**Epidermal growth factor receptor (EGFR) status and K-Ras mutations in colorectal cancer**


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Received 7 April 2008; revised 2 June 2008; accepted 6 June 2008

**Background:** In advanced colorectal cancer, K-Ras somatic mutations predict resistance to mAbs targeting epidermal growth factor receptor (EGFR). Relationships between K-Ras mutations and EGFR status have not been examined so far. We analyzed relationships between K-Ras mutations and EGFR expression based on EGFR germline polymorphisms, gene copy number, and expression.

**Methods:** Eighty colorectal tumors (stage 0–IV) and 39 normal mucosas were analyzed. K-Ras mutations at codons 12 and 13 were detected by a sensitive enrichment double PCR–restriction fragment length polymorphism (RFLP) assay, EGFR gene polymorphisms at positions −216G>T, −191C>A and 497Arg-Lys were analyzed (PCR–RFLP), along with CA repeat polymorphism in intron 1 (fluorescent genotyping) and EGFR gene copy number (PCR amplification). EGFR expression was quantified by Scatchard binding assay.

**Results:** The number of EGFR high-affinity sites, dissociation constant (Kd), gene copy number, interon 1, −216G>T, −191C>A or 497Lys-Arg genotypes was not different between K-Ras-mutated or K-Ras-non-mutated tumors. No relationship was observed between any of the analyzed EGFR genotypes and EGFR expression. EGFR expression was not related to gene copy number. EGFR gene copy number in tumor and normal tissue was not correlated. The mean value of the tumor/normal mucosa gene copy number ratio was 1.16.

**Conclusions:** Present data clearly show that EGFR status is independent of K-Ras mutations in colorectal tumors.

**Key words:** EGFR, K-Ras mutations, colorectal cancer, scatchard assay, gene polymorphism, gene copy number

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**introduction**

One of the most promising targeted therapies in colorectal cancer treatment involves the epidermal growth factor receptor (EGFR) which controls signaling pathways involved in cell differentiation, proliferation, and angiogenesis. EGFR is expressed in 80% of colorectal cancers. It has been demonstrated that the anti-EGFR mAb cetuximab, alone or combined with irinotecan, improved responsiveness in patients with irinotecan-refractory colorectal cancer [1, 2]. However, these trials failed to establish a significant link between EGFR tumor expression and K-Ras mutation status. Of note, EGFR expression was correlated with K-Ras mutation status. The aim of this study was thus to examine in a series of 80 primary colorectal tumors the relationships between EGFR and K-Ras mutation status. Of note, EGFR expression was quantified with a Scatchard binding assay that distinguishes biologically active high-affinity sites from low-affinity sites that do not contribute to EGFR binding [11]. In addition, we also analyzed the EGFR gene copy number, as well as the main EGFR germinal polymorphisms described so far.

The first intron of the EGFR gene contains a polymorphic microsatellite sequence (CA repeats) that may influence gene transcription [12, 13], and skin toxicity has been reported to be linked to the number of CA repeats [14]. Additional single-nucleotide polymorphisms (SNPs) have been described on the EGFR gene: one at position −216G>T (promoter region) [15], one at position −191C>A close to the Sp1 recognition site in the promoter region [15] and one at codon 497 (Lys>Arg) in the extracellular domain of the receptor [16]. Since the links...
between these SNPs and EGFR expression have not been fully analyzed, an additional objective of the present study was to explore them thoroughly. Moreover, the possibility of obtaining paired samples of tumoral tissue and normal mucosa led us to compare these different parameters in both tumor and normal mucosas.

**patients and methods**

**patients**

This retrospective study was conducted on a series of 80 colorectal cancer patients (48 men, 32 women, mean age 69, extremes 46–90) already explored for EGFR expression quantified by a Scatchard binding assay with human $^{125}$I-EGF [17]. The Scatchard analysis allows both high- and low-affinity sites to be quantified separately, along with their corresponding dissociation constant (Kd) values. Tumor staging was 4 stage 0, 13 stage I, 22 stage II, 23 stage III and 18 stage IV. Cancer localizations were 27 right colon (RC), seven transverse colon (TC), 12 left colon (LC), 21 sigmoid (S) or rectosigmoid junction (RSJ) and 11 rectum (R). For each patient, a biopsy of the primary tumor was taken at the time of initial surgery, before starting any chemotherapy. In addition, for 39 patients, a biopsy of adjacent normal mucosa was also carried out. This study obtained approval from the ethics committee and patients gave consents for sample collection and analysis. Biopsy samples were immediately frozen and stored in liquid nitrogen.

**EGFR gene polymorphisms**

CA repeat polymorphism in intron 1 of EGFR gene was analyzed by fluorescent genotyping on CEQ-8000 Beckman-Coulter, as previously described [13]. The EGFR gene polymorphisms at positions $-216G>T$ and $191C>A$ (promoter region) were analyzed by PCR–restriction fragment length polymorphism (RFLP), as previously described [15]. The $497Lys>Arg$ polymorphism in the extracellular domain of the receptor was analyzed by PCR–RFLP, as previously described [18].

**EGFR gene copy number**

Amplification of the EGFR gene was carried out by real-time PCR on LightCycler (Roche Meylan, France). The primers used were those described by Takano et al. [19] and the 36B4 gene was chosen as the reference gene [20]. The calibrator was generated from DNA extract of CAL33 human head and neck cancer cell line (DSMZ: ACC 447). The results, calculated using RelQuant software, were expressed as the ratio of EGFR gene expression in the tumor to EGFR gene expression in the calibrator after normalization by the reference gene. Gene copy number was thus expressed as relative units.

**K-Ras mutation analyses at codons 12 and 13**

Mutations at K-Ras codons 12 and 13 (exon 1) were detected by a sensitive enrichment double PCR–RFLP assay, as previously described [21]. DNA from tumor cell lines, SW620 (mutated at codon12), LOVO (mutated at codon 13) and WIDR (wt at codons 12 and 13), were used as controls.

**statistics**

Comparisons between the observed genotype frequencies and those expected from the Hardy–Weinberg equilibrium were tested by means of chi-square tests (http://innateimmunity.net). Linkage disequilibrium was tested by means of the Fischer’s exact test. For statistical purposes, we defined the CA sum as the sum of the short + long CA repeats. Cancer localizations were merged as follows: RC + TC versus LC + S + RSJ versus R. Nonparametric tests were applied. Statistics were carried out on SPSS 15.0 software (Chicago, IL).

**results**

**EGFR status**

As previously reported by us [17], the majority of tumors (62 of 80) expressed a single class of high-affinity binding sites, with a median site number at 98 fmol/mg prot (extremes 7–310) and a median Kd value at 0.76 nM (extremes 0.15–8.8). In the 18 tumors with two classes of sites, the median high-affinity site number and Kd were 28 fmol/mg prot (extremes 10–142) and 0.26 nM (extremes 0.14–0.68), respectively. The number of EGFR high-affinity sites in tumors was not related to tumor staging, tumor localization or patient age.

Figure 1 illustrates the distribution of intron 1 polymorphism versus the number of high-affinity EGFR sites in tumors. No relationship was observed between intron 1 gene polymorphism and EGFR expression in tumors ($P = 0.48$ between the CA-sum and the number of high-affinity sites). Genotype distribution of the three analyzed SNPs is depicted in Table 1. All concorded with the Hardy–Weinberg equilibrium. A linkage disequilibrium was noted between $-216G>T$ and $-191C>A$, the rare allele of one genotype being associated with the frequent allele of the second ($P = 0.011$). Also, $-216G>T$ genotype and CA repeat polymorphism were not independent: the median CA sum was 36 in GG, 33 in GT and 32 in TT tumors (Kruskal–Wallis test, $P = 0.002$). These three SNPs did not influence the number of high-affinity EGFR sites in tumors ($P = 0.77$, 0.52 and 0.72 for $-216G>T$, $-191C>A$ and 497Lys/Arg, respectively). Superimposable genotypes were observed between tumor and normal mucosa and none of the analyzed EGFR gene polymorphisms had a significant influence on the number of high-affinity sites or Kd measured in normal mucosa.

EGFR gene copy number varied from 0.44 to 2.06 relative units (mean 0.79, median 0.74) in tumors and from 0.45 to 1.29 relative units (mean 0.70, median 0.65) in normal mucosa. EGFR gene copy number was not significantly different between tumor and normal mucosa (Wilcoxon paired test, $P = 0.078$). The mean value of the tumor/normal mucosa amplification ratio was 1.16 (median 1.08, range 0.55–2.68). EGFR gene copy number did not correlate between tumor and normal mucosa ($P = 0.70$). No correlation was observed between gene copy number and the number of EGFR high-affinity sites either in tumors (Figure 2, $P = 0.074$) or in normal mucosa ($P = 0.16$). EGFR gene copy number in tumors was not related to tumor staging or tumor localization, but a trend for a negative correlation was observed with patient age (Figure 3, $P = 0.028$). A trend for a positive correlation between CA repeat polymorphism (CA sum) and EGFR gene copy number was observed in tumors (Figure 4, $P = 0.021$). Regarding the $-216G>T$ polymorphism, greater EGFR gene copy number was observed in GG patients than in others ($P = 0.014$).

**K-Ras mutation status**

Among the 80 analyzed primary tumors, 24 were K-Ras mutated (i.e. 30%). Twenty cases were mutated at codon 12, five cases were mutated at codon 13 and one tumor was mutated at both codons 12 and 13. The presence of a K-Ras mutation was independent from primary cancer localization but was linked to tumor staging, with a significantly higher
mutation rate in stages 0–1 relative to stages II–IV (52.9% versus 23.8%, \( P = 0.035, \) Table 1). The presence of a K-Ras mutation did not influence the high-affinity EGFR site numbers (\( P = 0.91 \)) or high-affinity Kd values (\( P = 0.38 \)) (Table 1). No relationship was observed between \(-216G>T, -191C>A, 497Lys>Arg \) or intron 1 EGFR genotypes and the presence of a K-Ras mutation (Table 1). Also, the level of EGFR gene copy number in tumors was independent of the presence of a K-Ras mutation (Table 1).

**discussion**

EGFR expression and gene modifications have been examined in colorectal cancer as possible predictive markers of treatment outcome under anti-EGFR therapies. Ras proteins, localized on the cell membranes, function as signal switches that link receptor tyrosine kinase activation to downstream effectors. K-Ras mutations usually lead to constitutive activation of the signaling pathways, stimulating cell proliferation [22]. Two recent studies conducted on advanced colorectal cancer patients receiving anti-EGFR mAbs have confirmed that the presence of a K-Ras mutation allows unresponsive patients to be identified [9, 10]. The main objective of the present study was thus to study the influence of K-Ras somatic mutations on EGFR status. In addition, we thoroughly analyzed the links between EGFR expression, EGFR germline polymorphisms and EGFR gene copy number.

The EGFR gene exhibits several germline polymorphisms, among which the CA repeat polymorphism in intron 1 influences EGFR expression on the basis of experimental data, with the lower the number of CA repeat the greater the EGFR expression [12]. At the clinical level, the impact of the CA repeat polymorphism on EGFR tumoral expression was reported in breast cancer [23, 24] and in head and neck cancer by us [13]. However, in a recent study, Liu et al. [25]

**Table 1.** Demographic data and EGFR analysis according to K-Ras mutation status in primary tumors

<table>
<thead>
<tr>
<th>Primary localization</th>
<th>Wild-type K-Ras (( N = 56 ))</th>
<th>Mutated K-Ras at codon 12 or 13 (( N = 24 ))</th>
<th>Statistics (( P ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right + transverse colon</td>
<td>26 (76.5%) 8 (23.5%)</td>
<td>Fischer’s exact test; ( P = 0.45 )</td>
<td></td>
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<tr>
<td>Left colon + sigmoid + rectosigmoid junction</td>
<td>21 (63.6%) 12 (36.4%)</td>
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<tr>
<td>Rectum</td>
<td>9 (81.8%) 2 (18.2%)</td>
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<tr>
<td>Tumor staging</td>
<td></td>
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<tr>
<td>0–I</td>
<td>8 (47.1%) 9 (52.9%)</td>
<td>Fischer’s exact test; ( P = 0.035 )</td>
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<tr>
<td>II–IV</td>
<td>48 (76.2%) 15 (23.8%)</td>
<td></td>
<td></td>
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<tr>
<td>High-affinity EGFR site (fmol/mg prot)a</td>
<td>80 (7–282) 68 (10–310)</td>
<td>Mann–Whitney; ( P = 0.91 )</td>
<td></td>
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<tr>
<td>High-affinity EGFR Kd (nM)a</td>
<td>0.57 (0.14–8.81) 0.58 (0.16–3.76)</td>
<td>Mann–Whitney; ( P = 0.38 )</td>
<td></td>
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<tr>
<td>EGFR gene copy number (relative unit)a</td>
<td>0.74 (0.44–2.06) 0.74 (0.51–1.09)</td>
<td>Mann–Whitney; ( P = 0.43 )</td>
<td></td>
</tr>
<tr>
<td>Intron 1 EGFR polymorphism (sum of CA repeat)a</td>
<td>34.5 (31–41) 33.5 (31–39)</td>
<td>Mann–Whitney; ( P = 0.156 )</td>
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<table>
<thead>
<tr>
<th>(-216G&gt;T) EGFR polymorphism</th>
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<tbody>
<tr>
<td>GG (( N = 36 ))</td>
<td>27 (75%) 9 (25%)</td>
<td>Fischer’s exact test; ( P = 0.82 )</td>
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<tr>
<td>GT (( N = 36 ))</td>
<td>24 (66.7%) 12 (33.3%)</td>
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<tr>
<td>TT (( N = 7 ))</td>
<td>5 (71.4%) 2 (28.6%)</td>
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<th>(-191C&gt;A) EGFR polymorphism</th>
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<tbody>
<tr>
<td>CC (( N = 63 ))</td>
<td>43 (68.3%) 20 (31.7%)</td>
<td>Fischer’s exact test; ( P = 0.67 )</td>
<td></td>
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<tr>
<td>CA (( N = 15 ))</td>
<td>12 (80%) 3 (20.0%)</td>
<td></td>
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<tr>
<td>AA (( N = 1 ))</td>
<td>1 (100%) 0 (0%)</td>
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<th>(497Lys&gt;Arg) EGFR polymorphism</th>
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<tr>
<td>Lys/Lys (( N = 41 ))</td>
<td>26 (63.4%) 15 (36.6%)</td>
<td>Fischer’s exact test; ( P = 0.50 )</td>
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<tr>
<td>Lys/Arg (( N = 31 ))</td>
<td>23 (74.2%) 8 (25.8%)</td>
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<tr>
<td>Arg/Arg (( N = 7 ))</td>
<td>6 (85.7%) 1 (14.3%)</td>
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*Medians along with extreme values are given.

EGFR, epidermal growth factor receptor.
demonstrated on 58 cancer cell lines of the NCI that CA repeat polymorphism did not significantly influence EGFR expression. Interestingly, in 30 colorectal cancer patients treated with gefitinib, it was shown that patients with lower numbers of CA repeat frequently developed skin toxicity [14] which has been unanimously reported to be a response predictor to anti-EGFR therapies [26]. In the present series of 80 primary colorectal tumors, the number of high-affinity EGFR sites was not linked to the CA repeat polymorphism in intron 1 (Figure 1) or to the three common SNPs at positions $-216G>T$, $-191C>A$ and $497Lys/Arg$. In contrast, Liu et al. reported that $-216G>T$ polymorphism was associated with both EGFR protein (Western blot) and mRNA levels (real-time PCR or microarray) [25]. This discrepancy may arise since measurement of mRNA or EGFR protein differs from exact quantification of high-affinity binding sites as carried out in the present study. In our opinion, the latter is a more pharmacologically relevant approach.

Present data show that the two SNPs at positions $-216G>T$ and $-191C>A$ are not independent. These two SNPs, located in the promoter region of the gene, have been shown to influence protein expression [15, 27]. Since colorectal cancer is subject to multiple gene rearrangements, it was interesting to compare EGFR genotypes between tumoral and normal tissues. For the four analyzed gene polymorphisms, genotypes were identical in tumors and normal mucosa. This observation is thus of practical interest for future pharmacogenetic studies in which genotyping is carried out on normal tissues, such as peripheral blood mononuclear cells for instance.

Recent clinical studies have reported that a large number of EGFR gene copies may be predictive of clinical responsiveness to EGFR target therapies [3, 28]. Since high-affinity EGFR sites represent the biological target of anti-EGFR therapies [11], we felt justified in examining the possible relationship between EGFR gene copy number and high-affinity EGFR sites in the present series of colorectal tumors. EGFR gene copy number in tumors varied up to 4.7-fold between patients (from 0.44 to 2.06 units). Clearly, no correlation was observed between EGFR gene copy number and the number of high-affinity EGFR sites (Figure 2). Comparisons between EGFR expression and gene copy number analyzed by a FISH or chromogenic in situ hybridization (CISH) technique have been previously reported in colorectal cancer with contrasting conclusions. For instance, Shia et al. [29] reported a positive relationship between expression and amplification. However, only a small fraction of EGFR-positive tumors detected by IHC were associated with gene amplification [29]. Ooi et al. [30] observed that EGFR overexpression was frequently accompanied by gene amplification. To our knowledge, an analysis comparing EGFR expression quantified by a ligand-binding assay, such as that presently used, with EGFR gene copy number quantified by a FISH or CISH technique has not been reported so far. Such an analysis deserves to be carried out since discrepancies between the FISH technique and the presently used PCR-based...
technique may be possible. Even though the present study failed to show a link between high-affinity EGFR sites and gene amplification, a trend for a positive correlation was presently reported between EGFR gene copy number and the number of CA repeat (Figure 4), suggesting that germlinal gene characteristics such as EGFR CA repeat polymorphism may influence gene amplification in the tumor.

The core of the present study was to focus on K-Ras mutation. In this series of 80 colorectal cancer patients, 30% of tumors were K-Ras mutated. This figure is representative of K-Ras mutation status in colorectal tumors [31]. K-Ras mutations are involved in the activation of the EGFR pathway. Deactivation of EGFR signaling via EGFR internalization, feedback inhibition of regulatory proteins or transiently activated Ras-GAP proteins has been proposed [32]. It is thus conceivable that the presence of K-Ras mutations may influence the level of EGFR expression in the tumor. To our knowledge, the only study reporting EGFR expression analysis according to K-Ras mutation status is that of Hilbe et al. [33] conducted on a series of 66 non-small-cell lung cancers. The authors showed that EGFR expression was not different between tumors bearing a K-Ras mutation at codon 12 (n = 7) and the remaining wild-type tumors (n = 59). However, the conclusion of this latter study is limited by the IHC method used to measure EGFR expression since it has been reported that IHC is questionable for EGFR application [34]. Also, it is regrettable that EGFR analysis using a biochemical assay, as presently used, has never been applied to test the value of EGFR for predicting anti-EGFR responsiveness. The present study, using a specific ligand-binding assay, clearly establishes on a large set of patients that the number of high-affinity EGFR sites and Kd values are independent of the presence of K-Ras mutations in colorectal cancer tumors. The independence between K-Ras mutation status and EGFR status is particularly relevant in the current context of anti-EGFR therapies in colorectal cancer [26] and suggests that resistance mechanisms linked to the presence of K-Ras mutations are not associated with a quantitative change in the EGFR target itself. This result strongly suggests that EGFR status will not provide any relevant information enabling identification of candidate patients for anti-EGFR therapy.

funding
French Research Ministry (PHRC).

acknowledgement
Presented in part at the 2007 annual ASCO meeting.

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