THE EXPRESSION OF *MICRORNA-744* (*MIR-744*) IN NASOPHARYNGEAL CARCINOMA: A PRELIMINARY STUDY

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ABSTRACT

The aim of this study was to investigate the level of miR-744 expression in nasopharyngeal carcinoma (NPC) tumour tissue and to provide initial clue on its potential as biomarkers for early detection of NPC in a preliminary analysis. Total miRNAs was extracted from NPC tissue as well as normal nasopharynx tissue taken from Hospital Tengku Ampuan Afzan (HTAA), Kuantan and converted into cDNA. The level of miR-744 expression in the cDNA was quantified using quantitative reverse transcription polymserase chain reaction (RT-qPCR) technique. The expression level of *SNORD48* was measured simultaneously for each sample, which served as endogenous control. The difference in the expression of miR-744 in NPC and normal nasopharynx tissue were analysed using relative quantification, $2^{-\Delta\Delta CT}$. In this preliminary analysis, this study found that miR-744 was upregulated in NPC as compared to normal nasopharynx tissue by 2.5 fold changes, respectively suggesting it may involve in progression of tumour. However, the finding is not significant and may not accurately reflect the overall population, due to small sample size involved in the study. Findings from the current study suggest the potential of miR-744 to serve as useful diagnostic and prognostic biomarker in NPC.

KEYWORDS: Nasopharyngeal carcinoma, MicroRNAs, MiR-744, Tumour tissue, RT-qPCR

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is the fourth most common cancer among Malaysians (Ferlay et al., 2014; Zainal Ariffin & Nur Saleha, 2011). In the context of ethnicity, the incidence and prevalence of nasopharyngeal carcinoma was found to be higher in ethnic Chinese, followed by Malays and very rare among Indian population in the country (Devi, Pisani, Tang & Parkin, 2004; Zainal Ariffin & Nur Saleha, 2011). In addition, a deeper ethnic-based study revealed that the sub-ethnic Bidayuh, one of the native in West Malaysia showed the highest rate of NPC as compared to any other population in the world (Devi *et al.*, 2004).

Furthermore, the majority of NPC cases were diagnosed at advanced stage due to trivial presentation of symptoms related to NPC at early stage such as neck masses and symptoms associated with dysfunction of the Eustachian tube (Tabuchi, Nakayama, Nishimura, Hayashi & Hara, 2011). This complicates the management and treatment of the patients, which leads to the poor overall 5-year survival rate (Chen *et al.*, 2009; Feinmesser *et al.*, 1992). Current treatment on NPC involves combination of radiotherapy and chemotherapy. Although chemoradiotherapy is superior to combat NPC rather than radiotherapy alone, patient is exposed to various side effects such as hair lose, fatigue, constipation, salivary gland failure and loss appetite (Cancer Research UK, 2015; Lin *et al.*, 2003).

Current diagnosis of NPC requires invasive technique through histological examination to recognize different types of cancer and computerized tomography (CT) scan for cancer staging performed by highly trained specialist (Abdullah, Alias & Hassan, 2009 ; Tabuchi *et al.*, 2011). Due to its complex structure and unspecific symptoms, scanning with fused positron emission tomography, which is efficient to detect certain solid cancers, seems notoriously difficult for NPC diagnosis and staging (Cho, 2007). This has encouraged the efforts to develop early detection strategies on NPC through molecular approach.

Recent studies explored relationship between prognostic values of microRNAs (miRNAs) involved in progressing of NPC (Liu et al., 2012; Zhao, Chen & Cao, 2010). While miRNAs showed high association with Epstein-Barr virus-encoded latent membrane protein 1 activated signal transduction pathways, it has been suggested that certain miRNAs are also associated with angiogenesis factor, invasion and migration of NPC cells (Zhao et al., 2010).

Several evidences indicated that *microRNA-744* (*miR-744*) have been involved in various cancers including hepatocellular carcinoma and gastric cancer to cause tumourigenesis (Song et al., 2012; Tan et al., 2015). Furthermore, a profiling study on miRNAs expression conducted on head and neck cancer patients from Perak, Malaysia found that *miR-744* was up-regulated, suggested it may act as potential diagnostic biomarkers (Nurul-Syakima et al., 2011). However, no continuous study was done to

explore more on these miRNAs in NPC tissue. Therefore, this study is proposed to validate the expression of *miR-744* in NPC tissue samples as potential biomarkers.

MATERIALS AND METHODS

Study subject

This study involved five eligible patients who were diagnosed with NPC in Hospital Tengku Ampuan Afzan (HTAA), Kuantan, Pahang. The inclusion criteria include the NPC cases reported in HTAA, newly diagnosed with NPC case and histologically confirmed to have NPC. Patients excluded from this study are those who were free from history of cancer (other) and the NPC case is not recurrent. The patient should also does not have any known genetic and/or autoimmune disease. The protocol has been reviewed and approved by IIUM Research Ethic Committee (IREC) (IREC 530) and National Medical Research Register (NMRR-15-1976-27156). All patients were given proper explanation regarding the purpose and procedures of the research before they gave written informed consent. The interview sessions with the NPC patients and sample collection from HTAA were made by postgraduate students. The demographics data of patients are available in Table A (appendix).

Sample collection

Tumour tissue was dissected and collected by HTAA surgeons during tissue biopsy from suspected NPC patient (indicated with lump found in nasopharyngeal area during endoscopy procedure). On the other hand, normal nasopharynx tissue (>2 cm away from NPC tissue) was cut <0.5 cm in at least one dimension to be fixed with formalin right after surgical removal conducted by medical officer in ear, nose and throat clinic followed by histological verification from pathologist (Nurul-Syakima et al., 2011). The NPC samples and normal samples were labelled as nasopharynx cancer (NC) and nasopharynx normal (NN) respectively. However, two samples from two different patients were not received from HTAA.

Histological procedures

Upon receiving sample block of formalin-fixed paraffin-embedded (FFPE) tissue from HTAA, the tissue was sectioned into 4-6μm thick sections using microtome and placed on water bath (46°C) before putting it onto glass slide and dried on hot plate (55°C). Then the slide was stained with hematoxylin and eosin (H&E) staining procedure before sent to an expert histopathologist, who observed and verified the malignant part on the tissue sample. After histological verification, the samples were classified according to their group as in Table B (appendix).

Total RNA isolation

The extraction procedure was performed as instructed in the innuPREP FFPE total RNA kit (Analytik Jena, Germany). Prior to RNA extraction, Starting material of FFPE was prepared by sectioning six sections of ten µm thicknesses and each of 300 mm^2 area of sample block using microtome and put into 1.5 ml of reaction tube. 400 µl of lysis solution and 40 µl of Proteinase K were added to the tube and it was incubated at 65°C for 30 minutes followed by 80°C for 30 minutes with regular vortexing before collecting its supernatant into a new tube. To determine the extraction procedure was conducted appropriately, exogenous control, *ath-miR-159a* was included and analysed together in RT-qPCR. Ten pmol of *ath-miR-159a* was put into the reaction tube and 600 µl of absolute ethanol (96-99%) were added to the sample. Subsequent RNA extraction steps followed the manufacturer's instruction. The purity and yield of extracted RNA was measured at absorbance of A260/A280 using NanoDrop 1000 spectrophotometer (Thermo Scientific, Germany).

Validation of miRNAs using quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Reverse transcription reaction was performed using miScript HiSpec Buffer to selectively convert mature miRNAs into complementary DNA (cDNA) as instructed in miScript PCR system Handbook 10/2011 (Qiagen, Germany). Total RNA (50ng/µl) was subjected to a reverse transcription reaction according to the protocol.

The preparation of reaction mixture for RT-qPCR analyses for the expression of *miR-744* in NPCs and normal nasopharynx tissues were performed according to the protocol provided by miScript PCR system handbook 10/2011 (Qiagen, Germany). This protocol enables quantification of mature miRNAs by RT-qPCR using target-specific miScript Primer Assay (forward primers) of *miR-744*, miScript SYBR Green PCR Kit containing miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix. The housekeeping gene *SNORD48* (*RNU48*) served as endogenous control as recommended in previous study (Nurul Syakima et al., 2011). Another primer assay for exogenous control of *ath-miR-159a* was also used to detect the expression of spike-in inserted during extraction of NPC and normal nasopharynx tissues.

The qPCR reaction was run in triplicate using CFX96 Real-Time System (Bio-RAD, USA) in a 25 µl of volume reaction according to manufacturer's protocol. No cDNA template was also included to assess the specificity of qPCR reaction. Relative quantification of $2^{-\Delta\Delta Ct}$ was used to indicate the fold change of NPC relative to the normal nasopharynx as in equation 1 in appendix (Livak & Schmittgen, 2001).

Statistical analysis

The data obtained from the assessment of extracted RNA yield from FFPE samples to theoretical value and gene expression were analysed using SPSS version 22. Non-parametric statistical analysis was utilized due to abnormal distribution of data used in this study. One

sample Wilcoxon signed rank test was used to determine the median difference of RNA yield extracted from FFPE samples as compared to median of theoretical value (Beer-Lambert Law). The gene expression was determined using Mann Whitney U test to compare the significance between the distribution of *miR-744* expression in NPC tissue and normal nasopharynx tissue with the data was shown as mean rank. A *p*-value less than 0.05 were assumed to indicate significant difference.

RESULTS AND DISCUSSION

Assessment of NPC Tissue and Normal Nasopharynx Tissue Using Haematoxylin and Eosin (H&E) Staining

Assessment of nasopharyngeal tissues using H&E staining was verified by an expert pathologist. They were assessed for features of NPC, while for normal tissues they were examined in order to ascertain that the tissue is normal i.e. absence of tumour tissue. There are two types of NPC that were found in the samples; keratinizing squamous cell carcinoma and non-keratinizing squamous cell carcinoma as illustrated in Figure 1.

Figure 1 A shows NPC tissue that featured the criteria of keratinizing squamous cell carcinoma found in sample of NN 9 with the sheets of malignant polygonal cells exhibited abundant cytoplasm with keratinization, prominent nuclei, distinct cell border and desmoplastic stroma infiltrated with inflammatory cells of lymphocytes, neutrophils and plasma cells. The tissue was diagnosed with moderately differentiated invasive keratinizing squamous cell carcinoma of NPC or tumour metastasized to nasopharynx, though later it was treated as NPC. On the other hand, Figure 1 B represents non-keratinizing squamous cell carcinoma of undifferentiated type NPC as observed in NC 10 tissue sample, which was characterized by syncytial-appearing large tumour cells with indistinct cell border and appears crowded or overlapping. Large central nucleoli were also featured with scant cytoplasm. There was also lymphoplasmacytic infiltration. Similar characteristics were observed in sample of NN 10, NN 13 and NN 15 which diagnosed as non-keratinizing squamous cell carcinoma of undifferentiated type. Our observations, thus verified the earlier diagnosis made in the hospital on these patients.

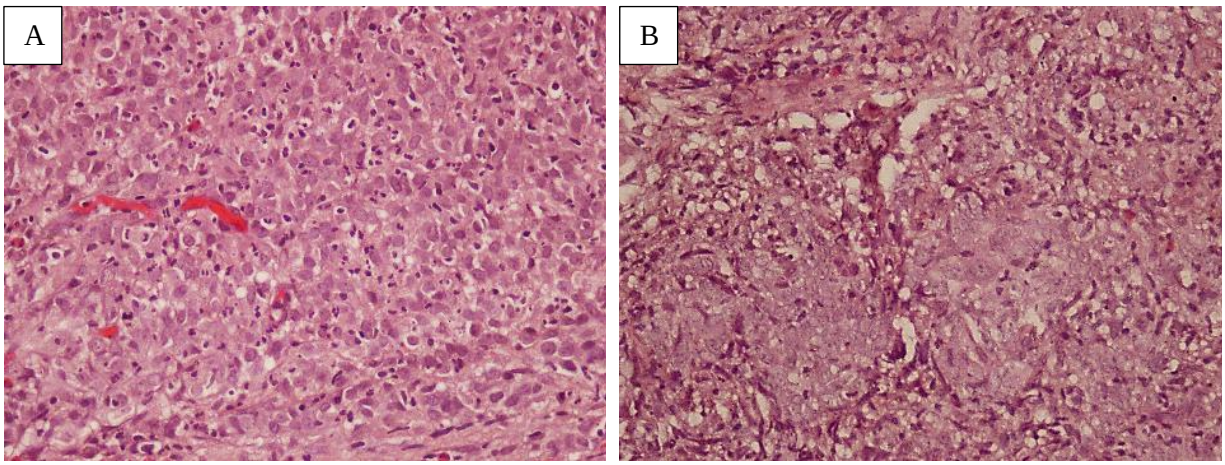

Figure 1. Representative histologic sections of NPC tissue as observed under 40 x magnification. **A** Keratinizing squamous cell carcinoma (moderately differentiated) in sample of NN9. **B** Non-keratinizing squamous cell carcinoma (undifferentiated type) in sample of NC 10.

There are also three samples of NC 9, NC 11 and NN 11 that showed normal nasopharynx tissue characteristics of loose connective tissue, pseudostratified mucosal lining which forms approximately 40% of the tissue and non-epithelial of goblet cells. The tissue also consisted of lymphoid cells collection with no signs of malignancy or dysplasia. Similar features were found in both NC 11 and NN 11 as well.

The finding demonstrates different types of NPC of keratinizing squamous cell carcinoma and non-keratinizing squamous cell carcinoma (undifferentiated type) by observing prominent features of nuclear appearance, formation of cell border and infiltration of inflammatory cells in NPC tissue. Keratinizing squamous cell carcinoma usually developed locally advanced tumour growth with lower propensity for lymph node metastasis. Nevertheless, non-keratinizing squamous cell carcinoma was commonly found in high incidence areas with 90% of it represented with undifferentiated type. This type of NPC is more radiosensitive and has stronger relationships with EBV as compared to type one (Li & Zong, 2014). The result showed the samples were confirmed as five NPC tissue samples and three normal nasopharynx tissue samples thus verified the earlier diagnosis made by HTAA.

Determination of Quality and Efficiency of miRNA Extraction from NPC Tissue and Nasopharynx Tissue using RT-qPCR

The evaluation of quality and efficiency of miRNA extraction from FFPE samples was determined using expression analysis of RT-qPCR. Exogenous control, *ath-miR-159a* was used in RNA isolation process to distinguish RNA process failure of FFPE samples from samples with poor RNA quality. Endogenous control, *SNORD48* was also utilized as normalization to relative quantification. Figure 2 shows the interrogation of expression levels of *miR-744*, *ath-miR-159a* and *SNORD48* by RT-qPCR in a representative FFPE RNA samples (NC 9, NN 9, NC 10, NN10, NC 11, NN11 and NN 15) using SYBR-GREEN detection with equal input of total RNA for each sample (50 ng/µl). It was observed that miRNAs were able to be isolated from all of the triplicate samples as detected by average cycle threshold (Ct) values. However, the analysis identifies NN 11 as a sample failure (expected *ath-miR-159a*, low *SNORD48*) and NN 9 as a process failure (low *ath-miR-159a* and *SNORD48*).

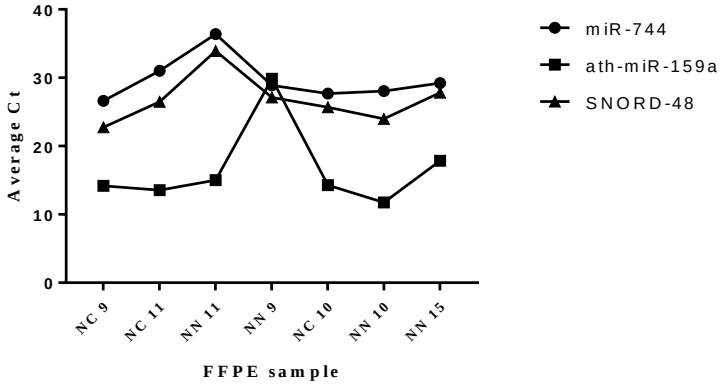

Figure 2. Line graphs illustrates the average Ct of *miR-744*, *ath-miR-159a* and *SNORD48* across FFPE samples using RT-qPCR

The implementation of RNA isolation control, *ath-miR-159a* served to monitor potential of process failure during RNA isolation which also identifies the sample failure as shown in the result. *Ath-miR-159a* was utilized in several RNA isolations of different samples including plasma and other biofluids (Du et al., 2015). It is also useful to evaluate the quality of cDNA synthesis which shows *ath-miR-159a* was expressed in all samples except in NN 9. The current finding suggested the potential of *ath-miR-159a* is useful for RNA isolation from FFPE samples for biomarker discovery and indicates most of the cDNAs were in good quality.

Gene Expression Analysis of NPC Tissue against Normal Nasopharynx Tissue

Gene expression analysis of *miR-744* in NPC tissue and normal nasopharynx tissue was analyzed in triplicate with a negative control without cDNA template using RT-qPCR. The fold change of relative quantification was calculated using the 2-ΔΔCt. Table 1 shows normalization of NPC and normal nasopharynx tissue to reference gene, *SNORD48*. There was no significant difference between distribution of normalized Ct values in NPC tissue and distribution of normalized Ct values in normal nasopharynx tissue (p=0.220) in Mann-Whitney U test. Figure 3 also depicts the expression of *miR-744* in NPC tissue was increased by fold change of 2.5 as compared to normal nasopharynx tissue. However, a sample of NPC (NN 13) was excluded due to the result of negative extracted RNA purity and very low RNA yield. Repeated extraction could not be done due to inadequate remaining of NPC tissue.

Table 1. Comparison of *miR-744* expression in NPC tissue and normal nasopharynx tissue relative to *SNORD48*

Sample	*miRNA-744*	*SNORD48*	**ΔCT (Avg. miR C_T - Avg. *SNORD48* C_T)**	**ΔΔCT (Avg. miR C_T - Avg. *SNORD48* C_T)**	**Normalized Cancer tissue amount relative to Normal tissue $2^{-\Delta\Delta CT}$**
Normal					
NC 9	26.63	22.75	3.88		
NC 11	31.01	26.47	4.54		
NN 11	36.38	33.91	2.47		
Average	31.34 ± 4.9	27.71 ± 5.7	3.63 ± 1.1	0.00 ± 1.1	1 (0.5-2.1)
Cancer					
NN 9	28.87	27.12	1.75		
NC 10	27.68	25.69	1.99		
NN10	28.05	23.99	4.06		
NN 15	29.23	27.82	1.41		
Average	28.46 ± 0.7	26.16 ± 1.7	2.30 ± 1.2	-1.33 ± 1.2	2.5 (1.1-5.8)

*=significant at $p<0.05$

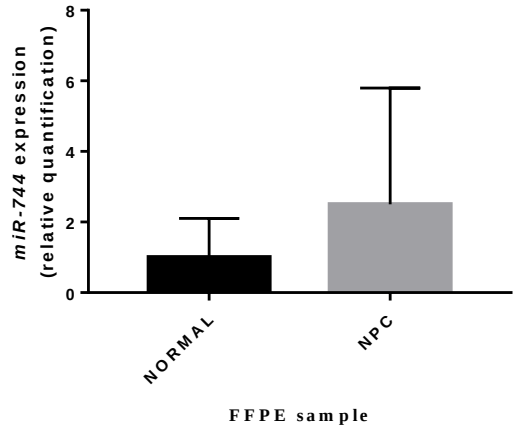

Figure 3. Bar graphs of fold change in *miR-744* expression in NPC tissue and normal nasopharynx tissue

The finding of gene expression demonstrates no significant difference between distribution of normalized Ct values in NPC tissue as compared to the distribution of normalized Ct values in normal nasopharynx tissue (p=0.220) in Mann-Whitney U test. However, the result shows that *miR-744* expression is higher in NPC tissue as compared to normal nasopharynx tissue by 2.5 fold change using relative quantification, $2^{-\Delta\Delta CT}$ analysis. The finding supports an earlier study by Nurul-Syakima et al. (2011) that found *miR-744* to be overexpressed in cancer patients, indicating that *miR-744* might be involved in the pathogenesis of head and neck cancers.

It is noted that the average Ct of endogenous control, *SNORD48* across the samples were variable and not consistent throughout the study. Primer design may be an issue for this observation, as indicated in the primer efficiency test, although the primer was commercially acquired. Therefore future study may take this into account and having multiple endogenous controls included in the analysis may provide more reliable outcome of the analysis.

Nonetheless, this is the first study that investigate the expression of mir-744 in nasopharyngeal carcinoma. The findings in this study indicates the level of *miR-744* is higher in NPC tissue as compared to normal nasopharynx tissue which may suggest the potential of *miR-744* as oncogenic biomarker in NPC. However, the finding is not significant and may not accurately reflect the overall population, due to small sample size involved in the study. Thus, further study is required to prove the relevance of *miR-744* as potential non-invasive biomarker in NPC by a larger population.

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Appendices

A. Equation

$2^{-\Delta\Delta Ct} = 2^{\wedge} -[(C_t\ miR\text{-}744 - C_t\ SNORD48)$ NPC sample] – [($C_t\ miR\text{-}744 - C_t\ SNORD48$) normal nasopharynx sample] (Equation 1)

B. Table

Table A: Demographics of patients with nasopharyngeal carcinoma.

Characteristics	No.
Gender	
Male	5
Age	
Year, median (range)	51 (16-70 year old)
Race	
Malay	5

Table B: Types of tissue samples as verified by histopathologist

Samples	Type
NC 9	Normal
NN 9	NPC
NC 10	NPC
NN 10	NPC
NC 11	Normal
NN 11	Normal
NN 13	NPC
NN 15	NPC