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Circulating Tumor DNA Outperforms Circulating Tumor Cells for *KRAS* Mutation Detection in Thoracic Malignancies

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BACKGROUND: Circulating biomarkers, such as circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), are both considered for blood-based mutation detection, but limited studies have compared them in a head-to-head manner. Using *KRAS* (Kirsten rat sarcoma viral oncogene homolog), we performed such a comparison in patients who underwent surgery for suspected lung cancer.

METHODS: We recruited 93 patients, including 82 with lung cancer and 11 with benign diseases of the lung. Mutations were detected in codons 12 and 13 of *KRAS* in DNA extracted from CTCs, plasma, and matched tumors or lung tissues with custom-designed coamplification at lower denaturation temperature (COLD)-PCR assays, high-resolution melt analysis (HRM), and commercial assays (Roche Cobas® *KRAS* mutation test and Qiagen Therascreen® pyrosequencing *KRAS* kit).

RESULTS: With the Cobas mutation test, we identified *KRAS* mutations in 21.3% of tumors. Mutation analysis in matched CTC DNA and ctDNA samples by COLD-PCR/HRM assay revealed mutations in 30.5% (ctDNA) and 23.2% (CTC DNA) of patients with lung cancer. Combined results of different tests revealed *KRAS*-positive cases for 28% of tumors. The diagnostic sensitivity and specificity of *KRAS* mutation detection in tumors achieved with ctDNA was 0.96 (95% CI 0.81–1.00) and 0.95 (0.85–0.99), respectively. The diagnostic test performance was lower for CTC DNA, at 0.52 (0.34–0.73) and 0.88 (0.79–0.95).

CONCLUSIONS: Our results support ctDNA as a preferential specimen type for mutation screening in thoracic malignancies vs CTC DNA, achieving greater mutation detection than either CTCs or limited amounts of tumor tissue alone.

Circulating tumor cells (CTCs)[3] and circulating tumor DNA (ctDNA) represent a valuable source of genetic information about the tumors from which they originate. Their detection and molecular characterization, often denoted as a form of "liquid biopsy," is one of the most actively developing areas in translational research in cancer *(1, 2)*.

The clinical utility of liquid biopsy is based on the hypothesis that CTCs and ctDNA harbor the same profile of somatic mutations and genomic rearrangements as the source (primary tumor). Therefore, their molecular analysis in the blood may reveal the mutation status of the primary tumor without the need for invasive tissue biopsy. A number of successful studies have been performed to validate this concept, but except for a few examples *(3–5)*, the substrate was either CTCs or (separately) ctDNA.

One potential benefit of CTCs is their DNA integrity, since the DNA is extracted from viable intact cells, whereas ctDNA is fragmented and degraded. However, tumor heterogeneity may be a drawback, because the isolated circulating cancer cells may not adequately represent the primary tumor in its entirety. Furthermore, capture of CTCs remains challenging *(6)*. At the same time, ctDNA may represent a summation of the overall mutation status of the primary tumor, since the DNA is shed into the bloodstream; however, this occurs in minute amounts (1–100 ng/mL) and is diluted among abundant DNA from nontumor cells without somatic mutations.

Although highly sensitive methods, such as digital PCR and BEAMing (beads, emulsification, amplifica-

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[3] Nonstandard abbreviations: CTC, circulating tumor cell; ctDNA, circulating tumor DNA; BEAMing, beads, emulsification, amplification, and magnetics; COLD-PCR, coamplification at lower denaturation temperature PCR; HRM, high-resolution melt analysis; FFPE, formalin-fixed paraffin-embedded; Tc, critical temperature.

tion, and magnetics), have been developed, their use in routine clinical practice is limited by associated costs and access to specialized equipment *(7)*. Coamplification at lower denaturation temperature (COLD)-PCR has been effectively applied to samples with low abundance of mutated DNA molecules to detect clinically relevant mutations, e.g., in *KRAS* (Kirsten rat sarcoma viral oncogene homolog),[4] *EGFR* (epidermal growth factor receptor), *BRAF* (B-Raf proto-oncogene, serine/threonine kinase), and *TP53* (tumor protein p53) *(8–10)*. Subsequent detection of mutations can be done by relatively inexpensive and straightforward methods such as Sanger sequencing, pyrosequencing, or high-resolution melt analysis (HRM) *(11, 12)*.

The aim of our study was to compare the diagnostic test performance of blood-based CTC DNA vs ctDNA to detect mutations in codons 12 and 13 of *KRAS* genes. We used COLD-PCR coupled with HRM on formalin-fixed paraffin-embedded (FFPE) primary tumor samples (as reference) in patients with thoracic malignancies.

Material and Methods

The study was performed at the Royal Brompton and Harefield NHS Foundation Trust. The project was approved under the auspices of the National Institute of Health Biomedical Research Unit Advanced Disease Biobank (NRES 10/H0504/9). Signed informed consent to participate in the study was obtained from all the patients.

Patients were enrolled consecutively and without prior knowledge of diagnosis or tumor mutation status.

From each patient, peripheral blood was drawn into EDTA-containing Vacutainer tubes. We used 6 mL blood to capture CTCs with ScreenCell® Molecular Biology devices according to the manufacturer's instructions. The capture of CTCs by these devices is based on filtration of fixed blood through filters with pores of 7.5-μm diameter. With the QIAamp DNA Micro kit (Qiagen), we carried out DNA extraction from the CTCs trapped in the filters according to the protocol modification recommended by ScreenCell.

We processed 9 mL blood to obtain plasma, which was stored at −80 °C until further use. Matched FFPE tissues from tumor resection were obtained as well.

We extracted ctDNA from plasma with the QIAamp DNA Blood Mini kit (Qiagen). Before extraction, 1 mL plasma was added to a 2-mL nonstick polypropylene tube and centrifuged at 600*g* for 10 min, followed by removal of a 900-μL aliquot that was used for DNA extraction according to the manufacturer's protocol [adapted to the volume of plasma used by a proportional increase of the volumes of Qiagen protease, buffer AL (guanidinium chloride), and ethanol as recommended by the manufacturer]. Final elution was carried out in 50 μL buffer AE (10 mM Tris-Cl and 0.5 mM EDTA, pH 9.0).

We carried out DNA extraction from FFPE tissues with the QIAamp DNA FFPE Tissue kit (Qiagen). We initially assessed the concentration and purity of DNA spectrophotometrically with the NanoDrop™ Lite instrument (Thermo Scientific) and also by a custom-designed quantitative real-time PCR assay.

For the detection of mutations in codons 12 and 13 of *KRAS*, we developed a COLD-PCR assay coupled with HRM analysis. The primers were designed to flank a 138-bp region surrounding *KRAS* codons 12 and 13. Critical temperature (Tc) for COLD-PCR was initially identified with uMelt software *(13)* and then adjusted experimentally (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue10). Both fast and full COLD-PCR assays were developed. COLD-PCR/HRM was carried out on an ABI 7500 fast instrument with MeltDoctor master mix (Life Technologies). We used the following protocol for fast COLD-PCR: initial denaturation for 10 min at 95 °C; 20 cycles of 15-s denaturation at 95 °C and 1-min primer annealing/amplification at 60 °C; and 45 cycles of 3-s denaturation at Tc and 1-min annealing/amplification at 60 °C. We used the following protocol for full COLD-PCR: initial denaturation for 10 min at 95 °C; 20 cycles of 15-s denaturation at 95 °C and 1-min primer annealing/amplification at 60 °C; and 45 cycles of 15-s denaturation at 95 °C, 5-min heteroduplex formation at 70 °C, 3-s denaturation at Tc, and 1-min annealing/amplification at 60 °C. After PCR, we carried out HRM with the following protocol: 15-s denaturation at 95 °C followed by 1-min renaturation at 60 °C followed by melting from 60 °C to 95 °C at 1% ramp speed.

The analysis of melting curves was performed with High Resolution Melt Software, version 3.0.1 (Life Technologies). All tests were performed in duplicate, with separate DNA samples for the technical replicates, and the results of fast and full COLD-PCR were combined.

We established the analytical sensitivity of the COLD-PCR assays with A549 and H358 cell lines, both carrying *KRAS* codon 12 mutations (c.34G>A and c.34G>T, respectively). The DNA extracted from these cells was mixed with wild-type DNA of TT1 noncancerous lung epithelium cell line in different proportions followed by the COLD-PCR/HRM assay. We found the analytic sensitivity of fast and full COLD-PCR to be as

[4] Human genes: *KRAS,* Kirsten rat sarcoma viral oncogene homolog; *EGFR,* epidermal growth factor receptor; *BRAF,* B-Raf proto-oncogene, serine/threonine kinase; *TP53,* tumor protein p53; *KRASP, KRAS* pseudogene.

little as 0.025% and 0.4% of *KRAS* mutant DNA, respectively, with DNA input of 1–10 ng (see online Supplemental Fig. 2). No amplification of the *KRAS* pseudogene (*KRASP*) was observed under the specified conditions as confirmed by pyrosequencing.

We analyzed DNA extracted from FFPE samples with the Cobas® *KRAS* mutation test and Cobas® 4800 instrument (Roche Molecular Diagnostics). Also, pyrosequencing was carried out on selected samples with Therascreen® *KRAS* kit and PyroMark Q24 instrument (Qiagen).

Statistical analysis included sensitivity and specificity tests as well as Cohen κ statistics estimates and was carried out with *bdpv* and *epicalc* packages in R *(14, 15)*.

Results

The study was designed to compare the performance of the use of DNA extracted from either CTCs or plasma to detect mutations in matched tumors.

From January 2012 to January 2013, 93 patients who underwent surgery for suspected lung cancer were enrolled into the study (Table 1). Among them, cancer was diagnosed in 82 (88.2%), with primary lung cancer in 47 (57.3%). Eleven patients (11.8%) were diagnosed with benign lung diseases, such as pneumothorax or pneumonia. These patients served as a negative control for *KRAS* mutation detection. There were 52 men and 41 women (55.9% and 45.1%, respectively); mean (SD) age was 60 (16) years.

Mutations in codons 12 and 13 of *KRAS* are prioritized markers in the framework of the Cancer Research UK Stratified Medicine Program. The initiative aims to establish the feasibility of molecular genetic testing of drug-targetable mutations in lung cancer patients in the UK. Within the Cancer Research UK Stratified Medicine Program, the Cobas *KRAS* mutation test was chosen to detect mutations in *KRAS*; therefore, we considered this method as a clinically accepted standard. Ninety-three DNA samples extracted from FFPE were subjected to *KRAS* codon 12 and 13 mutation detection by the Cobas mutation test. Eighty-nine tests were successful and revealed mutations in 17 cases (19.1% for all samples and 21.3% for cancer cases only) (Table 2). All mutations were found in patients with cancer.

Considering these results as a reference, we performed mutation analysis in matched CTC DNA and ctDNA samples with our custom-designed COLD-PCR/HRM assay. This assay revealed 26 mutations in ctDNA and 20 mutations in CTC DNA (30.5% and 23.2%, respectively, for cancer patients). In 1 case of benign disease (reactive, nonspecific fibrosis), mutations were found in both ctDNA and CTC DNA.

There were more *KRAS* mutations detected in the blood-derived ctDNA with COLD-PCR/HRM assay than in the reference Cobas testing of FFPE. To resolve the discrepancy, we used the Therascreen *KRAS* pyrosequencing kit to screen the 8 FFPE DNAs samples that were negative for *KRAS* mutations according to Cobas *KRAS* mutation test but positive in matched ctDNA. Seven of 8, including a case classified as benign on formal histopathology, were positive for *KRAS*, confirming the false-negative results for Cobas *KRAS* testing. The mutant allele prevalence in these samples was approximately 1.5% as detected by pyrosequencing, which is below the detection limit for the Cobas test (3%).

Finally, we performed COLD-PCR/HRM analysis in DNA extracted from primary tumors and obtained 22 *KRAS*-positive cases; again, the benign disease case appeared positive. That the mutation was discovered in all tested tissues and in FFPE tissue, that the presence of the mutation was confirmed by the well-established commercial Therascreen *KRAS* test, and that no mutations

Table 1. Patient demographics.[a]

Variable	Value
n	93
Age, years	60.0 (16.2)
Males	52 (56)
Females	41 (45)
Pathology	
Primary lung cancer	47 (50.5)
Adenocarcinoma	27 (57.4)
Squamous cell carcinoma	8 (17.0)
Carcinoid tumor	9 (19.1)
Large cell undifferentiated carcinoma	1 (2.1)
Pleiomorphic carcinoma	1 (2.1)
Small cell carcinoma	1 (2.1)
Metastatic cancer[b]	30 (32.3)
Other cancer[c]	5 (5.4)
Benign	11 (11.8)
Clinical stage[d]	
1	27 (36.0)
2	9 (12.0)
3	8 (10.7)
4	31 (41.3)

[a] Data are n (%) or mean (SD).
[b] Includes 9 colorectal adenocarcinomas, 2 breast adenocarcinomas, 2 chondrosarcomas, 2 large bowel adenocarcinomas, 2 myosarcomas, 2 pleomorphic sarcomas, cancer of bladder or larynx origin, gastrointestinal cancer, haemangiothelioma, liposarcoma, myxofibrosarcoma, osteosarcoma, prostate cancer, teratoma of unspecified origin, left ulna sarcoma, melanoma, and B-cell lymphoma.
[c] Includes 2 cases of mesothelioma, chondrosarcoma of chest wall, ganglioneuroma, and atypical lipomatous tumor of chest wall.
[d] Stage was not reported in 7 cases.

Table 2. Samples with mutations in codons 12 and 13 of KRAS.[a]

Mutation detection method	n	FFPE DNA	ctDNA	CTC DNA
Cobas *KRAS* mutation test				
Total	89[b]	17 (19.1)		
Cancer	80	17 (21.3)		
Benign	9	0 (0.0)		
Therascreen *KRAS* pyrosequencing test[c]				
Total	8	7 (87.5)		
Cancer	7	6 (85.7)		
Benign	1	1 (100.0)		
Custom COLD-PCR/HRM assay				
Total	93	20 (21.5)	26 (28.0)	20 (21.5)
Cancer	82	19 (23.2)	25 (30.5)	19 (23.2)
Benign	11	1 (9.1)	1 (9.1)	1 (9.1)
All methods combined				
Total	93	24 (25.8)		
Cancer	82	23 (28.0)		
Benign	11	1 (9.1)		

[a] Data are n (%).
[b] Test failed in 2 cancer and 2 benign cases.
[c] These samples were negative on Cobas *KRAS* test, but mutations were detected in matched ctDNA or CTC specimens.

were found in other benign disease cases (which confirmed the diagnostic specificity of our test), implied that this mutation was genuine.

Altogether, by combining results of the Cobas *KRAS* test, pyrosequencing, and COLD-PCR/HRM, 24 *KRAS*-positive cases were revealed for FFPE tissue, including 23 in cancer patients (28.0%).

Moderate concordance between ctDNA and CTC DNA was found (Cohen $\kappa = 0.445$). High diagnostic sensitivity and specificity of *KRAS* mutation detection in tumors were achieved with ctDNA, whereas these statistics, especially sensitivity, were lower for CTC DNA (Table 3). This remained the same with results of all mutation detection methods combined for FFPE tissues and with the COLD-PCR/HRM method alone. The latter comparison was performed to compare "like with like," since ctDNA and CTC DNA were not tested by other methods.

There was a moderate increase of *KRAS* mutations in patients with advanced cancer compared with patients with early-stage cancer (Fig. 1); however, this trend was not statistically significant (Fisher exact $P > 0.05$).

Discussion

Both ctDNA and CTCs are currently subjects of many studies looking at biomarkers in cancer. The concentrations of ctDNA and CTCs correlate with disease staging and progression. Inherent genetic abnormalities are considered by some to be potentially useful in relation to mutation status of tumors for personalized medicine.

Our study results show that, compared with CTCs, ctDNA has a much better concordance with FFPE tumor tissue (95.1% for ctDNA vs 78% for CTCs) (Table 3) and is the specimen type of choice for blood-based mutation testing in thoracic malignancies, a finding corroborated in other cancer types *(4)*.

We observed more *KRAS* mutations in ctDNA and CTCs than in the primary FFPE tumor tissue. Discrepancies have also been reported in which *TP53* mutations have been detected in CTCs but not in the corresponding breast cancer tumors *(16)*. This highlights an important issue when using FFPE for mutation detection: intratumoral heterogeneity. Scrolling of a tumor sample from an FFPE block may result in sampling part of the tumor that contains no mutations in a target gene, whereas other parts may contain drug-targetable mutations *(17)*. Mutation detection in this sample would produce false-negative results and, therefore, lead to an incorrect exclusion of the patient from the targeted treatment group.

Conversely, situations in which the primary tumor was positive for mutations but CTCs were negative were also noted in our study (Table 3) and others *(16)*. Apart from tumor heterogeneity, the lack of blood-based mu-

Table 3. Breakdown and diagnostic statistics of concordance between mutation detection in DNA obtained from tumors and blood-derived DNA.[a]

	All mutation detection methods combined[a]		Cold PCR/HRM assay	
DNA source	Positive	Negative	Positive	Negative
ctDNA				
Positive	22	3	18	7
Negative	1	56	1	56
Sensitivity (95% CI)	0.957 (0.810–0.999)		0.947 (0.774–0.999)	
Specificity (95% CI)	0.949 (0.874–0.989)		0.889 (0.801–0.954)	
CTC				
Positive	12	7	10	9
Negative	11	52	9	54
Sensitivity (95% CI)	0.522 (0.335–0.732)		0.526 (0.320–0.756)	
Specificity (95% CI)	0.881 (0.789–0.951)		0.857 (0.764–0.933)	

[a] Cobas *KRAS* mutation test, Therascreen *KRAS* kit, and custom-designed COLD-PCR/HRM assay.

tations when assessed by CTCs could be attributed to the absence of the CTCs in the actual blood sample. We have recently reported *(18)* that, from 3 mL blood, the ScreenCell devices were able to trap the CTCs in ≤72% of patients with thoracic malignancies in the standard clinical settings by use of cytomorphologic criteria (nucleated cells with high nuclear:cytoplasmic ratios larger than resting lymphocytes; cells may also have irregular nuclear outline). Most of the cells were singletons, with a few clusters identified. Although in some cases CTCs likely were genuinely absent in the blood samples, other technical issues such as small sample processing volume may have led to the lack of CTC capture. ScreenCell devices are also occasionally subject to clogging as blood passes through them, resulting in failure to trap CTCs. In our study, blockage occurred in several samples, and we repeated the capture of these cases by use of new devices so that the clogging would not affect our results. Finally, CTCs smaller than 7.5 μm could have passed though the filters. All these reasons could contribute to the observed low diagnostic sensitivity of CTCs for mutation detection. This issue is much less pronounced for ctDNA, and in our study it occurred in only 1 case (Table 3).

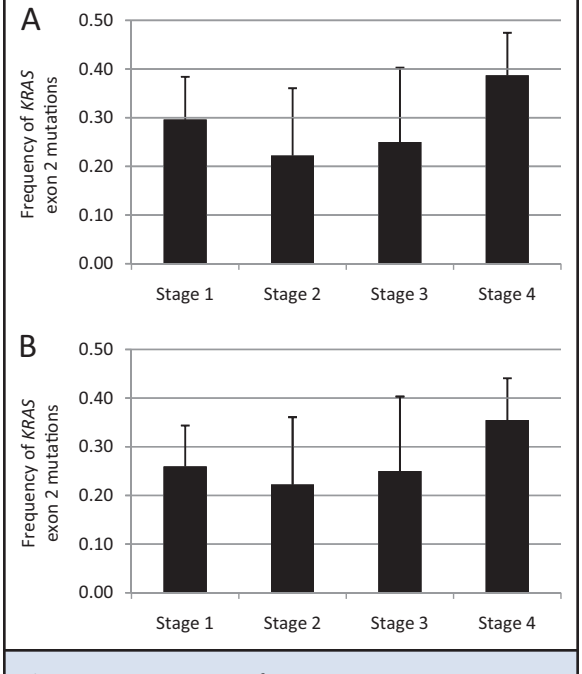

Fig. 1. Frequency (SE) of *KRAS* exon 2 mutations in ctDNA (A) and tumors (B) of lung cancer.

Our method of COLD-PCR/HRM has a lower limit of detection than some other conventional methods in clinical use. This is consistent with other studies that use COLD-PCR combined with different detection methods such as Sanger sequencing, pyrosequencing, and HRM *(8–12)*. A further increase of analytic sensitivity of COLD-PCR is possible, for instance with use of different strategies of wild-type blocking *(19)*. Other high-analytical-sensitivity methods, such as digital PCR, BEAMing, and ultradeep sequencing, may also reveal mutations at a very low level. Although the clinical significance of ultralow-level mutations is currently unclear, since the complexity of diagnosis in thoracic tumors continues to increase, it will become ever more important to establish the overall mutation frequency. Although the

appropriate methods of highly analytically sensitive mutation detection are being widely accepted, it would be crucially important to establish the critical level of mutations, which remains beneficial for predictive testing for targeted therapy.

We also made an important observation of no significant increase of *KRAS* mutation load in patients with advanced lung cancer compared with patients at early stages (Fig. 1). This is consistent with the data that *KRAS* mutations are a rather early driving event in tumor development *(20)*. Practically, it means that detection of *KRAS* mutations alone in liquid biopsy for early cancer diagnosis is insufficient, and a multigene signature reflecting tumor progression will be required.

Overall, our study revealed that ctDNA has considerably higher concordance with FFPE primary tumor tissue than DNA extracted from CTCs for blood-based *KRAS* mutation screening in patients with underlying thoracic malignancies. This makes ctDNA the substrate of choice for liquid biopsy in predictive mutation testing of thoracic malignancies.

We hypothesize that the detection of *KRAS* mutations in the blood is more relevant as a global marker of *KRAS* load, even in the absence of the mutations in the FFPE samples due to tumor and processing heterogeneity (pathologists may have submitted a section of tumor with low *KRAS* mutation load). However, it must be noted that, at present, whereas there are a number of trials for treatment of patients with *KRAS*-positive tumors, none have yet reached conventional clinical practice, and as such there are no present clinical implications of *KRAS* mutation detection in liquid biopsy for cancer.

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