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EXPRESSION OF MICRORNA-101 IN FORMALIN-FIXED PARAFFIN- EMBEDDED SAMPLES OF NASOPHARYNGEAL CARCINOMA

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Abstract

Nasopharyngeal carcinoma (NPC) is among the five most common malignancies in Malaysia. Most NPC patients are diagnosed at late stages of the disease which complicates the clinical management of the patients. Identification of new reliable biomarker is crucial to improve early diagnosis of NPC and increase the survival rate of patients. Recent study found that microRNAs (miRNAs), particularly miR-101, were involved in the tumorigenesis of head and neck cancer where NPC samples were included in the study. This study was conducted to observe the expression of miR-101 in NPC tumour tissues and compare its consistency with previous study as a step towards finding the new biomarker for NPC. The biopsy samples were obtained from hospitals and verified histologically using hematoxylin and eosin method for tissue classification. Total RNA was extracted from NPC tissues and normal nasopharyngeal epithelium tissues. The expression of miR-101 in NPC was quantified using quantitative polymerase chain reaction (qPCR) method. The differential expression of miR-101 in NPC as compared to normal nasopharyngeal epithelium tissues was analysed using $2^{-\Delta\Delta CT}$ calculation. The significance of the differential expression was analysed using SPSS software. Five samples have been verified as NPC and three samples were normal nasopharyngeal epithelium. The differential expression calculation found that miR-101 was downregulated in NPC as compared to normal nasopharyngeal epithelium tissues, which consistent with previous study. However, the differential expression was not significant. Therefore, our finding provides a preliminary result towards embarkment of a larger and comprehensive study.

INTRODUCTION

The recent trend on the statistics of nasopharyngeal carcinoma (NPC) in Malaysia is quite worrying. This cancer has been categorized as the five most common malignancies in the country. According to the Malaysian National Cancer Registry Report for 2007 until 2011 period, the ethnic Chinese had been affected the most, followed by the Malays and very rare among the Indians [1]. Most patients who suffered from this cancer have less than five-year survival rate [1,2]. This might be due to late diagnosis of the cancer whereby most NPC patients are diagnosed at late stages of the disease [1 – 3]. Therefore, this might complicate the clinical management of the patients. Combination treatment by radiotherapy and chemotherapy is needed for the late stage NPC whereas only radiotherapy alone is needed for an early stage of the cancer [3]. Therefore, early detection is an important factor to decrease the burden of the patient and determine the chance of success in the treatment [4]. Nowadays, the available method for diagnosis of NPC only relies on biopsy and histological examinations, which is invasive and requires highly trained specialists [5]. In addition, there is no effective and feasible method for NPC screening available. Due to this fact, a molecular approach is suggested to enable the early detection of NPC which may be very helpful in improving diagnosis [6]. In a previous study by Nurul-Syakima et al. [6] found that miR-101 was down-regulated

in biopsies of head and neck cancer Malaysia patients where NPC biopsies were also included in the studied samples. Therefore, in the current study, we sought to observe the expression of miR-101 in independent biopsy samples of Malaysia NPC patients as a preliminary study before embarking with a more comprehensive study.

MATERIALS AND METHODS

Subject recruitment

The protocol was reviewed and approved by IIUM Research Ethic Committee (IREC 530) and Medical Research Ethic Committee (NMRR15-1976-27156). The subjects were recruited in two Pahang government hospitals, which were Hospital Tengku Ampuan Afzan, Kuantan and Hospital Sultan Haji Ahmad Shah, Temerloh. The consent informs have been obtained the subjects who fulfilled the inclusion and exclusion criteria of the study. The personal details of the subjects have been kept confidential and the names of subjects have been replaced with identification number. Based on the subject recruitment, five male patients who met the inclusion and exclusion criteria were recruited for sample collection. All the subjects have been briefed about the study and given with written informed consent. During the recruitment, demographic data have been taken from each patient. **Table 1** summarizes the demographic data of the patients.

Table 1. Demographics of patients with nasopharyngeal carcinoma

Characteristics	Number
Gender	
Man	5
Age	
Year, median (range)	51 (16 – 70 years old)
Race	
Malay	5

Biopsy sample collection

The NPC tissues have been obtained from the patients who have been suspected for NPC during their visits to Ear, Nose and Throat Clinic of HTAA and HOSHAS. The tissue biopsies have been taken from the patients during the endoscopic procedure. The tumor tissues have been cut <0.5 cm in at least one dimension and the specimens were fixed using formalin immediately after the surgical removal. Normal tissues have been taken at >2 cm away from the tumor part or from the other side of the nasopharynx of the same patient [6]. The biopsy tissues have been put into formalin immediately after the dissection for fixation. The status of the tissues, either NPC tissue or normal nasopharyngeal epithelium tissues, have been verified by a pathologist through histopathological examination. The tissues have been labeled as "NN" for NPC tissues and "NC" for normal nasopharyngeal epithelium tissues.

Histopathological examination

After obtaining the formalin-fixed biopsy specimens, tissue processing was carried out using an automated tissue processor. The samples have been placed in the aqueous environment and taken through a series of dehydration using 50% alcohol for 1 hour, 70% alcohol for 1 hour, 80% alcohol for 1 hour, 95% alcohol for 1 hour and two times in 100% alcohol for 2 hours. Finally, the samples have been cleared twice by clearing solvent, xylene for another 2 hours before the samples have been placed in molten wax to form formalin-fixed paraffin-embedded (FFPE) tissue blocks. Then, the blocks have been sectioned into 4 µm thickness sections using a rotary microtome, placed onto glass slides and stained using hematoxylin & eosin (H&E) method. Finally, histopathological examination was performed by a pathologist to verify the status of the tissues.

Total RNA isolation

The total RNA isolation from FFPE tissue samples have been conducted using innuPREP FFPE Total RNA Kit (Analytik Jena, Germany) according to the manufacturer's protocol. Approximately six sections of 10 µm thickness in each of 300 mm² area or four sections of 10 µm thickness in each of 400 mm² of FFPE tissue samples have been used as starting material for total RNA isolation. The FFPE starting material was placed into a 1.5 ml reaction tube and centrifuged at maximum speed for 1 minute to push the sample to the bottom of the tube. The sample was lysed using 400 µl Lysis Solution MA and 40 µl Proteinase K and mixed vigorously for 10 seconds. The reaction tube was incubated at 65°C for 30 minutes and vortexed 4 times during the incubation period. After lysis step, the sample was centrifuged at maximum speed for 3 minutes. Then, the supernatant was transferred into a new 1.5 ml reaction tube. The procedure was repeated with incubation at 80°C for 30 minutes. Then, the supernatant was transferred into a new 1.5 ml reaction tube. The supernatant was added with 600 µl absolute ethanol, spiked-in with 2 µl of ath-miR-159a mimic as an exogenous control and mixed vigorously for 10 seconds. Then, 600 µl of the mixture was transferred into a Spin Filter with a 2.0 ml receiver tube and centrifuged at 10.000 × g for 1 minute. The receiver tube was discarded with the filtrate and placed the Spin Filter into a new 2.0 ml receiver tube. The Spin Filter was subjected to washing steps with 500 µl of Washing Solution C, 650 µl of Washing Solution BS and 650 µl of absolute ethanol with centrifugation at 10.000 × g for 1 minute for each washing step. Then, the Spin Filter was centrifuged at maximum speed for 3 minutes to remove all traces of ethanol. Finally, the sample was eluted by adding 50 µl RNase-free water into the Spin Filter with a 1.5 ml Elution Tube, incubated at room temperature for 1 minutes and centrifuged at 10.000 × g for 1 minute. RNA yield and purity assessment was performed using NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA). Finally, the total RNA extract was stored at -80°C prior to usage.

Reverse transcription

Reverse transcription (RT) has been carried out on the total RNA extract using the miScript II RT Kit (Qiagen, Germany) according to the manufacturer's protocol. The initial concentration of RNA template was standardized to 50 ng/µl for all samples. Preparation of master mix for each sample was performed by mixing 4 µl 4× miScript HiSpec Buffer, 2 µl 10x miScript Nucleics Mix, 2 µl miScript Reverse Transcriptase Mix & RNase-free water to make up the total reaction volume to 20 µl. The reaction master mix was incubated at 37°C for 60 minutes, followed by 95°C for 5 minutes and immediately cooled down to 4°C using CFX96 Touch™ Real-

Time PCR Detection System (Bio-Rad, USA). Then, the cDNA product was diluted into 200 µl of RNase-free water prior to qPCR procedure.

Quantitative Polymerase Chain Reaction

The qPCR has been performed using miScript SYBR Green PCR kit (Qiagen, Germany) and miScript Primer Aassay (Qiagen, Germany) of hsa-miR-101-3p as a primer of interest, SNORD48 as endogenous control and ath-miR-159a as an exogenous control. The reaction mixture for each sample was prepared by mixing 2.5 µl of cDNA with 12.5 µl 2× QuantiTect SYBR Green PCR Master Mix, 2.5 µl 10× miScript Universal Primer, 2.5 µl 10× miScript Primer Assay and 5 µl RNase-free water. Each sample was prepared with triplicate. The sample has been amplified at 95°C for 15 minutes, 94°C for 15 seconds, 55°C for 30 seconds and 70°C for 30 seconds for 40 cycles using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Then, the differential expression of miR-101 was calculated using $2^{-\Delta\Delta Ct}$ method (7).

Statistical analysis

Statistical analysis was performed by using SPSS software version 22.0.1. The Mann-Whitney U test was used to compare the mean between NPC and normal samples.

RESULTS AND DISCUSSION

Histopathological examination

The histological sections have been examined by a highly trained pathologist using the light microscope. The NPC tissues have been examined for features of malignancy, while for normal tissues they have been examined to ascertain that the tissue is normal i.e. absence of tumor tissue. After the histological verification, the samples have been classified according to their respective grouping as in **Table 2**. However, two NPC samples have no their counterpart normal samples, which were NC 13 and NC 15. The histopathological examination found that out of eight nasopharynx biopsy samples, five samples have been confirmed as NPC and three samples have been confirmed as normal nasopharyngeal epithelium. The results were illustrated in **Table 2** and **Figure 1**.

Table 2. Status of histologically verified tissues

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

Total RNA purity and yield

The total RNA yield and purity have been determined by observing the absorbance ratio reading at for A260/280 and A260/230 as illustrated in **Table 3**. The ratio of A260/280 indicates the protein contamination of a nucleic acid sample. For pure nucleotide ratio, A260/280 ratio should be in the range of 1.8-2.0. A lower ratio indicates the sample is protein contaminated. The presence of protein contamination may affect the downstream applications. The A260/230 ratio indicates the presence of organic contaminants, such as phenol, chaotropic salts and aromatic compounds. Samples with A260/230 ratios below 2.0 were considered to have a significant amount of these contaminants that will interfere with downstream applications. **Table 3** showed the purity and yield of our total RNA extraction. The RNA purity showed a reliable range of reading for downstream application. However, only one sample showed poor reading of A260/230 ratio, which was NN13, that indicated the presence of organic contaminants and this sample has been excluded from further analysis. Meanwhile, the rest of the samples showed the good purity to be eligible for downstream procedures. Our result showed that there was no issue regarding poor total RNA recovery due to formalin-induced cross-linking and degradation in FFPE samples, as mentioned previously, by using innuPREP FFPE Total RNA Kit (Analytik Jena, Germany) for total RNA isolation [8,9].

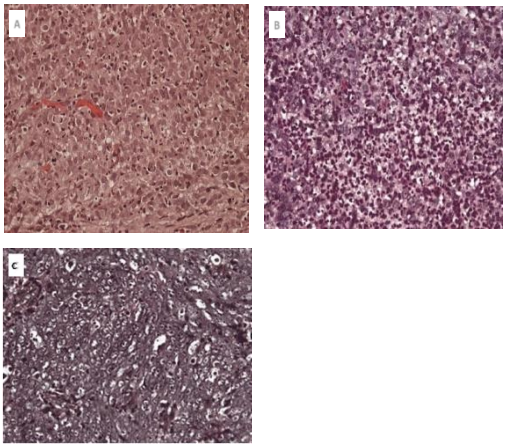

Figure 1. Representative histologic sections for NPC of sample (A) NN 9, (B) NN10 and (C) NN 15 (Type of specimen: nasopharynx, original magnification:×40, stain: H&E).

Table 3. The readings of total RNA yield, purity and contaminants in different tissue samples

Sample	Type	A260/A280	A260/A230	ng/µl
NC 9	Normal	2.12	2.2	28.57
NN 9	NPC	2.00	1.86	143.23
NC 10	NPC	2.12	2.49	76.82
NN 10	NPC	2.18	2.92	43.18
NC 11	Normal	2.09	3.35	5.42
NN 11	Normal	1.91	3.29	38.53
NN 13	NPC	2.82	-1.53	4.75
NN 15	NPC	2.04	2.41	88.95

Differential expression of miR-101

qPCR is one of the gold standards for quantifying gene expression. The sensitivity and specificity in measuring gene expression, even at low RNA quantity, make qPCR is largely selected for analysis [10]. Our analysis showed that miR-101 has been down-regulated in NPC as compared to normal nasopharyngeal epithelium

samples. However, the statistical analysis using Mann- Whitney U test demonstrated that there was no significant difference between miR-101 expression in NPC as compared normal nasopharyngeal epithelium samples (p=0.23), as illustrated in **Figure 2**. Interestingly, the down-regulation of miR-101 expression in NPC tissue as compared to normal nasopharyngeal epithelium tissue in our study was consistent with earlier study by Nurul-Syakima et al. [6]. The down-regulation of miR-101 in NPC, either in clinical samples or cell lines, also has been reported in previous studies, which supporting our finding, even with small sample size [11 – 13]. Our differential expression results indicate the potential of miR-101 to be further studied comprehensively on its utilization as a biomarker for NPC.

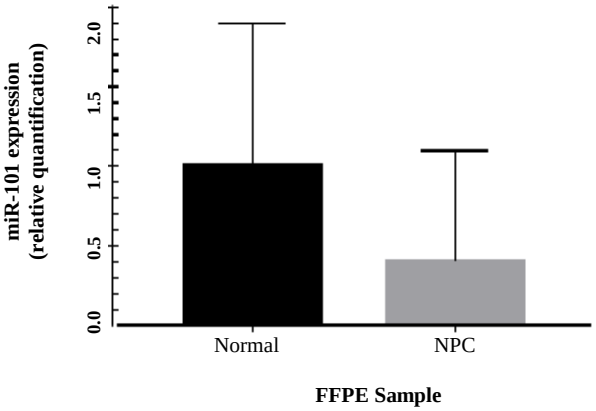

Figure 2. Relative quantification of miR-101 in normal nasopharyngeal epithelium tissues and NPC tissues

For future study, certain improvement must be made to obtain a more reliable and valid result. The use of precise sectioning instrument, such as microdissection, might reduce the surround non- cancerous tissues from being included in the extraction procedure, thus, giving a more accurate picture of miRNA quantification. Furthermore, increasing the small sample size could give more significant and valid result to reject the null hypothesis.

CONCLUSION

We have successfully showed that miRNA can be quantified from FFPE samples. We also demonstrated that the extracted miR-101 from FFPE samples was consistent with previous studies, indicating the potential of miR-101 to be further studied for NPC. However, certain improvements are warranted to increase the significance and reliability of the result.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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