, 7 pairs, namely miR-1260 and miR-526b, miR-1260 and miR-138, miR-138 and miR-149, miR-138 and miR-526b, miR-139-5p and miR-141, miR-139-5p and miR-526b, miR-141 and miR-526b, were observed to commonly regulate target genes that were enriched in cell cycle- and apoptosis-related GO terms (**Table 3**).

Discussion

In the present study, miR-139-5p and miR-141, miR-1260 and miR-526b, miR-1260 and miR-138, miR-138 and miR-149, miR-138 and miR-526b, miR-139-5p and miR-526b, miR-141 and miR-526b were supposed to have functional synergy in NPC pathogenesis, via commonly regulating the expression of target genes that are involved in cell cycle regulation and apoptosis. Uncontrolled cell cycle progression and reduced programmed cell death resulting from the abnormality in miRNAs or proteins involved in cell cycle control and apoptosis are two typical characteristics of tumor cells [16]. An *in silico* analysis by Luo et al. using miRNA samples extracted from NPC cells has also revealed that the predicted target genes of miRNA are mostly enriched in GO terms of cell proliferation, cell migration and cell matrix adhesion [17].

Among the 33 miRNAs forming 515 interaction pairs, miR-141 shared the most common GO terms with miR-139-5p, indicating their close synergy in NPC pathogenesis. Additionally, miR-141 was also assumed to have a synergistic interaction with miR-526b. In the present study, miR-141 expression was observed to be up-regulated, which was also reported by Luo et al. [17] and Zhang *et al.* in NPC [5]. Using luciferase reporter assay and Western blot analysis, Zhang *et al.* have pointed out that miR-141 plays a role as oncogene in NPC cells via influencing cell cycle, migration and invasion, as inhibition of miR-141 can arrest NPC cells in the G_0-G_1 phase and increase the apoptosis rate. They further proposed three putative direct targets genes of this miRNA, namely *BRD3*, *UBAP1* and *PTEN*, by using web-based bioinformatics software. And the involvement of *BRD3* and *UBAP1* in the development of NPC has been demonstrated by Zhou *et al.* previously [18].

Interestingly, down-regulated expression was found in either miR-139-5p or miR-526b our study, which was both supposed to have func-

Table 3. The Gene Ontology terms enriched with the common target genes of the top 10 miRNA-miRNA pairs

GO ID	Term	Count
GO: 0000278	mitotic cell cycle	9
GO: 0007049	cell cycle	61
GO: 0007050	cell cycle arrest	30
GO: 0007346	regulation of mitotic cell cycle	3
GO: 0010564	regulation of cell cycle process	4
GO: 0010948	negative regulation of cell cycle process	2
GO: 0022402	cell cycle process	37
GO: 0044770	cell cycle phase transition	1
GO: 0044772	mitotic cell cycle phase transition	2
GO: 0045786	negative regulation of cell cycle	32
GO: 0045787	positive regulation of cell cycle	4
GO: 0051726	regulation of cell cycle	62
GO: 0090068	positive regulation of cell cycle process	1
GO: 1901987	regulation of cell cycle phase transition	1
GO: 1901988	negative regulation of cell cycle phase transition	1
GO: 2000045	regulation of G1/S transition of mitotic cell cycle	1
GO: 2000134	negative regulation of G1/S transition of mitotic cell cycle	1

tional synergy with miRNA-141, indicating that their roles are opposite to miR-141 in NPC pathogenesis. However, the functional synergy between each of the two down-regulated genes and miR-141 has never been reported in NPC before. But the concurrent up-regulation of miR-141 and down-regulation of miR-139-5p has been observed in malignant bladder tissue samples by Ratert et al. [19]. Chen *et al*. who studied miRNA deregulation and pathway alterations in NPC, have also observed the down-regulation of miR-139-5p [20]. According to Zhang *et al*., miR-139-5p inhibits cell proliferation and metastasis, and promotes apoptosis and cell cycle arrest by targeting a oncogenic *NOTCH1* in colon cancer cell lines [21]. For miR-526b, aberrant up-regulation and down-regulation have been reported in breast cancer [22] and gastric cancer [23] respectively. However, its expression change in NPC has not been reported yet.

Furthermore, miR-139-5p was also speculated to have a close synergy with miR-526b in the present study, indicating complicated relationships between the three miRNAs: miR-141, miR-139-5p and miR-526b.

MiR-138 is another down-regulated miRNA that was observed to have extensive functional synergisms with other miRNAs in NPC, such as miR-141, miR-526b, miR-1260 and miRNA-149.

Its down-regulation here was not consistent with the significant up-regulation of its expression in NPC tissue biopsies reported by Chen et al. [20] and Lin [24]. A further functional analysis by Lin suggested that miR-138 overexpression in the undifferentiated NPC cell line HONE1 could enhance proliferation, migration and invasion of NPC cells [24]. However, Liu et al. have also reported its down-regulation in NPC specimens and NPC cell lines, and they further presented that *CCND1* (Cyclin D1encoding gene), a widely up-regulated gene in NPC tumors, might be a direct target of miR-138 [25]. Thus, the change in miRNA-138 expression needs to be further validated by experimental methods.

MiRNA-1260 (previously denoted as miRNA-1260a) expression was observed to be up-regulated in our study. Its up-regulation has also been reported by Peng et al. in formalin fixed paraffin embedded NPC tissue samples [26]. This agrees with the opinion that it acts as an oncogene in breast tumorigenesis [27]. However, there has not been any report on its synergy with miRNA-138 in NPC pathogenesis, although the simultaneous down-regulation of miRNA-138-5p and miRNA1260a expression has been reported in late-onset Alzheimer's disease patients [28].

MiRNA-149 expression was observed to be up-regulated in our study. Additionally, Luo et al. have observed the significant up-regulation of miR-149 together with miR-141 in NPC biopsies [17]; however, this concomitance was not observed in our study. The concomitant change in miRNA-149 and miRNA-138 expression has not been reported in NPC either, but their simultaneous down-regulation in expression has been reported by Wong et al. in patients with tongue carcinomas using real-time quantitative PCR [29]. Furthermore, Wang et al. have report-

ed *ZBTB2* as a target of miRNA-149 during its inhibition of proliferation and cell cycle progression in human gastric cancer [30]. This further suggests miRNA-149 expression change may depend on tumor type.

Taken together, it is speculated that miR-141 and miRNA-138 may have functional synergy with many other miRNAs during their regulation of the expression of genes that are involved in cell cycle regulation and apoptosis in NPC pathogenesis, thus these two miRNAs may have critical roles in NPC pathogenesis. However, miRNA expression changes observed in the present study were not always consistent with previous findings. Additionally, as mRNAs of a certain gene are always regulated by more than one type of miRNA, and the target mRNAs of one miRNA may vary with disease type, the relationships between genes and miRNAs can be complicated. Thus, the conclusions drawn in the present study need further experimental validation. Whatever, this study has provided some novel information for NPC pathogenesis.

Disclosure of conflict of interest

None.

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