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Optimizing Single Agent Panitumumab Therapy in Pre-Treated Advanced Colorectal Cancer[1,2]

Giampietro Gasparini*[,3,4], Fiamma Buttitta[†,3], Mario Rosario D'Andrea*, Salvatore Tumolo[‡], Angela Buonadonna[§], Ida Pavese[¶], Stefano Cordio[#], Michele De Tursi**, Stefania Mosconi[††], Luciano Stumbo*, Lara Felicioni[†] and Antonio Marchetti[†,4]

*Department of Oncology, San Filippo Neri Hospital, Rome, Italy; [†]Center of Predictive Molecular Medicine, University-Foundation, CeSI Biotech Chieti, Italy; [‡]Oncology Unit Santa Maria degli Angeli, Pordenone, Italy; [§]Oncology Unit B IRCCS Centro di Riferimento Oncologico, Aviano, Italy; [¶]Oncology Unit Ospedale Villa San Pietro Fatebenefratelli, Rome, Italy; [#]Oncology Unit Ospedale Garibaldi, Catania, Italy; **Oncology Unit Department of Clinical and Experimental Science, University of Chieti, Chieti, Italy; [††]Oncology Unit Ospedali Riuniti di Bergamo, Bergamo, Italy

Abstract

PURPOSE: To improve the selection of advanced colorectal cancer patients to panitumumab by optimizing the assessment of *RAS (KRAS-NRAS)* mutations. *EXPERIMENTAL DESIGN:* Using a centralized pyrosequencing *RAS* assay, we analyzed the tumors of 94 patients, wild-type for *KRAS* mutations (codons 12 to 13) by Sanger sequencing (SS), treated with panitumumab. *RESULTS:* By SS analysis, 94 (62%) of 152 patients were wild-type and their objective response rate to panitumumab was 17%. We first optimized the *KRAS* test, by performing an accurate tissue-dissection step followed by pyrosequencing, a more sensitive method, and found further mutations in 12 (12.8%) cases. Secondly, tumors were subjected to *RAS* extension analysis (*KRAS*, exons 3 to 4; *NRAS* exons 2 to 4) by pyrosequencing that allowed to identify several rare mutations: *KRAS* codon 61, 5.3%; codon 146, 5.3%; *NRAS*, 9.5%. Overall, *RAS* mutation rate was 32.9%. All patients with additional *RAS* mutations had progressive or stable disease, except 3 patients with mutations at codon 61 of *KRAS* or *NRAS* who experienced partial (2 cases) or complete response. By excluding from the analysis 11 cases with mutations at codons 61, no patient was responsive to treatment ($P = .021$). *RAS* wild-type versus *RAS* mutated cases had a significantly better time to progression ($P = .044$), that resulted improved ($p = .004$) by excluding codon 61 mutations. *CONCLUSION:* This study shows that by optimizing the *RAS* test it is possible to significantly improve the identification of patients who do not gain benefit of panitumumab. Prospective studies are warranted to determine the clinical significance of rare mutations.

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Address all correspondence to: Prof. Giampietro Gasparini, Department of Oncology, San Filippo Neri Hospital, Via G. Martinotti 20, Rome, Italy. E-mail: gasparini.oncology@hotmail.it
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[3] Contributed equally to the study.
[4] G Gasparini and A Marchetti are co-senior authors.
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Introduction

The major challenge related to the optimal administration of target therapy in oncology is the identification of the subgroups of patients who are more likely to be responsive or resistant to a selective agent [1]. The proper selection of patients to be treated by any targeted drug should be based on the availability of a specific predictive tool to be detected in each single patient allowing a favorable cost/efficacy ratio [2].

The therapeutic paradigm in metastatic colorectal cancer (mCRC) changed after the discovery that mutated tumor *KRAS* status is associated with lack of response to therapy with epidermal growth factor receptor (EGFR) monoclonal antibodies (mAbs). [3–8]. Specifically, patients with *KRAS* mutations in exon 2 are resistant to anti-EGFR. *KRAS* is a small transducer G protein and acquired *KRAS* codon 12 or 13 gain-of-function mutations leads to constitutive signaling through the EGF pathway and to downstream activation of MAPK and PI3K dependent pathways [9,10]. However, *KRAS* determination alone does not have an absolute predictive capability. In fact, even among wild-type tumors the percentage of responsiveness to anti-EGFR agents is approximately 15% to 25% [3–8,11].

Recent results by Douillard et al. [12] demonstrated that additional *RAS* mutations predicted resistance in patients treated with first-line panitumumab and FOLFOX4. They [12] found that 17% of patients with non-mutated *KRAS* exon 2 had other mutually exclusive mutations associated with lack of response to therapy. Similarly, Peeters et al. [13] evaluated, in a retrospective analysis, response to panitumumab monotherapy by using a parallel multigene sequencing technique to determine *KRAS*, additional *RAS* activating mutations (*KRAS* codon 61; *NRAS* codons 12-13-61) and *BRAF*. The authors [13] found improved response rate among the patients with wild-type *KRAS, NRAS,* and *BRAF* tumors.

We evaluated a cohort of 152 patients of whom 94 with tumors treated with panitumumab, on the base of on-site assessment of *KRAS* wild-type, after failure of at least 2 previous schedules of treatment to determine: 1) the impact of a centralized analysis of *KRAS* status by a more accurate tissue dissection and the more sensitive pyrosequencing method, and 2) by extending the study to additional *RAS*-activating mutations, with the aim to identify more potentially non-responsive patients initially classified *KRAS* wild-type. We correlated these analyses with objective response rate (ORR) and time-to-progression (TTP).

Patients and Methods

Patients

Eligible patients for the RASMES multicentre study had pathologic diagnosis of colorectal carcinoma and radiological documentation of metastatic disease (Stage IV; Dukes D). All the patients had received administration of fluoropyrimidine, oxaliplatin and irinotecan, and had disease progression. Other key eligible criteria included: determination of *KRAS* status by Sanger sequencing, 18 years or older, Eastern Cooperative Oncology Group performance status score of 0 to 2, at least two prior chemotherapeutic inclusive adjuvant therapy regimens, measurable disease, adequate bone marrow, hepatic, and renal functions. Exclusion criteria included symptomatic brain metastases, interstitial pneumonitis or pulmonary fibrosis, prior anti-EGFR agent treatment, and *KRAS* mutated tumors.

Panitumumab was administered by a 60-minute intravenous infusion at the dose of 6 mg/kg once every 2 weeks until patients progressed or unacceptable toxicity developed.

All patients with measurable disease at the baseline central review had their objective tumor response assessed by the investigator and blinded central radiology review using modified Response Evaluation Criteria in Solid Tumors (RECIST) [14] every 8 weeks, and, thereafter until disease progression, and confirmed no less than 4 weeks after the criteria for response were first met. At the discretion of the investigator, patients could be evaluated for radiographic tumor assessment after developing symptoms consistent with disease progression. All patients were followed for survival approximately every 3 months up to 2 years.

Time to progression was calculated as the time (in weeks or months) from the basal evaluation of disease before starting panitumumab treatment to the time to the first radiological or clinical progression of disease. Local and central reviews were conducted for all assessments.

The study protocol was approval by The San Filippo Neri Hospital institutional review board and by independent Ethical Committees at each participating Center. Informed consent was obtained from each subject included in the study.

Biomarker Analysis

From a cohort of 152 patients with advanced colon-rectal cancer assessed on-site for *KRAS* codon 12 to 13 mutation status by laboratories who had undergone external quality program, 94 patients with wild-type *KRAS* were treated with panitumumab after failure of at least 2 previous schedules of treatment. These 94 cases were re-analyzed in a centralized laboratory (University of Chieti) for *RAS* (*KRAS/NRAS*) mutations status. Tissue blocks from all these 94 patients obtained from primary surgical resection and before all lines of therapy were sent to the referral laboratory where 3 serial 4-μm-thick histological sections were obtained for morphological and molecular analysis. One section was stained with hematoxylin-eosin and reviewed by 2 pathologists (A.M.and F.B.) to select the optimal tumor areas to be analyzed. The other two sections were carefully micro-dissected under a dissecting microscope to exclude surrounding normal tissues, stromal and inflammatory areas, and to assure the presence of at least 70% of neoplastic cells in the selected areas which were scraped off the glass slide by a sterile needle and then xylene de-paraffinized before digestion with proteinase K at 56 °C overnight. DNA extraction was performed using the spin column procedure (QIAamp DNA Mini Kit; Qiagen, Valencia, CA) on the QiaCube platform (Qiagen, Valencia, CA) and the recovered DNA quantified by NanoDrop 2000 (Thermo Scientific).

DNA samples from the 94 *KRAS* wild type patients were analyzed for codon 12 to 13 of *KRAS* [15] and for additional mutations in *KRAS* (exon 3 at codon 61, exon 4 at codons 117 and 146) and *NRAS* (exon 2 at codons 12 to 13, exon 3 at codon 61, and exon 4 at codons 117 and 146). *RAS* mutational analysis was performed by pyrosequencing using the *KRAS* Pyro Kit, *NRAS* Pyro Kit, *RAS* Extension Pyro Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Sequencing was performed on the PyroMark Q24 platform (Qiagen) that utilizes a pyrosequencing data analysis software (PyroMark Q24 software (Qiagen)) developed to increase the efficiency and consistency of pyrosequencing data analysis.

Statistical Analysis

The variables measured in the study were investigated for association by using the Fisher's exact test or χ^2 test as appropriate. Progression-free survival was measured for each patient from the day

of treatment to the first event of progressive disease. Survival curves were estimated using the Kaplan–Meier method, and differences among them evaluated by the log-rank Mantel Cox test. A $P < .05$ was considered as significant. All statistical analyses were performed using the SPSS pack version 15.

Results

Patients

KRAS status was ascertained in mCRC tumors from 152 patients evaluated for the study of whom 94 (62%) were wild-type and 58 (38%) had *KRAS* codon 12 or 13 mutations.

The clinic-pathologic characteristics of the cohort of 94 patients with *KRAS* wild-type tumors, as determined by a direct sequencing method on site in each single participating center, are reported on Table 1. Severe drug-related side effects were rare particularly regarding gastrointestinal, hematological, and metabolic toxicities. As expected, the majority of grade 3 to 4 toxicities were related to dermatological side effects (12%).

As a first step analysis we re-evaluated in a central referral laboratory (A.M. and F.B.) all the tumor samples of the 94 *KRAS* wild-type patients by an accurate tissue dissection and by a more sensitive pyrosequencing method of exon 12 and 13. This analysis was capable to detect 12 more (12.8%) *KRAS* mutated tumor cases.

Secondly, we evaluated other *RAS* mutations by a comprehensive pyrosequencing method and we found a further 19 (20.2%) mutations (5 in *KRAS* codon 61, 5 in *KRAS* codon 146; 6 in *NRAS* codon 61 and 3 in *NRAS* codon 12 to 13) (Table 2). Overall, by applying both the above analyses we were able to identify, besides the standard direct sequencing *KRAS* method performed on-site in each Center and used to enroll the patients for therapy, a total of 31 *RAS* mutated tumors (32.9%).

Table 1. Patient Clinico-Pathologic Characteristics.

Characteristic	No. of patients (%)
SEX	
Male	53 (56%)
Female	41 (44%)
Eastern Cooperative Oncology Group PS	
0	57 (61%)
1	36 (38%)
2-3	1 (1%)
Primary site	
Colon	75 (80%)
Rectum	19 (20%)
Metastatic sites:	
Liver (only)	20 (21%)
Lung (only)	14 (15%)
Lymph nodes (only)	2 (2%)
Skeletal (only)	2 (2%)
Peritoneum (only)	4 (5%)
Multiple sites	52 (55%)
Number of target lesions	
1 *	26 (28%)
2	26 (28%)
3	18 (19%)
>3	24 (25%)
Number of previous chemotherapy lines	
1	25 (27%)
2	53 (56%)
3	14 (15%)
>3	2 (2%)

* All treated with oxaliplatin-based adjuvant therapy.

Table 2. Frequency of All *RAS* Mutations.

MUTATION	Number of patients (%)
KRAS codons 12-13	12 (13%)
KRAS codon 61	5 (5%)
KRAS codon 146	5 (5%)
NRAS codon 61	6 (6%)
NRAS codons 12-13	3 (3%)
TOTAL	31 (32.9%)

Efficacy According to Tumor RAS Status

The ORR to panitumumab was 17% with 3 patients achieving a complete response (CR) and 13 patients with a partial response (PR). Another 28 cases (29.7%) had a stable disease (SD) lasting at least 3 months. The clinical benefit was 46.7%.

The overall cohort of patients assessed in each Center for *KRAS* analysis, for the central laboratory review and extended *RAS* evaluation is shown in Figure 1.

In the series of the 12 patients with *KRAS* codon 12 to 13 mutations, as detected by the central pyrosequencing method, 10 cases had PROGRESSION DISEASE (PD), and 2 cases had SD.

In the cohort of the 19 tumors in which the extended *RAS* analysis showed further mutations, 11 patients experienced PD, 1 patient achieved a CR, other 2 a PR and 5 had a SD. In detail, of the 5 cases with *KRAS* codon 146 mutation, 3 had PD and 2 had stable disease. Of the 5 cases with *KRAS* codon 61 mutations, 4 had a PD and one a PR. Among the 9 patients with *NRAS* mutated tumors, 7 showed a mutation at codon 61 and 2 at codon 12 to 13. Of the 7 cases with *NRAS* codon 61 mutations, 3 had a PD; 2 SD, one a PR, and one a CR; of the 2 cases mutated at codon 12 to 13 of *NRAS*, 1 had a PD and 1 a SD. In summary, responses (1 CR and 2 PR) were observed only in patients having tumors with *KRAS* and *NRAS* mutations at codon 61. In Table 3 are reported the ORR of the 11 patients with tumors harboring *KRAS* and *NRAS* codon 61 mutations.

By Fisher's exact 2-sided test we correlated ORR with the *RAS* gene status. In all the 31 cases with *RAS* mutations we found no statistically significant difference on response *versus* non response between *RAS* mutated and wild-type tumors ($P = .25$).By excluding from the analysis the 11 cases harboring *RAS* codon 61 the difference in ORR between patients with wild-type tumors versus *RAS* mutated ones reached statistical significance ($P = .02$).

Regarding TTP, the patients with wild-type tumors had a statistically significant better TTP as compared to those with *RAS* mutated disease with 7.4 ± .85 weeks versus 5.2 ± .91 weeks (log rank–Mantel Cox, $P = .044$) (Figure 2*A*). Similar to the results observed on ORR also regarding TTP the deletion from the analysis of the cases harbouring *RAS* codon 61 mutations enhanced the significance in outcome between wild-type versus mutated tumors with 7.4 ± .85 weeks versus 3.94 ± .43 weeks ($P = .04$) (Figure 2*B*).

Discussion

Panitumumab was approved for third-line therapy in mCRC after publication of the results of phase III trial by Van Cutsem et al. [16] demonstrating that this anti-EGFR agent significantly improved progression-free survival, with a good toxicity profile, as compared to best supportive care.

The subsequent retrospective analysis by Amado et al. [3] showed that the patients with mutant *KRAS* codon 12 and 13 tumors are unlikely to benefit from panitumumab therapy. Consequently, clinical guidelines up to 2012 suggested that only mCRC patients

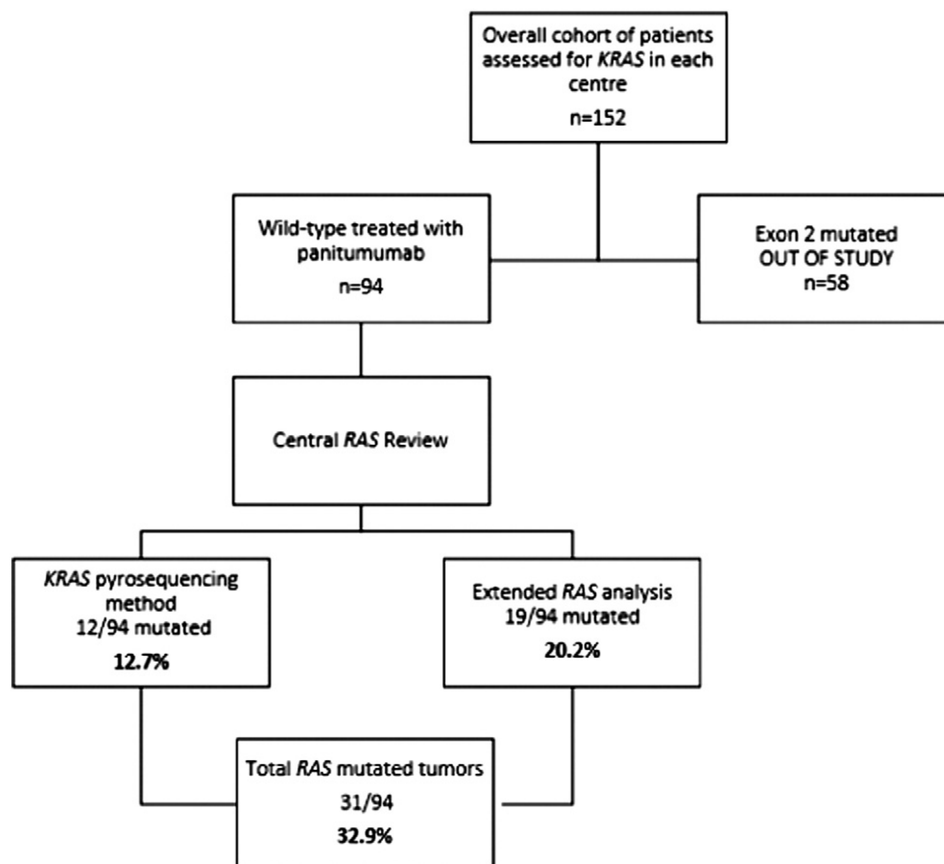

Figure 1. Overall cohort of patients assessed in the study.

with wild-type *KRAS* tumors are to be treated with this anti-EGFR agent. Recently another two phase III trials added relevant information on the relationship between *RAS* gene and efficacy to panitumumab. The first study by Peeters et al. [11] confirmed the better efficacy of the FOLFIRI-panitumumab versus FOLFIRI alone as second-line treatment in patients with mCRC and wild-type *KRAS* tumors. The second study by Douillard et al. [12] allowed a further significant step in the identification of the subpopulation of patients potentially resistant to the regimen FOLFOX4-panitumumab by extending the analysis to all known *RAS* mutations. The authors [12] showed that *RAS* mutations, in addition to *KRAS* exon 2 mutations, improved the benefit-risk profile of panitumumab-FOLFOX4 as first-line treatment. The study [12] identified 17% more patients to be excluded to therapy and allowed for a more selective selection of patients for such a therapeutic regimen.

Table 3. Correlation Between *RAS* Codon 61 Mutations and ORR.

Case	TYPE of mutation	ORR
01	*KRAS* Q61H	PD
02	*KRAS* Q61L	PD
03	*KRAS* Q61H	PD
04	*KRAS* Q61H	PD
05	*KRAS* Q61H	PR
06	*NRAS* Q61R	CR
07	*NRAS* Q61H	PD
08	*NRAS* Q61H	PD
09	*NRAS* Q61R	PR
10	*NRAS* Q61R	SD
11	*NRAS* Q61L	PD

In the present study the patients were prospectively treated with panitumumab monotherapy on the basis of determination of *KRAS* performed on-site in each single Center. The additional molecular analyses performed in the centralized laboratory allowed to improve the results of identification of *KRAS* mutations by using a more sensitive pyrosequencing method and by assessing other *RAS* mutations. Among the 94 patients with *KRAS* wild-type tumors as for the standard on-site direct sequencing technique we identified a further 31 *RAS* mutations (32.9%).

Recent reports have suggested that rare *KRAS* mutations may have different significance with respect to responsiveness to anti-EGFR mAbs.

In particular, controversial data are reported on *KRAS* codon G13D with a number of small retrospective studies that have reported improved outcomes in some patients with such mutations [17–19,11,20–23]. In our series of cases all the 4 patients with G13D mutations had PD in accordance to the results recently reported by Schirripa et al. [24].

Interestingly, in the cohort of tumors analyzed we revealed a relatively high frequency of rare *RAS* mutations possibly due to the high sensitive methodology applied. This allowed us to correlate a relatively high number of tumors harboring rare mutations with clinical outcome.

There is some evidence suggesting that mutations at *KRAS* and *NRAS* codons 61 and 146 have similar impact of mutations at codons 12 and 13. We found that among the 5 tumors harboring codon 146 mutations we observed 2 SD and 3 PD. More controversial was the correlation between codon 61 mutations and outcome. Among the 11 cases we observed 3 responsive patients (1 CR;

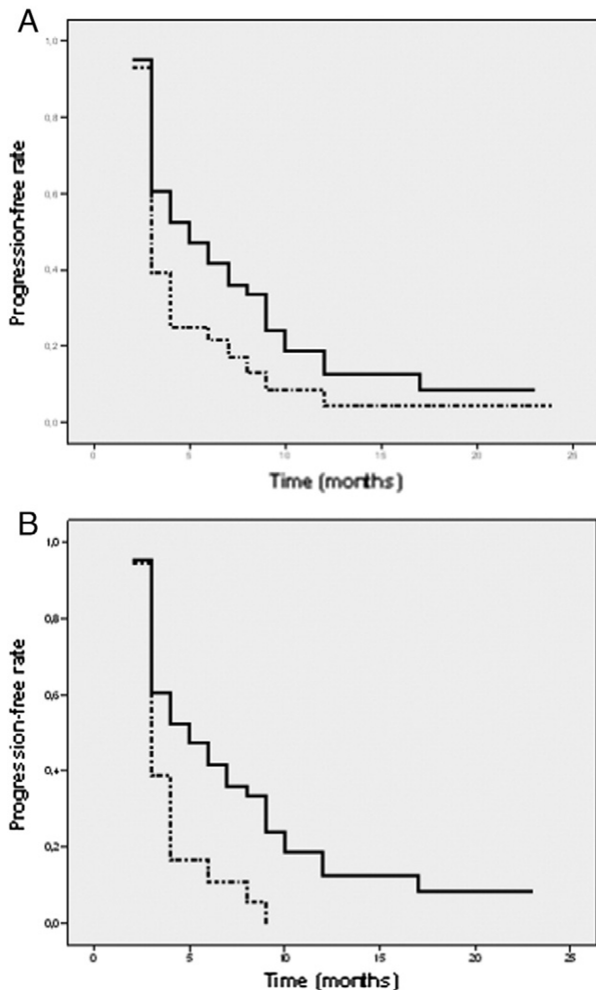

Figure 2. (A) Time to progression by *RAS* status: *RAS* wild type tumors (————), *RAS* mutated tumors (--------------). (B) Time to progression by *RAS* status: *RAS* wilde type tumors (————), *RAS* mutated tumors by excluding codon 61 mutations from the analysis (--------------).

2 PR), 1 SD and 7 PD. Similarly Peeters et al. [13] reported that 1 patient with codon Q61H out of the 6 patients carrying *KRAS* codon 61 mutations had a PR.

These results, as the clinical significance of 61 codon mutations is concerned, if confirmed in a larger prospective series, may suggest that their determination may be not useful for clinical making decisions on the administration of panitumumab treatment.

The analysis of correlation between *RAS* wild-type versus the 31 cases mutated and response rate found no statistically significant difference in outcome. However, when the 11 tumors harboring the 61 codon mutations were excluded from the analysis the difference in ORR reached a statistically significant level. Similarly, regarding TTP the deletion of 61 codon mutants allowed to enhance the significance in outcome between wild-type versus mutated tumors. It should be taken into account that our analysis was retrospective and exploratory in nature and performed in a small number of patients, therefore data needs to be interpreted with caution.

The correlation between *RAS* mutations with low frequencies such as codon 61 and 146 and clinical outcome of patients treated with anti-EGFR mAbs, needs prospective [23] large predictive pooled trials or meta-analyses of more studies in order to better define their clinical significance and possible predictive value to future innovative therapeutic strategies for MCRC resistant to anti-EGFR therapy [25,26].

Conclusions

The results of our study agree with Douillard's data [10] on the relevance of determination of *KRAS* as a negative predictive tool for panitumumab-based treatment. The high sensitive methods applied allowed us to identify more *RAS* mutations than the standard methods useful to spare unnecessary treatments to primary resistant tumors in the future.

On the basis of the most available data and consistently with current treatment guidelines for administration of panitumumab in mCRC, only the patients with extended *RAS* wild-type tumors are likely to be responsive and are eligible for anti-EGFR targeted therapy.

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