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Patient-centric thresholding of Cobas® EGFR mutation Test v2 for surveillance of EGFR-mutated metastatic non-small cell lung cancer

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Cobas EGFR mutation Test v2 was FDA-approved as qualitative liquid biopsy for actionable EGFR variants in non-small cell lung cancer (NSCLC). It generates semiquantitative index (SQI) values that correlate with mutant allele levels, but decision thresholds for clinical use in NSCLC surveillance are lacking. We conducted long-term ctDNA monitoring in 20 subjects with EGFR-mutated NSCLC; resulting in a 155 on-treatment samples. We defined optimal SQI intervals to predict/rule-out progression within 12 weeks from sampling and performed orthogonal calibration versus deep-sequencing and digital PCR. SQI showed significant diagnostic power (AUC 0.848, 95% CI 0.782–0.901). SQI below 5 (63% of samples) had 93% (95% CI 87–96%) NPV, while SQI above 10 (25% of samples) had 69% (95% CI 56–80%) PPV. Cobas EGFR showed perfect agreement with sequencing (Kappa 0.860; 95% CI 0.674–1.00) and digital PCR. SQI values strongly (r: 0.910, 95% 0.821–0.956) correlated to mutant allele concentrations with SQI of 5 and 10 corresponding to 6–9 (0.2–0.3%) and 64–105 (1.1–1.6%) mutant allele copies/mL (VAF) respectively. Our dual-threshold classifier of SQI 0/5/10 yielded informative results in 88% of blood draws with high NPV and good overall clinical utility for patient-centric surveillance of metastatic NSCLC.

Keywords Diagnostic techniques and procedures, Clinical laboratory techniques, Liquid biopsy, Circulating tumor DNA, Biomarkers, Tumor, Statistics and numerical data, Carcinoma non-small-cell lung, Disease progression, Likelihood functions, ROC curve

Abbreviations

AUC	Area under the Receiver Operating Characteristics (ROC) curve
ctDNA	Circulating tumor DNA
CI	Confidence interval
dPCR	Digital PCR
EGFR	Epithelial growth factor receptor gene
LR	Likelihood ratio
NPV	Negative predictive value
NSCLC	Non-small cell lung cancer
PCR	Polymerase chain reaction
PPV	Positive predictive value
PSS	Plasma-SeqSensei™
SQI	Semiquantitative index

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Circulating tumor (ctDNA) is increasingly used for cancer diagnosis and monitoring. ESMO and ASCO recognize the clinical utility of liquid biopsy for diagnostic purposes and to identify actionable mutations, but position tumor tissue genotyping still as first choice given its higher sensitivity[1,2]. For surveillance of advanced cancers and monitoring of therapy efficacy, ASCO and ESMO call for more data on assay interoperability and clinical validity of ctDNA levels[1,3,4]. Most studies on ctDNA for surveillance of advanced cancers have two limitations. First, ctDNA was mostly used qualitatively, without leveraging the diagnostic value of ctDNA concentrations. Second, data analysis was limited to proportional hazards modeling at cohort level. The latter statistical approach is useful to investigate the prognostic value of increasing ctDNA levels[5] or to monitor therapeutic efficacy, but conveys no guidance for individual patients.

We recently introduced a simple and patient-centred statistical approach to exploit the diagnostic value of absolute ctDNA concentrations for surveillance of metastatic breast cancers[6]. In a prospective observational trial (PrecisionTrack), we performed long-term (90 weeks), intensive (5-weekly) molecular counting of ctDNA concentrations by digital PCR and deep sequencing and confirmed that rising ctDNA resulted in earlier detection of progression with a median lead time of 10–12 weeks as compared to standard of care[7]. We also showed that primary driver mutation concentrations below 10 copies/mL achieved 90% negative predictive values (NPV) for cancer progression within 12 weeks, while levels above 100 copies per mL had 80–90% positive predictive value (PPV). This 0/10/100 copies per mL model yielded clinically informative ctDNA results in 90% of blood draws.

Due to the high cost of ctDNA analysis, real-world clinical ctDNA data sets are often granular. Since our statistical approach with dichotomization for absence/presence of progression within 12 weeks is theoretically applicable to any quantitative ctDNA data, we here challenged it in another advanced cancer type, using another liquid biopsy assay: Cobas® EGFR Mutation Test v2 (hence Cobas EGFR test) in *EGFR*-mutated metastatic non-small cell lung cancer (NSCLC). In 2016, this test was FDA approved for qualitative detection of EGFR variants actionable by tyrosine kinase inhibitors (TKI)[8,9]. It uses allele (mutation)-specific PCR, with automated calculation of a semiquantitative index (SQI) value. SQI is a measure of cycle threshold of the multiplex PCR and shows a positive, exponential correlation with actual mutant *EGFR* allele concentrations or ratios of mutated allele to wild type allele (variant allele frequency, VAF). Several[9–11] but not all[12,13] studies confirmed this quantitative power of SQI. Cohort analyses established SQI as surrogate indicator for therapeutic response to TKI[10–12]. However, thus far no study proposed actual SQI decision thresholds to rule-in or rule-out impending progression in individual patients. Here, we first conducted an orthogonal method validation of SQI to mutant allele copy numbers and VAF by BEAMING digital PCR and deep-sequencing using Safe-SeqS technology[14,15]. Next, we performed ROC analysis of SQI values to predict impending cancer progression and defined clinically valid result intervals for patient-centric surveillance.

Methods

Study cohort

Subjects were recruited by the department of Pulmonary Diseases at the AZ Delta Hospital (Roeselare, Belgium) during multidisciplinary tumor board discussions. Inclusion criteria were the presence of advanced stage NSCLC with activating EGFR mutation and TKI treatment. Exclusion criterion was presence of EGFR mutation not covered by Cobas EGFR test. This real-world clinical data set included a total of 155 liquid biopsy time points from 20 subjects with *EGFR*-mutated NSCLC, consisting of 6 (30%) males and 14 (70%) females of median (IQR) age 68 (33–92). An overview of baseline patient characteristics is provided in Tables 1, and S1 (TNM stages, smoking status, EGFR variant, age, sex, duration of follow up, number of progression, survival at interim analysis). Standard of care follow up consisted of physical examination and CT-imaging (median every 12 weeks). 13 patients died during follow-up by tumor progression, one patient died of cirrhosis. Median follow-up was 25 months. On each liquid biopsy time point, blood draws consisted of 2 tubes (A, B tube) using Cell-Free DNA BCT® (Streck, La Vista, NE, USA) tubes. Plasma was separated within 3 h as follows: centrifugation 10 min at $1600 \times g$ at 10 °C, harvest 5 mL plasma for second centrifugation for 10 min at $3200 \times g$ at 10 °C and storage at − 80 °C prior to analysis. All measurements on humans were performed as part of standard of care and data handled in accordance with the WMA Declaration of Helsinki. Data were pseudonymized according to GDPR rules to safeguard privacy and confidentiality. A waiver of informed consent was granted by the Ethics Committee AZ Delta) considering the study was based on secondary use of pseudonymized data and samples obtained as standard of care (Clinical Trial Number/IRB: B117202000040, approval date 23/11/2020).

Molecular assays for quantification of *EGFR* variants

Cobas® EGFR Mutation Test v2 (Hoffman-La Roche, Basel, Switzerland) was performed following manufacturer's recommendations. cfDNA was isolated by Cobas® cfDNA Sample Preparation kit from 2 mL plasma. 25 µL cfDNA (cfDNA concentration not measured) was loaded into the reaction wells. Amplification and detection were done on Cobas® z 480 analyser and data were interpreted by Cobas® z 480 software if negative and positive controls showed valid results. This assay qualitatively detects a total of 42 mutations (Single Nucleotide Variants (SNV) and indels) in exons 18, 19, 20 and 21 of the *EGFR* gene and provides a semiquantitative index (SQI) that was derived from a dilution series of known copy numbers of mutated EGFR alleles and a fixed amount of wild-type EGFR[10]. The limit of detection (LOD) declared by the manufacturer for EGFR Exon 19 deletions (Ex19del) is 75 copies/mL. Independent verification[16] indicated LOD of 5–27 copies/mL (0.1–0.5% VAF), 18–36 copies/mL (0.4–0.8% VAF) and 35–70 copies/mL (0.4–0.8% VAF) for EGFR Ex19del, T790M and L858R, respectively. Cobas EGFR test was orthogonally validated to two methods capable of absolute quantification of mutant alleles: Plasma-SeqSenseiTM Solid Cancer IVD Kit and BEAMing digital PCR (Sysmex Inostics GmbH, Hamburg, Germany). For both *Plasma-SeqSenseiTM Solid Cancer IVD Kit* (hence PSS) and BEAMing digital PCR, cfDNA was extracted from the available amount of plasma (2.7–4 mL) of tube B (N = 28 samples) using the QIAmp

Case	Gender	Age	TNM – classification[a]	EGFR variant	Tissue DNA VAF%[b]	Treatment	Tumor/ctDNA profile at progression	Duration of follow-up
1	F	68	T4N1M0	Ex19 Del	14	Afatinib + radiotherapy	No progression	60 months
2	F	54	T4N2M1a	Ex19 Del	22	Afatinib	SQ-I increase + secondary T790M	23 months (†)
3	F	43	T3N2M1c	Ex19 Del	6	Osimertinib	Conversion to SCLC	20 months
4	M	54	T2N3M1c	Ex19 Del T790M	13	Erlotinib	SQ-I increase	19 months (†)
5	V	81	T3N2M1c	Ex21 L861Q	88	Osimertinib	SQI-increase	29 months
6	V	84	T4N2M1a	Ex19 Del	46	Osimertinib	CT: local progression	42 months (†)
7	V	69	T2bN0M1c	Ex19 Del	90	Pancranial RTO, lobectomie + osimertinib	SQI-increase	28 months
8	V	81	T2bN2M1c	Ex19 Del	43	Osimertinib	SQ-I increase	12 months (†)
9	M	61	T4N1M0	Ex19 Del	22	Gefitinib	SQ-I increase	29 months (†)
10	V	76	T2aN3M1b	Ex19 Del	36	FLAURA study[c]	SQ-I increase + MET-amplification	26 months (†)
11	V	33	T4N2M1c	Ex21 L858R	54	Osimertinib	SQ-I increase	31 months (†)
12	M	61	T4N2M1c	Ex19 Del	43	Pancranial RTO + Osimertinib	No progression	7 months
13	V	61	T3N2M1c	Ex21 L858R	91	Osimertinib	SQ-I increase + METamplification	18 months
14	V	68	T4N3Mc	Ex21 L858R	9.3	Osimertinib	SQ-I increase + S768I mutation	12 months (†)
15	V	60	cT2aN2M1b	Ex19 Del	46	Osimertinib	No progression	20 months
16	V	92	T3N4M1b	Ex19 Del	4.5	Erlotinib trial	SQ-I increase	14 months (†)
17	M	72	T2N2M1b	Ex19 Del	27	Afatinib	No progression	48 months (†)
18	M	57	T2N2M1c	Ex20 S768I	7	Afatinib	SQ-I increase	32 months (†)
19	V	77	T2bN3M1b	Ex21 L858R	58	Osimertinib	SQ-increase + secondary T790M	24 months (†)
20	M	83	T1N2M1c	Ex19 Del + T790M	70	Osimertinib	SQ-I increase of Ex19Del	46 months (†)

Table 1. Baseline patient characteristics. [a]TNM classification: tumor, node and metastasis classification. [b]Tissue DNA variant allele frequency (VAF%) on FFPE of primary tumor in diagnostic staging. [c]Flaura study = Osimertinib versus Erlotinib or Tarceva. (†) Died during follow-up.

Circulating Nucleic Acid kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cfDNA was quantified utilizing a LINE-1 qPCR assay. Half of the cfDNA (4.6–63.3 ng) was loaded into the PSS assay, and half of each sample was used for the BEAMing digital PCR assay. PSS provides amplicon-based deep sequencing of EGFR exons 18–21, BRAF, KRAS and PIK3CA with end-to-end bioinformatic analysis of SNV and indels. Digital error correction allows ultrasensitive detection (LOD around 0.07% VAF, 7 mutant allele copies/mL) with absolute quantification of mutant alleles through internal calibrators (Quantispike). During an initial PCR step, cfDNA molecules were tagged with unique identifiers (UIDs). Afterwards, a subsequent round of amplification was performed, integrating distinct indices in each reaction. The generated libraries were sequenced on a NextSeqTM 500/550 platform utilizing Mid/High Output kit (Illumina, San Diego, USA). Identification of the somatic alterations was performed using Plasma-SeqSenseiTM IVD Software v1.0.1 (Sysmex Inostics GmbH, Hamburg, Germany). For *BEAMing digital PCR*, the first steps include a multiplex and nested PCR, which are followed by amplification of tumour-specific targeted DNA sequences using magnetic beads in an emulsion PCR. These sequences were then hybridized with fluorescent probes targeting wild-type or mutant sequences. To distinguish between wild-type and mutant beads the samples were run on a C6 flow cytometer (Becton Dickinson) and analysed using FCS Express v4.0 software (De Novo Software, Glendale, CA). For identification of potential mutant-positive calls, samples with a mutant bead population exceeding 0.02% of total beads carrying the target PCR product were considered tentatively positive. For validation of mutant positivity, the number of mutant DNA molecules per sample were calculated (mutant allelic fraction multiplied by input of cfDNA as Genomic Equivalence (GE)). Samples were classified as true positive when at least one mutant molecule was detected. SQI values were additionally correlated to variant allele frequencies in a set of artificial recovery samples, obtained by a *laboratory-developed hybrid capture sequencing test* using SeqCap EZ HyperCap workflow (Roche, Basel, Switzerland)[6]: DNA was extracted from archived FFPE samples from NSCLC cancers with known EGFR variants, using the Cobas DNA sample Preparation kit. DNA was quantified by Qubit, quality controlled and enzymatically fragmented using the KAPPA HyperPlus Library preparation kit. After amplification, libraries were multiplexed and overnight hybridized with the custom SeqCap EZ Probe pool (Roche, Basel, Switzerland). Finally, captured DNA was amplified using LM-PCR. Sequencing of the libraries was performed on a MiSeq sequencer (Illumina), using a Miseq Reagent kit V2 (2×150). Primary and secondary analysis (demultiplexing, FASTQ generation, alignment (GRCh37), generation of VCF files) was performed using the MiSeq Reporter Software (MRS) and variants called by the Seqnext module of the Sequence Pilot (Seqpilot) software (version 5.0.0. Build 503, JSI medical systems, Ettenheim, Germany).

Data analysis strategy and statistical methods

Primary outcome was clinical progression, defined as radiological progression according to standard of care confirmed by the treating physician and/or a change in therapeutic regimen, blinded to ctDNA test results. Routine imaging was done by chest/abdomen CT, bone scan with MRI or PET-CT in selected cases and reviewed according to RECIST 1.1. Each blood sampling time point was dichotomized with a value of 1 if clinical progression was recorded within 12 weeks from its sampling date and 0 if not (Px12w)[6]. A similar dichotomization was done for progression or not within 4 weeks from sampling date (Px4w). Sample size for outcome analysis (Px12w) was 155 (26% positive, with positive indicating presence (outcome label = 1) of clinical progression within 12 weeks from sampling date), consisting of 99 samples with Ex19del-type (28% positive) and 56 single nucleotide variant (SNV)-type (23% positive) *EGFR* mutations (L858R, T790M). The diagnostic value of SQI values were analysed by ROC analysis and selection of result intervals guided by likelihood ratios (LR). Spearman rank correlation and Bland–Altman was used to evaluate the association between SQI by Cobas EGFR test and VAF and mutant allele copies/mL by PSS. Cohen Kappa statistics were used to analyse qualitative agreement between Cobas EGFR and PSS. Results were considered statistically significant with a P value < 0.05. Statistical analyses were performed using MedCalc® Statistical Software version 19.6.4 (MedCalc Software Ltd, Ostend, Belgium). Data used for logistic regression modeling is provided as Supplementary information file Table S2 (excel format).

Results

Study cohort

20 subjects with EGFR-mutated non-squamous cancers were enrolled for longitudinal monitoring of EGFR mutations in cell-free DNA as part of standard of care. Two patients had stage IIIA disease (T4N1M0. The other (90%) had stage IV disease (Tables 1, S1). All patients were treated with an EGFR-TKI (afatinib, erlotinib or osimertinib) two of them in a clinical trial. One patient had radiotherapy on primary tumor, two had pancranial radiotherapy and one a lobectomy. In total, 155 on-treatment plasma samples were collected for ctDNA monitoring by Cobas EGFR test. Median (range) follow-up was 25 (IQR 7–60 months) months with a clinical and radiological evaluation every twelve weeks (median). 13 patients died during follow-up by tumor progression, one patient died to cirrhosis. At inclusion, 70% of subjects had EGFR Ex19del variants and 40% single nucleotide variants (SNV), with L858R (20%), T790M (10%) (Table S1). Secondary appearance of T790M during TKI therapy was observed in 10%, MET-amplification in 15%. One patient had a transformation to small cell lung cancer and was treated with additional chemotherapy. Subjects had a median (range) of 1 (1–3) progression, with a total of 21 progression events in the data set. Three subjects had multiple progression events. Over the course of these progressions, Cobas EGFR test SQI (median (IQR, range) decreased significantly ($P = 0.0001$) from peak during progression (11.8 (8.8–14.1, range 2.0–18.4) to nadir of 0.0 (0.0–0.0, range 0.0–5.6) at best treatment by ctDNA analysis on median (IQR) 71 (56–208) days after the peak during progression.

Diagnostic value of Cobas EGFR test for *cancer* progression

ctDNA data points (N = 155) were dichotomized for presence (1) or absence (0) of clinical progression within 4 weeks (Px4w, Fig. 1A,C) or 12 weeks (Px12w, Fig. 1B) from sampling date. Time points not followed by progression within 4 or 12 weeks had median (IQR) SQI value of 0 (0–0) with range up to 19.6. Samples with progression within 4 and 12 weeks showed higher ($P < 0.0001$) median (IQR) SQI values of 11.59 (8.92–13.63) and 11.54 (10.10–13.51), respectively (Fig. 1A,C). ROC analysis showed significant diagnostic value of SQI value for progression within 4 weeks (AUC 0.845, 95% CI 0.777–0.900) (Fig. 1C) and within 12 weeks (AUC 0.848, 95% CI 0.782–0.901) (Fig. 1D). Diagnostic value was similar ($P = 0.1085$) for *EGFR* Ex19del variants (AUC 0.812, 95% CI 0.722–0.884, N = 99, 28% positive) and *EGFR* single nucleotide variants (SNV, L858R or T790M) (AUC 0.911, 95% 0.804–0.970, n = 56, 23% positive) in contrast with a previous study showing higher sensitivity for EGFR Ex19del variants[16]. Increasing SQI values were associated with increasing positive likelihood ratios for the risk of progression (insets Fig. 1C,D) up to SQI values around 12 for any type of *EGFR* variant.

Correlation of Cobas EGFR test SQI value ctDNA concentrations measured by PSS and digital PCR

To investigate the correlation of SQI value with ctDNA concentration, a blinded orthogonal validation of Cobas EGFR test (on the A-tube) to two different quantitative methods was performed (both on the B-tube) on 31 patient blood draws: targeted deep-sequencing by PSS and BEAMing digital PCR (dPCR) (scatter diagrams in Fig. 2). In addition to variant allele frequency (VAF) by both PSS and dPCR, PSS also provides the absolute concentrations of mutant alleles in copies/mL through the use of internal calibrators. Cohen's kappa analysis indicated perfect inter-rater agreement for qualitative results (mutation detected/not detected) between Cobas EGFR test and PSS with Weighted Kappa of 0.860 (95% CI 0.674–1.00) with only 2 false negative Cobas EGFR results at mutant allele concentrations around 10 copies/mL (0.1% VAF), well below the Cobas EGFR test's limit of detection (LOD)[16]. BEAMing dPCR showed 2 false positive results versus both Cobas EGFR test and PSS, resulting in overall perfect agreement with PSS (Kappa 0.842) but only substantial agreement (Kappa 0.706) with Cobas EGFR test (Fig. 2). SQI values were strongly correlated with mutant allele copies/mL by PSS (Fig. 2A, rho: 0.910, 95% 0.821–0.956) and VAF by both PSS (Fig. 2B) and BEAMing dPCR (Fig. 2C) with also excellent correlation between the latter (Fig. 2D). SQI values were exponentially related to both copies/mL and VAF with a significant linear relation between SQI and log transformed copies/mL and VAF (Cusum, $P = 0.78$). Due to limitations in cfDNA input in PSS/BEAMing dPCR, LOD of the various assays could not be formally evaluated.

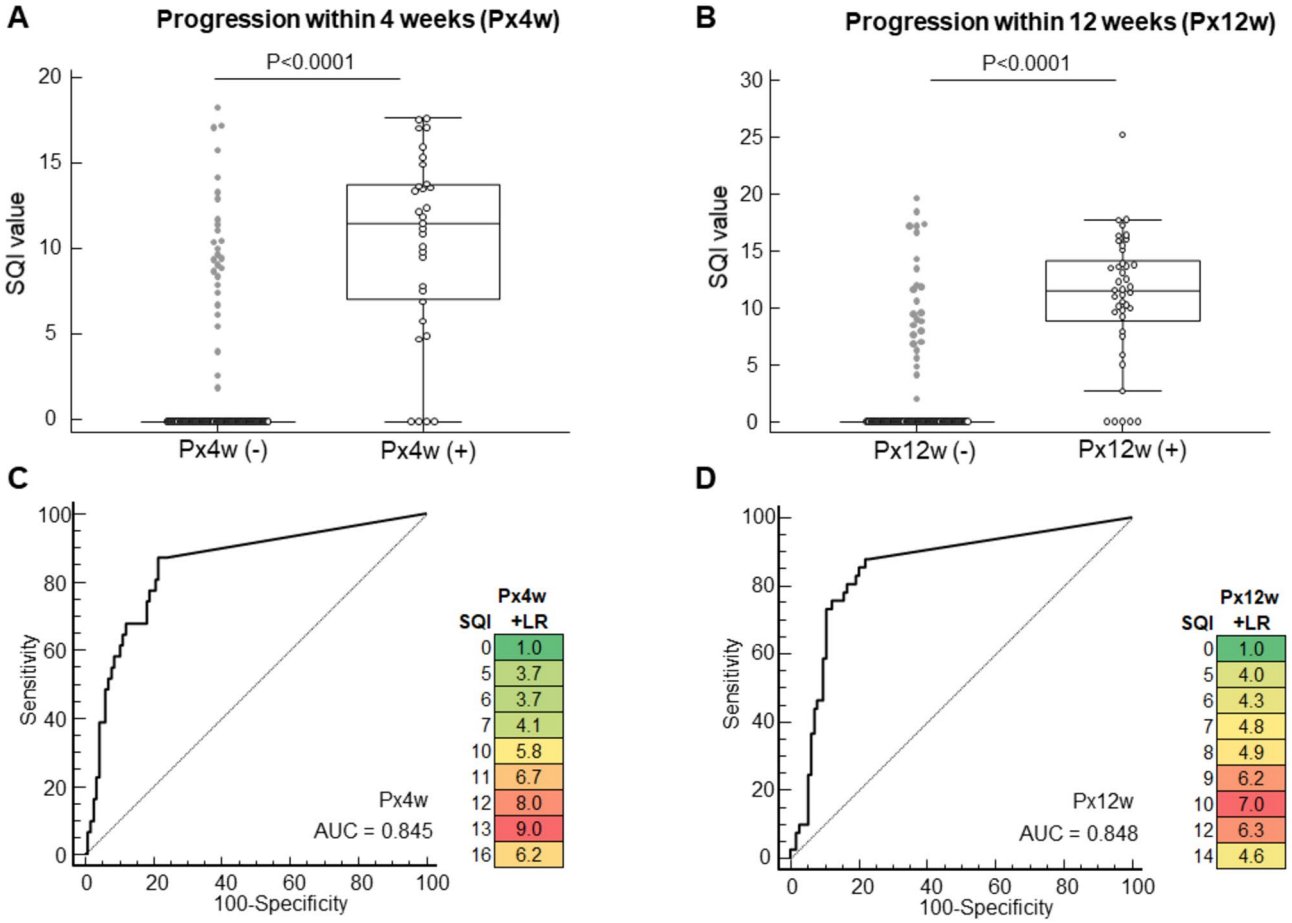

Figure 1. Diagnostic power of SQI values of the Cobas EGFR test for disease progression in metastatic NSCLC cancer. (**A**) Distribution of SQI values in samples followed (Px4w (+) or not followed (Px4w (−)) by disease progression within 4 weeks from sampling date. (**B**) Distribution of SQI values in samples followed (Px12w (+)) or not followed (Px12w (−)) by disease progression within 12 weeks from sampling date. *P* value by Mann–Whitney U test. (**C**) ROC curve of SQI value for presence of progression within 4 weeks (Px4w). (**D**) ROC curve of SQI value for presence of progression within 12 weeks (Px12w). Inset in (**C**)–(**D**) indicate the positive likelihood ratio (+LR), reflecting the probability of progression to probability of no progression within 4 or 12 weeks for SQI values above the indicated value.

Differential calibration of sequencing-derived VAF and the Cobas SQI values for SNV- and Ex19del-type of *EGFR* mutations

Since Cobas EGFR test relies on mutation-specific primers, the efficiency of PCR reactions and SQI are influenced by the type of mutation. This results in mutation-specific LOD[16] and regression curves[10] with the most prominent difference noted between all exon 19 deletions (Ex19del)-type of *EGFR* mutations and the single nucleotide variant (SNV)-type EGFR variants (L858R, T790M). To derive VAF estimates from SQI values, pooled regression analysis was performed of SQI values and VAF, using next generation sequencing (NGS) data from three different sources (Supplementary Fig. 1, Fig. S1): (1) ultra-deep sequencing of NSCLC patient samples previously reported by Marchetti et al.[10] (N = 34, VAF% 0.020–72.1%) (Fig. S1A); (2) our PSS on samples from NSCLC patients (N = 31, VAF% 0.10–70.8%) (Fig. S1B) and (3) in-house hybrid-capture NGS on artificial samples (N = 37, VAF% 0.31–63.5%) (Fig. S1C) prepared from enzymatically fragmented DNA from FFPE specimens of NSCLC cancers. In all 3 data sets, a high (> 0.80) or very high (> 0.90) positive linear correlation was obtained with SNV- and Ex19del-regression curves between SQI and log transformed VAF values. After pooling of the three data sets, Passing-Bablok regression showed a linear correlation between SQI values and log transformed VAF ranging from high (r = 0.818) for SNV-type to very high (r = 0.959) for Ex19del-type of EGFR mutations (Fig. S1C).

Rational selection of SQI result intervals to rule-out or rule-in impending progression

To derive clinical guidance from SQI values in individual blood draws, SQI result intervals were defined that were associated with high negative predictive value (NPV) or high positive predictive value (PPV) for progression within 12 weeks. Dual threshold models with lower threshold around SQI of 4 or 5 and upper threshold around SQI 9 or 10 came out as optimal models for both EGFR Ex19del and SNV variants. As shown in Table 2, SQI values lower than 5, present in 63% of data points, had a likelihood ratio (LR) of 0.214 (95% CI 0.108–0.423) and

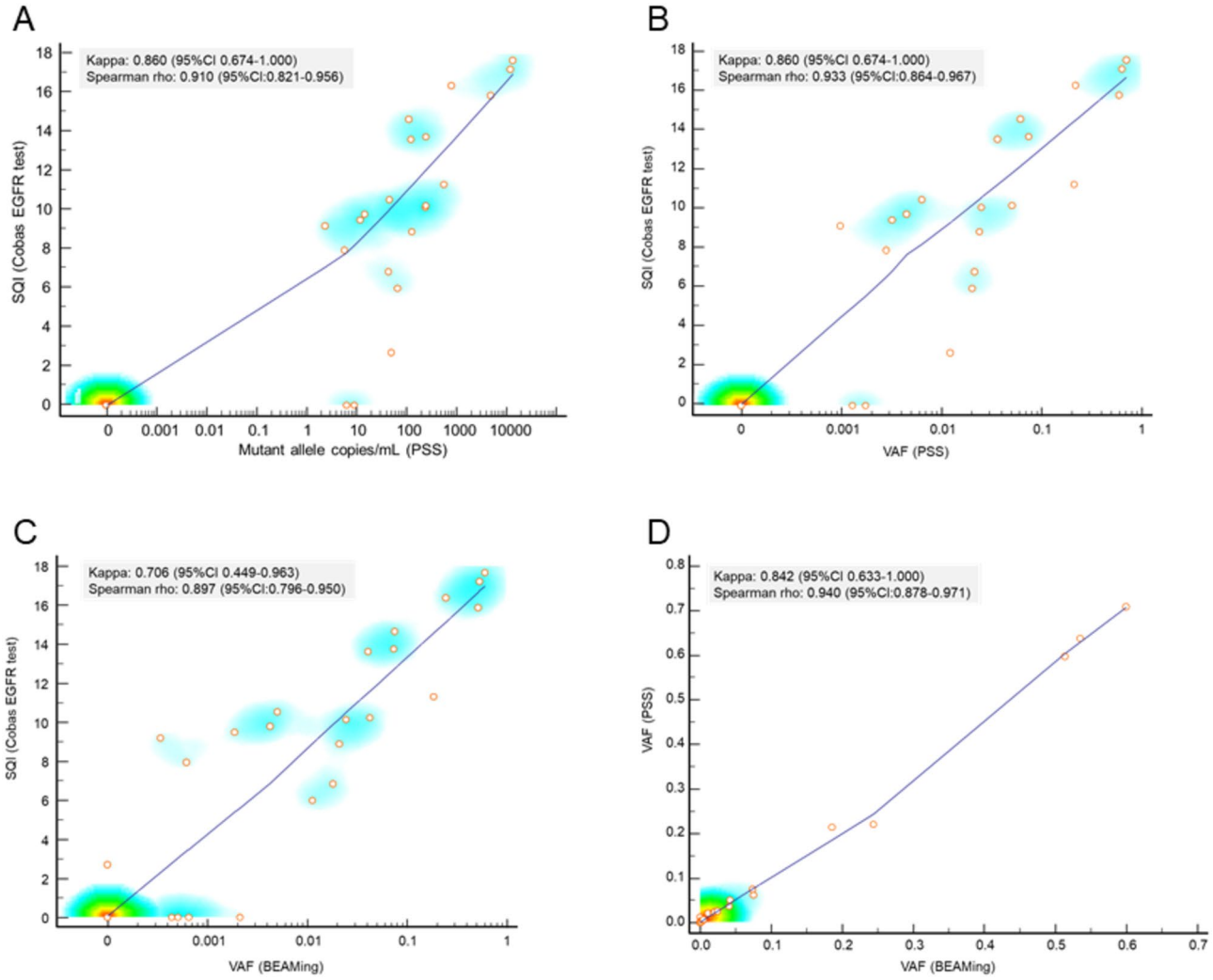

Figure 2. Orthogonal method comparison between Cobas EGFR test, Plasma-SeqSensei (PSS) NSCLC RUO kit and BEAMing digital PCR. Qualitative (mutation detected/not detected) and quantitative (SQI, mutant allele copies/mL, VAF) method comparison of Cobas EGFR test (A-tube) versus deep sequencing by PSS and targeted digital PCR by BEAMing technology (both on B-tube, blinded analysis) on 31 patient samples (of which 21 with ctDNA detected by any technology). Scatter plots (MedCalc) of A. Cobas SQI value versus mutant allele copies/mL by PSS; B. Cobas SQI values versus Variant Allele Frequency (VAF) by PSS; C. Cobas SQI value versus VAF by BEAMing digital PCR and D. VAF by PSS versus VAF by BEAMing. Insets indicate per comparison the qualitative inter-rater agreement by Cohen Kappa (95% CI) and Spearman's rank correlation coefficient rho (95% CI). All 4 correlations showed linear relation (cumulative sum chart analysis, MedCalc) between SQI value and logarithm of VAF or copies/mL.

a 93% (95% CI 87–96%) NPV for progression within 12 weeks (Table 2). SQI values above 10, present in 25% of data points, had a LRR of 6.256 (95% CI 3.507–11.160) and a PPV of 69% (56–80%). For the SNV-type mutations (L858R and T790M), the NPV and PPV were 94% and 75%, respectively, for values below 5 or higher than 10. For Ex19del-type of mutations, the corresponding NPV and PPV were 92% and 67%. SQI values between 5 and 10, encountered in only 12% of samples, were diagnostically meaningless (LR 1.711, 95% CI 0.765–3.828). Based on regression analysis in our PSS data set (data set B, Fig. 2A), the lower decision threshold of SQI values 4–5, corresponds to 6–9 mutant allele copies/mL, while the upper threshold of SQI 9–10 corresponds to 64–105 copies/mL. The estimated VAF associated to lower threshold, based on mutation type-specific regression (Fig. S1D) are 0.01–0.02% for Ex19del and 0.6–0.8% for SNV. The upper thresholds correspond to an estimated VAF of 0.14–0.23% for Ex19del and 3.1–4.4% for SNV. In a simplified regression combining both type of mutations, the upper and lower threshold are 0.2–0.3% and 1.1–1.6% respectively.

Discussion

In NSCLC, ctDNA testing has shown clinical validity for longitudinal monitoring of treatment response[17], identification of actionable variants including resistance mechanisms at progression[18,19] and for minimal residual disease screening in stage I-III cancers treated with curative intent[20,21]. Due to its near maximal specificity for

SQI interval	Progression within 12 weeks		LR	95% CI	Sample distribution (%)	Predictive value	Copies/mL	VAF%
	Positive	Negative					Estimated	
All EGFR variants								
0–5	**7**	**91**	**0.214**	**0.108–0.423**	**63**	**NPV: 93% (95% CI 87–96%)**	**9**	**0.3**
5–10	*7*	*11*	*1.769*	*0.735–4.257*	*12*			
>10	***27***	***12***	***6.256***	***3.507–11.160***	***25***	***PPV: 69% (95% CI 56–80%)***	***81***	***1.6***
SNV-type variants (L858R, T790M)								
0–5	**2**	**33**	**0.200**	**0.0554–0.725**	**63**	**NPV: 94% (95% CI 82–98%)**	**34**	**0.8**
5–10	*2*	*7*	*0.945*	*0.223–4.004*	*16*			
>10	***9***	***3***	***9.923***	***3.142–31.339***	***21***	***PPV: 75% (49–90%)***	***307***	***4.4***
Ex19del-type variants								
0–5	**5**	**58**	**0.219**	**0.0980–0.487**	**64**	**NPV: 92% (95% CI 84–96%)**	**1**	**0.0**
5–10	*5*	*4*	*3.170*	*0.917–10.952*	*9*			
>10	***18***	***9***	***5.071***	***2.595–9.911***	***27***	***PPV: 67% (95% CI 51–80%)***	***16***	***0.2***

Table 2. Cobas EGFR test SQI result intervals to rule-out or rule-in progression within 12 weeks. Dual threshold model for SQI values for all EGFR variants combined (top), Single Nucleotide Variant (SNV)-type variants (middle) and Exon 19 deletion (Ex19del)-type variants (bottom). Left columns shows the three SQI result intervals and number of samples in that interval: SQI 0–5, associated with high negative predictive value (NPV) for progression within 12 weeks (rule-out, bold); SQI 5–10 representing a italic zone with no diagnostic value (likelihood ratio confidence interval encompassing 1) and SQI above 10, associated with high positive predictive power (PPV) for progression (rule-in, bolditalic). Sample distribution indicates the percentage of samples falling in the respective result intervals. Right columns indicate the estimated mutant allele concentration (copies/mL) and variant allele frequency (VAF), derived respectively from calibration curves by targeted deep sequencing (copies/mL) and a pooled data set (VAF) as described in Results.

cancer cells, ctDNA analysis is theoretically superior to protein-type biomarkers such as CEA, NSE and CA15-3. Also, its analytical sensitivity is no longer limiting: molecular counting assays such as digital PCR or deep sequencing achieve detection limits down to a few molecules of ctDNA per mL. A recent seminal study by Assaf et al. (Phase 3 IMpower150), with longitudinal analysis of 466 NSCLC patients using FoundationOne Liquid CDx assay, showed that ctDNA outperforms radiographic imaging to predict progression and overall survival[22]. Cost issues aside, it is therefore remarkable that the clinical use of ctDNA for surveillance of metastatic cancers remains very limited. In our opinion, two obstacles remain.

First, more studies are needed that investigate ctDNA as a quantitative marker. This requires assays capable of absolute quantification ctDNA concentrations as mutant allele copies per mL plasma. Most studies still report ctDNA levels expressed as variant allele frequencies. The latter is a relative unit that is additionally biased by fluctuations in wild type alleles shed from non-cancerous tissues that suffer bystander injury from chemo- or radiotherapy. Second, clinicians lack guidance on reference values for ctDNA concentrations so that clinical actions can be coupled to a ctDNA concentration in an individual patient sample. We were the first to propose a simple dual threshold classifier for surveillance of metastatic breast cancer[6]: ctDNA levels below 10 copies/mL (0.25% VAF) were reassuring with 90% NPV for progression within 12 weeks from sampling and levels above 100 copies/mL (2.5% VAF) showing PPV above 80%. A gray zone result of 10–100 copies/mL was diagnostically meaningless but only encountered in only 10% of blood draws.

The key novelty of present study, is that we confirm the inter-operability of this 0/10/100 copies per mL (0%/0.25%/2.5% VAF) classifier, using another liquid biopsy technology, in another cancer type. Our data thus suggest similar absolute ctDNA concentration thresholds of 10/100 copies mutant allele per mL to rule-out/rule-in impending progression in breast and lung cancers. This is remarkable and requires independent confirmation for multiple reasons. First, because of known analytical limitations of Cobas EGFR test[12,13]. Molecular counting, by digital PCR or molecular barcode-enabled deep sequencing, is by far more accurate. Second, the correlation between the amount of ctDNA shed into the circulation and tumor progression is influenced by tumor type, vascularization, oxygenation, metabolic activity and tumor mass that all affect cancer cell turnover[23,24]. Generally, ctDNA concentrations are proportionate to the overall tumor burden and number of metastatic sites[17], but some cancers (e.g. glioma, renal cell and thyroid carcinoma) are notoriously poor shedders[18,24]. Interestingly, a recent cross-sectional analysis of 23,482 deep sequencing liquid biopsies across 25 solid tumor types[24] indicated similar circulating tumor fractions in NSCLC and breast cancer, giving support to our observation of similar decision thresholds in both cancer types. Other common cancer types such as colorectal, prostate and bladder cancer showed higher ctDNA levels, so caution is warranted in generalizing our classifier. Of note, the latter study consisted of diagnostic liquid biopsies sampled at baseline or progression, and the median tumor fraction was 2.2%, in line with our observed upper threshold around 2.5% VAF close to progression.

Follow up of metastatic lung cancers now relies on periodic imaging, clinical symptoms and low-cost biomarkers such as CEA, NSE or CYFRA21-1. Our data indicate that follow-up could be shifted to upfront, frequent (e.g. monthly) ctDNA quantification in an outpatient setting and the use of ctDNA concentrations for risk-informed scheduling of care. In 63% of blood draws, very low ctDNA levels (below 10 mutant copies/mL) could be used to reassure patients and its 93% NPV for progression is sufficient to delay imaging and free up scan time in systems with limited capacity. High ctDNA levels (above 100 mutant copies per mL) result in earlier recognition of impending progression, buying time to optimally select the next therapeutic line, advance radiology or trigger test escalation to comprehensive genomic testing on a re-biopsy or cell-free DNA.

Our study had limitations. Its size was comparable to previous studies[10,11,13] but better powered, multicentric and prospective confirmations are required before its conclusions can be included in guidelines. The originality of our ctDNA data analysis, with dichotomization of each data point/ctDNA level to predict progression within a chosen time frame from sampling date, is that it can be applied, also retrospectively, to available real-world data or clinical trial data, as long as a quantitative liquid biopsy assays were used. It thus represents a simple and intuitive approach for multicentric data pooling. Also the choice for 12 weeks as prediction horizon was arbitrarily. For some cancer types with lower relapse rates, longer periods might be better. The observed PPV around 70–75% of SQI above 10 is moderate. This is, however, an underestimation of the real PPV for two reasons: (1) biologically, because subjects with very high tumor burden are clinically/radiologically classified as stable disease but still have continuously elevated ctDNA levels above 100 copies/mL; and (2) methodologically, because the standard of care often fails to timely recognize cancer progression: ctDNA data points with SQI above 10 were not always followed within 12 weeks by patient visits and imaging in our real-world data set, and are thus falsely classified as negative. The PPV of ctDNA might also be boosted by combining with conventional biomarkers such as NSE or CEA, as we previously reported for the ctDNA/CA15-3 combo in breast cancer[6].

More studies are needed on health economic impact of frequent ctDNA monitoring. The cost for highly sensitive ctDNA analysis varies greatly from 50 euro for singleplex targeted PCR to more than 1500 euro for gene panel sequencing. The former is only achievable for labs capable of setting up tumor-informed and patient-personalized assays. The latter allows more consolidation in a tumor agnostic approach, but is not affordable in even the advanced health care systems. Patients' wellbeing should also be taken into account: the collection of patient-reported experience measures is much needed e.g. to investigate if outpatient liquid biopsy relieves anxiety induced by medical scan procedures[25].

In conclusion, this study shows that the SQI value of Cobas EGFR test has significant diagnostic power for cancer progression in metastatic EGFR-mutated lung cancers. Its broader impact is that it provides a simple approach to data handling that can be retrospectively applied by all centres worldwide using this FDA-approved liquid biopsy and thus stimulate the broader use of ctDNA quantification for surveillance of metastatic cancers.

Data availability

Data used for logistic regression modeling is provided as Supplementary information file Table S2 (excel format).

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Author contributions

CRediT Statement: J.C.: Data curation; Investigation; Methodology; Validation; Writing—original draft. D.D.S.: Conceptualization; Data curation; Formal review and editing draft. J.B.: Data curation; Methodology; Resources; Software; Supervision; Writing—review and editing. J.W.: Data curation; Methodology; Writing—review and editing. U.H.: Data curation; Writing—review and editing. I.D.: Data curation; Writing—review and editing. G.A.M.: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing—original draft. Jonas Claus and Dieter De Smet contributed equally (shared first authors).

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Competing interests

The authors declare no competing interests.

Additional information

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