Impact of KRAS, BRAF, PIK3CA Mutations, PTEN, AREG, EREG Expression and Skin Rash in ≥2nd Line Cetuximab-Based Therapy of Colorectal Cancer Patients

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Abstract

Background: To investigate the predictive significance of KRAS, BRAF, PIK3CA mutational status, AREG- EREG mRNA expression, PTEN protein expression and skin rash in metastatic colorectal cancer (mCRC) patients treated with cetuximab containing salvage chemotherapy.

Methods: Primary tumors from 112 mCRC patients were analyzed. The worst skin toxicity during treatment was recorded.

Results: KRAS, BRAF and PIK3CA mutations were present in 37 (33%), 8 (7.2%) and 11 (9.8%) cases, respectively, PTEN was lost in 21 (19.8%) cases, AREG and EREG were overexpressed in 48 (45%) and 51 (49%) cases. In the whole study population, time to tumor progression (TTP) and overall survival (OS) was significantly lower in patients with KRAS (p = 0.001 and p = 0.026, respectively) or BRAF (p = 0.001 and p < 0.0001, respectively) mutant tumors, downregulation of AREG (p = 0.018 and p = 0.013, respectively) or EREG (p = 0.002 and p = 0.004, respectively) and grade 0-1 skin rash (p < 0.0001 and p < 0.0001, respectively). In KRAS wt patients TTP and OS was significantly lower in patients with BRAF (p = 0.0001 and p < 0.0001, respectively) mutant tumors, downregulation of AREG (p = 0.021 and p = 0.004, respectively) or EREG (p = 0.001 and p < 0.0001, respectively) and grade 0-1 skin rash (p < 0.0001 and p < 0.0001, respectively). TTP was significantly lower in patients with PIK3CA mutations (p = 0.01) or lost PTEN (p = 0.002). Multivariate analysis revealed KRAS (Hazard Ratio [HR] 4.3, p < 0.0001), BRAF mutation (HR: 5.1, p < 0.0001), AREG low expression (HR: 1.6, p = 0.021) and absence of severe/moderate skin rash (HR: 4.0, p < 0.0001) as independent prognostic factors for decreased TTP. Similarly, KRAS (HR 2.9, p = 0.01), BRAF mutation (HR: 3.0, p = 0.001), EREG low expression (HR: 1.7, p = 0.021), absence of severe/moderate skin rash (HR: 3.7, p < 0.0001) and the presence of unfavourable tumours (HR: 2.2, p = 0.001) were revealed as independent prognostic factors for decreased OS.

Conclusions: These results underscore that KRAS-BRAF mutations and EREG expression can be used as biomarkers to further select patients undergoing anti-EGFR treatment.

Introduction

Despite the progress made in the management of metastatic colorectal cancer (mCRC) over the last few years, the disease remains a major public health problem in the western world with an estimated 146,970 new CRC cases and 49,920 deaths for 2009 in the United States [1].

Two monoclonal antibodies targeting EGFR (anti-EGFR moAbs), both by binding to the extracellular domain, and thus, leading to inhibition of its downstream signaling, the chimeric IgG1 moAb cetuximab and the fully humanized IgG2 moAb panitumumab, have entered clinical practice in the mCRC setting and have proven to provide a modest clinical benefit in pretreated patients, either used alone or in combination with chemotherapy [2–5]. Nevertheless, from the beginning became clear that not all patients derive a benefit from the incorporation of these agents into the treatment combinations; indeed, non-randomized retrospective studies [6–11] as well as retrospective analysis of prospective randomized trials [12–16] demonstrated that the presence of KRAS mutations were predictive of resistance to anti-EGFR moAbs therapy and were associated with a worse prognosis and a shorter survival. Based on this knowledge, a primary tumor’s KRAS mutational status is now mandatory for the treatment of metastatic disease with an anti-EGFR moAb (European Medicine Agency – EMEA-H-C-741 and H-C-558 and U.S. Food and Drug Administration - FDA Application No. (BLA) 125084 and No. (BLA) 125147).

However, not all patients with KRAS WT tumours benefit from anti-EGFR moAbs treatment, meaning that additional genetic
determinants of resistance exist [7,9,17–19]. Indeed, from three sporadic mCRC retrospective studies [20–22], the BRAF V600E mutation has been shown to identify a subgroup (<10%) of patients that not only present resistance to anti-EGFR MoAbs therapy, but, is also characterized by particularly unfavorable prognosis regardless of treatment administration [20–22]. Furthermore, although not entirely clear yet, PIK3CA-mutant tumor seem to derive no or little benefit from anti-EGFR MoAbs treatment [20,23–26].

Besides the KRAS-BRAF-PIK3CA mutational status, EGFR epiregulin (EREG) and ampiregulin (AREG) ligands’ expression in primary CRC tumours has been shown to significantly predict clinical outcome in KRAS WT mCRC patients treated with cetuximab, indicating ligand-driven autocrine oncogenic EGFR signaling [27,28]. In addition, PTEN [phosphatase and tensin homolog] protein expression, and specifically its loss, seems to be associated in a number of studies with resistance to treatment with anti-EGFR MoAbs treatment [21,29–31]. Furthermore, from a clinical point of view, the only parameter which has been constantly associated with a high probability of response, prolonged progression-free survival (PFS) and median Overall Survival (mOS) to anti-EGFR MoAbs treatment is the development of skin rash [2,5,32].

Clinical parameters seem to be inadequate for patient selection, but, biomarkers’ analyses have already been incorporated in the treatment of CRC patients. The aim of the present study was to simultaneously ascertain and investigate the clinical relevance of all known biomarkers, KRAS exon 2, BRAF V600E, PIK3CA exon 9 and 20 mutational status in conjunction with AREG, EREG mRNA expression, PTEN immunohistochemical protein expression, as well as, skin rash development, in mCRC patients treated with cetuximab containing salvage combination chemotherapy.

Materials and Methods

Patient population and study design

One hundred and twelve consecutive patients, with histologically confirmed mCRC and available tumor material for molecular analysis, who were treated with cetuximab containing salvage chemotherapy at the Department of Medical Oncology, University Hospital of Heraklion (Crete, Greece) between 1/2005 - 12/2008, were enrolled. The study was approved by the Ethics and Scientific Committees of the University General Hospital of Heraklion and all patients gave their written informed consent for the use of the tissue material for translational research.

Materials and Methods

Tissue selection, DNA and RNA extraction

Formalin-fixed, paraffin-embedded (FFPE) tumor sections were reviewed by a pathologist (MT) to confirm the diagnosis and define tumor-enriched areas for dissection. Ten serial sections of 5 μm thickness were stained with nuclear fast red (Sigma-Aldrich, St Louis, MO, USA) and scrape dissection under a binocular microscope was performed for samples with ≥80% tumor cells; for samples with <80% malignant cells, microdissection with the piezoelectric Eppendorf microdissector (Eppendorf, Hamburg, Germany) was performed. DNA extraction was performed with the use of the Epicentre® Biotechnologies MasterPure™ Complete DNA and RNA Purification Kit according to the manufacturer’s instructions (Epicentre, Madison, WI, USA) after the isolated cancer cells were lysed in buffer containing Proteinase K at 60°C for 72 h. For RNA extraction, cancer cells were re-suspended in 400 μl RNA lysis buffer supplemented with 300 mg proteinase K (QIAGEN, Valencia, CA, USA) and incubated at 60°C for 16 hours until the tissue was completely solubilized. RNA was purified by Trizol LS (Invitrogen, Carlsbad, CA, USA) and subsequently, treated with DNase (DNA-free, Ambion, Austin, TX, U.S.A.) in order to avoid genomic DNA contamination and stored at -80°C until used.

KRAS and PIK3CA mutational analysis

KRAS and PIK3CA mutational analysis was performed by Sanger sequencing after PCR amplification of KRAS exon 2 and PIK3CA exons 9 and 20. PCR conditions with primers sets which have been previously reported [22].

BRAF mutational analysis

The V600E, BRAF mutation was detected by real-time PCR using the allelic discrimination method as previously described [33,34]. In brief, the DNA extracted from tumoral cells was amplified with the use of a set of primers and two hydrolysis probes in the ABI PRISM 7900HT Sequence Detection System (AB; Applied Biosystems, Forest City; CA; USA). The two hydrolysis probes were labeled at 5 with VIC and FAM fluorophores reporters for the wt and the mutant allele, respectively. The SDS 2.3 software was used for the analysis of the results.

AREG and EREG mRNA expression

The SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) was used to prepare cDNA from 50 ng of total RNA for each gene analyzed as previously described [35]. Relative cDNA quantification for AREG, EREG and both β-actin and PGK as internal reference genes was done using the ABI Prism 7900HT Sequence Detection System (AB), as described previously [35]. The primers and probe sets were designed using Primer Express 2.0 Software (AB), according to the Ref Seq NM_001657.2 for AREG and NM_001432.2 for EREG [http://www.ncbi.nlm.nih.gov/LocusLink]. The sequence of the primers and 5’ labeled fluorescent reporter dye (6FAM) probes for all reference and target genes are shown in Table 1.

Relative gene expression quantification was performed according to the comparative Ct method using β-actin and PGK as endogenous controls and commercial RNA controls (Stratagene, La Jolla, CA, USA) as calibrators. Final results were determined as follows: 2^ΔΔCt sample-ΔCt calibrator, where ΔCt values of the

Table 1. Sequence of the primers and probes of all references and target genes.

| Gene | Forward Primer | 5’-labeled (FAM) probe | Reverse Primer |
|------|----------------|------------------------|---------------|
| β-actin | 5’-GCC CAG CAC AAT GAA G-3’ | 5’TCA AGA TTG TCT CTC CTG AGC GC-3’ | 5’-GCC GAT CCA CAC GGA GTA CT-3’ |
| PGK | 5’-GCC GAT CCA CAC GGA GTA CT-3’ | 5’TGCAGGCGGGCAGAAAG-3’ | 5’TGCAGGCGGGCAGAAAG-3’ |
| AREG | 5’-CGGCTAGGGAGGCAATGACGAT-3’ | 5’-GCC GAT CCA CAC GGA GTA CT-3’ | 5’-GCC GAT CCA CAC GGA GTA CT-3’ |
| EREG | 5’-TGCATCTATCCGAGAGTGAATG-3’ | 5’-AAACACTTGCAAGTGAAGT-3’ | 5’-AAACACTTGCAAGTGAAGT-3’ |

doi:10.1371/journal.pone.0015980.t001
cumulative score of the primary antibody. Prostate cancers and endothelial cells were
tham, MA, USA). Negative control slides were prepared by omitting
Immunostaining was performed using the UltraVision LP Large
previously described [28,36]. After deparaffinization and hydration
PTEN protein expression
Three- to 4-μm tumor tissue sections of paraffin-embedded
specimens from each patient were selected for PTEN IHC staining
using the 17.A mouse monoclonal antibody (1:25 dilution,
Neomarkers; ThermoFisher Scientific Inc, Fremont, CA), as
previously described [20,36]. After deparaffinization and hydration of sections, antigens were unmasked by heat in EDTA buffer. Immunostaining was performed using the UltraVision LP Large Volume Detection System AP Polymer (Thermo Scientific, Waltham, MA, USA). Negative control slides were prepared by omitting the primary antibody. Prostate cancers and endothelial cells were used as external and internal positive controls, respectively.
PTEN staining was mainly cytoplasmatic. As previously described [28], intensity was scored according to a four-tier system:
0, no staining; 1, weak; 2, moderate; and 3, strong. One, two or
three additional points were attributed if the percentage of positive
expression was $\leq 25\%$, 25–50% or $>50\%$, respectively. The specimens with a cumulative score of $\geq 4$ were characterized as positive [28].

Study Design and Statistical analysis
The present study was a retrospective analysis aiming to explore the predictive value of extensive biomarkers analysis in the outcome of patients with mCRC treated with cetuximab plus chemotherapy as salvage treatment. All available biopsies of the primary tumor with more than 100 cells per section were included in the analysis. RT-qPCR analysis yielded values that were expressed as ratios between two absolute measurements (gene of interest: mean of internal reference genes). CART analysis has been used for the estimation of the cut-off points of AREG and EREG mRNA expression, in order to classify cases into groups of a dependent (TTP and mOS) variable. Samples with mRNA expression above or equal to the cut-off point were considered as samples with high expression, while those with value below the median as samples with low expression. Associations between KRAS, BRAF, PIK3CA mutation status, AREG and EREG mRNA expression and PTEN IHC expression with baseline characteristics were assessed using the Fisher’s exact test for categorical variables or logistic regression for continuous variables. Spearman’s exact test was used to evaluate the correlation between AREG and EREG mRNA expression. Time to tumour progression (TTP) and overall survival (OS) were measured from the date of the cetuximab containing treatment line initiation to the first radiographic documentation of disease progression or death, respectively. Kaplan-Meier curves were used to describe the proportion of patients who remained free of events over the follow-up period. Associations between prognostic factors and TTP or OS were examined using Cox proportional hazards regression models. All reported $p$-values are two-sided and not adjusted for multiple testing.

Results

Patient demographics
The mutational status for KRAS exon 2, BRAF exon 15, and PIK3CA exons 9 and 20 was detected in all 112 consecutive patients with mCRC whereas, AREG and EREG mRNA expression was determined in 106 and 105 patients for whom tumour material was available respectively, while PTEN expression was evaluated in 106 patients. All patients were treated with cetuximab in combination with chemotherapy (73% in combination with Irinotecan, 27% with Oxaliplatin) as salvage treatment (Table 2). Sixty-six (39%) patients had received the treatment in the 2nd line setting and the remaining 46 (41%) as 3rd line treatment. There was no patient who received the anti-EGFR moAbs in the 1st line setting. Disease characteristics were typical for mCRC in the western world; the patients’ median age was 66 years and 60% of them were male (Table 2). The median PFS from 1st line treatment was 8.9 months (95% CI 8.1–9.9) and the median time from relapse to previous treatment line until the cetuximab administration was 1.1 months (95% CI 0.7–1.0).

Mutational status and expression values results
KRAS mutations were detected in 37 (33%), BRAF mutations in eight (7.2%) and PIK3CA mutations in 11 (9.8%, 8 in exon 9 and 3 in exon 20) primary tumours, respectively. KRAS and BRAF mutations were mutually exclusive, whereas, three tumours carried both KRAS and PIK3CA mutations. AREG and EREG were overexpressed in 48 (45%) and 51 (49%) patients, respectively, whereas, PTEN was scored as negative (i.e. loss of function) in 21 (19.8%) patients (Figures 1A and 1B). When PIK3CA mutations and PTEN expression were analyzed together, activation of the pathway (defined as loss of PTEN or PIKECA mutation) was detected in 25 (23.5%) patients. A trend for decreased incidence of

| Table 2. Patients’ and tumors’ characteristics. |
| --- |
| Feature | N | % |
| Median Age (Range) | 66(23–83) | 78 |
| ≤70 years | 76 | 78 |
| >70 years | 36 | 32 |
| Gender | | |
| Male | 68 | 60 |
| Female | 44 | 40 |
| Stage at diagnosis | | |
| I-II | 61 | 54 |
| IV | 51 | 46 |
| Tumor Location | | |
| Colon | 83 | 74 |
| Rectum | 29 | 26 |
| Tumor differentiation | | |
| Well moderate | 66 | 59 |
| Undifferentiated | 46 | 41 |
| Mucinous Features | | |
| Yes | 18 | 16 |
| No | 94 | 84 |
| Cetuximab administration line | | |
| 2nd | 66 | 59 |
| 3rd | 46 | 41 |
| Chemotherapy administered with Cetuximab | | |
| Irinotecan-based | 82 | 73 |
| Oxaliplatin-based | 30 | 27 |

Biomarkers to Anti-EGFR moAbs in mCRC

doi:10.1371/journal.pone.0015980.t002
KRAS mutations in rectal tumors was observed ($p = 0.097$) since 31 of the 83 (37%) tumours located at the colon and six of the 29 (20%) tumours located at the rectum harbored a KRAS mutation. There was no correlation between the presence of KRAS mutations with the patients’ gender, age (>70 years old versus ≤70 years old), stage at diagnosis, histological grade, mucinous status, PTEN loss and AREG-EREG expression (all $p$-values > 0.05). Also, a statistically significant correlation was observed between the presence of BRAF mutations and the histological grade (well/moderate versus undifferentiated) ($p = 0.049$) and EREG mRNA downregulation ($p = 0.013$). There was no correlation between the presence of BRAF and PIK3CA mutations with the patients’ gender, age (>70 years old versus ≤70 years old); stage at diagnosis, tumour location, mucinous status, PTEN loss and AREG expression (in both cases all $p$-values > 0.05).

**Impact of mutational status and expression values on the outcome of salvage cetuximab therapy**

**Results in the whole patients’ population** (Table 3). Tables 3 and 4 summarize the impact of genetic alterations on the outcome of cetuximab-containing salvage treatment. The median TTP of the whole group of patients was 4.9 months (95% CI 4.1–5.7) and the corresponding median overall survival (OS) 14.5 months (95% CI 10.0–18.9). TTP and OS were significantly lower among patients whose tumours carried KRAS mutations (3.1 vs. 6.4 months, $p = 0.001$ and 10.6 vs. 16.3 months, $p = 0.026$, respectively) (Figure 2A and 2B).

Similarly, TTP and OS were significantly lower among patients whose tumours carried BRAF mutations (2.1 vs. 5.2 months, $p = 0.001$ and 4.3 vs. 15.1 months, $p<0.0001$, respectively) (Figure 3 and 4).

There was no significant correlation in terms of TTP according to PIK3CA mutational status or PTEN expression in all treated patients (4.9 vs. 5.7 months, $p = 0.427$ and 5.2 vs. 6.03 months, $p = 0.102$, respectively) (Figure 4A and 4B); similarly, there was no difference in terms of median OS between patients with PIK3CA mutant (15.6 months) and wt (15.0 months) primary tumours ($p = 0.41$; Figure 5A), as well as between patients with lost (14.3 months) or normal (15.1 months) PIK3CA function ($p = 0.82$; Figure 5B). Nevertheless, when PIK3CA mutational status and PTEN expression were taken into consideration together, activation of the pathway through PIK3CA mutations and/or PTEN loss was correlated with a trend for decreased TTP in all patients (3.8 vs. 5.0 months, $p = 0.051$) (Figure 4E), while no difference was observed in the median OS (13.9 vs. 14.5 months, $p = 0.878$) (Figure 5E).

A highly significant correlation between AREG and EREG mRNA expression was observed (Spearman $r^2 = 0.736$, $p<0.001$). In the whole group of patients, AREG mRNA overexpression was significantly correlated with increased TTP and OS (5.0 vs. 3.8 months, $p = 0.018$ and 20.2 vs. 10.7 months, $p = 0.013$, respectively) (Figures 6A and 6B). Furthermore, EREG mRNA overexpression was also correlated significantly with increased TTP and OS (6.1 vs. 3.6 months, $p = 0.002$ and 17.6 vs. 10.7 months, $p = 0.004$, respectively) (Figures 7A and 7B).

**Table 3 and Figures 8A and 8B** demonstrate the differences in TTP and OS according to KRAS-BRAF mutational status and AREG expression. It is shown that the KRAS-BRAF-WT and AREG overexpression profile was correlated significantly with increased TTP and OS compared with any other combination. Similarly, Figures 8C and 8D and Table 3 illustrate the differences in TTP and OS according to KRAS-BRAF mutational status and EREG expression; again, the KRAS-BRAF WT and EREG overexpression profile was correlated significantly with increased TTP and OS compared with any other combination.

Finally, we correleted the impact of cetuximab induced skin rash with treatment outcome. Patients with severe or moderate (grade 2–3) skin rash presented significantly higher TTP (7.5 months) in comparison with those with mild (grade 1) (4.5 months; $p = 0.013$, respectively) (Figures 6A and 6B).

**Results in the KRAS WT patients’ population** (Table 4). When only KRAS WT cases were analyzed patients whose tumours carried the BRAF mutation had even more significantly lower TTP and OS (TTP: 2.1 vs. 6.4 months, $p<0.0001$; OS: 4.3 vs. 16.3 months, $p<0.0001$) (Figure 3C and 3D) compared with the results in the whole population. In addition, when only the KRAS WT cases were considered, decreased TTP was significantly associated with the presence of PIK3CA mutation (4.3 vs. 6.4 months, $p = 0.01$) (Figure 4C) and PTEN downregulation (3.7 vs. 5.0 months, $p = 0.002$) (Figure 4D). Nevertheless, in this particular group of patients with KRAS WT tumors, no significant correlation was found in the median OS between patients with or without PIK3CA mutations (13.5 vs. 16.3 months, respectively; $p = 0.345$) or those with downregulated or functional PTEN (15.3 vs. 14.5 months, respectively; $p = 0.382$) (Figures 5C and 5D). But, in KRAS WT patients when PIK3CA mutational status and PTEN expression were taken into consideration together, a significantly decreased TTP was observed with the activation of the pathway through PIK3CA mutations and/or PTEN loss, compared with its inactivated presence with wt PIK3CA and/or functional PTEN (3.8 vs. 6.4 months, $p = 0.001$) (Figure 4F); conversely, such a correlation could not be revealed in terms of median OS (13.9 vs. 16.2 months; $p = 0.987$) (Figure 5F).

**Figure 1. Assessment of PTEN expression by immunohistochemistry.** Panel A: Sample of a moderate differentiated adenocarcinoma of the colon scored as PTEN positive (x100) Panel A: Sample of a moderate differentiated adenocarcinoma of the colon scored as PTEN negative (x100). doi:10.1371/journal.pone.0015980.g001
In KRAS WT patients, AREG mRNA overexpression was significantly correlated with increased TTP and OS (5.8 vs. 4.3 months, \( p = 0.021 \) and 23.2 vs. 10.7 months, \( p = 0.004 \), respectively) (Figures 6C and 6D), as well as, EREG mRNA overexpression (7.0 vs. 3.6 months, \( p = 0.0001 \) and 20.2 vs. 10.5 months, \( p < 0.0001 \), respectively) (Figures 7C and 7D).

### Univariate and Multivariate analysis

As far as TTP was concerned, the univariate analysis (Table 3 and 4) demonstrated significant associations with: i) KRAS mutations \( (p = 0.001) \); ii) BRAF mutations \( (p = 0.001) \); iii) AREG mRNA expression \( (p = 0.018) \); iv) EREG mRNA expression \( (p = 0.002) \) and v) the development of moderate severe skin rash \( (p < 0.0001) \). In addition, TTP in KRAS wt patients was significantly correlated with PIK3CA mutation \( (p = 0.01) \), PTEN expression \( (p = 0.002) \) and the PIK3CA-PTEN axis activation \( (p = 0.001) \). As far as OS was concerned the univariate analysis (Table 3 and 4) demonstrated significant associations with: i) KRAS mutations \( (p = 0.026) \); ii) BRAF mutations \( (p < 0.0001) \); iii) AREG mRNA expression \( (p = 0.013) \); iv) EREG mRNA expression \( (p = 0.004) \) and

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**Table 3. TTP and OS to the \( \geq 2 \)nd line cetuximab-containing treatment according to KRAS, BRAF, PIK3CA mutations status, PTEN protein expression, AREG and EREG mRNA expression and grade of skin rash in the whole patient’ population.**

| Feature | Patients’ population (No of patients) | Time to Tumor Progression (months) | Overall survival (months) |
|---------|--------------------------------------|-----------------------------------|--------------------------|
|         |                                       | Median (months) (95% CI) | HR (95% CI) | \( \rho \) value | Median (months) (95% CI) | HR (95% CI) | \( \rho \) value |
| KRAS status | n = 112 Mutant (n = 37) | 3.1 (2.0–4.2) | 3.3 (2.4–5.1) | 0.001 | 10.6 (5.7–15.5) | 2.2 (1.7–2.8) | 0.026 |
|           | WT (n = 75) | 6.4 (5.4–7.4) | 16.3 (12.7–19.6) | 0.0001 |
| BRAF status | n = 112 Mutant (n = 8) | 2.1 (0.8–3.3) | 4.9 (2.2–10.9) | 0.001 | 4.3 (0.3–10.3) | 3.6 (1.7–7.5) | 0.0001 |
|           | WT (n = 104) | 5.2 (4.3–6.1) | 15.1 (12.2–17.9) | 0.0001 |
| PIK3CA status | n = 112 Mutant (n = 11) | 4.9 (2.9–6.9) | 1.9 (0.9–4.1) | 0.427 | 13.6 (4.9–19.2) | 1.3 (0.7–2.9) | 0.44 |
|           | WT (n = 101) | 5.7 (4.8–6.8) | 15.0 (13.2–22.2) | 0.82 |
| PTEN expression | n = 106 Loss (n = 21) | 5.2 (4.1–6.3) | 1.7 (0.9–2.8) | 0.102 | 14.3 (2.6–18.8) | 1.1 (0.6–1.8) | 0.82 |
|           | Preserved (n = 85) | 6.0 (4.9–7.2) | 15.1 (9.8–24.3) | 0.013 |
|           | Activated (n = 25) | 3.8 (2.7–4.9) | 1.6 (1.0–2.6) | 0.051 | 13.9 (7.8–20.0) | 1.1 (0.7–1.7) | 0.878 |
|           | Normal (n = 81) | 5.0 (3.9–6.1) | 14.5 (9.6–19.4) | 0.013 |
| AREG expression | n = 106 Downregulated (n = 58) | 3.8 (2.7–4.9) | 1.7 (1.1–3.2) | 0.018 | 10.7 (9.5–11.9) | 1.7 (1.1–2.6) | 0.013 |
|           | Overexpressed (n = 48) | 5.0 (3.9–6.1) | 20.2 (12.8–27.6) | 0.0001 |
| EREG expression | n = 105 Downregulated (n = 54) | 6.1 (3.9–8.3) | 2.1 (1.3–3.1) | 0.002 | 10.7 (9.3–11.9) | 1.8 (1.2–2.8) | 0.004 |
|           | Overexpressed (n = 51) | 3.6 (2.0–5.3) | 17.6 (12.6–22.7) | 0.0001 |
| Skin rash | n = 112 None (n = 24) | 2.3 (1.9–2.7) | 5.1 (2.9–9.1) | <0.0001 | 4.9 (2.8–6.9) | 5.3 (3.0–9.4) | <0.0001 |
|           | Grade 1 (n = 40) | 4.5 (3.3–5.7) | 2.5 (1.5–4.0) | <0.0001 | 13.2 (8.9–17.5) | 2.2 (1.4–3.7) | <0.0001 |
|           | Grade 2–3 (n = 48) | 7.5 (6.0–9.0) | 24.1 (21.4–26.7) | 0.001 |
| KRAS - BRAF -AREG genotype | KRAS or BRAF mutant AREG downregulated (n = 25) | 2.3 (1.8–2.9) | 7.0 (3.8–12.9) | <0.0001 | 9.9 (6.1–13.7) | 3.1 (2.1–3.6) | 0.001 |
|           | KRAS or BRAF WT and EREG overexpressed (n = 7) | 1.5 (1.2–2.0) | 13.6 (9.7–17.5) | 0.0001 |
|           | KRAS or BRAF WT and EREG overexpressed (n = 33) | 4.6 (3.8–5.4) | 2.5 (1.5–4.2) | <0.0001 | 10.2 (8.8–11.6) | 2.0 (1.1–3.8) | 0.019 |
|           | KRAS or BRAF WT and EREG overexpressed (n = 34) | 9.9 (7.6–12.2) | 23.3 (21.3–25.2) | 0.0001 |
| KRAS - BRAF -AREG genotype | KRAS or BRAF mutant EREG downregulated (n = 19) | 2.2 (1.9–2.5) | 16.8 (11.8–31.4) | <0.0001 | 9.2 (3.2–15.1) | 3.5 (2.5–4.4) | <0.0001 |
|           | KRAS or BRAF WT and EREG overexpressed (n = 17) | 3.5 (2.4–4.6) | 6.8 (3.4–13.8) | <0.0001 | 10.1 (5.6–14.7) | 2.2 (1.2–3.9) | 0.013 |
|           | KRAS or BRAF WT and EREG overexpressed (n = 35) | 5.0 (4.3–5.8) | 2.6 (1.5–4.3) | <0.0001 | 10.2 (9.1–11.3) | 2.1 (1.1–3.8) | 0.015 |
| KRAS - BRAF -AREG genotype | KRAS or BRAF WT and EREG overexpressed (n = 34) | 8.2 (5.3–11.1) | 23.2 (17.8–28.7) | 0.0001 |

*95% CI: Confidene Interval, \( ^{\mathrm{HR}} \): Hazard Ratio, WT: Wild Type.*

\( ^{\dagger} \): Skin rash grade 2–3 vs. grade 1, \( ^{\ddagger} \): Skin rash grade 2–3 vs. none, \( ^{\mathcal{B}} \): Skin rash grade 2–3 vs. grade 1, \( ^{\mathcal{C}} \): KRAS or BRAF WT and EREG overexpressed vs. KRAS or BRAF mutant and EREG downregulated, \( ^{\mathcal{D}} \): KRAS or BRAF WT and EREG overexpressed vs. KRAS or BRAF mutant and EREG downregulated, \( ^{\mathcal{D}} \): KRAS or BRAF or EREG overexpressed vs. KRAS or BRAF WT and EREG downregulated, \( ^{\mathcal{D}} \): doi:10.1371/journal.pone.0015980.t003
the development of moderate severe skin rash ($p<0.0001$). Finally, tumor differentiation (undifferentiated tumors) was significantly correlated with decreased median OS (Hazard Ratio: 1.9; $p=0.003$).

In the multivariate analysis, KRAS (HR 4.3, $p<0.0001$), BRAF (HR 5.1, $p<0.0001$) mutation and low EREG mRNA expression (HR 1.6, $p=0.021$) emerged as independent factors associated with reduced TTP. Furthermore, the absence of severe and moderate (grade 2–3) skin rash emerged as well, as an independent prognostic factor for decreased TTP (HR 4.0, $p<0.0001$) (Table 5). In addition, KRAS (HR 2.9, $p=0.01$), BRAF (HR 3.0, $p=0.001$) mutation and low EREG mRNA expression (HR 1.7, $p=0.021$) emerged as independent factors associated with reduced OS. In addition, tumor differentiation grade 3 emerged, as well, as an independent prognostic factors for reduced OS (HR 2.2, $p=0.001$). Furthermore, the absence of severe and moderate (grade 2–3) skin rash emerged as an independent prognostic factor for decreased OS (HR 3.7, $p<0.0001$, respectively) (Table 5).

**Discussion**

Following the discovery of KRAS mutations in association with anti-EGFR moAbs resistance, the KRAS mutational characterization of mCRC tumours is, currently, preformed in routine basis before any treatment decision. Although the presence of KRAS mutations is a specific predictive biomarker for lack of anti-EGFR moAbs efficacy [6-9,14,37] there is convincing evidence that additional genetic events are involved in this process, since approximately half of the KRAS wt patients are resistant to such a treatment [30]. In addition, several biomarkers have been proposed in association with KRAS mutations as predictive markers for the efficacy of the anti-EGFR moAbs including BRAF [19,22] or PIK3CA mutations [21], EGFR ligands overexpression [23,27], PTEN protein expression [28] and EGFR copy numbers [10,11]. In the current study we evaluated the predictive significance of other common mutations observed in CRC in conjunction with PTEN protein expression and EGFR ligands (EREG and AREG) mRNA expression as well as the impact of skin rash in a cohort of patients with mCRC treated with anti-EGFR plus chemotherapy as salvage treatment. To the best of our knowledge this is the first study which combines all these parameters together. Patient’s characteristics, the incidence of mutations and the treatment regimens were all typical for mCRC [22,37]; therefore, the results of our analysis could serve as a useful guide for clinical practice.

The data presented here are consistent with previous reports demonstrating that KRAS and BRAF mutations are mutually exclusive; the prevalence of BRAF mutations (7.2%) is, practically, similar with that reported in other patients’ series from a first-line setting [39], but higher than that described in heavily pre-treated colorectal cancer patients [21,37], indicating that its prognostic significance mainly depends on the studied patients’ population. The presence of BRAF mutations has been correlated with resistance to anti-EGFR moAbs treatment [19,22,34]. In accordance with these previous reports, in the current study we also observed that patients with tumours that harboured BRAF mutations had a significantly worse TTP and shorter OS compared to BRAF wt tumours. Furthermore, in our series of tumours, a statistically significant correlation was observed between BRAF mutations and the undifferentiated histological grade reflecting that this mutation seems to characterize a subgroup of patients with poor prognosis since they carry a significant higher risk of progression and death due to disease.

Biomarkers to Anti-EGFR moABS in mCRC

**Table 4. TTP and OS to the ≥2nd line cetuximab-containing treatment according to KRAS, BRAF, PIK3CA mutations status, PTEN protein expression, AREG and EREG mRNA expression and grade of skin rash in the KRAS WT patients’ population.**

| Feature | Patients’ population (No of patients) | Time to Tumor Progression (months) | Overall survival (months) |
|---------|--------------------------------------|----------------------------------|--------------------------|
|         |                                      | 6.4 months (95% CI 5.4–7.4)       | 16.3 months (95% CI 12.7–19.6) |
| KRAS WT | n = 75                               | Median (months) (95% CI)         | Median (months) (95% CI) |
|         |                                      | HR<sup>1</sup> (95% CI)         | HR<sup>1</sup> (95% CI) |
|         |                                      | $p$ value                        | $p$ value |
| BRAF status | n = 75 Mutant (n = 8) | 2.1 (0.2–3.4) | 9.5 (3.9–23.3) | <0.0001 | 4.3 (0.2–10.3) | 4.6 (2.1–10.0) | <0.0001 |
|          | WT (n = 67)                           | 6.4 (5.3–7.5)                   |                           | 163 (13.6–19.1) |       |
| PIK3CA status | n = 75 Mutant (n = 8) | 4.3(2.3–6.2) | 3.3 (1.4–7.7) | 0.01 | 13.5 (4.9–18.8) | 1.5 (0.8–3.3) | 0.345 |
|          | WT (n = 67)                           | 6.4 (5.3–7.4)                   |                           | 163 (4.9–18.8) |       |
| PTEN expression | n = 74 Loss (n = 14) | 3.7 (2.9–4.5) | 2.7 (1.4–5.1) | 0.002 | 15.3 (6.2–22.8) | 1.1 (0.7–2.0) | 0.862 |
|          | Preserved (n = 60)                    | 5.0 (4.0–6.0)                   |                           | 14.5 (11.8–21.3) |       |
| PIK3CA-PTEN axis | n = 74 Activated (n = 17) | 3.8 (2.4–5.2) | 2.9 (1.6–5.3) | 0.001 | 13.9 (11.0–18.9) | 1.1 (0.7–1.8) | 0.987 |
|          | Normal (n = 57)                       | 6.4 (5.7–7.0)                   |                           | 162 (13.3–19.1) |       |
| AREG expression | n = 75 Downregulated (n = 39) | 4.3 (2.8–5.7) | 2.0 (1.3–2.5) | 0.021 | 10.7 (11.9–18.2) | 2.2 (1.3–3.8) | 0.004 |
|          | Overexpressed (n = 36)                | 5.8 (4.0–7.6)                   |                           | 23.2 (18.5–27.9) |       |
| EREG expression | n = 75 Downregulated (n = 39) | 3.8 (1.6–5.9) | 2.3 (1.4–3.9) | 0.001 | 10.5 (9.4–11.6) | 2.9 (1.7–5.0) | <0.0001 |
|          | Overexpressed (n = 36)                | 7.0 (4.8–9.2)                   |                           | 20.2 (13.4–27.0) |       |

*CI: Confidence Interval, 
<sup>1</sup>HR: Hazard Ratio, 
<sup>2</sup>WT: Wild Type, 
<sup>3</sup>Skin rash grade 2–3 vs. none, 
<sup>4</sup>Skin rash grade 2–3 vs. grade 1, 
<sup>5</sup>KRAS or BRAF WT and EREG overexpressed vs. KRAS or BRAF mutant and EREG downregulated, 
<sup>6</sup>KRAS or BRAF WT and EREG overexpressed vs. KRAS or BRAF WT and EREG expressed, 
<sup>7</sup>KRAS or BRAF WT and EREG overexpressed vs. KRAS or BRAF WT EREG downregulated.

doi:10.1371/journal.pone.0015980.0004
Figure 2. Patients’ outcome according to KRAS mutational status. Panel A: Time to Tumor Progression (TTP) Panel B: Median Overall Survival (OS).

doi:10.1371/journal.pone.0015980.g002

Mutations in PIK3CA and PTEN protein expression loss have also been suggested as biomarkers of anti-EGFR mAbs resistance. The role of PIK3CA mutational status on the anti-EGFR mutational status is conflicting. In the current study, PIK3CA mutations were identified in 11 tumors (9.8%) and, more especially, in exon 9 than in exon 20; this observation is in contrast with that observed in the Sartore-Bianchi’s et al [21] cohort but in agreement with that reported by Prenen et al [26]. A significant negative correlation between PIK3CA mutations and response to anti-EGFR mAbs has been documented in the Sartore-Bianchi’s et al [21] and the Perone’s et al [30] reports, whereas, Prenen et al [26] could not find a clear association between the presence of PIK3CA mutation status and an impaired efficacy of anti-EGFR mAbs. Our data demonstrate that there was no significant correlation between the TTP and OS and the PIK3CA mutational status when the analysis was performed in the whole group of patients; however, when only KRAS wt patients were analyzed, PIK3CA mutational status was correlated with a significantly lower TTP. Nevertheless, this lower TTP could not be translated into differences in OS between wt KRAS patients with mutant and wt PIK3CA alleles in their primary tumours, as previously described by our group [22]. In a very recent study by De Roock et al [40], where a large cohort of patients has been evaluated, the role of PIK3CA mutational status has been more clearly revealed. Exon 9 and exon 20 PIK3CA mutations were able to be analyzed separately and, indeed, only exon 20 mutations were found to be associated with a worse outcome after cetuximab administration. This seems to be a possible explanation for the reported conflicting results published in the literature, since there could be more than one interpretation when two events (exon 9 and exon 20 mutations) have different and opposite effects. However, the lack of efficacy of EGFR mAbs which is observed in patients with mutant KRAS extends to other common mutations that deregulate the cellular signaling pathway, especially BRAF and, probably, PIK3CA [41].

The role of PTEN loss and consecutive over-activation of the AKT pathway and its evaluation is still under investigation, as far as response to anti-EGFR mAbs is concerned. Five relatively small, retrospective studies [26,28–30] have provided evidence that PTEN status is associated with objective responses in cetuximab-treated mCRC patients suggesting that PTEN-positive tumours tend to have a better outcome than negative ones; however, another study failed to confirm this observation [21]. This probably could be due to several methodological differences such as the used anti-PTEN antibodies, the IHC scoring algorithms and cut-off criteria [31,42]. In the present study, the significantly lower TTP which was observed in patients with wt KRAS and PIK3CA according to the down- and up-regulation of PTEN could not be translated into differences in OS. Nevertheless, since PTEN IHC is not yet adequately validated, it cannot be considered for immediate routine clinical use, but, it should be kept in mind in the planning process of prospective biomarkers studies.

EGFR ligands AREG and EREG were quite recently found by biomarker exploratory analysis using Affimetrix to be the top genes associated with efficacy to anti-EGFR mAbs [27]. In the group of patients with wt KRAS we found a statistically significant correlation of AREG and especially EREG mRNA overexpression with increased TTP and OS in accordance with previous reports [23]. Our data also seem to identify a subgroup of KRAS wt patients who could be considered to more EGFR-dependent and, thus, have a higher probability of responding to EGFR inhibition as already previously has been reported [23]. Patients whose tumours were characterized by ligands’ downregulation behaved like KRAS mutants upon treatment with anti-EGFR mAbs.

The most frequently reported side effect of EGFR inhibitors is a dose-dependent acneiform skin rash occurring in more than 50% of patients [42]. A number of studies have suggested that from a clinical point of view, the severity of skin rash is positively correlated with clinical outcome (response rates, progression free survival and OS) and, thus, it could be used in order to distinguish mCRC patients more likely to be sensitive to anti-EGFR treatment [2,32,42]. Particularly, the analysis of the PRIME trial showed that the patients with KRAS mutated tumours and moderate or severe skin rash presented better outcome in comparison with those with KRAS wt tumours and no or mild skin rash [32]. In our study as well, mCRC patients with severe and moderate skin rash presented significantly higher TTP and OS compared with those with mild and no rash. Indeed, in the multivariate analysis the absence of severe and moderate (grade 3 and 2) skin rash formation emerged as an independent predictive factor for reduced TTP and OS. Although skin toxicity seems to be an important clinical surrogate marker of anti-EGFR mAbs
Figure 3. Patients’ outcome according to BRAF mutations status. Panel A: Time to Tumor Progression (TTP) in the whole patients’ population. Panel B: Median Overall Survival (OS) in the whole patients’ population. Panel C: Time to Tumor Progression (TTP) in patients with KRAS wt primary tumors. Panel D: Median Overall Survival (OS) in patients with KRAS wt primary tumors. doi:10.1371/journal.pone.0015980.g003

Figure 4. Time to Tumor Progression (TTP) according to PIK3CA mutations status and PTEN expression. Panel A: according to PIK3CA mutations status in the whole patients’ population. Panel B: according to PTEN expression in the whole patients’ population. Panel C: according to PIK3-PTEN axis activation status (PIK3CA mutations status and PTEN expression) in the whole patients’ population. Panel D: according to PIK3CA mutations status in patients with KRAS wt primary tumors. Panel E: according to PTEN expression in patients with KRAS wt primary tumors. Panel F: according to PIK3-PTEN axis activation status (PIK3CA mutations status and PTEN expression) in patients with KRAS wt primary tumors. doi:10.1371/journal.pone.0015980.g004
Figure 5. Median Overall Survival (OS) according to PIK3CA mutations status and PTEN expression. Panel A: according to PIK3CA mutations status in the whole patients' population. Panel B: according to PTEN expression in the whole patients' population. Panel C: according to PIK3-PTEN axis activation status (PIK3CA mutations status and PTEN expression) in the whole patients' population. Panel D: according to PIK3CA mutations status in patients with KRAS wt primary tumors. Panel E: according to PTEN expression in patients with KRAS wt primary tumors. Panel F: according to PIK3-PTEN axis activation status (PIK3CA mutations status and PTEN expression) in patients with KRAS wt primary tumors. doi:10.1371/journal.pone.0015980.g005

Figure 6. Patients' outcome according to AREG mRNA expression. Panel A: Time to Tumor Progression (TTP) in the whole patients' population. Panel B: Median Overall Survival (OS) in the whole patients' population. Panel C: Time to Tumor Progression (TTP) in patients with KRAS wt primary tumors. Panel D: Median Overall Survival (OS) in patients with KRAS wt primary tumors. doi:10.1371/journal.pone.0015980.g006
Figure 7. Patients’ outcome according to EREG mRNA expression. Panel A: Time to Tumor Progression (TTP) in the whole patients’ population. Panel B: Median Overall Survival (OS) in the whole patients’ population. Panel C: Time to Tumor Progression (TTP) in patients with KRAS wt primary tumors. Panel D: Median Overall Survival (OS) in patients with KRAS wt primary tumors.
doi:10.1371/journal.pone.0015980.g007

Figure 8. Patients’ outcome according to KRAS-BRAF mutations status and AREG or EREG mRNA expression. Panel A: Time to Tumor Progression (TTP) according to KRAS-BRAF mutations status and AREG mRNA expression. Panel B: Median Overall Survival (OS) according to KRAS-BRAF mutations status and AREG mRNA expression. Panel C: Time to Tumor Progression (TTP) according to KRAS-BRAF mutations status and EREG mRNA. Panel D: Median Overall Survival (OS) according to KRAS-BRAF mutations status and EREG mRNA.
doi:10.1371/journal.pone.0015980.g008
efficacy, the biological correlation is still unknown and the elucidation of the biologic mechanisms will be of great value.

The multivariate analysis revealed that the presence of KRAS or BRAF mutations and EREG downregulation are the only biomarkers which are independent prognostic factors for decreased TTP and OS. In a recently published study, the mutational analysis of KRAS, BRAF, NRAS and PIK3CA exon 20, in that specific order, has been proposed as the most effective approach [40]. The common finding between the two studies is that multigene models seem to be more effective than single-gene analysis for the selection of patients who could gain the maximum benefit from the administration of anti-EGFR moAbs. The important issue of cost for the molecular analysis and the limited amount of tumour cells available in FFPE specimens for all potential biomarkers testing could be tackled with the development of multiplex assays [43]. Furthermore, the severity of skin rash during the treatment with anti-EGFR moAbs has been constantly reported as a predictive factor for response and survival [2,16], and this was also the case in the present study, since the severity of skin rash was an independent predictive factor for TTP.

Figure 9. Patients’ outcome according to severity of skin rash during the cetuximab administration. Panel A: Time to Tumor Progression (TTP) according to the worst skin rash grade developed during the treatment with cetuximab + chemotherapy. Panel B: Median Overall Survival (OS) according to the worst skin rash grade developed during the treatment with cetuximab + chemotherapy.

doi:10.1371/journal.pone.0015980.g009
and OS. The biologic mechanism which links the development of severe skin rash and tumor response is not yet elucidated, and very few data are published regarding this issue [44].

In summary, the genetics underpinnings of CRC are established [45] and the results of the present study support the idea that advanced application of CRC genetic profiling could lead to informed treatment decisions. Despite the fact that the results of a retrospective study should be interpreted with caution, it seems that the determination of the KRAS-BRAF mutational status, with additional screening of CRC tumours for their EREG mRNA expression, could help stratify patients likely to benefit from a regimen containing an anti-EGFR moAb. Studies which focus in the elucidation of the mechanism which links the development of skin rash with tumors response are urgently warranted. Nevertheless, since most available data come from retrospective studies, validation in prospective randomized clinical trials is imperative in order to formally confirm the predictive and prognostic value of these biomarkers.

**Author Contributions**

Conceived and designed the experiments: ZS JS. Performed the experiments: ZS MZ CP MS AK ET MT IM. Analyzed the data: ZS MZ. Wrote the paper: ZS JS. Critically revised article: MT ES ZS MZ CP JS. Final approval of the version to be published: ZS VG JS.

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