PLASMA MICRORNA-21 EXPRESSION IN PATIENTS WITH NON-SMALL CELL LUNG CANCER

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ABSTRACT

Lung cancer is one of the most common malignancies and is also the leading cancer-related death worldwide. Majority of which are non-small cell lung cancers. Five year survival rate of stage IV non-small cell lung cancer is approximately only 10% whereas for stage I disease, it can be as high as 80%. These data provide the rationale to improve the diagnosis of non-small cell lung cancer and to detect the disease at an earlier stage where the cure of the disease can be expected.

The aim of this study was to study the role of plasma miRNA-21 expression in patients with non-small cell lung cancer. One hundred patients were recruited from the Department of Respiratory Medicine, Yangon General Hospital and Yangon Specialty Hospital from February 2013 to May 2014. Among them, 50 patients were confirmed non-small cell lung cancer patients and 50 were patients with non-malignant respiratory diseases: the study population selected for comparison in this cross-sectional comparative study.

Demographic data were taken, radiological and other diagnostic investigations necessary to confirm the respective diagnoses were performed in all patients. By using quantitative reverse transcriptase PCR technique, the plasma expression of miRNA-21 was determined in all studied patients. Clinical staging was carried out in all non-small cell lung cancer patients and categorized into stage I-IV diseases (TNM staging).

The mean (SD) of plasma miRNA-21 in the patients with non-small cell lung cancer was 64.07 ± 137.17 ($\times 10^5$ copies/µl) and that in the patients with non-malignant respiratory diseases was 2.35 ± 2.29 ($\times 10^5$ copies/µl). Plasma miRNA-21 level measured in the patients with non-small cell lung cancer was significantly higher than that determined in the patients with non-malignant respiratory diseases ($p < 0.05$). The diagnostic value of plasma miRNA-21 was improved after applying the ROC curve analysis. In this study, plasma miRNA-21 yielded the area under the ROC curve (AUC) value of 0.86 (95% CI: 0.79-0.93) with both sensitivity and specificity 86%, and likelihood ratio of 38.37 when the optimum cut-off level of plasma miRNA-21 was selected at 5.01 ($\times 10^5$ copies/µl). Importantly, plasma miRNA-21 levels were significantly related to the stages of non-small cell lung cancer ($p < 0.05$). It was found that plasma miRNA-21 levels were related to the stages of non-small cell lung cancer in such a way that as the clinical staging increased from I to IV, the expression levels of plasma miRNA-21 became higher. Moreover, the demographic parameters; age, sex, pack-year and histological types of non-small cell lung cancer patients did not exhibit significant association with miRNA-21 levels (all $p > 0.05$). Altered expressions of plasma

miRNA-21 were not shown to be effected by these clinico-pathological variables.

Plasma miRNA-21 is a potential blood based biomarker which could be implied clinically to support the diagnosis of non-small cell lung cancer. Plasma miRNA-21 also plays an important role in the tumour progression and it could be a prognostic marker of non-small cell lung cancer as well.

INTRODUCTION

Lung cancer is one of the most common malignancies worldwide since the beginning of the 20th century and its occurrence has increased rapidly. By the year 2012, the occurrence of new lung cancer has increased up to 1.8 million. Among the cancer deaths as well, it is one of the top list cancer deaths, responsible for 1.6 million deaths in 2012[1]. In Myanmar, lung cancer also stood as the third commonest cancer between the years 1993 and 1999 and it was in a rising trend towards the 21st century [2].

It is evidently seen that the magnitude of the disease is rising but the chance of cure and the survival rate of the disease are still not promising. The reason behind this challenge is most of the lung cancers do not usually become clinically apparent until they reach an advanced stage. Successful surgical resection is the only curative treatment for non-small cell lung cancer only when they are diagnosed at an early stage. The proportion of the patients with lung cancer that could aim curative intent is only 15-25%[3].

These facts highlight the need for further search of novel diagnostic and therapeutic strategies for early diagnosis and better survival of lung cancer. In the earlier 20th century, there have been many researches which actively investigate the development of reliable, non-invasive tests for early detection of lung cancer[4]. Researchers had focused on the tumor markers and then explored again to the proteomics and genomics for early detection of lung cancer.

miRNAs are small non-coding RNAs that post-transcriptionally regulate the translation of target genes. An increasing number of studies have shown that miRNAs have links with cancers. It was found that miRNAs behave either as tumor suppressor genes or oncogenes[5]. miRNA-21 is one of the first microRNAs to be identified as an "oncomir" and its role in the tumorogenesis and tumour promotion as an oncogene has already been demonstrated. The mechanisms of microRNA-21 in cancer pathogenesis are by means of oncogenic K-ras activation, decreasing apoptosis and activating Epidermal Growth Factor Receptor (EGFR) signaling pathway[6]. Moreover, they are found to have special properties; high stability in many clinical specimens, resistance to enzymatic cleavage and reliably measurable in plasma[7]. So, miRNA-21 becomes one of the attractive proteomics as a potential blood biomarker for lung cancer diagnosis.

In this study, we tried to find out the role of plasma miRNA-21 in non-small cell lung cancer. The diagnostic value of plasma miRNA-21 expression in detecting non-small cell lung cancer was determined and the association between the levels of plasma miRNA-21 expression among different stages of non-small cell lung cancer was

further explored, thereby evaluated its role in tumour progression.

MATERIALS AND METHOD

Patients A total of 100 patients were included in this study. Among 50 patients with confirmed non-small cell lung cancer, the mean age was 61.48 ± 8.71 years, their ages ranged between 45-82 years and the ratio of male to female was 2.8:1. Among them, majority was smokers (84%) and the ratio of smoker to never-smoker was 5.25:1. The mean pack-year of smokers was 9.97 ± 6.08 pack-years. On the other hand, among 50 patients with non-malignant respiratory diseases, the mean age was 56.94 ± 10.93 years and their range of ages was 27-78 years. The ratio of male and female patients was 1.63:1 and that of smoker to never-smoker was 3.1:1. Mean pack year of smoker in this patient group was 10.55 ± 5.87 pack years.

Materials for detection of miRNA expression

miRNeasy Serum/Plasma Kit (for purification and extraction of miRNA), miRNeasy Serum/Plasma Spike-In Control (for normalization control for miRNA expression), miScript Reverse Transcription Kit, miScript Primer Hs-miR-21 (for reverse transcription) and miScript SYBR Green PCR Kit, real time cycler (for qRT- PCR) were used accordingly.

METHODS

Study procedure Clinical information was collected form all patients. Necessary laboratory tests, radiological investigations including CT (chest), USG (abdomen), (CT brain, isotopic bone scan when clinically

indicated) were carried out in suspected lung cancer patients. In this patients' group, histological diagnosis was confirmed by one or more of the procedures; flexible fiberoptic bronchoscopic biopsy and bronchial washing, percutaneous needle biopsy, pleural biopsy in patients having suspicious malignant pleural effusion with underlying primary lung cancer, biopsy of lymph nodes suspicious of metastasis from primary lung cancer and surgical lung specimens in patients who underwent surgery. Among lung cancer patients, squamous cell carcinoma was found to be the most common histological type (82 %) followed by adenocarcinoma (14 %) and large cell anaplastic carcinoma (4%). These patients were staged clinically with bronchoscopy and appropriate radiological investigations according to the American Joint Committee on Cancer[8]. Most of the patients were found to have advanced staged cancer at the time of diagnosis; stage III (46%), stage IV (42%) while only 4% of patients belonged to Stage I and 8% to stage II. 5 ml of blood was collected from the patients with lung cancer before giving necessary treatment and also from the patients of other group after appropriate investigations.

All the blood samples were further processed for quantitative determination of miRNA expression levels. Whole blood samples collected in EDTA was subsequently centrifuged by 3000 rpm for 10 min at room temperature and plasma was collected and stored at -20°C until use. Total RNA was extracted by using miRNA extraction kit (miRNeasy Serum/Plasma kit, Catalog no. 217184, Qiagen). Reverse

transcription of miRNA was performed using the miScript Reverse Transcription kit (Catalog no. 218061, Qiagen) and RT primers of miR-21(miScript primer assay HsmiR21 with the sequence of 5'UAGCUUAUCAGACUGAUGUUGA3', Catalog no. 218300, Qiagen). The expression of miRNA-21 was detected by using miScript SYBR Green PCR kit (catalog no. 218073, Qiagen). To monitor RNA recovery, reverse transcription efficacy and amplification efficacy, synthetic Caenorhabditis elegans miRNA-39-1 (Catalog no. 219610, Qiagen), which had no homologus gene in human, was added into each sample. Subsequently, real-time PCR was performed in RT-PCR machine (Rotor gene 6000). Finally, the absolute concentration of microRNA into the reverse transcriptase reaction was converted to amount of miRNA, expressed as copies of miRNA per microliter of plasma by using the software, Rotor-Gene Q Series 2.0.2 (Build 4) 2008 Corbett Life Science, Qiagen.

Statistical design & analysis

The study design was cross sectional comparative study. Data was analyzed by using the EpiData 3.1 statistical software. Due to the magnitude and range of miRNA level observed, the absolute concentration of miRNA-21 detected was expressed and analyzed in the data as $\times 10^5$ copies of miRNA-21/µl of plasma. Means levels of miRNA-21 between two patients group were analyzed by independent sample t test. ROC curve was applied to evaluate the best cut-off value. Univariate analysis was undertaken to determine the association between plasma miRNA and demographic data and Fisher's exact test and test of variances for association between plasma miRNA and stages of lung cancer. All p values of < 0.05 were considered statistically significant.

RESULTS

Comparison of plasma miRNA-21 expression levels in two groups of patients

The mean (SD) value of miRNA-21 level in non-small cell lung cancer patients was 64.07 ± 137.17 ($\times 10^5$ copies/ µl) and that of the patients with non-malignant respiratory disease was 2.35 ± 2.29 ($\times 10^5$ copies/ µl). It was found that plasma miRNA-21 level was significantly higher in non-small cell lung cancer patients than the patients with non-malignant respiratory diseases ($p < 0.05$).

The diagnostic value of plasma miRNA-21 in the diagnosis of non-small cell lung cancer

The receiver-operator characteristic (ROC) curve demonstrated the sensitivity and specificity values (86%) when the miRNA-21 level was set at 5.01×10^5 copies/ µl. Plasma miRNA-21 yielded an area under the receiver operator characteristics curve (AUC) of 0.86 (95 CI: 0.79-0.93). Positive likelihood was 6.14 and negative likelihood was 0.16 and the likelihood ratio was found to be 38.37 at that cut-off value. The best cut-off point of plasma miRNA-21 found in this study was 5.01 ($\times 10^5$ copies/ µl) for the diagnosis of non-small cell lung cancer.
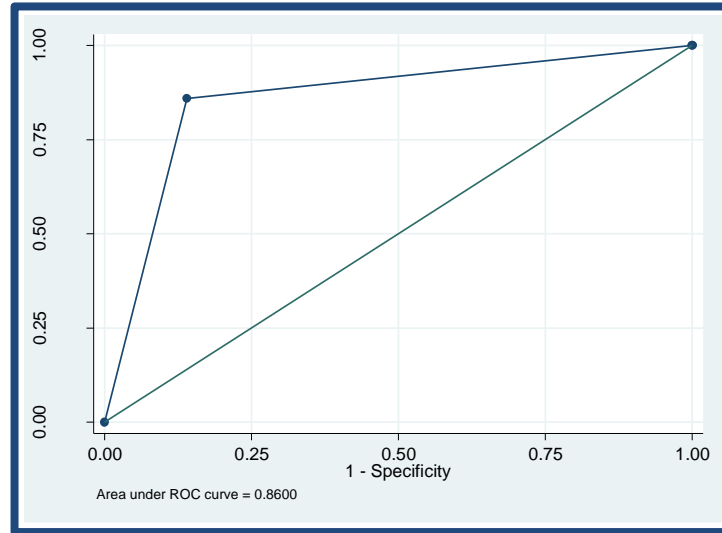
Figure (1) AUC of plasma miRNA-21 at the best cut-off value (5.01×10^5 copies/ µl)

Association between plasma miRNA-21 expression and clinico-pathological data in patients with non-small cell lung cancer

There was no association between the expression of the miRNA-21 and the demographic data of the patients with non-small cell lung cancer (all p > 0.05). The demographic variables such as age (p = 0.33), gender (p = 0.49) and pack year (p = 0.42) of the patients did not have significant effect on miRNA-21 expression levels. The means of plasma miRNA-21 expression among different histological types were also found to be not differ significantly (p>0.05).

Correlation of plasma miRNA-21 levels and stages of non-small cell lung cancer

It was found that the expression levels of miRNA-21 were significantly elevated in the more advanced-stages of lung cancer patient (p < 0.05). The respective means and median levels of plasma miRNA were also found to be higher as the stages of lung cancer became more advanced.

DISCUSSION

miRNAs have generated much interest as novel biomarkers for molecular diagnosis of malignancies[9]. The documented mechanisms of miRNA-21 involvement in cancer oncogenesis are by blocking apoptosis, silencing, and activation of caspases and by targeting tumor suppressor genes[10,11].

After understanding the molecular pathogenesis of miRNA-21 in cancers, the researchers have been trying to investigate its usefulness for diagnosis, prognosis and therapeutic target in non-small cell lung cancer.

miRNAs can be reproduced from different clinical specimens by molecular techniques such as Northern blot analysis, real-time PCR, miRNA microarray analysis [11,12].
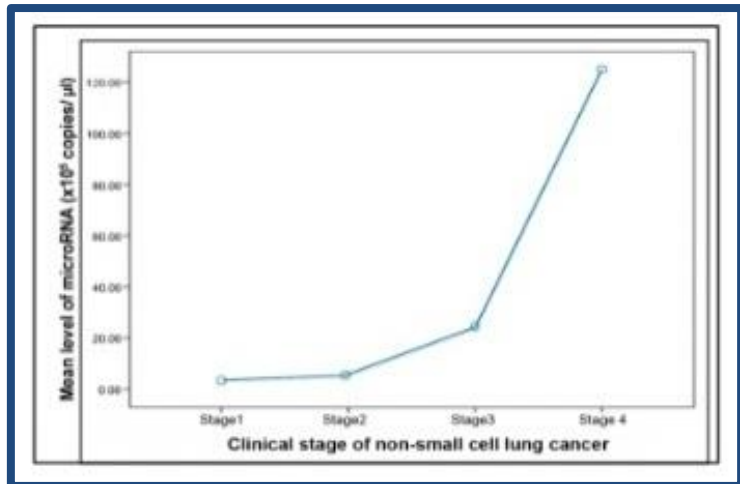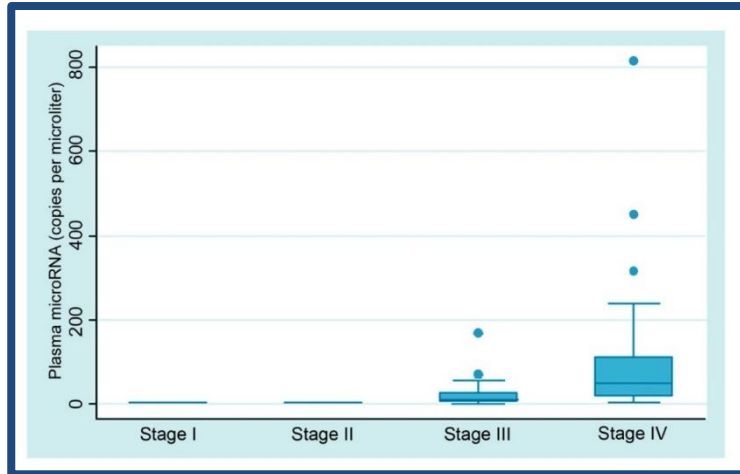
Figure (2, 3)   Relationship of plasma miRNA-21 levels with different clinical stages of non-small cell lung cancer patients

But to identify miRNAs in blood is more attractive than others because blood is an easily accessible and rich body fluid.

In this study, we used quantitative real time PCR technique to measure plasma miRNA-21 expression. It was found that it can be specifically measured in the plasma by reverse transcriptase polymerase chain reaction with good validity. This fact supports further researches on miRNAs as a potential blood based biomarkers.

This study pointed out that plasma miRNA-21 expression was found significantly higher in non-small cell lung cancer patients compare to the patients with non-malignant respiratory diseases (p <0.05). It also signified the clinical implication of plasma miRNA-21 by providing its value in terms of promising sensitivity and specificity (both values 86%) at the optimal cut-off point $(5.01 \times 10^5$ copies/ µl). Plasma miRNA-21 also exhibited the area under the ROC curve (AUC) value of 0.86 (95% CI - 0.79-0.93) at that best cut-off value.

The value of miRNA-21 in the diagnosis of lung cancer is also evidently seen in the recent systematic review and meta-analysis of miRNA-21 by Yang et al [13] in which the pooled sensitivity was 0.71 (95% CI: 0.57–0.82) and the pooled specificity was 0.84 (95% CI: 0.76–0.89) for the diagnosis of lung cancer. It could be concluded that plasma miRNA-21 could be used as a potential blood biomarker for the diagnosis of non-small cell lung cancer.

There are experimentally proved mechanisms of miRNAs by which they play roles in many aspects of cancer biology, such as proliferation, invasion/metastasis,

and angiogenesis[11]. So, the next important part of this study is finding out the association between plasma miRNA and stages of lung cancer in order to explore its role in tumour progression. In the current study, it was found that miRNA-21 level was significantly related with stages of non-small cell lung cancer. As the clinical stages of lung cancer were getting higher up, the levels of miRNA-21 became higher. In the retrospective analysis of three cohorts from Maryland, Norway and Japan, consisting of 317 patients recruited from 1987 to 2008, it was proved that elevated miRNA- 21 expression in snap-frozen lung tissue specimens of early stage, non-small cell lung cancer patients was independently associated with disease progression and poor survival [3]. Another study carried out by Wei et al also supports the pathogenic mechanism of miRNA-21 in tumour proliferation and progression[16]. And this significance may also be helpful for further researches in the fields of prediction of disease progression, prognosis and therapeutic response of non-small cell lung cancer.

Another fact about plasma miRNA-21 in this study was that it independently up-regulated and was not influenced by other clinico-pathological parameters of non-small cell lung cancer patients. There were discrepancies among the findings on the association between miRNA-21 and age, gender, smoking status and histology of lung cancer patients [3, 14, 17]. The heterogeneity of findings in this aspect might be influenced by ethnic origins of the study population.

Although many challenges remain, the current study could point out some

significant findings on the role of miRNA-21 in non-small cell lung cancer, for the diagnosis and prognosis. More researches, systematic reviews and meta-analyses should be performed to evaluate its implication particularly as an early diagnostic biomarker for non-small cell lung cancer so that better survival of lung cancer patients would be achieved.

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