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*Original Article*

# Identification and prognostic analysis of the cetuximab resistance-related gene *REV1* in *RAS* wild-type metastatic colorectal cancer

Ning Zhu[1*], Xuefeng Fang[1*], Dan Li[1], Mengyuan Yang[1], Lizhen Zhu[1], Liping Zhong[1], Shanshan Weng[1], Juan Wang[1], Ying Yuan[1,2,3]

*[1]Department of Medical Oncology, Key Labaratory of Cancer Prevention and Intervention, Ministry of Education, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China; [2]Cancer Institute, Key Laboratory of Cancer Prevention and Intervention, Ministry of Education, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China; [3]Cancer Center, Zhejiang University, Hangzhou, Zhejiang, China. [*]Equal contributors and co-first authors.*

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**Abstract:** The survival of patients with *RAS* wild-type metastatic colorectal cancer (mCRC) has improved markedly since the introduction of cetuximab, which is an anti-epidermal growth factor receptor monoclonal antibody. However, not all RAS wild-type patients respond to cetuximab treatment. Although some genetic alterations associated with cetuximab resistance have been identified, they cannot fully explain all cases of cetuximab resistance. Thus, in this research, we aimed to identify new genetic alterations associated with resistance to this treatment. The study retrospectively analyzed 70 patients diagnosed with RAS wild-type mCRC at our hospital between November 2009 and July 2018. First, five progression-free survival (PFS)-longest and 5 PFS-shortest tumor deoxyribonucleic acid were analyzed by whole-exome sequencing (WES) to identify differentially mutated genes. Then, PFS analysis of the 70 patients was used to verify the correlation between the candidate gene and cetuximab sensitivity. Finally, data from public databases were used to further verify the relationship between the mRNA expression level of the candidate gene and cetuximab responsiveness. The WES results indicated *REV1*: c.2108G > A was a candidate gene mutation related to the effectiveness of cetuximab. Survival analysis suggested *REV1*: c.2108G > A was associated with rapid disease progression (median PFS time, *REV1* mutant vs. *REV1* wild-type: 4.4 months vs. 8.7 months, P = 0.034). Data from the Genomics of Drug Sensitivity in Cancer and the Gene Expression Omnibus databases suggested low REV1 mRNA levels might be related to the poor response of CRC cells and reduced cetuximab efficacy among mCRC patients. In conclusion, *REV1* expression levels and the *REV1*: c.2108G > A mutation may be related to cetuximab resistance in *RAS* wild-type mCRC.

**Keywords:** Colorectal cancer, whole-exome sequencing, cetuximab, resistance, *REV1*

## Introduction

Colorectal cancer (CRC) has been identified as one of the most common gastrointestinal malignancies worldwide; in fact, it has the third-highest global incidence rate among malignant tumors [1]. Metastasis to distant organs is the main cause of death in patients diagnosed with CRC, accounting for approximately 90% of CRC deaths [2]. Because clinical signs and symptoms of CRC are non-specific at an early stage, approximately 25% of patients are diagnosed at an advanced disease stage, and 40% of patients will develop metastatic disease during follow-up [3]. The 5-year survival rate of patients with metastatic CRC (mCRC) is as low as 13.1%, while the rate in patients with non-mCRC is 90.1% [2, 3].

Cetuximab, which is an anti-epidermal growth factor receptor (EGFR) monoclonal antibody, inhibits the growth of cancer cells and promotes apoptosis in tumor cells by competitively inhibiting EGFR ligand binding and downstream signaling [4]. Studies have shown that cetuximab monotherapy can induce long-lasting responses in 12-17% of patients with *RAS* wild-type mCRC [5] and that the objective response

rate (ORR) was as high as 72% when cetuximab is combined with chemotherapy [6]. The FOLFOX or FOLFIRI chemotherapy regimens plus cetuximab are currently the standard first-line treatment regimens for *RAS* wild-type mCRC [7].

Despite these advances in *RAS*-wild-type mCRC treatment, some initially responsive patients exhibit resistance within 3-18 months of starting cetuximab treatment, a situation known as secondary drug resistance [8, 9]. Other patients experience rapid (within 3 months) disease progression after cetuximab treatment, a situation known as primary drug resistance. Genetic alterations related to primary cetuximab resistance include mutations in the *RAS* (*KRAS* and *NRAS*), *BRAF*, and *PIK3CA* genes and the amplification of *HER2* and *MET* [10]. Mutations in the EGFR extracellular domain (S492R), *RAS*, and *BRAF* and amplification of *KRAS*, *HER2,* and *MET* discovered during treatment are associated with secondary resistance to cetuximab [10]. Mutation in *KRAS* is a primary mechanism of cetuximab resistance and is found in 30-40% of patients with mCRC [11]. The authoritative diagnosis and treatment guidelines of NCCN and CSCO all unanimously recommend that patients with mCRC undergo RAS (KRAS and NRAS) and BRAF gene testing before treatment; furthermore, a broad consensus accepts that patients with the above gene mutations are not recommended for cetuximab treatment [12, 13]. However, some patients with cetuximab resistance do not present any of these genetic alterations suggesting that other alterations may exist. Therefore, in order to improve the prognosis of patients with mCRC, it is of great practical significance that cetuximab-sensitive patients be screened more accurately by finding new gene alterations related to cetuximab resistance.

In this current study, we aimed to identify new genetic alterations associated with cetuximab resistance. We screened gene mutations associated with sensitivity to cetuximab in *RAS* wild-type mCRC by whole-exome sequencing (WES). Tumor samples and the clinicopathological characteristics of patients at our center as well as data from public databases were used to verify the correlation between the candidate gene identified and cetuximab sensitivity as well as the relationship of this gene with prognosis. Alterations in the candidate gene may serve as a potential new predictive biomarker for cetuximab efficacy in patients with *RAS* wild-type mCRC.

## Method

### *Patient selection and sample preparation*

In this study, 70 patients diagnosed with *RAS* wild-type mCRC and treated with cetuximab combined with chemotherapy at the Second Affiliated Hospital of Zhejiang University School of Medicine (China) between November 2009 and July 2018 were retrospectively reviewed. The inclusion criteria were as follows: (1) patients histologically confirmed with primary colorectal adenocarcinoma; (2) distant metastasis was confirmed by imaging examinations or by pathology assessment; (3) tumor tissue could be obtained from the tissue bank of the hospital's department of pathology; (4) patients with measurable lesions according to the Response Evaluation Criteria in Solid Tumors (RECIST, 1.1) criteria; and (5) patients with wild-type *RAS* (*KRAS* and *NRAS*) and *BRAF*. Meanwhile, the exclusion criteria were as follows: (1) patients with familial adenomatous polyposis; (2) patients with second primary malignant tumors; and (3) patients with incomplete clinical data or inadequate follow-up information.

Frozen (-80°C) and paraffin-embedded tumor tissue samples from the 70 included patients who underwent resection for sporadic CRC were obtained from the Department of Pathology of the Second Affiliated Hospital of Zhejiang University School of Medicine. This study was approved by the ethics committee of the Second Affiliated Hospital of Zhejiang University School of Medicine.

### *Deoxyribonucleic acid extraction and sequencing*

Deoxyribonucleic acid (DNA) was extracted from the paraffin-embedded and frozen tumor tissue samples using the QIAamp DNA Tissue Kit (QIAGEN, Germany), as per the manufacturer's protocols. WES was then performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing) using DNA extracted from frozen tumor tissue. The sequencing depth was 200×. Direct Sanger sequencing was performed by Tsingke Biological Technology Co., Ltd. (Hang-

zhou) to validate the candidate gene variant identified by WES using DNA extracted from paraffin-embedded tumor tissue. The forward primer sequence was 5'-ATGCTTTATAGGTTCT-GCCGTG-3', whereas the reverse primer sequence was 5'-GGCTCTGGGGATACAACAGTTT-3'.

*Clinicopathological and follow-up data*

Patient demographic characteristics (sex and age at cetuximab initiation), tumor characteristics (primary tumor site; tumor (T), lymph node (N), and metastasis (M) stages; histological type and histological grade), treatment information (chemotherapy regimen and line), and progression-free survival (PFS) data were retrospectively reviewed in our hospital using medical and imaging records. The pathological stages of mCRC were determined according to the American Joint Committee on Cancer (AJCC; 8$^{th}$ ed.) TNM staging system and the RECIST 1.1 criteria were applied to assess treatment response. Two pathologists reviewed all the pathology results.

Follow-up was conducted primarily through telephone interviews and outpatient clinic visits. The follow-up data collected included the survival status, disease status and date of tumor progression if applicable. The ORR was then defined as being complete response (CR) + partial response (PR), while the disease control rate (DCR) was defined as being CR + PR + SD. Additionally, PFS was defined as the duration of cetuximab treatment initiation until disease progression or death. The final follow-up time point was November 2019.

*Statistical analysis*

Data analysis was conducted using the SPSS Statistics software (21.0) program, and a two-sided $P$ value < 0.05 was considered statistically significant. Survival curves were plotted using the Kaplan-Meier method, and a log-rank test was performed to evaluate survival in the different groups. The log-rank test and Cox proportional hazards models were used for univariate and multivariate analyses, respectively, and variables with P < 0.10 in the univariate analysis were included in the multivariate analysis. Subgroup analysis was performed using the Stata (v.13.0) software. Forest plots of subgroup analysis data were generated using GraphPad Prism (v.7.0). Correlation analysis was determined by linear fitting. A boxplot of the differences in the candidate gene's mRNA levels among different groups was generated with package ggplot2 in the R for statistical computing.

## Results

*A novel cetuximab resistance-related gene mutation: REV1: c.2108G > A*

*Identification of cetuximab resistance-related genes by WES:* In total, 70 patients diagnosed with mCRC and treated with cetuximab and chemotherapy at our hospital between November 2009 and July 2018 were able to meet our inclusion criteria. For all patients, the median follow-up time was 21.5 months (range, 3.1-106.7 months). The PFS times of the 70 patients were calculated (median PFS time, 8.6 months; range, 0.7-65.8 months).

We then selected the five patients with the longest PFS times as the long group (labeled L1-L5; median PFS, 24.5 months) and the five patients with the shortest PFS times as the short group (labeled S1-S5; median PFS, 3.2 months). The DNA extracted from frozen tumor tissue samples of L1-L5 and S1-S5 was used for WES. Genes with mutations specific to the long or short groups were considered candidate genes related to cetuximab sensitivity in *RAS* wild-type mCRC. First, we looked for mutations in currently known resistance-related genes in the genomes of patients in the long and short groups. No currently known genetic alterations in genes related to cetuximab resistance such as mutations in *RAS*, *BRAF*, or *PIK3CA,* or amplification of *HER2* or *MET* were found in these two groups (**Figure 1**). That is, no patients in either the long and/or short group carried a molecular alteration currently known to be related to cetuximab resistance.

To identify cetuximab-related mutations, we next compared the WES data between the two groups of patients and identified differentially mutated genes. Two gene alterations (in *REV1* and *PCDH12*) were then detected in the short group but not in the long group, while no gene alterations were detected in only the long group. The gene alteration carried by all patients in the short group (S1-S5) was a missense mutation in the *REV1* gene (NM_001037872:

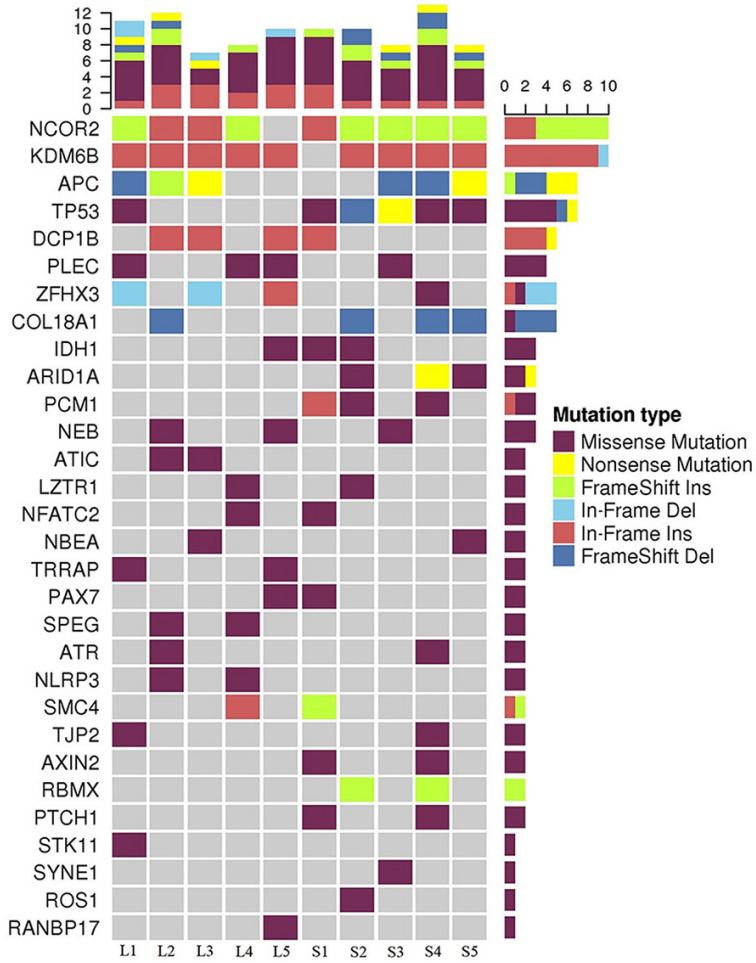


**Figure 1.** Heat map of known driver genes in the long and the short groups. Known driver genes of each tumor sample were identified by comparing the gene alternations detected by WES with known driver genes in the database. No currently known genetic alterations in genes related to cetuximab resistance were found in these two groups.

exon13, c.G2108A: p.R703Q). An additional gene alteration carried by patients S1, S2, and S4 was an insertion mutation in the *PCDH12* gene (NM_016580: exon4, c.3526-3544In_Frame_Ins) (**Figure 2**). Because the *REV1* mutation was present in all five patients, we selected this mutation for further analysis.

*Shortened progression-free survival in RAS wild-type mCRC patients treated with cetuximab is associated with REV1 mutation:* Up to this point, our results suggested that the c.2108G > A mutation in *REV1* may be associated with cetuximab resistance and shorter PFS in patients treated with this drug. To corroborate these findings we performed direct Sanger sequencing on genomic DNA from the paraffin-embedded tumor tissue samples of the 70 patients diagnosed with *RAS* wild-type mCRC and treated with cetuximab and chemotherapy. The clinicopathological characteristic and prognostic data of these 70 patients were then used to analyze the correlation between the candidate gene and cetuximab sensitivity and the relationship of this gene with prognosis. Sanger sequencing results showed that 10 of the 70 (14.3%) patients carried *REV1*: c.2108G > A (**Figure 3**). The PFS analysis of the 70 patients showed that patients with the *REV1*: c.2108G > A mutation had significantly shorter PFS than patients without this mutation (median PFS time, *REV1* mutant vs. *REV1* wild-type, 4.4 months vs. 8.7 months, P = 0.034; **Figure 4D**), suggesting that *REV1*: c.2108G > A was associated with rapid disease progression.

The clinicopathological characteristics of the 70 patients with *RAS* wild-type mCRC are presented in **Table 1**. Among these patients, the median age at cetuximab initiation was 58 years (range, 39-87 years). The majority of patients (62.9%) received an irinotecan-based chemotherapeutic regimen (FOLFIRI) plus cetuximab; one patient received single-agent fluorouracil, while the remaining 25 patients were treated with an oxaliplatin-based chemotherapeutic regimen (FOLFOX) plus cetuximab. According to the RECIST 1.1 criteria, 40 patients (57.1%) achieved a PR, 20 had SD, and 10 had progressive disease (PD). The ORR was 57.1%, and the DCR was 85.7%.

As shown in **Table 1**, univariate analysis results suggested that the tumor lymph node stage (N) (P = 0.030, **Figure 4A**), tumor metastasis stage (M) (P = 0.001, **Figure 4B**), tumor histological grade (P < 0.001, **Figure 4C**), and *REV1* mutation status (P = 0.034, **Figure 4D**) were disease progression-related factors in *RAS* wild-type mCRC patients treated with cetux-

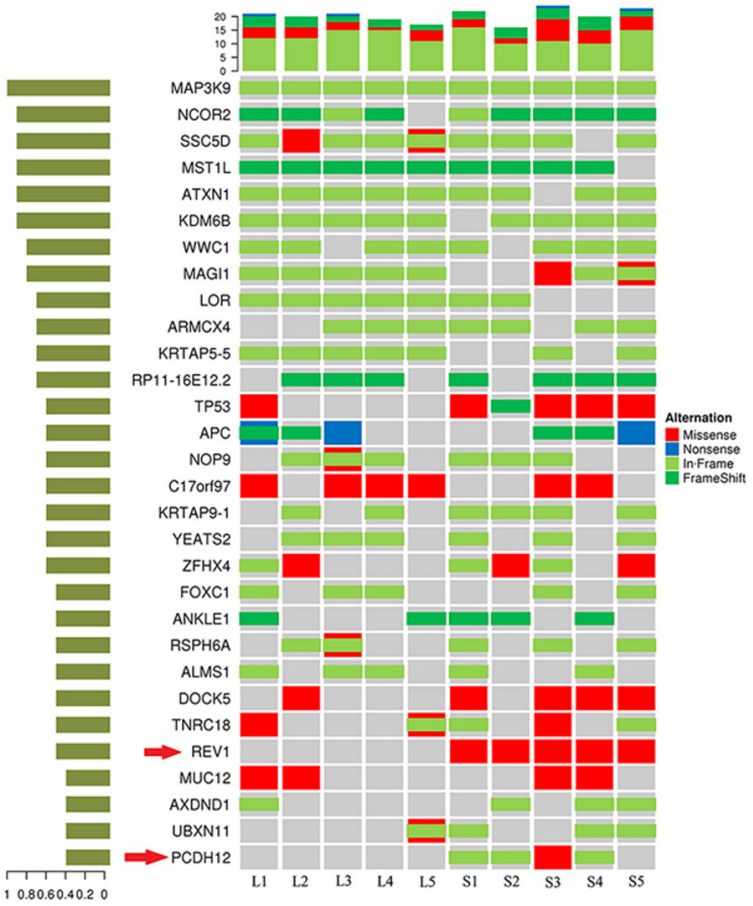


**Figure 2.** Heat map of the significantly mutated genes in the long and the short PFS groups. A missense mutation in the *REV1* gene (NM_001037872: exon13 c.G2108A: p.R703Q) was carried by all patients in the short group (S1-S5). An insertion mutation in the PCDH12 gene (NM_016580: exon4 c.3526-3544In_Frame_Ins) was carried by patients S1, S2, and S4.

imab. Thus, the N and M stages, tumor histological grade, chemotherapy regimen, and *REV1* mutation status were included in the multivariate analysis. The results of multivariate Cox regression analysis (**Table 1**) revealed that the patients carrying mutant *REV1* had shorter PFS times than those with wild-type *REV1* (HR = 3.589, 95% CI, 1.511-8.526, P = 0.004). In other words, *REV1* gene mutation was an independent risk factor for disease progression in patients with *RAS* wild-type mCRC treated with cetuximab.

To explore the effect of *REV1* gene mutation on prognosis in different subgroups with mCRC, we performed subgroup analyses. As shown in **Figure 5**, the patients with mutant *REV1* had shorter PFS times than those with wild-type *REV1* in the following subgroups: female sex (HR = 0.208, P = 0.01), age at cetuximab initiation ≥ 58 years (HR = 0.351, P = 0.019), T4 (HR = 0.33, P = 0.027), N1-N2 (N1, HR = 0.32, P = 0.043; N2, HR = 0.213, P = 0.036), M1a (HR = 0.232, P = 0.001), common adenocarcinoma (HR = 0.421, P = 0.017), irinotecan-based chemotherapeutic regimen (FOLFIRI) plus cetuximab treatment (HR = 0.394, P = 0.03), and first-line treatment (HR = 0.395, P = 0.04). That is, the effect of *REV1* gene mutation on prognosis was determined to be more significant in patients in these subgroups than in those in other subgroup(s) within each category.

*Cetuximab sensitivity and REV1 mRNA level*

Due to the limited sample size derived from our hospital, *REV1*-related data from the Genomics of Drug Sensitivity in Cancer (GDSC) and the Gene Expression Omnibus (GEO) databases were included in our study. Because no data on *REV1*: c.2108G > A were found in public databases, only *REV1* mRNA levels were analyzed.

*Genomics of Drug Sensitivity in Cancer database analysis:* To explore the effect of REV1 mRNA levels on the sensitivity of CRC cells to cetuximab, we explored the correlation between the *REV1* mRNA level and the inhibitory concentration (IC) 50 of cetuximab in CRC cells based on data for 29 CRC cell lines (SNU-C1, CL-34, SNU-61, LS-1034, SNU-C2B, SW620, SK-CO-1, CL-40, HT-29, LS-513, NCI-H508, MDST8, CL-11, KM12, SNU-1040, HCT-116, SW837, RKO, LS-180, LS-411N, LS-123, NCI-H747, SW1463, LoVo, CCK-81, COLO-678, C2BBe1, SW1116, and SW48) retrieved from the GDSC database. We were able to determine a negative correlation between *REV1* mRNA expression levels and cetuximab IC50 in these cell lines (correlation factor, -0.3725, P = 0.0466, **Figure 6**). This suggested that low *REV1* mRNA levels may be related to the poor response of CRC cells to cetuximab.

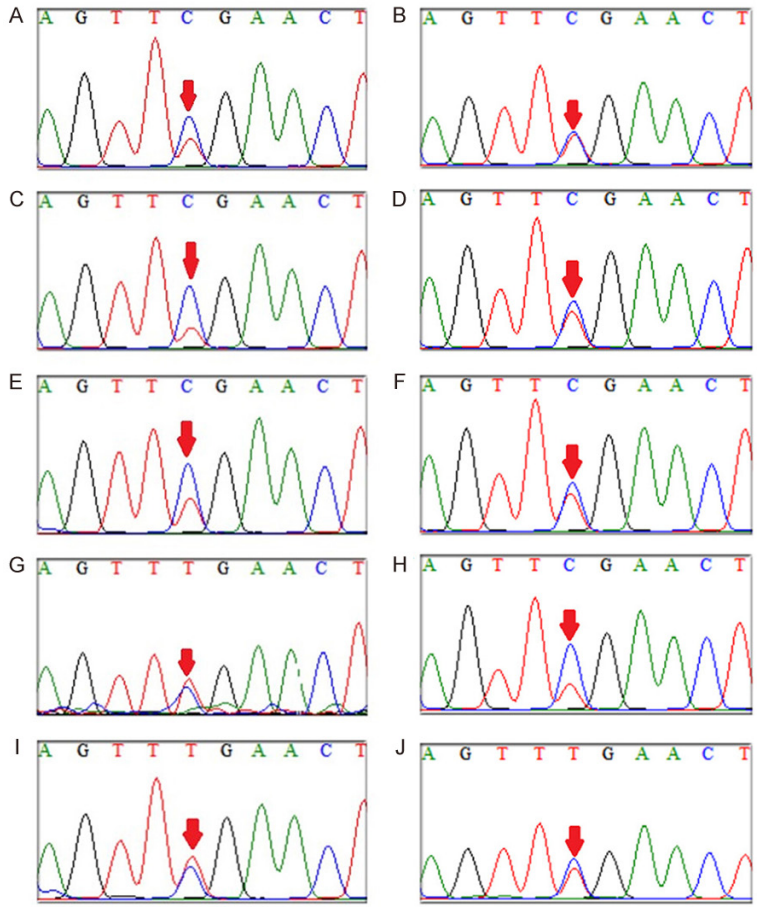


**Figure 3.** The DNA sequencing chromatograms of 10 patients with the *REV1* gene mutation. Ten of the 70 (14.3%) patients carried *REV1*: c.2108G > A.

*Gene Expression Omnibus database analysis:* To evaluate whether *REV1* expression levels were associated with responses to cetuximab monotherapy, we compared the *REV1* mRNA levels in patients from the GSE5851 dataset of the GEO database classified as PR, SD, and nonresponders. The GSE5851 dataset was derived from a phase II exploratory pharmacogenomics study [14] of cetuximab monotherapy in patients with advanced metastatic CRC. Transcriptional profiling was then conducted on RNA from pre-treatment metastatic site biopsies to identify genes whose expression correlated best with clinical responses. We then extracted the data of the 43 *KRAS* wild-type patients in this dataset, whose clinical characteristics are shown in **Table 2**. Five patients achieved PR, 15 achieved SD, 19 achieved PD, and 4 patients died before their first response evaluation; thus, their responses were unable to be determined (UTD).

The 43 patients with wild-type *KRAS* were then divided into 2 groups, i.e., the disease control group (DCG) and the nonresponder group. The DCG included patients with PR or SD response evaluations, while the nonresponder group included patients with a PD or UTD response evaluation. As per our findings, a decreasing trend can be noted in the *REV1* mRNA level among patients with PR and nonresponders (PD + UTD) (**Figure 7**; **Table 2**). The *REV1* mRNA levels were lower in the nonresponder group than in the DCG (median *REV1* mRNA level, 1775.4 vs. 1945.05, P = 0.025, **Figure 8**). This suggested that low *REV1* mRNA levels may be related to the poor efficacy of cetuximab. Additionally, the REV1 expression level was not statistically different in terms of age, sex, race, puncture site, and efficacy evaluation subgroups among patients with RAS wild-type mCRC in the GSE5851 dataset (**Table 2**).

## Discussion

The ongoing development of molecular targeted therapy provides new options for the personalized management of advanced solid tumor, including mCRC. Drugs targeting EGFR, such as cetuximab, are highly important for improving the survival and prognosis of patients with *RAS* wild-type mCRC. However, the clinical use of cetuximab is limited due to drug resistance, and the mechanisms underlying this resistance are yet to be entirely understood. To explore the potential mechanism of cetuximab resistance, we performed WES and identified two candidate genes (*REV1* and *PCDH12*) that may be associated with sensitivity to cetuximab. We selected *REV1* gene mutation for further analysis and exploration.

In the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP), although at least 132 non-synonymous single-nucleotide variants of *REV1* have been described, the functional effects of these gene variants have not been

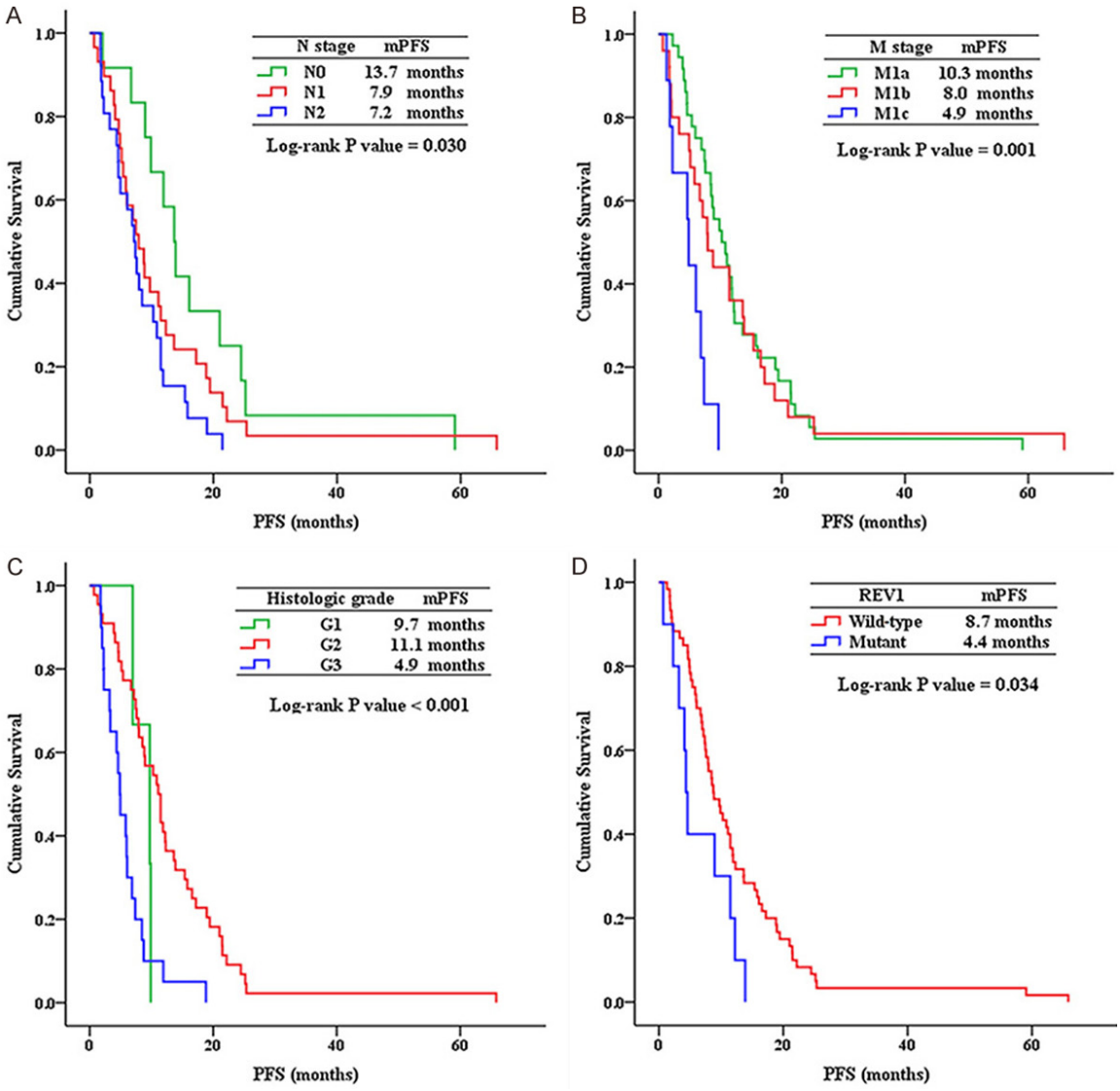


**Figure 4.** Kaplan-Meier survival curves. The tumor lymph node stage (N) stage (A), tumor metastasis stage (M) stage (B), tumor histological grade (C), and REV1 mutation status (D) were disease progression-related factors in *RAS* wild-type mCRC patients treated with cetuximab (mPFS: median progression-free survival).

described. In existing studies, two single-nucleotide polymorphisms (SNPs) in the *REV1* gene, in codons 257 and 373, have also been reported to significantly correlate with cancer risk. Although the specific mechanisms are not yet clear, the N373S SNP is associated with an increased risk of cervical cancer, while the F257S SNP is associated with a reduced risk of cervical cancer but an increased risk of lung cancer in people with severe smoking habits [15, 16]. Some studies have suggested the V138M SNP in the *REV1* gene to be associated with poor cisplatin efficacy in patients with malignant mesothelioma and osteosarcoma [17, 18]. One of these studies has also revealed a link between *REV1* gene SNPs and hematotoxicity after the administration of cisplatin for malignant mesothelioma [17]. The above literature suggests that *REV1* SNPs may alter the function of *REV1*, thereby altering the individual risk of cancer and the individual outcomes of cancer treatment.

In our study, the multivariate analysis suggested the identified *REV1*: c.2108G > A mutation as an independent risk factor for disease progression in patients with *RAS* wild-type mCRC treated with cetuximab. This finding suggests that this *REV1* gene mutation is related to the poor therapeutic efficacy of cetuximab in

**Table 1.** Clinicopathological characteristics and disease progression-related factors in *RAS* wild-type mCRC patients from our center who were treated with cetuximab and evaluated by univariate and multivariate analyses

| Clinical characteristics | Number of patients (%) | Univariate analysis | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | | mPFS (months) | *P*-value | HR | 95% CI | *P*-value |
| Sex | | | 0.299 | | | |
| Male | 48 (68.6%) | 8.5 | | | | |
| Female | 22 (31.4%) | 8.5 | | | | |
| Age at cetuximab initiation | | | 0.462 | | | |
| < 58 years old | 34 (48.6%) | 8.7 | | | | |
| ≥ 58 years old | 36 (51.4%) | 8.5 | | | | |
| Primary tumor site | | | 0.758 | | | |
| Right colon | 19 (27.1%) | 7.4 | | | | |
| Left colon and rectum | 51 (72.9%) | 9.0 | | | | |
| T stage | | | 0.223 | | | |
| T1 | 0 (0.0%) | - | - | | | |
| T2 | 3 (4.3%) | 16.1 | | | | |
| T3 | 19 (27.1%) | 11.5 | | | | |
| T4 | 45 (64.3%) | 7.6 | | | | |
| Unoperated | 3 (4.3%) | - | | | | |
| N stage | | | 0.030 | | | |
| N0 | 12 (17.1%) | 13.7 | | Reference | | 0.129 |
| N1 | 29 (41.4%) | 7.9 | | 1.479 | 0.657-3.330 | 0.344 |
| N2 | 26 (37.1%) | 7.2 | | 2.389 | 0.971-5.873 | 0.058 |
| Unoperated | 3 (4.3%) | - | | - | - | - |
| M stage | | | 0.001 | | | |
| M1a | 36 (51.4%) | 10.3 | | Reference | | 0.261 |
| M1b | 25 (35.7%) | 8.0 | | 1.248 | 0.705-2.210 | 0.447 |
| M1c | 9 (12.9%) | 4.9 | | 2.173 | 0.813-5.812 | 0.122 |
| Histological type | | | 0.060 | | | |
| Common adenocarcinoma | 60 (85.7%) | 9.0 | | Reference | | |
| Special adenocarcinoma | 10 (14.3%) | 5.0 | | 1.074 | 0.422-2.738 | 0.880 |
| Histological grade | | | < 0.001 | | | |
| G1 | 3 (4.3%) | 9.7 | | Reference | | 0.007 |
| G2 | 44 (62.9%) | 11.1 | | 0.383 | 0.094-1.569 | 0.182 |
| G3 | 20 (28.6%) | 4.9 | | 1.291 | 0.298-5.592 | 0.733 |
| Uncertain | 3 (4.%) | - | | - | - | - |
| Chemotherapy regimen | | | 0.092 | | | |
| Irinotecan-based | 44 (62.9%) | 8.5 | | Reference | | 0.207 |
| Oxaliplatin-based | 25 (35.7%) | 9.0 | | 1.716 | 0.939-3.136 | 0.079 |
| Single-agent fluorouracil | 1 (1.4%) | 3.2 | | 1.432 | 0.155-13.231 | 0.752 |
| Line | | | 0.177 | | | |
| First | 50 (71.4%) | 9.7 | | | | |
| Second | 19 (27.1%) | 6.9 | | | | |
| Third | 1 (1.4%) | 5.8 | | | | |
| REV1 mutation status | | | 0.034 | | | 0.004 |
| Wild-type | 60 (85.7%) | 8.7 | | Reference | | |
| Mutant | 10 (14.3%) | 4.4 | | 3.589 | 1.511-8.526 | |

| Baseline Characteristic | No. of patients REV1 wild-type | No. of patients REV1 mutant | Hazard Ratio for PFS (95% CI) | P Value |
|---|---|---|---|---|
| Total | 60 | 10 | 0.481 (0.241-0.962) | 0.038 |
| Sex | | | | |
| Male | 42 | 6 | 0.619 (0.256-1.495) | 0.286 |
| Female | 18 | 4 | 0.208 (0.063-0.691) | 0.01 |
| Age, years | | | | |
| < 58 | 31 | 3 | 0.717 (0.213-2.410) | 0.591 |
| ≥ 58 | 29 | 7 | 0.351 (0.146-0.842) | 0.019 |
| Primary tumor location | | | | |
| Right | 18 | 1 | | |
| Left | 42 | 9 | 0.489 (0.230-1.040) | 0.063 |
| T stage | | | | |
| T2 | 3 | 0 | | |
| T3 | 15 | 4 | 0.367 (0.110-1.228) | 0.104 |
| T4 | 40 | 5 | 0.33 (0.123-0.884) | 0.027 |
| N stage | | | | |
| N0 | 10 | 2 | 0.46 (0.089-2.392) | 0.356 |
| N1 | 25 | 4 | 0.32 (0.106-0.967) | 0.043 |
| N2 | 23 | 3 | 0.213 (0.050-0.904) | 0.036 |
| M stage | | | | |
| M1a | 29 | 7 | 0.232 (0.095-0.570) | 0.001 |
| M1b | 22 | 3 | 0.705 (0.203-2.453) | 0.583 |
| M1c | 9 | 0 | | |
| Histological type | | | | |
| Common adenocarcinoma | 50 | 10 | 0.421 (0.207-0.856) | 0.017 |
| Special adenocarcinoma | 10 | 0 | | |
| Histological grade | | | | |
| G1 | 3 | 0 | | |
| G2 | 38 | 6 | 0.541 (0.220-1.327) | 0.179 |
| G3 | 16 | 4 | 0.282 (0.074-1.073) | 0.063 |
| Chemotherapy | | | | |
| Irinotecan-based | 37 | 7 | 0.394 (0.170-0.915) | 0.03 |
| Oxaliplatin-baesd | 23 | 2 | 0.894 (0.204-3.923) | 0.882 |
| Other | 0 | 1 | | |
| Line | | | | |
| First | 44 | 6 | 0.395 (0.162-0.959) | 0.04 |
| Second | 15 | 4 | 0.764 (0.244-2.396) | 0.645 |
| Third | 1 | 0 | | |

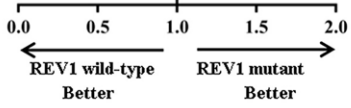


**Figure 5.** Forest plots of subgroup analyses. The patients with mutant *REV1* had shorter PFS times than those with wild-type *REV1* in the subgroups of the female sex, age at cetuximab initiation ≥ 58 years, T4, N1-N2, M1a, common adenocarcinoma, irinotecan-based chemotherapeutic regimen (FOLFIRI) plus cetuximab treatment, and first-line treatment.

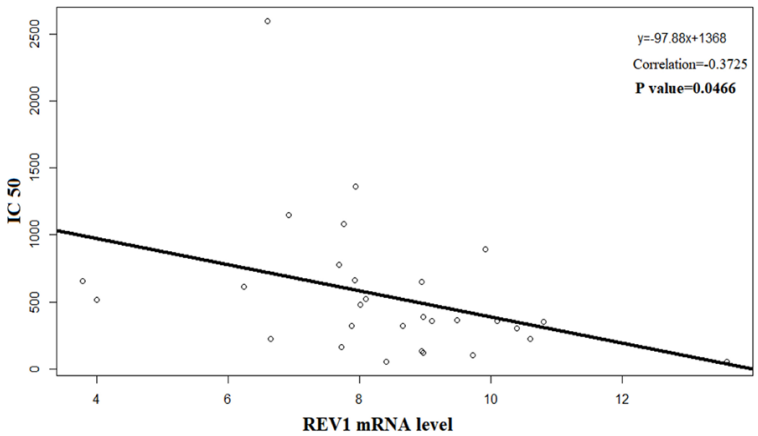


**Figure 6.** Correlation between the *REV1* mRNA level and the IC50 of cetuximab in 29 CRC cell lines from the GDSC database. The linear fitting suggests a negative correlation (correlation factor, -0.3725, P = 0.0466).

patients with *RAS* wild-type mCRC. In addition, subgroup analysis based on *REV1* mutation status suggested that the effect of *REV1* gene mutation on prognosis was determined

**Table 2.** General clinical characteristics and *REV1* expression levels of patients with *RAS* wild-type mCRC in the GSE5851 dataset

| Clinical characteristics | No. of patients | Mean expression of *REV1* | *P* value |
|---|---|---|---|
| Age (median age: 61 years) | | | 0.896 |
| ≤ 61 years old | 22 | 1763.905 | |
| > 61 years old | 20 | 1778.13 | |
| NA | 1 | 2085.4 | |
| Sex | | | 0.337 |
| Male | 25 (58.1%) | 1734.688 | |
| Female | 18 (41.9%) | 1838.15 | |
| Race | | | 0.601 |
| White | 37 (86.0%) | 1757.586 | |
| African-American | 5 (11.6%) | 1926.22 | |
| Asian | 1 (2.3%) | 1792.1 | |
| Puncture site | | | 0.397 |
| Liver | 36 (83.7%) | 1740.481 | |
| Lung | 1 (2.3%) | 1940.8 | |
| Colon or rectum | 2 (4.7%) | 1848.7 | |
| Others | 4 (9.3%) | 2039.6 | |
| Efficacy evaluation | | | 0.105 |
| PR | 5 (11.6%) | 2035.74 | |
| SD | 15 (34.9%) | 1858.313 | |
| PD | 19 (44.2%) | 1682.379 | |
| UTD | 4 (9.3%) | 1608.825 | |

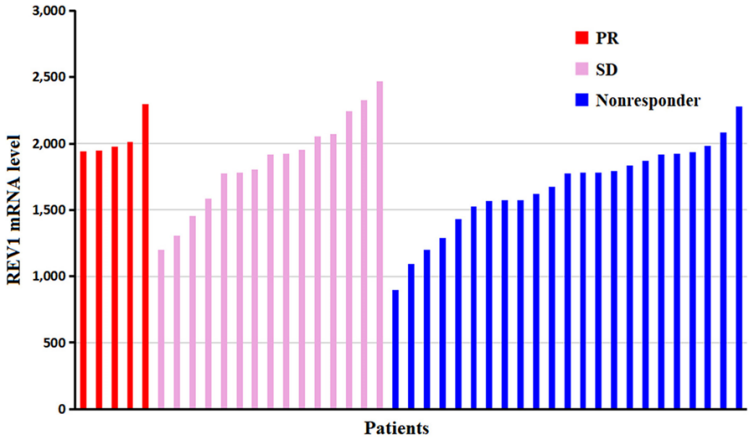


**Figure 7.** The *REV1* mRNA levels in patients with different response evaluations. There is a decreasing trend in the REV1 mRNA level among patients with a PR to nonresponders (PD + UTD).

to be more significant in female patients aged ≥ 58 years at cetuximab initiation, who had stage T4, N1-N2, or M1a disease, had common adenocarcinoma, were being treated with FOLFIRI + cetuximab, or were undergoing first-line treatment. This finding underscores that if this *REV1* gene mutation is a cetuximab resistance mutation in *RAS* wild-type mCRC, cetuximab should not be recommended for patients with the REV1: c.2108G > A mutation, particularly for patients who fall into the above subgroups.

There are no reports in the literature indicating the REV1 gene or REV1: c.2108G > A mutation as being related to RAS mutation. The patients from our center that were included in this study were all representative of wild-type KRAS, NRAS, and BRAF genes, indicating that REV1 is a new potential gene associated with cetuximab resistance.

The *REV1* gene is a member of the eukaryotic Y-polymerase family and plays a role in DNA translesion synthesis (TLS). *REV1* acts as a scaffolding protein for regulating protein-protein interactions with proliferating cell nuclear antigen, panproteins, and other polymers to coordinate the TLS process [19-21]. In addition, as a deoxycytidyl transferase, *REV1* has intrinsic catalytic activity and is thus unique among polymerases [22, 23]. The *REV1* protein (1,251 amino acids long) comprises the catalytic core (amino acids 330-833), while the N- and C-terminal regions include the protein interaction domains (e.g., BRCT, UBM, and PID) [23, 24].

Based on the above information indicating that the catalytic core of REV1 is located in amino acids 330-833, the *REV1* gene missense mutation (*REV1*-NM_001037872: exon13, c.G2108A: p.R703Q), identified by WES in our study, is located in the catalytic core of the *REV1* protein (amino acid 703). Furthermore, the results of a study by Yeom et al. suggest that the *REV1* gene with this missense mutation encodes a low-activity variant

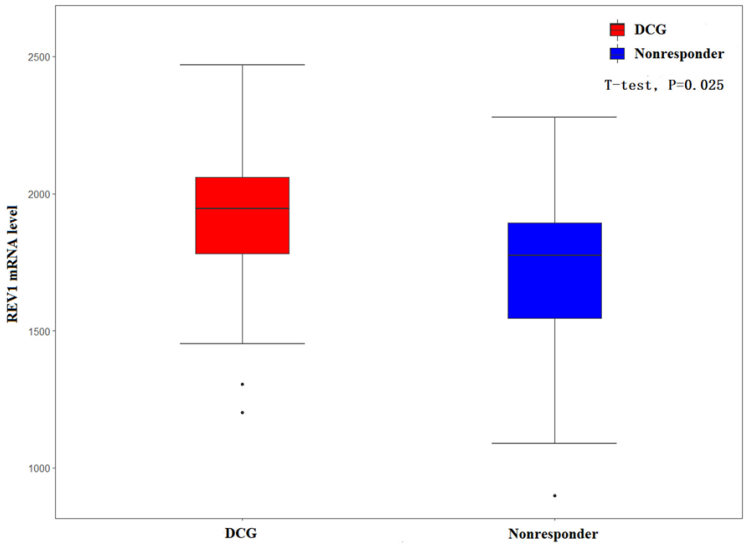


**Figure 8.** Differences in *REV1* mRNA levels between the DCG and the nonresponder group. The DCG included patients with PR or SD response evaluation, while the nonresponder group included patients with a PD or UTD response evaluation. The REV1 mRNA levels were lower in the nonresponder group than in the DCG group (median REV1 mRNA level, 1775.4 vs. 1945.05, P = 0.025).

protein that significantly weakens the catalytic activity of REV1. This missense mutation, which is located in the linker region or the finger domain of the *REV1* protein, results in the loss of positively charged arginine residue facing or remote from the sugar-phosphate DNA backbone, which may result in a decrease in DNA binding affinity, thus directly or indirectly affecting the interaction of REV1 with DNA [25]. The *REV1* SNP identified in our study occurred the same amino acid alteration. We thus speculate that the *REV1* R703Q SNP identified in our study may have reduced the catalytic activity of REV1 through the same mechanism, which affects TLS, DNA damage repair and mutagenic events in response to cellular DNA damage [25], thereby influencing an individual's sensitivity to selected therapies.

To date, studies have suggested that the overexpression of *REV1* can promote the development of carcinogenic intestinal adenoma. A possible mechanism in this context is that *REV1* overexpression promotes mutagenic TLS to maintain the replication of damaged templates [26]. In addition, some studies suggested that the sensitivity of non-small-cell lung cancer cell lines to some anticancer drugs, such as MEK and AKT inhibitors, is associated with the *REV1* expression level [27]. In the present study, we analyzed the relationship between the *REV1* expression level and cetuximab sensitivity in CRC patients and cell lines via the analysis of data from the GEO and GDSC databases, respectively. The results of our GEO GSE5851 dataset analysis showed that a reduced mRNA level of *REV1* was associated with reduced sensitivity to cetuximab in patients with *RAS* wild-type mCRC. The analysis of CRC cell line data from the GDSC database indicated that decreasing *REV1* expression among the CRC cell lines correlated with increasing IC50s for cetuximab, indicating that a reduced expression level of *REV1* was associated with reduced sensitivity of CRC cell lines to cetuximab. Accordingly, these results suggest that both the mutation affecting REV1 function and reduced *REV1* expression levels may underlie cetuximab resistance in *RAS* wild-type mCRC.

Our study included some limitations. First, this was a retrospective single-center study, and due to the limitation of the small sample size, the study conclusions must be confirmed by multicenter clinical studies with large sample sizes. Furthermore, although the combination of data from our center with public database data enhanced our analysis, we did not conduct in vivo and in vitro validation experiments to explore the specific molecular mechanisms by which the *REV1* gene caused cetuximab resistance. In our future studies, a larger sample size comprising multiple centers will be incorporated and the *REV1*: c.2108G > A mutant CRC cell lineage will be generated using CRISPR/Cas9 technology to confirm the roles of the *REV1* gene in resistance and disease progression. Moreover, the potential exploration of PCDH12 in mCRC cetuximab resistance also will be conducted.

To the best of our knowledge, this study is the first to find that the *REV1* gene may be a predictive gene in patients with *RAS* wild-type mCRC

treated with cetuximab. We speculate that the G2108A mutation in exon 13 of the *REV1* gene may affect the catalytic activity of the REV1 protein or cause cetuximab resistance by impacting the *REV1* mRNA level. The results of our study reveal that *REV1* gene mutation is a potential new predictive biomarker for cetuximab efficacy in patients with *RAS* wild-type mCRC.

## Conclusion

The *REV1* expression levels and the *REV1*: c.2108G > A mutation may be related to cetuximab resistance in *RAS* wild-type mCRC. The presence of this mutation is associated with rapid disease progression in these patients. The REV1 gene mutation is a potential new predictive biomarker for cetuximab efficacy in patients with *RAS* wild-type mCRC. Cetuximab may thus not be suitable for patients with the REV1: c.2108G > A mutation.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Ying Yuan, Department of Medical Oncology, Key Labaratory of Cancer Prevention and Intervention, Ministry of Education, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China. Tel: +86-571-87784795; Fax: +86-571-87767088; E-mail: yuanying1999@zju.edu.cn

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