Pathology & Oncology Research (2020) 26:1595–1603
https://doi.org/10.1007/s12253-019-00744-8

ORIGINAL ARTICLE

External Quality Assurance of Current Technology for the Testing of Cancer-Associated Circulating Free DNA Variants

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Received: 29 July 2019 / Accepted: 29 August 2019 / Published online: 5 September 2019

Abstract

Liquid biopsy testing is rapidly emerging as a diagnostic means of identifying circulating free DNA (cfDNA) disease-associated variants. However, the reporting of cfDNA variants remains inconsistent due in part to the application of multiple testing pipelines which raise uncertainty about current cfDNA detection efficiency. External quality assurance (EQA) programs are required to monitor, evaluate and help improve laboratory performance for cfDNA variant detection and in clinical interpretation. This study therefore evaluated the performance of diagnostic laboratories currently performing cfDNA testing in China, Australia and New Zealand. A total of 89 laboratories participated in this EQA program. Reference testing material comprised of cfDNA manufactured to contain six different genotypes in four different genes (*EGFR, KRAS, BRAF, NRAS*). The predicted genotypic variant allelic frequencies ranged between 0.5% - 2.5%. Proficiency testing used a z-score on the laboratory consensus allelic frequency data to compare laboratory performance for the detection of the different genotypes. Allelic frequency genotyping data were received from 88 of the 89 laboratories. Next generation sequencing and digital PCR testing platforms were primarily used by participants in this pilot EQA. The average consensus data for each cfDNA genotype identified allelic frequencies ranging between 0.39% - 4.4%. Z-score proficiency testing found that >92% of clinical laboratories were concordant for detecting the cfDNA variants. The data from this pilot study suggest that current cfDNA testing platforms can detect cfDNA allelic frequency variants from 0.39% and above with high levels of confidence. In addition, these data highlight the importance of laboratories enrolling on EQA programs so that proficiency in cfDNA diagnostic testing can be determined and potential sources of error identified and addressed.

Keywords Circulating free DNA (cfDNA) · Next-generation sequencing · Digital PCR · External quality assurance · Liquid biopsy

Introduction

Liquid biopsy is emerging as key material for early diagnostic evaluation of disease. The liquid biopsy material comprises of (but not limited to) blood plasma, urine, saliva, and cerebrospinal fluid. However, blood plasma remains the most common, containing circulating cell-free DNA (cfDNA) that are representative of small DNA fragments ranging approximately 160–200 base pairs in length. These specific cfDNA fragments are released into the blood circulation by healthy cells and tumour cells through the processes of apoptosis, necrosis, autophagy, necroptosis, and other physiological mechanisms [1, 2]. However, in cancer, treatment resistance tumour-specific cfDNA variants have been detected in various genes and can therefore be differentiated from normal cellular wild-type cfDNA. A primary clinical focus of cfDNA testing is to therefore identify and monitor specific variants that are associated with pharmaceutical treatment resistance in cancer [3–16]. For example, the *EGFR* c.2369C > T (p.Thr790Met) variant is commonly found in the tumours of non-small cell lung cancer patients where resistance to tyrosine kinase

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12253-019-00744-8) contains supplementary material, which is available to authorized users.

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inhibitor therapy is detected [17, 18]. Monitoring the onset of resistance through observing an increase in cfDNA *EGFR* c.2369C > T copy number allows for earlier intervention and the application of next generation therapeutic inhibitors. Analyses of plasma-derived cfDNA has therefore been proposed as an alternative test for cancer diagnostics [6, 19–23]. The key advantage of blood plasma cfDNA testing is that it is minimally invasive making it a very attractive technique over the invasiveness of surgery and of the risks associated with this. Additionally, cfDNA analyses also avoids inconsistencies in data arising from solid tumour heterogeneity [24]. However, globally accepted cfDNA clinical standardisation guidelines are not yet available which makes global clinical adoption of cfDNA testing challenging. In particular, data derived from different testing platforms can be inconsistent and may in part be reflective of the procedures used to collect blood plasma, in the isolation of cfDNA, and in identifying a specific platform's limit of detection (LoD) for low copy number variants [25, 26]. The accuracy of cfDNA variant identification and subsequent data interpretation can therefore be problematic [26–31].

Recent external quality assurance (EQA) reports on liquid biopsy testing have identified shortcomings in the detection efficiency of cfDNA genotypes [25, 26, 32]. However, current EQA schemes also include cfDNA extraction as part of the proficiency testing process. The issue here is that errors arising from the extraction phase, or low levels of cfDNA recovery, may be reflected in the quality of the data output and would likely have an impact on clinical interpretation [32, 33]. It is therefore difficult to ascertain whether laboratory issues relating to incorrect reporting of cfDNA genotypes are a consequence of the cfDNA extraction process or in the measuring platform used. Given that liquid biopsy analysis is in its infancy, a technology specific EQA needs to offer reference testing material that contains natural cfDNA characteristics with known precise genotypic allelic frequency distributions across multiple genes in a format that does not require cfDNA extraction. The use of such reference testing material allows for the efficiency of liquid biopsy testing platforms to be fully evaluated.

In the present study, the National Centre for Clinical Laboratories (NCCL, Beijing, China) and Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP, Sydney, Australia) developed a pilot cfDNA EQA program to evaluate the technical performance of multiple diagnostic laboratories to detect common cancer-associated cfDNA variants that are reflective of variants found in patient serum. Specific cfDNA reference testing standards were synthetically manufactured to contain different allelic frequency variants in four common cancer-associated genes. The aim of this study was to determine the ability of laboratories to accurately detect cfDNA genotypes, and to compare the detection efficiency of different cfDNA testing platforms. A total of 89 clinical testing laboratories participated in this pilot program.

Methods

EQA Program Design

This EQA program was designed to assess laboratory technical performance for detecting cfDNA variant allelic frequencies that are associated with cancer. Laboratories were requested to perform routine analysis for the detection of cfDNA variants that were of clinical interest to them. In this way, multiple clinical testing laboratories could enroll for proficiency assessment for the detection of cfDNA variants that are associated with different cancers. This therefore represents a more efficient and cost effective EQA program since multiple cfDNA variants associated with multiple cancers can be assessed at the same time.

Extraction of cfDNA was not required to be performed by any laboratory for this program. Instead, laboratories were sent Tris EDTA (TE) buffer containing synthetically derived cfDNA which served as the reference testing material and consisted of four testing samples designed to contain six clinically relevant cfDNA variant genotypes in the *EGFR, KRAS, NRAS,* and *BRAF* genes [17, 18, 34]. Any potential issues relating to laboratory-specific cfDNA extraction processes were therefore removed. The cfDNA genotypic predicted allelic frequencies ranged from 0.5% to 2.5%. Each reference testing standard consisted of 125 ng of cfDNA in 25ul TE buffer (pH 8). All reference testing standards were distributed to each laboratory with accompanying instructions for storage and assay procedures. Genomic DNA extracted from healthy blood cells were also distributed to participants for use as a mutation negative control (for all variants tested for). Participating laboratories were requested to provide key information relating to the specific cfDNA detection methodology used including assay kits and limit of detection (LoD) of their testing platforms.

Participants

A total of 89 genetic testing diagnostic laboratories (83 from China, 5 from Australia, 1 from New Zealand) enrolled in this EQA cfDNA variant detection pilot program.

Generation of Reference Testing Standards

The cfDNA liquid biopsy reference testing standards were generated as previously reported [35]. The derived reference testing material was designed to reflect commonly reported cfDNA variants identified in various cancers including adenocarcinoma (colon), non-small cell lung cancer,

and melanoma [36–39]. This allowed laboratories to test for variants found in specific cancers that were of clinical interest to them. Laboratories were therefore not required to test for all variants in all cancer types.

Validation of Reference Testing Standards

Validation of each predicted cfDNA variant allelic frequency was performed using next generation sequencing (NGS) and/or digital PCR (dPCR) as previously reported [35]. The RCPAQAP also performed additional confirmation of the genotypes using digital PCR according to Zhang et al., (2017) [35]. Stability testing was performed by shipping samples at room temperature to a Singapore laboratory and having the samples returned to the RCPAQAP for repeat digital PCR analyses.

Assessment Criteria of Technical Performance

Z-score calculations using the participant consensus mean for each gene allelic frequency were used to determine laboratory performance. Z scores (z = (individual laboratory data – laboratory mean) / standard deviation) between −2.0 to 2.0 were considered to be acceptable and therefore concordant. In contrast, z scores less than −2.0 or greater than 2.0 were considered discordant. Participants who did not test for specific genotypes, or where the LoD of their testing platform was above the allelic frequency range being tested for, where not assessed or penalized.

Results

Participant Data

EQA cfDNA allelic frequency data were received from 88 of the 89 enrolled clinical testing laboratories. Key information relating to specific platforms, assay kits and LoD of testing platforms used for data analyses were also received (Table 1).

Reference Testing Standards

Confirmation of predicted cfDNA genotypes in the reference testing standards was obtained as previously reported [35]. Predicted allelic frequencies are provided in Table 2.

Diagnostic Testing Platforms

Various diagnostic testing platforms were used in this EQA proficiency testing program for cfDNA variant detection. NGS was the most commonly used technology with 76.1% (67/88) of laboratories performing analyses on an NGS platform, 20.5% (18/88) using digital PCR, 2.3% (2/88) using mass array technology and 1.1% (1/88) using allele specific PCR (ASP). The reported LoD of all cfDNA detecting platforms ranged from 0.01% - 5% (Table 1).

Proficiency Assessment

To evaluate laboratory technical performance of cfDNA variant detection, the laboratory consensus allelic frequency mean for each individual variant tested for was derived and used to generate a z-score for each individual laboratory (Supplementary Table 1). The derived laboratory consensus mean for each cfDNA allelic frequency closely matched that of the predicted allelic frequency (Table 2). Of the 89 participating laboratories, one laboratory did not report any allelic frequency data and was therefore not assessed. The most common cfDNA gene variant tested was the *EGFR* c.2235_2249del15 (p.Glu746_Ala750del) variant in sample 1 with 98.8% (87/88) of laboratories testing for this. Of the remaining variants, 82.9% (73/88) of laboratories tested for the *BRAF* c.1799 T > A (p.Val600Glu) variant in sample 5, 80.6% (71/88) of laboratories tested for the *KRAS* c.35G > A (p.Gly12Asp) variant in sample 2, 65.9% (58/88) of laboratories tested for the *EGFR* c.2310_2311insGGT (p.Asp770_Asn771insGly) variant in sample 5, 60.2% (53/88) of laboratories tested for the *NRAS* c.181C > A (p.Gln61Lys) variant in sample 4, and 51.1% (45/88) of laboratories tested for the *NRAS* c.181C > A (p.Gln61Lys) variant in sample 5 (Table 2).

Laboratory concordance ranged from 92.5% for detecting the *NRAS* c.181C > A variant (consensus allelic frequency of 4.4%) in sample 4 to 98.3% for detecting the *EGFR* c.2310_2311insGGT variant (consensus allelic frequency of 0.54%) in sample 5 (Table 2). In contrast, for sample 1, 2.3% (2/87) of laboratories were discordant for testing the *EGFR* c.2235_2249del15 variant. For sample 2, 4.2% (3/71) of laboratories were discordant for testing the *KRAS* c.35G > A variant. For sample 4, 7.5% (4/53) of laboratories were discordant for testing the *NRAS* c.181C > A variant. For sample 5, 4.1% (3/73) of laboratories were discordant for testing the *BRAF* c.1799 T > A variant, 4.4% (2/45) of laboratories were discordant for testing the *NRAS* c.181C > A variant, and 1.7% (1/58) of laboratories were discordant for testing the *EGFR* c.2310_2311insGGT variant. Two laboratory assays were not assessed due to the LoD of their testing platform being above the consensus value for that variant (Table 1 and Supplementary Table 1). The individual discordant laboratories are provided in Supplementary Table 1.

In total, 387 cfDNA variant genotype assays were performed in this EQA program with 370 (95.6%) being concordant for detecting the consensus variants.

Table 1 Circulating free DNA testing platforms used by 88 clinical diagnostic laboratories

Laboratory	Platform	Company	LoD	Assay Kit
1	NextSeq 500	Illumina	0.10%	Burning Rock Dx
2	Ion torrent PGM	Thermo fisher	0.50%	Thermo Fisher Scientific
3	Ion Torrent S5XL	Thermo fisher	0.10%	Thermo Fisher Scientific
4	HiSeq X Ten	Illumina	0.50%	Novogene
5	NextSeq CN500	Illumina	0.50%	Berry Genomics
6	HiSeq X Ten	Illumina	0.10%	Agilent
7	HiSeq X Ten	Illumina	0.05%	NEB
8	NextSeq CN500	Illumina	0.20%	3DBiopharm
9	Ion torrent PGM	Thermo fisher	0.20%	MP33 Panel
10	NextSeq 500	Illumina	0.50%	IDT
11	NextSeq CN500	Illumina	0.10%	Amoy Dx
12	NovaSeq 6000	Illumina	0.30%	Roche
13	HiSeq X Ten	Illumina	0.10%	Agilent
14	GeneReader	QIAGEN	0.10%	QIAGEN
15	NextSeq CN550	Illumina	0.10%	Kapa Biosystems
16	Ion torrent PGM	Thermo Fisher	0.10%	Ion Ampliseq Custom DNA Panel
17	HiSeq X Ten	Illumina	0.20%	SureSelect XT Library Prep Kit (Agilent)
18	NextSeq 500AR	Illumina	0.50%	QIAseq Targeted DNA Panel(QIAGEN)
19	HiSeq X Ten	Illumina	0.20%	Roche
20	Hiseq4000	Illumina	0.10%	LDT
21	Hiseq4000	Illumina	0.10%	LDT
22	NextSeq CN500	Illumina	0.10%	IDT
23	Ion torrent PGM	Thermo Fisher	0.30%	MP33 Panel
24	NovaSeq 6000	Illumina	0.20%	SeqCap EZ Hyb and Wash Kit (Roche)
25	NextSeq 550AR	Illumina	0.30%	Roche Nimblegen SeqCap EZ hybridization and Wash Kit
26	Ion S5	Thermo Fisher	0.03%	Oncomine Lung Cell-Free Total Nucleic Acid Research Assay (Thermo fisher)
27	NextSeq 500	Illumina	0.10%	AnchorAIM
28	NextSeq CN500	Illumina	0.25%	PlasAim
29	HiSeq3000	Illumina	0.10%	xGEN Lockdown custom Probe (IDT)
30	NextSeq 500	Illumina	0.10%	PlasAim
31	BioelectronSeq 4000	Thermo Fisher	0.10%	Oncomine Lung cfDNA assay (Thermo Fisher)
32	HiSeq X Ten	Illumina	0.30%	NimbleGen SeqCap Hybridization and Wash Kit (Roche)
33	NextSeq CN500	Illumina	0.10%	Pillar™ High Sensitivity cfDNA Lung Cancer Hot Spots Panel
34	Nextseq 500	Illumina	0.30%	LDT
35	HiSeq 2500	Illumina	0.20%	Seq Cap EZ Hybridization and Wash Ki (Roche)
36	MiSeq	Illumina	0.20%	VariantPro cancer Kit(LDT)
37	NextSeq CN500	Illumina	0.05%	IDT xGen® Lockdown Probes
38	NextSeq CN500	Illumina	0.50%	SureSelect QXT Reagent kit for 96 samples (Agilent)
39	NextSeq CN500	Illumina	0.10%	Pillar™ High Sensitivity cfDNA Lung Cancer HotSpots Panel
40	NextSeq CN500	Illumina	0.20%	SureSelect Target Enrichment Box(Agilent)
41	HiSeq 2500	Illumina	0.50%	Seq Cap EZ Hybridization and Wash Kit (Roche)
42	NextSeq CN500	Illumina	0.10%	xGen Universal Blockers-TS Mix, 96rxn (IDT)
43	NextSeq CN500	Illumina	0.10%	xGen Universal Blockers-TS Mix, 96rxn (IDT)
44	HiSeq X Ten	Illumina	0.30%	Labseq Target Enrichment for Illumina Platform
45	NextSeq 500	Illumina	0.10%	xGen Universal Blockers-TS Mix, 96rxn (IDT)
46	DA8600	Thermo Fisher	0.50%	LDT blockers and xGen® Hybridization and Wash Kit (IDT)
47	NextSeq CN500	Illumina	0.30%	SLIMamp™ Pan-Cancer HotSpots Panel
48	NextSeq CN500	Illumina	0.10%	LDT
49	NextSeq CN500	Illumina	0.10%	LDT

Table 1 (continued)

Laboratory	Platform	Company	LoD	Assay Kit
50	NextSeq CN500	Illumina	0.30%	NimbleGen SeqCap Hybridization and Wash Kit(Roche)
51	NextSeq CN500	Illumina	0.10%	LDT
52	NextSeq CN500	Illumina	0.50%	xGen Universal Blockers-TS Mix, 96rxn (IDT)
53	NextSeq CN500	Illumina	0.10%	LDT
54	NextSeq CN500	Illumina	0.10%	LDT
55	NovaSeq 6000	Illumina	0.30%	SeqCap EZ Library(Roche)
56	NextSeq 500	Illumina	0.10%	LDT
57	NextSeq CN500	Illumina	0.30%	NimbleGen SeqCap Hybridization and Wash Kit(Roche)
58	HiSeq X Ten	Illumina	0.50%	xGen Lockdown Probes and Reagents (IDT)
59	NextSeq CN500	Illumina	0.10%	LDT
60	NextSeq 500	Illumina	0.20%	Human Actionable Solid Tumor Panel (QIAGEN)
61	Ion S5XL	Thermo Fisher	0.10%	ION Ampliseq Library kit 2.0-96LV (Thermo Fisher)
62	NextSeq 500	Illumina	0.20%	96 rxn xGen® Lockdown® Reagents (IDT)
63	HiSeq X Ten	Illumina	0.10%	LDT
64	NextSeq CN500	Illumina	0.20%	xGen® Lockdown® Reagents (IDT)
65	HiSeq 2500	Illumina	0.10%	SeqCap® EZ Hybridization and Wash Kits (Roche NimbleGen)
66	NextSeq CN500	Illumina	0.50%	xGen Lockdown Reagents(IDT)
67	dPCR	Thermo Fisher	0.50%	Custom Probes
68	dPCR	Bio-Rad	0.10%	EGFR gene mutation detection kits (yuanqi bio)
69	dPCR	Bio-Rad	0.04%	LDT (AmoyDx)
70	dPCR	Bio-Rad	0.10%	ddPCRTM Supermix for Probes(Bio-rad)
71	dPCR	Bio-Rad	0.10%	EGFR gene mutation detection kits (yuanqi bio)
72	dPCR	Thermo Fisher	0.10%	QuantStudio 3D Digital PCR probes
73	dPCR	Bio-Rad	0.20%	PrimePCR ddPCR Mutation Assay
74	dPCR	Bio-Rad	0.20%	PrimePCR ddPCR Mutation Assay
75	dPCR	Bio-Rad	0.10%	PrimePCR ddPCR Mutation Assay
76	dPCR	Bio-Rad	0.10%	PrimePCR™ ddPCR™ Mutation Detection Assay Kit
77	dPCR	Bio-Rad	0.05%	PrimePCR™ ddPCR™ Mutation Detection Assay Kit
78	dPCR	Bio-Rad	0.10%	PrimePCR™ ddPCR™ Mutation Detection Assay Kit
79	dPCR	Bio-Rad	0.01%	EGFR T790 M detection kits
80	dPCR	Bio-Rad	0.10%	PrimePCR™ ddPCR™ Mutation Detection Assay Kit
81	dPCR	Thermo Fisher	0.50%	QuantStudio 3D Digital PCR probes
82	dPCR	Bio-Rad	0.10%	PrimePCR™ ddPCR™ Mutation Detection Assay Kit
83	Mass array	Agena Bioscience	5.00%	Oncofocus panel (EGFR, RAS, BRAF)
84	dPCR	BioRad	0.50%	BRAF, EGFR detection kits
85	dPCR	BioRad	0.10%	TaqMan probe
86	ARMS	Roche	0.1–1%	Cobas EGFR assay
87	Mass array	Agena Bioscience	1%	Lung panel, colon panel
88	MiSeq	Illumina	0.50%	Accel-Amplicon 56G Oncology Panel v2 by Swift Biosciences

Discussion

Diagnostic testing of liquid biopsy cfDNA material is of emerging interest to clinical oncology testing laboratories given that cancer-associated DNA variant biomarkers can be easily detected and isolation of the material is representative of a noninvasive process [16, 23, 40–45]. However, liquid biopsy clinical testing guidelines have yet to be devised and the performance of cfDNA testing platforms have not been fully externally assessed. EQA performance monitoring of cfDNA diagnostic testing laboratories is presently challenging given that there is a current lack in the availability of appropriate cost-effective reference testing material. In addition, currently offered liquid biopsy cfDNA EQA schemes do not

Table 2 Proficiency testing data and laboratory performance for cfDNA allelic frequency (AF) detection

Sample	Gene	Transcript	cDNA variant	Protein variant	Predicted AF	Participant consensus AF	Number of laboratories testing each variant	Concordant laboratories
1	*EGFR*	NM_005228.3	c.2235_2249del15	p.Glu746_Ala750del	2.50%	3.72%	87	84 (96.6%)
2	*KRAS*	NM_033360.3	c.35G > A	p.Gly12Asp	0.50%	0.80%	71	68 (95.8%)
3	Control DNA	–	–	–	0%	0%	88	88 (100%)
4	*NRAS*	NM_002524.4	c.181C > A	p.Gln61Lys	2.50%	4.40%	53	49 (92.5%)
5	*BRAF*	NM_004333.4	c.1799 T > A	p.Val600Glu	1%	0.50%	73	70 (95.9%)
5	*NRAS*	NM_002524.4	c.181C > A	p.Gln61Lys	0.50%	0.39%	45	42 (93.3%)
5	*EGFR*	NM_005228.3	c.2310_2311insGGT	p.Asp770_Asn771insGly	1%	0.54%	58	57 (98.3%)

differentiate between the cfDNA extraction phase and the genotype identification phase. This can be problematic for laboratories provided with discordant EQA proficiency testing reports since the EQA provider will be unable to fully ascertain where the sources of error may be occurring [25, 26, 32]. This is important given that different cfDNA extraction processes have been reported to differ in cfDNA recovery rates and these are likely to impact on clinical data interpretation [33]. To eliminate this potential source of error, the reference testing material used in this study were supplied in TE buffer and can be used directly for platform testing without the need to extract the cfDNA. The data generated are therefore more directly reflective of platform efficiency. Once this EQA test is fully established, a specific cfDNA extraction scheme can then be devised and offered.

The data from this study identified that NGS based testing platforms are most commonly used for cfDNA analysis. This is reflective of NGS platforms evolving for sensitive sequencing of cfDNA [46, 47]. In addition, the derived consensus allelic frequency mean for each genotype tested for closely matched that of the predicted allelic frequencies in the EQA reference testing material, thus indicating that the reference testing material was suitable for use in this EQA program (Table 2). Importantly, the laboratory z-score data for each of the six allelic frequency variants analysed suggests that 92.5% - 98.3% of diagnostic testing laboratories were concordant for detecting cfDNA genotypic variants that are at allelic frequencies between 0.39% - 4.4% (Table 2). This allelic frequency range encompasses the cfDNA variant frequency threshold of 2.5% for identifying actionable variants in multiple cancers [48]. The data from this pilot EQA study therefore suggest that clinical testing laboratories are capable of operating at high levels for detecting cfDNA allelic frequency variants that are within a clinical actionable threshold.

In contrast, laboratory discordance ranged from 1.7% (1/58) for detecting the *EGFR* c.2310_2311insGGT variant in sample 5, to 7.5% (4/53) for detecting the *NRAS* c.181C > A variant in sample 4 (Supplementary Table 1). The discordant laboratories primarily reported higher cfDNA genotypic allelic frequencies in comparison to the consensus mean values (Supplementary Table 1). The reporting of higher allelic frequency may be reflective of differences in platforms used, in platform calibration setup, and/or in interpreting the allelic frequency data output. For example, none of the discordant laboratories used the technique of digital PCR (Supplementary Table 1). However, digital PCR testing technology was only used by 18 of the 88 (20.5%) laboratories and allelic frequency testing was limited to a small number of variants (Supplementary Table 1). Nonetheless, these data highlight the importance of participating in an EQA program so that potential sources of error can be readily identified, and key information relayed back to the participating laboratory so that improvements can be implemented. Importantly, the

clinical reporting of false high allelic frequency genotypes could have severe implications for patients undergoing tumour pharmaceutical management since these data would suggest that tumour cells are becoming therapeutically resistance to treatment [49, 50]. However, this is unlikely to occur at this stage since liquid biopsy testing is not clinically accepted as a stand-alone test. As such, tumour biopsy and cytology tests would still be required for confirmation.

In conclusion, this study found that participating clinical laboratories primarily used NGS technology for cfDNA variant genotyping. Importantly, proficiency testing identified that at least 92% of laboratories testing for a specific cfDNA variant were proficient for clinical analyses. The finding of discordant laboratories further highlights the importance of enrollment on EQA programs so that proficiency in diagnostic testing can be determined and potential sources of error identified and addressed. These data may further support the development of cfDNA technology testing guidelines which would help raise confidence for the use of cfDNA liquid biopsy testing in clinical practice [32, 48].

Author Contributions **SYC** acquired and performed data analysis, assisted in the drafting and in revisions of the updated document, approved final version and agreed to be accountable for all aspects of the work performed.

RP co-developed the EQA material used in this study, acquired and performed data analysis, assisted in the drafting and in revisions of the updated document, approved final version and agreed to be accountable for all aspects of the work performed.

RZ co-developed the EQA material used in this study, acquired and performed data analysis, assisted in the drafting and in revisions of the updated document, approved final version and agreed to be accountable for all aspects of the work performed.

LZ co-developed the EQA material used in this study, acquired and performed data analysis, assisted in the drafting and in revisions of the updated document, approved final version and agreed to be accountable for all aspects of the work performed.

NP acquired and performed data analysis, assisted in the drafting and in revisions of the updated document, approved final version and agreed to be accountable for all aspects of the work performed.

KHT acquired and performed data analysis, assisted in the drafting and in revisions of the updated document, approved final version and agreed to be accountable for all aspects of the work performed.

TB performed interpretation of data, assisted in the drafting and in revisions of the updated document, approved final version and agreed to be accountable for all aspects of the work performed.

JL co-developed the EQA material used in this study, performed interpretation of data, assisted in the drafting and in revisions of the updated document, approved final version and agreed to be accountable for all aspects of the work performed.

MPH developed initial manuscript, performed interpretation of data, assisted in the drafting and in revisions of the updated document, approved final version and agreed to be accountable for all aspects of the work performed.

Funding Information This work was supported by the Beijing Natural Science Foundation (No. 7174345; R.Z.), the National Nature Science Foundation of China (No. 81601848; R.Z.), and the Quality Use of Pathology Program (QUPP) funding, Australia.

Compliance with Ethical Standards No human or animal was used in this study. All reference testing material were synthetically derived using commercially available cells.

Conflict of Interest The authors declare no conflict of interest.

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