A genetic variant in Rassf1a predicts outcome in mCRC patients treated with cetuximab plus chemotherapy: results from FIRE-3 and JACCRO 05 and 06 trials

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The Hippo pathway is involved in colorectal cancer (CRC) development and progression. The Hippo regulator Rassf1a is also involved in the Ras signaling cascade. In this work, we tested single nucleotide polymorphisms within Hippo components and their association with outcome in CRC patients treated with cetuximab. Two cohorts treated with cetuximab plus chemotherapy were evaluated (198 RAS wild-type (WT) patients treated with first-line FOLFIRI plus Cetuximab within the FIRE-3 trial and 67 Ras WT patients treated either with first-line mFOLFOX6 or SOX plus Cetuximab). In these two populations, Rassf1a rs2236947 was associated with overall survival (OS), as patients with a CC genotype had significantly longer OS compared with those with CA or AA genotypes. This association was stronger in patients with left-side CRC (hazard ratio (HR): 1.79 (1.01–3.14); P = 0.044 and HR: 2.83 (1.14–7.03); P = 0.025, for Fire 3 and JACCRO cohorts, respectively). Rassf1a rs2236947 is a promising biomarker for patients treated with cetuximab plus chemotherapy.

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INTRODUCTION

Salvador–Warts–Hippo pathway controls organ size by regulating tissue growth. In recent times, several studies have highlighted the implication of deregulated Hippo signaling in cancer development and progression. This novel pathway acts as a complex tumor suppressor network controlling cell growth, proliferation, stem-cell maintenance and epithelium mesenchymal transition. Hippo’s signaling core consists of a complex of kinases whose activation ultimately leads to the phosphorylation of the oncoproteins YAP and TAZ preventing their translocation to the nucleus. On the contrary, if YAP/TAZ are not phosphorylated they can translocate to the nucleus, where they regulate the activity of several transcription factors that control the expression of the Hippo target genes. These target genes include amphiregulin, Sox2 or Birc5 among others. Additionally, Hippo pathway interacts with other pathways such as Wnt, TGFβ or Notch. These pathways connections are of particular relevance for colorectal cancer (CRC) development and progression. Moreover, some of Hippo’s upstream regulators like Rassf1a are also crucial players in CRC. Rassf1a is a tumor suppressor that interacts with Ras signaling through a Ras interaction domain and with the Hippo pathway, specifically with MST, through a SARAH interaction domain. Rassf1a is also involved in microtubule stability, cell-cycle regulation and apoptosis. Rassf1a is methylated in a high percentage of CRC samples (12–81% depending on the series), representing an alternative mechanism of aberrant Ras signaling and, interestingly, a mutually exclusive relationship with KRAS mutations has been reported. Rassf1a has also been found to regulate the EGFR ligand amphiregulin by Hippo activation.

The growing interest in the Hippo pathway in cancer is slowly translating into multiple translational research works that underscore the clinical relevance of this pathway in CRC tumors. The expression of Hippo’s oncoproteins YAP and TAZ has been correlated with the prognosis of CRC patients. A potential explanation for this correlation could be that TAZ/YAP signaling contributes to chemoresistance conferring cancer stem cell-related traits. Recently, in colon cancer cell lines YAP was reported to contribute to 5-Fluorouracil resistance by inducing cellular quiescence as well as contributing to a stem cell-like phenotype. Not only the expression of YAP and TAZ appear to be useful in predicting the patients’ prognosis in CRC. Single nucleotide variations within genes involved in the Hippo pathway have also been investigated as biomarkers in colorectal cancer patients. In stages II and III colorectal cancer polymorphisms located within TAZ and Rassf1a were found to be associated with the recurrence risk. However, in the metastatic colorectal cancer (mCRC) setting to our knowledge genetic variants within genes involved in the Hippo pathway have not been evaluated. In mCRC, a combination of anti-EGFR therapies plus chemotherapy is considered as a standard of care in Ras wild-type (WT) patients. Despite of the presence of Ras mutations as strong biomarkers to select the patients that benefit the most from anti-EGFR, ~ 25–30% of the patients do not respond to treatment and,

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moreover, survival among responders can vary significantly. The mechanisms for this lack of response and survival differences remain unknown. We hypothesized that the critical role of the Hippo pathway in CRC development and progression might play a role in these differences. In this work, we evaluated single nucleotide polymorphisms within the Hippo pathway as biomarkers in mCRC patients treated with cetuximab plus chemotherapy.

**MATERIAL AND METHODS**

Selected polymorphisms

A total of four single nucleotide polymorphisms (SNPs) were selected based on previously reported results and based on their potential relevance in cetuximab treated patients. The selected polymorphisms were: rs2073498 and rs2236947 located in the Rassf1a gene, rs558614 located in the LATS2 gene and rs3811715 located in the TAZ gene (also known as WWTR1). Rassf1 rs2073498 polymorphism is a missense change (Ala133Ser) located in exon three. LATS2 rs558614 polymorphism is also a missense change (Ala324Val) located in exon four. The rest of the analyzed polymorphisms are located intronicly.

DNA was extracted from FFPE tissue samples and genotypes were obtained using PCR-based direct sequencing. Five percent of the samples were re-sequence to ensure the accuracy of the results revealing a concordance higher than 99%. The author that performed the genotyping was blinded to the clinical data set.

Patients’ clinical characteristics

These four SNPs were tested first in cohort one that comprised of all Ras WT patients enrolled in the arm A of Fire three trial. Those SNPs significantly associated with survival were subsequently evaluated in an independent cohort two that included all Ras WT patients enrolled in JACCRO 05 and JACCRO 06 trials.

Cohort one consisted of a total of 199 Ras WT patients enrolled in the arm A of Fire three trial (NCT00433927) treated with FOLIRI plus cetuximab. Cohort two consisted of a total of 67 patients enrolled in JACCRO 05 (UMIN000004197) or 06 (UMIN000007022), who received oxaliplatin based chemotherapy (FOLFOX or SOX) plus cetuximab. The clinical characteristics of these two cohorts have been described in detail somewhere else.13,17,18

This study was performed following the REMARK recommendations for the reporting of biomarkers.19 The study was approved by the ethics committees and all patients signed an informed consent.

Statistical analysis

The endpoints of the current study included overall survival (OS), progression-free survival (PFS), and tumor response per RECIST 1.0. Overall survival was measured as the time from randomization or registration to death from any cause. PFS was defined as the time from the date of randomization in FIRE 3 and registration in JACCRO 05 or 06 to disease progression or death from any cause. PFS and OS were censored at the last follow-up if progression and death were not observed.

Deviations from distribution of the Hardy–Weinberg equilibrium were examined using $\chi^2$ test. The true inheritance mode of the candidate polymorphisms had not been known yet, therefore a codominant, dominant or recessive model was assumed whenever appropriate. The associations of the SNPs and PFS or OS were analyzed using Kaplan Meier curves and log-rank tests. In the multivariatable Cox regression analysis, the model was adjusted by baseline prognostic factors. The associations between the SNPs and tumor responses were examined using $\chi^2$ tests.

All analyses were conducted using SAS statistical package version 9.4 (SAS Institute, Cary, NC, USA). All tests were two-sided at a significance level of 0.05. P values were adjusted for multiple testing using the false discovery rate. The false discovery rate-adjusted $P$ values $< 15\%$ were considered as statistically significant.

**RESULTS**

The median follow up for cohort one was 34.1 months (range $0.03–70.8$) and the median OS reached 33.1 months. For the JACCRO 05 and 06 cohort, the median follow up was 31.6 months (range $5.5–42.9$) and the median survival was 33.9 months.

Of all the analyzed samples, genotypes were achieved in at least 90% of the cases for each polymorphism. In those failed cases, genotypes were not obtained due to a limited DNA quantity or poor DNA quality.

The four analyzed polymorphisms were within the probabilities limits of the Hardy–Weinberg equilibrium ($P>0.05$). For the Fire three cohort, the minor allele frequency was 47% and for the Japanese cohort 27% (expected 46% and 21% respectively, according to www.Ensembl.org).

In cohort one, the rs2236947 polymorphism was associated with overall survival. In the dominant model, patients with a CC genotype had a median OS of 46.3 months (95% CI; 21.8–70.8), whereas patients with a CA or AA genotypes had a median OS of 30.6 (95% CI, 23.9–38.3); $P=0.023$. In the multivariable Cox regression model adjusting for sex, ECOG performance status (0 vs 1–2) and primary tumor site (right, left vs NA) and number of metastatic sites (1–2 vs 3 or more) the hazard ratio (HR) was 1.50 (95% CI, 0.94–2.38); $P=0.088$. This SNP did not associate with the response rate or the PFS in this population.

The rest of the analyzed polymorphisms did not yield any association regarding response rate, PFS or OS. Table 2 shows in detail all the analyzed associations.

#### Table 1. Baseline characteristics of the two cohorts

|                | Cohort 1: Fire-3 Arm A | Cohort 2: JACCRO 05 and 06 |
|----------------|------------------------|---------------------------|
|                | N = 297                | N = 77                     |
| Age, years     |                        |                           |
| Median (range) | 64 (38–79)             | 63 (39–79)                |
| ≤ 65           | 158                    | 45                        |
| > 65           | 139                    | 32                        |
| Sex            |                        |                           |
| M              | 213                    | 44                        |
| F              | 84                     | 33                        |
| ECOGPS         |                        |                           |
| 0              | 154                    | 69                        |
| 1–2            | 143                    | 6                         |
| Primary tumor site |                |                           |
| Right          | 54                     | 11                        |
| Left           | 236                    | 64                        |
| Unknown        | 7                      | 2                         |
| Metastatic sites, n |          |                           |
| 1              | 123                    | 33                        |
| > 1            | 174                    | 44                        |
| Time to mets   |                        |                           |
| Synchronous    | 217                    | 59                        |
| Metachronous   | 75                     | 18                        |
| Unknown        | 5                      |                           |
| Adjuvant therapy |                    |                           |
| No             | 226                    | 71                        |
| Yes            | 66                     | 6                         |
| Unknown        | 5                      |                           |
| Mutation status |                   |                           |
| All RAS wildtype | 199                   | 67                        |
| Mutant         | 39                     | 10                        |
| Unknown        | 59                     |                           |
The rs2236947 located in the Rassf1a gene was analyzed in the second cohort of patients. In this population, the rs2236947 was also associated with OS: patients harboring a CC genotype had a median OS of 42.8 months (95% CI, 27.1–42.8) compared with the patients with a CA or AA genotypes whose median OS was 19.0 months (95% CI, 13.4–42.9); \( P = 0.057 \). In the multivariable Cox regression model adjusting for ECOG performance status the HR was 2.72 (95% CI, 1.23–6.04); \( P = 0.014 \). In this cohort, an association was found also regarding PFS. Table 3 shows in detail these results.

These polymorphisms were also evaluated in an exploratory cohort of 190 patients enrolled in the arm B of the FIRE 3 arm and treated with FOLFIRI plus Bevacizumab. In this population no associations were found regarding response, PFS or OS based on the rs2236947 genotype (Online only Supplementary Table 1).

Subgroup analysis

The association of Rassf1a rs2236947 with OS was stronger in patients bearing left-side tumors. In cohort one, patients with a CC genotype had a median OS of 59.0 months (95% CI, 23.8–70.8) compared with 38.3 (95% CI, 23.4–70.1) months for the patients with a CA or AA genotypes, \( P = 0.013 \). In multivariable analysis this association remained statistically significant with a HR of 1.79 (1.01–3.14); \( P = 0.044 \) (Figure 1, Table 4). No association was found regarding Rassf1a rs2236947 genotype in patients harboring right-side colon tumors.

Table 2. Hippo pathway SNPs and clinical outcomes in patients with all RAS WT mCRC treated with first-line FOLFIRI+Cetuximab in FIRE-3

| Tumor response, RECIST | Progression-Free survival (PFS) | Overall survival (OS) |
|------------------------|-------------------------------|-----------------------|
| SNP                    | N    | CR+PR | s.d.+PD | Median, ms (95% CI) | HR (95% CI) | Median, ms (95% CI) | HR (95% CI) |
| RASSF1a rs2073498      |      |       |         |                   |            |                       |              |
| C/C                    | 155  | 98 (74%) | 34 (26%) | 10.0 (8.0, 11.5) | 1 (reference) | 29.8 (23.7, 38.3) | 1 (reference) |
| C/A                   | 31   | 26 (84%) | 5 (16%) | 11.1 (9.5, 14.3) | 0.74 (0.50, 1.10) | 0.84 (0.56, 1.28) | 0.003 (0.14) |
| A/A                   | 4    | 3 (75%) | 1 (25%) | 10.5 (8.0, 13.0) | 0.95 (0.67, 1.34) | 0.91 (0.64, 1.29) | 0.002 (0.14) |
| P value\(^d\)           |      |       |         | 0.35          | 0.13          | 0.42           | 0.20          |
| RASSF1a rs2236947      |      |       |         |                   |            |                       |              |
| C/C                    | 57   | 37 (66%) | 12 (24%) | 10.1 (7.8, 11.1) | 1 (reference) | 36.3 (21.8, 70.8) | 1 (reference) |
| C/A                   | 132  | 88 (78%) | 25 (22%) | 10.5 (9.3, 13.0) | 0.95 (0.67, 1.34) | 0.91 (0.64, 1.29) | 0.002 (0.14) |
| A/A                   | 1     | 1 (100%) | 0 (0%)  | 13.0 (6.1, 70.8) | 0.53 (0.23, 1.20) | 0.58 (0.25, 1.36) | 0.016 (0.20) |
| P value\(^d\)           |      |       |         | 0.04          | 0.17          | 0.36           | 0.11          |
| LATS rs228614          |      |       |         |                   |            |                       |              |
| A/A                    | 120  | 80 (78%) | 22 (22%) | 10.4 (9.2, 13.0) | 1 (reference) | 38.7 (27.1, 49.8) | 1 (reference) |
| A/G                    | 55   | 35 (63%) | 13 (27%) | 10.0 (7.8, 11.8) | 1.15 (0.81, 1.63) | 1.10 (0.77, 1.57) | 0.023 (0.14) |
| G/G                    | 11   | 5 (45%) | 3 (27%) | 13.0 (6.1, 70.8) | 0.53 (0.23, 1.20) | 0.58 (0.25, 1.36) | 0.002 (0.14) |
| P value\(^d\)           |      |       |         | 0.46          | 0.17          | 0.36           | 0.11          |
| TAZ rs3811715          |      |       |         |                   |            |                       |              |
| C/C                    | 124  | 77 (78%) | 26 (25%) | 10.4 (9.0, 12.2) | 1 (reference) | 33.4 (24.4, 45.0) | 1 (reference) |
| C/T                   | 57   | 42 (75%) | 12 (22%) | 10.6 (8.0, 13.3) | 0.98 (0.70, 1.36) | 1.03 (0.73, 1.43) | 0.017 (0.20) |
| T/T                   | 4    | 4 (100%) | 0 (0%)  | 13.0 (6.1, 70.8) | 0.53 (0.23, 1.20) | 0.58 (0.25, 1.36) | 0.017 (0.20) |
| P value\(^d\)           |      |       |         | 0.84          | 0.89          | 0.88           | 0.50          |

\(^a\) Wald test for PFS and OS in the multivariable Cox regression model. \(^b\) Adjusting for sex (male vs female), ECOG performance status (0 vs 1–2), primary tumor site (right, left vs NA) and number of metastatic disease (1, 2 vs 3+). \(^c\) A dominant model was used. \(^d\) \( P \) value was based on Fisher’s exact test for response, log-rank test for PFS and OS in the univariable analysis. \(^e\) \( P \) value adjusted by false discovery rate.

Table 3. Rassf1a rs2236947 and clinical outcomes in Japanese patients with all RAS WT mCRC treated with first-line oxaliplatin+ cetuximab in JACCRO 05 and 06

| Tumor response, RECIST | Progression-Free survival (PFS) | Overall survival (OS) |
|------------------------|-------------------------------|-----------------------|
| SNP                    | N    | CR+PR | s.d.+PD | Median, ms (95% CI) | HR (95% CI) | Median, ms (95% CI) | HR (95% CI) |
| All patients           |      |       |         |                   |            |                       |              |
| C/C                    | 35   | 26 (74%) | 6 (19%) | 13.8 (6.6, 17.4) | 1 (reference) | 42.8 (27.1, 42.8) | 1 (reference) |
| C/A, A/A\(^d\)          | 27   | 20 (77%) | 6 (23%) | 9.4 (5.8, 11.3) | 1.44 (0.81, 2.54) | 1.69 (0.93, 3.07) | 0.005 (0.14) |
| P value\(^d\)           |      |       |         | 0.18          | 0.088        | 0.014          | 0.014          |
| Left-sided CRC          |      |       |         |                   |            |                       |              |
| C/C                    | 31   | 24 (77%) | 4 (14%) | 15.2 (8.8, 18.0) | 1 (reference) | 32.3 (13.4, 42.9) | 1 (reference) |
| C/A, A/A\(^d\)          | 21   | 17 (81%) | 4 (19%) | 10.0 (8.5, 11.7) | 1.75 (0.91, 3.34) | 1.98 (1.02, 3.84) | 0.283 (1.14, 7.03) |
| P value\(^d\)           |      |       |         | 0.71          | 0.059        | 0.045          | 0.025          |

\(^a\) Wald test for PFS and OS in the multivariable Cox regression model. \(^b\) Adjusting for ECOG performance status (0 vs 1), and regimen (FOLFOX vs SOX). \(^c\) A dominant model was used. \(^d\) \( P \) value was based on Fisher’s exact test for response, log-rank test for PFS and OS in the univariable analysis.
In cohort 2, patients harboring a CC genotype had a median OS of 42.8 months (95% CI, 30.5–42.8) whereas patients with a CA or AA genotypes had a median OS of 23.2 (13.4–42.9), \( P = 0.056 \). In the multivariable analysis the HR was 2.83 (1.14–7.03); \( P = 0.025 \) (Figure 2, Table 3).

In this cohort, the rs2236947 SNP was also associated with PFS in patients harboring left-side tumors. Patients with a CC genotype had a median PFS of 15.2 months (95% CI, 8.8–18.0) compared with 10.0 months (95% CI, 8.5–11.7) for the patients with a CA or AA genotype, \( P = 0.059 \). In multivariable analysis the HR was 1.98 (95% CI, 1.02–3.84); \( P = 0.045 \).

**DISCUSSION**

The polymorphism rs2236947 located in the Rassf1 gene was found to be associated with overall survival in two independent cohorts of patients treated with chemotherapy plus the anti-EGFR monoclonal antibody cetuximab. Moreover, this association appears to be stronger in patients bearing left-sided tumors. Additionally, in the JACCRO population this SNP was also associated with progression-free survival.

**Rassf1a is a tumor suppressor frequently methylated in colorectal cancer. Rassf1a is involved not only in Ras signaling, but also it is a recognized upstream regulator of Hippo signaling.**

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**Figure 1.** Rassf1a rs2236947 is associated with OS in Ras WT left-sided mCRC patients treated with FOLFIRI plus cetuximab in Fire 3. *Wald test in the multivariable Cox Regression model adjusting for sex, ECOG and number of metastatic sites.

**Figure 2.** Rassf1a rs2236947 is associated with OS in Ras WT left-sided mCRC patients treated with oxaliplatin-based chemotherapy plus cetuximab. *Wald test in the multivariable Cox Regression model adjusting for ECOG and regime (FOLFOX vs SOX).

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**Table 4.** Hippo pathway SNPs and clinical outcomes in patients with all Ras WT left-sided mCRC treated with first-line FOLFIRI+Cetuximab in Fire-3

| SNP           | N   | CR+PR | s.d.+PD | Median, ms (95%CI) | HR (95%CI)a | HR (95%CI)b |
|---------------|-----|-------|---------|--------------------|-------------|-------------|
| RASSF1a rs2073498 | C/C | 120   | 83 (79%) | 22 (21%)         | 10.4 (9.3,12.9) | 1 (reference) | 1 (reference) |
|               | C/A§| 33    | 23 (82%) | 5 (18%)          | 12.2 (9.6,14.3) | 0.81 (0.53,1.24) | 0.82 (0.53,1.28) |
| P valuec      |     | 0.80  |         |                   | 0.32         | 0.39         |
| RASSF1a rs2236947 | C/C | 47    | 32 (80%) | 8 (20%)         | 10.4 (9.2,12.2) | 1 (reference) | 1 (reference) |
|               | C/A,A/A | 103 | 74 (81%) | 17 (19%)        | 11.5 (9.6,14.1) | 0.98 (0.66,1.45) | 0.92 (0.62,1.38) |
| P valuec      |     | 1.00  |         |                   | 0.92         | 0.69         |
| LATS rs558614 | A/A  | 95    | 68 (83%) | 14 (17%)        | 12.2 (9.7,14.1) | 1 (reference) | 1 (reference) |
|               | A/G  | 42    | 29 (76%) | 9 (24%)         | 9.9 (7.8,11.8)  | 1.23 (0.82,1.83) | 1.23 (0.80,1.89) |
|               | G/G  | 10    | 5 (63%)  | 3 (38%)         | 13.0 (6.1,10.8) | 0.49 (0.20,1.22) | 0.51 (0.20,1.28) |
| P valuec      |     | 0.27  |         |                   | 0.12         | 0.19         |
| TAZ rs3811715 | C/C  | 98    | 65 (76%) | 20 (24%)        | 10.4 (8.1,12.2) | 1 (reference) | 1 (reference) |
|               | C/T§ | 47    | 35 (85%) | 6 (15%)         | 12.9 (10.3,14.1)| 0.87 (0.60,1.28) | 0.90 (0.61,1.32) |
| T/T§          |     | 0.35  |         |                   | 0.48         | 0.59         |

*Wald test for PFS and OS in the multivariable Cox regression model. Adjusting for sex (male vs female), ECOG performance status (0 vs 1–2), and number of metastatic disease (1, 2 vs 3+). \( P \) value was based on Fisher’s exact test for response, log-rank test for PFS and OS in the univariable analysis. Value adjusted by false discovery rate.

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In cohort 2, patients harboring a CC genotype had a median OS of 42.8 months (95% CI, 30.5–42.8) whereas patients with a CA or AA genotypes had a median OS of 23.2 (13.4–42.9), \( P = 0.056 \). In the multivariable analysis the HR was 2.83 (1.14–7.03); \( P = 0.025 \) (Figure 2, Table 3).

In this cohort, the rs2236947 SNP was also associated with PFS in patients harboring left-side tumors. Patients with a CC genotype had a median PFS of 15.2 months (95% CI, 8.8–18.0) compared with 10.0 months (95% CI, 8.5–11.7) for the patients with a CA or AA genotype, \( P = 0.059 \). In multivariable analysis the HR was 1.98 (95% CI, 1.02–3.84); \( P = 0.045 \).
interacting with MST through its SARAH domain. The critical importance of Ras signaling in mCRC is widely known. Regarding Rassf1a is implicated in Ras signaling, and Ras signaling is of high importance in tumors could be associated to these molecular differences. Nonetheless, due to the low value of rs2236947 patients treated with chemotherapy plus cetuximab regardless of conclusion that this SNP has no value in this population.

(rs13100173) located in the HYAL3 gene. However, whether this intronically and its functionality is not known. This SNP is in the Japanese cohort was also associated with PFS whereas no association of Rassf1a regulation and the expression of target genes. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

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CONFICT OF INTEREST

The authors declare no conflict of interest.
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