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Original Research Article

Role of liquid biopsy in non small cell lung cancer

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ABSTRACT

Introduction: Lung cancer is the leading cause of morbidity and mortality worldwide. It is usually diagnosed in advance stage. miRNA present in serum and pleural fluid can be studied for early diagnosis of lung cancer. Present study was carried out to evaluate whether miRNA can be used as biomarkers in diagnosis of non small cell lung cancer.

Aims and Objectives: The study was intended to find the non-invasive tumour biomarkers for presence of lung malignancy with the intent of instituting early diagnosis to reduce lung cancer related mortality. The aim of the study was to evaluate circulating microRNA expression in adenocarcinoma and squamous cell carcinoma lung in comparison with age and sex matched healthy controls. The expression of these miRNA was correlated with histopathology and/or immunohistochemistry. The circulating miRNA expression in age and sex matched non-smoking healthy controls was also noted.

Materials and Methods: It was a Prospective observational study in which 50 cases of non small cell lung cancer was included. 50 healthy non smoker volunteers (control group, well adjusted to the patients according to the age and sex) were also included in the study. About 5 ml of serum and wherever possible pleural fluid was collected in the sterile container. The sample was allowed to stand at room temperature for one hour, and then samples were centrifuged at 1300g for ten minutes at room temperature.

RNA was extracted using miRNeasy mini kit (Cat no. 217004) and quantative PCR was done. The patients age, sex, histopathological results, clinical staging, immunohistochemistry, presence of pleural effusion. Expression of mi RNA (miRNA 21, miRNA 17-92 cluster, miRNA 221/222, miRNA Let- 7, miRNA 34 and miRNA 200) were studied.

Results: Out of 50 patients of suspected lung cancer 17 were females (34%) and 33 (66%) were males. Mean age of presentation was 63.26 years. 37 patients gave history of smoking (74%) while 13 patients were non Smokers (26%). 29 patients (58%) showed histomorphological features suggestive of adenocarcinoma whereas 21 patients (42%) showed histomorphological features of squamous cell carcinoma. EGFR mutation was seen in 10 patients (34%). Pleural effusion was present in 20 cases.

Statistically significant correlation was found between the expression of miRNA in healthy controls and in lung cancer patients. All the tested miRNAs were significantly correlated with the corresponding expression in the healthy control. As compared to healthy controls that let-7, miR-34 and miR-200 were downregulated in lung cancer patients whereas miRNA-221, miRNA 17-92, miRNA-21 were upregulated in lung cancer patients. miR 34, miR 200 and let 7 was detected in healthy controls also. No statically significant correlation of miRNA with age, sex, smoking, histopathological type, grade of tumor, stage of disease, EGFR mutation and IHC was found. Stastically significant correlation was found between miRNA 200 and pleural effusion patients.

Conclusions: Present study concludes that miRNA can be a potential biomarker for diagnosing lung cancer. To date, there is convincing evidence supporting the potential role of miRNAs as biomarkers for lung cancer diagnosis and prognosis. However, further research is required in this aspect.

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1. Introduction

Lung cancer is one of the leading causes of cancer deaths worldwide. Approximately 1.5 million new cases of lung cancer are diagnosed every year. The morbidity and mortality of lung cancer has increased over past few years.[1] Five year survival rate of patients with lung cancer is around 16%.[2] As patients remain asymptomatic in early stages it is usually detected in advanced stage.[3] Various risk factors associated with lung cancer are age gender, occupation, status, susceptibility to ionizing radiation, cigarette smoking, exposure to environmental carcinogenic chemical and air pollution. Despite emerging advances in early diagnosis and novel targeted agents, the prognosis of lung cancer remains poor. Patients with lung cancer are often diagnosed at advanced stages, and usually more than 60% of patients are at stages III or IV before treatment. Therefore, the overall 5-year survival rate of lung cancer remains low at 16.8%, and is less than 5% among those with metastatic disease.[4] Early diagnosis and treatment of lung cancer can increase the survival rate. It can be achieved by developing an advanced approach for diagnosis. Presently available diagnostic techniques include CT guided fine needle aspiration cytology from lung lesion, pleural fluid cytology and lung biopsy.

Liquid biopsy using miRNA in pleural fluid and serum could be a new non invasive diagnostic modality for early diagnosis of lung lesion. In 1993, first non coding miRNA lin -4 was identified in Caenorhabditis Elegans.[5] Presently 2500 mature miRNAs have been identified in the miRBase database.[6] The pri-miRNAs are the precursors of miRNA. They have more than 100 nucleotides. Transcription of miRNA is carried by RNA Polymerase II. RNase III acts on these miRNA's and form pre- miRNA. Exportin 5 transports these pre-miRNAs are transported into cytoplasm.[7] In the cytoplasm, Dicer, processes the pre-miRNAs into the miRNA: :miRNA duplex . One chain of the miRNA duplex binds to RNA-induced silencing complex (RISC). It acts like a template and recognizes the complementary mRNA and then it can negatively regulate mRNA expression.[8–10] miRNAs play a important for genomic and epigenomic interaction. One-third of human genes are regulated by miRNAs.[11] miRNA are involved in gene regulation, apoptosis, hematopoietic development, and maintenance of cell differentiation.[12] miRNA act as both tumor suppressors and as protooncogenes. The tumor suppressor miRNA will be downregulated in patients with lung cancers and miRNA encoding for protooncogenes will be upregulated in lung cancer. In present study, we have studied the role of tumor suppressor and oncogenic miRNA in non small cell lung cancer patients The present study was aimed to find the non-invasive tumour biomarkers for presence of lung malignancy with the intent of instituting early diagnosis to reduce lung cancer related mortality. Circulating microRNA expression in adenocarcinoma and squamous cell carcinoma lung in compared with age and sex matched healthy controls and correlation of miRNA expression with histopathology and immunohistochemistry was also done. Circulating miRNA expression in age and sex matched non-smoking healthy controls was also studied.

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2. Materials and Methods

It was a Prospective observational study in which 50 cases of non small cell lung cancer was included. Lung biopsy was done in all cases. Cases with diagnosis of non small cell lung cancer on histopathological examination were included in the study. Informed consent was taken from all patients. Cases with prior treatment, history for lung cancer were excluded from the study. 50 healthy non smoker volunteers (control group, well adjusted to the patients according to the age and sex) were also included in the study. Serum samples were collected in accordance with the protocol approved by the ethical committee of our centre. Pleural fluid was also collected from the NSCLC patients who had pleural effusion. About 5 ml of serum and wherever possible pleural fluid was collected in the sterile container. The sample was allowed to stand at room temperature for one hour, then samples were centrifuged at 1300g for ten minutes. This centrifugation was done at room temperature. The resultant serum and pleural fluid samples were stored at-80 C.

Samples which showed hemolysis were discarded. None of the patients had received any treatment prior to collection of sample. RNA was extracted using miRNeasy mini kit (Cat no. 217004) and quantative PCR was done.The patients age, sex, histopathological results, clinical staging, immunohistochemistry, presence of pleural effusion, EGFR mutation in cases of adenocarcinoma were noted. The serum miRNA level was expressed as –delta Ct to maintain the normal distribution of the parameter. Normality of data was checked using Shapiro Wilk test. miRNA expression in different groups were compared using wilcoxon two sample test and Krushal Walis test. Statistical software R 4.1.2 was used for analysis.

3. Results

Out of 50 biopsy proven cases of lung cancer 17 were females (34%) and 33 (66%) were males. Age of presentation ranged from 34 years to 84 years. Mean age of presentation was 63.26 years. 37 patients gave history of smoking (74%) while 13 patients were non Smokers (26%). 29 patients (58%) showed histomorphological features suggestive of adenocarcinoma whereas 21 patients (42%) showed histomorphological features of squamous cell carcinoma. Among 29 patients of adenocarcinoma-05 patients each showed features of well differentiated and moderately differentiated adenocarcinoma (17%). 19

patients were of poorly differentiated adenocarcinoma (66%). Grades of Squamous cell carcinoma- among 21 patients of squamous cell carcinoma -02 patients were of poorly differentiated squamous cell carcinoma (10%). 15 patients were of moderately squamous cell carcinoma (71%). 04 patients were of well differentiated squamous cell carcinoma (19%) Immonohistochemical profile- P 40 showed strong positivity in 21 cases. TTF and napsin was strongly positive in 29 cases . EGFR testing was done in all 29 cases of adenocarcinoma . EGFR mutation was seen in 10 patients (34%). Pleural effusion was present in 20 cases and was not seen in 30 cases.01 patient was in stage I and stage IIA each, 8 cases of stage IIB, 13 cases of stage IIIA, 8 cases of stage IIIB and 19 cases were in stage IV at the time of presentation.(Table 1)

Statistically significant correlation was found between the expression of miRNA in healthy controls and in lung cancer patients (Table 2). All the tested miRNAs were significantly correlated with the corresponding expression in the healthy control. As compared to healthy controls that let-7, miR-34 and miR-200 were downregulated in lung cancer patients whereas miRNA-221, miRNA 17-92, miRNA-21 were upregulated in lung cancer patients. miRNA 34, miRNA 200 and let 7 was detected in healthy controls also. All the studied miRNA did not show statistically significant correlation with the EGFR mutation, histopathological types of lung cancer, the immunohistochemistry markers for lung cancer, smoking. Median value for let 7 in patients without pleural effusion was 0.052 and median value for let 7 in patients with pleural effusion was 0.0312. p value was 0.3416. All miRNA were studied in the pleural fluid also.Statistically significant correlation was found with miRNA 200.

Median value for miRNA 200 in patients without pleural effusion was 0.04 and median value for miRNA 200 in patients with pleural effusion was 0.0015. p value was 0.007 and was statistically significant. There was a positive correlation with correlation coefficient of 0.396 between let-7 expression in serum and pleural fluid

but it was not statistically significant. p value for let 7 was 0.084. There was a positive correlation of all studied miRNA except miRNA 200 in the serum and in pleural fluid. However it was not statistically significant. There was a positive correlation with correlation coefficient of 0.681 between miRNA 200 in serum and pleural fluid and it was statistically significant. p value for miRNA 200 was 0.0009.

4. Discussion

Non small cell lung cancer is characterized by its rapid progression and poor outcome. The heterogeneity of these tumors as seen in histopathological examination and various mutation and gene expression pattern associated with NSCLC makes this malignancy difficult to diagnose and treat. The present study investigated the effect of six

Table 1: The clinicopathological characteristics of patients

	NSCLC	Healthy volunteer
Age		
< 65years	24	27
>65 years	26	23
Gender		
Male	33	30
Female	17	20
Histological subtype		
Adenocarcinoma	29	
Squamous cell Carcinoma	21	
Grade of adenocarcinoma differentiation		
Well	5	
Moderately	5	
Poorly	19	
Grade of squamous cell differentiation		
Well	4	
Moderately	15	
Poorly	2	
IHC		
P40 positive	21	
TTF1 positive	29	
Napsin A positive	29	
EGRF mutation in adenocarcinoma	10	
Pleural effusion	20	
Clinical stage		
Stage I	1	
Stage IIA	1	
Stage II B	8	
Stage III A	13	
Stage III B	8	
Stage IV	19	
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Non smokers	37	
Smokers	13	

Table 2: Comparison of miRNA expression in patients and healthy volunteers.

miRNA	Case (median)	Control (median)	P value
Let-7	0.037	1.00000	0.00283
miRNA- 17/92	54.627	0.000488	<2.2e-16
miRNA-21	62.27	0.000488	<2.2e-16
miRNA-221	37.144	0.000488	=6.646e-15
miRNA-34	0.0092	0.5000	0.00017
miRNA-200	0.086	1.0000	0.0000042

miRNA miR-21 and let-7 family members as biomarkers in diagnosis of NSCLC patients. We demonstrated the role of three tumor suppressor genes and three proto- oncogenes in non small as lung cancer patients. We concluded that the tumor suppressor miRNA (Let 7, miRNA 34 and miRNA 200) were downregulated in NSCLC and proto-oncogenes (miRNA 221, miRNA 17, miRNA 21) were upregulated in NSCLC patients. All six miRNA were detected in the serum as well as in the pleural fluid of NSCLC patients. Detection of let 7 in different body fluid has been done in the past and it has been confirmed that it is a potential non invasive marker, which can be used in early detection of cancer. Prior studies have concluded that let-7 plasma levels are increased in patients with breast, prostate, liver, colon,renal, gastric and liver cancer.[13–15]

Decreased serum levels of let 7 have also been reported by some researchers. The decreased serum let-7 levels were seen in colon, lung, prostate, gastric, ovarian, and breast cancers.[16] Study conducted by Cecelia showed that let-7 family, specifically let-7a/b/e/f is downregulated in lung cancer patients and its

downregulation was associated with poor outcome for NSCLC patients .In present study, let 7 was downregulated in NSCLC. But no statistically significant correlation was found with any of the clinicopathological parameters studied.

Study conducted by Yanaihara et al[17] examined the miRNA expression in frozen lung adenocarcinoma tissues and normal lung tissues. They studied the expression of miRNA-155, miRNA-17, miRNA-21, miRNA-145 and let-7a, and concluded that the levels of these miRNA differed significantly among the various tissues from NSCLC patients. According to their study, the expression of miRNA- 21, miRNA-17 and miRNA-155, was significantly elevated in the lung adenocarcinoma patients. Study conducted by Saito et al showed that high miR-21 expression was associated with the prognosis and progression of stage I NSCLC,[18] thus concluding that abnormal miR-21 expression might be a potential biomarker for the prognosis prediction of NSCLC . Study conducted by Tian et all found that the level of miRNA-21 expression was significantly increased in the NSCLC tissues compared with the normal tissues.[19] This is in concordance with our study. But according to study done by Tian et al the miR-21 expression in late NSCLC (stage IIb-IIIa) tissue was much higher than that in early NSCLC (stage Ia-IIa) tissues. In present study no such correlation could be established. Various studies have been done in past to find the role of miRNA 34 in NSCLC.

Study done by Tafsiri et al showed that the serum levels of miRNA 34 were significantly reduced in lung cancer patients as compared to the healthy adults.[20] Stahlhut et al studied the levels of miRNA34 and Let 7 in non small cell lung cancer and he also concluded that serum levels of miRNA 34 was decreased in lung cancer patients as compared to healthy adults.[21] Study conducted by Zhao et all on plasma and biopsy tissue of lung cancer patient showed that high expression of miR-34a and miR-34c was associated with prolonged overall survival.[22] In present study miRNA 34 was downregulated in NSCLC patients in both serum as well as pleural fluid but no correlation could be established with other clinical and pathological parameters. The microRNA-200 family includes miR-200a, 200b, 200c, 141 and 429. It regulates epithelial mesenchymal transition. miRNA 200 suppresses the EMT-inducing transcription factors zinc finger E-box binding homeobox 1 and 2 (ZEB1 and ZEB2).[23]

Study done by Burk et al showed that miR-200 regulate cancer cell invasion by suppressing the expression EB1 and ZEB2 genes.[24] In present study miRNA 200 was significantly downregulated in pleural fluid as well as serum in patients of NSCLC. Not many studies have done to see the expression of miRNA 200 in lung cancer. Zheng et al. showed that miRNA-221 was significantly up-regulated in several types of human cancers like prostate, hepatocellular carcinoma, and colorectal carcinoma, suggesting its oncogenic role in tumor causation and progression. However, the effect of miR-221 on cancer cell growth could be changeable in different cell types. Thus miR-221 might exhibit either pro- or anti-oncogenic roles in different cancer cell lines. Study conducted by Ning Wang et al. demonstrated that miR-221 might play a important role in chemotherapy resistance in NSCLC. In present study miRNA 200 levels were upregulated in NSCLC. In both

pleural fluid as well as in plasma as compared to healthy adults. miRNA 17-92 cluster is an oncogenic miRNA which is upregulated in many malignancies like lung, breast and colon. Study conducted by Hayashita et al showed that miR-17-92 cluster is overexpressed in small cell lung cancer.[25]

Study conducted by RAPONI et al. found 15 miRNAs that were differentially expressed between normal lung and squamous cell lung carcinomas. They included miRBNA 17/92 cluster. They found that as compared to m RNA based signature miRNA profiling has a better clinical utiility in predicting the prognosis of patients

with squamous cell lung carcinomas.[26] Study conducted by Monila et al. was the first study which demonstrated an overexpression of the miR-17-92 cluster in patients with lung adenocarcinoma.[27] In present study miRNA 17/92 cluster was upregulated in NSCLC patients as compared to healthy adults.

5. Conclusion

Liquid biopsy has emerged as a new diagnostic modality in lung cancer. MiRNA can help in early diagnosis of lung cancer. If performed on fluid it is less invasive and safer alternative to more traditional methods. A liquid biopsy has not replaced tissue based diagnosis till now, further research

is required in this aspect. Studied miRNA can be used as the potential biomarker for detection of non small cell lung cancer.

6. Limitation

Due to small sample size no statistically significant correlation could be found between the studied miRNA 's and clinical profile of the patients

7. Conflict of Interest

The authors do not have any conflicts of interest.

8. Source of Funding

None.

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