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Original article

Exosomal microRNA-214 expression and its prognostic significance in non-small cell lung cancer patients

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ABSTRACT

Altered miRNAs expression have been found to be linked with several solid tumors and expression levels detected in serum, plasma or other body fluids. Thus the current study aimed to explore the serum based exosomal microRNA-214 expression in NSCLC patients.

Present study recruited 100 NSCLC case and 100 healthy controls to examine the serum based exosomal miRNA-214 expression in NSCLC patients. Patients blood sample were collected in plain vials to separate serum for exosome isolation using precipitation buffer and then total RNA were extracted form exosomes. 100 ng of total extracted RNA were used to make the cDNA using microRNA specific kit. Synthesized cDNA were used to quantify the miRNA-214 expression using taqman probe for miRNA-214 and ΔΔ ct method was applied to determine the miRNA-214 fold change in expression. In patients, more than 17 fold increased miRNA-214 expression was observed compared to controls. Increased serum based exosomal miRNA-214 expression was observed in advanced TNM stage of patients (18.38), in the same way more than 19 fold increased serum based exosomal miRNA-214 expression was observed in distant organ metastatic patients. Patients who had pleural effusion showed more than 19 fold up-regulated in expression. Higher AUC was observed for early/advanced TNM patients (0.79), present/absent distant organ metastases (0.79) and present/absent pleural effusion (0.66) suggested that can be used for prognostic indicator. Patients with more than 17 fold increased miRNA-214 expression could be used as indicator for poor NSCLC patients' survival. Study concluded higher serum based miRNA-214 expression is associated with advancement of disease and it suggested that serum based miRNA-214 expression could be used as prognostic and poor survival indicator in NSCLC patients.

1. Introduction

Lung cancer is the most important reason for cancer associated mortality worldwide, Genetic and epigenetic smash up caused by tobacco smoke is the main etiology (Travis et al., 2004)). Systematic analysis of RNA and proteins expression in the molecular network of thousand genes has contributed in lung carcinogenesis. MicroRNAs are noncoding RNAs about 22 nucleotides long which showed numerous genetic processes. MicroRNAs can target the untranslated region of corresponding sequences and mediate to down-regulation of target mRNA to control protein synthesis (Pasquinelli, 2012), miRNAs have been associated to play a significant role in cause of cancer and tumor metastasis to distant organ. The function aspect of miRNAs is well-known in tumor growth (Nagadia et al., 2013), angiogenesis, cellular outburst, differentiation, migration and invasion to other organs (Mueller and Bosserhoff, 2009). Dysregulated miRNAs expressions were found to linked with different types of cancer and their functions was associated with specific tissues. The expression levels of miRNAs in different body fluids such as serum, plasma were found to be associated as important diagnostic/prognostic indicator for disease (Bovell et al., 2012). MicroRNA-214 (miR-214) stand within the *DNM3* gene located on human chromosome number arm q24.3 and is around 6 kilobase in size (Weber, 2005). Several evidences revealed that altered miR-214 regulation can be contributory factor for variety of several solid tumors such as hepatocellular carcinoma, breast cancer, osteosarcoma, gastric cancer, carcinoma of lung, pancreatic cancer, cervical cancer, cancer of ovary, melanoma, bladder cancer and prostate cancer. Micro RNA-214 showed

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mutual actions in diverse tumor tissue types that provide multifaceted function as tumor suppression and tumorigenesis in multiple solid tumors. Abnormal miR-214 expression levels in several tumors found to have influence on altered cellular propagation and control of cell cycle, however present data's are contentious. MicroRNA-214 was found to promote AKT signalling pathway, cellular explosion and survival in gastric tumors via PTEN signalling pathway as well as lactoferrin and Bim signalling pathway in nasopharyngeal carcinomas (Yang et al., 2013).

MicroRNA-214 has been associated to be important circulating miRNA, and its expression in serum have shown to be diagnostic importance. Serum miR-214 expression in 100 patients of breast cancer were enough to distinguish between advanced stage tumors and early stage tumors, as well as in healthy subjects, Significantly decreased expression was observed in post-operative sera but increased expression level were found to be association with distant organ metastatic and spread to local lymph nodes (Schwarzenbach et al., 2012). Similarly, it has been observed that circulating exosomal miRNA-214 were over-expressed in primary tumor mass isolated from ovarian carcinoma patients and the same observation was found in lung cancer patients. Thus the present study mainly focused on to explore the clinical significance of serum based exosomal microRNA-214 regulation profiles in Non Small Cell Lung Cancer (NSCLC).

2. Materials and methods

2.1. Blood sample collection and exosomes isolation from serum

This study recruited newly diagnosed histopathologically confirmed cases of NSCLC. Patients previously with any history of cancer or any other body organ metastasized cancer were excluded from study. 4 mililitter of patient's peripheral blood were collected in plain vials after confirmed diagnosis as well as 3 ml of peripheral blood from healthy individuals as controls. Blood samples collected in plain vials were centrifuged at 1500 rpm to separate the serum were collected and stored at −70 °C. Before exosomes isolation sample were thawed and centrifuged at 3000x g to for 5–10 min to pellet cells, debris and platelets. 1.5 ml of serum were mixed in 600 µl of precipitation buffer A (miRCURY Exosome Kits, Qiagen) and mixed for 10 s. Mixture was incubated for 60 min at 2–8 °C and centrifuges at 1500×*g* for 30 min at 20 °C. After centrifugation pellet were saved and resuspended in 240 µl of resuspension buffer to the tube containing pellet and further used for total RNA exosomal RNA extraction.

2.2. Total RNA extraction

Total RNA from exosomes suspended in resuspension buffer was isolated by using Trizol and stored at −70 °C in RNase-free eppendorf tubes. The quality and purity of RNA were determined by the A260/280 ratio.

2.3. Polyadenylation and cDNA synthesis

10 ng of total RNA was used for Polyadenylation and cDNA synthesis using Advanced microRNA cDNA Synthesis Kit (TaqMan, Thermo Scientific) by following manufacturer protocol. Reverse Transcriptase enzyme and other essential reagents were added subsequently for cDNA synthesis to switch in poly (A) - tailed miRNAs into cDNA using an universal RT primer supplied with the manufacturer kit.

2.4. qPCR for miRNA-214 expression

Quantitative real-time PCR (qPCR) was performed to compute the serum based exosomal microRNA-214 expression level. qPCR was performed in Quant Studio 6 using advanced taqman master mix (4444556), Taqman probes (478768_mir) for miRNA-214 for quantification and hsa-miR-16-5p (477860_mir) were used as internal control as normaliser to calculate the expression.

2.5. Statistical analysis

All the data analysis was done by using SPSS 20.0 and Graph Pad Prism 5 version of software. ΔΔ ct method was applied to compute the fold change in expression of miRNA-214 in NSCLC patients. Parametric (*t* test and ANOVA) and nonparametric (Mann Whitney *U* test and Kruskal Wallis) test were used to compare the different groups of variables. The Kaplan–Meier analysis was used to compute the overall survival of Non Small Cell Lung Cancer patients. ROC curve was plotted to check the prognostic importance of miR-214 in NSCLC patients and p value < 0.05 was considered to be statistically significant.

3. Results

3.1. Demographics

All demographic and clinical features of NSCLC patients and healthy control were depicted in Table 1. In brief total 100 NSCLC patients and 100 healthy control subjects were included in present

Table 1
Demographic and clinical characteristic of Non Small Cell Lung Cancer patients.

Variables	NSCLC cases Number (%)	Healthy controls
Total	100 (100%)	100 (100%)
Age		
≤50 years	37 (37%)	35 (45%)
greater than50 years	63 (63%)	65 (65%)
Gender		
Male	71 (71%)	70 (70%)
Female	29 (29%)	30 (30%)
TNM Stages		
Early Stage (I & II)	17(17%)	
Advanced Stage (III & IV)	83 (83%)	
Distant organ metastases		
Yes	50 (50%)	
No	50 (50%)	
Histological type		
Adenocarcinoma	42 (42%)	
Squamous cell carcinoma	58 (58%)	
Pathological grade		
Well differentiated	16 (16%)	
Moderately differentiated	34 (34%)	
Poorly differentiated	50 (50%)	
Smoking status		
Ex-Smoker	23 (23%)	
Current Smoker	45 (45%)	
Non Smoker	32 (32%)	
Mode of smoking		
Cigarette	35 (36%)	
Pipe	26 (26%)	
Cigarette + Pipe	7 (7%)	
Level of smoking (Pack year)		
Mild smokers (≤10)	22 (22%)	
Moderate smokers (≤40)	32 (32%)	
Heavy smokers (greater than40)	14 (14%)	
Pleural effusion		
Yes	24 (24%)	
No	76 (76%)	

study. Patients with ≤50 years were 37% and greater than 50 years of age were 63%, males were (71%) and females (29%). 17% patients were in early stage and 83% patients were in advanced stage, while 50% patients had distant organ metastases metastases. Patients with different histological type, adenocarcinoma patients were 42% and 58% were squamous cell carcinoma, however more details were depicted in Table 1.

3.2. Serum based exosomal miRNA-214 expression in NSCLC patients:

In NSCLC patients, overall more than 17 fold increase expression was observed in serum based exosomal miR-214. NSCLC patients in early stage showed more than 14 fold increased serum based exosomal miR-214 expression while advanced stage patients had more than 18 fold increased serum based exosomal miR-214 expression and differences was found to be significant ($p = 0.0002$). Patients who had distant organ metastases such as adrenal gland, skeletal, liver, lymph node etc had 19.88 fold while no distant organ metastases showed 15.56 fold increased serum based exosomal miR-214 expression and the differences was found to be significant ($p < 0.0001$). It has been observed NSCLC patients who showed pleural effusion, showed 19.35 fold while those who did not had any pleural effusion showed 17.15 fold increased serum based exosomal miR-214 expression and differences was found to be significant ($p = 0.01$) (Table 2).

3.3. Association of microRNA-214 expression with smoking parameters

No such association was observed in serum based exosomal miR-214 expression with any smoking parameters. However, patients who smoked pipe and cigarette together had increased miRNA-214 expression. Cigarette and pipe smoker had more than 17 and 16 fold increased serum based exosomal miRNA-214 expression while pipe and cigarette smoked together had more than 21 fold increased serum based exosomal miRNA-214 fold increased expression (Table 3).

3.4. Prognostic importance of microRNA-214 expression in NSCLC patients

To calculate the importance of serum based exosomal microRNA-214 as prognostic/predictive molecular marker for NSCLC patients, several clinical parameters such as TNM stage, distant organ metastases and lymph node involvement were divided into two groups and ROC curves were plotted. Patients with early vs advanced stage, patients with vs without distant organ metastases and lymph node involvement (Fig. 1a–c) (Table 4).

ROC curves plotted between early and advanced stages and at cut-off value of 14.90-fold increased in serum based exosomal microRNA-214 expression, sensitivity and specificity were 75 and 77%, respectively (AUC was 0.79, $p < 0.0001$). ROC curve w.r.t. distant organ metastases at cut-off value of 16.65-fold change serum based exosomal microRNA-214 expression, sensitivity and specificity were 81 and 71%, respectively (AUC was 0.79, $p < 0.0001$). ROC curve between without lymph node involvement and with lymph node involvement at cut-off value of 17.15-fold change serum based exosomal microRNA-214 expression, sensitivity and specificity were 79 and 60%, respectively (AUC was 0.66, $p = 0.01$).

Table 2
Association of miR-214 with different clinical features of NSCLC patients.

Clinical Feature	miR-214 expression (fold change in mean ± SD)	p value
Overall expression	17.68 ± 4.43	–
TNM stages		
Early stage (I&II)	14.23 ± 3.61	**0.0002**
Advanced stage (III&IV)	18.38 ± 4.26	
Histological type		
Adenocarcinoma	17.81 ± 4.32	0.65
Squamous cell carcinoma	17.57 ± 4.54	
Pathological grade		
Well differentiated	18.20 ± 5.76	0.74
Moderately differentiated	17.01 ± 3.65	
Poorly differentiated	17.96 ± 4.47	
Distant organ metastases		
Yes	19.88 ± 4.07	**<0.0001**
No	15.56 ± 3.68	
Pleural effusion		
Yes	19.35 ± 3.94	**0.01**
No	17.15 ± 4.46	

Table 3
Association of miR-214 with different smoking features of NSCLC patients.

Smoking parameters	miR-214 expression (In fold change)	p value
Smoking status		
Smokers	17.75 + 4.39	0.72
Non-smokers	17.50 + 4.59	
Current-smokers	17.55 + 4.31	0.79
Ex-smokers	18.09 + 4.61	
Mode of smoking		
Cigarette	17.76 ± 3.52	0.19
Pipe	16.79 ± 4.52	
Pipe + cigarette	21.09 ± 6.51	
Pack years		
Mild (≤10)	17.52 ± 4.26	0.65
Moderate (≤40)	17.34 ± 3.70	
Heavy (greater than40)	18.96 ± 5.94	

3.5. Serum based exosomal miRNA-214 expression and OS (overall survival) of NSCLC patients

OS of NSCLC patients were calculated and found to be statistically significant variation with respect to ≤ 17-fold miRNA-214 expression and greater than 17-fold increased serum based exosomal miRNA- 214 expression (Fig. 2). Patients with ≤17-fold microRNA-214 expression showed 21.2 months of median OS time while greater than 17-fold serum based exosomal microRNA-214 expression showed 15.4 months of median OS time and the difference was found to be statistically significant ($p = 0.005$). It was observed that patients in early stage (I & II) had long median OS time (32.9 months) in contrast to advanced stage patients (III & IV) (23.6 months) and the difference was observed to be statistically significant ($p < 0.0001$) (Fig. 2).

4. Discussion

MicroRNAs have been demonstrated diverse levels of expression pattern and its prognostic importance across different ethnic groups. Several studies reported that miR-214 targets multiple genes, which involved in regulation numerous biological processes, such as cellular differentiation, tumor progress and tumor angiogenesis (Shrestha et al., 2014). Dysregulation of miRNA-214 was observed in different types of solid tumors (Chen et al., 2015) and it has been indicated that altered expression of miRNA-214 could participate to develop tumors. Exploration of altered miRNAs in different cancer types and its stages could give novel insight into the potency of miRNA- 214 as a prognostic and diagnostic molecular marker. (De Guire et al., 2013).

In answer to examine the serum based exosomal miRNA-214 as prognostic marker, survival and other clinical significance as

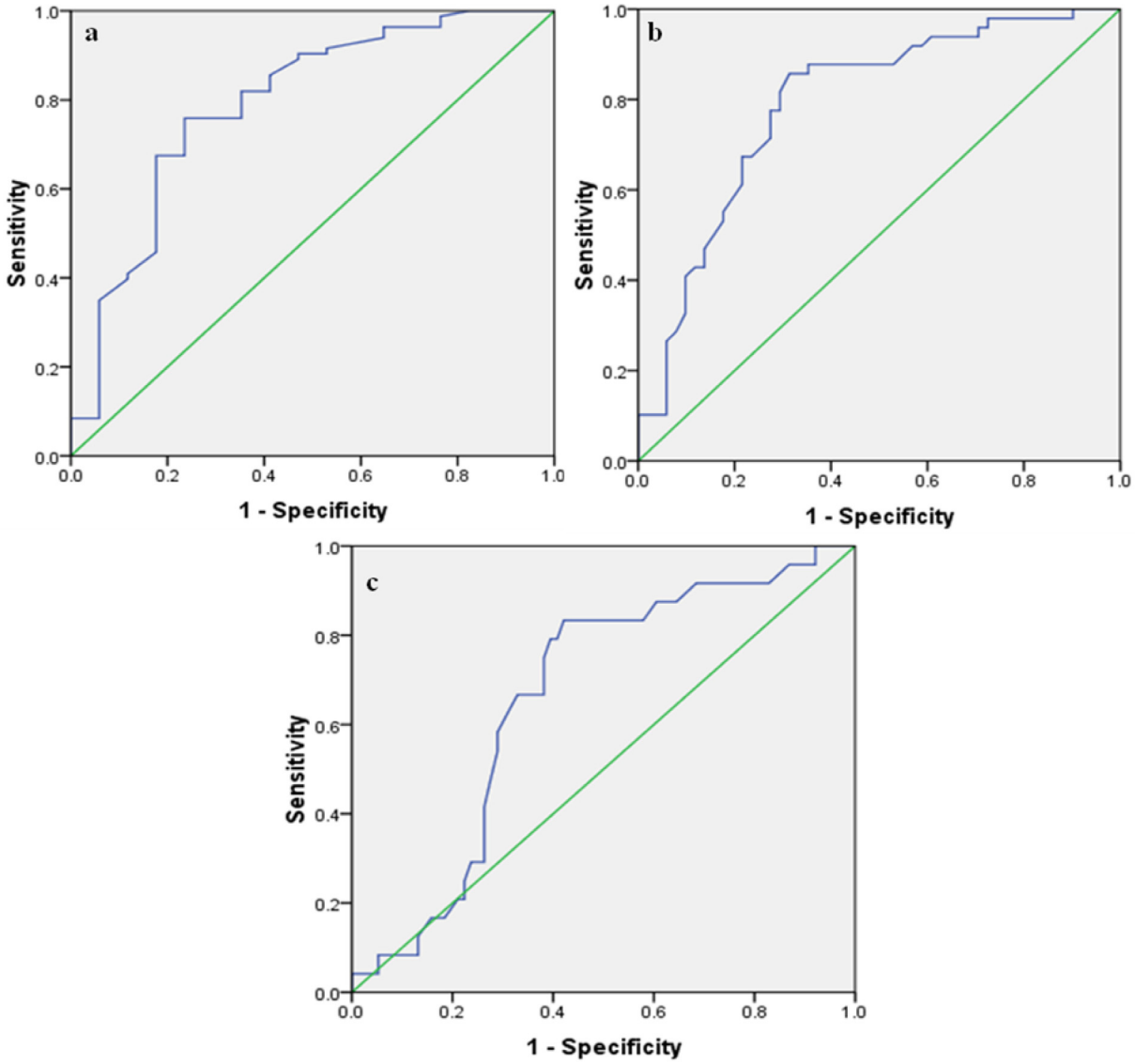

Fig. 1. Serum based exosomal miRNA-214 expression and ROC curve w.r.t. different variables of NSCLC (a) ROC curve between early and advanced stage (b) ROC curve between without and with distant organ metastases (c) ROC curve between without and with pleural effusion.

Table 4
Serum based exosomal miRNA-214 expression and ROC curve between different variables in NSCLC patients.

AUC (CI) (Early stage vs Advanced stage)	**Sensitivity**	**Specificity**	**Cut off value**	**p value**
0.79 (0.66–0.92)	75%	77%	14.90 fold	<0.0001
AUC (CI) (With Distant organ and without Distant organ metastases)	**Sensitivity**	**Specificity**	**Cut off value**	**p value**
0.79 (0.77–0.88)	81%	71%	16.65 fold	<0.0001
AUC (CI) (With lymph node and without lymph node involvement)	**Sensitivity**	**Specificity**	**Cut off value**	**p value**
0.66 (0.54–0.77)	79%	60%	17.15 fold	0.01

molecular marker in NSCLC, we conducted this study to identify potential role in NSCLC patients. We found up-regulation of serum based exosomal microRNA-214 expression in NSCLC patients compared to control. It has been found that patients who were in advanced stage of disease had more than 4 fold higher serum based exosomal microRNA-214 expression compared to early TNM stage of NSCLC patients. It has been observed that patients who had distant organ metastases showed 4 fold higher serum based exosomal microRNA-214 expression in NSCLC patients compared to patients without distance organ metastases. It was observed that patients who had pleural effusion showed 2 fold higher serum based exosomal microRNA-214 expression compared to patients who did not showed any pleural effusion. It has been found that miRNA-214 were highly expressed and linked with worse prognosis and increases the risk for metastatic in ocular melanomas. miRNA-214 up-regulation was associated with bad prognosis, poor OS and disease invasiveness, metastatic behaviour and poor therapy response (Yang et al., 2013). High expression of microRNA- 214 in ovarian carcinoma was significantly related with high grade tumor and metastatic behaviour of tumor, as well as with poor OS (Yang et al., 2008). Interestingly, miRNA-214 was reported to be cell free miRNA and its high levels shown to have diagnostic

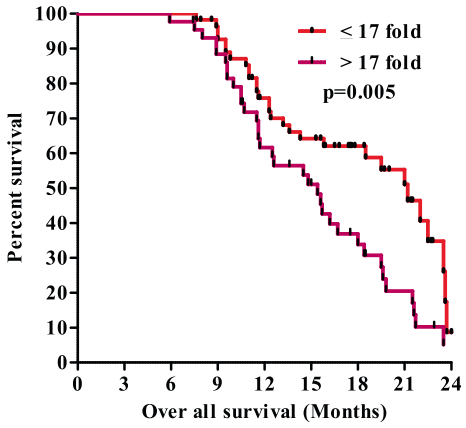

Fig. 2. Kaplan–Meier curve for OS: miR-214 ≤ 17 and greater than 17 fold increased serum based exosomal microRNA.

value. In fact, miR-214 expression levels were able to discriminate the malignant tumor and benign tumor and decreased level were observed in post operative patients, however increase miRNA-214 was found in metastatic cancer of breast (Schwarzenbach et al., 2012). MicroRNA-214 expression in blood sample was capable to predict the patients carrying malignant tumor of peripheral nerve sheath. Similarly, A study by Taylor DD et al in 2008 found that circulating exosomes miRNA-214 was found to be over-expressed in primary tumor mass in ovarian carcinoma patients (Taylor and Gercel-Taylor, 2008) as well as same results was observed in lung cancer patients (Rabinowits et al., 2009). Furthermore, miRNA-214 demonstrated to be a prognostic / diagnostic molecular biomarker for prostate cancer and bladder cancer (Kim et al., 2013). These explanations emphasize that cell free miRNA-214 could be used as a diagnostic / prognostic indicator in cancer.

In the same way for miRNa-214, higher AUC was observed for early/advanced TNM stage (0.79) and with present/absent distant organ metastases (0.79) after ROC curve analysis. At cut-off value of 14.90 fold serum based exosomal miRNA-214 expression for early/advanced TNM stage, sensitivity and specificity was 75% and 77%. While 16.65 fold serum based exosomal miRNA-214 expression, sensitivity and specificity was 81% and 71% could be used as molecular prognostic marker for NSCLC patients. It was observed that higher serum based exosomal miRNA-214 expression was linked with reduced OS of NSCLC patients.

MiRNA-214 has been found to be significantly associated with osteosarcoma invasion by transcriptional silencing of LZTS1 gene. MiRNA-214 shown to involved in metastatic properties of mammary tumor cells (Penna et al., 2011), In particular, cisplatin-induced apoptosis in human ovarian cancers were linked with miRNA-214 over-expression by targeting PTEN mRNA silencing for AKT pathway activation for cell survival. Moreover, miRNA-214 was verified to be associated with cisplatin resistance in squamous cell carcinomas of tongue in human (Yu et al., 2010). Increased miRNA-214 expression was found to be associated with gefitinib-resistant in NSCLC. It has been demonstrated that miRNA-214 knockdown is sufficient to normalize AKT signalling pathway to resensitize cells to different therapies. Furthermore, miRNA-214 over-expression was found to be associated with gemcitabine resistance or reduced sensitivity in pancreatic cancer, probably by targeting ING4 gene which involved in cell death, repair of DNA and cell cycle arrest (Zhang et al., 2010).

5. Conclusion

Current study concludes that serum based exosomal microRNA-214 was significantly expressed in NSCLC patients w.r.t. advanced stage, distant organ metastases and pleural effusion positive patients. ROC curve and survival analysis suggested that serum based exosomal microRNA-214 could be used as prognostic indicator and poor survival indicator for NSCLC patients.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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