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RESEARCH ARTICLE

Her-2/neu mRNA Expression by quantitative reverse transcriptase realtime PCR (qRT-PCR) on samples of bronchial wash is a biomarker for early diagnosis of non-Small Cell Lung Cancer

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Abstract

Even though lung cancer is the leading cause of cancer related death worldwide, there is no effective method for early detection and screening of the high risk groups. The diagnosis in more than 75% of cases is usually achieved after the appearance of symptoms which are late in the course of the disease. The currently used methods of the annual chest X-rays or sputum cytology are not sensitive screening tests. The new advances in the molecular genetics studies have opened new ways for implementation of biomarkers in the diagnosis, therapeutics and prognosis. Human Epidermal Growth Factor Receptor-2 gene (Her-2/neu gene) amplification and/or overexpression is seen in non-small cell lung cancer (NSCLC) and cancer of other organs. In this study, twenty-four samples of bronchial wash positive for NSCLC by cytopathology and thirty samples negative (control) were studied for expression of messenger RNA (mRNA) of Her-2/neu gene by quantitative reverse transcriptase realtime polymerase chain reaction (qRT-realtime PCR). Her-2/neu mRNA was expressed in all samples of bronchial wash (positive and control), and relative quantification of mRNA of Her-2/neu gene showed a significant increase of expression in bronchial wash samples positive for NSCLC compared to control (negative) samples after normalization with GAPDH as a housekeeping gene. In conclusion, Her-2/neu gene could be used as a biomarker for the early detection of NSCLC in bronchial wash in addition to its therapeutic significance.

1. INTRODUCTION

Lung cancer is one of the leading causes of death from cancer, if not the first among various cancers, all over the world [1,2,3,4,5,6,7,8]. There is increase in the incidence of lung cancer starting from the fifties of the last century as a result of widespread tobacco smoking [9,10,11,12]. There are two types of lung cancer; the non-small cell (NSCLC) and the small cell lung cancer (SCLC) [5,9,10]. NSCLC is the commonest type of lung cancer worldwide, including Iraq, comprising about 85% of all lung cancer types [3,4,5,7,8]. NSCLC consists of three major histological types: squamous cell carcinoma (SqCC), adenocarcinoma (AC), and large cell undifferentiated carcinoma (LCC). The disease is frequently diagnosed at advanced and terminal inoperable stage in more than 75 % of cases [9,10]. Finding of NSCLC at earlier stage reduces the mortality and improves the outcome [9,10]. Nowadays, the diagnosis of lung cancer depends mostly on the development of symptoms, which are usually late in the course of disease, especially worrying symptoms such as repeated cough with or without hemoptysis and/or chest pain [9,10]. Therefore, developing a minimally invasive technique by taking advantages of the recent developments in molecular genetics

for diagnosis of NSCLC at an early stage is important clinically. By taking the advantages of the recent advances in molecular genetics, we aimed in this study to develop a non-invasive molecular method for screening of high risk groups and early detection of lung cancer. We chose the study of qRT-PCR expression of mRNA of Her-2/neu gene in samples of bronchial wash positive for NSCLC by cytopathology and compared them to that of negative samples (control cases). Her-2/neu gene is located in normal human cells as a single copy on the long arm of chromosome 17q21, its protein is necessary for the regulation of normal cell growth and differentiation, and it is associated with multiple signal transduction pathways [9,10,13]. The study of mRNA of Her-2/neu has a therapeutic importance by implementing anti-Her-2/neu targeted therapy [14]. Furthermore, amplification of the Her-2/neu gene leads to overexpression of its receptor and formation of Her-2/neu heterodimers that may results in the development of many types of tumors [14,15,16].Overexpression of Her-2/neu gene is seen in a wide variety of human epithelial malignancies including breast, ovary, salivary gland, gastrointestinal tract, prostate, lung, kidney, liver and bladder carcinomas [9,10]. Suggesting that its overexpression could play a critical role in the development and progression of these tumors [15,16,17]. Her-2/neu protein is overexpressed in 20-30% of non-small-cell lung cancers (NSCLC) and particularly in adenocarcinoma [9,10].

2. MATERIALS AND METHODS

2.1 Study Design and samples collection

This is a prospective case-control study in which 54 patients were enrolled. Patients were recruited from the Thoracic Surgical Unit in the Specialized Surgery Hospital/Medical City during the period from March 2012 to April 2014.

2.2 Study groups

1. Twenty-four selected patients with NSCLC lung cancer proved by cytopathology of bronchial wash samples (15 male and 9 female patients).
2. Thirty selected patients with benign pulmonary lesions (controls) proved by cytopathology of bronchial wash (20 patients males and 10 patients females).

2.3 Inclusion Criteria

1. Patients were presented for the first time complaining from chest problem (cough, sputum, hemoptysis, tightness, chest pain etc...), proved to be dueto pulmonary diseases malignant or benign.
2. Patients underwent Fiberoptic bronchoscopy with bronchial wash.

2.4 Exclusion Criteria

1. All cases that have received any form of lung cancer treatment (radical surgery, chemotherapy and/or radiation therapy) prior to sample collection were excluded from the study.

2. All cases known to have a second primary tumor other than lung cancer were excluded from the study.

3. All cases with uncertain diagnosis, whether benign or malignant were excluded from the study.

2.5 Ethical considerations

Ethical approval for this work was obtained from Baghdad Medical College Ethics Committee. All patients participated in the study have received a written information sheet explaining to them the aim of the study and then a signed consent form was taken from each one before participating in the study and the right was given to them to withdraw from this study at any time.

2.6 Samplescollection

In a labeled nuclease free tube (2 – 3) milliliters of bronchial wash was taken directly from bronchoscope and kept on ice until transferred to the laboratory for separation. The separation was performed by centrifugation at a speed of 1500 g for 15 minutes at a temperature of 4 degree centigrade. The supernant fluid was discarded and the sediment was re-suspended in 5 times volume of RNALater solutionfrom Applied Biosystem to prevent degradation of RNA. The specimens then kept in a deep freeze at- 80 degree centigrade (Ć) for total RNA extraction. The rest of the bronchial wash specimens were taken for cytopathological study.

2.7 Materials

2.7.1 Equipment and Instruments

Table 2.1: Equipment, instruments used in the study and their companies.

Items		Company
1.	Computer Laptop	*hp* Agilent (USA)
2.	Cabinet	Bio-SAFETY Class II (USA)
3.	Deep Freeze	GFL (Germany)
4.	Incubator	Memmert (Germany)
5.	Mask	Yama (Japan)
6.	Microfuge (cold)	Jouan MIR 23 I (USA)
7.	Micropipettes	DRAGON (Korea)
8.	Nano drop	Bio-drop company (USA)
9.	Nitrate gloves	Broche (Germany)
10.	Realtime PCR	STRATAGENE,Mx-3000PAgilent, technologies (USA)
11.	Thermal cycler	Veriti 96 wells Applied Biosystem (USA)
12.	Vortex mixer	Gemmy Industrial group (Korea)

2.7.2 Reagents and solutions

The specific reagents and general solutions used in the study are shown in tables [2.2 & 2.3].

Table 2.2: Specific Realtime PCR reagents, companies and countries of origin.

Reagent		Company	Country
1.	KAPA CYBR Master mix Universal	Biosystem	USA
2.	DEPEC treated water	Ambion	USA
3.	DNA Free Kit	Ambion	USA
4.	Ependorf tubes	Ependorf	Germany
5.	mirVana Isolation Kit with phenol	Ambion	USA
6.	Nucleases Free Water	Ambion	USA
7.	Nucleases Free Tips	Promega	USA
8.	High capacity cDNA reverse transcriptase	Applied Biosystem	USA
9.	Primers for cDNA amplification	Applied Biosystem	USA
10.	RNA Later	Ambion	USA
11.	Superase Spray	Ambion	USA
13.	TBE Buffer	Promega	USA

Table 2.3: General solutions

Items		Company	Country
1.	Chloroform	BDH	UK
2.	Ethanol	BDH	UK
4.	Formaldehyde	BDH	UK
5.	Methanol	BDH	UK

2.8 Cytopathological diagnosis

2.8.1 Stains for cytological examination

The smears taken from the deposit of bronchial washs were stained with Papaniculaou stain and/or Hematoxlin - Eosin stain.

2.8.2 Criteria of cytological diagnosis of NSCLC

2.8.2.1 Criteria for cytopathological diagnosis of squamous cell bronchogenic carcinoma

The cells are enlarged with a raised nucleo-cytoplasmic ratio (N/C ratio), the nucleus of malignant cell exhibits hyperchromatism, abnormal chromatin pattern, and irregular nuclear membrane. The cytoplasm of malignant cells is abundant and dense, in the well differentiated type it is cyanophilic, while basophilic in less mature type. Other features of malignant cells are also seen such pleomorphism, bizarre shaped cells and, giant tumor cells [9,10,18,19,20,21].

2.8.2.2 Criteria for cytopathological diagnosis of bronchogenic adenocarcinoma

There is moderate hyperchromasia of their nuclei with fine granular chromatin pattern, prominent nucleoli with occasional mitotic figures. The cytoplasm is amphophilic with fine or coarse vacuoles which are due to degeneration rather than mucus secretion, and cells are never ciliated. The presence of papillary clustering or three dimensional appearances may be seen as well [9,10,18,19,20,21].

2.8.2.3 Criteria for cytopathological diagnosis of large cell bronchogenic carcinoma

LCC is characterized by syncytial clusters of cell and dispersed cells. The cells have irregular nuclei with striking chromatin clearing, prominent, often multiple nucleoli and ill-defined, feathery cytoplasm [9,10,18,19,20,21].

2.9 Steps of qRT-Realtime PCR

Before starting any step, the inside of hood, instruments, and pipettes were be cleaned with detergent, alcohol and followed by Superase spray from Applied Biosystem to remove any RNase enzyme present, then followed by ultraviolet sterilization of hood and instruments. All those working in any of the steps from collection of samples to extraction, cDNA reverse transcription and PCR amplification should wear Powderless (nitrate) nuclease free gloves and mask with frequent changing of the gloves.All steps of total RNAs extraction and preparation of solutions for cDNA reverse transcription and PCR amplification were performed under hood. Solutions and samples and reagents were kept on ice during preparation. The steps qRT-realtime PCR after are:

2.9.1 Total RNA Extraction and purification

The extraction started after thawing of specimen and removing of RNALater by centrifugation and then followed by washing cell sediments by iced phosphate buffer sulphate solution(PBS)three times to remove contaminants, normal saline, local anesthetics and remaining RNALater from cell pellets. The extractions was performed by the use of extraction kit (mirVana™ miRNA Isolation Kit, with phenol) from Ambion according to the manufacturer's instructions [22]. The extracted total RNAtreated by DNA-free kit to get rid of any contaminant DNA using DNA free kit from Ambion and according to manufacturer's instructions [23]. The purity and concentration of total RNA were measured by Nano-drop spectrophotometer and those with ratio of A260/A280 measuring 1.9 – 2 considered as a good samples. The concentration of samples were unified to 100µg/ml andstored at a temperature of (– 80C) for reverse transcription into cDNA.

2.9.2 Reverse Transcription of total RNAs to cDNAs

Two samples from DNA free total RNA (each containing 1 µg of total RNA) were taken for reverse transcription of mRNAs into cDNAs using (High Capacity cDNA reverse transcriptase kit from Biosystem) according to manufacturer's instructions[24]. A pool of mix reagents was also used to reduce pipetting errors and the reverse transcription was performed in a thermal cycler for forty cycles. The concentration and purity of cDNA was also tested with Nano-drop spectrophotometer. The quality of cDNA as a template for real time PCR amplification of mRNA was assessed by qRT-PCR amplification of (GAPDHhousekeeping gene).

2.9.3 Primers design for realtime PCR amplification of Her-2/neu and GAPDH genes

We chose Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization of mRNAs as a housekeeping gene because it is ubiquitously expressed in body tissue, its expression remains constant in cancerous and non-cancerous samples [25], table [2.4]. The primers design was performed using software of Applied Biosystem [26], taking into account the followings; primer length should be 18 – 22 bps, primers Melting Temperature™ range from

52 – 58C, GC bps content of primer should be 40 – 60%, presence of G or C bp in the last five bases from the 3' end of primers promotes stronger binding of primers, and avoiding repeats.

Table 2.4: Sequence of GAPDH & Her-2/neu PCR primers

	Forward primer	**Reverse primer**
GAPDH	5'TGCACCACCA ACGGTTGC-3'	5'GGCATGGACTGTGGTCTGAG-3'
Her-2/neu	5'-ACGGACGTGGGATCCTGCA-3'	5'CTTCTCACACCGCTGTGTTCCAT-3'

2.9.4 Optimization of primers for qRT-PCR amplification of cDNAs from mRNAs

The optimum concentration of primers was assessed by serial dilutions of primersand performing realtime PCR amplification of targeted genes and then choosing the most appropriate concentration. The optimum annealing temperature also investigated by test changing annealing temperature and performing PCR runs. The results of optimum primers concentrations and annealing temperatures are shown in table [2.5].

Table 2.5: Concentrations of primer and the annealing temperature.

	Property	**GAPDH**	**Her-2/neu**
Forward primer	**(amount of use)**	15 pmol	10 pmol
Reverse primer	**(amount of use)**	15 pmol	10 pmol
Optimized annealing temperature		55.5 C	53.4 C

2.9.5 Realtime qRT-PCR amplification of cDNA

The qRT-realtime PCR amplification of mRNAs of (Her-2/neu and housekeeping gene GAPDH) in the samples taken from bronchial wash was performed in duplicate using KAPA SYBR FAST qPCR Kit Master MIX (2X) Universal from KAPA BIOSYSTEMS with primers from Applied Biosystems. The assays were performed according to the manufacturer's instructions [27], with the use of master mix pool to reduce pipetting errors and with a non-template control (NTC), non-primer control and negative control. The thermal profile was designed into three stages of; denaturation, annealing and extension for forty cycles, and then followed by a dissociation curve and melting point for assessment of specificity of the amplified DNA [28], figure [2.1]. At the end of amplification the result of Ct values for each specimen was recorded for further analysis of gene expression.

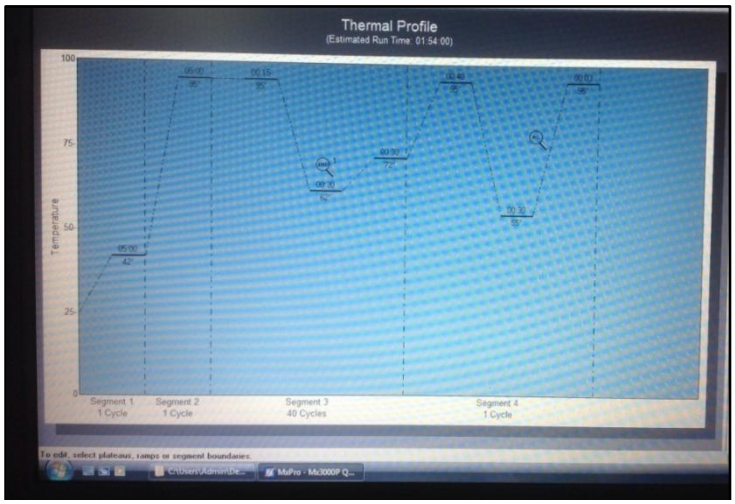

Figure 2.1: Thermal profile of real time qRT-PCR amplification of cDNA from mRNAs.

2.10 Analysis of the results

IBM SPSS version 22 software was used for the data statistical analyses. Numerical data wereformulated as mean (M), standard deviation (SD), and standard error (SE). Comparison between malignant and benign (control) samples was carried out by *t*-test, while comparison between more than two group was carried out using ANOVA test. The results were considered significant when the *p*-values < 0.05.

3. RESULTS

3.1 Result of cytopathological examination

3.1.1 Result of cytopathological examination of bronchial wash positive for non-small cell lung cancer

Bronchogenic squamous cell carcinoma was the most frequent type of lung cancer accounting 19/24 (79.17 %) of whole NSCLC cases, subdivided according to sex into 13/24(54.17%) were men, and 6/24 (25 %) were women. Bronchogenic adenocarcinoma subtype of NSCLC were present in 4/24 (16.67 %), subdivided into 1/24 (4.17%) was in males and 3/24 were (12.0%) in females.While, large cell bronchogenic carcinoma was 1/24 (4.16%), figure [3.1]. The cytopathological results of bronchial wash that were positive for lung cancer according to age groups showed that, cancer was more common in the age group (60 – 69 years), and followed by the age group (70 – 79 years) and in most of age groups the NSCLC was more common in males than females or equal to them, figure [3.2].

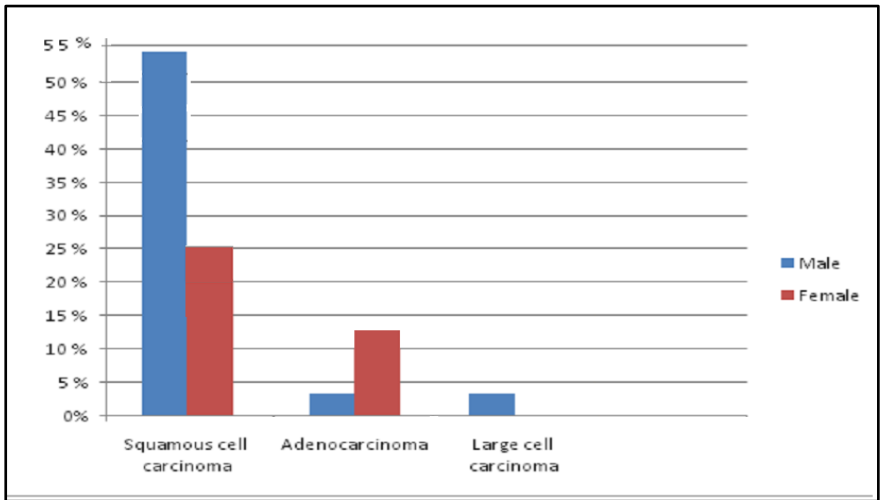

Figure 3.1: Cytopathological results of bronchial wash samples positive for NSCLC, the frequency of cytopathological type according to sex.

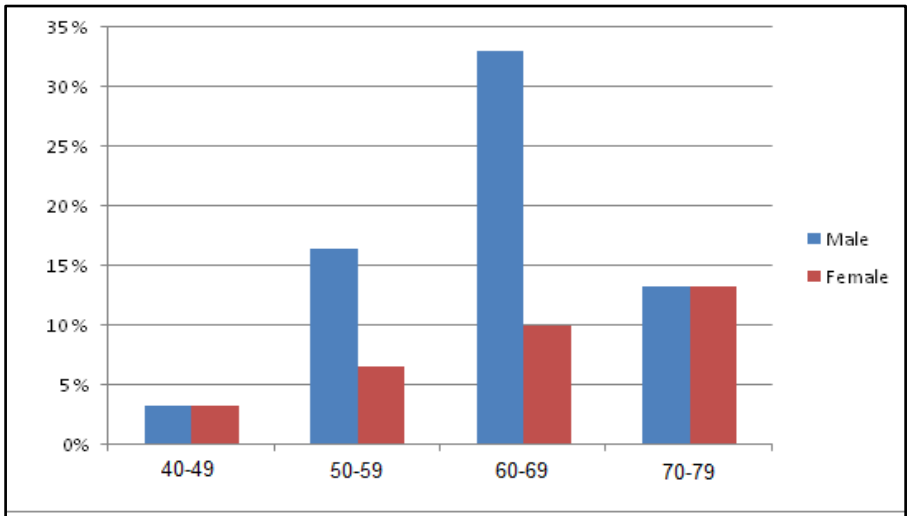

Figure 3.2: Cytopathological results of bronchial wash positive for malignant Cells, the frequency according to the age groups and sex.

3.1.2 Result of cytopathological examination of bronchial wash samples negative for lung cancer cells

Cytopathological examination of non-malignant bronchial wash samples revealed acute and/or chronic inflammatory cells together with macrophages and benignlooking bronchial epithelial cells. However, further diagnosis of these cases based on bronchoscopic findings, bronchial biopsy and/or bacteriological examination. The results revealed chronic bronchitis in 12/30 (40%) of cases, emphysema in 6/30 (20%), bronchiectasis in 5/30 (16.6 %), lung fibrosis in 3/30 (10%), tuberculosis in 2/30 (6.7%), and asthma in 2/30 (6.7%).Male sex was predominated in cases with chronic bronchitis 11/30(36.67%) compared with one female 1/30(3.33%) of cases. Emphysema was shown in 6/30 (20%) of whole cases, subdivided into 2/30(6.67%) were males and in 4/30(13.33%) were females. Bronchiectasis was seen in 5/30 (16.67%) of which 3/30(10%) was in males and 2/30 (6.67%) of cases in females. Lung fibrosis was seen in 3/30 (10%) of cases with 2/30(6.67%), in males and 1/3 (3.33%) in females. Pulmonary tuberculosis and asthma were equally distributed in both sexes, one case for each (3.3%).The mean age was 57.7 years and SD was 9.732.The highest frequency for males was noticed in the age group(50 – 59) and (60 – 69), and the highest frequency for female patients was noticed in the age group (40 – 49), figures [3.3& 3.4].

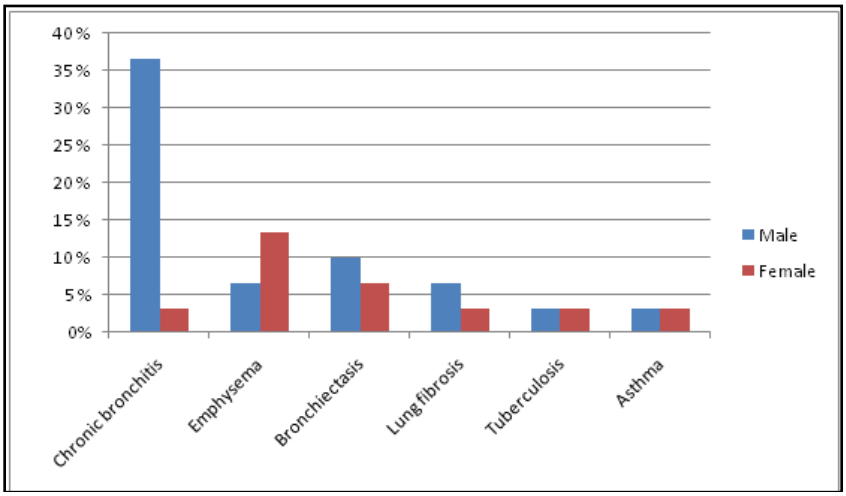

Figure 3.3: Clinical diagnosis of patients with bronchial wash negative for lung cancer cells distributed according to sex, CD (chronic bronchitis), EM (emphysema, BE (bronchiectasis), LF (lung fibrosis), Tb (tuberculosis) & AS (asthma).

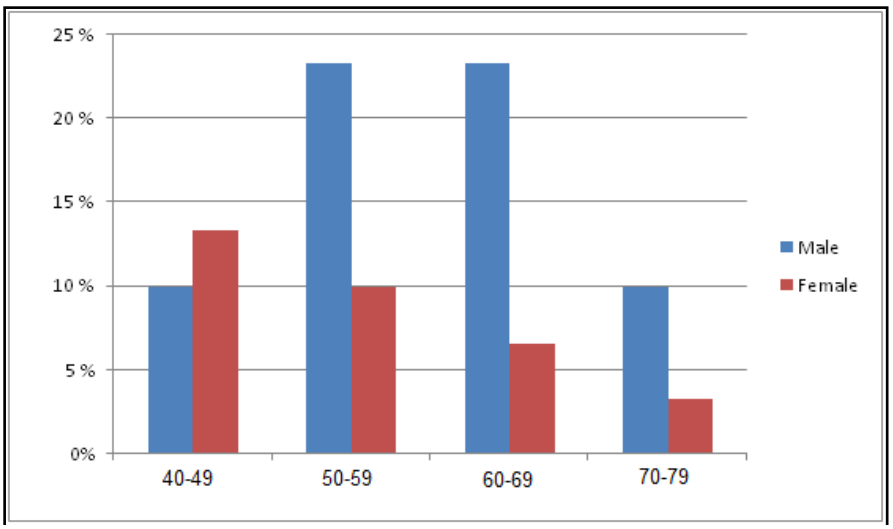

Figure 3.4: Clinical diagnosis of patient with bronchial wash negative for lung cancer cells distributed according to the age groups and sex.

3.2 Result of measurement of concentration and purity of total RNAby nano-drop

The total RNA concentration and purity of each sample, after total RNA extraction by mirVana Isolation kit and treatment with DNA free kit, was measured by nano-drop and samples with ratio of A260 / A280 equal to 1.9 – 2.0 were considered as good samples. The mean ± SD for concentration of whole samples of bronchial wash positive for lung cancer cells was 4346 ± 53.147µg/ml, for males it was 4785.88±65.174 µg/ml and for females it was 3466.96±23.201µg/ml. The mean ± SD of concentration of whole samples of bronchial wash that were negative for lung cancer cells was 3230.45±22.404 µg/ml, for males it was 3205.995±23.908 µg/ml and for females it was 3279.36±19.078 µg/ml.The mean of purity ± SD of whole samples of bronchial wash positive for lung cancer cells was 1.9602±0.03616, for males 1.9553±0.0386606 and for females 1.970±0.0299629. The mean of purity ± SD of whole samples of bronchial wash that were negative for lung cancer cells was 1.95510±0.0372479, for males it was 1.95035±0.395731 and 1.96460±0.0318441for females, Appendices [A1 & A2].

3.4 Results of realtime qRT-PCR amplification (Ct value)

The Ct value of qRT-PCR amplification of replica of bronchial wash samples (control non-malignant and malignant conditions) were performed in duplicate with melting curve and the mean results of Ct values were taken. All bronchial wash samples submitted for amplification by realtime qPCR, after total RNA extraction and cDNA reverse transcription, revealed amplification curves for mRNA of both Her-2/neu & GAPDH genes included in the studyand continued without forming a platue at the end. The amplification curves were separated with a specific Ct value for each, appendices [B1&B2].

3.5. Analysis of Ct values

3.5.1. Analysis of raw Ct values

3.5.1.1 Comparison of raw Ct values between samples of bronchial positive for lung cancer and control wasusing t- test

The difference in mean raw Ct value between malignant and control (benign) cases was statistically non-significant, *p*-value was >0.05 with mRNA of GAPDH. While, in case of mRNA of Her-2/neu it was significant, the *p*-value was <0.05, table [3.1].

Table 3.1: The *t*-test of controls and lung cancer cases, for non-normalized Ct-values.

	Study group		
	Control group	**Cases (lung Ca)**	**P (t-test)**
Ct value – GADPH			0.31[NS]
Range	(21.4 to 25.17)	(22.4 to 26)	
Mean	23.90	24.17	
SD	1.08	1.00	
SE	0.20	0.18	
Ct value - Her-2/neu			0.004
Range	(27.79 to 30.69)	(23.69 to 29.5)	
Mean	29.01	27.94	
SD	0.71	1.84	
SE	0.13	0.34	

3.5.1.2 Comparison of raw Ct values between different histopathological types of lung cancer and control in samples of bronchial positive for lung cancer and control wasusing ANOVA test and p-LSD.

The mean Ct-value for difference between different types of lung cancer and control was statistically non-significant (*p*-value >0.05) on studying of mRNA of GAPDH in samples of bronchial wash that were positive and negative for lung cancer cells. The *p*-LSD test for differences between C and SqCC, C and AC, and SqCC and AC were also statistically non-significant, *p*-values were>0.05. While, the same result were statistically significant, $p<0.05$. Her-2/neu in the same specimens on comparing malignant and benign cases using ANOVA test and comparing C and AC and SqCC and AC. But it was became statistically non-significant on comparing C and SqCC, table [3.2].

Table 3.2: Bronchial wash specimens, ANOVA test and *p*-LSD tests for non-normalized Ct-values.

	Final diagnosis				***P*- (ANOVA)**
	SqCC	**AC**	**SCC**	**Control**	
Ct value – GADPH					**0.39[NS]**
Range	(22.4 to 25.7)	(22.8 to 25.87)	(23.4 to 26)	(21.4 to 25.17)	
Mean	24.03	24.68	24.47	23.90	
SD	0.96	1.31	0.93	1.08	
SE	0.22	0.66	0.38	0.20	
P- (LSD) for difference in mean between:					
C X SqCC = 0.67[NS]					
C X AC = 0.17[NS]					
SqCC X AC = 0.26[NS]					
Ct value - Her-2/neu					**<0.001**
Range	(26.4 to 29.5)	(23.69 to 26.98)	(24.1 to 29.3)	(27.79 to 30.69)	
Mean	28.90	25.51	26.91	29.01	
SD	0.89	1.38	2.19	0.71	
SE	0.20	0.69	0.89	0.13	
P- (LSD) for difference in mean between:					
C X SqCC = 0.73[NS]					
C X AC <0.001					
SqCC X AC <0.001					

3.5.2 Analysis of normalized Ct values

3.5.2.1 Comparison of normalized Ct values between samples of bronchial positive for lung cancer and control wasusing t- test

The differences in the mean normalized Ct values between malignant and control cases was statistically significant, *p*-value was < 0.05 with mRNAs of GAPDH&Her-2/neu genes using *t*-test, table [3.3].

Table 3.3: Recorded results for normalized Ct values of studied markers (GAPDH & Her-2/neu genes), compared between lung cancer cases and control, as obtained from bronchial wash specimens.

	Study groups		
	Control group	**Cases (lung Ca)**	***P*-(*t*-test)**
Ct value - GAPDH			**<0.001**
Range	(22.08 to 25.17)	(24.1 to 26.21)	
Mean	23.63	25.41	
SD	0.99	0.48	
SE	0.18	0.09	
Ct value - Her-2/neu			**0.01**
Range	(26.49 to 30.69)	(26.38 to 32.3)	
Mean	28.95	28.12	
SD	0.94	1.52	
SE	0.17	0.28	

3.5.2.2 Comparison between different histopathological types of lung cancer and using ANOVA test

The mean of normalized Ct values were compared between different histopatho-logical types of lung cancer and control cases for each of the studied markers in bronchial wash samples using ANOVA test. The *P*-(LSD) was also studied for differences in mean between control and different histopathological types of lung cancer and also between different histopathological types themselves. ANOVA testing for mRNA of GAPDH gene between all histopathological types of lung cancer and control wasstatisticallysignificant, *P*-value was <0.05. The *P*-(LSD) for

difference in mean normalized Ct values of mRNA of Her-2/neu gene was statistically significant, *P*-value was < 0.05, between C and SqCC, and between C and AC. The *P*-(LSD) for differences between the types of lung cancer was statistically not significant, *p*-value was > 0.05, between SqCC and AC.ANOVA testing for mRNA of Her-2/neu gene between all histopathological types of lung cancer and control was significant, *P*-value was <0.05. The *P*-(LSD) for difference in mean normalized Ct values of mRNA of Her-2/neu gene was statistically significant, *P*-value was < 0.05, between C and AC. While, the *P*-(LSD) for differences between the types of lung cancer was statistically not significant, *P*–value was > 0.05, between C and SqCC, and between SqCC and AC, table [3.4].

Table 3.4: ANOVA and LSD for differences in means of normalized Ct values of different markers as studied in bronchial wash samples, according to histopathological types of lung cancer.

	Final diagnosis			***P* - (ANOVA)**
	SqCC	**AC**	**Control**	
Ct value – GAPDH				<0.001
Range	(24.84 to 26.21)	(24.1 to 26)	(22.08 to 25.17)	
Mean	25.46	25.34	23.63	
SD	0.60	0.46	0.99	
SE	0.25	0.10	0.18	
P -(LSD) for difference in mean between:				
C x SqCC <0.001				
C x AC <0.001				
SqCC x AC = 0.74[NS]				
Ct value - Her-2/neu				0.037
Range	(26.38 to 30.41)	(26.54 to 32.3)	(26.49 to 30.69)	
Mean	27.94	27.98	28.95	
SD	1.38	1.53	0.94	
SE	0.57	0.34	0.17	
P- (LSD) for difference in mean between:				
C x SqCC = 0.08[NS]				
C x AC = 0.01				
SqCC x AC = 0.95[NS]				

3.5.3 Comparative CT Method

Calculation of expression by comparative Ct method using the formula, expression = ($2^{-\Delta\Delta C}$), the result of expression = $2^{-\Delta\Delta C}$ {[Ct of target gene – Ct of housekeeping gene]-[Ct of control – Ct of housekeeping gene]}[28].
As shown in table [3.5], GAPDH gene qualifies as a best (housekeeping) gene, since the calculated carcinoma (Ca) case to control ratio for the mean Ct gene expression value was the closest to one (ratio = 0.78), indicating that is expression is not noticeably different between cases with Ca and controls (non-malignant). While in case of Her-2/neu the case to control ration was (4.8).

Table 3.5: Case to control ratio with mean and SD of markers studied in samples of bronchial wash.

	Ca cases		**Controls**		**Case to control Ratio**
$2^{(-Ct)}$	**Mean**	**SD**	**Mean**	**SD**	
Ct-GAPDH	6.63E-08	4.52E-08	8.55E-08	7.45E-08	0.78
Ct-Her2/neu	9.95E-09	1.69E-08	2.07E-09	1.01E-09	4.8

The genes are overexpressed when its comparative Ct value is over (1), and under expressed when its comparative Ct value is less than (1). Her-2/neu was overexpressed in samples of bronchial wash positive for NSCLC, the comparative Ct value is (4.8) compared to control with *p*-value of <0.05, table [3.6].

Table 3.6: Mean Ct value with SD of cancer cases and control cases with comparative Ct values of studied markers and their P-values, in samples of bronchial wash.

	Ca cases		**Controls**		**Comparative Ct- value**	***P*- value**
	Mean	SD	Mean	SD		
GAPDH	Reference (control gene)		Reference (control gene)			
Ct-Her2/neu	0.245	0.518	0.042	0.037	5.9	0.004

3.5.3.1 Expression of Her-2/neu gene in samples of bronchialwashpositive for Squamous Cell Carcinoma (SqCC) and controls

The mRNA of Her-2/neu gene was only slightly overexpressed compared to control in samples of bronchial wash positive for bronchogenic squamous cell carcinoma (1.4 times) more than control, table [3.7].

Table 3.7: Comparative Ct values with mean and SD of Her-2/neu gene in samplesof bronchialwash positive for lung squamous cell carcinoma by cytopathology.

	SqCC cases		**Controls**		**Comparative Ct Value**
	Mean	**SD**	**Mean**	**SD**	
Ct-Her2/neu	0.058	0.083	0.042	0.037	1.4

3.5.3.2 Expression of Her-2/neu gene in samples of bronchial wash positive for Lung Adenocarcinoma (AC) by cytopathology

The expression of mRNA of Her-2/neu gene in samples of bronchial wash positive for squamous cell lung (AC) compared to controls cases using comparative Ct method was high (26.1) times more than controls, table [3.8].

Table 3.8: Expression of Her-2/neu gene in bronchial wash samples positive for lung adenocarcinoma.

	AC cases		**Controls**		
Ct-Her2/neu	**Mean**	**SD**	**Mean**	**SD**	**Comparative Ct**
	1.084	1.141	0.042	0.037	26.1

The expression of mRNA of Her-2/neu gene in sample of bronchial wash positive for NSCLC of large cell undifferentiated carcinoma with neglected because it was only one case.

3.5.4 The ROC area occupied by studies markers and *p*-value in samples of bronchial wash after normalization

The *p*-value for ROC curves was statistically significant,< 0.05 for mRNA of GAPDH and non-significant, > 0.5 for mRNA of Her-2/neu gene, table [3.9].

Table 3.9: The ROC area occupied by each test and the *p*-value of markers studied from bronchial wash samples after normalization.

	ROC area	***p*-value**
Normalized Ct value – GAPDH	0.707	0.006
Normalized Ct value - Her-2/neu	0.527	0.72[NS]

3.5.5Expression of Her-2/neu mRNA in individual samples

Her-2/neu mRNA was overexpressed in 8/24 (33.33%) from whole NSCLC cases, 4/24 (16.66%) were males and 4/24 (16.66%) were females. According to histopathological type 3/24 of cases (12.5%) were squamous cell carcinoma (2caes were males and one female). Adenocarcinoma cases were 4/24 (16.66%) (3 cases were females and 1 case was male). Large cell carcinoma was 1/24 forming (4.16%) of NSCLC and it was male, details in appendix-C.

3.6 The Specificity and sensitivity of Her-2/neu gene test in bronchial wash samples

When standardized Ct value was 0.965 the sensitivity of Her-2/neu was 26.7% and the specificity was 100 %, and when the standardized Ct value was 1.075 the sensitivity was 93.3%. The specificity was 13.3%, table [3.10], figure [3.5].

Table 3.10: Specificity, sensitivity, accuracy, positive predictive value and negative predictive value of the test

Positive if < cut-off value	Sensitivity	Specificity	Accuracy	Optimal if minimum	PPV at pretest probability =		NPV at pretest probability = 10%
					50%	90%	
Standardized Ct value - Her-2/neu							
0.965	26.7	100.0	63.3	0.733	100.0	100.0	92.5
0.975	46.7	96.7	71.7	0.534	93.3	99.2	94.2
0.985	50.0	96.7	73.3	0.501	93.8	99.3	94.6
0.995	56.7	93.3	75.0	0.438	89.5	98.7	95.1
1.005	56.7	83.3	70.0	0.464	77.3	96.8	94.5
1.015	70.0	73.3	71.7	0.401	72.4	95.9	95.7
1.025	76.7	63.3	70.0	0.435	67.6	95.0	96.1
1.035	80.0	60.0	70.0	0.447	66.7	94.7	96.4
1.045	83.3	40.0	61.7	0.623	58.1	92.6	95.6
1.055	83.3	30.0	56.7	0.720	54.3	91.5	94.2
1.065	90.0	20.0	55.0	0.806	52.9	91.0	94.7
1.075	93.3	13.3	53.3	0.869	51.9	90.6	94.7

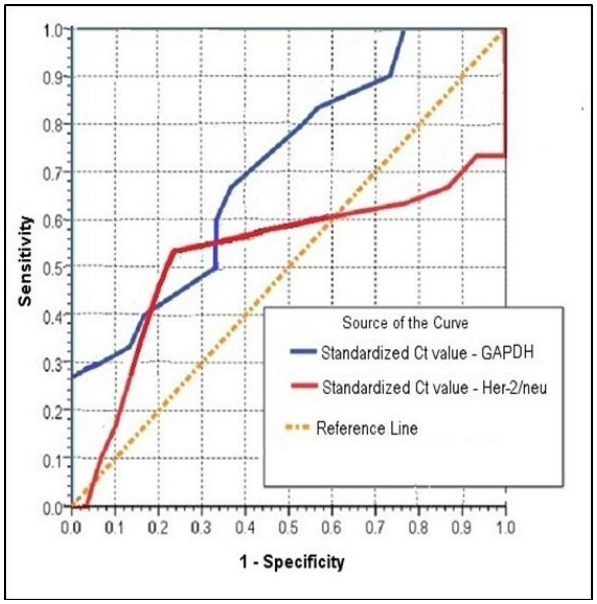

Figure 3.5: The curves for sensitivity and specificity of studied markers (mRNAs of GAPDH and Her-2/neu gene), in samples of bronchial wash positive for lung cancer.

4. DISCUSSION

In the present study, we found that the mRNAs of GAPDH and Hert-2/neu genes were stably present and readily measurable in the bronchial wash samples of lung cancer cases and controls. Additionally, mRNA of Her-2/neu gene was significantly over-expressed in specimens from cancer patients compared with those of controls.The frequency of overexpression of Her-2/neu in malignant lesions was only 40%, occupied mostly by 100% expression in adenocarcinoma. These findings were consistent with similar studies. Menkhi [29] reported overexpression Her-2/neu gene in 36.6% of lung cancer cases employing FISH technique. While, Giltnane et al. [30], Bunn et al. [31], and Hirsch et al. [15], reported overexpressionof Her-2/neu gene in 26%, 50%, and 47% of cases of lung cancer

respectively. In these researches, different sample sizes and different methodologies were used. Like other markers, normalization of Ct values help much in differentiating malignant from benign samples, being statistically significant, *p*-value<0.05. The ROC area for Her-2/neu was not significant before and after normalization. Although, mRNA of Her-2/neu gene was overexpressed in samples of bronchial wash positive for bronchogenic squamous cell carcinoma and bronchogenic adenocarcinoma, it was more overexpressed in samples from cases of adenocarcinoma than in samples of squamous cell carcinoma. There are several limitations in this study, Inspite of our result appears to be promising. First, the sample size of the study groups was too small, so further launching of mRNA of Her-2/neu gene in a large sample seize and independent studies is clearly required. Secondly, in this current study the cases were in advanced stages, and we are not sure whether the expression in early stages of lung cancer is the same as that of late stages however, investigations in the future may give an answer. Thirdly, our present study was on expression of one marker (mRNA of Her-2/neu gene), launching of a panel of mRNAs will increases the sensitivity and the specificity of the study.

In conclusion, we found that the expressions of the mRNA of Her-2/neu gene in samples of bronchial wash could readily and specifically measured to be used as a minimally invasive diagnostic biomarker for NSCLC. Nonetheless, further independent cohort is required for validating the utility of this potential biomarker.

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