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Original scientific article

IS THERE AN ADVANTAGE OF MONITORING VIA EXOSOME-BASED DETECTION OF EGFR MUTATIONS DURING TREATMENT IN NON-SMALL CELL LUNG CANCER PATIENTS?

Ege Riza KARAGUR[1], Aydin DEMIRAY[1], Nedim KARAGENC[1], Esra ELVER[3], Onur TOKGUN[1], Arzu YAREN[2], Gamze G. DOGU[2], Hakan AKCA[1*]

[1]Departmnet of Medical Genetic, School of Medicine Pamukkale University, Pamukkale, 20160 Denizli, Turkey
[2]Department of Medical Oncology, Pamukkale University, Pamukkale, 20160 Denizli, Turkey
[3]Graduate School of Health Sciences, Molecular Cancer Biology, Pamukkale University, Pamukkale, 20160 Denizli, Turkey

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We know that detection of EGFR mutations is very important for individual therapy. Nowadays FFPE samples are commonly using to detect the EGFR mutation status. But it has a few handicaps such as, tumor heterogeneity and non-repeatable, it is need to examine mutation statues of EGFR after each treatment regimen for individually treatment of NSCLC patients. Therefore, there is still need to develop non-invasive and useable over and over again approach for monitoring EGFR mutation statues and other genes for individual therapy. So, we aim to examine whether exosomes are good target for detection of EGFR mutation status or not. Pyrosequencing was used to detect, EGFR mutation in FFPE and exosome samples in some NSCLC patients. For the patients given different chemotherapy regime (n=28), PFS was evaluated before and after treatment. In patients who were EGFR positive before treatment, the median PFS for EGFR mutation-positive patients after treatment was 101.7 weeks (95% CI: 0.09-3.21), while for patients

Corresponding author: Hakan Akca, Pamukkale Ünivesitesi Tıbbi Genetik A.D. dekanlık binası kat:3 Pamukkale/Denizli-Turkey, Phone: 0(258) 296 16 68, E-mail: hakca@pau.edu.tr

who were negative after treatment, the median PFS was 42.43 weeks (95% CI: 0.31-10.52). Likewise, in patients who were EGFR negative before treatment and EGFR mutation negative after treatment, the PFS was median 52 weeks (95% CI: 0.17-2.84), while in patients who were positive after treatment, the median PFS was 27.57 weeks (95% CI: 0.35-5.58). We show that exosomes are good tools for monitoring EGFR mutation status and exosomes can be use as semi-invasive method for isolation of tumor DNAs for detection of mutation statues for individually treatment of NSCLC patients.

Keywords: Exosome RNA, Lung cancer, *EGFR*, NSCLC, PFS

INTRODUCTION

One of every five cases of cancer related death worldwide are caused by lung cancer (SUNG *et al*., 2021). Lung cancer is histologically divided into two classes as non-small-cell lung cancer and small cell lung cancer (NSCLC). NSCLC has been the most common subtypes of lung cancer and it is responsible for 80-85 % of all lung cancer diagnoses (OSER *et al*., 2015). Five-year survival rate of NSCLC has been only 15% ("Cancer Statistics Review, 1975-2016 - SEER Statistics," n.d.) because more than half of patients with NSCLC already have advanced disease at diagnosis. In the past decade, new therapeutic approaches about on mutation profiles, genetic rearrangement defects to diagnosis and therapy of NSCLC have been discovered (ARBOUR and RIELY, 2019). Epidermal growth factor receptor activating mutations take the first place in lung cancer. Researchers have obtained that there are epidermal growth factor receptor (EGFR) mutations 30% of NSCLC cases.

Tumor mutation burden is very important to determine therapy regime. Determining tumor mutation load from tumor biopsy has several disadvantages. Firstly, the procedure is invasive and can lead to insufficient sample collection, thus preventing proper analysis and requiring more intervention. Secondly, these samples have high tumor heterogeneity, since the mutated cells and the non-mutated cells are together in tumor biopsy. Biopsies from tumor tissue have been still the gold standard for both diagnosis and classification of lung cancer, although it has many disadvantages. Recently liquid biopsy concepts have been used to diagnosis as a non-invasive method in clinic. Currently, there are four major strategies for liquid biopsy using blood samples, namely circulating tumor cells, circulating tumor DNA (ctDNA), tumor-educated platelet and extracellular vesicles (EVs). Small membranous vesicles have been called as EVs and are classified into three subclasses: micro vesicles, exosomes and apoptotic bodies (RAPOSO and STOORVOGEL, 2013). Recently, certain exosomes, which are cancer derived EVs, were identified as large oncosomes. The size of this population was larger than those described previously (1–10 μm diameter) (CUI *et al*., 2018). Since, large oncosomes are secreted by tumor cell and tumor microenvironment, exosomes have a potential as predictive, prognostic, or diagnostic biomarker for lung cancer.

In this study, here we clearly showed that exosomes are more useful and more sensitive than tumor biopsy and have more tumor homogeneity than tumor biopsy. Therefore, exosome-based studies are crucial in the diagnosis and determination of the therapy regime for lung cancer.

MATERIAL AND METHOD

Assay design

The study consists of three steps: First step is DNA isolation (Qiagen) from tumor tissue of lung cancer patients to perform EGFR mutation analysis by pyrosequencing. The second step is the isolation of exosomal RNA from patient's plasma. There are two groups in this step. The first group is EGFR mutation positive patients receiving Tyrosine Kinase Inhibitor (TKI) therapy and the second one is EGFR mutation negative patients receiving at least three cure of platinum-based chemotherapy therapy. The third step is the assessment of the EGFR mutation profile analyzed from both tumor tissue and exosome with clinical data.

Clinical Samples

This study included a total 28 patients who enrolled the Pamukkale University Hospital. Patiens DNAs were isolated from Paraffin-embedded tissues (FFPE) by using Qiamp DNA FFPE Tissue kit (Qiagen GmBH, Hilden, Germany) and Exosomal RNA of identical patients were isolated from plasma by using Qiagene Exosoma RNA isolation Kit (Qiagen GmBH, Hilden, Germany) Quality of DNAs and ExoRNA was checked nanodrop (Thermo).

Reverse-Transcriptase PCR/PCR

RT^2 First Strand Kit (Qiagene) were used to perform cDNA synthesis of isolated ExoRNA and then quality/concentration of DNAs was adjusted in the range of 20-100 ng/uL. Samples were performed PCR from exon 18 codon 718, exon 19 del 19, exon 20 codon 768-790 and exon 21 codon 858+861 with biotin labeled primers to sequencing Pyrosequencing method according to the instructions of the manufacturer.

Pyrosequencing

To perform pyrosequencing reactions, biotinylated PCR products were immobilized onto streptavidin-coated beads (Streptavidin Sepharose GE Healthcare USA) by mixing the PCR product with Streptavidin Sepharose suspension and the appropriate amount of the binding buffer according to manufacturer's directions. Samples were denatured and cleaned both non-biotinylated DNA strand and artifacts, after immobilize pure single strand DNA was transferred to PyroMark Q24 plate which have in stock specific sequencing primer by using Workstation Tool (Qiagen). According to pre-run report enzymes, substrates and nucleotides were put down in a PyroMark Q24 Cartridge (Qiagen), pyrosequencing was performed by using PyroMarkQ24 instrument software after PyroMark Q24 plate and PyroMark Q24 Cartridge were placed in PyroMarkQ24 instrument. The runs were analyzed by using PyroMark report software.

Statistical Analyses

All statistical analyses were done using the Statistical Package for the Social Sciences (SPSS) version 23.0 (SPSS® IBM Corporation, Armonk, NY, USA) and Prism 7.2. For descriptive analyses, the $\chi 2$(Fisher-exact) were performed. Progression-free survival (PFS) analyses, according to EGFR mutations' status were done using the Kaplan Meier Method (McNemar) and estimating the survival difference with the use of long rank test. A value of $p < 0.05$ was accepted to be statistically significant.

RESULTS

We used the 28 patients with NSCLC in this study. The training cohort consist of 14 (50 %) squamous, 14 (50 %) adenocarcinoma and 13 (46.4 %) early stage and 15 (53.5%) advanced stage NSCLC patients. The mean age of the training cohort was 63 (range 29-77) years. 26 patients were male (91.7%) and 2 females (8.3%) and 23 (82.1%) had a history of smoking. Clinical and demographic characteristics of the patients were shown in Table 1.

Table 1. Clinical and demographic characteristics of the patients

Characteristics	Patients n= 28 (%)
Age (years)	
<62	9 (%33.3)
>62	19 (%66.6)
Sex	
Male	26 (91,7)
Female	2 (%8,3)
Smoking status	
Smokers	23 (%66.6)
Non-smokers	5 (%33.3)
Histopathology	
Adenocarcinoma	14 (50)
Squamous cell carcinoma	14 (%50)
Stages	
Early stage (I; II; IIIA)	13 (%33.3)
Advanced stage (IIIB; IV)	15 (%66.6)

EGFR mutations profiles (exon 18, 19, 20 and 21) of this patient cohort consisted of 28 was determined from FPEE tissue biopsy by using pyrosequencing method. Five patients had EGFR mutation (Δ746-750, L858R, L861Q and L861R) and twenty-three patients were wild type (Figure 1). All EGFR mutation positive patients have received first generation TKI. Others have received prior treatment at least three cure platinum-based combination chemotherapy.

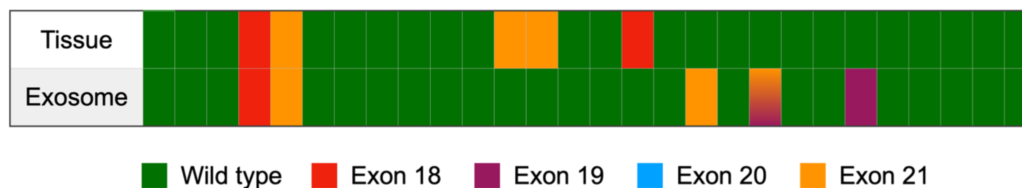

Figure 1. The mutation profiles of *EGFR* gene in patients with NSCLC (n=28).

Patients who received TKI were tested isolated exosomes from their blood for EGFR mutation analysis after the first progression, while the two patients' results are consistent with their pre-treatment results, the mutation profiles of the three patients differ (Figure 1). When we analyze relationship clinic data with mutation-based therapy, we have determined that patients who have not change mutation status have 101.7 weeks median of PFS (95% CI: 0.09-3.21) and the patient group with a difference in the mutation profile has a 42.43-week median of PFS (95% CI: 0.31-10.52) (Figure 3a).

EGFR wild type patients were tested exosomes from their blood for EGFR mutation analysis after at least three cure of platinum-based combination chemotherapy. And then we determined that three patients have new mutations. So, we have compared the PFS average of patients who have changed and not changed mutation status. This comparison shown that patients whose pre-treatment and post-treatment mutation status have not changed have 52 weeks median of PFS (95% CI: 0.17-2.84). Meanwhile, patients whose mutation status changes have low PFS times (median 27.57 weeks) because they cannot get full efficiency from the treatment regimen (Figure 3b).

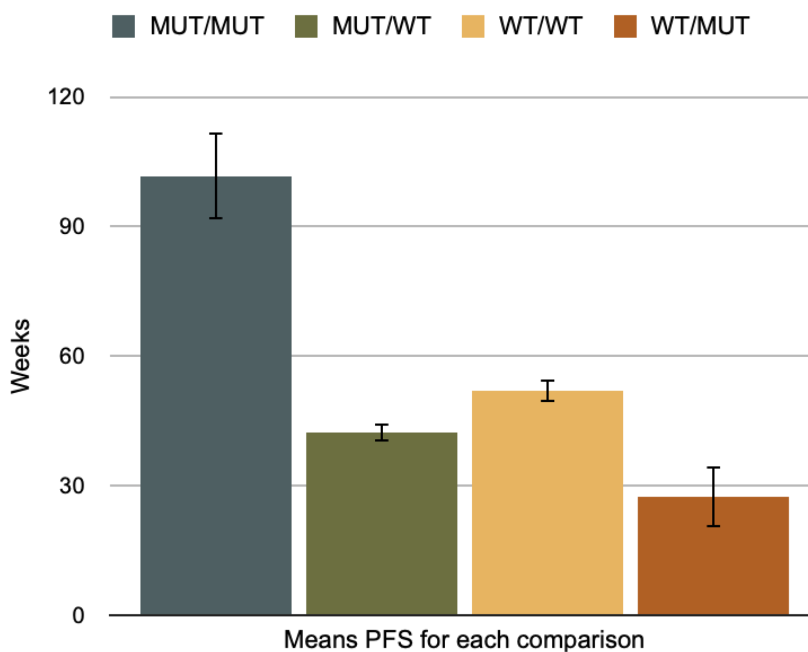

Figure 2. The graphic represent PFS means according to mutation profiles in patients with NSCLC (p<0.0001).

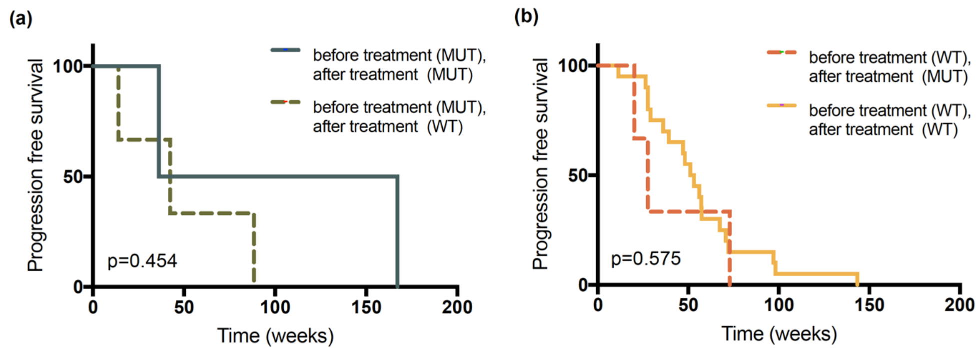

Figure 3. Kaplan-Meier progression-free survival curve of patients in the different two groups. (a) In the group of patients with positive EGFR before treatment, patients with positive and negative EGFR after treatment were compared. (b) In the group of patients with negative EGFR before treatment, patients with positive and negative EGFR after treatment were compared.

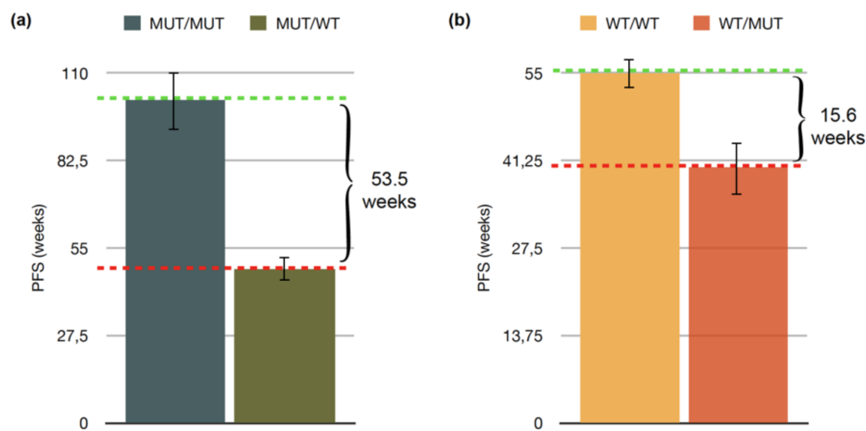

Figure 4. The graphic represents average PFS gain according to binary mutation profiles comparison in patients with NSCLC. (a) In the group of patients with positive EGFR before treatment, patients with positive and negative EGFR after treatment were compared ($p<0.0001$). (b) In the group of patients with negative EGFR before treatment, patients with positive and negative EGFR after treatment were compared ($p=0.0006$).

In this study, when the mutation profile of the patients' post-treatment and the PFS of the patients were evaluated, patients with the same mutation profile in both pre-treatment and post-treatment had higher PFS were determined (Figure 2). Moreover, when we evaluated the PFS gain in EGFR positive patients, we also observed almost 53.5 weeks of PFS gain (Figure 4a). On the other hand, when EGFR negative patients were analyzed, the results showed that this was 15.6 weeks PFS gain (Figure 4b).

As a result, monitoring the mutation status before and after treatment, even in different treatment regimens, will provide PFS or overall survival (OS) gain in patients, as it will enable more efficient treatment regimens to be determined.

DISCUSSION

Previous studies have shown that more than 300 mutations have been identified in lung cancer. In addition, the lung tumorigenesis was promoted by a few genes including Epidermal Growth Factor Receptor, Anaplastic Lymphoma Kinase (ALK), Hepatocyte Growth Factor Receptor (HGFR) (GELSOMINO *et al.*, 2014; SHAW and ENGELMAN, 2013; STEUER and RAMALINGAM, 2015; VOGELSTEIN *et al.*, 2013). It is well known that the presence of mutations in the kinase domain of the EGFR correlate with sensitivity to first generation TKIs. Therefore, determination of EGFR mutation status is important for more effective therapy in patients (STEUER and RAMALINGAM, 2015).

Nowadays both clinic researchers and pharmaceutical companies have focused on health gain, OS gain, and PFS gain (EVANS *et al.*, 2016). So, we questioned whether there was a gain or not, when EGFR mutation status of patients was followed regularly after the treatment. In line with this objective, we compared to the average PFS of patients with and without concordant mutation status before and after treatment. Firstly, we separated to main independent two groups as wild type and EGFR mutation positive. And then we followed up EGFR mutation status of each patient to determine evolution the mutation status after the treatment.

In our study, when we assessed EGFR mutations status in both tumor biopsies and exosomes, we have determined ten EGFR mutations (Δ746-750, G719S, G719C, L861R, L858R, L861Q) on three different exons in samples obtained by two different methods from the patient cohort. It was also detected that some patients did not have same mutation in their tumor biopsies and exosomes and some patients' mutations changed after treatment. This evolution of the mutation profile might be due to elimination of cells with mutations because the target specific treatment focused on to exterminate mutated cells generally.

In 23 patients with EGFR negative status before treatment, it has been identified 20 EGFR negative and 3 EGFR positive mutation (L861Q, G12S&L861Q and G719C) after treatment. When progression free survival of these 23 patients was compared, it was identified that PFS of the patients who haven't changed their mutational profile was 55.78 weeks and PFS of patients who changed their mutation profile was 40,19 weeks. Since, these three patients do not take an effective chemotherapy because of changed mutation profiles, they have poor PFS than others.

In 5 patients with EGFR positive before treatment, while two patients have been still EGFR positive (L858R and Δ746-750), three patients are EGFR negative. We compared to these 5 patients for PFS, as they have taken same chemotherapy regime since initial diagnosis. We notice that the patients being EGFR positive before and after treatment have high benefit for the

chemotherapy regime. The patients who are EGFR positive before and after treatment (101.50 weeks) have more prolonged PFS than the patients who have a discordant mutation profile before and after treatment (48 weeks) (Figure 4a).

We compared the PFS between two main groups. The patients having stable mutation status before and after the treatment have average 34.54 weeks PFS gain (Figure 2). We evaluated PFS of the patients for each main group detailed. Patients with EGFR mutations took a TKI treatment. Patients whose EGFR mutation profile did not change during treatment had a longer PFS (53.5 weeks) than patients whose mutation profile changed (Figure 4a). Likewise, patients without mutations before and after treatment regime have a longer PFS (15.6 weeks) than patients who maintained a mutation during treatment (Figure 4b).

Tumor biopsies were the only option to investigate mutations within patients and used to determine the EGFR mutations; however, despite advances in the biopsy methodology and technique, tumor biopsies have several disadvantages, such as the difficulty of taking tumor biopsies due to the location of the tumor, as well as possible errors in mutation profiling due to tumor heterogeneity, and it cannot be used as a monitoring method due to non-repeatable (CASTRO-GINER *et al*., 2018). Tumor heterogeneity in lung cancer is a common condition, especially in the early stage (stage I and stage II). This is because clones can generate distinct subpopulations or subclones that create tumor heterogeneity during tumor mass formation and expansion (MARUSYK and POLYAK, 2010). This situation emerges as one of the most pivotal challenges in detecting precancerous lesions, namely early diagnosis of lung cancer (KNIGHT *et al*., 2017). The recently studies show that the novel methods based on a liquid biopsy such as cfDNA, ctDNA and ExoRNA have been developed to determine mutation in lung cancer (LAZZARI *et al*., 2018). These new medical advances can make possible to detect and manage early stage cancer from a body fluid as well as non-invasive rather than a traditional biopsy (NIEVA and KUHN, 2012).

We have planned to do with exosomes in our study, because exosomes have strategic advantages in cancer treatment in that both glycosylated peptides on the exosome's surface are resistant to proteasome-mediated degradation in circulation and it contains specific RNAs, DNAs, proteins, lipids from the tumor microenvironment (HUNG and LEONARD, 2015; ZHOU *et al*., 2017). Also, exosomes are stable in the body fluid and have not tumor heterogeneity, it is a promising material for characterizing tumor behavior. Thus, early diagnosis of lung cancer can make thanks to exosome-derived proteins and traceable RNAs or DNAs (ZHANG *et al*., 2019). Moreover, the tumor cells are release abundant exosomes in comparison with non-tumor cells. These exosomes are known to be found in most body fluids such as blood, serum, urine, and cerebrospinal fluid (ZHAO *et al*., 2018). These results shown that the tumor-derived exosomes can be used as monitoring materials and, monitoring the mutation status before and after treatment, even in different treatment regimens, has advantages to determine more efficient treatment regimens and owing to the true treatment regimens can provide PFS or OS gain in patients.

CONCLUSION

The results show a high concordance between the molecular data described in this study and its post-treatment, a clinical evaluation. This compatibility allows us to confirm that an exosome, a variant of liquid biopsy (LB), is a valid technique today. In addition, according to

international guidelines, first-line EGFR-TKI treatment can be prescribed based on the results of liquid biopsy in patients for whom the surgical procedure or biopsy is not suitable or the amount of tumor tissue is insufficient. The emergence of new mutations related to drug resistance or the extinction of mutated tumor cells during treatment with anti-EGFR-TKI drugs correlated with PFS. Although this study has a small number of patients, these results suggested that monitoring for EGFR mutations during treatment in NSCLC can provide the clinician with an estimate of treatment responses, disease progression, and survival. For these reasons, we believe that determination of EGFR mutational status from the exosome during treatment will be used as a validated tool of clinical importance.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The patients' samples were obtained with the informed consent of all participants. The Pamukkale University review board of the Ethics committee for non-invasive Clinical Research approved, code 60116787-020/20928.

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DA LI POSTOJI PREDNOST MONITORINGA PREKO DETEKCIJE EGFR MUTACIJA TOKOM LEČENJA PACIJENATA SA RAKOM PLUĆA

Ege Riza KARAGUR[1], Aydin DEMIRAY[1], Nedim KARAGENC[1], Esra ELVER[3], Onur TOKGUN[1], Arzu YAREN[2], Gamze G. DOĞU[2], Hakan AKCA[1*]

[1]Departman za medicinsku genetiku, Medicinski fakultet Univerziteta Pamukale, Pamukale, 20160 Denizli, Turska
[2]Odsek za medicinsku onkologiju, Univerzitet Pamukale, Pamukale, 20160 Denizli, Turska
[3]Fakultet zdravstvenih nauka, molekularna biologija raka, Univerzitet Pamukale, Pamukale, 20160 Denizli, Turska

Izvod

Znamo da je detekcija EGFR mutacija veoma važna za individualnu terapiju. Danas se uzorci FFPE obično koriste za otkrivanje statusa mutacije EGFR. Ali zbog nekoliko nedostataka kao što su heterogenost tumora i neponovljivost, potrebno je ispitati mutacije EGFR nakon svakog režima lečenja za individualni tretman pacijenata sa NSCLC. Stoga, još uvek postoji potreba da se razvije neinvazivan i upotrebljiv pristup za praćenje mutacija EGFR i drugih gena za individualnu terapiju. Dakle, cilj nam je da ispitamo da li su egzozomi dobra meta za otkrivanje statusa mutacije EGFR ili ne. Pirosekvencija je korišćena za otkrivanje mutacije EGFR u FFPE i uzorcima egzozoma kod nekih pacijenata sa NSCLC. Za pacijente kojima je dat različit režim hemoterapije (n=28), PFS je procenjen pre i posle tretmana. Kod pacijenata koji su bili pozitivni na EGFR pre tretmana, medijana PFS za pacijente pozitivne na EGFR mutaciju nakon tretmana bila je 101,7 nedelja (95% CI: 0,09-3,21), dok je za pacijente koji su bili negativni nakon tretmana, medijana PFS bila 42,43 nedelje (95 % CI: 0,31-10,52). Slično, kod pacijenata koji su bili EGFR negativni pre tretmana i EGFR negativni nakon tretmana, PFS je bio medijana 52 nedelje (95% CI: 0,17-2,84), dok je kod pacijenata koji su bili pozitivni nakon tretmana, medijana PFS bio 27,57 nedelja (95 % CI: 0,35-5,58). Pokazali smo da su egzozomi dobri alati za praćenje statusa mutacije EGFR i da se mogu koristiti kao poluinvazivna metoda za izolaciju DNK tumora za detekciju mutacija za individualni tretman pacijenata sa NSCLC.

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