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ORIGINAL ARTICLE

# EGFR Protein Expression of KRAS Wild-Type Colorectal Cancer: Predictive Value of the Sidedness for Efficacy of Anti-EGFR Therapy

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**Abstract**
Right- and left-sided colorectal cancers (RSCRC and LSCRC, respectively) are different developmentally, genetically and prognostically. Clinical data also indicate that they respond differently to anti-EGFR therapies. The role of EGFR protein expression in KRAS wild type colorectal cancer is also controversial. Here we have used a cohort of anti-EGFR antibody treated KRAS-wild type colorectal cancer patients ($n = 97$) to analyse the prognostic role of EGFR protein expression in relation to sidedness. In our cohort EGFR copy number, determined by FISH, was not associated with the level of EGFR protein, assessed by immunohistochemistry and measured by H-scoring. There was a significantly higher EGFR H-score detected in RSCRC as compared to LSCRC in primary tumors ($p = 0.04$). Furthermore, in a proportion of cases ($n = 31$) metastatic tissues were also available and their analysis also found a significantly higher EGFR H-score in metastases of RSCRC compared to LSCRC ($p = 0.018$). Kaplan Meyer survival analysis demonstrated that anti-EGFR antibody therapies were more effective in case of LSCRC compared to RSCRC. Although in case of progression-free survival data just indicated a trend ($p = 0.065$), in case of overall survival the difference was significant favouring LSCRC ($p = 0.047$). These data demonstrated for the first time that the EGFR protein expression is significantly higher in KRAS wild type RSLCL as compared to LSCRC. Meanwhile it is somewhat unexpected that the lower EGFR protein expression was found to be associated with better efficacy of anti-EGFR antibody therapies of colorectal cancer, the finding of which must be further validated.

**Keywords** Colon cancer · EGFR protein · Sidedness · Anti-EGFR therapy

## Introduction

The large intestine is developing from the midgut and the hindgut providing anatomical histological and functional differences. The coecum, ascending colon, hepatic flexture and the proximal 2/3rd of the transverse colon are developed from the midgut, while the distal 1/3rd of the transverse colon, the lineal flexure, the descending colon, the sigmoid colon and the rectum are developed from the hindgut. Although this developmental curiosity is well known for centuries its clinical significance was not recognized in oncology for a long time. Than large clinical analyses, focusing either on early stage or metastatic colon cancer reported that right-sided tumors has a worse prognosis compared to left-sided ones [1, 2].

Detailed clinicopathological and molecular analyses than revealed that the right-sided colorectal cancer (RSCRC) profoundly different from the left-sided colorectal cancer (LSCRC). RSCRC is characterized by mucinous histology, occur in older ages, predominant in females, tend to disseminate on the peritoneum, contains a strong lymphocytic infiltrate, frequently MSI-high (mismatch deficient), immunogenic and respond well to immunotherapy. On the other hand, LSCRC tend to be polipoid, has a tubular or villous morphology, occur in younger ages and more frequent in males, tend to metastatize to the liver and lung, characterized by chromosomal imbalances (CIN-high) and poorly immunogenic and respond poorer to immunotherapies but well to chemotherapies [3–5]. The importance of sidedness, location of primary tumor, as a predictive factor for anti- EGFR antibody therapy

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has been confirmed by the results of large clinical trials. In case of right sided tumors the expected PFS and OS are far more worse than in case of left sided location [6–9]. Anti-EGFR- monoclonal antibodies, cetuximab and panitumumab are considered as a part of standard treatment of metastatic colorectal cancer. However, the role of EGFR expression as a *predictive* factor for anti EGFR treatment is not proven, therefore the RAS mutation status serves as a strong negative predictor. Interestingly the prognostic value of sidedness has not been proven in a cohort of chemotherapy refractory, cetuximab treated metastatic colorectal patients, as the reevaluation of the NCIC CO.17 clinical trial reported [7] but it was considered as a predictive factor for cetuximab therapy [8, 9].

In a large cohort ($N = 1424$) of primary tumors authors have already compared the EGFR protein expression of right and left sided tumors, as a part of comparative molecular analyses of left-sided colon, right-sided colon, and rectal cancers. Using a simple low level positive/negative thresholding they have found that EGFR expression of right sided tumors is significantly higher than left sided ones [10]. However, this protein expression pattern was not correlated with the corresponding KRAS mutation statuses of tumors. Based on these results we attempted to answer the question if there is a difference between the EGFR expression of the left-, and right-sided tumors, and how it is correlated with the efficacy of anti-EGFR therapies.

## Materials and Methods

### Patients

We collected data for analysis of 97 metastatic colorectal cancer patients who received anti EGFR-therapy at the Hungarian Defence Forces Medical Center between 2008 and 2014. The analysis was approved by the local IRB (19/1043). The end of the data collection was defined as16-th August 2017. At that time 5 patient were still alive.

The median age of the 65 male and 32 female was 62,6 years (24–79). The primary tumor was reseceted in 84 cases, and in 13 synchronous metastatic patient only biopsy were performed from the primary. The diagnosis of metastatic disease was considered as synchronous (within 3 months after the diagnosis of primary) in 66 cases and metachronous in 31 cases. The characteristics of location, TNM stage and Grade of the primary tumors, and the sites of their corresponding metastasis are included into Table 1.

Eighty-eight patients were treated with cetuximab-FOLFIRI combination, one with cetuximab- De Gramont and 8 patients with panitumumab monotherapy. The anti-EGFR therapy was administered as second line therapy per cetuximab-FOLFIRI protocol in most of the cases ($N = 63$). In 8 cases it was delivered as first line therapy. All other patients ($N = 26$) were treated anti -EGFR treatment in later lines.

**Table 1** Patients

| | | | |
|---|---|---|---|
| Sex | | [N] | [%] |
| | Male | 65 | 67,0 |
| | Female | 32 | 33,0 |
| Age (years) | Median | 62,6 | |
| | Range | 24–79 | |
| Primary tumour location | | [N] | [%] |
| | Rectum | 28 | 28,9 |
| | Rectosigmoid | 6 | 6,2 |
| | Sigma | 32 | 33,0 |
| | Descending colon | 7 | 7,2 |
| | Lienal flexure | 2 | 2,1 |
| | Transverse colon | 4 | 4,1 |
| | Hepatic flexure | 1 | 1,0 |
| | Ascending colon | 9 | 9,3 |
| | Coecum | 8 | 8,2 |
| Primary tumour T | | [N] | [%] |
| | NA | 12 | 12,4 |
| | 1 | 0 | 0,0 |
| | 2 | 7 | 7,2 |
| | 3 | 59 | 60,8 |
| | 4 | 19 | 19,6 |
| Primary tumour N | | [N] | [%] |
| | NA | 12 | 12,4 |
| | 0 | 23 | 23,7 |
| | 1 | 30 | 30,9 |
| | 2 | 31 | 32,0 |
| | 3 | 1 | 1,0 |
| Primary tumour grade | | [N] | [%] |
| | NA | 8 | 8,2 |
| | 1 | 4 | 4,1 |
| | 2 | 66 | 68,0 |
| | 3 | 19 | 19,6 |
| Time of the diagnosis of metastatic disease | | [N] | [%] |
| | Synchron | 66 | 68,0 |
| | Metachron | 31 | 32,0 |
| Organs involoved, diagnosis by CT | | [N] | [%] |
| | Liver | 48 | 49,5 |
| | Lung | 9 | 9,3 |
| | Peritoneum | 7 | 7,2 |
| | Soft tissue | 1 | 1,0 |
| | Stomach | 1 | 1,0 |
| | Multi organ | 28 | 28,9 |

### Left-Right Deffinition

Right-sided CRC (RSCRC) was defined as primary tumors originating in the appendix, cecum, ascending colon, hepatic

flexure, or transverse colon. Primary tumors originating in the splenic flexure, descending colon, sigmoid colon, or rectum were classified as left-sided CRC (LSRCRC) according to the stratification was used for the evaluation of the role of sidedness in Crystal and Fire-3 [6].

### KRAS Mutation Testing

Primary or metastatic FFPE samples have been macrodissected for optimal tumor tissue content, the tumor/normal ratio was determined under light microscope and were tested for KRAS exon2 mutation as described [11].

### EGFR Protein Expression

EGFR protein expression of colorectal cancer tissues was determined by immunohistochemistry on Benchmark Ultra automatic stainer (Ventana, Tucson, Ar). EGFR protein was detected by the Ventana Confirm anti-EGFR and Ultraview Universal DAB detection kits. Membranous EGFR protein expression level of tumor cells was determined by light microscopy by evaluating the % of positive cells and the intensity of the reaction in the range of 1–3 applying the industrial standard H-score (0–300) semiquantitative methodology [12].

### Evaluation of EGFR Gene Copy Number Using Interphase Fluorescence In Situ Hybridization (iFISH)

iFISH analysis was used to evaluate the copy number status of *EGFR* gene. 5 μm-thick FFPE tissue sections were mounted onto Superfrost Plus positively charged slides, deparaffinized and rehydrated in distilled water. For antigen retrieval, sections were incubated in citric acid-based antigen unmasking solution (Vector Laboratories, Inc. Burlingame, CA, USA) at 95 °C for 20 min. Sections were then incubated in Triton X-100 (AppliChem GmbH, Ottoweg 4, 64,291 Darmstadt, Germany) –SSC solution at 65 °C for 30 min to lyse cells, following by the digestion in pepsin solution for 12 min at 37 °C, and washing twice in SSC for 5–5 min. ZytoLight® FISH-Tissue Implementation Kit (ZytoVision GmbH, Bremerhaven, Germany) was used in prehybridizational steps. Sections were air-dried prior to denaturation at 73 °C for 10 min. Hybridization was performed using 7 μl of ZytoLight SPEC EGFR/CEN 7 Dual Color Probe (ZytoVision Gmbh, Bremerhaven, Germany) per slide at 37 °C overnight in an automated hybridization chamber (ZYTOMED Systems GmbH Berlin, Germany). Slides were then washed in buffer SSC for 30 min at 45 °C to remove unbound probes, rinsed in water for 10 min and air-dried. Cell nuclei were counterstained with DAPI in antifade solution (Vector Laboratories, Inc. Burlingame, CA, 94010, USA). The Leica DM RXA fluorescent microscope equipped with Leica DFC 365FX high-performance CCD camera (Leica Microsystems GmbH, Wetzlar, Germany) and appropriate filters was used to evaluate the hybridization results. Areas with well-separated cell nuclei and overall good hybridization signals were selected for analysis. Minimum two FISH images per case were digitally captured at 63x magnification. For each case, green (EGFR) and red (CEN7 centromeric region) fluorescent signals were counted separately in at least 50 non-overlapping interphase nuclei. Based on these data, the following parameters were calculated: average *EGFR* copy number/cell, average CEN7 copy number/cell, *EGFR*/CEN7 ratio, average *EGFR* copy number/cell in amplified cell population, and percentage of polysomic or amplified cells.

### Statistical Analysis

H-score of EGFR (left vs. right) was analyzed by Mann-Whitney test. Overall and progression-free survival analyses were done using the Kaplan-Meier method. Overall and progression-free survival intervals were determined as the time period from initial diagnosis to the time of death and prognosis, respectively. The comparison between survival functions for different strata was assessed with the log-rank statistics. Statistical significance level was determined when $P$ values were $< 0.05$. Statistical analysis was performed using Statistica 12.0 software (StatSoft, Tulsa, OK).

## Results

KRAS wild type RSCRC composed the majority of our 97-patient cohort (Table 1). EGFR H-scores were broadly distributed in KRAS-wt primary as well as metastatic tumors (Fig. 1) from 0 to 300. In selected small proportion of the cases ($n = 7$) we have compared the EGFR copy numbers (CN) in tumor cells to the corresponding EGFR protein scores looking for association. We have found that in these cases CN/cell varied between 1.9 (diploid) and 5.04 (amplified) and the EGFR H-scores between 5 to 250 without any clear association. Extremely low protein scores have been associated with amplified tumors and high scores with near diploid statuses. (Table 2.)

Next we have evaluated the EGFR-H-scores of KRAS-wt RS- and LS-CRCs. Analysis was performed on primary tumors as well as on their metastases. (Fig.2.) In case of the primary tumors we have found that the EGFR H-scores of the LSCRC is significantly lower than the RSCRC ($p = 0.04$). (Fig.2a) It came as surprise, that a similar comparison of the right-sided tumor metastases to the left-sided ones also indicated that the EGFR scores of left-sided CRCs are significantly lower than the right-sided ones. ($p = 0.018$) (Fig.2b).

Finaly, we have analysed the survival of RS- and LSCRC patients treated with anti-EGFR antibody therapies using Kaplan-Meyer analysis. (Fig.3) In both cases RSCRC patients

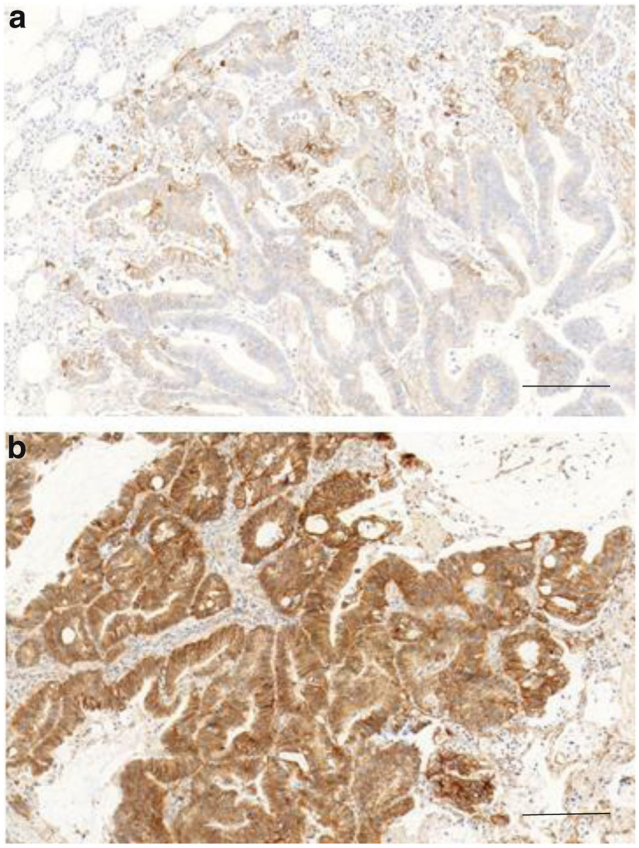


**Fig. 1** EGFR protein detection in CRC by immunohistochemistry. **a** Primary tumor of low EGFR protein expressor LSCRC (H-score = 61). Note the occasional tumor cell membrane positivity (brown colour) at the invasion edges. **b** Lung metastasis of a RSCRC characterized by high EGFR protein expression in the majority of tumor cells (H-score = 263). Bar =200 μm. LSCRC = left-sided colorectal cancer, RSCRC = right-sided colorectal cancer

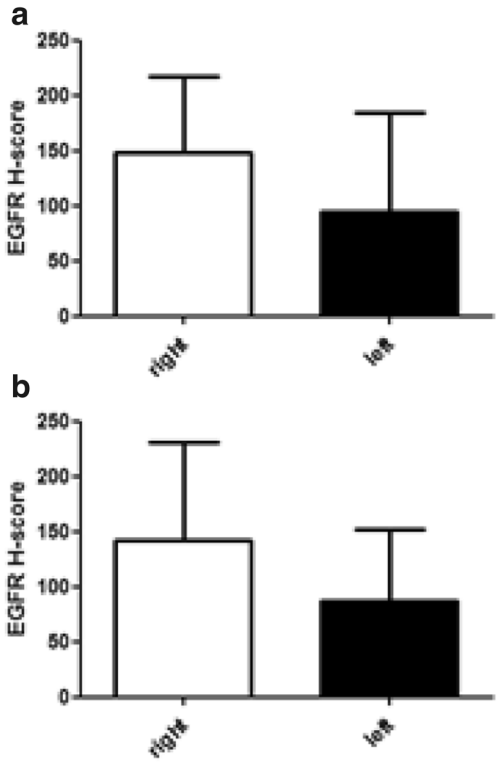


**Fig. 2** Comparison of EGFR protein expression levels of right- and left-sided colorectal cancers measured by H-scoring. **a** Comparison of primary tumors ($n = 97$). **b** Comparison of metastases ($n = 31$). Data are expressed as mean± SD. *$p = 0.04$(A), ** $p = 0018$(B)

have a poorer survival with significant difference found for overall survival (OS, Fig.3b) ($p = 0.04$) and close trend for progression-free survival (PFS, Fig3a) ($p = 0.064$). These differences can be translated to median survival in days: in case of PFS of LSRCR the median was 189d as compared to 117d (RSCRC) and in case of OS of LSCRC the median was 423d as compared to 265d (RSCRC).

**Table 2** Lack of association between the EGFR copy number and EGFR protein H-score in KRASwt colorectal cancer cases

| Case No | H-score | EGFR CN/ tumor cell | % of tumor cells with amplified EGFR |
|---|---|---|---|
| 1 | 5 | 4.44 | 22.81 |
| 2 | 25 | 1.9 | 0 |
| 3 | 30 | 4.77 | 13.33 |
| 4 | 70 | 4.08 | 5.77 |
| 5 | 70 | 4.26 | 40.0 |
| 6 | 200 | 2.73 | 16.13 |
| 7 | 250 | 5.04 | 7.69 |

CN = copy number, EGFR amplification = EGFR/cen7 ratio > 2

## Discussion

Our study confirmed those previous reports that metastatic KRAS-wt LSCRC respond significantly better to anti-EGFR antibody therapy than RSCRC [6–9]. This is a unique characteristics of LSCRC since there is no difference in response to anti-VEGF antibody therapy, [13] raising the issue of a selective EGFR-signaling related pathomechanism which is independent of RAS mutation. Previous study analysed the molecular profiles of LSCRC as compared to RSCRC and using a simple ± thresholding it was found an increased EGFR protein expression in the RSCRC as compared to LSCRC, however an association with KRAS mutation status was not performed [10]. Here we were able to study the EGFR protein expression of KRAS-wt CRC. We have used a semi-quantitative measurement of H-scoring for EGFR protein assessment [12]. Our data confirmed that RSCRC has a significantly higher EGFR protein expression level than LSCRC even in case of KRAS-wt setting. However, we have an access to metastatic tissues as well and that analysis also indicated that even in

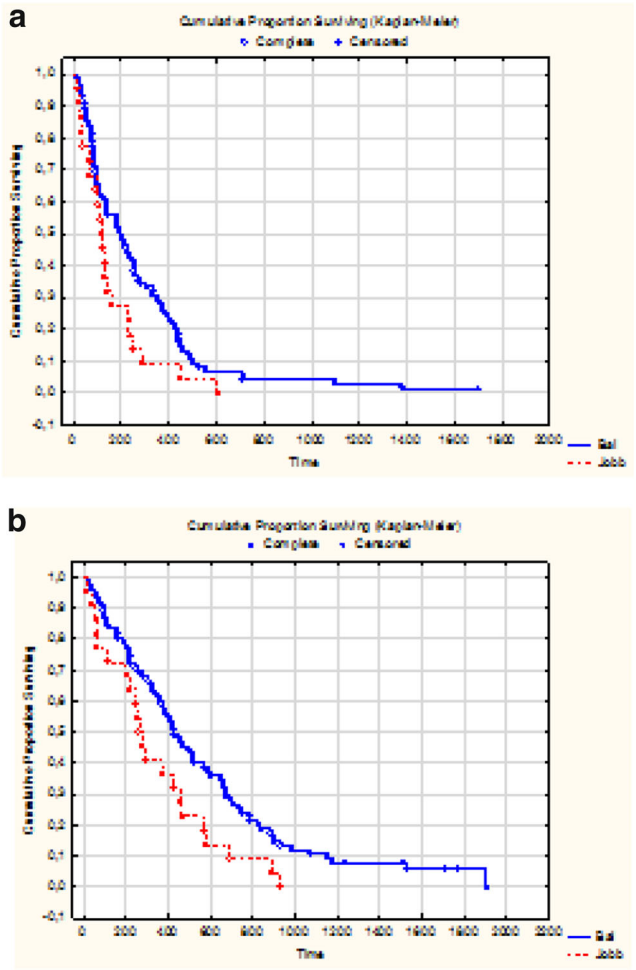


**Fig. 3** Survival of colorectal cancer patients treated with anti-EGFR antibody therapies (Kaplan-Meier analysis). **a** Presentation of progression free survival. $p = 0.065$. **b** Presentation of overall survival. $p = 0.047$. Y axis: Cummulative proportion surviving, X axis = days. Circules: left-sided tumors, dotted line: right-sided tumors

visceral tumor metastases RSCRC maintains a higher EGFR protein expression level as compared to LSCRC. Previous study suggested the association between EGFR copy number, EGFR protein level and efficacy of anti-EGFR treatment [14]. In our cohort we were not able to found any correlation between EGFR copy number and EGFR protein levels.

Our observations on the other hand, are relatively unexpected since in our cohort a higher EGFR protein expression was found to be associated with a significantly poorer efficacy of anti-EGFR antibody therapies measured by PFS and OS. Previous data already discredited EGFR protein expression as a predictive marker for anti-EGFR therapies in CRC [15]. Even there is a report which found that EGFR protein expression is a negative predictor of the anti-EGFR therapies in CRC [16]. Another study also documented that EGFR-negative CRC can respond to anti-EGFR antibody therapy [17]. Our cohort is not qualified to analyse wheather EGFR protein expression of CRC is a strong negative prognostic factor or a negative predictive factor for anti-EGFR antibody therapies. Therefore further studies are needed to clarify these issues.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors do not report any conflict of interest concerning this manuscript.

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