High Intra- and Inter-Tumoral Heterogeneity of RAS Mutations in Colorectal Cancer

Marion Jeantet 1,2,3, David Tougeron 1,4,5, Gaëlle Tachon 1,2, Ulrich Cortes 1,2, Céline Archambaut 1,2, Gaëlle Fromont 3 and Lucie Karayan-Tapon 1,2,6,*

1 Faculté de Médecine Pharmacie, Université de Poitiers, 86021 Poitiers, France; marion_doc@hotmail.fr (M.J.); david.tougeron@chu-poitiers.fr (D.T.); gaelle.tachon@chu-poitiers.fr (G.T.); ulrich.cortes@chu-poitiers.fr (U.C.); labonco@gmail.com (C.A.)
2 Département de Cancérologie Biologique, Centre Hospitalo-Universitaire de Poitiers, 86021 Poitiers, France
3 Département d’anatomopathologie, Centre Hospitalo-Universitaire de Poitiers, 86021 Poitiers, France; Gaelle.fromont-hancard@univ-tours.fr
4 Département de Gastroentérologie, Centre Hospitalo-Universitaire de Poitiers, 86021 Poitiers, France
5 Laboratoire Inflammation, Tissus Epithéliaux et Cytokines, EA 4331, Université de Poitiers, 86021 Poitiers, France
6 INSERM1084, Laboratoire de Neurosciences Expérimentales et Cliniques, Université de Poitiers, 86021 Poitiers, France
* Correspondence: l.karayan-tapon@chu-poitiers.fr; Tel.: +33-5-4944-4988

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Abstract: Approximately 30% of patients with wild type RAS metastatic colorectal cancer are non-responders to anti-epidermal growth factor receptor monoclonal antibodies (anti-EGFR mAbs), possibly due to undetected tumoral subclones harboring RAS mutations. The aim of this study was to analyze the distribution of RAS mutations in different areas of the primary tumor, metastatic lymph nodes and distant metastasis. A retrospective cohort of 18 patients with a colorectal cancer (CRC) was included in the study. Multiregion analysis was performed in 60 spatially separated tumor areas according to the pathological tumor node metastasis (pTNM) staging and KRAS, NRAS and BRAF mutations were tested using pyrosequencing. In primary tumors, intra-tumoral heterogeneity for RAS mutation was found in 33% of cases. Inter-tumoral heterogeneity for RAS mutation between primary tumors and metastatic lymph nodes or distant metastasis was found in 36% of cases. Moreover, 28% of tumors had multiple RAS mutated subclones in the same tumor. A high proportion of CRCs presented intra- and/or inter-tumoral heterogeneity, which has relevant clinical implications for anti-EGFR mAbs prescription. These results suggest the need for multiple RAS testing in different parts of the same tumor and/or more sensitive techniques.

Keywords: colorectal cancer; RAS mutation; intra-tumoral heterogeneity; inter-tumoral heterogeneity

1. Introduction

Colorectal cancer (CRC) is the third deadliest of all cancers [1]. Nearly one-third of the patients will eventually die of the disease. Targeting the epidermal growth factor receptor (EGFR), an important component in CRC carcinogenesis, is one of the major therapeutic options in metastatic CRC (mCRC). Two anti-EGFR monoclonal antibodies (mAbs), cetuximab and panitumumab, are commonly used in mCRC. Clinical trials have shown the benefit of anti-EGFR mAbs alone or in combination with chemotherapy in mCRC [2–4].

Several studies have demonstrated that KRAS mutation in exon 2 is a predictive marker of resistance to anti-EGFR mAbs [5]. More recently, other activating RAS mutations (KRAS exons 3 and 4 and NRAS exons 2, 3 and 4) were also shown to confer resistance to anti-EGFR mAbs [3,4].
Approximately 50% of mCRC harbor mutations in exons 2, 3 or 4 of either KRAS or NRAS genes [6]. The most frequent mutations are detected in exon 2 (codons 12 and 13) of KRAS (40%), and, to a lesser extent, in exon 3 (codons 59 and 61) and exon 4 (codons 117 and 146) of KRAS (≈7% of cases). Activating mutations of NRAS occur only in a subset of mCRC (≈5% of cases), mostly at codons 12, 13 and 61 [6]. The \( \text{BRAF}^{V600E} \) mutation occurs in 10%–15% of mCRC [7,8]. \( \text{BRAF}^{V600E} \) mutant mCRC is associated with poorer outcomes. However, whether this mutation is predictive of resistance to anti-EGFR mAbs is uncertain [7].

Only wild-type (WT) RAS mCRCs benefit from treatment with anti-EGFR mAbs. Nevertheless, nearly 35% of patients with WT RAS tumors do not respond to anti-EGFR treatment [3,4,6]. Several molecular mechanisms underlying the development of treatment resistance have been reported in the literature [9]. One possible explanation lies in tumor heterogeneity with regard to RAS mutations [8,10]. There is a general consensus that progression of cancer develops from a single mutated cell, followed by clonal expansion associated with genetic alterations. The acquisition of these alterations can result in the emergence of new tumor subclones with different genotypes [11]. Intra-tumoral heterogeneity is defined by the presence of at least two different tumoral subclones within the same tumor mass. Inter-tumoral heterogeneity consists in the presence of at least two different tumor subclones at different tumor sites in a single patient (i.e., primary tumor, metastatic lymph nodes or metastases) [12]. Both intra- and inter-tumoral heterogeneity are important to identify since they could affect response to targeted therapies.

Different levels of tumoral heterogeneity have already been observed in several tumor types [13–15]. Nevertheless, there are few data concerning intra- and inter-tumoral heterogeneity in CRC. KRAS, NRAS and BRAF mutations are considered to be mutually exclusive in CRC [16]. Inter-tumoral heterogeneity seems to be relatively low between primary and metastatic lesions in mCRC since concordance of KRAS and BRAF status is over 95% [17–19]. Nevertheless, these previous works used sequencing methods with low sensitivity and did not study complete RAS status. In addition, few data have been available concerning inter-tumoral heterogeneity of RAS and BRAF mutations between primary tumors and lymph node metastasis. Data concerning intra-tumoral heterogeneity of RAS and BRAF mutations between different areas of primary tumor data are lacking. In the present study, we investigated intra- and inter-tumoral heterogeneity of RAS and BRAF mutations in 60 tumor areas from 18 CRCs.

2. Results

2.1. Population

We retrospectively analyzed tumors from 18 patients with CRC (twelve colons and six rectums). Mean age at diagnosis was 66.5 ± 9.0 years (Table 1). Tumor stages were stage I (\( n = 1, \text{5\%} \)), stage II (\( n = 3, \text{17\%} \)), stage III (\( n = 5, \text{28\%} \)) and stage IV (\( n = 9, \text{50\%} \)). According to the pathological tumor node metastasis (pTNM) staging, tumors were pT2 (\( n = 1, \text{5\%} \)), pT3 (\( n = 14, \text{78\%} \)) and pT4a (\( n = 3, \text{17\%} \)).

Tumors were assessed for KRAS mutations in codons 12 and 13 (\( \text{KRAS}^{12–13} \)) on the initial transparietal section (ITS). Five tumors were \( \text{KRAS}^{12–13} \) mutant (MUT), seven tumors were \( \text{KRAS}^{12–13} \) WT and six tumors had potential low level (PLLM) \( \text{KRAS}^{12–13} \) mutations (Table 2). Tumors with PLLM are defined as tumors with mutant allele frequency \( \geq \) limit of detection (LOD) and \( \leq \text{LOD + 3\%} \) as described in the materials and methods section [8]. One WT KRAS tumor had a \( \text{BRAF}^{V600E} \) mutation.
| Patient | Age  | Sex | Tumor Site   | Stage | pTNM 2009 | Initial KRAS | Initial BRAF | Recurrence | OS       | Status |
|---------|------|-----|--------------|-------|-----------|--------------|--------------|------------|----------|---------|
| 2       | 77.9 | F   | right colon  | III   | pT3N1bM0  | WT           | WT           | yes        | 48.68    | dead    |
| 3       | 75.9 | M   | left colon   | III   | pT4aN2bM0 | WT           | WT           | no         | 2.50     | alive   |
| 4       | 79.2 | F   | right colon  | II    | pT3N0M0   | WT           | V600E        | no         | 59.14    | alive   |
| 5       | 55.9 | M   | left colon   | I     | pT2N0M0   | WT           | WT           | no         | 62.50    | alive   |
| 6       | 67.3 | M   | left colon   | III   | pT3N1bM0  | G12S         | WT           | yes        | 69.21    | dead    |
| 7       | 72.9 | F   | left colon   | IV    | pT3N1bM1  | G12V         | WT           | -          | 26.35    | dead    |
| 8       | 58.5 | M   | left colon   | III   | pT4aN0M0  | G12S         | WT           | yes        | 82.86    | dead    |
| 9       | 77.8 | F   | left colon   | IV    | pT4aN0M1  | G12D         | WT           | -          | 22.60    | dead    |
| 10      | 76.4 | F   | right colon  | IV    | pT3N1aM1  | G12D         | WT           | -          | 48.39    | dead    |
| 11      | 77.7 | M   | left colon   | IV    | pT3N2bM1  | G12D         | WT           | -          | 14.41    | dead    |
| 12      | 74.7 | F   | right colon  | II    | pT3N0M0   | G12V         | WT           | no         | 37.70    | alive   |
| 13      | 66.0 | M   | left colon   | II    | pT3N0M0   | G12V         | WT           | -          | 49.00    | alive   |
| 14      | 61.9 | M   | rectum       | IV    | pT3N2bM1  | WT           | WT           | -          | 22.5     | dead    |
| 15      | 58.9 | M   | rectum       | IV    | pT3N2bM1  | WT           | WT           | -          | 52.34    | dead    |
| 16      | 57.1 | F   | rectum       | IV    | pT3N2bM1  | WT           | WT           | -          | 44.80    | dead    |
| 17      | 65.2 | F   | rectum       | IV    | pT3N2bM1  | G12D         | WT           | -          | 17.96    | dead    |
| 18      | 54.9 | F   | rectum       | III   | pT3N2aM0  | G12V         | WT           | yes        | 41.22    | alive   |
| 19      | 58.6 | M   | rectum       | IV    | pT3N2M1   | G12R         | WT           | -          | 43.82    | dead    |

M: male; F: female; OS: overall survival; WT: wild-type; pTNM: pathological tumor node metastasis.
### Table 2. RAS and BRAF mutations in the initial transparietal sections (n = 13) and spatially separated tumor areas (n = 43) in 13 colorectal cancers.

| Heterogeneity                          | Patients | pTNM | %TC | KRAS        | Other Genes |
|----------------------------------------|----------|------|-----|-------------|-------------|
|                                        |          |      |     |             |             |
| Intra-tumoral heterogeneity            | 4        | ITS  | 70  | WT          | BRFV600E (PLLM) |
|                                        |          | pT1  | 30  | WT          | WT          |
|                                        |          | pT2  | 90  | WT          | BRFV600E (MUT) |
|                                        | 9        | ITS  | 70  | G12S (PLLM)/Q61R (PLLM) | N Ras-Q61R (MUT) |
|                                        |          | pT1  | 95  | WT          | WT          |
|                                        |          | pT2  | 70  | WT          | N Ras-Q61R (MUT) |
|                                        |          | pT3  | 25  | WT          | N Ras-Q61R (MUT) |
|                                        | 18       | ITS  | 80  | G12R (MUT) | WT          |
|                                        |          | pT1  | 30  | G12R (MUT) | WT          |
|                                        |          | pT2  | 35  | G12R (MUT) | WT          |
|                                        |          | pT3  | 20  | G12R (MUT) | WT          |
| Intra-tumoral and Inter-tumoral        | 2        | ITS  | 80  | WT          | N Ras: A59T (PLLM) |
| heterogeneity                          |          | pT2  | 15  | WT          | WT          |
|                                        |          | pT3  | 20  | G13D (MUT) | WT          |
|                                        |          | N    | 5   | WT          | WT          |
| Intra-tumoral heterogeneity only       | 7        | ITS  | 60  | G12S (PLLM) | FA (N Ras61 et Kras146) |
|                                        |          | pT1  | 70  | G12D (MUT)/Q61L (PLLM) | N Ras:Q61R (MUT) |
|                                        |          | pT2  | 25  | G12S (MUT) | N Ras:Q61K (MUT) |
|                                        |          | pT3  | 40  | Q61L (PLLM) | N Ras:Q61K (MUT) |
|                                        |          | N    | 25  | WT          | N Ras:Q61K (MUT) |
| Inter-tumoral heterogeneity only       | 12       | ITS  | 40  | G12D (PLLM)/A146T (MUT) | FA (Braf) |
|                                        |          | pT2  | 15  | G12D (MUT) | FA (Braf) |
|                                        |          | pT3  | 20  | Q61H(PLLM)/A146T (MUT) | FA (Braf) |
|                                        |          | N    | 25  | FA (Braf)    | FA (Braf) |
| Mutation without heterogeneity        | 14       | ITS  | 30  | G12V (MUT) | WT          |
|                                        |          | pT1  | 5   | G12V (MUT) | WT          |
|                                        |          | pT2  | 40  | G12V (MUT) | WT          |
|                                        |          | pT3  | 5   | G12V (MUT) | WT          |
|                                        |          | N    | 5   | WT          | WT          |
|                                        | 8        | ITS  | 30  | G12V (MUT) | WT          |
|                                        |          | pT1  | 60  | G12V (MUT) | WT          |
|                                        |          | pT2  | 75  | G12V (MUT) | WT          |
|                                        |          | pT3  | 60  | G12V (MUT) | WT          |
|                                        |          | N    | 80  | G12V (MUT) | WT          |
|                                        | 11       | ITS  | 5   | G12D (PLLM) | WT          |
|                                        |          | pT1  | 5   | G12D (MUT) | WT          |
|                                        |          | pT2  | 40  | G12D (MUT) | WT          |
|                                        |          | pT3  | 5   | G12D (MUT) | WT          |
|                                        |          | N    | 5   | G12D (MUT) | WT          |
|                                        | 13       | ITS  | 70  | G12V (MUT) | WT          |
|                                        |          | pT1  | 70  | G12V (MUT) | WT          |
|                                        |          | pT2  | 10  | G12V (MUT) | WT          |
|                                        |          | pT3  | 15  | G12V (MUT) | WT          |
|                                        | 15       | ITS  | 40  | G12D (MUT) | WT          |
|                                        |          | pT1  | 60  | G12D (MUT) | WT          |
|                                        |          | pT2  | 15  | G12D (MUT) | WT          |
|                                        |          | pT3  | 20  | G12D (MUT) | WT          |
|                                        |          | N    | 40  | G12D (MUT) | WT          |
|                                        | 16       | ITS  | 40  | G12V (MUT) | WT          |
|                                        |          | pT1  | 80  | G12V (MUT) | WT          |
|                                        |          | pT2  | 40  | G12V (MUT) | WT          |
|                                        |          | pT3  | 10  | G12V (MUT) | WT          |
|                                        | 17       | ITS  | 30  | G12V (MUT) | WT          |
|                                        |          | pT1  | 30  | G12V (MUT) | WT          |
|                                        |          | pT2  | 20  | G12V (MUT) | WT          |
|                                        |          | pT3  | 10  | G12V (MUT) | WT          |
|                                        |          | N    | 20  | G12V (MUT) | WT          |

%TC: percentage of tumoral cells; ITS: initial transparietal section; N: metastatic lymph node; M: metastasis; WT: wild-type; PLLM: potential low level mutation; MUT: mutant; FA: failed analysis.

#### 2.2. Intra-Tumoral and Inter-Tumoral Heterogeneity of KRAS and NRAS Mutations

Thirty-nine percent of mCRC studied harbored intra- and/or inter-tumoral heterogeneity (Table 2). Among the six tumors with intra-tumoral heterogeneity, only two were KRAS12−13 WT in ITS (cases 2 and 4). Three tumors (cases 2, 7, 12) presented KRAS12−13 PLLM or MUT in at least one of the tumor areas selected, but they also presented other KRAS or NRAS MUT or PLLM in other areas of
the tumor (case 2) or in the same area (case 7, 12). An additional NRAS\textsuperscript{117} PLLM in the submucosa (pT1) was observed for one tumor (case 18) (Table 2 and Figure S1). All in all, 33\% of tumors (6/18) showed intra-tumoral heterogeneity for RAS mutation. The others presented either a WT pattern (cases 1, 3, 5, 6 and 10) or the same mutation with variation in allele frequency (cases 8, 11, 13–17) for all intra-tumoral areas (Table 2 and Table S1).

To evaluate the inter-tumoral heterogeneity, we tested the metastatic lymph nodes and distant metastases for KRAS, NRAS and BRAF mutations when available. Among 11 cases analyzed, four presented inter-tumoral heterogeneity (36\%). Three mutated primary tumors were WT in the metastatic lymph nodes (N+) or in the visceral metastases (M+) (cases 2, 12, 14) (Table 2).

It is worth noting that all patients with mCRC with RAS PLLM and/or RAS mutation in a limited area of the tumor (i.e., intra- or inter-tumoral heterogeneity) treated with anti-EGFR mAbs had disease progression at 2–3 months (n = 5, cases 2, 7, 9, 11 and 12).

2.3. Mutational Intra-Tumoral Heterogeneity

We focus on mutational intra-tumoral heterogeneity, defined as subclones with different mutant allele frequencies. Therefore, we calculated mutant allele frequency in neoplastic cells (MAFnc) and heterogeneity score (HS) for cases 7–9 and 13–18, which harbored the same mutation from the submucosa (pT1), the muscular layer (pT2) to the subserosa (pT3) (Figure 1). MAFnc is the mutant allele frequency normalized to 100\% tumoral cells and HS corresponds to the fraction of neoplastic cells carrying a specific mutation and was calculated assuming that somatic mutations are usually heterozygous events [20]. Mean HS increased with T stage, 128.4 ± 46.1 standard deviation (SD) in pT1, 234.1 ± 186.8 SD in pT2 and 296.2 ± 156.9 SD in pT3 (p = 0.03) (Figure S2). We observed that HS was higher in pT3 areas in 55\% of cases (n = 5/9) regardless of the mutation. Mean total HS (sum of HS for each mutation in one tumor) was 692.6 ± 262.6 SD. Total HS score was associated neither with tumor stage (p = 0.78), nor with tumor location (p = 0.90), nor with tumor recurrence (p = 0.56).

![Figure 1](image)

**Figure 1.** Mutation allele frequency and heterogeneity score. Variability of mutation allele frequency and heterogeneity score between tumoral zone selections in cases which harbored the same mutation in pT1 to pT3. MAF: mutation allele frequency; HS: heterogeneity score.

2.4. RAS Mutations Are Not Exclusive

RAS mutations are considered to be mutually exclusive. Only a few articles have reported cases of coexisting KRAS and NRAS mutations [21]. However, in our study, we observed that different KRAS mutations as well as KRAS and NRAS mutations may coexist. Five tumors had multiple RAS mutated subclones in the same tumor (28\%). Moreover, four presented, in one of their tumor selections, at least
two RAS mutated clones (22%) (cases 7, 9, 12, and 18) (Table 2). Indeed, we observed the coexistence of different KRAS mutations in three tumors (17%) (cases 7, 9, 12) as well as KRAS and NRAS mutations in three tumors (17%) (cases 7, 9 and 18).

Tumors with two or more RAS mutations (cases 2, 7, 9, 12, and 18) as compared with tumors showing one RAS mutation (cases 8, 11, 13 to 17) were associated neither with tumor stage ($p = 0.29$) nor with tumor recurrence ($p = 0.07$).

3. Discussion

This study revealed a high proportion of intra- and inter-tumoral heterogeneity for RAS mutations in metastatic colorectal cancer. Nearly 40% of the mCRC studied harbored intra- and/or inter-tumoral heterogeneity. We also demonstrated the coexistence of different RAS mutations within the same tumor. These results have relevant clinical implications for anti-EGFR monoclonal antibodies prescription in mCRC since RAS mutations confer resistance to this treatment. Hence, testing of KRAS and NRAS mutations (codons 12, 13, 59, 61, 117 and 146) is a prerequisite for anti-EGFR mAbs used in mCRC. In daily practice, this testing usually relies on a single tumor sample with high tumor cell content. However, some patients with RAS WT tumor have primary resistance to anti-EGFR mAbs. One explanation is the limited sensitivity of testing methods, leading to false negative results [6,8,10]. Nevertheless, intra- and/or inter-tumoral heterogeneity of RAS mutations could be the most important cause of therapeutic failure.

Indeed, recently, the “Big Bang” model emphasized that clonal alterations and subclonal events are an early event in CRC carcinogenesis with subclone mixing [22]. Several neoplastic subclones with co-existing mutations in different genes (as well as different molecular alterations) could be present in a single primary tumor with different mutant allele frequencies [19,20,23–25]. In our study, by testing KRAS and NRAS mutations in histologically relevant macrodissected zones according to pTNM, we observed the presence of (i) mutational intra-tumor heterogeneity with at least two co-existing KRAS and/or NRAS mutations within the same tumor areas and (ii) spatial intra-tumoral heterogeneity with coexistence within the same tumor of KRAS and/or NRAS mutated zones and WT zones. This intra-tumoral heterogeneity was found in 33% of cases in our study. Kosmidou et al. reported similar results (44%) of intra-tumoral heterogeneity for KRAS mutations when they compared tumor center and tumor periphery [23]. Recently, Kim et al. observed a substantial level of intra-tumoral heterogeneity (46% to 80%) on multiregion biopsies from five mCRCs [26]. Up until now, our study is one of the largest study concerning intra-tumoral heterogeneity and the only one concerning an extended RAS status.

KRAS, NRAS and BRAF mutations are considered to be mutually exclusive in CRC [16]. Nevertheless, in our study, we observed the coexistence of two different RAS mutations in 28% of cases. We identified the coexistence of different KRAS mutations as well as KRAS and NRAS mutations. Coexistence of different KRAS$^{12–13}$ mutations has been reported by others on small series [24–26]. To our knowledge, the present study is one of the first reports concerning the coexistence of mutations in codons 12–13, 61 and 146 of KRAS (17% of cases). In addition, we demonstrated the coexistence of mutations in KRAS and NRAS, which has been reported only once in Vagaja, N.’s article [21].

Concordance of KRAS and BRAF status between primary and metastatic lesions in mCRC has been considered to be over 95% [17–19]. To our knowledge, few studies have evaluated inter-tumoral heterogeneity of RAS mutations between primary tumors and lymph nodes or distant metastatic lesions. We observed 36% of inter-tumoral heterogeneity, with RAS mutated primary tumors being WT in lymph nodes and/or distant metastatic lesions. In addition, metastatic lymph nodes may have different RAS mutations as compared to primary tumors. Concerning metastatic lymph nodes, only limited data concerning KRAS mutations are available. In one study, heterogeneity in KRAS mutations between primary tumors and lymph node metastases was found in approximately 30% of cases [27]. To summarize, in some tumors, KRAS or NRAS mutations were “universal” as they were present in all macrodissected areas in primary lesions and in metastases. In other cases, we observed multiple
subclones with mutations present only in one area or present in all areas in primary lesions but not in metastases, and they could be classified as “primary-private” and as “primary-clonal”, respectively, as described by others [26] (Figure 2).

In daily practice, RAS mutation testing is usually carried out on a single transparietal section of a given location with a high fraction of neoplastic cells. However, our results have shown that not all RAS mutations present in the tumor can be detected by the current sampling method. One alternative suggestion might be the preparation of a DNA mix obtained after macrodissection of multiple histologically relevant areas, as previously proposed [28]. Moreover, for patients with multiple tumor biopsies and/or surgeries, it is important to perform RAS analyses on each sample. In addition, all KRAS and NRAS mutations must be sought simultaneously in first-line testing as they are not mutually exclusive.

![Figure 2. Subclone distribution in primary tumor and metastatic lymph node. In some cases, KRAS or NRAS mutations were universal/common as they were present in all macrodissected regions in primary tumors (PTs) and in metastatic lymph nodes (MLNs). In other cases, we observed multiple subclones with mutations present only in several regions but not all, or present in all regions in PT but not in MLN, and they could be classified as primary-clonal. In other cases, mutation was present only in one area of the tumor and could be classified as primary-private.](image-url)

All of these results concerning intra- and inter-tumoral heterogeneity are dependent on the sensitivity of the technique used. The more sensitive the sequencing technique, the higher the probability of detecting minority subclones when assessing intra- and inter-tumoral heterogeneity [8,10,29]. However, the clinical relevance of these minority subclones called “potentially low-level mutants” is unknown (i.e., impact on anti-EGFR mAbs resistance). In a previous work, we demonstrated that low-frequency KRAS mutations (2.3% to 10%) are associated with resistance to anti-EGFR mAbs [8]. More recently, Laurent-Puig et al., using highly sensitive picodroplet digital PCR (detection of one mutant KRAS allele in 200,000 WT KRAS alleles), suggested that patients with mCRC showing KRAS-mutated subclones lower or equal to 1% were benefiting from anti-EGFR therapies, while others were not (i.e., >1%) [10]. Due to the limited number of patients treated with anti-EGFR mAbs in our series, we cannot reliably assess the impact of intra- and inter-tumoral heterogeneity on anti-EGFR mAbs efficacy. Nevertheless, all patients with a mCRC harboring RAS PLLM and/or
RAS mutations in a limited part of the tumor (i.e., intra- or inter-tumoral heterogeneity), and treated with anti-EGFR mAbs showed disease progression \((n = 5)\). To conclude, RAS mutated subclones (>1%) partially explain primary anti-EGFR mAbs resistance. In contrast, secondary resistances to anti-EGFR mAbs are partially due to RAS and EGFR mutation [30–32].

One limitation of our study is the limited number of patients included, even though 78 samples from 18 tumors were analyzed. Moreover, in some cases, PPLM was found only in ITS but not in selected macrodissected areas in primary tumors. Interestingly, these results involved high tumoral cellularity cases, thereby confirming that the mutation is present but remains undetectable in the selected macrodissected areas. The major strengths of this study are: (1) analysis of extended RAS status; (2) multiple testing in primary tumors, metastatic lymph nodes and metastasis; and (3) use of a highly sensitive method.

With regard to tumor infiltration, the mutations were more frequently found in subserosa selections (pT3) and heterogeneity score increased with wall invasion; thus, pT3 selection seemed to be the best sampling zone for molecular analysis. In addition, we observed that HS was very high in several areas with a low fraction of neoplastic cells, thereby suggesting possible bias due to low tumoral cellularity or genomic amplification of the mutant allele or loss of the wild-type allele as well as mutation in both alleles [33].

4. Materials and Methods

4.1. Tumor Samples

Tumoral zone selection was performed under HES (Haematoxylin-Erythrosin-Saffron) staining by two experienced pathologists (Marion Jeantet and Gaelle Fromont) according to the 2009 pTNM classification [34]. For each tumor, selections involved the submucosa (pT1), the muscular layer (pT2), the subserosa (pT3), the metastatic lymph nodes (N+) and/or the visceral metastases (M+) when available. For instance, for a pT2 tumor, we performed pT1 and pT2 selections. For a pT3 or pT4 tumor, we performed pT1, pT2 and pT3 selections (Figure 3). The corresponding area of each tumoral zone selection was macrodissected and included in a new paraffin block. Another HES staining was performed to verify correspondence with the originally selected area. The percentage of tumor cells was assessed and selections with less than 5% of tumor cells were excluded.

A total of 60 tumoral macrodissections were analyzed. Among these samples, 15 were in pT1, 18 in pT2, 16 in pT3, 10 in N+ and 1 in M+. Four to six 10 µm thick sections were used for molecular analysis.

4.2. KRAS, NRAS and BRAF Mutation Testing

Genomic DNA from macrodissected sections was extracted with the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany). The 18 initial samples and the 60 newly macrodissected samples were tested for KRAS and NRAS mutations in codons 12–13 (KRAS\(^{12-13}\) and NRAS\(^{12-13}\)), codon 59 (KRAS\(^{59}\) and NRAS\(^{59}\)), codon 61 (KRAS\(^{61}\) and NRAS\(^{61}\)), codon 117 (KRAS\(^{117}\) and NRAS\(^{117}\)) and codon 146 (KRAS\(^{146}\) and NRAS\(^{146}\)). BRAF was tested for mutation in codon 600 (BRAF\(^{V600E}\)). Mutations were detected by pyrosequencing using TheraScreen KRAS PyroKit CE-IVD kit (Qiagen) for KRAS\(^{12-13}\) and KRAS\(^{59}\) or the PyroMark PCR kit (Qiagen) with homemade primers using PyroMark Assay Design 2.0 software (Qiagen, Hilden, Germany) for other RAS [35] and BRAF mutations (Table S2). PCR was carried out using 50 ng of DNA in a total volume of 25 µL according to the Qiagen supplier’s instructions. Each series included a known-mutated and a known wild-type sample, as positive and negative controls. Pyrosequencing was performed using a Pyromark Q24 MDx according to the manufacturer’s instructions (Qiagen).
4.4. Statistical Analyses

Differences in HS according to clinicopathological parameters were analyzed using the Mann–Whitney U test (comparison of two groups) or Kruskal–Wallis test (comparison of more than two groups). For dichotomous variables, Fisher’s exact test was used. Statistical analyses were carried out as follows: when the mutant allele frequency was <LOD, the sample was considered as PLLM and when the mutant allele frequency was ≥LOD + 3%, it was considered as MUT. All mutations (PLLM and MUT) were confirmed at least twice, in two independent experiments.
out with a two-sided test with a significance value of 0.05. All analyses were performed using Statview software (Statview for Windows, SAS Institute, version 5.0, Cary, NC, USA).

5. Conclusions

Concordance of KRAS and BRAF status between primary and metastatic lesions in mCRC has been considered to be over 95% [17–19]. To our knowledge, the present study is one of the first reports concerning inter-tumoral heterogeneity of RAS mutations between primary tumors and lymph nodes or distant metastatic lesions. In conclusion, we have demonstrated the tumoral heterogeneity of RAS mutation and the co-existence of different RAS mutations in CRC, which may have major clinical implications. Our results question our daily practice and expose the limits of single transparietal sampling to ensure optimal molecular analysis. Macrodissection of multiple histologically relevant areas can be an interesting solution as proposed by Richman [28]. The use of new sensitive new generation sequencing methods can also be an alternative option [36]. However, the best solution may arise from peripheral blood testing since circulating tumor cells and circulating tumor DNA are the reflection of the whole tumor [37,38]. These technologies provide another avenue to detect mutations, especially in patients with lymph nodes or distant metastasis.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/17/12/2015/s1.

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