Detection of HER2 polymorphism and expression using circulating DNA and RNA as a tool in lung adenocarcinoma patients: a case control study

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Background: Circulating DNA and RNA is an important prognostic tool for noninvasive malignant disease detection and in disease prognosis. Study aimed to evaluate the possible prognostic role of HER2 (-3444C/T) promoter polymorphism and its mRNA expression in Lung adenocarcinoma patients using circulating DNA and RNA.

Methods: One hundred newly diagnosed lung adenocarcinoma patients and 100 age and sex matched healthy controls were included and allele specific (AS) polymerase chain reaction (PCR) was used for genotyping and expression was analyzed by quantitative real time PCR. Overall survival of patients was analyzed by Kaplan-Meier method.

Results: We observed a statistically significant difference in the frequency of HER2 CC, CT, and CT genotype among lung adenocarcinoma cases *vs.* healthy controls (P=0.001). Compared to the CC genotype, OR 2.51 (1.4–4.51), 5.97 (1.17–30.41) and RR 1.56 (1.17–2.07), 2.83 (0.82–9.73) for heterozygous CT and homozygous TT genotypes suggesting possible dominant effect on risk of lung adenocarcinoma. Cases with CC genotype showed 9.29 fold increased mRNA expression while cases with heterozygous CT and homozygous TT genotype showed 16.26, 16.72 fold increased mRNA expression (P<0.0001). We observed 13.92 fold increased HER2mRNA expression Lung adenocarcinoma patients. Patients in different TNM stages showed significant difference in HER2 mRNA expression which was found to be significantly associated (P<0.0001). Patients with distant metastases and without distant metastases had 17.44 and 11.16 fold increased HER2 mRNA expression was also found to be significantly associated (P<0.0001). It was also observed that patients with pleural effusion and without pleural effusion showed significant difference in HER2 mRNA expression (P=0.03). We also analysed patients with CC, TT, CT (P=0.02) and CT + TT (P=0.008) genotype showed 15.8, 7.9, 9.5 and 7.9 months of overall median survival time and found to be significantly associated, respectively. Patients with >13 and ≤13 fold increased HER mRNA expression also showed 7.9 and 11.5 months of overall median survival time was also found to be significantly associated (P=0.01).

Conclusions: Our work provides evidence that circulating DNA and RNA may be a potential prognostic tool in Lung adenocarcinoma patients. Promoter polymorphism of HER2 (-3444C/T) gene had significant impact on higher HER2 mRNA expression could be a predictive factor for patients' worse overall survival and metastatic behaviour.

Keywords: Lung adenocarcinoma; HER2 (-3444C/T) genotype; circulating HER2 mRNA; overall survival

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Introduction

Globally, every year million patients are diagnosed with lung cancer, making it most common type of cancer worldwide (1). Non-small cell lung cancer (NSCLC) accounts nearly 80% of all lung cancers and became the major cause of mortality (2,3). Patients detected at late stage of disease limits the treatment option for NSCLC patients and most of the cancer patients diagnosed at the metastatic stage (2). HER2 aberrations are more prevalent in adenocarcinoma patients (4) and it was observed that HER2 is associated with tumorigenesis, metastases of disease and worse clinical prognosis in patients. Ligand binding causes receptor dimerization and passes signal by auto-phosphorylation of HER2 tyrosine kinase domain and activates the target proteins, such as mTOR, Src, STAT, MAPK (5). *HER2* gene amplification and overexpression occurs in lung cancer and contributed to worse prognosis (6-8). Functional implications of HER2 polymorphisms might result in increased autophosphorylation and tyrosine kinase activity (9,10), single nucleotide polymorphisms (SNPs) are the most common genetic variation, and that may contribute to an individual's cancer susceptibility (11,12). It was analysed that HER2 is highly expressed in several solid tumours and increased *HER2* gene expression has been associated with poor prognosis of patients (13,14). *HER2* gene activation, overexpression and its potential prognostic relevance in NSCLC is still under evaluation (15,16). The over-expression of HER2 has found to be associated with disease aggressiveness, increased mortality and higher relapse ratio (17,18), and over-expression of HER2 also an indicative of increased metastatic behaviour cancer cells (7). Main features of cancer cells are the apoptosis resistance (19) and HER2 over-expression defines apoptosis suppression in breast cancer patients (20). Apoptosis suppression, over-expression of HER2 has been linked to disrupt both, intrinsic and extrinsic apoptotic pathways and it is also required to maintain HER2 expression for HER2 mediated suppression of apoptosis (21). The potential clinical relevance of *HER2* gene expression in NSCLC is still under evaluation (16), however, the current role of *HER2* gene amplification in the resistance to tyrosine kinase inhibitor is reported in 12–13% of patients (22). Thus the present study aimed to analyze the clinical usefulness of circulating DNA and RNA in detection of HER2 promoter polymorphism and HER2 mRNA expression in Lung adenocarcinoma patients is first study from India in which *HER2* (-3444C/T) gene promoter polymorphism were analysed with its expression.

Methods

Study population and sample collection

This study was conducted in a cohort of 100 histopathologically confirm newly diagnosed lung adenocarcinoma patients and 100 age and sex matched healthy controls. Study was approved by institutional ethics committee of Maulana Azad Medical College & Associated Hospitals and All India Institute of Medical Sciences New Delhi. After informed consent, patients' 3 mL blood sample was collected from each subject before any treatment and serum was separated and stored at –80 ℃ until circulating DNA, RNA extraction from cases as well as from healthy controls, patients and healthy controls serum sample processed and stored in similar manner. Patients included in study were followed from the 2013 to 2015 for overall survival analysis.

Circulating DNA, RNA isolation and cDNA synthesis

Circulating DNA was extracted using commercially available kit (Epigentech, USA) following manufacturer's protocol and circulating RNA was extracted by Trizol reagent according to the manufacturer's protocol (AMRESCO, USA) of lung adenocarcinoma cases as well as from healthy control serum stored at –80 ℃. Quality of DNA and RNA was checked by Nanodrop and cDNA was synthesized by using 100 ng total RNA following manufacturers protocol (Verso, Thermo Scientific, USA). Briefly, 100 ng of total RNA, 5X cDNA synthesis buffer, dNTPs (5 mM each), RT enhancer, Verso enzyme mix and random hexamers (400 ng/μL) in the total volume of 20 μL incubated for 60 min at 42 ℃.

Genotyping and quantitative real time polymerase chain reaction (PCR)

The -3444C/T (rs2643194) promoter polymorphism of *HER2* gene was genotyped using allele specific (AS) PCR using circulating DNA with forward wild type primer 5'-ATGGC GTCCACAGTAGCTTTC-3'; forward mutant type primer 5'-ATGGCGTCCA CAGTAGCTTTT-3'; and common reverse primer 5'-CTTAGAGGCCATCGGGATGTTA-3'. PCR was performed in 25 μL reaction volume containing 3 μL

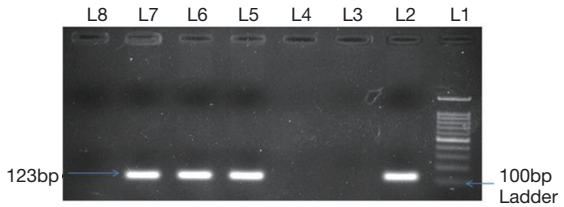

Figure 1 Agarose gel electrophoresis image of *HER2* (-3444C/T) gene polymorphism. AS-PCR HER2 (-3444C/T): L1—100 bp ladder; L2, L3—homozygous CC (123bp); L4, L5—homozygous TT (123 bp); L6, L7—heterozygous CC (123 bp); L8—NTC (non template control).

of 100 ng template circulating DNA, 0.25 µL 25 pmol each primer, 10 µL of mastermix containing 10 mM dNTPs, 20 mM $MgCl_2$, 5 U/µL Taq polymerase with 10× Taq Buffer (Fermantas) and 25 µL reaction volume was maintained by adding nuclease-free ddH_2O followed by programme 10 min of initial denaturation at 95 ℃ and 40 cycles at 95 ℃ for 40 s, 60 ℃ for 40 s and 72 ℃ for 40 s with a final 10 min extension step at 72 ℃ and PCR product of 123 bp was visualized on 2% agarose gel containing ethidium bromide (*Figure 1*). HER2 mRNA expression was studied by QRT-PCR (SYBR Green I technology) with β-actin gene as internal control. The primer sequences for HER2 mRNA expression were forward primer 5'-AGTACCTGGGTCTGGACG TG-3', reverse primer 5'-CTGGGAACTCAAGCAGG AAG-3' (23), for β-actin were forward primer 5'-CGACAACGGCTCCGGCATGTGC-3', reverse primer 5'-GTCACCGGAGTCCATCACGATGC-3'. The expression of HER2 and β-actin was performed by PCR programme for 40 cycles, denaturation at 94 ℃ for 40 s, annealing at 60 ℃ for 40 s, extension at 72 ℃ for 40 s and reaction volume was 20 µL. A final extension step at 72 ℃ for 5 min to complete the reaction and melting curve analysis was performed between the range 40 to 90 ℃ to ensure the specific amplification. A control without cDNA was included in each experiment as non template control and all reaction were performed in duplicate. The relative quantification method ($2^{-\Delta\Delta CT}$) was used to analyse the circulating HER2 mRNA expression level by using β-actin as internal control and final results were expressed as mean fold change in circulating HER2 mRNA expression in lung adenocarcinoma patients as compared to control.

Statistical analysis

Differences in select demographic variables and HER2 genotype frequencies between the cases and controls were evaluated by using the Chi-square test. The associations between HER2 variant genotypes and risk of lung adenocarcinoma were estimated by computing the odds ratios (ORs) and risk ratio (RR) with 95% confidence intervals (CIs). Allele frequencies between the cases and controls were evaluated using Hardy-Weinberg equilibrium test. Mann Whitney and Kruskal Wallis test were used to analyze the association of gene expression with different variables included in study. The Kaplan-Meier method was used to calculate the survival of lung adenocarcinoma patients. A P value <0.05 was considered indicative of a statistically significant difference. All statistical analyses were performed using the SPSS 16 and Graph Pad version 6.0.

Results

Demographics

All demographic features of the subjects are depicted (*Table 1*). In brief, total of 100 lung adenocarcinoma patients were analyzed and healthy controls were age, sex and history of smoking status and type was also matched. This study included both males (71%) and females (29%) and mean age of 54.37 years. A total of 44% patients were in stage IV and 15%, 15%, 26% patients in stage I, II and III respectively while 44% patients had distant metastases. Patients with different pathological grade, grade 1 (well differentiated) includes 24%, grade 2 (moderately differentiated) includes 41% and grade 3 (poorly differentiated) includes 35% cases. We included smoker 45% as well as non smoker 55% with different smoking type as cigarette, bidi, and hukka, 18% cases smoked cigarette, 16% cases smoked bidi and 11% cases smoked hukka.

Case-control genotype distribution

The genotype and allele distribution of HER2 (-3444C/T) in cases and controls are summarised in *Table 2*.

We observed a statistically significant difference in the frequency of HER2 CC, CT, and CT genotype among lung adenocarcinoma cases *vs.* healthy controls (P=0.001). The frequency of T allele (fT) was found to be higher in lung adenocarcinoma cases (0.33) whereas, the higher frequency of C allele (fC) was observed among healthy controls (0.78).

Table 1 Demographic characteristic of lung adenocarcinoma patients

Variables	Lung adenocarcinoma patients (%)	Healthy controls (%)
Total No. of cases	100	100
Gender		
Males	71	71
Females	29	29
Age (years)		
≤55	56	56
>55	44	44
Mean ± SD age (years)	54.5±10.77 (range, 32–75)	55±10.82 (range, 30–70)
Smoking status		
Non smoker	55	55
Smoker	45	45
Current smoker	24	24
Ex. smoker	21	21
Smoking type		
Cigarette	18	18
Bidi	16	16
Hukka	11	11
Smoking level (pack year)		
Mild (<10)	23	
Moderate (≤40)	18	
Heavy (>40)	4	
TNM stage		
Stage I	15	
Stage II	15	
Stage III	26	
Stage IV	44	
Distant metastases		
Positive	44	
Negative	56	
Histopathological grade		
Grade 1	24	
Grade 2	41	
Grade 3	35	
Pleural effusion		
Yes	15	
No	85	

Table 2 Genotype distribution and allele frequencies of HER2 (-3444C/T) among lung adenocarcinoma patients and controls

Variables	CC	CT	TT	C allele frequency	T allele frequency	P value
Patients (n=100)	34 (34%)	59 (59%)	7 (7%)	0.63	0.37	0.001
Controls (n=100)	58 (58%)	40 (40%)	2 (2%)	0.78	0.22	

HER2 (-3444C/T) polymorphism and lung adenocarcinoma risk

The degree of association between the HER2 (-3444C/T) genotype and risk of lung adenocarcinoma was estimated by calculating OR, RR with 95% CIs and hazard ratio with different stages was also depicted in *Tables 3*,*4* respectively. Compared to the CC genotype, OR 2.51 (1.4–4.51), 5.97 (1.17–30.41) and RR 1.56 (1.17–2.07), 2.83 (0.82–9.73) for heterozygous CT and homozygous TT genotypes were estimated, suggesting possible dominant effect on lung adenocarcinoma risk in Indian population.

HER2 (-3444C/T) genotypes and HER2 expression

HER2 genotype was found to be significantly associated with expression of HER2 mRNA depicted in *Table 5*.

Lung adenocarcinoma cases with CC genotype showed 9.29 fold increased mRNA expression while cases with heterozygous CT and homozygous TT genotype showed 16.26, 16.72 fold increased mRNA expression (P<0.0001).

HER2 mRNA expression and lung adenocarcinoma patients

We analysed circulating HER2 mRNA expression with several variables of lung adenocarcinoma cases in this study and observed increased gene expression was 13.92 fold in NSCLC (adenocarcinoma) patients. Patients in stage I showed 7.3 fold increased HER2 mRNA expression, stage II showed 7.62 fold increased gene expression while in stage III showed 15.42 fold increased HER2 mRNA expression and stage IV showed 17.44 fold increased gene expression which is found to be significantly associated (P<0.0001). Patients with distant metastases had 17.44 fold increased HER2 mRNA expression while patients without metastases

Table 3 Risk of lung adenocarcinoma associated with HER2 (-3444C/T) genotype

Genotype	Healthy controls (n=100)	Lung adenocarcinoma patients (n=100)	OR (95% CI)	RR (95% CI)	P value
CC (ref)	58 (58%)	34 (34%)	1	1	–
CT	40 (40%)	59 (59%)	2.51 (1.4–4.51)	1.56 (1.17–2.07)	0.001
TT	2 (2%)	7 (7%)	5.97 (1.17–30.41)	2.83 (0.82–9.73)	0.02
CT + TT	42 (42%)	66 (66%)	2.68 (1.51–4.75)	1.62 (1.22–2.15)	0.0007

OR, odd ratio; RR, risk ratio; CI, confidence interval.

Table 4 Hazard ratio with respect to different stages

TNM stages	Hazard ratio (95% CI)	P value
Stage I	Reference	–
Stage II	0.07 (0.01–0.33)	0.0006
Stage III	0.10 (0.04–0.24)	<0.0001
Stage IV	0.15 (0.08–0.30)	<0.0001

CI, confidence interval.

Table 5 Serum HER2 mRNA expression of patients with different genotypes

Genotype	Lung adenocarcinoma patients (n=100)	Mean ± SD	Range	P value
CC	34 (34%)	9.29±8.11	2–32.67	<0.0001
CT	59 (59%)	16.26±8.83	2.27–43.11	
TT	7 (7%)	16.72±8.32	3.10–30.18	

had 11.16 fold increased HER2 mRNA expression also showed significant differences (P<0.0001). Patients who had pleural effusion showed 18.46 fold increased HER2 mRNA expression while patients without pleural effusion showed 13.12 fold increased HER2 mRNA expression was also found to be significantly associated (P=0.03) data showed in *Table 6*.

Patient's overall survival with respect to HER2 (-3444C/T) genotype and HER2 expression

Survival analysis of 100 lung adenocarcinoma patients included in study was analysed by Kaplan-Meier method, and it was observed that the mean follow-up time of the patients was 8.79 months (median, 8.1 months). There were 70 (70%) lung adenocarcinoma-related death events in the patients with a mean follow-up time of 6.6 months (median, 5.3 months), and for the 30 (30%) patients who survived, the mean follow-up time was approximately 13.88 months (median, 14.15 months). Patients with CC, TT, CT (P=0.02) and CT + TT (P=0.008) genotype showed 15.8, 7.9, 9.5 and 7.9 months of overall median survival time and found to be significantly associated, respectively (*Figure 2A*,*B*). Patients with >13- and ≤13-fold increased HER mRNA expression showed 7.9 and 11.5 months of overall median survival time was observed and found to be significantly associated (P=0.01) (*Figure 2C*). It was also observed that patients with >13 fold increased mRNA expression showed 6 months of progression free survival while patients with <13 fold increased showed 12.62 months (*Figure 2D*). Patients in stage III (5.1 months) and IV (5.6 months) showed reduced overall survival while stage II and I showed 12 and 14.88 months of overall survival respectively (*Figure 2E*).

Discussion

Single nucleotide alterations are the most common form of genetic variation in human genome, and may contribute individual's cancer susceptibility (11,12). Several studies demonstrated that polymorphisms in gene alters the gene expression are associated with the risk of lung cancer (24-26). HER2 is a oncogene belonging to the EGFR family of genes, play major role in proliferation, growth, cellular differentiation and apoptosis (27,28) controlled by any of the ErbB-receptor family genes (7). It was found that HER2 overexpression has been associated with disease aggressiveness, increased mortality and higher relapse ratio (17,18). Present study investigated the role of circulating DNA and RNA to evaluate the *HER2* gene promoter polymorphism and HER2 mRNA expression in Lung adenocarcinoma patients and statistically significant difference was observed in genotype of HER2 (-3444C/

Table 6 Serum HER2 mRNA expression of patients with different variables

Variables	Mean ± SD	Range	P value
NSCLC patients	13.92±9.11	2.00–43.11	–
Gender			0.73
Males	13.85±9.70	2.00–43.11	
Females	14.11±7.64	2.19–29.24	
Age (years)			0.74
≤55	13.87±8.62	2.19–34.54	
>55	13.99±9.80	2.00–43.11	
TNM stage			<0.0001
Stage I	7.30±6.50	2.11–18.13	
Stage II	7.62±6.30	2.00–22.79	
Stage III	15.42±10.91	2.27–43.11	
Stage IV	17.44±7.22	2.41–32.67	
Distant metastases			<0.0001
Positive	17.44±7.22	2.41–32.67	
Negative	11.16±9.54	2.00–43.11	
Histopathological grade			0.22
Grade 1	14.92±9.11	2.22–34.54	
Grade 2	12.35±9.64	2.11–43.11	
Grade 3	15.15±8.10	2.00–27.47	
Pleural effusion			0.03
Yes	18.46±7.30	11.08–32.67	
No	13.12±9.20	2.00–43.11	
Smoking status			0.56
Non smoker	14.58±9.67	2.11–43.11	
Smoker	13.12±8.41	2.00–32.50	
Smoking status			0.77
Non smoker	14.58±9.67	2.11–43.11	
Current smoker	12.74±8.45	2.00–32.45	
Ex. smoker	13.55±8.55	2.27–32.50	
Smoking type			0.11
Cigarette	15.14±9.00	2.27–32.50	
Bidi	13.97±8.15	3.94–30.18	
Hukka	8.57±6.45	2.00–18.13	
Smoking level (pack year)			0.54
Mild (<10)	12.81±7.98	2.41–32.45	
Moderate (≤40)	14.42±9.15	2.00–32.50	
Heavy (>40)	9.00±7.89	2.36–18.13	

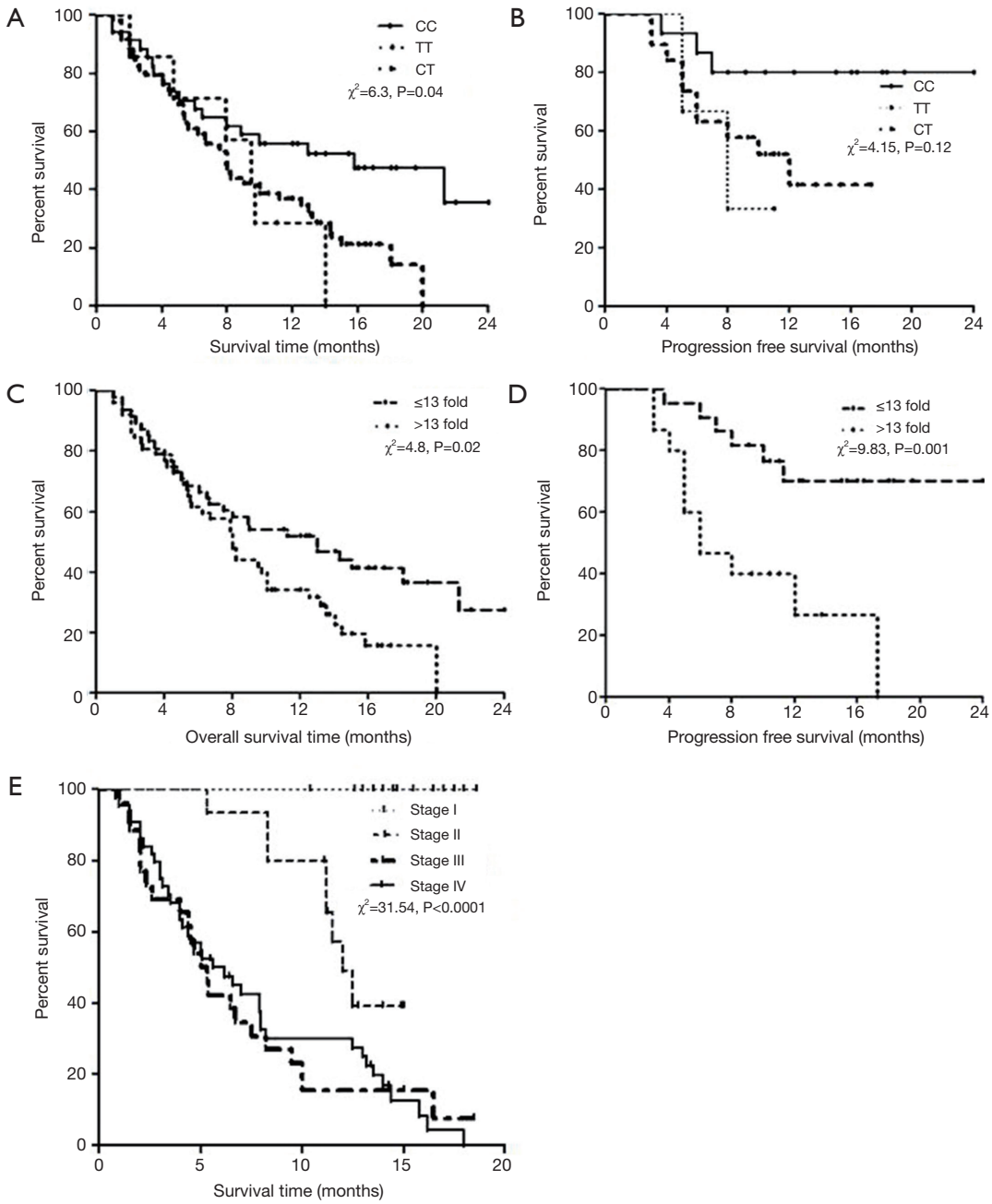

Figure 2 Kaplan-Meier survival curves with respect to HER2 (-3444C/T) genotype. (A) Overall survival with respect to CC, CT and TT genotype; (B) progression free survival with respect to CC, CT and TT genotype; (C) overall survival with respect serum HER2 mRNA expression; (D) progression free survival with respect serum HER2 mRNA expression; (E) overall survival with respect to TNM stage of NSCLC patients.

T) in lung adenocarcinoma cases *vs.* healthy controls. It was observed that heterozygous HER2-3444CT and homozygous TT genotype had more than 2 and 5 fold increased risk of developing lung adenocarcinoma than CC genotype respectively and more death events was observed with homozygous TT and heterozygous CT genotype compare to homozygous CC genotype. To best of our knowledge, this is the first report in genetic association of HER2 (-3444C/T) polymorphism with the lung adenocarcinoma risk and prognosis, suggesting possible role of *HER2* gene in the pathogenesis of malignancy. Jo *et al.* in 2008 (29) found that the HER2 (-3444C/T) polymorphism is associated with the risk of lung cancer in Korean population. Han *et al.* in 2005 (30) also investigated that the 5'-untranslated *HER2* (rs2643195-3444C/T) gene polymorphism was found to be associated with HER2 protein expression and disease outcome in breast cancer and has functional impact on cancer aggressiveness. On the other hand, it was also found that HER2 overexpression is an independent prognostic factor in patients survival outcome and became important target in lung cancer, using an EGFR tyrosine kinase inhibitor along with HER2 dimerization inhibitors (7,8,31). This study also explored the circulating HER2 mRNA expression in lung adenocarcinoma patients and it was found to be significantly associated with TNM stages, metastatic behaviour, pleural effusion and overall survival. The major findings in the study was higher circulating HER2 mRNA gene expression observed in patients with stage III and stage IV in comparison of stage I and stage II and found to be significantly associated. Patients with distant metastases showed 1.56 times higher circulating HER2 mRNA expression in contrast of patients without any distant metastases. It was also observed that the lung adenocarcinoma patients had 1.4 times higher HER2 mRNA expression in patients with pleural effusion *vs.* patients without pleural effusion. HER2 mRNA expression had significant impact on patients' overall survival, patients with >13 fold increased gene expression was found to be significantly associated with reduced lung adenocarcinoma patients overall survival. In favour of present statement, Schneider *et al.* found 4–32-fold higher *HER2* gene expression in NSCLC cell lines while 8–32 fold higher *HER2* gene expression levels were observed in adenocarcinomas cell lines (A549, H322, H522, and H596) (32). Prognostic relevance of *HER2* gene overexpression found to be associated with poor patients' survival in NSCLC patients (33) and increased HER2 expression levels also have been reported in a number of malignant tumor types, and its gene amplification induces gene overexpression (34). *HER2* gene overexpression regulates invasive growth of cancer in association with the cadherin-catenin complex (35) and found to be associated with increased migration capacity of tumor cells (36). HER2 overexpression has been reported in NSCLC and particularly in adenocarcinoma patients (37) and overexpression is associated with poor clinical prognosis and worse patients' survival (38). It has been also suggested that HER2 overexpression is associated with metastatses behaviour of cells and poor prognosis of patients (39). Alterations of the HER2 proto-oncogene have been defined in the carcinogenesis and prognosis of many cancers, mainly breast cancer and HER2 overexpression leads to apoptosis suppression in breast cancer cells (20,40). The overexpression of HER2 has been found to be associated with disease aggressiveness, increased mortality and higher relapse ratio (17). High expression of HER2 had been shown to be associated with poor prognosis in breast cancer (41) endometrial cancer (42) and ovarian cancer (43).

Conclusions

In conclusion, our work provides evidence that circulating DNA and RNA may be a potential prognostic tool in management and monitoring of Lung adenocarcinoma patients. Promoter polymorphism of *HER2* (-3444C/T) gene had significant impact on higher HER2 mRNA expression could be a predictive factor for patients' worse overall survival, metastatic behaviour of lung adenocarcinoma patients. Understanding the molecular biology of *HER2* gene will help in progress of molecular-based treatment of cancer patients having HER2 overexpression. The findings of present study need to be validated further independent and prospective studies on larger population and further investigations would be necessary to clarify the efficacy of HER2 as molecular prognostic tool in lung adenocarcinoma patients.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest

to declare.

Ethical Statement: The study was approved by institutional ethics committee of Maulana Azad Medical College & Associated Hospitals and All India Institute of Medical Sciences New Delhi and written informed consent was obtained from all patients.

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