Polymorphisms in genes involved in EGFR-turnover are predictive for cetuximab efficacy in colorectal cancer

Sebastian Stintzing1,2, Wu Zhang1, Volker Heinemann2,3, Daniel Neureiter4, Ralf Kemmerling4,5, Thomas Kirchner3,8, Andreas Jung6, Matthias Folwaczny7, Dongyun Yang8, Yan Ning1, Ana Sebio1,9, Stefan Stremitzer1, Yu Sunakawa1, Satoshi Matsusaka1, Shinichi Yamauchi1,10, Fotios Loupakis11, Chiara Cremolini11, Alfredo Falcone11, Heinz-Josef Lenz1,8

Corresponding Author:
Sebastian Stintzing, MD
Department of Hematology and Oncology, University of Munich, Marchioninistrasse 15, 81377 Munich, Germany,
P: +49 89 4400 752208; F: +49 89 4400 75256;
E-mail: sebastian.stintzing@med.uni-muenchen.de

Authors’ Affiliations:

1. USC/Norris Comprehensive Cancer Center, Keck School of Medicine, Sharon Carpenter Laboratory
2. Department for Medical Oncology and Comprehensive Cancer Center, University of Munich, Munich, Germany
3. German Cancer Consortium (DKTK); German Cancer Research Centre (DKFZ), Heidelberg, Germany
4. Institute of Pathology, SALK and Medical University of Salzburg, Austria
5. Medizinisches Versorgungszentrum für Histologie, Zytologie und Molekulare Diagnostik, Trier, Germany
6. Institute of Pathology, University of Munich, Munich, Germany
7. Department of Preventive Dentistry and Periodontology, University of Munich, Munich, Germany
8. Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California
9. Hospital de la Santa Creu i Sant Pau, Barcelona, Spain
10. Graduate School, Tokyo Medical and Dental University, Tokyo Japan
11. U.O. Oncologia Medica 2 – Aziende Ospedaliero-Universitaria Pisana, Pisa, Italy
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Abstract

Transmembrane receptors such as the epidermal growth factor receptor (EGFR) are regulated by their turnover, which is dependent on the ubiquitin-proteasome-system (UPS). We tested in two independent study cohorts whether single nucleotide polymorphisms (SNPs) in genes involved in EGFR turnover predict clinical outcome in cetuximab treated metastatic colorectal cancer patients. The following SNPs involved in EGFR degradation were analyzed in a screening cohort of 108 patients treated with cetuximab in the chemorefractory setting: c-CBL (rs7105971; rs4938637; rs4938638; rs251837), EPS15 (rs17567; rs7308; rs1065754), NAE1 (rs363169; rs363170; rs363172), SH3KBP1 (rs7051590; rs5955820; rs1017874; rs11795873); SGIP1 (rs604737; rs6570808; rs7526812); UBE2M (rs895364; rs895374); UBE2L3 (rs5754216). SNPs showing an association with response or survival were analyzed in BRAF and RAS wild-type samples from the FIRE-3 study. 153 FOLFIRI plus cetuximab treated patients served as validation set, 168 patients of the FOLFIRI plus bevacizumab arm served as controls. EGFR FISH was done in 138 samples to test whether significant SNPs were associated with EGFR expression. UBE2M rs895374 was significantly associated with PFS (logrank-p = 0.005; HR 0.60) within cetuximab treated patients. No association with bevacizumab treated patients (n=168) could be established (p= 0.56, HR: 0.90). rs895374 genotype did not affect EGFR FISH measurements. EGFR recycling is an interesting mechanism of secondary resistance to cetuximab in mCRC. This is the first report suggesting that germline polymorphisms in the degradation process predict efficacy of cetuximab in patients with mCRC. Genes involved in EGFR turnover may be new targets in the treatment of mCRC.
Introduction

Metastatic colorectal cancer (mCRC) is one of the most frequent malignancies in the Western world with an estimated 136,830 new cases and an estimated 50,130 deaths in the US in 2014 (1). Despite the progress that has been achieved in terms of prolonging median overall survival times during the last two decades, biomarker-driven targeted treatment is still limited to the anti-epidermal growth factor receptor (EGFR) antibodies cetuximab and panitumumab. The only established biomarker in the treatment of mCRC is the negative predictive biomarker represented by the RAS (rat sarcoma) mutations (2-5). In first-line treatment the combination of standard doublet chemotherapy with anti-EGFR antibodies results in response rates of 60-70%. In single agent trials in further-line treatment, anti-EGFR antibodies have a response rate of 20-30%. But even in first-line treatment almost one third of the tumors do not respond to treatment, and ultimately most tumors develop resistance. Therefore, other mechanisms of anti-EGFR-resistance needs to be identified. Receptor protein turnover regulated by the ubiquitin-proteasome-system (UPS) is a potential mechanism of resistance (6).

Receptor dependent signaling relies on the quantity of ligands binding to the respective receptors, the amount of receptors on the cell surface and the presence of activating mutations in the intracellular signal pathway (7). To predict EGFR-antibody resistance beyond RAS and BRAF mutations in mCRC the expression levels of the ligands amphiregulin and epiregulin (8), and the receptor gene copy number (9) appeared to be of predictive value. But methodological problems to standardize intra-tumor protein measurements are unresolved. SNPs in contrast are offering a reliable and easy to access possibility of biomarker determination. Genomic DNA of white blood cells can be collected by standard blood draw and tumor and genomic SNP genotypes are equivalent (10). The amount of EGFR receptors on the cell surface available for ligand binding is also regulated by processes of receptor turnover as antibody binding to the EGFR induces receptor internalization (11). Receptor turnover has not been can be divided into three steps: (i) receptor internalization, (ii) ubiquitination and (iii) recycling, or degradation (12). In mCRC, those
processes are important for both, VEGFR (13) and EGFR (14) signaling. In short, after activation of the receptor tyrosine kinase through ligand binding and dimerization, the activated receptor is internalized by clathrin dependent endocytosis and ubiquitinated. This terminates the tyrosine kinase activity of the activated EGFR and regulates the amount of receptors expressed on the cell-surface. The final step of degradation is done by the proteasome, however ubiquitinated receptors can be de-ubiquitinated by desubiquitinases and are then recycled towards the cell membrane (15). Three classes of enzymes (E1-E3) are needed for poly-ubiquitination. E1 enzymes are responsible for activating ubiquitin, E2 enzymes are transferring the activated ubiquitin towards the protein structure and E3 enzymes are ligases that are able to add ubiquitin to each other. Whereas there are only some dozens of E1 and E2 enzymes, some thousands of E3 ligases are known and responsible for the substrate specific sorting (Figure 1). For the EGF-receptor ubiquitination the most important E3 ligase is c-CBL (casitas B-lineage lymphoma). C-CBL belongs to the class of cullin-RING ligases that are regulated by the addition of NEDD8 (neddylation) (16). N neddylation is driving the turnover of EGFR towards degradation by accelerating poly-ubiquitination through c-CBL (17).

The following enzymes play important roles in EGFR turnover and were selected for the following study: The activated receptor is internalized by the help of c-CBL (Casitas B-lineage Lymphoma), c-CBL interacting protein of 85kDa (CIN85) and endophilin. After being transferred into an early endosome, ubiquitination starts with the activation of ubiquitin by ubiquitin activating enzyme (UAE1), which is then transferred to the receptor by the help of ubiquitin conjugating enzyme (UbcH7). Poly-ubiquitination is done by the help of c-CBL. Epidermal growth factor receptor pathway substrate 15 (EPS15) is supporting the ligation process (18). UBC12 (NEDD8-conjugating enzyme) neddylates c-CBL by transferring activated NEDD8 to the cullin-RING motif of c-CBL (19, 20). To become functional NEDD8 needs be activated by NAE1 (neddylation activating enzyme 1).
This analysis aimed to (i) define subgroups of patients by single nucleotide polymorphisms (SNP) within genes of EGFR turnover being predictive for cetuximab efficacy and to (ii) test the predictive or prognostic value in a control cohort not treated with cetuximab.

**Material and methods:**

**Patients and Material:**

SNPs with a minor allele frequency of more than 10% in genes involved in EGFR degradation were evaluated for association with overall response rate, progression-free survival (PFS) and overall survival (OS). Multivariate testing was adjusted for gender, age, primary tumor location and treatment regimen. The training set consisted of patients from two phase II trials with histologically confirmed, KRAS exon 2 and BRAF wild-type metastatic adenocarcinoma of the colon and rectum (21, 22). Patients of the screening cohort were treated with irinotecan plus cetuximab (21) or cetuximab mono-therapy (22) in further-line treatment of mCRC. SNPs with significant data in multivariate analysis in the screening cohort were tested prospectively in 321 FIRE-3 BRAF and RAS (exon 2, 3, 4) wild-type specimens. Patients treated with FOLFIRI plus cetuximab (n=153) served as validation cohort and FOLFIRI plus bevacizumab (n= 168) treated patients were used as negative control.

Patients within the training set were either treated with FOLFIRI plus cetuximab (180 mg/m² of body-surface area (BSA) irinotecan; 400 mg/m² BSA leucovorin; 400 mg/m² BSA 5- fluorouracil as an intravenous bolus followed by a continuous infusion over 46 hours of 2400 mg/m² BSA) and cetuximab (400mg/m² BSA initial dose followed by a weekly dose of 250mg/m² BSA) (21) or cetuximab single agent (400mg/m² BSA initial dose followed by a weekly dose of 250mg/m² BSA) (22).

Patients within the FIRE-3 trial were treated with FOLFIRI (180 mg/m² of BSA irinotecan; 400 mg/m² BSA leucovorin; 400 mg/m² BSA 5- fluorouracil as an intravenous bolus followed by a
continuous infusion over 46 hours of 2400 mg/m² BSA) and either bevacizumab (5mg/kg) or cetuximab (400mg/m² BSA initial dose followed by a weekly dose of 250mg/m² BSA) (5).

All patients had given their written informed consent.

Methods:

Formalin-fixed, paraffin embedded tissue samples or venous blood samples were used to extract genomic DNA. DNA extraction was carried out using the QIAamp® DNA easy kit (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. DNA was stored at -20°C until use.

Common and potentially functional polymorphisms within the genes being crucial for EGFR turnover regulation were selected using the Ensemble database (www.ensemble.org). If no functional SNPS were identified within targeted genes, tagging (Tag) SNPs were selected by SNPinfo (http://snpinfo.niehs.nih.gov/snpinfo/snptag.htm). The following criteria were used to select the candidate gene polymorphisms: (a) a minor allele frequency (MAF) ≥10%; (b) located in the 3´UTR, 5´UTR or coding regions of the tested genes and/or were shown to be of biological significance according to the location within the gene or according to literature review.

The tested SNPs for EGFR turnover and their forward and reverse primers used for PCR amplification are shown in table 1.

PCR products were analyzed using direct sequencing. The investigator (SS) reading the sequence was blinded to the clinical results.

EGFR FISH analysis was done in 138 FFPE samples according to a standard protocol of the Institute of Pathology, University of Munich. In short, after dewaxing, rehydration, and a pepsin digestion, a commercially available test kit (Zytomed, Berlin, Germany) was used following the manufacturers recommendations. Expression analysis was done by two pathologists (DN, RK).

In 100 cells in each sample signals of EGFR and the corresponding chromosome 7 were counted and were set in relation with each other.
Statistics:

Treatment efficacy was measured by objective response rate (ORR), progression-free (PFS) and overall survival (OS) in both cohorts. Survival times were measured from start of chemotherapy until progression or death. ORR was assed using response criteria in solid tumor (RECIST) as assessed by the referring physician.

Allelic distribution of all polymorphisms was tested for deviation from Hardy-Weinberg equilibrium using \( \chi^2 \)-test with 1 degree of freedom. To evaluate the prognostic value of the polymorphisms on endpoints, the associations were examined using by Kaplan-Meier curve methods and tested by the log-rank test. The Cox proportional hazards regression model with stratification factors were fitted to re-evaluate the association between polymorphisms and outcomes considering the imbalanced in the distributions of baseline patient characters in both cohorts. The baseline demographic and clinical markers that remained significantly associated with endpoints in the multivariable analyses (p<0.1) were included in the final model.

With 108 patients (98 and 73 events for PFS and OS, respectively) in the screening cohort we would have 80% power to detect a minimum hazard ratio of 1.79-2.17 and 1.94-2.32 on PFS and OS, respectively, for a SNP with a range of the variant allele frequencies (0.1–0.5) in a dominant model using a 0.05 level two-sided log-rank test. The validation set consisted of 153 patients (126 and 81 events for PFS and OS, respectively) would give us 80% chances to find the SNPs with hazard ratio of 1.71-2.03 for PFS and 1.94-2.25 for OS using the same test.

As a control arm 168 patients (138 and 105 events for PFS and OS, respectively) not treated with cetuximab were used. With this number we would have 80% power to detect a minimum hazard ratio of 1.69-2.00 and 1.82-2.12 in PFS and OS, respectively for a SNP across a range of the variant allele frequencies (0.1–0.5) in a dominant model using the same test.

Expression levels were compared using nonparametric Kruskal-Wallis test.
The level of significance was set to 0.05, and all statistical tests were two-sided and performed using the SAS statistical package version 9.3 (SAS Institute, Cary, NC, USA). Linkage disequilibrium was tested for significant SNPs.

Results

Baseline characteristics and primary outcome measurements of both cohorts have been published before (5, 21, 22) and are summarized in table 2.

All tested SNPs were within the Hardy-Weinberg equilibrium and close to the expected minor allele frequencies for the Caucasian population. For quality control purposes a random selection of 10% of the samples was re-examined for each polymorphisms and genotype concordance rate was 100%.

Within the screening set of 108 patients only rs895374 a SNP in the UBE2M gene encoding for the NEDD8 conjugating (E2) enzyme was able to separate significantly (p= 0.02) different patient groups. Another four SNPs (rs379464, rs7051590, rs2511837, and rs17567) showed a trend towards a possible selection and were selected to be tested on FIRE-3 samples. The results are shown in detail in table 3A and 3B.

Rs895374 polymorphisms defined different patient subgroups for PFS with patients being homozygous for the major allele C reaching 7.9 months and 12.3 months for those bearing the minor allele A (logrank test p= 0.005; HR 0.60 (Figure 2A)). The median OS was not different reaching 38.3 months and 33.5 months respectively (logrank test p= 0.85; HR 0.96). After adjusted for sex, age, ECOG performance status, number of metastatic sites, and acneiform exanthema, using Cox regression analysis, PFS was still significantly in favor of the minor allele A (p= 0.002; HR 0.54). For OS even after multivariate adjustment, no difference for the different genotypes could be established (p=0.50, HR 0.78).
To distinguish whether this value is prognostic or predictive, the same SNP was tested in 168 bevacizumab treated patients. It was not able to separate between the patients treated with bevacizumab PFS was 12.4 months and 10.5 months (logrank test p= 0.56, HR= 0.90) (Figure 2B) and median OS was 28.7 months and 25.4 months respectively (logrank test p=0.53, HR= 0.88).

Within both arms of FIRE-3 no difference could be established regarding the overall response rate, reaching 78.3% for CA and AA and 76.6% for CC in the cetuximab (two sided Fisher’s exact p =0.83) and 63.4% and 71.7% in the bevacizumab arm (two sided Fisher’s exact p= 0.38) respectively.

All associations between UB2M rs895374 and clinical outcomes in first-line FOLFIRI combination with either cetuximab or bevacizumab in FIRE-3 are shown in supplemental table 1.

To test whether rs895374 genotypes are associated with EGFR gene copy number, EGFR FISH analysis was carried out. EFGR FISH readout was possible in 126 of 138 (91%) of all samples. EGFR FISH expression in relation to chromosome 7 was in median 1.022 (95% CI 1.006 – 1.038) and was comparable for the rs895374 genotypes (Kruskal-Wallis test p= 0.09). Data of the association of EGFR FISH and response or survival is not shown.

There was no association between the rs895374 genotype and the occurrence of acneiform exanthema (p= 0.48) (supplemental table 2).
Discussion:
This is the first report of SNPs in genes involved in EGFR turnover to be analyzed for their predictive and prognostic value in the treatment of metastatic colorectal cancer with the anti-EGFR antibody cetuximab. Rs895374 could be validated as a predictive biomarker for progression-free survival in cetuximab treated RAS and BRAF wild-type patients. For bevacizumab treated patients, used as control group, neither a predictive nor a prognostic value could be demonstrated for rs895374. EGFR FISH results were not influenced by rs895374 genotypes in this analysis. The use of anti-EGFR antibodies has been restricted to KRAS and NRAS exon 2, 3 and 4 wild-type tumors (2, 5). But even in the RAS wild-type population, according to RECIST, about 30% of patients do not respond to treatment. Predictive biomarkers for treatment efficacy beyond the RAS mutational analysis would be useful to further personalize therapy (23). Attempts using gene expression analysis for the EGFR ligands amphiregulin and epiregulin (8) or the expression of the EGFR (24, 25) have shown promising results, but methodological issues are, until now, unresolved. SNPs, on the other side, provide an ideal possibility for biomarker development as they are not influenced by the technique used for analysis, are easy to access by venous blood draw and do not change under treatment. Rs895374 was able to predict PFS but not tumor response or overall survival. In other words, tumors with different genotypes of rs895374 initially respond to cetuximab the same way but the duration of response was different between patients bearing any “A” allele as reflected by the significantly different PFS times. This can be interpreted as a sign of developing secondary resistance to the initially successful regimen. EGFR turnover has been recognized as a possible pathway for acquired anti-EGFR resistance (6), but until now, only preclinical models have been published (26, 27). After binding to the extracellular domain of the receptor, EGFR and the respective antibody (cetuximab or panitumumab) are internalized (28). The amount of receptor to be recycled back to the cell surface is important for resistance to cetuximab/panitumumab treatment. Antibody mixtures like the novel
drug Sym004 are known to enhance EGFR degradation (29) and can overcome cetuximab resistance (30).

The ubiquitin-proteasome system (UPS) has been increasingly recognized as a potential pathway for biomarker and drug development. Drugs inhibiting enzymes important for degradation have been developed (31) and inhibitors of neddylation are in early clinical development (32).

Rs895374 is an intergenic SNP located next to UBE2M which is coding for UbcH12 the NEDD8 conjugating enzyme important for neddylation of c-CBL. Neddylation of c-CBL increases the ability of c-CBL to poly-ubiquitinate EGFR and therefore drives the balance of EGFR recycling and degradation towards degradation (17). In colorectal adenocarcinoma the Colon Cancer Atlas Network (33) revealed 5.56% UBE2M mutant tumors, but the function of those mutations remain unclear. Two studies found elevated expression of UBE2M in colon adenocarcinoma when compared to normal tissue (34), but again, relevance with regard to treatment efficacy is unclear. Furthermore, no knockout mouse model has been described so far.

In silico information provided by ENSMBL and REGULOME databases (35, 36) indicates that rs895374 alters transcriptional regulation. The polymorphism is found in a motif modulating the protein binding of the transcription factor CREBB (CREB regulated transcription coactivator 1 binding protein) (37, 38). Furthermore, rs895374 is a cis expression quantitative trait loci (eQTL) for the gene MGC2752 (CENPB DNA-binding domains containing 1 pseudogene) (40, 41). A search of the GEO-Profiles database (42) revealed that MGC2725 is associated with recurrence in UICC II CRC patients linking rs895374 to CRC recurrence.

As an intergenic SNP rs895374 is also upstream CHMP2A (Charged multivesicular body protein 2a) which is a core component of the endosomal sorting required transport complex-III (ESCRT-III). The ESCRT machinery mediates the intracytoplasmatic sorting from endocytic bodies towards lysosomes via multivesicular bodies (MVBs) (43). In particular ESCRT-III is involved in deubiquitination, clathrin-dependent endocytosis of ubiquitinated proteins, and MVB forming. All of which interfere with EGFR turnover and potentially the efficacy of cetuximab.
Although the predictive value of rs895374 could be demonstrated, several limitations have to be stated. Due to missing patient material, we cannot exclude that other mechanisms of secondary resistance such as the outgrowth of low frequent RAS mutant tumor subclones (44), the occurrence of cetuximab specific EGFR mutations (45), or the up-regulation of other growth factor receptor pathways (26) interfere with the value of rs895374. The functional mechanism of rs895374 remains unclear and this NEDD8 SNP needs to be tested in preclinical models to understand the biological relevance. Therefore the predictive value of rs895374 should be prospectively valued taking other factors of secondary resistance into account.

Receptor recycling is an important mechanism of anti-EGFR antibody resistance. EGFR receptor turnover is mediated by the ubiquitin-proteasome-system (UPS) and modulated by the process of neddylation. This study validated for the first time the predictive value of a SNP associated with neddylation for the treatment with cetuximab in two different and independent study populations. No relation to treatment outcome could be revealed in a bevacizumab treated control group. The antibody mixture Sym004 targeting the EGFR may overcome cetuximab resistance by urging EGFR turnover towards degradation. Inhibitors of the UPS and neddylation are already developed and in early clinical development (15). For mCRC the UPS offers multiple targets for drug and biomarker development and should be further evaluated.

**Acknowledgements:**

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References:


Table 1: Analyzed single nucleotide polymorphisms within degradation

<table>
<thead>
<tr>
<th>gene SNP</th>
<th>MAF</th>
<th>allele location</th>
<th>function</th>
<th>forward (f) and reverse (r) primer</th>
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</thead>
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<tr>
<td>Casitas B-lineage Lymphoma (c-CBL)</td>
<td></td>
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<tr>
<td>rs2511837</td>
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<td>C/T downstream</td>
<td>tag SNP</td>
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<td>rs4938637</td>
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<tr>
<td>rs4938638</td>
<td>44%</td>
<td>A/G upstream</td>
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<tr>
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<td>25%</td>
<td>G/A intron</td>
<td>tag SNP</td>
<td>f: TGGTTGGAAATACTTGTTGAATTA</td>
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<tr>
<td>Epidermal growth factor receptor substrate 15 (EPS15)</td>
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<td>rs7308</td>
<td>21%</td>
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<td>rs17567</td>
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<tr>
<td>rs1065754</td>
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<td>NEDD8- activating enzyme (NAE1)</td>
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<td>rs363169</td>
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<td>NEDD8-conjugating enzyme (UBE2M)</td>
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<td>rs895374</td>
<td>39%</td>
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<td>Downstream UBE2M, Upstream CHMP2A</td>
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**Legend:** SNP = single nucleotide polymorphism, A = adenine, G = guanine, T = thymine, C = cytosine; f = forward primer, r = reverse primer, tag SNP = tagging SNP, NEDD8 = ubiquitin like molecule NEDD8, MAF = minor allele frequency.
Table 2: Baseline characteristics and primary outcome measurements of the tested cohorts

<table>
<thead>
<tr>
<th></th>
<th>Training set further-line cetuximab N= 108</th>
<th>FIRE-3 FOLFIRI + cetuximab N= 153</th>
<th>FIRE-3 FOLFIRI + bevacizumab N= 168</th>
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<tr>
<td>female</td>
<td>39.8%</td>
<td>24.8%</td>
<td>30.4%</td>
</tr>
<tr>
<td>male</td>
<td>60.2%</td>
<td>75.2%</td>
<td>69.6%</td>
</tr>
<tr>
<td><strong>ECOG PS (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>62.1%</td>
<td>53.6%</td>
<td>55.4%</td>
</tr>
<tr>
<td>1</td>
<td>37.9%</td>
<td>45.8%</td>
<td>43.5%</td>
</tr>
<tr>
<td>2</td>
<td>0.7%</td>
<td>0.7%</td>
<td>1.2%</td>
</tr>
<tr>
<td><strong>localization</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>midgut</td>
<td>N/A</td>
<td>80.4%</td>
<td>78.5%</td>
</tr>
<tr>
<td>hindgut</td>
<td></td>
<td>3.3%</td>
<td>3.6%</td>
</tr>
<tr>
<td>unknown</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ORR</strong></td>
<td>20%</td>
<td>77.7%</td>
<td>60.7%</td>
</tr>
<tr>
<td><strong>PFS (months) (95% CI)</strong></td>
<td>3.7 (2.8 – 4.6)</td>
<td>10.5 (9.1 – 12.0)</td>
<td>11.3 (10.1 – 12.5)</td>
</tr>
<tr>
<td><strong>OS (months) (95% CI)</strong></td>
<td>10.5 (7.7 – 13.3)</td>
<td>36.4 (27.7 – 45.1)</td>
<td>27.7 (24.4 – 31.0)</td>
</tr>
</tbody>
</table>

**Legend:** ORR = objective response rate; PFS = progression-free survival, OS = overall survival, 95% CI = 95% confidence interval
### Table 3A: Results for FOLFIRI plus cetuximab treated patients (n= 153)

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype (N)</th>
<th>ORR (%)</th>
<th>PFS months</th>
<th>p**</th>
<th>HR</th>
<th>OS months</th>
<th>p**</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs895374</td>
<td>CC (55)</td>
<td>76.6</td>
<td>7.9</td>
<td>0.018</td>
<td>38.3</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC (75)</td>
<td>79.0</td>
<td>12.2</td>
<td>0.62</td>
<td>33.1</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA (23)</td>
<td>76.2</td>
<td>12.7</td>
<td>0.57</td>
<td>49.8</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>rs379464</td>
<td>CC (10)</td>
<td>60.0</td>
<td>7.8</td>
<td>0.59</td>
<td>39.4</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT (45)</td>
<td>90.0</td>
<td>12.2</td>
<td>1.4</td>
<td>33.8</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT (83)</td>
<td>77.3</td>
<td>12.8</td>
<td>1.1</td>
<td>38.4</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>rs7051590</td>
<td>CC (116)</td>
<td>78.4</td>
<td>10.6</td>
<td>0.37</td>
<td>36.4</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CG (7)</td>
<td>71.4</td>
<td>13.3</td>
<td>1.7</td>
<td>41.3</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG (14)</td>
<td>91.7</td>
<td>14.1</td>
<td>1.8</td>
<td>58.5</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>rs2511837</td>
<td>CC (23)</td>
<td>75.0</td>
<td>12.9</td>
<td>0.58</td>
<td>41.0</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT (67)</td>
<td>75.9</td>
<td>11.3</td>
<td>0.80</td>
<td>36.4</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT (86)</td>
<td>86.8</td>
<td>10.6</td>
<td>0.82</td>
<td>33.1</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>rs17567</td>
<td>CC (5)</td>
<td>100</td>
<td>10.4</td>
<td>0.75</td>
<td>18.6</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT (49)</td>
<td>78.0</td>
<td>13.1</td>
<td>1.2</td>
<td>36.4</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT (88)</td>
<td>80.8</td>
<td>10.0</td>
<td>0.88</td>
<td>38.3</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3B: Results for FOLFIRI plus bevacizumab treated patients (n=168)

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype (N)</th>
<th>ORR (%)</th>
<th>PFS months</th>
<th>p**</th>
<th>HR</th>
<th>OS months</th>
<th>p**</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs895374</td>
<td>CC (65)</td>
<td>71.7</td>
<td>10.5</td>
<td>0.84</td>
<td>25.4</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC (79)</td>
<td>59.2</td>
<td>12.5</td>
<td>0.90</td>
<td>28.6</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA (24)</td>
<td>76.7</td>
<td>10.8</td>
<td>0.91</td>
<td>25.0</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>rs379464</td>
<td>CC (6)</td>
<td>80.0</td>
<td>6.9</td>
<td>0.16</td>
<td>13.0</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT (45)</td>
<td>65.0</td>
<td>14.5</td>
<td>1.7</td>
<td>26.1</td>
<td>1.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT (86)</td>
<td>67.1</td>
<td>18.0</td>
<td>1.4</td>
<td>28.9</td>
<td>1.49</td>
<td></td>
</tr>
<tr>
<td>rs7051590</td>
<td>CC (96)</td>
<td>67.4</td>
<td>11.3</td>
<td>0.65</td>
<td>25.9</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CG (9)</td>
<td>77.8</td>
<td>14.9</td>
<td>1.1</td>
<td>nr</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG (21)</td>
<td>73.7</td>
<td>9.8</td>
<td>0.77</td>
<td>30.8</td>
<td>0.22</td>
<td></td>
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<tr>
<td>rs2511837</td>
<td>CC (47)</td>
<td>65.9</td>
<td>11.3</td>
<td>0.41</td>
<td>29.1</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT (48)</td>
<td>69.0</td>
<td>12.4</td>
<td>0.87</td>
<td>24.9</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT (45)</td>
<td>64.3</td>
<td>10.2</td>
<td>0.47</td>
<td>27.5</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>rs17567</td>
<td>CC (6)</td>
<td>50.0</td>
<td>12.8</td>
<td>0.36</td>
<td>27.7</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT (48)</td>
<td>55.8</td>
<td>9.8</td>
<td>0.91</td>
<td>25.0</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT (86)</td>
<td>77.2</td>
<td>11.5</td>
<td>1.3</td>
<td>28.1</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

**Legend:** ORR = objective response rate; PFS = progression-free survival, OS = overall survival, 95% HR= Hazard ratio, p*= CHI-square test p; p**= logrank test p, A= adenine, C= cytosine, G= guanine, T= thymidine, nr = not reached
Figure legends:

Figure 1: Growth factor receptor turnover principle

Legend: EGF = epidermal growth factor; EGFR = epidermal growth factor receptor, P = phosphorylated, c-CBL = casitas B-lineage lymphoma; CIN85 = c-Cbl interacting protein of 85 kDa; UAE1 = ubiquitin activating enzyme, NAE1 = NEDD8 activating enzyme; EPS15 = Epidermal growth factor receptor substrate 15; UB = ubiquitin; N8 = NEDD8, UbcH7 = ubiquitin-conjugating enzyme; Ubc12 = NEDD8-conjugating enzyme

Figure 2: Value of rs895374 in FOLFIRI plus cetuximab and FOLFIRI plus bevacizumab treated patients for progression-free survival and overall survival in FIRE-3

Legend: UBE2M = gene for NEDD8-conjugating enzyme; A= adenine, C = cytosine; PFS = progression-free survival, OS = overall survival, N = number of patients; m = months, 95% CI = 95% confidence interval
Figure 1: Growth factor receptor degradation

EGF

Cell membrane

Recycling

Endocytosis

Early endosome

Ubiquitination

Degradation

Lysosome

NEDDylation
Estimated probability of Progression-free Survival

**FOLFIRI plus Cetuximab**

- **N**
- **median PFS, m (95%CI)**
  - **C/A or A/A**: 98, 12.3 (10.2 – 14.4)
  - **C/C**: 55, 7.9 (5.7 – 10.0)

  *logrank -test p = 0.005
  HR: 0.60 (0.42-0.86)*

**FOLFIRI plus Bevacizumab**

- **N**
- **median PFS, m (95%CI)**
  - **C/A or A/A**: 103, 12.4 (10.5 – 14.3)
  - **C/C**: 65, 10.5 (9.1 – 11.9)

  *logrank -test p = 0.56
  HR: 0.90 (0.64-1.27)*

Estimated probability of Overall Survival

**FOLFIRI plus Cetuximab**

- **N**
- **median OS, m (95%CI)**
  - **C/A or A/A**: 98, 33.5 (23.0 – 43.9)
  - **C/C**: 55, 38.3 (22.6 – 54.0)

  *logrank -test p = 0.85
  HR: 0.96 (0.61-1.51)*

**FOLFIRI plus Bevacizumab**

- **N**
- **median OS, m (95%CI)**
  - **C/A or A/A**: 103, 28.7 (24.4 – 32.9)
  - **C/C**: 65, 25.4 (23.1 – 27.7)

  *logrank -test p = 0.53
  HR: 0.88 (0.59-1.31)*
Polymorphisms in genes involved in EGFR-turnover are predictive for cetuximab efficacy in colorectal cancer

Sebastian Stintzing, Wu Zhang, Volker Heinemann, et al.

Mol Cancer Ther Published OnlineFirst July 23, 2015.

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