EGFR in colorectal cancer: more than a simple receptor


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Background: Advances in the understanding of tumor biology have led to the development of targeted therapies allowing progress in colorectal cancer treatment. One of the most promising targets is the epidermal growth factor receptor (EGFR).

Method: The presence and distribution of high- and low-affinity EGFR was investigated retrospectively in a group of 82 colorectal cancer samples (43 normal colon–colon cancer paired samples) using a specific ligand binding assay (Scatchard Analysis).

Findings: A large majority of tumor samples exhibited one class of high-affinity binding sites (78%). Eighteen cases (22%) exhibited both high- and low-affinity binding sites. A wide interpatient variability was observed for the site number, with physiologically-relevant high-affinity sites ranging from 7 to 310 fmol/mg protein in tumors and from 6 to 313 fmol/mg protein in normal mucosa. A significant positive correlation was demonstrated between tumor and normal mucosa for the high-affinity Kd values and for the number of high-affinity sites, suggesting a common regulation for both tumor and normal tissue.

Interpretation: These observations (i) could explain recently-reported clinically-active EGFR targeting in colorectal tumors apparently negative for EGFR, and (ii) may offer a plausible explanation for the link observed between toxicity in normal tissue (cutaneous rash) and clinical outcome of patients treated with anti-EGFR drugs. Present data extends our understanding of EGFR identity in colorectal cancer which could be useful in reconsidering the predictive tools for the identification of tumors putatively responsive to EGFR targeted therapy.

Key words: colorectal cancer, EGFR, epidermal growth factor receptor

Introduction

Colorectal cancer is still carrying a high morbidity and mortality. Significant improvements have been made in the management of this disease mainly through the introduction of active chemotherapeutic agents, oxaliplatin and irinotecan, combined with fluorouracil [1, 2]. More recently, advances in the understanding of tumor biology have led to the development of targeted therapies [3] allowing progress in the treatment of colorectal cancer [4, 5]. One of the most promising targets is the epidermal growth factor receptor (EGFR), a member of the subclass I of the receptor tyrosine kinase superfamily [6] which is overexpressed in different solid cancers reaching up to 80% overexpression in colorectal cancer [7, 8].

The major clinical impact of EGFR targeting is illustrated by the results of the irinotecan-cetuximab trials showing that cetuximab alone or combined with irinotecan provided an additional response in patients with irinotecan-refractory colorectal cancer [4, 9]. Both trials failed to establish a significant link between EGFR tumor expression based on immunohistochemistry (IHC) and response to treatment [4, 9]. The authors hypothesized that even EGFR negative tumors might be responsive to cetuximab [4, 9]. In a small, retrospective study, Chung et al. thus provocatively reported that a 25% objective response rate was observed in EGFR-IHC negative colorectal cancer patients receiving cetuximab-based therapy and concluded that selection or exclusion of patients for cetuximab therapy on the basis of currently available EGFR IHC does not seem warranted [10]. Interestingly, the authors formulated the hypothesis that tumors with a low overall level of EGFRs, below the threshold of IHC detection, may have receptors with high affinity and that they are responsive to EGFR-targeted therapy. This consideration is based on the fact that high-affinity EGFRs are the predominant biologically active receptors leading to tyrosine kinase activation whereas low-affinity EGFRs do not contribute significantly to intracellular signal activation [11–13]. Our knowledge regarding the existence of two classes or EGFR receptors in colon cancer is based on a limited series of 10 primary...
canceroma [14]. As no data are available concerning the presence and distribution of high- and low-affinity EGFR in a large series of colorectal cancer samples, we investigated retrospectively a group of 82 colorectal cancer samples (43 normal colon–colon cancer-paired samples) using a specific ligand binding assay (Scatchard Analysis) for this purpose.

**material and methods**

**patients**

This retrospective study included a group of 82 colorectal cancer patients. These patients were part of a larger prospective multicentric French study conducted between 1998 and 2002, in which a tumor sample was taken at the time of initial surgery, before starting any chemotherapy. For the 82 patients, one part of these collected samples was used for the present EGFR analysis. This group of patients was selected so as to cover all disease stages with a sufficient number of cases and with the prerequisite to dispose of at least 100 mg tissue sample so as to perform the Scatchard analysis. In addition, for 43 patients EGFR analysis was also performed in a sample of adjacent normal mucosa. Both tumor and normal mucosa samples were systematically controlled by histology. Tumors and normal tissue biopsies (100–200 mg) were immediately frozen and stored in liquid nitrogen until analysis. This study obtained approval from the local ethics committee and patients gave consent for tissue collection and analyses. The population comprised 49 men and 33 women (mean age 69, extremes 46–90). The tumor localization was as follows: 30 right colon, seven transverse colon, 13 left colon, 14 sigmoid, seven recto-sigmoid junctions and 11 rectum. Tumor staging was the following: four stage 0 (*in situ* carcinoma), 14 stage I (T1N0 + T2N0), 22 stage II (T3N0 + T4N0), 24 stage III (all T, N1 to N3) and 18 stage IV (metastatic disease). Tumor histological analysis showed four, 34 and 35 cases of poor, moderately and well differentiated adenocarcinomas, respectively; five cases corresponded to mucinous carcinomas and four adenocarcinomas were not documented for their differentiation status.

**EGFR assay**

The level of EGFR was determined by means of a Scatchard analysis, according to a previously published method [15], with human recombinant EGF as the ligand. 125I-labeled EGF (900 to 1300 Ci/mmol) was from Amersham (Orsay, France) and unlabeled EGF from Biosource (Sigma, St Quentin Fallavier, France).

Samples were homogenized in liquid nitrogen and the resulting powder was mixed in 10 volumes of TED buffer (10 mM Tris–HCl, 1 mM EDTA, 0.5 mM DTT, 10 mM sodium molybdate, pH 7.4). After centrifugation for 10 min at 800 g (2°C), the pellet (crude membranes) was washed with 1 ml TED buffer and resuspended in TMP buffer (10 mM Tris–HCl, 10 mM MgCl2, 1 mM phenyl methylsulfonyl fluoride, 0.02% Na3VO4, pH 7.4); an aliquot was taken for protein assay according to the technique of Bradford [16]. The membrane preparation was then adjusted to a protein concentration of 0.5 to 1.5 mg/ml. One hundred μl of 125I-EGF (0.16 nM final concentration) was incubated with 100 μl of the membrane preparation, along with 50 μl of increasing concentrations of unlabeled EGF ranging between a 0.26 and 168 nM final concentration. The highest unlabeled EGF concentration (168 nM) allowed for determination of the non-specific binding. EGF solutions were prepared in TMB buffer containing 0.1% bovine serum albumin to reduce non-specific binding. Incubations were performed for 90 min at room temperature. The reaction was stopped by adding 1 ml 0.1% BSA-TMB cold buffer, and the tubes were immediately centrifuged for 15 min at 11 000 g (4°C). The resulting pellet was washed with 1 ml 0.1% BSA-TMB cold buffer and centrifuged for 15 min at 11 000 g (4°C). The supernatant was then discarded and the y radioactivity in the pellet was counted (total EGF binding). Specific EGF binding was obtained by subtracting the non-specific binding. The Scatchard analysis [17] was performed using MicroPharm Binding software version 5.1 (Dr Urien, INSERM, Paris), by plotting the specific EGF binding/free EGF (Bs/F ratio) against the specific EGF binding (Bs).

Scatchard modeling led to the identification of one or two classes of binding sites, depending on the shape of the plotted curve. Validation of the chosen model, i.e. one or two classes of binding sites, was based on the Akaike parameter (the lower the Akaike value, the better the modeling). The quality of the estimated parameters (site number and dissociation constant (Kd)) was evaluated by their coefficient of variation (CV). For the highest affinity sites in all analysed samples, mean CV was 16.0% for the site number and 36.4% for the Kd (n = 125, corresponding to tumor and normal mucosa samples together). The intra-assay reproducibility was evaluated by measuring 8 aliquots of the same membrane preparation within the same serie. Scatchard analysis identified one class of sites with a CV at 8.1% for the number of sites and 10.9% for the Kd.

**statistics**

Non-parametric tests were performed (Spearman rank correlations, Wilcoxon paired test, Mann-Whitney or Kruskal-Wallis tests) since the analyzed variables (high and low affinity sites, high and low Kd) did not match a Gaussian distribution. Frequency comparisons were performed by means of the chi-square test, or Fischer’s Exact test when appropriate. Statistics were obtained using SPSS software version 13.0 (Chicago, USA).

**results**

**description of EGFRs**

Figure 1a illustrates a typical example of a Scatchard representative of a tumor/normal mucosa paired-sample exhibiting one class of sites. Figure 1b illustrates a Scatchard representative of a tumor sample with two classes of sites. The characteristics of the tumor and normal mucosa samples with respect to EGFRs are shown in Table 1. A majority of tumor samples exhibited one class of binding sites (64/82, i.e. 78%). Eighteen cases (22%) exhibited two classes of binding sites. A similar pattern of distribution was observed in normal mucosa samples, with 34/43 samples (i.e. 79.1%) showing one class of binding sites and 9 samples (i.e. 20.9%) having two classes of binding sites.

In tumors exhibiting one class of binding sites, the median Kd value was 0.75 nM, indicating the presence of high-affinity sites only, even though a wide variability was observed with Kd ranging from 0.15 to 8.8 nM (Table 1). In tumors with two classes of sites, the high-affinity sites had Kd values comprised between 0.14 and 0.68 nM, whereas the low-affinity sites had Kd values between 4.8 and 67.2 nM (Table 1). In normal mucosa, the Kd values were in the same range as in the tumors (Table 1).

A wide inter-patient variability was also observed for the site number, with high-affinity sites ranging from 7 to 310 fmol/mg protein in tumors and from 6 to 313 fmol/mg protein in normal mucosa (Table 1). Comparison of one class versus two classes EGFR revealed that the number of high-affinity sites was significantly greater in the one class than in the two class of binding sites (Mann-Whitney: P < 0.001 and P = 0.029 in tumor and normal mucosa, respectively).
For the two class EGFR, the greater the number of low-affinity sites, the greater the number of high-affinity sites (Spearman correlation: \( P = 0.040 \) in tumor and \( P = 0.019 \) in normal mucosa). The number of low-affinity sites was 1.3 to 18.5-fold higher than the number of high-affinity sites (median value of the ratio low/high-affinity site was 2.2 in tumors and 3.2 in normal mucosa).

EGFR comparison between tumor and normal mucosa

EGFRs were analyzed both in tumor and normal mucosa samples from 43 patients. There was no correspondence for the class of binding sites between tumor and normal mucosa (Fisher’s Exact test: \( n = 43, P = 0.24 \)). A significant positive correlation was demonstrated between tumor and normal mucosa for the high-affinity \( K_d \) values (Figure 2, Spearman rank correlation: \( P < 0.001 \)) and for the number of high-affinity sites (Figure 3, \( n = 43 \), Spearman correlation: \( P = 0.013 \)).

A paired-sample analysis showed that high-affinity \( K_d \) values were not significantly different between tumor and corresponding normal mucosa. Accordingly, the median of the ratio of high-affinity \( K_d \) values between normal mucosa and tumor was 0.99 (range 0.2–3.6).

The number of high-affinity binding sites was on average 37% lower in tumors as compared to normal tissues (Wilcoxon paired test: \( P < 0.001 \), \( n = 43 \)). The median of the ratio of high-affinity sites between normal mucosa and tumor was 1.7 (range 0.1–30.3). A sub-group analysis performed on samples from 22 patients with one family sites in both tumor and normal tissue confirmed that the number of high-affinity EGFR was significantly lower in tumors as compared to normal tissue (38% on average, Wilcoxon paired test: \( P = 0.003 \)).

influence of tumor staging, histology and localization on EGFRs

The proportion of the one class of binding sites in tumor samples was not significantly different according to tumor histology (moderate versus well differentiated), localization (right + transverse versus others) or staging (0-I versus II-III-IV). High-affinity \( K_d \) values and site numbers, in tumors, were not significantly different according to tumor histology or localization. Table 2 describes tumoral EGFR as a function of tumor staging. Even though no correlation was demonstrated between the number of high-affinity sites and the tumor staging (Spearman correlation: \( P = 0.19 \)), comparison of stage 0-I versus stage II-III-IV showed on average 1.9-fold lower high-affinity site number in the higher stage group (Mann-Whitney: \( P = 0.01 \)). Of note, the greater the tumor staging, the lower the high-affinity \( K_d \) value (Spearman correlation: \( P = 0.054 \)).

Table 1. Characterization of the EGFR in tumor and normal tissue samples

<table>
<thead>
<tr>
<th></th>
<th>Tumor</th>
<th></th>
<th>Normal mucosa</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_d ) (nM)</td>
<td>Site number (fmol/mg prot)</td>
<td>( K_d ) (nM)</td>
<td>Site number (fmol/mg prot)</td>
</tr>
<tr>
<td>One class of binding sites (High-affinity sites)</td>
<td>( n = 64 )</td>
<td>0.99 ± 1.16, 107 ± 71</td>
<td>0.81 ± 0.58, 139 ± 86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mean ± SD</td>
<td>median = 95</td>
<td></td>
<td>median = 123</td>
</tr>
<tr>
<td></td>
<td>median = 0.15–8.81</td>
<td>extremes = 7–310</td>
<td></td>
<td>extremes = 0.15–2.32</td>
</tr>
<tr>
<td></td>
<td>( n = 18 )</td>
<td>0.32 ± 0.15, 44 ± 36</td>
<td>0.32 ± 0.24, 73 ± 48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mean ± SD</td>
<td>median = 28</td>
<td></td>
<td>median = 63</td>
</tr>
<tr>
<td></td>
<td>median = 0.14–0.68</td>
<td>extremes = 10–142</td>
<td></td>
<td>extremes = 0.10–0.74</td>
</tr>
<tr>
<td>Two classes of binding sites</td>
<td>( n = 18 )</td>
<td>17.79 ± 16.96, 135 ± 105</td>
<td>21.13 ± 17.73, 300 ± 346</td>
<td></td>
</tr>
<tr>
<td>High-affinity sites</td>
<td>mean ± SD</td>
<td>median = 114</td>
<td></td>
<td>median = 179</td>
</tr>
<tr>
<td></td>
<td>median = 12.57</td>
<td>extremes = 35–418</td>
<td></td>
<td>extremes = 5.65–62.78</td>
</tr>
<tr>
<td>Low-affinity sites</td>
<td>mean ± SD</td>
<td>median = 12.57</td>
<td></td>
<td>median = 179</td>
</tr>
<tr>
<td></td>
<td>median = 4.80–67.21</td>
<td>extremes = 35–418</td>
<td></td>
<td>extremes = 5.65–62.78</td>
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Figure 1. Scatchard representations: (a) Typical Scatchard representation for a tumor/normal mucosa paired-sample exhibiting one class of binding sites (normal mucosa, dotted line; tumor mucosa, full line). \( K_d \) values were 3.76 nM and 2.32 nM, in the tumor and normal mucosa, respectively. The number of binding sites was 120 and 302 fmol/mg prot, in the tumor and normal mucosa, respectively. (b) Typical Scatchard representation for a tumor sample with two classes of binding sites. For high-affinity sites (first class), the \( K_d \) was 0.26 nM and the number of sites was 30 fmol/mg prot. For low-affinity sites (second class), the \( K_d \) was 16.97 nM and the number of sites was 155 fmol/mg prot.
One of the main findings of the present study is the existence of a heterogeneous population of EGFRs in colorectal tumors with both one class and two classes of binding sites. The presence of an EGFR class with a mixed presence of low- and high-affinity sites with, for the latter, a putative capacity for higher biological activity [11–13], corroborate a recent IHC study by Cunningham and coworkers showing that there was a lesser proportion of phosphorylated (active) EGFR compared to the total expression of the EGFR [8]. The present study reveals the existence of a majority, 78%, of colorectal tumors with a single class of EGFRs. Our data at this stage confirm the finding of a previous study by Moorghen and co-workers on a limited series of 10 cases that showed the existence of colorectal tumors with one and two binding sites [14]. Noteworthy, the present study indicates that EGFR levels were measurable in all cases. Also, concerning the single class EGFRs, there was a marked variability in the tumoral expression of the receptor from 7 to 310 fmol/mg protein. These findings have several implications. First, and above all, from an analytical point of view, it is conceivable that immunological measurement of EGFR may underestimate the presence of functional receptors below their limit of detection since high-affinity EGFRs preferentially represent the biologically active receptors [11–13]. This could explain, at least in part, why Chung and coworkers reported efficient EGFR targeting with cetuximab-based therapy in tumors apparently negative in IHC for EGFR [10]. In support of this opinion, it should be stressed that a recent structural study pointed out that cetuximab interacts exclusively with domain III of EGFR constituting the specific ligand binding region [18]. Another implication is that, for tumors carrying two classes of EGFRs, i.e. with both low- and high-affinity sites, as the low-affinity site number is superior to the high-affinity site number, the result from immunologically-based methods can be positive, due mainly to the presence of a majority of potentially inactive receptors with a low affinity for the ligand. In support of this view, most commercially available anti-EGFR antibodies for IHC, including the Dako EGFR kit, were obtained with A431 cells, which contain both low- and high-affinity EGFR [19]. This may explain the fact that there is no relationship between the response rate to cetuximab-based therapy and EGFR expression as reported so far [4, 9].

Other interesting findings in the present study derive from a comparison between tumor and paired normal tissue. Although pathological analysis was systematically performed to demonstrate the ‘normality’ of the control samples, it is not impossible that some molecular pre-tumor abnormalities may be present in these apparently normal mucosa samples. Bearing this in mind, there was first a significant positive correlation between the tumor and the corresponding normal tissue for the number of high-affinity sites (Figure 2). This means that...
common regulation of EGFR may exist for both tumor and normal tissue. One explanation for such a mechanism of common regulation could be of genetic origin. Indeed, there is a germinal polymorphism in the CA repeats of intron 1 of EGFR which has been demonstrated to be correlated to in vitro EGFR expression [20] and in vivo in tumors obtained by others [21] and by us [22]. This positive correlation between tumor and normal tissue for pharmacologically-relevant high-affinity EGFR may be one of the plausible explanations for the link observed between toxicity in normal tissue (cutaneous rash) and clinical outcome of patients treated with anti-EGFR drugs [7]. The second provocative finding arising from the comparison between tumor and normal adjacent tissue is that there was a significant lower number of EGFR in tumor compared to normal mucosa samples. A similar comparison was made previously by other investigators studying colorectal cancer and was based on similar EGFR binding assays. The conclusions varied, reporting either no statistically significant difference [14, 23, 24] or tumor expression higher than that of normal counterpart [25–27] or, as in the present study, a lower EGFR number in tumor tissue compared to the normal intestinal mucosa [28]. These discrepancies may be explained, to a large extent, by the limited number of samples analyzed as well as by the use in these studies of an EGF ligand which was not of human origin. The present study, using human EGF, covered a large series of 82 cases with 43 paired samples with tumor and corresponding normal mucosa. This counter-intuitive finding cannot be explained by chromosomal loss since, on the contrary, there are reports showing a gain in chromosome 7, which carries the EGFR gene, in colorectal cancer samples [29, 30]. A more plausible explanation in our opinion may be a ligand-mediated down-regulation of the EGFR since it has been shown that TGF is expressed at higher levels in colorectal carcinomas than in normal colorectal tissue [31]. The high expression of EGFR in normal mucosa may be one of the explanations for the digestive toxicity (diarrhea) frequently described during anti-EGFR therapy [32].

Taking into account the distribution of EGFRs through tumor staging there was no significant change in the EGFR number according to the severity of the malignancy (Table 2), although comparison of stage 0-1 versus stages II-III-IV showed on average a 1.9-fold lower high-affinity site number in the higher stage group. This subgroup analysis was performed on relatively small cohorts of patients and this can limit the power of the statistical analysis. More important in our opinion than the quantitative difference in EGFR through the increase in tumor staging was the qualitative difference. Interestingly, Kd values were lower in tumors with higher stages pointing to the fact that receptor affinity increased in more aggressive tumors. This observation strengthens the putative role of EGFR in the progression of colon cancer.

In conclusion, the present study points to the existence of a heterogeneous population of EGFR in colorectal cancer with a majority of tumors falling in the one binding site category with high-affinity and with potential biological significance and a minority of tumors with mixed receptors showing a preponderance of low-affinity sites with a lower biological activity. These data, along with the fact that immunologically-derived methods have limited sensitivity and are unable to distinguish between the two classes of EGFRs, may explain the disappointing results reported in the recent literature concerning the lack of predictiveity of the response to cetuximab-based therapy by IHC-derived methods. These data strongly suggest the use of alternative analytical methods such as gene copy number [33] or ligand binding assays as used in the present study requiring however to put the sample in liquid nitrogen. Moreover, there is the fact that, contrary to immunologically-based methods, preclinical studies have shown that tumor EGFR expression determined by a ligand binding assay was able to predict for anti-EGFR responsiveness from both murine studies [34, 35] and in vitro data [36]. It is hoped that the present report will be useful in assisting reconsideration of the predictive tools available for the identification of tumors putatively responsive to EGFR targeted therapy and thus improve management of this new treatment strategy.

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references


