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Study of mir-141 and its Potential Targeted mRNA PTEN Expression in Nasopharyngeal Carcinoma: From in Silico to Initial Experiment Analysis

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Abstract

Recently, accumulating evidences indicated that microRNA-141 (miR-141) is associated with NPC due to their abilities to affect the expression of genes that modulate tumorigenesis. Unfortunately, there is still limited publication about miR-141 expression in Vietnamese nasopharyngeal cancer patients. In this study, we adopted bioinformatics tools, such as Pictar, Target Scan, miRDB, etc. to predict its target gene. As the results, PTEN (phosphatase and tensin homolog gene), acts oncogenic role associated with biological processes lead to the nasopharyngeal carcinogenesis, was identified as the direct target of miR-141. Experimentally, we reported the evaluation of miR-141 and PTEN expression in NPC biopsy samples and non-cancerous swab samples. The present study demonstrated that miR-141 was upregulated 9.38 times, and PTEN expression was significantly lowered in NPC biopsy samples compared to non-cancerous epithelial swab samples. Our finding demonstrated that the miR-141 was upregulated and PTEN was down regulated in NPC biopsy samples. In the upcoming research, a larger clinical sample size from patients at each stage of the NPC will be performed to understand the expression pattern of the miR-141 and PTEN for further applied in early diagnosis and prognosis as well as therapeutic of NPC.

Keywords: miR-141, Nasopharyngeal Carcinoma, PTEN

1. Introduction

In Vietnam, NPC is the fifth most common cancer, resulting in 86,691 cases (Age-standardized rate-ASR = 1.2/100,000) and 50,831 (ASR = 0.7/100,000) deaths annually in Vietnam[1,2]. Although diagnosis of NPC has been improved in recent, most of NPC patients are still diagnosed with the advantage stage of NPC, due to ambiguous symptoms. Improved identification of potential biomarkers which promoting the NPC progression is not only essential for the development of new diagnosis strategies but also promising therapy. It is well known that miRNAs (micro RNAs) are short non-coding RNA molecular within about 20-23 nucleotides (nts) in length that involved in post-transcriptional regulation of multiple genes[3,4]. miRNAs are highly conserved and widely detected in animals, plants, protists and viruses, that involved in numerous cellular processes including cell proliferation, differentiation, metabolism, stress response and apoptosis[4,5]. miRNAs control cell functions by binding to sequences in 3'-untranslated region (3'-UTR) of their target mRNAs, lead to turnover

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and/or repression of mRNA expression[3,4,6]. In the past decades, growing evidences indicated that miRNAs have been implicated as both oncogenes and tumor suppressors that the abnormal expression (positive regulation or downregulation) of miRNAs contributes to various human tumor pathogenesis[4,7–9]. Overexpression of cancer-causing miRNAs, known as onco-miRNAs, have been detected in various types of human cancers, including Nasopharyngeal Carcinoma (NPC), thus, represents not only therapeutic targets, but also pivotal biomarkers for cancer detection and management[10–12]. Among the known onco-miRNAs, miR-141, which is a member of the oncogenic miRNA clustered with miR-200c, located at 12p13.31 (nt: 6964097-6964191, [+]), stands out as the important role in human tumorigenesis[13–17]. However, numerous studies have shown the conflicting results regarding to whether the role of miRNA is an oncogenic miRNA or tumor suppressor miRNA in different types of cancer. Thus, determining the role of miR-141, especially on clinical specimens, leads to more understanding of cancers and the determination of potential diagnostic and prognostic biomarker as well as the therapeutic in clinical applications in different populations. In the case of miR-141 profile in NPC, only a few publications are reported. miR-141 is reported to be up-regulated in NPC specimens in comparison with normal nasopharyngeal epithelium, resulting in affecting cell cycle, apoptosis, cell growth, migration and invasion in NPC cells[13]. On the other hand, the prediction of its target gene, for which the target gene acts as an oncogene or a tumor suppressor gene, combined to miR-141, give a hand for development of the novel molecular target diagnosis and treatments. In previous studies, targets of miR-141 were identified, includes *BRD3, PTEN,* and *UBAP1*, involved in a gene-miR-141 network to contribute to NPC development[13]. Another report indicated that expression of miR-141 was also remarkably increased in NPC tissues and involved in BRD7-medidate cell progression and tumor formation through suppression of PTEN/AKT axis in nasopharyngeal carcinoma[15]. Therefore, miR-141 and its target gene expression profile has become an important tool to study the biomarker for prognosis and diagnosis as well as the therapeutic target for NPC. In this initial study, for further study of miR-141 and its target gene could be served as a potential biomarker for NPC in the Vietnamese population, we adopted many bioinformatics software to identify whether the targets of miR-141 is upregulated or downregulated, then, miR-141 and its target gene were experimentally identified from NPC biopsy samples and normal individuals.

2. Materials and Methods

2.1 Patients' Samples Collection and Ethics Statement

For the miRNA expression study, 20 NPC biopsy samples and 20 normal nasopharyngeal epithelium swab samples were enrolled into current study. Both samples were collected form patients in the Cho Ray Hospital. All samples were stored at -20°C until further assays. All of those samples were submitted to the histopathological department, subsequently, were proved histologically to have NPC by Immunohistochemistry (IHC) confirmed. The NPC biopsy samples were positively confirmed as NPC by hematoxylin and eosin for histological examination (Figure 1).

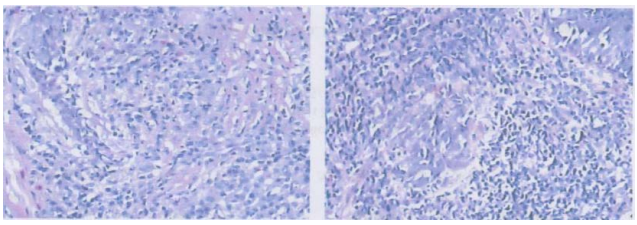

Figure 1. Histological examination of undifferentiated nasopharyngeal carcinoma.

Institutional Ethics Board approval was obtained from the Medical Ethics Committee of the Cho Ray Hospital, Ho Chi Minh City, Vietnam. (The decision number of the permission from Ethical committee: 516/BVCR-HDDD, Cho Ray Hospital, Ho Chi Minh City, Vietnam). All the samples used in this study were agreed by Cho Ray Hospital and obtained from all participants in current study. The patients are required to be agreed and sign on the consent forms.

2.2 Bioinformatics Analysis

The miRBase database (http://www.mirbase.org/) was used for finding out the basic information of miR-141. Possible target genes and signal pathways were validated by utilizing Pictar (https://pictar.mdc-berlin.de/), TargetScan 7.2 (http://www.targetscan.org/vert_72/), and miRDB (http://mirdb.org/), which identifies binding sites targeted by single miRNA.DAVID 2008 Functional Annotation Bioinformatics Microarray Analysis Tools

(http://niaid.abcc.ncifcrf.gov/) was used to classify the functions of the target genes, which were predicted from Pictar, TargetScan 7.2 and miRDB online softwares. We used the Cancer Gene Census (https://cancer.sanger.ac.uk/cosmic/) and Tumor suppressor database (https://bioinfo.uth.edu/TSGene/) to identify whether the target gene is an oncogene or a tumor suppressor gene.

2.3 Total RNA Isolation, Reverse Transcriptase PCR Assay and Detection of miR-141 Expression

miRNAs were isolated from NPC biopsy samples and non-cancerous samples by applying mirVana™ miRNA Isolation Kit (Ambion, Life Technology) according to the manufacturer's instructions. cDNA was reverse transcribed from approximately 5 ng of Total RNA by using TaqMan® Advanced miRNA cDNA Synthesis Kit. The detection of miR-141 was determined by qualitative Real-time PCR (qRT PCR) assay with Taqman™ Advanced miRNA assays kit (ThermoFisher Scientific). 5 µl of RT reaction product was used to each reaction for detection of miR-141. The UniSp6 rRNA was used as the internal control candidate to normalize the Ct values because of the non-differential expression level in tumor and health adjacent samples.

2.4 Evaluation of mRNA PTEN Expression

For evaluation of PTEN expression, the detection of target cDNAs was determined by Reverse-Transcriptase PCR. cDNA from total of RNA template were generated by SensiFAST™ cDNA Synthesis. The cDNA synthesis assay was performed by following steps: 25°C for 10 mins, 42°C for 15 mins, 85°C for 5 mins, and 4°C hold. The cDNA was stored at -20°C for further PCR assay. The cDNA *GAPDH* was used as the internal control candidate in this assay. The sequence of primers used in current study was shown in (Table 1).

3. Statistical Analysis

Data were analyzed using Medcalc® Version 12.7.0.0. All p values were two-side, and values less than 0.05 were considered significant. All values were reported as mean ± SD. The relative expression of miR-141 as determined using q-PCR was analyzed using the $2^{-\Delta\Delta Ct}$ method. Finding was greater and less than 1 was determined to classify up-regulation and down-regulation, respectively. Chi-test was used to determine the association between the expression of miR-141 and NPC status. Moreover, the association between expression of miR-141 and risk of NPC was estimated by computing OR (Odd ratios), RR (Relative risk) and 95% Confidence Intervals (CI).

4. Results

4.1 Bioinformatics Analysis of miR-141 and Putative Targets

miR-141 is belonged to the miR-200c family, concludes five members: miR-200b, miR-200c, miR-429, miR-141 and miR-200a, divided into two sub-families which classified based on the sequence homology in their seed regions, and the highly conversed nucleotides of miR-141 is observed in many species, such as *Pan troglodytes*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Macaca mulatta*, *Gorilla gorilla*, *Pongo pygmaeus*, *Pan paniscus*, *Monodelphis domestica*[17]. The target genes are predicted by the online softwares, includes Pictar, TargetScan 7.2, miRDB. TargetScan predicted that 896 genes are possible targets of miR-141, Pictar predicted that 422 genes are possible targets, whereas miRDB predicts that 759 genes are target of miR-141. The functions of predicted genes, which were predicted from above three online softwares, were classified by using DAVID 2008 Functional Annotation Bioinformatics Microarray Analysis Tools. The results revealed that most target genes of miR-141 are involved in many signal pathways, which are considered

Table 1. The sequence of primers used in current study

Gene	Primer	Sequences (5' – 3')	Reference
PTEN	Forward	CAGAAAGACTTGAAGGCGTAT	18
	Reversed	AACGGCTGAGGGAACTC	
GAPDH	Forward	AATCCCATCACCATCTTCCA	19
	Reversed	CCTGCTTCACCACCTTCTTG	

to be important role in tumorigenesis, such as: cell cyle, p53 signaling pathway, tight junction, Wnt signaling pathway, MAPK signaling pathway, Jak-STAT signaling pathway, etc. (Figure 2).

In the list of miR-141-targert genes, PTEN (phosphatase and tensin homolog gene) was attracted our attention. Cancer Gene Census and Tumor suppressor database were used to identify the whether PTEN is a suppressor gene or an oncogene. As the result, *PTEN* is tumor suppressor gene in human cancer. PTEN encoded protein inhibits the activity of PI3K and then acts as a vital of tumor suppressor gene, that involved in multiple biological processes such as cell growth, proliferation, metabolism, apoptosis, etc… Additionally, PTEN regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. Hallmark page, based on the Cancer Gene Census analysis, explains the role of PTEN in cancer by highlighting, which of the classic behavior are displayed by the gene product and whether they are promoted or suppressed (Table 2).

Confirmed the reliability of PTEN is the target gene of miR-141, consequential pairing of 3'-untranslated region of PTEN and miR-141-3p were performed by TargetScan 7.2. The results revealed that the site type of binding between miR-141-3p and PTEN 3'-UTR were 7mer-m8, 7mer-A1 (Table 3).

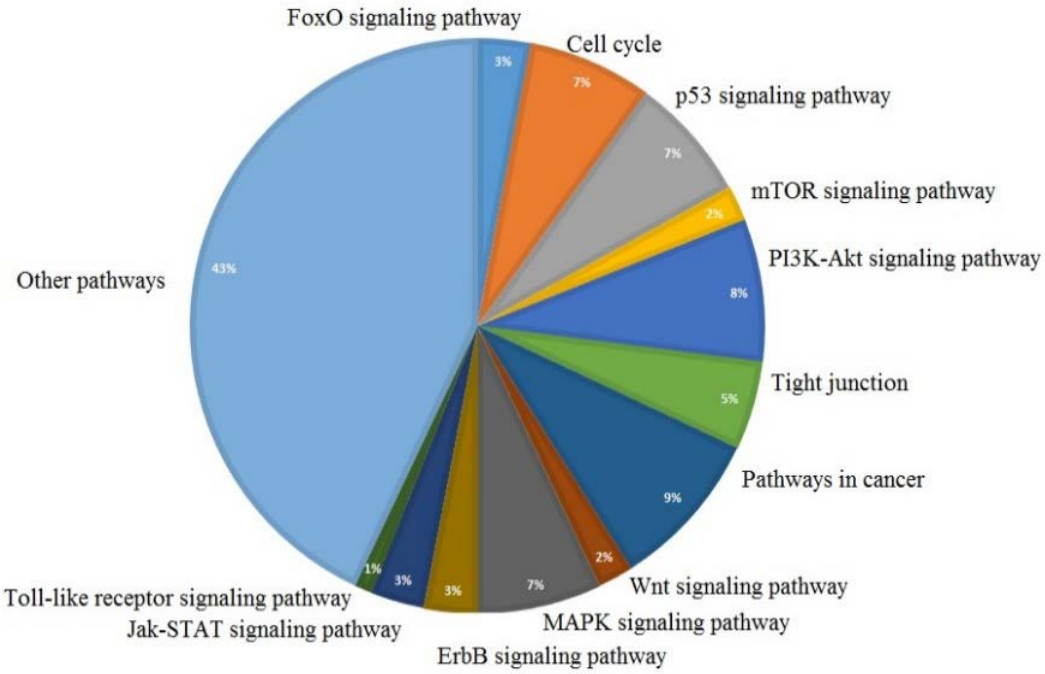

Figure 2. Functions of predicted miR-141 target genes.

Table 2. A concise overview of cancer hallmark of PTEN with associated references

Hallmarks	Promote or Suppress	Reference
Suppression of growth	Promote	20-22
Escaping immunic response to cancer	Suppress	23
Cell replicative immortality	Suppress	24
Invasion and metastasis	Suppress	25, 26
Genome instability and mutations	Suppress	27, 28
Escaping programmed cell death	Suppress	29, 30
Change of cellular energetics	Promote	31, 32

Table 3. Predicted conserved consequential pairing of miR-141 and PTEN 3'-UTR

Position of miR-141 binding PTEN 3'-UTR	Predicted consequential pairing of PTEN (top) and miR-141-3p (bottom)	Site type
1467 – 1473	5' . . .UUUUUUUUAAAUGUGCAGUGUUG. . . |||| ||||||| 3' GGUAGAAAUGGUCU-GUCACAAU	**7mer-m8**
3257 – 3263	5' . . .AACCCUUUAUCUCUUAGUGUUAU. . . |||| ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	**7mer-A1**
4252 – 4258	5' ...GAAUUUAAAACUCACCAGUGUUU. . . |||| ||||||| 3' GGUAGAAAUGGUCU---GUCACAAU	**7mer-m8**

Note: the binding site was in black frame

4.2 miR-141 was Higher Expression in NPC Biopsy Samples

cDNA U6 (UniSp6 rRNA) was used as internal control for the evaluation of miR-141 expression in the comparison between the NPC group (case group) and non-cancerous group (control group). The cDNA U6 was positive in both the case and control group by Real-time PCR, the Ct value of cDNA U6 were 30.55 ± 0.63 and 30.55 ± 0.75in the case and control group, respectively. The p value (p = 0.86>0.05) indicated the expression of U6 was no difference between those two groups (Figure 3A). In current study, the proportion of miR-141 expression in NPC and non-cancerous samples were detected by qRT-PCR. The positive rate of miR-141 was 40% (8 of 20 cases) and 10% (2 of 20 cases) in case and control group, respectively. The p value (p = 0.03) indicated the significant correlation between the miR-141 expression and NPC. The results of odds ratio and relative risk values were 6.00 (95% CI = 1.08 to 33.28), 2.00 (95% CI = 1.17 to 3.42), respectively. The mean of Ct value of miR-141 expression in the case and control group were 27.27 ± 1.14 and 34.50 ± 2.72, (p = 0.0116) (Figure 3B). The relative quantification of miR-141 expression between the case group and control group was analyzed by the $2^{-\Delta\Delta Ct}$ method, as the result, the expression of miR-141 levels was 9.38 times (p = 0.003) higher in tumor samples in comparison with the non-cancerous samples (p = 0.022) (Figure 3C).

4.3 PTEN was Downregulated in NPC Biopsy Samples

To access whether PTEN is upregulated or downregulated in case group, we explored the relationship between the

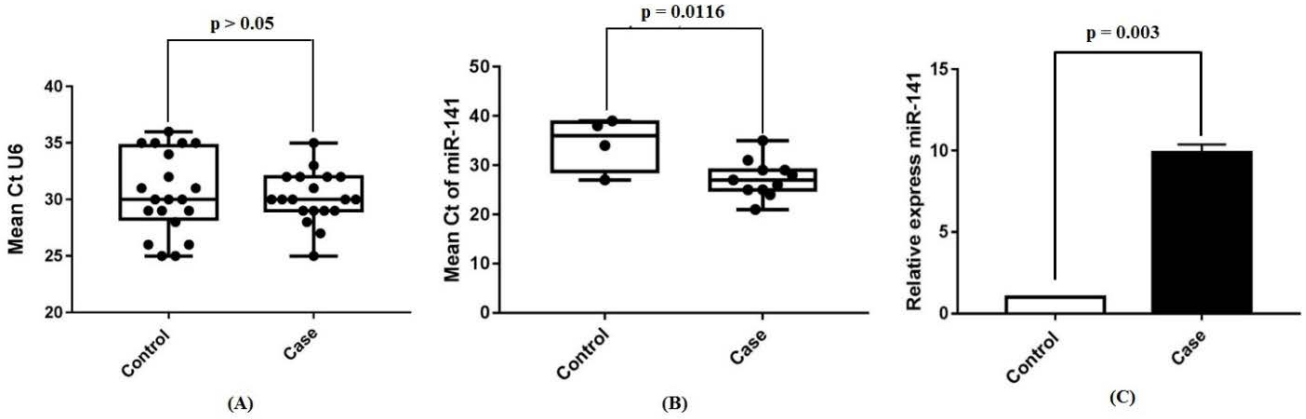

Figure 3. The mean of Ct value (mean ± SE) of (A) UniSp6 rRNA; (B) miR-141 in the case group and control group. Each black dot was indicated the Ct value of each sample; (C) qRT-PCR showed the level expression of miR-141 was up-regulated in NPC biopsy samples compared with the non-cancerous samples.

expression of PTEN in biopsy samples and non-cancerous samples by Reverse-transcriptase PCR. The PCR products obtained in distinctly size, yielded a PCR product of 625 bps (Figure 4A). The amplification of PTEN was determined by Sanger sequencing. As the result, the sequenced results of target genes indicated the clear and single peak of each nucleotide (Figure 4B). According to BLAST results, candidate gene sequence was similar to Homo sapiens Phosphate and Tensin homolog (PTEN), mRNA within accession number: NM_001304717, Total score = 939, Ident = 98%, e-value = 0.0. The examination showed that the proportion of positive PTEN were 45.0% (9 of 20 cases) and 85.0% (17 of 20 cases) in case and control group, respectively. The p value (p = 0.02) indicated the significantly higher rate in control group than in the case group. The results of odds ratio and relative risk values were 0.14 (95% CI = 0.03 to 0.65), 0.44 (95% CI = 0.2430 to 0.7987), respectively. Those results indicated that the expression of PTEN may serve as the tumor suppressor genes by reduction of odds ratio and relative risk.

5. Discussion

NPC is the most common cancer originated from the nasopharynx with high occurrence in Southern China and Southern Asia. According to the global registry of cancer incidence and mortality, NPC ranked 5th among all malignancies in Vietnam. Up to date, accumulating evidences indicated the dysregulation of miRNAs has crucial roles in the progression of multiple cancers[3,4,7,33]. The dysregulation of miR-141, located on chromosome 12p13.31, was observed in wide range of human cancers, nasopharyngeal carcinoma, hepatocellular carcinoma, colon cancer, prostate cancer, bladder cancer, ovarian cancer, breast cancer, etc.[17], acting the dual roles either as oncogene or as tumor suppressor via several molecular mechanisms. In current study, we confirmed that miR-141 is expressed significantly higher in NPC biopsy samples in the comparison with non-cancerous nasopharyngeal epithelial swab samples by qRT-PCR. The positive rate of miR-141 expression in NPC biopsy samples was 40.0% (Sensitivity = 40.0%), it implied that the characteristic of 40.0% of NPC biopsy samples will be positive of miR-141 expression, compared to 10.0% of non-cancerous swab samples. The $2^{-\Delta\Delta Ct}$ value ($2^{-\Delta\Delta Ct}$ = 9.38) is once confirmed that the overexpression of miRNA levels was once confirmed by 9.38 times higher in tumor samples in comparison with the non-cancerous sample. The up-regulation of miR-141 was strongly correlated with NPC risk with the significant statistic through the calculating the RR and OR ($p < 0.05$) The odds for a positive expression of miR-141 in NPC was 6.00 times higher than in the case of cancer without expression of miR-141 (OR = 6.00, 95%CI = 1.08 to 33.28). Additionally, the expression of miR-141 was 2.00 times higher than no miR-141 expression in NPC (RR = 2.00, 95%CI = 1.17 to 3.42). Thus, all of these results imply that, even though the limit amount of samples enrolled in current study, miR-141 might play a pivotal role as oncogene in NPC tumorigenesis. Compared to previous studies, the up-regulated expression of miR-141 was also observed in NPC samples, which were obtained from The Second Xiangya Hospital of Central South University (Changsha, China), The Second Xiangya Hospital and Hunan Province Tumor Hospital, and NPC cell lines compared

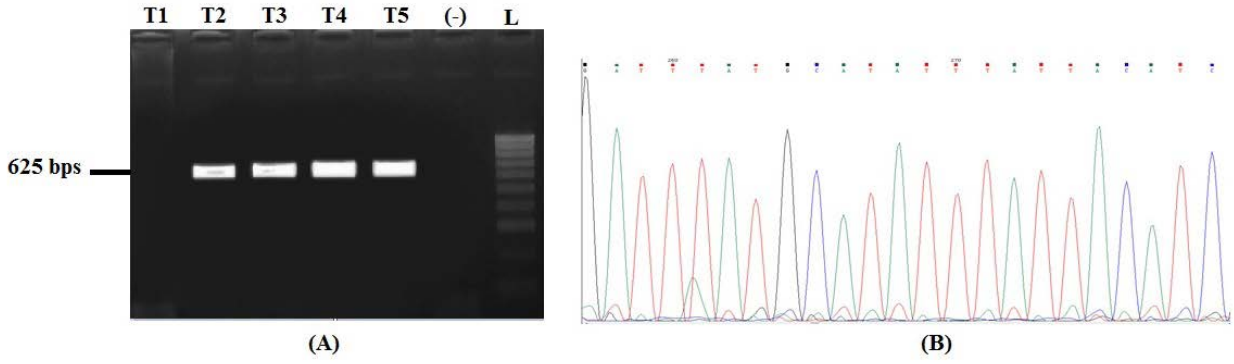

Figure 4. (A) Agarose gel electrophoresis of the PCR products of representative NPC biopsy samples. (-): negative control; L: 100-bps DNA marker. (B) The part of cDNA sequencing of PTEN in representative sample.

to non-cancerous samples[13,15,34]. Notably, in Vietnam, no study about of miR-141 in NPC has been reported. This is a follow-up study on previous findings in the application of using biopsies to assess miRNA properties. Therefore, this study was the first case-control study that we focus on the evaluation of miR-141 profile in local NPC patients. By the prediction of bioinformatics tools, such as Pictar, TargetScan, miRDB, a wide range of possible target genes of miR-141, which are linked to many signaling pathways considered to be important role in tumorigenesis, were predicted. Bioinformatics analysis results showed PTEN confirmed that PTEN act as direct target of miR-141 through the consequential pairing of 3'-untranslated region of PTEN and miR-141-3p, includes 7mer-8, 7mer-A1 site type. With the prediction of Cancer Gene Census and Tumor suppressor database, PTEN acts as a tumor suppressor gene, that involved in multiple biological processes lead to the carcinogenesis, based on the analysis of cancer hallmark. The relationship between miR-141 and PTEN to contribute to NPC development has been reported. According to Zhang et al., the expression of miR-141 may increase the phosphorylation of AKT, which mechanism plays an important role to result in the activation of cascade of several proteins involved in cell proliferation, invasion and promote tumorigenesis. Notably, the ATK phosphorylation levels are increased by miR-141 through the inhibition of PTEN expression[13]. Therefore, understanding of both expression of miR-141 and PTEN may lead to find out possible prognosis and early diagnosis biomarkers for NPC. In current study, the expression of PTEN in both case and control group were determined by RT-PCR. As the results, the expression of PTEN in NPC biopsy samples with the lower positive rate of 45.0% compared to the positive rate in non-cancerous swab samples with the rate of 85.0%. The down-regulation of PTEN in NPC samples increased the risk of NPC through the OR and RR value. This observation was similar to previous studies that PTEN is downregulated in NPC samples. Therefore, based on those results, we implied that miR-141 is the negative regulator of PTEN. Finally, we suggested that the same concept of miR-141 role with previous studies is that: miR-141 was up-regulated, acted as an oncogenic role, and also reported as negative regulator of PTEN, acted as an tumor suppressor in NPC. For further study, identification of miR-141 as well as PTEN expression in larger amount samples, as well as the non-invasive specimens, to characterize the miR-141 and PTEN expression profile and severed as the biomarkers for the screening and early diagnosis of NPC in Vietnamese population, will be continuously studied.

6. Conclusion

In summary, we demonstrated that PTEN is the direct target gene of miR-141, which was confirmed by bioinformatics tools prediction, multiple biological processes lead to the nasopharyngeal carcinogenesis. Experimentally, we confirmed that miR-141 was upregulated 9.38 times in NPC samples compared with the non-cancerous samples. The percentage of miR-141 detection in cancerous and non-cancerous samples were 40.0%, and 10.0%, ($p = 0.03$), respectively. Additionally, PTEN was determined as the negative regulated by miR-141, in detail, the down-regulation of PTEN was observed in NPC samples compared to non-cancerous samples. Therefore, all of those findings suggest that the determination of both stage of miR-141 and PTEN might serve as possible biomarkers in the diagnosis and prognosis of NPC. In further research, the amount of samples have to be enlarged, as well as different stages of cancers, will increase the application of using miR-141 and PTEN properties in the diagnosis and prognosis of NPC.

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8. Author's Contribution

Thuan LD 35%; Danh NH 30%; Thuy LHA 35%. All authors read and approved the final manuscript.

9. Ethics Approval and Consent to Participate

All patients signed inform consent before entering into the study. No study drug or procedure was applied. This is an observational study.

10. Conflict of Interest

The authors declared that they have no competing interests.

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