Comparison between cryobiopsy and forceps biopsy in detection of epidermal growth factor receptor amplification in non-small-cell lung cancer

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Background Non-small-cell lung cancer (NSCLC) represents 85% of lung cancer cases. Genotyping is now considered as a cornerstone in proper management and better results of such cancers, especially with targeted therapy. Cryobiopsy is a promising tool in NSCLC to obtain larger samples, with well-preserved tissue sufficient for accurate histopathological and gene detection.

Aim To compare cryobiopsy and ordinary forceps results in detection of epidermal growth factor receptor (EGFR) amplification in NSCLC.

Materials and methods Samples from 34 patients with proven NSCLC by cryobiopsy versus forceps biopsy were compared for size, quality, and diagnostic yield of EGFR gene amplification.

Results The samples obtained by cryoprobe had larger size and better artifact-free areas with more diagnostic yield of EGFR gene amplification (29.4%) versus with forceps biopsy (8.8%), with gene amplification showing higher statistical significance in younger patients, never smokers, and women ($P<0.001$).

Conclusion Cryobiopsy is an excellent tool for larger, better-quality sampling and for higher diagnostic yield of EGFR amplification in NSCLC.
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Keywords: cryobiopsy epidermal growth factor receptor, cryobiopsy, EGFR, non-small-cell lung cancer

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Introduction

Lung cancer is the most common cancer worldwide, representing 13% of total cancers, with non-small-cell lung cancer (NSCLC) representing ∼85% of lung cancer cases [1].

In personalized medicine era, the advent of molecular biology has moved lung cancer histological classification beyond simple division into small cell lung cancer and NSCLC, making genotyping of NSCLCs a crucial aspect of management [2,3].

Moreover, detection of specific genetic mutations has made targeted therapies more effective when these mutations are present. Tumors having somatic mutations in gene that encodes for tyrosine kinase domain of the cell surface protein, epidermal growth factor receptor (EGFR), were found to have better outcome to tyrosine kinase inhibitor (EGFR TKI) drugs, gefitinib and erlotinib [3].

Therefore, selecting adequate diagnostic biopsy tools to get reliable sufficient tissue biopsy with good quality, less risk, and discomfort for patient all are important for histologic characterization and mutation analysis, as a step for proper treatment and optimal targeted therapy in NSCLC [1].

The traditional diagnostic tool in lung cancer is through forceps biopsy via a flexible bronchoscope. One way to obtain larger tumor tissue sample is either by multiple biopsies or using cryobiopsies [4].

Flexible cryoprobe was first used for cryoablation as an alternative for mechanical debulking of malignant endobronchial stenosis, and because of larger size and less artifact-free tissue samples, the technique has been efficiently used for biopsy of endobronchial lesions [5–7].

This study aims to compare between cryobiopsy and ordinary forceps samples in detection of EGFR gene amplification by fluorescence in-situ hybridization technique (FISH).

Materials and methods

This prospective case study was done during the period from September 2015 to December 2016 on 50 patients recruited from Chest Department Tanta

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University Hospitals, but only 34 patients with endobronchial lesions were able to be sampled by three forceps biopsy attempts and one cryobiopsy from each patient, which were compared for size, quality of tissue, and complications of each type of biopsy. Moreover, the samples were compared for diagnostic yield of EGFR amplification.

Inclusion criteria

Adult patients with endobronchial lesion, previously pathologically confirmed as having NSCLC by clinical, radiological, and histological examination were included.

Exclusion criteria

Bleeding diathesis, anticoagulants use, oxygen saturation less than 90%, hereditary diseases, congenital anomalies, severe comorbidity such as severe cardiac disease and other malignancy, and also any patient with contraindication(s) for flexible bronchoscopy as stated by British Thoracic Society guidelines in adults 2013were excluded from the study [8].

The study protocol was approved by the institutional ethics committee, and a written informed consent was taken from all patients before study.

Bronchoscopy and biopsy instruments used and obtained from were as follows: fiberoptic bronchoscopy (EB-1975k; Pentax, Tokyo, Japan), forceps biopsy (reusable fenestrated forceps FB-21C or FB-52C-1), and cryoprobe (Erbokryo; Erbe Elektromedizin GmbH, Tubingen, Germany).

Patients were first deeply sedated using intravenous midazolam and 10% lidocaine spray intranasal and oropharyngeal, and 2% xylocaine solution within airways was used for local anesthesia. A cryoprobe was placed over endobronchial lesion and used for freezing for 3–5 s, and then the probe was pulled out en bloc with the bronchoscope. The sample on the probe's tip was thawed in sterile 0.9% sodium chloride water bath and placed in formalin [5,7].

Histopathology

Samples were fixed in 10% formalin for 24 h. Pathological analysis was performed. Biopsies were measured using centimeters, before sectioning for microscopic evaluation.

EGFR amplification method was as follows: detection of EGFR amplification was done using FISH technique, using Cytocell aquarius probe (cat. no. LPS 003-SA/LPS 003-A), with probe specification EGFR, 7p11.2, Red, and D7Z1, 7p11.1–q11.1, Green [9].

Sample preparation

Formalin-fixed paraffin-embedded tissue sections were prepared according to laboratory or institution guidelines. For FISH, 4–6-μm-thick formalin-fixed paraffin-embedded tissue sections were cut.

Tissue sample pretreatment

For pretreatment, Aquarius tissue pretreatment kit (LPS 100) was used.

Pre-denaturation steps

Probe was removed from the freezer and allowed to warm to room temperature.

Denaturation of specimen DNA and probe hybridization

Overall, 10–15 μl of probe sample was added to a target area, and then covered and sealed with (FixoGum) rubber cement. The slide was placed on the hybrid surface (Vysis, Vysis hybrite CA 92641, California, USA). The hybrid was heated to 80°C for 10 min (for denaturation) and then cooled to 37°C overnight for hybridization.

Post-hybridization washes

The coverslip and traces of glue were removed carefully and then the slide was immersed in 0.4×SSC with NP40 (pH 7.0) at 74°C for 2 min, and the slide then transferred to 2×SSC with NP40 at RT (pH 7.0) for 2 min. Overall, 10–15 μl of DAPI was applied onto each sample and covered with a coverslip, and the slide was placed in a dark box in a fridge for 20–30 min before screening.

Interpretation of result and image capture

Slides were examined with an epifluorescence Olympus, BX60 microscope (New York, USA). The images were captured with scan IAI camera fitted with PC Applied Image System (Pittsburgh, USA) analysis software. Only interphases with clear signals, with no overlapping or splitting, were analyzed [9].

Statistical analysis

Statistical analysis was done using SPSS version 20 (SPSS Inc., Chicago, Illinois, USA). Continuous data were expressed as mean±SD and categorical variables as percentages. Multivariant correlation was used for correlation, P value was considered significant if less than 0.05.

Results

From 50 patients who attended the Chest Department of Tanta University Hospitals, 16 patients tissue specimens were not of good quality for FISH analysis and no signals could be detected for interpretation. Therefore, 34 patients were enrolled in the study, comprising 14 males and 20 females, with mean age of 57.44±5.12 years. The study population data are illustrated in Table 1.

On comparing the samples taken by forceps biopsy and cryobiopsy, cryoprobe samples were of better quality with larger size and artifact-free areas. The median size of the cryoprobe and forceps biopsies was 1.51±0.41 and 0.89±0.31 cm, respectively, whereas cryobiopsies artifact-free tissue was significantly larger (1.45±0.43 vs. 0.58±0.22 cm, P<0.001; Tables 2 and 3 and Figs 1 and 2).

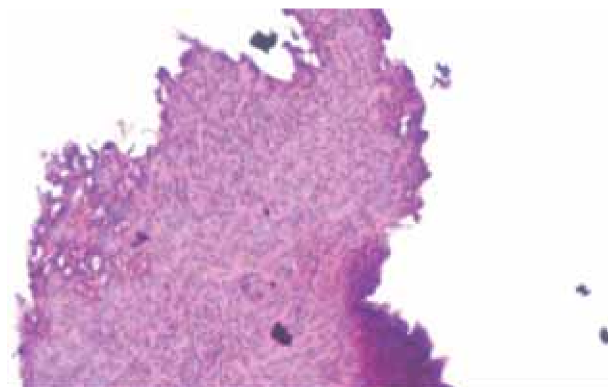
Table 1 Basic demographic data of patients in both groups

	Frequency (%)
Age (mean±SD)	57.44±5.12
Sex	
Male	14 (41.2)
Female	20 (58.8)
Smoking	
Smoker	12 (35.3)
Ex-smoker	5 (14.7)
Nonsmoker	17 (50)
Tumor size (cm)	
>3	20 (58.8)
<3	14 (41.2)
Tumor site	
Right main	7 (20.6)
Left main	44 (11.8)
RLL	10 (29.4)
RUL	1 (2.9)
LLL	12 (35.3)
Pathology	
Adenocarcinoma	14 (41.2)
Sq C C	20 (58.8)

LLL, left lower lobe; RLL, right lower lobe; RUL, right upper lobe; Sq C C, squamous cell carcinoma.

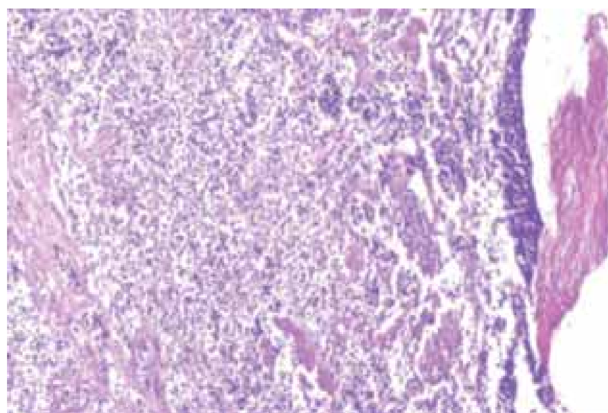
On comparing the complications of procedure in both groups, no reported cases of pneumothorax or respiratory failure was seen. There was only mild bleeding (i.e. treated with suctioning and cold saline) in 11 (32.4%) of 34 cases in cryobiopsy group compared with nine (26.5%) of 34 cases in forceps biopsy group, with no statistically significant difference between the two groups.

Figure 1



A case of non-small-cell lung cancer (H&E 400) by forceps with crash artifact.

Figure 2



A case of non-small-cell lung cancer (H&E 400) by cryobiopsy with no crash artifact.

Table 2 Size of sample of cryobiopsy versus forceps biopsy

	Cryobiopsy		Forceps biopsy		P value
	Range	Mean±SD	Range	Mean±SD	
Size of sample	0.7–2.2	1.51±0.41	0.2–1.4	0.89±0.31	<0.001

Table 3 Artifact-free tissue areas of cryobiopsy versus forceps biopsy

	Cryobiopsy		Forceps biopsy		P value
	Range	Mean±SD	Range	Mean±SD	
Artefact free area	0.8–2.1	1.45±0.43	0.2–0.9	0.58±0.22	<0.001

EGFR gene status was classified into six categories based on tumor cells frequency with specific gene copy numbers and chromosome seven centromere. These categories were further classified into (a) FISH negative (disomy, low trisomy, high trisomy, and low polysomy) and (b) FISH positive (high polysomy and gene amplification) [10,11].

As for diagnostic yield of EGFR amplification (gene copy number), FISH positivity (amplification and high polysomy) was detected by bronchoscopic cryobiopsy in 10 (29.4%) of 34 patients whereas forceps biopsy showed FISH positivity in only three (8.8%) cases ($P<0.001$; Table 4 and Figs 3 and 4).

Samples taken with cryobiopsy that showed FISH positivity were three (8.8%) cases with EGFR amplification, and seven (20.5%) cases with high polysomy (Fig. 5).

There was a correlation between EGFR and tumor histopathological type, smoking status, and sex. Of the 10 cases of EGFR amplification using FISH technique detected with cryobiopsy, there were seven cases of adenocarcinoma and three cases of squamous cell carcinoma (Fig. 6). However, in the samples taken by forceps biopsy. Of the three cases of EGFR gene amplification, there were two cases of adenocarcinoma and one case of squamous cell carcinoma. There was a positive correlation between adenocarcinoma histopathological type, smoking status, and sex, so EGFR gene amplification was predominant in females, and nonsmokers with adenocarcinoma (Table 5).

Discussion

Advances in molecular biology are improving our understanding of lung cancer and changing approach to treatment. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society guideline recommends mutational testing in nonsquamous NSCLC [2,12,13].

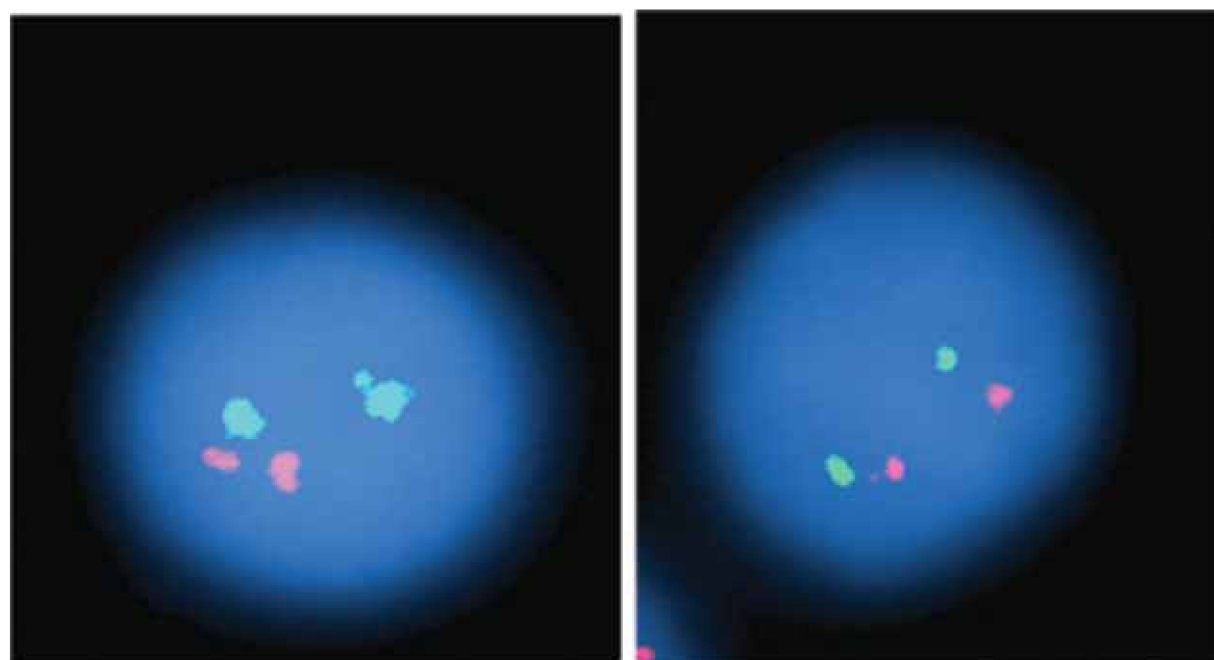
In general, the larger the biopsy sample the more accurate the diagnosis. However, increasing biopsy

Table 4 Diagnostic yield of epidermal growth factor receptor gene copy number by fluorescence in-situ hybridization analysis in cryobiopsy versus forceps biopsy

	Cryobiopsy [*N* (%)]	Forceps biopsy [*N* (%)]	*P* value
Fluorescence in-situ hybridization positive	10 (29.4)	3 (8.8)	0.031*
Fluorescence in-situ hybridization negative	24 (70.6)	31 (91.2)	

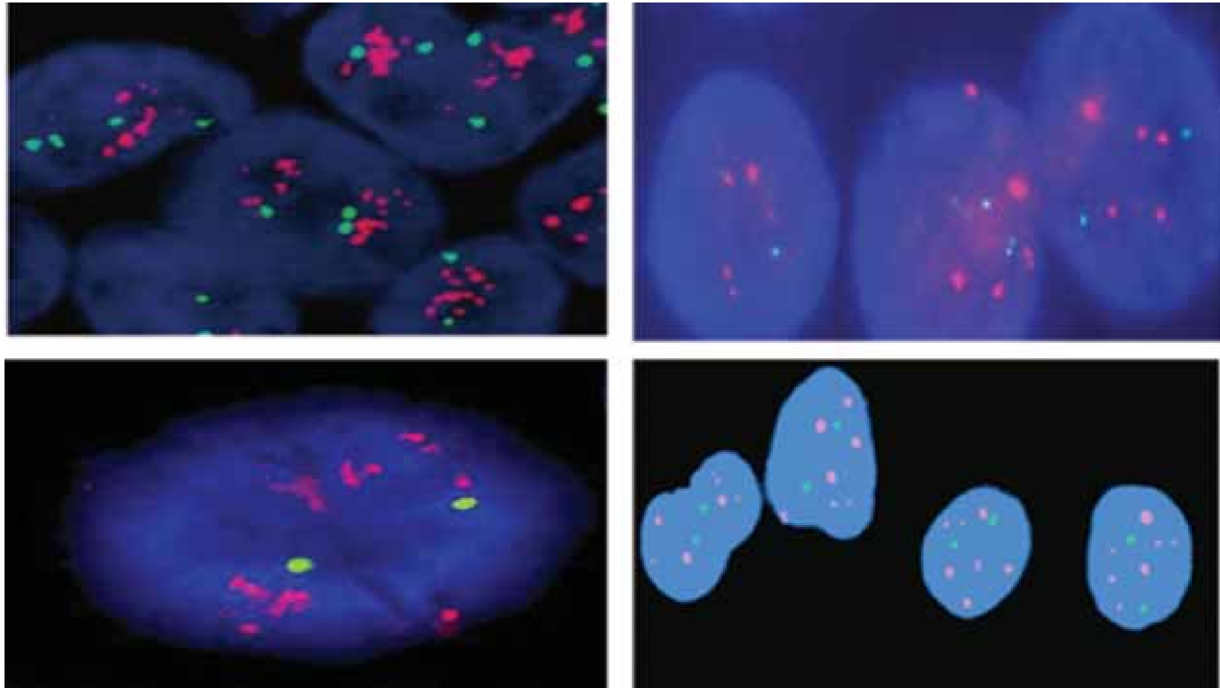
*$P\leq0.05$, statistically significant.

Figure 3



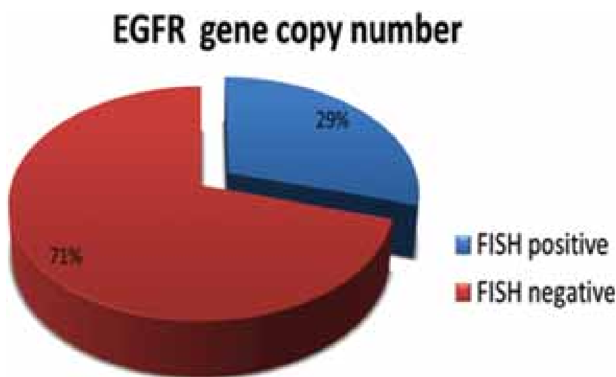
A case of non-small-cell lung cancer show interphase cell with normal epidermal growth factor receptor gene gain (two orange signals and two green signals).

Figure 4



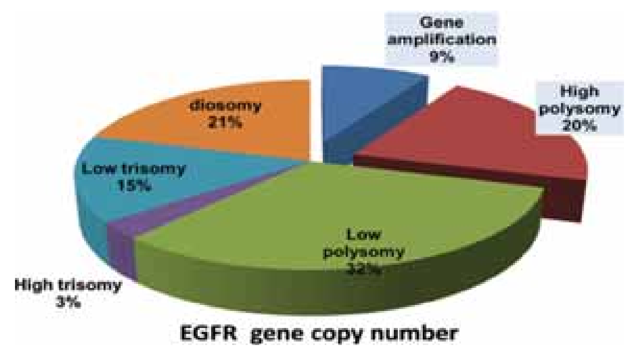
A cases of non-small-cell lung cancer show interphase cell with epidermal growth factor receptor gene amplification (multiple orange signals).

Figure 5



Epidermal growth factor receptor gene copy number by fluorescence in-situ hybridization analysis of studied cases.

Figure 6



Categories of epidermal growth factor receptor gene copy number of studied cases.

Table 5 Multivariant analysis of correlation between epidermal growth factor receptor gene amplification and different characteristic of studied cases

	Multivariate		*P* value
	Odds ratio	95% confidence interval	
Pathology (adenocarcinoma)	0.452	0.236–0.857	0.025*
Smoking (nonsmoker)	0.294	0.033–0.416	0.019*
Sex (female)	0.536	0.364–0.842	0.043*
Age	0.657	0.208–2.365	0.321
Size	0.854	0.415–3.652	0.256
Site	0.698	0.306–2.067	0.218

*$P\leq 0.05$, statistically significant.

size also increases the risk of complications. Even on obtaining multiple specimens, these may be not enough to perform more detailed molecular analysis. So biopsy tool of choice should be safe and able to get large

biopsies without sample morphologic changes, thereby reducing the number of additional sampling and repeated bronchoscopies [1,4,14].

An inexpensive method for increasing biopsy yield is flexible cryobiopsy, which has most of these advantages. Cryobiopsy of endobronchial lesions has better diagnosis and less complications and operating time. Cryoprobe also facilitates sampling of tangentially positioned lesions to bronchoscope, which are difficult to sample with ordinary forceps [4,15–17].

Regarding the histological quality of cryobiopsies, in our study, we founded that tissue samples obtained by cryoprobe had larger size and better artifact-free areas compared with forceps biopsies ($P<0.001$). Our results were in accordance with our previous work that demonstrated cryotechnique gives both larger and also better quality specimens with less mechanical damage [5].

The same results were proved in many studies, such as Hetzel and colleagues, who stated that artifact-free area and molecular markers were well-preserved in cryobiopsies, enabling better immunohistochemical diagnostics whereas forceps biopsies exhibit crush artifacts, making diagnosis difficult, especially in small biopsy samples. Moreover, Schumann and colleagues showed the same advantage for cryobiopsy over forceps biopsy, with larger and qualitatively better quality specimens. Another study showed the mean volumes of sample by cryobiopsy significantly provided high diagnostic accuracy, in addition to sampling both exophytic and flat lesions. Other authors also described the total area of cryobiopsies samples was twice larger than forceps biopsies [4,7,18–21].

This may be explained by three factors: first, cryoprobe only touches the tissue, with no grasping of the sampled tissue, whereas forceps biopsy needs lesion squeezing; second, it applies cold effect at the probe's tip creating hemostasis, which results in artifact-free area, and also more freezing time increases size of sample, whereas forceps biopsies are limited by forceps claws size; and third, specimens are released in fresh frozen condition [7,22,23].

Regarding complications of cryobiopsy, as cartilage tissue is insensitive to freezing, the only complication is bleeding. Bleeding risk significantly increases with extra biopsies. A cooling time of 3 s is enough to gather a diagnostic sample without bleeding risk [24].

In our study, only mild bleeding occurred and was not statistically significant different between the two groups. This may be explained by vasoconstrictory effect and short bleeding duration, despite the larger defects created at the site of tissue sampling. Our results were in accordance with many previous studies. Most of these studies showed risk of bleeding was same with cryobiopsy and conventional methods ranging from 5, 6–18, 10 up to 28% as reported by Schumann and colleagues, Segmen and colleagues, Jabari and colleagues, and Eldahdouh and colleagues, respectively. On the contrary, only Hetzel and colleagues, showed mild bleeding (no intervention need for control) was significantly higher in cryobiopsy, but more severe bleeding was similar in both techniques [4,7,24–26].

EGFR is a cell surface tyrosine kinase receptor much expressed on both epithelial and stromal cells. EGFR overexpression occurs in many solid tumors, including NSCLC, with a prognostic value and correlation to disease progression and survival [27,28].

Our study showed that cryoiopsy was a safe method, with a better diagnostic yield (29.4%) compared with 8.8% forceps biopsy for determination of EGFR copy number.

FISH positivity of 29.4% in our study was in accordance with previous studies that reported high EGFR gene amplification in 7–45% of lung cancer. This wide range may be owing to variable methods and positivity criteria [8,29–32], but on adopting the same criteria in previous studies similar to our study, values ranged from 31 to 48% [8,33–35].

In agreement with our results, but with much higher results, was the study by Lee and colleagues regarding EGFR FISH positivity, as represented in 45% of patients with NSCLC, and also Pinter and colleagues concluded that EGFR gene copy number was increased in 40% of 118 patients. Unlike our study, Zhang and colleagues reported FISH was positive in 62% of cases [36–38].

We analyzed EGFR in relation to age and sex and revealed that amplification can be presented with lung cancer in younger patients and never smokers and in women more than men; these differences were statistically significant. These findings were in accordance with a previous work by Zhang *et al.* [38] who showed gene amplification was related to mutation, primarily occurring in females, nonsmokers, and patients with adenocarinomas (all $P<0.001$).

On the contrary, Gaber *et al.* [28] founded FISH positivity was more in adencarcinoma than squamous cell carcinoma but was not statistical significance and was not associated with age, sex, smoking, or tumor size. Other study by Sone *et al.* [34] observed FISH-positive results in adenocarcinomas and nonsmokers, not associated with sex, but were not statistically significant.

Conclusion

Bronchoscopic cryobiopsy might provide samples with better material than forceps biopsy allowing better diagnostic yield in detection of EGFR gene amplification.

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Conflicts of interest

There are no conflicts of interest.

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