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Detection of *KRAS* Exon 2 Mutations in Circulating Tumor Cells Isolated by the ISET System from Patients with *RAS* Wild Type Metastatic Colorectal Cancer[1]

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Abstract

INTRODUCTION: The presence of *KRAS* mutations in patients with metastatic colorectal cancer (mCRC) predicts poor response to agents targeting the EGFR. Even in patients with *RAS* wild type (WT) tumors, resistance eventually develops due to multiple mechanisms, including the expansion of previously undetected *KRAS* mutated clones. In this feasibility study, we aimed to detect *KRAS* exon 2 mutations in serial samples of circulating tumor cells (CTCs) of *RAS* WT patients with mCRC captured by the Isolation by Size of Epithelial Tumor cells (ISET) system. *METHODS:* CTC isolation using the ISET system was performed from prospectively collected blood samples obtained from patients with *RAS* and *BRAF* WT mCRC prior to first-line therapy initiation, at first imaging assessment and on disease progression. CTCs were enumerated using hematoxylin & eosin and CD45 double stain on a single membrane spot. DNA was extracted from 5 spots and *KRAS* exon 2 mutations were detected using a custom quantitative Polymerase Chain Reaction (qPCR) assay. *RESULTS:* Fifteen patients were enrolled and 28 blood samples were analyzed. In 9 (60%) patients, at least one sample was positive for the presence of a *KRAS* exon 2 mutation. In 11 out of 28 samples (39.2%) with detectable CTCs a *KRAS* mutation was detected; the corresponding percentages for baseline and on progression samples were 27% and 37.5%, respectively. The most commonly detected mutations were G13D and G12C (n = 3). The presence of *KRAS* mutated CTCs at baseline was not prognostic for either PFS (P = .950) or OS (P = .383). CTC kinetics did not follow tumor response patterns. *CONCLUSION:* The results demonstrate that using a qPCR-based assay, *KRAS* exon 2 mutations could be detected in CTCs captured by the ISET system from patients with *RAS* WT primary tumors. However, the clinical relevance of these CTCs remains to be determined in future studies.

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Introduction

The elucidation of the underlying biology of colorectal cancer (CRC) has resulted in significant advances regarding the development of novel agents and, consequently, to the clinically meaningful prolongation of the overall survival (OS) of patients with metastatic disease (mCRC). Approximately 35% to 40% of patients with CRC harbor baseline somatic mutations at the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene exon 2 codon 12, 6% at exon 2 codon 13 and, less commonly, at exons 3 and 4 [1]. In

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addition, 5% to 10% of the patients harbor mutations at the neuroblastoma RAS viral (v-ras) oncogene homolog (*NRAS*) and another 5% to10% at the B-Raf proto-oncogene (*BRAF*) [2]. The presence of these mutations has important implications, since it affects treatment options in mCRC: *RAS* wild type (WT) patients derive benefit from anti-EGFR monoclonal antibodies such as cetuximab (CTX) and panitumumab (PAM) [3,4], in contrast with *RAS* mutated ones. Nevertheless, the emergence of resistance during the disease trajectory under the pressure of anti-EGFR treatment is inevitable [5]. Although multiple and diverse mechanisms have been implicated in the development of acquired resistance [6], the activation of the Mitogen Activated Protein Kinase (MAPK) pathway is almost universal, mainly through newly emerging *KRAS* mutations which may differ in their relative prevalence compared to baseline mutations [7,8].

It is clear that the appropriate selection of patients most likely to respond to anti-EGFR treatment and the timely adjustment of the therapeutic interventions could spare patients from unnecessary toxicity and possibly improve clinical outcomes. Serial biopsies are a useful, albeit cumbersome, tool with the possibility of serious adverse events due to their invasive nature [9]. Thus, the detection and analysis of circulating biomarkers such as circulating tumor cells (CTCs) is a viable alternative, which may accurately capture the spatial and temporal heterogeneity of the disease due to the ease of performing serial testing. Specifically, the enumeration of CTCs has been shown to be prognostic in both early and advanced CRC [10,11] and the genotypic analysis of CTCs has been shown to predict benefit from anti-EGFR treatment [12]. Moreover, the phenotypic and molecular characterization of CTCs can demonstrate the heterogeneity and polyclonality of CRC [13].

Currently, an abundance of methodologies for the detection of CTCs are available [14] but only one has received regulatory approval for use in CRC (CellSearch®, Menarini, Italy); the CellSearch platform can detect CTCs based on the expression of both the Epithelial cell adhesion molecule (EpCAM) and cytokeratins [10]. An inherent disadvantage of this approach is that CTCs undergoing epithelial-to-mesenchymal (EMT) transition, a subpopulation of cells with metastatic potential which are characterized by a down-regulation of epithelial marker expression such as EpCAM and cytokeratins, will not be detected (false negatives) [15]. In contrast, CTC enrichment methodologies based on the physical properties of CTCs such as their size, could improve detection yields. The recently developed Isolation by Size of Epithelial Tumor cells (ISET, Rarecells, France) system has been shown to improve the CTCs' detection rate compared to CellSearch assay in several tumor types [16–18]. As a result, we aimed to explore the feasibility of detecting *KRAS* mutations in CTCs isolated by the ISET system from mCRC *RAS* WT patients and to evaluate the evolving genetic heterogeneity of these cells, compared to both the primary tumor and the effects of treatment.

Patients and Methods

Study Design

This prospective observational study aimed to evaluate the enumeration and molecular characterization of CTCs in mCRC patients isolated by the ISET system and was conducted at the Medical Oncology Department of the University Hospital of Heraklion and the Laboratory of Translational Oncology of the University of Crete. The protocol was approved by the institutional review board (University Hospital of Heraklion Ethics and Scientific Committee, date of decision 11/4/2014, registration number 4399). The study was conducted in compliance with Good Clinical Practice Declaration of Helsinki. Written informed consent was required from all patients prior to enrollment.

Patients

Patients aged >18 years old with histologically confirmed mCRC were eligible for this study. Key eligibility criteria included the presence of at least one measurable lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 and the known absence of *KRAS, NRAS* and *BRAF* mutations either in the primary tumor or a metastatic lesion, as assessed using Sanger sequencing. Patients had to be eligible for combination treatment which included a monoclonal antibody, according to local clinical practice and contemporary guidelines. Exclusion criteria included a second active malignancy, prior administration of systemic treatment for metastatic disease and unstable central nervous system disease. Prior adjuvant chemotherapy was allowed if more than 6 months had elapsed since its completion.

Sample Collection, CTC Enrichment and Enumeration

Samples (10 ml blood in EDTA tubes) were collected immediately prior to the initiation of first line treatment, at the time of the radiologic assessment of response to treatment (either after 4 or after 6 cycles, depending on the treating physician's choice) and at the time of documentation of disease progression but before the initiation of second-line treatment. Samples were processed within 2 hours after their collection according to the manufacturer's instructions. One spot of the ISET membrane (1 ml of blood) was used for CTC enumeration following immunocytochemical (ICC) staining with (i) an anti-CD45 (clone 2B11 + PD7/26, Agilent Technologies, California USA) antibody for exclusion of hematopoietic cells and (ii) hematoxylin and eosin (HE) staining using standard protocols [16]. All ICH and HE stained samples were evaluated for the identification and enumeration of CTCs according to standard morphological criteria [19] by two observers (E.L, M.T).

DNA Extraction and Quantification

Five ISET membrane spots were placed in a 2 ml tube, where they were subjected to Proteinase K digestion at 65 °C for 4 hours. DNA extraction was performed using the MasterPure™ Complete DNA and RNA Purification Kit (Epicenter, Madison, Wisconsin, USA) following the manufacturer's instructions. DNA was quantified using the Qubit fluorometer 2.0 (Thermofisher Scientific, Waltham, Massachusetts, USA) and the samples were stored at -20 °C until their use.

Quantitative Polymerase Chain Reaction (qPCR)

The polypeptide nucleic acid (PNA) based qPCR assay that was used for the detection of *KRAS* mutations and its analytical validity, sensitivity and specificity have been previously described [20,21]. The following tumor cell lines, positive for each *KRAS* point mutation, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and used in control experiments: LS174T (Human colon adenocarcinoma): c.35G > A (p.G12D); HCT116 (Human colon adenocarcinoma): c.38G > A (p.G13D); HUP-T3 (Human pancreatic adenocarcinoma): c.34G > C (p.G12R);

KYSE410 (Human esophageal squamous cell carcinoma): c.34G > T (p.G12C); A549 (Human alveolar adenocarcinoma: c.34G > A (p.G12S); SW403 (Human colon adenocarcinoma): c.35G > T (p.G12 V) and RPMI8226 (Human myeloma): c.35G > C (p.G12A)] or wild type for KRAS (HCC827, Human lung adenocarcinoma) at 1:1 and 1:100 concentrations. [20,21]. In each run, positive and negative controls were included as well as non-template controls. All samples were run in triplicates and a sample was considered as positive if there was at least one positive signal, consistent with previously published studies [22].

Statistical Analysis

Because of the exploratory nature of the study it was not possible to define a sample size estimation. The primary end-point of the study was to evaluate whether CTCs harboring *KRAS* mutations could be detected during the treatment of mCRC *KRAS* WT patients. Summary tables (descriptive statistics and/or frequency tables) were provided for all baseline variables and efficacy variables, as appropriate. Continuous variables were summarized with descriptive statistics (n, mean, standard deviation, range, and median). Progression-free survival (PFS) was defined as the time elapsed between the date of the first chemotherapy administration and either the date of clinical or radiological progression or death from any cause. Overall survival (OS) was measured from the date of the first chemotherapy administration until the date of death from any cause or the date of last follow-up. Qualitative factors were compared by Fisher's exact test. Differences in terms of continuous variables, when comparing related samples, were assessed by the non-parametric Sign test for two medians and Wilcoxon signed-rank test for comparing means. PFS and OS for all patients were estimated using the Kaplan–Meier analysis and the comparisons were computed with the log-rank test. All statistical tests were two-sided, and $P < .05$ was considered statistically significant.

Results

Patient Characteristics

The clinical and demographic characteristics of patients enrolled in this study are summarized in Table 1. In total, 15 patients were enrolled, 7 males and 8 females; the median age was 63 years old (range, 48 to 83). Seven patients were diagnosed with de novo metastatic disease, while of the eight remaining patients seven had received prior systemic chemotherapy, either as adjuvant treatment or as a part of neoadjuvant chemoradiation for rectal cancer. At the time of enrollment, nine patients had liver-only disease and the remaining had lung-only (n = 2 patients), bone-only (n = 1 patient) or multiple sites of disease (n = 3 patients).

Table 1. Patients' Clinical and Demographic Characteristics

	N = 15	%
Age		
Median (range)	63,0 (48–83)	
Sex		
Male	7	46.6%
Female	8	53.3%
Stage at diagnosis		
II	2	13.3%
III	6	40%
IV	7	46.6%
Disease sites		
Liver	9	60%
Lung	2	13.3%
Bones	1	6.6%
Multiple	3	20%
Other treatments received		
Resection of primary tumor	11	73.3%
Neoadjuvant chemoradiotherapy	4	26.6%
Adjuvant chemotherapy	3	20%
Palliative radiotherapy	1	6.6%
Metastasectomy	2	13.3%

Administered Treatment and Outcomes

All patients received combination chemotherapy, either irinotecan-based (FOLFIRI, n = 9 patients) or oxaliplatin-based (FOLFOX, n = 6 patients) according to their physician's choice. Eight of the enrolled patients received an anti-EGFR monoclonal antibody (panitumumab) as part of their first-line treatment. The remaining seven patients received either bevacizumab (n = 4 patients) in the context of standard treatment or aflibercept (n = 3patients) as part of a non-randomized phase II trial evaluating the efficacy of aflibercept/FOLFIRI combination in the first-line setting. At the time of the first response evaluation by imaging studies, which was simultaneously performed with the second sample draw, no patients had experienced disease progression. Nine patients achieved a partial response and six disease stabilization, for an overall response rate of 60%. After a median follow-up of 22.7 months (range, 6.8 to 32.7 month), 5 patients (33.3%) had relapsed and the median PFS was 11.7 months (95% Confidence Interval [CI] 8.6 to 14.9 months) (Supplementary Figure S1). Ten patients were alive and five had died, for a 1-year survival rate of 92.9%. At the time of data cut-off, the median OS could not be estimated (range, 6.8 to 32.7 months).

Quantitative Assessment of CTCs

At least one CTC was isolated in all 28 of the evaluated samples, with a median of 4 CTCs / 1 ml of blood (range, 1 to 26). Clusters of at least 2 CTCs were identified in 14 samples, with a median of 1 cluster / 1 ml of blood (range, 1 to 6). There were no statistically significant differences regarding the number of CTCs between the 3 samples of each patient (Table 2). Although the number of patients was too low for any association between CTC kinetics and the observed tumor response, it should be noted that among the six responding patients with a baseline sample and an available CTC sample at the time of evaluation, two presented a CTC reduction and four a CTC increase in numbers. In addition, among the four patients with a blood sample at baseline and at the time of disease progression, the number of CTCs increased in three of them. Similarly, the evaluation of the kinetics of CTC clusters could not reveal any significant differences (Table 3).

Detection of KRAS Exon 2 Mutations in CTCs

The results regarding the detection of *KRAS* exon 2 mutations in CTCs are summarized in Table 4. In 6 out of 15 patients (40%) no mutations were detected in any sample, while in the remaining

Table 2. Median Values and Range of Circulating Tumor Cells and Comparisons between Samples

	A (n = 11)	B (n = 9)	C (n = 8)	*P* (A vs. B)	*P* (A vs. C)	*P* (B vs. C)
Median	3	6	4	0.508	0.625	1.000
Range	1 - 15	1–26	3–23			

A, baseline; B, at first radiology evaluation; C, at disease progression.
Sign test for two medians.

Table 3. Median Values and Range of Number of Clusters of Two or More Circulating Tumor Cells and Comparisons between Samples

	A (n = 11)	B (n = 9)	C (n = 8)	*P* (A vs. B)	*P* (A vs. C)	*P* (B vs. C)
Median	0	1	1	0.219	0.125	0.750
Range	0 – 2	0–5	0–6			

A, baseline; B, at first radiology evaluation; C, at disease progression.
Sign test for two medians.

patients (n = 9 patients; 60%) a mutation was detected in at least one sample. In total, in 11 (39.2%) out of the 28 samples with detectable CTCs, a *KRAS* exon 2 mutation was detected in these cells. At baseline, 3 out of 11 evaluable samples with CTCs were found to harbor a *KRAS* exon 2 mutation (27%); similarly, on disease progression the respective percentage was 37.5% (3 out of 8 evaluable samples). In all three of those patients, no mutations had been detected in previous samples (Table 4). The most commonly detected mutations were G13D (n = 3 samples) and G12C (n = 3 samples), followed by G12D (n = 2 samples) and G12R (n = 2 samples); the G12A mutation was detected in only one sample. The presence of *KRAS* mutated CTCs at baseline was neither predictive for response to treatment nor prognostic for PFS (*P* = .950) or OS (*P* = .383).

Discussion

The development of reproducible biomarkers that can be used to reliably monitor treatment efficacy in mCRC is a largely unmet need. Despite that radiological imaging is widely used, the attainment of an objective response to systemic chemotherapy has not been shown to correlate with survival outcomes [23]. Carcinoembryonic antigen (CEA) is the most commonly used circulating tumor marker and has been shown to be prognostic in mCRC [24]. Nevertheless, up to 40% of patients have CEA levels within normal range, thereby limiting its relevance for the entire patient population. As a result, liquid biopsy modalities, mainly CTCs and circulating tumor DNA (ctDNA) are being explored in a variety of clinical settings in CRC [25,26]. In this respect, the relative advantages of the ISET system, which captures a more diverse cell population and allows for the enumeration, genotypic and phenotypic characterization of CTCs, led us to explore its utility. In this proof-of-principle feasibility study, we aimed to demonstrate whether the detection of *KRAS* exon 2 mutations in CTCs isolated by the ISET system from patients with *RAS* WT primary tumors undergoing first-line treatment with chemotherapy and a monoclonal antibody was possible, since there is a paucity of data regarding genotyping CTCs captured by the ISET system. This is in keeping with our previously published study where we used the same qPCR methodology in EpCAM positive cells isolated by the CellSearch® system [21].

Table 4. Mutational Status of Patients' Circulating Tumor Cells

Patient Number	Primary Tumor Status	Time Point A	Time Point B	Time Point C
1	WT	-	-	WT
2	WT	-	-	G12R
3	WT	WT	G12C	-
4	WT	-	-	WT
5	WT	-	-	G12C
6	WT	WT	WT	-
7	WT	G13D	G12D	WT
8	WT	WT	WT	G12D
9	WT	WT	G12A	-
10	WT	WT	WT	-
11	WT	G12C	G12R	WT
12	WT	WT	G13D	-
13	WT	G13D	WT	-
14	WT	WT	-	-
15	WT	WT	-	WT

WT, wild type; Time point A, baseline; Time point B, at first radiology assessment; Time point C, at disease progression.

Currently, only the mutational status of the primary tumor cells is taken into account when treating mCRC [27]. Recently, the location of the primary tumor, presumed to be a surrogate for differences in the underlying biology, was also shown to differentiate patients most likely to benefit from anti-EGFR agents [28]. In addition, the emergence of resistance under the pressure of anti-EGFR therapy is unavoidable due to a complex interplay of multiple, often overlapping mechanisms, with newly detected *KRAS* mutations being the most common mechanism of resistance [29]. In accordance with these findings are the results of the current study which demonstrated that 60% of the patients with *RAS* WT primary tumors had detectable CTCs, isolated by ISET, which harbored *KRAS* exon 2 mutations at baseline, during treatment or at the time of disease progression. An interesting observation of the current study was the fact that in three patients, *KRAS* mutations were first detected only at the time of disease progression suggesting that this could be the resistance mechanism at play. In addition, although *KRAS* mutations were detected multiple times in serial samples of two patients, it was not the same mutation in each case, possibly indicating the heterogeneity of the CTC population. The small number of samples however hinders the ability to extract solid conclusions.

The detection of *KRAS* exon 2 mutated CTCs in patients with WT primary tumors corroborate the results of others, despite the use of different CTC capture techniques [30]. This observation further supports the hypothesis that clinically overt resistance in *RAS* WT mCRC frequently occurs due to the expansion of pre-existing *RAS* mutant subclones and, thus, it could represent a predetermined and anticipated event [31]. However, it is not known whether this phenomenon confers absolute or relative clinical resistance, which could be potentially mitigated or even reversed by intermittently withdrawing the selective pressure of anti-EGFR treatment [32]. If validated in a larger randomized trial, this hypothesis could have important therapeutic implications by pre-emptively adapting the administered therapy in order to circumvent or delay the emergence of resistance.

Previous studies have demonstrated that monitoring CTC counts isolated by an antibody-based assay is clinically relevant [33,34]. In our study, the total number of CTCs in the various phases of the disease course was not found to be predictive of clinical outcomes, while their kinetics also did not exhibit a statistically significant concordance with the results of anatomical imaging. Although this could be attributed to the small number of studied patients, we cannot exclude a true lack of correlation since the kinetics of CTC numbers that are captured by ISET are not well described in the literature. Indeed, two studies published by the same group showed conflicting results [13,35]. The lack of association of CTCs as assessed using cytomorphological criteria with the disease course is in contrast with the literature regarding the antigen-based assay and may be explained by a number of reasons, such as the small number of patients included in studies reporting on the ISET system that could have masked any association or the possibility of false positives due to the subjective nature of the enumeration process. Importantly, the ISET system has been shown to isolate a broader cell population

compared to the CellSearch® [17], as well as cells with possibly variable biologic aggressiveness and metastatic potential [36]. Intriguingly, a transient increase in CTC counts after the administration of systemic treatment has been recognized to occur in solid malignancies; this phenomenon seems to be caused by the mobilization of cancer cells from the tumor due to the cytotoxic effects of treatment and there is evidence that this phenomenon lacks any prognostic significance [37]. Therefore, the enumeration of the entire CTC population might not be biologically and clinically relevant and specific subpopulations ought to be recognized based on their comprehensive phenotypic and genotypic characterization.

This was a prospectively designed, proof-of-principle feasibility study, with pre-specified aims and with serial sampling in order to facilitate the detection of newly acquired mutations. Our study suffers from some obvious limitations, namely the low number of patients that were enrolled and the high drop-out rate, the inherently subjective nature of the CTC enumeration and the fact that only *KRAS* exon 2 mutations were tested for, leaving unaddressed the possibility that even more presumably *KRAS* WT CTC samples could potentially harbor *RAS* mutations. However, despite the above limitations our study clearly showed that it is feasible to use liquid biopsies to monitor the presence of *KRAS* mutations in WT mCRC patients undergoing first-line treatment, and therefore supports its use for mutational analysis. The emergence of reproducible ctDNA-based assays used for the diagnosis and treatment monitoring of mCRC offers yet another option [38]. The specific indications, such as treatment monitoring of patients with mCRC [39] or screening for presymptomatic disease [40], and optimal use of these complementary modalities remain to be evaluated in large scale comparative studies.

In conclusion, the detection of *KRAS* exon 2 mutations in CTCs isolated by the ISET system from patients with *RAS* WT mCRC treated with first-line chemotherapy and monoclonal antibodies is feasible and may provide novel insights on the resistance dynamics as depicted by the CTC subpopulations.

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Author contributions

AM and AV performed the PCR experiments. EL, MT and MT performed the CTC staining and enumeration. GS collected the clinical data. DM and VG designed the study. All authors participated in drafting the manuscript.

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