Clinical Chemistry 61:9
1191–1196 (2015)

Diagnostic Accuracy of Noninvasive Genotyping of *EGFR* in Lung Cancer Patients by Deep Sequencing of Plasma Cell-Free DNA

Junji Uchida,[1] Kikuya Kato,[2*] Yoji Kukita,[2] Toru Kumagai,[1] Kazumi Nishino,[1] Haruko Daga,[3] Izumi Nagatomo,[4] Takako Inoue,[1] Madoka Kimura,[1] Shigeyuki Oba,[5] Yuri Ito,[6] Koji Takeda,[3] and Fumio Imamura[1]

BACKGROUND: Genotyping of *EGFR* (epidermal growth factor receptor) mutations is indispensable for making therapeutic decisions regarding whether to use EGFR tyrosine kinase inhibitors (TKIs) for lung cancer. Because some cases might pose challenges for biopsy, noninvasive genotyping of *EGFR* in circulating tumor DNA (ctDNA) would be beneficial for lung cancer treatment.

METHODS: We developed a detection system for *EGFR* mutations in ctDNA by use of deep sequencing of plasma DNA. Mutations were searched in >100 000 reads obtained from each exon region. Parameters corresponding to the limit of detection and limit of quantification were used as the thresholds for mutation detection. We conducted a multi-institute prospective study to evaluate the detection system, enrolling 288 non–small cell lung cancer (NSCLC) patients.

RESULTS: In evaluating the performance of the detection system, we used the genotyping results from biopsy samples as a comparator: diagnostic sensitivity for exon 19 deletions, 50.9% (95% CI 37.9%–63.9%); diagnostic specificity for exon 19 deletions, 98.0% (88.5%–100%); sensitivity for the L858R mutation, 51.9% (38.7%–64.9%); and specificity for L858R, 94.1% (83.5%–98.6%). The overall sensitivities were as follows: all cases, 54.4% (44.8%–63.7%); stages IA–IIIA, 22.2% (11.5%–38.3%); and stages IIB–IV, 72.7% (60.9%–82.1%).

CONCLUSIONS: Deep sequencing of plasma DNA can be used for genotyping of *EGFR* in lung cancer patients. In particular, the high specificity of the system may enable a direct recommendation for EGFR-TKI on the basis of positive results with plasma DNA. Because sensitivity was low in early-stage NSCLC, the detection system is preferred for stage IIIB–IV NSCLC.

The efficacy of epidermal growth factor receptor (EGFR)[7] tyrosine kinase inhibitors (TKIs; e.g., gefitinib and erlotinib) in non–small cell lung cancers (NSCLCs) is correlated with the activation of somatic mutations in *EGFR* (epidermal growth factor receptor) *(1, 2)*. In Japan, first-line gefitinib for patients with mutation-positive NSCLC was demonstrated to prolong progression-free survival over standard chemotherapy *(3)*. Because of the high prevalence of mutation-positive NSCLCs in the Japanese population *(4)*, *EGFR* mutation testing, rather than a diagnostic test specific for EGFR-TKI therapy, is a routine clinical practice in many facilities.

Mutations are identified with biopsy samples obtained by bronchoscopy, computed tomography–guided biopsy, or surgical resection. For EGFR-TKI treatment, the presence of a resistant mutation, e.g., T790M *(5, 6)*, would be important clinical information. However, rebiopsy for recurrent/metastatic lesions is more complicated. Therefore, since the discovery of the correlation between the efficacy of EGFR-TKI and *EGFR* mutations, noninvasive procedures have been explored. In particular, circulating tumor DNA (ctDNA), which is cell-free DNA released from dying cancer cells into the blood, has been a focus of research. Because ctDNA includes DNA from metastatic lesions and primary lesions, it is

[1] Department of Thoracic Oncology, [2] Department of Molecular and Medical Genetics, Research Institute, and [6] Center for Cancer Control and Statistics, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan; [3] Department of Clinical Oncology, Osaka City General Hospital, Osaka, Japan; [4] Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, Osaka, Japan; [5] Graduate School of Informatics, Kyoto University, Japan Science and Technology, Kyoto, Japan.

* Address correspondence to this author at: Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3 Nakamichi, Higasninari-ku, Osaka, 537-8511, Japan. Fax +81-66-9735691; e-mail katou-ki@mc.pref.osaka.jp.

Received March 24, 2015; accepted June 24, 2015.
Previously published online at DOI: 10.1373/clinchem.2015.241414

[7] Nonstandard abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; NSCLC, non–small cell lung cancer; ctDNA, circulating tumor DNA; NGS, next-generation sequencing; PNA-LNA, peptide nucleic acid/locked nucleic acid; PM, plasma mutation; LOD, limit of detection; LOQ, limit of quantification.

expected to indicate mutations in lesions other than the biopsy sites. Recent metaanalysis of 27 studies involving 3110 participants demonstrated the feasibility of noninvasive genotyping with cell-free DNA *(7)*. However, although various technological approaches have been examined *(8–11)*, no approach has reached the level of practical use.

The current attention to and expectations for ctDNA have arisen mainly from technical developments, especially digital PCR *(12)* and related technologies, such as next-generation sequencing (NGS). Because massively parallel sequencers (the representative type of next-generation sequencers) use digital PCR in the template preparation step, we are able to use them in the same way as other digital PCR technologies. In addition to its high sensitivity, digital PCR could quantify the fraction of mutant alleles. In spite of the high expectations, there have been no clinical trials to demonstrate the utility of these technologies.

Previously, we constructed a detection system for *EGFR* mutations in ctDNA by use of deep sequencing with a massively parallel DNA sequencer *(13)*, the Ion Torrent PGM *(14)*. In this multi-institute prospective study, we demonstrated that genotyping with the system was practical, assessing the diagnostic sensitivity and specificity of the genotyping with a biopsy sample as a comparator. In particular, the high specificity of the system enables a direct recommendation for EGFR-TKI on the basis of positive results with plasma DNA.

Materials and Methods

PATIENTS

Lung cancer patients enrolled in the multi-institute prospective study (University Hospital Medical Information Network Clinical Trials Registry UMIN000006762) had no prior treatment and had visited 1 of the following 3 hospitals from November 24, 2011, to May 7, 2014: Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka City General Hospital, or Osaka University Hospital. Only patients whose biopsy samples contained cancer cells were registered. The target for patient recruitment was set at 320 to determine the 95% CI of overall sensitivity within a 20% range under the assumption that the overall sensitivity was 75% and the proportion of *EGFR* mutation–positive patients was 25%. The overall sensitivity was defined as the proportion of mutation-positive plasma samples in mutation-positive biopsy samples and did not require the samples to have an identical mutation type. We recruited 321 patients; 23 lacked biopsy samples for the *EGFR* mutation test, and 10 lacked plasma samples. Therefore, we obtained 288 pairs of samples.

The *EGFR* mutation status of biopsy samples was determined as routine medical practice by an outside service provider (LSI Medience Corp.) with the peptide nucleic acid/locked nucleic acid (PNA-LNA) clamp method *(15)*. Biopsy samples were frozen and transferred to the service provider after pathologic confirmation of cancer cells. The time interval between biopsy and blood sampling was ≤2 weeks. Written informed consent was obtained from all the patients. This study was approved by the ethics committees of each institution.

PLASMA DNA PREPARATION AND DEEP SEQUENCING

Approximately 5 mL blood, with EDTA as an anticoagulant, was taken from each patient. The plasma was separated from the blood cells via low-speed centrifugation within 3 h in clinical laboratories of the hospitals. The plasma was sent to the Research Institute at Osaka Medical Center for Cancer and Cardiovascular Diseases, where it was transferred to a fresh tube and recentrifuged at 15 100*g* for 10 min at room temperature and stored at −80 °C until DNA extraction. We allowed refrigerated overnight storage before the transfer; we previously confirmed no detectable deterioration of recovered DNA after overnight storage.

Laboratory procedures were the same as previously described *(13)* except that the current study used the latest versions of sequencing reagents. Deep sequencing was performed from November 2012 to July 2014 at a rate of 12–24 samples per week. The assay was performed on samples from various projects in order of arrival to the laboratory. The initial PCR amplification of *EGFR* exon fragments was successful with all the samples, and mutation data were obtained from all the samples. All laboratory work was performed by trained technical staff.

DATA ANALYSIS

We processed output data from the Ion Torrent PGM as previously described *(13)* except that we used revised templates for the identification of exon 19 deletions. We calculated 95% CIs by the method of Agresti and Coull *(16)*. All the clinical data and assay results of the PNA-LNA clamp method were collected by J. Uchida in the Department of Thoracic Oncology. All the assay results of plasma DNA were collected by Y. Kukita in the Research Institute. We combined the 2 data sets after completion of the data collection.

Results

PRINCIPLE OF MUTATION DETECTION

Exons 19, 20, and 21 of the *EGFR* gene were independently amplified with PCR from patient plasma DNA, and deep sequencing was performed with the Ion Torrent PGM *(14)*. More than 100 000 reads were obtained for each exon region. Because each read was of a single molecule, we were able to estimate the relative ratio of mutation alleles from the fraction of reads containing dele-

tions/substitutions. A diagnostic score, termed the plasma mutation (PM) score, was defined as the number of reads with deletions (exon 19 deletions) or substitutions (exon 20, T790M; exon 21, L858R) in 100 000 reads. In the previous study, we deduced parameters corresponding to the limit of detection (LOD) and limit of quantification (LOQ) *(13)*. The LOD was based on anomaly detection and was set as the PM score that was significantly higher than background error. Background error was determined from sequence reads of *EGFR* exon fragments obtained from white blood cells or plasma DNA from 48 healthy individuals. The probability of the PM score to be background error was calculated with statistical models, and the LOD was set at the probability level $P = 2 \times 10^{-5}$. The PM score of the LOD was 7 for both exon 19 deletion and L858R and was 60 for T790M. The LOQ was deduced from reproducibility experiments with plasma DNA from NSCLC patients. The PM score of the LOQ was set as 300 for all 3 mutations. Initially, the LOD was used for the threshold of mutation detection.

SENSITIVITY AND SPECIFICITY OF ACTIVATING MUTATION DETECTION

The major clinical characteristics of this patient population are shown in Table 1. The methods of biopsy sampling are summarized in Table 2.

Of the activating *EGFR* mutations in primary tumors, exon 19 deletion and L858R are the most common. The number of minor mutation types was too small for proper statistical analysis. Therefore, we focused on these 2 mutation types in the following analysis, classifying 1 G729A, 2 G729C, and 4 L861Q cases as wild-type.

To prevent potential confusion caused by double mutations or conflicting mutation types between biopsy and plasma samples, we evaluated exon 19 deletions and L858R individually. Unlike sensitivity, the evaluation of specificity is not straightforward. The biopsy samples categorized as wild-type included those judged as wild-type by successful sampling and assaying as well as samples that could not be categorized as mutants owing to failures in sampling/assaying. For an accurate evaluation of the specificity of the detection system, it is necessary to exclude the latter. Mutation-positive biopsy samples satisfied this criterion because these samples were guaranteed to have been successfully sampled and assayed. Because assays of exon 19 deletion and L858R are independent and the double mutation is rare, exon 19 deletion–positive and L858R-negative samples could be used to evaluate the specificity of L858R, and exon 19 deletion–negative and L858R-positive samples could be used to evaluate the specificity of the exon 19 deletion.

The mutation types of the 288 patients on the basis of the LOD or LOQ as the threshold for mutation detection are shown in Table 3. Applying the LOD for the

Table 1. Patient characteristics.

Characteristic	n
Sex	
Male	169
Female	119
Age, years	
≤49	11
50–59	55
60–69	109
70–79	92
80–89	20
Unknown	1
Stage	
I	64
IA	45
IB	18
IA or IB	1
II	19
IIA	13
IIB	6
III	53
IIIA	27
IIIB	26
IV	146
Undetermined	6
Histology	
Adenocarcinoma	274
Squamous cell carcinoma	7
Adenosquamous cell carcinoma	2
Other	5
Type of treatment	
Gefitinib	46
Gefitinib and pemetrexed	2
Erlotinib	15
Effect of EGFR-TKI	
Complete response	3
Mutation positive in plasma	1
Partial response	37
Mutation positive in plasma	24
Stable disease	12
Mutation positive in plasma	10
Progressive disease	3
Mutation positive in plasma	2
Not evaluable	6
Mutation positive in plasma	4
Unknown	2
Mutation positive in plasma	2

Table 2. Methods for biopsy sampling.

Tissue and method	n
Primary lesion	
Bronchoscopy	
Cytodiagnostic brushing/curette washing	230
FFPE	4
Tissue, no fixation	3
Unknown	1
Surgery	
FFPE	12
Tissue, no fixation	3
Needle biopsy	
Needle washing solution	3
FFPE	1
Metastatic lesion	
Tumor	
Bronchoscopy cytodiagnostic brushing/curette washing	12
Aspiration biopsy	2
Surgery, FFPE	2
Other lesions	
Pleural effusion	11
Lymph node	3
Bone	1

threshold, the sensitivity and specificity of the exon 19 deletion were calculated as follows:

Sensitivity (%) = 100 × (32 + 2 + 2)/(16 + 32 + 1 + 2 + 2) = 67.9;

Table 3. Biopsy and plasma mutation types.

	Plasma			
Biopsy	Wild-type	Exon 19 deletion	L858R	Double
LOD				
Wild-type	133	35	12	5
Exon 19 deletion	16	32	1	2
L858R	19	4	18	9
Double	0	2	0	0
LOQ				
Wild-type	177	7	1	0
Exon 19 deletion	25	26	0	0
L858R	31	0	18	1
Double	1	1	0	0

Data are n. Double, exon 19 deletion and L858R double mutation.

Table 4. Biopsy and plasma mutation types under the final threshold setting.

	Plasma			
Biopsy	Wild-type	Exon 19 deletion	L858R	Double
All cases				
Wild-type	163	5	15	2
Exon 19 deletion	23	25	2	1
L858R	23	0	26	1
Double	1	1	0	0
Stage I–IIIA				
Wild-type	65	0	8	1
Exon 19 deletion	13	2	2	0
L858R	15	0	3	1
Double	0	0	0	0
Stage IIIB–IV				
Wild-type	93	5	7	1
Exon 19 deletion	10	23	0	1
L858R	7	0	23	0
Double	1	1	0	0

Data are n.

Specificity (%): 100 × [1 − (4 + 9)/(19 + 4 + 18 + 9)] = 74.0.

This specificity was not adequate for practical use. High specificity is required for making treatment decisions regarding EGFR-TKI that are based directly on positive plasma results. Because we suspected that the background error rate of plasma DNA from cancer patients might be higher, we changed the threshold from the LOD to the LOQ for exon 19 deletions. The sensitivity and specificity of L858R with the LOQ were 36.5% and 100%, respectively. Because the number of false positives with the LOD was acceptable and loss of true positives with the LOQ was considerable, we continued to use the LOD for L858R as the threshold. Table 4 shows the number of patients classified with the final threshold setting. The sensitivity and specificity for exon 19 deletions were 50.9% (95% CI 37.9%–63.9%) and 98.0% (88.5%–100%), respectively. The sensitivity and specificity for L858R were 51.9% (38.7%–64.9%) and 94.1% (83.5%–98.6%). The overall sensitivity, as defined in Materials and Methods, was 54.4% (44.8%–63.7%).

By use of the specificity values, we estimated the number of false positives in the wild-type biopsy samples. There were 4 false positives for the exon 19 deletion and 11 for L858R. The observed false positives (Table 4, top) were slightly higher (7 and 17, respectively). These results might

indicate the presence of false-negative biopsy samples, although the number of these false negatives would be small.

Sensitivity had a strong bias with stage (Table 4). The overall sensitivity of stages IIIB–IV was 72.7% (60.9%–82.1%), and that of stages IA–IIIA was 22.2% (11.5%–38.3%). There were fewer mutation-positive plasma samples with patients who responded to EGFR-TKI (Table 1): complete and partial responses, 62.5% (47.0%–75.8%); stable and progressive diseases, 80.0% (54.1%–93.7%).

SPECIFICITY OF RESISTANT MUTATION DETECTION

The T790M mutation in primary lesions is rare *(17)*. In our patient population, there were no positive biopsy samples. Among 103 exon 19 deletion/L858R-positive cases with biopsy samples, the PM score exceeded the LOD in 6 cases and exceeded the LOQ in 1 case. Thus, the specificity of T790M is 94.2% (87.6%–97.6%) and 99.0% (94.2%–100%) with the LOD and LOQ as the thresholds, respectively.

Discussion

The objective of this multi-institute prospective study was to evaluate the detection system for 2 major purposes, i.e., genotyping of *EGFR* for treatment decision and monitoring of disease course with the expression levels of ctDNA. The results are worthy for cross-validation trials of different laboratories by use of the same preanalytic and analytic strategies. The high specificity of the system may enable direct recommendation of EGFR-TKI on the basis of positive results with plasma DNA. Because sensitivity was low in early-stage NSCLC, as observed in other cancers *(18)*, the detection system is preferred for stage IIIB–IV NSCLC. This feature does not affect practical use, because IA–IIIA NSCLC patients are not usually recipients of EGFR-TKI. Candidate recipients include patients with lesions difficult for biopsy sampling, those with obscure biopsy results, and those who prefer not to have biopsy. The presence of intertumor heterogeneity of *EGFR* mutations *(19)* indicates that the mutation status of primary lesions may not represent that of metastatic lesions. Because ctDNA includes DNA from both lesions, mutation screening with plasma DNA may be beneficial to detect mutations in lesions other than biopsy sites.

The detection system could be used for monitoring of disease course by performing assays at multiple time points with the same patient. The confidence of each data point being a true positive would be estimated from the specificity values obtained in this study. In particular, monitoring T790M is important to predict the presence of acquired resistance. A new generation of EGFR-TKIs, designed for T790M-positive EGFR, requires the selection of patients on the basis of their T790M status *(20)*. An emerging idea is the use of plasma DNA as a replacement for rebiopsy. PM scores over the thresholds could predict the presence of T790M in plasma DNA, with confidence represented by the specificity values.

Setting the threshold is the most important issue for mutation detection with digital PCR/NGS. The major focus of current studies *(21, 22)* is monitoring of disease courses, which does not require strict control of false positives. Such studies tend to use assays with increased sensitivity; an example is the assay in Bettegowda et al. *(18)*. Early detection and genotyping requires strict control of false positives. Increasing sensitivity is accompanied by decreasing specificity as a tradeoff, and vice versa, which is a common problem in the development of diagnostic tests. Determination of the optimum threshold with a learning data set is a common method. However, this approach is not preferable in our case because it requires an additional large number of patients for the learning data set. In addition, because the ctDNA assay is expected to detect mutations in lesions other than biopsy samples, results with biopsy samples are not necessarily a gold standard. Therefore, we used the intrinsic parameters of the detection system, i.e., the LOD and LOQ, for the threshold.

We changed the threshold value for the exon 19 deletion during the data analysis. The bias introduced by the change was small because the LOQ was already described in the previous study *(13)* and did not use any data from the current study. The performance of the detection system would be unchanged with future samples.

Like other analysis methods, the PNA-LNA clamp method is not free of false positives and negatives. However, the incidence of false positives in exon 19 deletion cannot be explained as a consequence of the false negatives in the biopsy samples. Because determination of the LOD was based on the samples from healthy individuals, the cause of the phenomenon is likely to be unidentified factors in plasma DNA of NSCLC patients that affect PCR/sequencing quality. The number of false positives in L858R and T790M is within the range explained by intertumor heterogeneity and false negatives of the reference. Because deep sequencing and the PNA-LNA clamp method were optimized for different sample types, it is not adequate to compare these technologies with our results.

Douillard et al. reported genotyping of *EGFR* in plasma DNA of lung cancer patients with a commercial kit for tissue samples *(23)*. That study was retrospective and was accompanied by changes in the assay protocol and by complicated patient selection processes. Although the actual performance of the approach awaits a prospective or prospective-retrospective analysis, the sensitivity and specificity were 65.7% and 99.8%, respectively. The pooled sensitivity and specificity deduced in the meta-analysis of 27 studies were 62.0% and 95.9%, respectively *(7)*. It should be noted that the patient populations

of these studies were different from that of our study: Douillard et al. enrolled locally advanced and metastatic lung cancer; 12 of the 27 studies in the metaanalysis enrolled advanced lung cancer only, but 8 studies enrolled all stages. The major drawback of the kit used by Douillard et al. *(23)* is the inability to quantify the data because this technology is unable to access the wealth of information obtained from the dynamics of ctDNA. In the metaanalysis, only 2 studies used quantitative analysis techniques.

Our approach presented in this study and the previous study could be applied to any rare mutation detection with digital PCR/NGS. Early detection requires better diagnostic accuracy and may require better methods to determine the LOD.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: This work was supported by the Osaka Foundation for the Prevention of Cancer and Cardiovascular Diseases.
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.
Acknowledgments: The authors thank Shiho Sasaki for her excellent technical assistance.

References

1. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
2. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004; 304:1497–500.
3. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010;362:2380–8.
4. Mitsudomi T, Yatabe Y. Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. Cancer Sci 2007;98:1817–24.
5. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
6. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:e73.
7. Qiu M, Wang J, Xu Y, Ding X, Li M, Jiang F, et al. Circulating tumor DNA is effective for the detection of EGFR mutation in non-small cell lung cancer: a meta-analysis. Cancer Epidemiol Biomarkers Prev 2015;24: 206–12.
8. Bai H, Mao L, Wang HS, Zhao J, Yang L, An TT, et al. Epidermal growth factor receptor mutations in plasma DNA samples predict tumor response in Chinese patients with stages IIIB to IV non-small-cell lung cancer. J Clin Oncol 2009;27:2653–9.
9. Brevet M, Johnson ML, Azzoli CG, Ladanyi M. Detection of EGFR mutations in plasma DNA from lung cancer patients by mass spectrometry genotyping is predictive of tumor EGFR status and response to EGFR inhibitors. Lung Cancer 2011;73:96–102.
10. Kimura H, Suminoe M, Kasahara K, Sone T, Araya T, Tamori S, et al. Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA). Br J Cancer 2007;97: 778–84.
11. Taniguchi K, Uchida J, Nishino K, Kumagai T, Okuyama T, Okami J, et al. Quantitative detection of EGFR mutations in circulating tumor DNA derived from lung adenocarcinomas. Clin Cancer Res 2011;17:7808–15.
12. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999;96:9236–41.
13. Kukita Y, Uchida J, Oba S, Nishino K, Kumagai T, Taniguchi K, et al. Quantitative identification of mutant alleles derived from lung cancer in plasma cell-free DNA via anomaly detection using deep sequencing data. PLoS One 2013;8:e81468.
14. Rothberg JM, Hinz W, Rearick TM, Schultz J, Mileski W, Davey M, et al. An integrated semiconductor device enabling non-optical genome sequencing. Nature 2011; 475:348–52.
15. Nagai Y, Miyazawa H, Huqun, Tanaka T, Udagawa K, Kato M, et al. Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. Cancer Res 2005;65:7276–82.
16. Agresti A, Coull BA. Approximate is better than 'exact' for interval estimation of binomial proportions. American Statistician 1998;52:119–26.
17. Inukai M, Toyooka S, Ito S, Asano H, Ichihara S, Soh J, et al. Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. Cancer Res 2006;66:7854–8.
18. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014;6:224ra24.
19. Chen ZY, Zhong WZ, Zhang XC, Su J, Yang XN, Chen ZH, et al. EGFR mutation heterogeneity and the mixed response to EGFR tyrosine kinase inhibitors of lung adenocarcinomas. Oncologist 2012;17:978–85.
20. Chong CR, Janne PA. The quest to overcome resistance to EGFR-targeted therapies in cancer. Nat Med 2013; 19:1389–400.
21. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;368: 1199–209.
22. Diaz LA Jr, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012;486:537–40.
23. Douillard JY, Ostoros G, Cobo M, Ciuleanu T, Cole R, McWalter G, et al. Gefitinib treatment in EGFR mutated Caucasian NSCLC: circulating-free tumor DNA as a surrogate for determination of EGFR status. J Thorac Oncol 2014;9:1345–53.