Detection of the *EGFR* G719S mutation in non-small cell lung cancer using Droplet Digital PCR

Margalida Esteva-Socias[1,2], Mónica Enver-Sumaya[3], Cristina Gómez-Bellvert[3,4], Mónica Guillot[5], Aitor Azkárate[3,5], Raquel Marsé[3,5], Víctor José Asensio[6,7], Josefa Terrassa[3,5] Antònia Obrador-Hevia[3,6]*

[1]Centro de Investigación Biomédica en Red in Respiratory Diseases (CIBERES), Plataforma Biobanco Pulmonar CIBERES, Hospital Universitari Son Espases, Palma, Spain.

[2]Grupo de Inflamación, reparación y cáncer en enfermedades respiratorias, Institut d'Investigació Sanitària de les Illes Balears (IdISBa), Hospital Universitari Son Espases, Palma, Spain.

[3]Group of Advanced Therapies and Biomarkers in Clinical Oncology, Institut d'Investigació Sanitària de les Illes Balears (IdISBa), Hospital Universitari Son Espases, Palma, Spain.

[4]Pathology Department, Hospital Universitari Son Espases, Palma, Spain.

[5]Oncology department, Hospital Universitari Son Espases, Palma, Spain

[6]Molecular Diagnosis and Clinical Genetics Unit, Hospital Universitari Son Espases, Palma, Spain.

[7]Grupo Genòmica de la Salut, Institut d'Investigació Sanitària de les Illes Balears (IdISBa), Hospital Universitari Son Espases, Palma, Spain.

*** Correspondence:**
Antònia Obrador Hevia
antonia.obrador@ssib.es

Keywords: *EGFR*, liquid biopsy, G719S, lung cancer, droplet digital PCR.

Word count: 2947

Abstract

Objectives: The main objectives of the study were 1) to set-up a droplet digital PCR (ddPCR) assay for the non-invasive detection of G719S EGFR mutation in NSCLC patients; 2) to determine the limits of detection of the ddPCR assay for G719S mutation and 3) to compare COBAS® and ddPCR System for G719S quantification in plasma.

Materials and methods: Blood samples were collected from 19 patients diagnosed with clinical stage IVA or IVB NSCLC according to the TNM Classification of Malignant Tumors. Then, plasma ctDNA was extracted with the Qiagen Circulating Nucleic Acids kit and quantified by QuantiFluor® dsDNA System. The mutational study of EGFR was carried out by digital droplet PCR (ddPCR) with the QX200 Droplet Digital PCR System with specific probes and primers.

Results: We observed the lowest percentage of G719S mutant allele could be detected in a wildtype background was 0,058%. In the specificity analysis, low levels of G719S mutation were detected in healthy volunteers with a peak of 21.65 mutant copies per millilitre of plasma and 6.35 MAFs. In those patients whose tissue biopsy was positive for G719S mutation, mutant alleles could also be detected in plasma using both ddPCR and COBAS® System. Finally, when mutational status was studied using both genotyping techniques, higher mutant copies/ml and higher mutant allele fraction (MAF) correlated with higher Semiquantitative Index obtained by COBAS®.

Conclusions: Although tissue biopsies cannot be replaced due to the large amount of information they provide regarding tumor type and structure, liquid biopsy and ddPCR represents a new promising strategy for genetic analysis of tumors from plasma samples. In the present study, G719S mutation was detected in a highly sensitive manner, allowing its monitorization with a non-invasive technique.

1 Introduction

Cancer is the second leading cause of death in developed countries (1) and lung cancer is the leading cause of cancer death in Europe. Metastatic lung cancer patients surviving for five years are less than 15% (2). 87% of all cases of lung cancer are non-small cell lung cancers (NSCLC). In order to improve survival of patients, research has focused on understanding the biology of tumors to develop targeted therapies and personalized medicine.

In NSCLC several recurrent mutations in genes involved in proliferation, apoptosis, cell survival and angiogenesis have been reported. One of the most important deregulated genes in NSCLC is *EGFR* (Epidermal Growth Factor Receptor). Genetic analysis of NSCLC tumors, especially adenocarcinomas, revealed that around 17% of them harbored *EGFR* mutations (3). About 90% of these mutations are small deletions in 5 amino acids from codon 746 to 750 of exon 19 or missense mutations at codon 858 of exon 21 (4,5). Moreover, less frequent mutations have been found like the mutation within the phosphate-binding loop (P-loop) that comprises part of the ATP-binding pocket which replaces Gly719 with Ser (G719S) (6,7). All of these mutations produce a gain of function. NSCLC cells become dependent on this aberrant signaling and inhibition with tyrosine kinase inhibitors (TKIs) specific for *EGFR* like erlotinib and gefitinib drive to cell death through intrinsic apoptosis (8,9). Unfortunately, TKIs effects are limited because of resistance occurrence due to several mechanisms, one of which being secondary resistance mutations in *EGFR* (normally T790M mutation) (10,11).

Screening for mutations in *EGFR* follows two objectives: selection of patients for treatment with TKIs and detection of resistance mechanisms. Tumor biopsies are the gold standard method for detecting these mutations. However, they are spatially and temporary limited due to: biopsies are invasive, often difficult to perform, do not reflect the entire tumor or different metastasis (12,13). Liquid biopsy for the study of circulating tumor DNA (ctDNA) is being developed to overcome some of these limitations (14,15). In this study, we have developed a method for detecting G719S mutation in liquid biopsy by means of digital droplet PCR technology.

2 Methods

2.1 Patients

Nineteen patients diagnosed with clinical stage IVA and IVB non-small cell lung cancer according to the TNM Classification of Malignant Tumours (8th Edition) were recruited to the study from

Hospital Universitari Son Espases (HUSE) from October 2015 to September 2016. The study was approved by the Clinical Research Ethics Committee of the Balearic Islands (CEIC-IB) and a written informed consent was acquired from all patients for specimen collection, clinical information collection and biomarker analysis in tissue and plasma samples. Clinical and pathological features of patients enrolled are provided in **Table 1.** Patients were eligible for the study according to the following selection criteria: histological confirmation of NSCLC in stages IIIB or IV ineradicable, functional state 0-2 according to Performance Status (PS) and patients of both sexes, aged over 18 and belonging to any ethnic group. Pregnant or breastfeeding women and patients with other antecedent of solid or haematological tumors in the previous five years, except for basal cell carcinoma, were excluded. Six healthy volunteers with no known significant health problems were also included in the study.

Tumor genotyping of *EGFR* mutation was carried out in the HUSE Pathology Department using DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue and COBAS ® 4800 system (Roche).

2.2 Plasma collection and DNA extraction

Blood samples were collected in Vacutainer EDTA tubes and immediately separated into plasma by centrifugation at 3000 rpm for 10 minutes at room temperature. Plasma samples were stored in 2 mL aliquots at -80ºC until ctDNA extraction. We analysed the samples corresponding to the dates of baseline, first month and third month after treatment and progressive disease.

ctDNA extraction was performed using 2 mL of plasma from each patient using the Qiagen Circulating Nucleic Acids kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. Extracted ctDNA from each plasma sample was twice eluted in 100 and 50 µL of AVE elution buffer and stored at -20ºC until mutation profiling. Quantification was performed by QuantiFluor® dsDNA System (Promega Corporation, Alcobendas, Madrid) using 4,8 µL of sample diluted 1/50 with TE 1x buffer (included in kit) following the manufacturer's instructions. Fluorescence measurement was carried out by multiple well spectrophotometer (BioTek, Winooski, VT, USA) and DNA concentrations were obtained in ng/µL.

2.3 G719S mutation detection in ctDNA

Mutation analysis was carried out with droplet digital PCR (ddPCR) System (Bio-Rad). The reaction mix was prepared using 10 µL from SuperMix for Probes without dUTP (Bio-Rad), 1 µL from each probe at 5 µM (HEX for the WT and FAM for the mutant), 1 µL from each primer at 9 µM (Table S1) and 6 µL from DNA extraction (concentration varies according to the sample used). A total of 20 µL were charged in the QX200 droplet generator (Bio-Rad) and immediately transferred to a 96-well plate through and amplified in a conventional thermal cycler. After PCR reaction, plate was placed in the QX200 reader (Bio-Rad) and data analysis was carried out with Quantasoft ™ Analysis Pro Software 1.0.596 (Bio-Rad). For each sample, detected droplets from triplicates were merged into 1 metawell. Wild-type and mutant allele concentrations present in the original blood samples were calculated using the following algorithm:

$$C_{ORIG} = \frac{20\ x\ C_I\ x\ V_E}{V_P\ x\ V_O}$$

where C_{ORIG} represents mutant allele concentration in the original plasma sample in copies/mL, V_E is the elution volume of ctDNA generated by the DNA extraction (100 mL); V_P is the volume of elution

of DNA used in the PCR reaction (µL); V_O is the volume of plasma used to extract ctDNA (2mL). The value of 20 located in the numerator of the equation corresponds to the final volume PCR mix, which was 20 µL.

Mutant-allele fraction (MAF) data was also calculated as (16):

$$MAF = \frac{mutant\ reads}{mutant\ reads + wild - type\ reads}$$

2.4 Limit of detection calculation

To determine the limit of detection (LOD), DNA template extracted from FFPE G719S mutant were serially diluted with wild-type DNA at levels of 0.003, 0.03, 0.3, 3 and 30% using a total of 25ng per well. The LOD was defined as the MAF or the lowest % of mutant allele that can be reliably detected (17).

2.5 Statistical analysis

Data analysis was carried out with the IBM SPSS Statistics 22 software and the graphical representation was performed with GraphPad Prism 5. For the comparative analysis of COBAS and ddPCR results, we applied the Kappa statistic to determine the measure of agreement between variables.

3 Results

3.1 Validation and sensitivity of G719S testing with ddPCR

G719S ddPCR assay was tested across an annealing temperature gradient to optimise thermocycling conditions. In order to perform it, we used DNA from positive tumor biopsies, confirmed by COBAS ®4800 System, of patients diagnosed with advanced stage of NSCLC. The temperature range studied came from 57 to 67ºC and the experiment was repeated twice. Decreasing annealing temperature increased FAM amplitude of the mutant probe and showed a good separation between the four droplet groups to plateau at 57.8ºC, allowing clear identification and quantification of both mutant and wild-type droplet groups (**Figure 1A**).

To calculate empirically the limit of detection we serially diluted positive mutant control DNA (from FFPE tissue samples) in a background of wild-type DNA. Mutant DNA was two-fold diluted, using 10 ng as initial amount. Total amount of DNA (mutant plus wild-type) was maintained in 25 ng per well.

The limit of detection was considered as the dilution that shows a statistically significant difference from the negative controls or the lowest mutant concentration detected where the lower error bar of the measured mutant concentration does not overlap with the upper error bar of the measured mutant concentration in the wild-type-only (mutation-negative) control. Taking this into consideration, the mutation G719S could be detected by ddPCR even 0,058% mutant fraction (**Figure 1B**).

3.2 Threshold setting for detection of true positives results

To optimize the specificity of the *EGFR* genotyping assay, we tested the incidence of false-positive reads in a healthy population of 6 volunteers. At least, we performed 6 independent reactions for each

individual. Low levels of *EGFR* G719S were detected in healthy volunteers with a peak level of 21.65 copies/mL (**Figure 2**) and 6.35 MAF. Using 22 mutant copies/mL as threshold for a positive result and MAF of 6.5%, 3 of 19 included in the study were real G719S positive patients.

3.3 Quantifying mutant load

Once a threshold and the sensitivity ddPCR for G719S mutation detection was stablished, MAF and mutant copies of G719S in plasma samples were calculated (**Figure 3**). It was observed that the patients whose tissue biopsy was G719S positive, mutation was also detected in plasma by COBAS and ddPCR systems. Higher values of Semiquantitative Index (SQI) obtained by COBAS System correlated with higher MAF and mutant copies/ml obtained by ddPCR. As agreement measure of both techniques used in the present work, it was calculated the Kappa coefficient (K=1), which indicated a perfect correlation between COBAS and ddPCR results.

4 Discussion

Targeted analysis for pathogenic variants in driver genes is the most promising approach for choosing personalized and more effective treatments to NSCLC patients. The number of FDA approved drugs targeting NSCLC driver genes has increased during the last decade. But routinely, only the most common mutations are studied. However, there are rarer mutations which also contribute to tumour heterogeneity that can also be attacked, as G719S and L851Q mutations in *EGFR*. Moreover, in recent years, liquid biopsy has been introduced as a tool of high potential for obtaining samples noninvasively of cancer patients in order to carry out this genetic diagnosis. Several technologies have been developed for the study of circulating tumour DNA, among which the ddPCR provides greater accuracy, sensitivity and absolute quantification in comparison to other conventional techniques used to date.

We herein describe the development of ddPCR technique for G719S rare mutation detection in advanced NSCLC patients using plasma samples. From a technical point of view, the ability to discriminate mutant sequences from wild-type is one of the critical steps of the study. The separation of the signal can be affected by both concentration DNA input and cross-reaction of probes to detect mutation and native sequence. For this reason, we performed temperature and concentration gradients using G719S positive FFPE samples to determine the conditions under which probes and primers work more efficiently in order to minimize false positive results. Because circulating tumor DNA represents 0.1% or even less of total circulating DNA (18), the sensitivity was evaluated by concentration gradients and using DNA from tissue samples. In our population, we had been able to detect G719S mutation even a dilution of 0.058%. This result is in agreement with those presented by Oxnard *et al*. and Zhu *et al*. who also determined ddPCR as a high sensitive technique showing >80% sensitivity when evaluating L858R and exon 19 deletion mutations.

The strategy of using healthy controls to test false positive results and to stablish a threshold to consider a result as true positive result has previously used by other groups (19–21). In the present study we used blood samples from 6 healthy people and the threshold was set in 22 mutant copies/mL as and MAF of 6.5%.

Despite sample size limitations, the present work shows a robust way to detect G719S mutation in NSCLC patients by ddPCR. However, it should be taken into account that if larger population could be tested, thresholds and correlations calculated may undergo slight variations. Thus, as more NSCLC patients with G719S mutation are detected in the Hospital, it would be advisable to include them into this study to validate the results.

One of the advantages of digital approaches is the quantification without the need for a standard curve. Taking advantage of this capacity, mutant allele load was calculated for three patients whose genetic diagnosis was positive for G719S in tissue biopsy. Also, the obtained values in plasma samples by ddPCR were compared with an approved genotyping methodology in clinical routine, COBAS 4800 System. We could observe that mutation studied values obtained by ddPCR corresponded with positive values in tissue biopsy using COBAS System. These results are comparable to those obtained by Zhu and Weber *et al*. which show a 90% of concordance between plasma and tissue determination in other *EGFR* mutations with K values of 0,75 and 0,62, respectively (22,23). Taking in consideration that in the current study sample size is limited, our results are in the same line as those published previously. In terms of correlation between COBAS and ddPCR in plasma samples, it has been shown higher rates obtained by COBAS correlates with greater mutant load in ddPCR that is statistically significant.

Until today, several studies have addressed a comparative analysis between digital and non-digital platforms. In general, digital techniques show greater sensitivity than non-digital techniques. This may be because, as detailed in the COBAS *EGFR* mutation test guide, the system is only capable of detecting mutations with a sensitivity of 5% (24). More specifically, as shown by the results of Thress *et al*. and Watanabe *et al*. ddPCR is one of the most sensitive techniques for genotyping ctDNA (25,26). However, due to the wide range of analytical techniques, laboratories will be able to select the optimal platform for their needs.

This research focuses on the development of G719S mutation detection using ddPCR in patients with advanced NSCLC without using commercial primers. Results obtained in the current study suggest ddPCR as a sensitive, specific and low cost genotyping tool for lung cancer patients and could also be applied to other cancers. That is why, if results are validated, the analysis of the mutational status of *EGFR*, specifically G719S mutation, could result in a new biomarker in NSCLC and could join gradually in clinical practice.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

AOH, CGB, MG, AA, RM, VJA and JT contributed conception and study design; MES1 and MES2 performed the experiments and organized the database; MES1 analyzed data, plotted the results and performed the statistical analysis. MES1 and AOH wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

7 Funding

This study was financed by Hospital Universitari Son Espases (HUSE) (Pilot Project, 2015) and HUESE Medical Oncology Department. MES1 is supported by Conselleria d'Innovació, Recerca i Turisme del Govern de les Illes Balears (TEC/002/2017). MES2 is supported by Programa Estrategia de Emprendimiento y Empleo Joven, Garantía Juvenil (Ministerio de Trabajo, Migraciones y Seguridad Social-SOIB).

8 Acknowledgments

The authors thank Genomics Unit of Institut d'Investigació Sanitària de les Illes Balears for their support to develop the project.

9 References

1. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol* (2007) doi:10.1093/annonc/mdl498

2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* (2011) doi:10.3322/caac.20107

3. Kris MG, Johnson BE, Kwiatkowski DJ, Iafrate AJ, Wistuba II, Aronson SL, Engelman JA, Shyr Y, Khuri FR, Rudin CM, et al. Identification of driver mutations in tumor specimens from 1,000 patients with lung adenocarcinoma: The NCI's Lung Cancer Mutation Consortium (LCMC). *J Clin Oncol* (2011) doi:10.1200/jco.2011.29.18_suppl.cra7506

4. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, 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) doi:10.1056/NEJMoa040938

5. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, et al. EGFR mutations in lung, cancer: Correlation with clinical response to gefitinib therapy. *Science (80-)* (2004) doi:10.1126/science.1099314

6. Shigematsu H, Gazdar AF. Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers. *Int J Cancer* (2006) doi:10.1002/ijc.21496

7. Yoshikawa S, Kukimoto-Niino M, Parker L, Handa N, Terada T, Fujimoto T, Terazawa Y, Wakiyama M, Sato M, Sano S, et al. Structural basis for the altered drug sensitivities of non-small cell lung cancer-associated mutants of human epidermal growth factor receptor. *Oncogene* (2013) doi:10.1038/onc.2012.21

8. Carey KD, Garton AJ, Romero MS, Kahler J, Thomson S, Ross S, Park F, Haley JD, Gibson N, Sliwkowski MX. Kinetic analysis of epidermal growth factor receptor somatic mutant proteins shows increased sensitivity to the epidermal growth factor receptor tyrosine kinase inhibitor, erlotinib. *Cancer Res* (2006) doi:10.1158/0008-5472.CAN-06-0453

9. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science (80-)* (2004) doi:10.1126/science.1101637

10. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* (2005) doi:10.1371/journal.pmed.0020073

11. Kobayashi S, Ji H, Yuza Y, Meyerson M, Wong KK, Tenen DG, Halmos B. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor.

Cancer Res (2005) doi:10.1158/0008-5472.CAN-05-1346

12. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* (2012) doi:10.1056/NEJMoa1113205

13. Zhang J, Fujimoto J, Zhang J, Wedge DC, Song X, Zhang J, Seth S, Chow CW, Cao Y, Gumbs C, et al. Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing. *Science (80-)* (2014) doi:10.1126/science.1256930

14. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer* (2011) doi:10.1038/nrc3066

15. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, Thornton K, Agrawal N, Sokoll L, Szabo SA, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med* (2008) doi:10.1038/nm.1789

16. Shen S, Wei Y, Zhang R, Du M, Duan W, Yang S, Zhao Y, Christiani DC, Chen F. Mutant-allele fraction heterogeneity is associated with non-small cell lung cancer patient survival. *Oncol Lett* (2018) doi:10.3892/ol.2017.7428

17. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev* (2008)

18. Alborelli I, Generali D, Jermann P, Cappelletti MR, Ferrero G, Scaggiante B, Bortul M, Zanconati F, Nicolet S, Haegele J, et al. Cell-free DNA analysis in healthy individuals by next-generation sequencing: a proof of concept and technical validation study. *Cell Death Dis* (2019) doi:10.1038/s41419-019-1770-3

19. van Ginkel JH, Huibers MMH, van Es RJJ, de Bree R, Willems SM. Droplet digital PCR for detection and quantification of circulating tumor DNA in plasma of head and neck cancer patients. *BMC Cancer* (2017) doi:10.1186/s12885-017-3424-0

20. Dewispelaere L, Bleret L, Van Acker T, Van Branteghem C, Cochaux P, Heimann P, El Housni H. One-Step Duplex Droplet Digital PCR for WT1 Overexpression. *J Mol Diagnostics* (2020) doi:10.1016/j.jmoldx.2020.05.010

21. Volik S, Alcaide M, Morin RD, Collins C. Cell-free DNA (cfDNA): Clinical significance and utility in cancer shaped by emerging technologies. *Mol Cancer Res* (2016) doi:10.1158/1541-7786.MCR-16-0044

22. Weber B, Meldgaard P, Hager H, Wu L, Wei W, Tsai J, Khalil A, Nexo E, Sorensen BS, Lynch T, et al. Detection of EGFR mutations in plasma and biopsies from non-small cell lung cancer patients by allele-specific PCR assays. *BMC Cancer* (2014) **14**:294. doi:10.1186/1471-2407-14-294

23. Zhu G, Ye X, Dong Z, Lu YC, Sun Y, Liu Y, McCormack R, Gu Y, Liu X. Highly sensitive droplet digital PCR method for detection of EGFR-activating mutations in plasma cell-free DNA from patients with advanced non-small cell lung cancer. *J Mol Diagnostics* (2015) **17**:265–272. doi:10.1016/j.jmoldx.2015.01.004

24. The cobas® EGFR Mutation Test. Available at: http://www.cobasegfrtest.com/

25. Watanabe M, Kawaguchi T, Isa S, Ando M, Tamiya A, Kubo A, Saka H, Takeo S, Adachi H, Tagawa T, et al. Ultra-Sensitive Detection of the Pretreatment EGFR T790M Mutation in Non-Small Cell Lung Cancer Patients with an EGFR-Activating Mutation Using Droplet Digital PCR. *Clin Cancer Res* (2015) **21**:3552–60. doi:10.1158/1078-0432.CCR-14-2151

26. Thress KS, Brant R, Carr TH, Dearden S, Jenkins S, Brown H, Hammett T, Cantarini M, Barrett JC. EGFR mutation detection in ctDNA from NSCLC patient plasma: A cross-platform comparison of leading technologies to support the clinical development of AZD9291. *Lung Cancer* (2015) doi:10.1016/j.lungcan.2015.10.004

10 Figure legends

Figure 1. Validation assays for G719S mutation detection in plasma samples. (A) Temperature gradient to determine the optimum annealing temperature; mutant positive events (top panel) and wild-type positive events (bottom panel) across the thermal gradient (57 to 67ºC). (B) Two-fold dilution series of mutant DNA into wild-type DNA: concentration is shown in copies per microliter for mutant (blue) and wild-type (green) events and Fractional abundance in percentage (orange). Error bars show 95% CI.

Figure 2. Detection of G719S in a healthy population using ddPCR. Concentration is represented in copies per millilitre of plasma in both healthy (●) and patient (◆) groups; where dashed line represents a candidate threshold for positive results with high sensitivity.

Figure 3. Summary of results obtained by COBAS and ddPCR and comparative evaluation for G719S detection in plasma. SQI, Semiquantitative Index obtained by COBAS® 4800 System.

*For sample LB010 SQI was not available.

11 Tables

Table 1.Outline of clinical and pathological features of patients. Cumulative smoking exposure was determined in terms of pack-years by multiplying the number of years smoked by the average number of packs per day.

Variable	Total (±SD)	Percentage (%)
Age (years)	63 ±14	-
Gender		

Male	7	37
Female	12	63
Smoking habit	11	58
Pack-year		
<20	3	27
>20	8	73
Stage		
IVA	8	42
IVB	11	58
NSCLC		
Primary	17	89
Secondary	2	11
Treatment		
None	4	21
First-line chemotherapy	7	37
Second-line chemotherapy	3	16
Third-line chemotherapy	2	11
TKIs [a]	7	37

337