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Abstract

Purpose The aim of this study was to investigate the association between expression of insulin-like growth factor-1 receptor (IGF1R) and its ligand, IGF-II, and disease-free survival (DFS) in patients with stage III colon cancer (CC).

Methods In this retrospective study we included consecutive patients who underwent curative surgery for stage III CC. IGF1R and IGF-II/IGF2 status were evaluated in tumour samples by immunohistochemistry and quantitative real-time PCR (qRT-PCR). Associations of markers with DFS were analysed using Cox proportional hazards models.

Results Hundred and fifty-one CC patients were included (median age, 66.6 years; female, 54.3%). Low levels of IGF1R and IGF-II protein expression were observed in 16.1% and 10.7% of the cases, respectively. No significant differences in clinicopathological characteristics between patients with tumours expressing low IGF1R or IGF-II protein levels and those with high levels were observed. A low IGF1R protein expression was found to be significantly associated with a shorter DFS (HR 3.32; 95% CI, 1.7-6.31; p = 0.0003), while no association was observed between IGF-II protein expression and DFS (HR 0.91; 95% CI, 0.28-2.96; p = 0.87). In a multivariate analysis, IGF1R protein status remained an independent prognostic factor for DFS (HR 2.73; 95% CI, 1.40-5.31; p = 0.003). Furthermore, we found that neither IGF1R nor IGF2 mRNA expression levels as measured by qRT-PCR correlated with the respective protein expression levels as assessed by immunohistochemistry. Neither of the mRNA expression levels was significantly associated with DFS.

Conclusions From our data we conclude that low IGF1R protein expression represents a poor prognostic biomarker in stage III colon cancer.

Keywords colon cancer • disease-free survival • IGF1R • IGF-II/IGF2 • MSI • KRAS/BRAF

1 Introduction

Colorectal cancer (CRC) is the second most common malignancy in developed countries [1]. Of patients with non-metastatic colon cancer (CC), though surgical resection offers a potential cure, approximately 30-50% with stage III disease develop recurrences and die from metastatic disease despite adjuvant chemotherapy [2]. The MOSAIC and NSABP-C07 phase III adjuvant trials have shown that adding oxaliplatin to 5-fluorouracil plus leucovorin (FL) resulted in improvements in survival of patients with stage III CC, thereby establishing FOLFOX as the standard of care [3,4]. More recent adjuvant trials failed to show survival benefits from adding the targeted agents bevacizumab (anti-VEGF) and/or cetuximab (anti-EGFR) to FOLFOX in patients with stage III CC [5-7], despite improved outcomes with these agents in metastatic CRC (mCRC) settings [8,9]. A better understanding of the molecular biology and signalling pathways underlying CRC oncogenesis may facilitate the identification of prognostic biomarkers and/or guide clinical decision-making for individual patients.

The insulin-like growth factor 1 receptor (IGF1R) signalling pathway has amply been shown to play a key role in regulating the growth and development of several cancer types, including CRC where its ligand IGF-II, produced in an autocrine manner, sustains the major proliferative effect [10-23]. Binding of the ligand to IGF1R activates its intrinsic tyrosine kinase activity, resulting in activation of intracellular signalling pathways, including the PI3K-AKT-TOR and RAS-RAF-MEK-ERK pathways [24]. It has been found that the percentage of CRCs positively expressing IGF1R protein may vary considerably between studies, i.e., from 28 to 99% in non-metastatic stage I-III CRC [13,14,17,20] and from 45 to 89 % in metastatic stage CRC [10,16,19,21]. Yet, several clinical trials using targeted therapies blocking IGF1R and/or epidermal growth factor receptor (EGFR) pathways conducted in patients with metastatic CRC have yielded disappointing results [19, 25-27].

In several studies it has been found that high IGF1R protein expression levels correlate with high-grades and advanced CRC tumour stages, increasing during tumour progression [13,14,22], and with CRC tumour size [20]. The prognostic impact of IGF1R protein expression has so far mainly been assessed in patients with metastatic disease [10,11,15,16,19,21]. These studies have

led to conflicting results with IGF1R overexpression being associated with a better prognosis in some studies [10,15,16], while others reported that high IGF1R expression levels or its phosphorylated form were associated with a shorter survival [11,2]. The prognostic impact of IGF1R expression in adjuvant settings has been poorly explored and the findings have remained controversial [17,28]. Nakamura et al. reported, for example, an increased risk of recurrence in patients with tumours with low IGF1R membrane expression levels, but not when considering total IGF1R protein expression [17]. Conversely, low IGF1R mRNA expression levels were found to be significantly associated with a longer disease-free survival (DFS), but not with a longer overall survival [28].

Here, we evaluated associations between DFS and the expression of IGF1R and its ligand IGF-II, at both protein and mRNA levels, in a series of patients receiving adjuvant chemotherapy for stage III CC.

2 Materials and methods

2.1 Study population

This retrospective study includes 151 patients who underwent curative surgical resection of histologically proven stage III CC between December 1997 and March 2006, either at the Saint-Antoine Hospital (n = 108) or at the Institut Mutualiste Montsouris (n = 43) (Paris, France). The follow-up protocol included physical examination with biological tests and measurement of the carcino-embryonic antigen (CEA) levels, pulmonary X-ray and abdominal ultrasonography or computed tomography every 3 months during the first 3 years after surgery, followed by every 6 months for 2 years.

2.2 IGF1R and IGF-II immunohistochemical analyses

Immunohistochemical analyses were performed on 4-µm sections of tissue microarrays constructed from 0.6-mm tissue cores obtained from formalin-fixed paraffin-embedded CC tissues comprising areas of normal tissues adjacent to the tumour tissues (3 cores per tumour). Sections

were dewaxed in xylene and rehydrated in a graded alcohol series. Haematoxylin and eosin staining was performed on each slide to confirm the quality of the tissues available for analysis. For IGF1R immunostaining, antigen retrieval was done by microwave heating in EDTA, pH 9.0. Next, the slides were incubated overnight at room temperature with a 1/100 dilution of a rabbit polyclonal anti-IGF1R antibody (#3027, Cell Signaling Technologies) and revealed using an Envision FLEX+ kit (Dako). For IGF-II immunostaining, antigen retrieval was done in a citrate buffer at pH 6.0, after which the slides were incubated with a 1/100 dilution of a rabbit anti-IGF-II polyclonal antibody (ab9574, Abcam) and revealed using a Novolink Polymer Detection System (Leica Biosystems). Expression was considered negative in case of complete absence of staining of neoplastic cells, provided that infiltrating normal cells showed positive staining. Otherwise labelling was considered not interpretable. Intensities were scored from 1 to 3 as follow: 1 for faint labelling (requiring varying microscope settings to be detected), 2 for clearly positive cells and 3 for very strong positive signals. For statistics analyses, 0 and 1 staining were classified as "low", while 2 and 3 were classified as "high". Examples of CC samples expressing various IGF1R levels are shown in Fig. 1.

2.3 IGF1R and IGF2 mRNA expression analysis by qRT-PCR

RNA extraction was performed on a subset of frozen tumour samples obtained from patients with stage III CC who were given FOLFOX. It should be noted that frozen samples from patients treated with 5FU were not available because surgery took place prior to establishing the collection of frozen samples. Samples were homogenized with a Mixer Mill MM300 (Qiagen) using a NucleoSpin TriPrep kit (Macherey-Nagel) with a DNA digestion step by DNase before RNA elution. RNA quality and quantity were assessed by electrophoresis on Experion™ RNA chips (Bio-Rad). Only RNA samples with an RNA quality indicator above 6 were subsequently used. Reverse transcription into cDNA was performed using M-MuLV (Moloney Murine Leukemia Virus) reverse transcriptase (Invitrogen) and random hexamers (Life Technologies). Quantitative measurements of transcripts were performed by real-time PCR on a LightCycler 480 instrument (Roche) using SYBR Green chemistry and specific primers. A 71-bp IGF1R region was amplified using 5'-AAAAACCTTCGCCTCATCC-3' and 5'-TGGTTGTCGAGGACGTAGAA-3' primers. A 153-bp IGF2

region was amplified using 5'-CGTCGCAGCCGTGGCATCGTTGA-3' and 5'-GCCCACGGGGTATCTGGGGAAG-3' primers. For each sample, gene expression was normalized to that of HPRT amplified as a 109-bp fragment using 5'-TAATTGGTGGAGATGATCT-3' and 5'-TGCCTGACCAAGGAAAGC-3' primers. The annealing temperature was 60°C, except for IGF2 for which it was 62°C. The relative quantity of each target gene mRNA was determined from replicate samples using the formula $2^{-\Delta\Delta Ct}$. The cut-off value to discriminate between high and low IGF1R expression groups was set at the median value.

2.4 Determination of MMR, *KRAS* and *BRAF* status

DNA mismatch repair (MMR) status was defined as previously described [29,30]. Also, the *KRAS* exon 2 and $BRAF^{V600E}$ mutation statuses were defined as previously described [31], except that DNA was purified from formalin-fixed paraffin-embedded tissue sections using a Maxwell® 16 FFPE Tissue LEV DNA Purification Kit (Promega). Real-time PCR was performed using TaqMan probes (Applied Biosystems) for *KRAS* exon 2 (c.34G>A/p.G12S, c.34G>C/p.G12R, c.34G>T/p.G12C, c.35G>A/p.G12D, c.35G>C/p.G12A, c.35G>T/p.G12V and c.38G>A/p.G13D) and *BRAF* (c.1799T>A/p.V600E) mutations, respectively [31].

2.5 Statistical analyses

Clinicopathological characteristics at baseline are described as quantitative variables with median and interquartile, and categorical variables with frequency and percentage. Comparisons between IGF1R and IGF-II/IGF2 expression levels in tumour tissues were made using a Mann Whitney test for medians and a χ^2 test (or Fisher test if appropriate) for proportions. DFS was defined as the date from curative tumour resection to the date of recurrence (local or distant) or death from any cause. Alive patients were censored at the date of last assessment. DFS curves were made using the Kaplan-Meier method and compared between groups using a log-rank test. Follow-up median times were estimated using the reverse Kaplan-Meier method. Hazard ratios and their 95% confidence intervals (95%CI) were estimated using Cox regression. All clinicopathological and molecular characteristics were investigated using a univariate Cox model. Correlations were

checked and significant variables at the 0.10 level in univariate analyses were subsequently introduced in a multivariate Cox model.

3 Results

3.1 Patient characteristics

The main demographic and clinicopathological characteristics of patients are listed in Table 1. A total of 151 stage III CC patients were included (median age, 66.6 years; female, 54.3%). According to the period of inclusion (before or after the results of the MOSAIC study), patients received adjuvant chemotherapy during six months of 5-fluorouracil plus leucovorin combination alone (5FU, n = 72) or with oxaliplatin (FOLFOX, n = 79). The IGF1R and IGF-II tumour protein expression levels were determined by immunohistochemistry (IHC) in 149 (98.7%) and 134 (88.7%) of the patient samples, respectively. Tumour IGF1R and IGF-II protein levels were considered as low in 24 (16.1%) and 13 (9.7%) patient samples, respectively (Table 1). The clinicopathological characteristics did not vary significantly according to IGF1R or IGF-II protein expression levels, but patients whose tumour expressed low IGF1R protein levels tended to be more frequently stage IIIC than stage IIIA/IIIB (65.6% *vs* 45.8%, $p = 0.07$) (Table 1). *KRAS* exon 2 and $BRAF^{V600E}$ mutations were detected in 27.2% and 8.2% of the cases, respectively, and were not found to be associated with the IGF1R and/or IGF-II expression levels. For the overall population, the median follow-up was 58.4 months (95%CI, 54.57-61.74). At the end of the follow-up 42 patients had relapsed or died.

3.2 IGF1R expression correlates with DFS

IGF1R expression analysis by IHC.

In tumours expressing high levels of IGF1R, the protein was predominantly found to be localized in the cytoplasm, with some tumours showing membrane labelling as well. In the overall population, the 3-year DFS rates were 80% and 52% for patients whose tumours expressed high and low IGF1R protein levels, respectively (HR 3.32; 95% CI, 1.75-6.31; log-rank test, $p = 0.0003$) (Fig.

2a). When analyses were performed according to adjuvant chemotherapy regimens, patients whose tumours expressed low IGF1R protein levels were found to exhibit shorter DFS for either the 5FU (HR 3.54; 95% CI, 1.56-8.04; log rank p = 0.0013) (Supplementary Fig. 1a) or the FOLFOX (HR 3.10; 95% CI, 1.08-8.92; log rank p = 0.027) (Supplementary Fig. 1b) groups. After adjustment to age at diagnosis and tumour stage, we found that low IGF1R protein expression remained significantly associated with a shorter DFS (HR 2.73; 95% CI, 1.40–5.31; p = 0.0031) (Table 2).

IGF1R mRNA expression analysis by qRT-PCR.

IGF1R mRNA expression levels were assessed in tumours from 69 patients with stage III CC treated with FOLFOX. We found that IGF1R mRNA expression had no impact on DFS (Fig. 3a). In keeping with this, no correlations between IGF1R mRNA levels and protein intensities evaluated by immunohistochemistry were observed (Fig. 3b).

3.3 IGF-II/IGF2 expression does not correlate with DFS

IGF-II protein expression analysis by IHC.

Using either the Cox univariate model or in the multivariate model, no association between IGF-II protein expression and DFS was detected (HR 0.94; 95% CI, 0.29-3.07, p = 0.92) (Table 2). The IGF-II protein expression level did not exhibit prognostic value when considering both the overall population (Fig. 2b) or subgroups of patients treated with 5FU or FOLFOX (Supplementary Fig. 2).

IGF2 mRNA expression analysis by qRT-PCR.

We additionally found that the IGF2 mRNA expression levels assessed in tumours from 72 patients with stage III CC treated with FOLFOX were not significantly associated with DFS (Fig. 4a). As reported for IGF1R, no correlation was observed between IGF2 mRNA levels and IGF-II protein levels evaluated by IHC (Fig. 4b).

4 Discussion

Our results indicate that low tumour IGF1R protein expression is associated with a shorter DFS compared to high IGF1R protein expression in CC patients. The prognostic value of IGF1R protein expression remained statistically significant after adjustment for usual prognostic factors and irrespective the regimen of adjuvant chemotherapy (5FU alone or combined with oxaliplatin).

The prognostic value of IGF1R expression, either at the protein or at the mRNA level, has been investigated in CRC patients at all stages of the disease, from colonic adenomas to metastatic tumour stages [13,14,22]. In a retrospective study including 48 patients, IGF1R protein expression was found to be higher in metastatic and primary CRCs (n = 36) compared to colon adenoma (n = 12) or normal colonic mucosa (n = 34) [13]. The authors found that a high IGF1R protein expression, as well as a high percentage of positive cells, correlated with a poor differentiation and high-stage tumours, but not with the survival of patients whose tumours were considered positive when more than 5% of the cells were immunoreactive. Two recent studies reported that IGF1R protein and/or mRNA expression increased with TNM stage, with the degree of differentiation and with lymphatic invasion, and that high IGF1R protein expression levels were associated with a worse prognosis [14,22]. In one of these studies, IGF1R protein expression was detected in all 98 CRC cases tested, half of them showing a high expression, in sharp contrast to adenomas of which < 10% were positive [22]. Univariate analysis revealed that patients whose tumours showed a high IGF1R expression exhibited significantly lower overall survival rates compared to patients with a low IGF1R expression. Although no multivariate analysis of IGF1R prognostic value was reported, we believe that these observations reflect the fact that patients with lymph node or distant metastasis, known to have a worse prognosis than nonmetastatic patients, were predominantly in the group of patients with a high IGF1R protein expression [22]. IGF1R expression increased with disease progression, with two thirds of stage III and IV cases being highly positive, compared to only one-fourth of stage I and II cases [22]. The second study included 121 Chinese patients with CC, half of them having local (stage III) or distant (stage IV) metastases [14]. High IGF1R protein expression was observed in 62% and 85% of stage I-II and stage III-IV cases, respectively. Multivariate Cox proportional hazard regression analysis revealed that high IGF1R protein expression served as an independent poor prognostic factor. Because the survival of patients with lymph node metastasis is much longer compared to patients with distant metastasis, it would be interesting to investigate the prognostic value of IGF1R in stage III and in stage IV patients,

separately. So far, only two clinical studies investigated the prognostic value of IGF1R protein expression in patients with nonmetastatic CRC [17,18]. In one of these studies IGF1R and IGF-II protein expression was analysed in tumour samples obtained from 713 patients with stage II (70%) or stage III (30%) colon (45%) or rectal (55%) cancer [18]. In this study, IGF1R protein expression was scored positive when more than 50% of the tumour cells showed specific staining, which was observed in nearly all cases (99.6%), thus hampering assessment of its impact on survival. Conversely, these authors reported that IGF-II protein expression, which was positive in 12.6% of the cases, was significantly associated with a worse clinical outcome in a univariate analysis, though it lacked prognostic relevance in a multivariate analysis. In the second study, IGF1R tumour protein expression was analysed in 161 patients with curatively resected stage III CRC, who had not received chemo- and/or radiotherapy before or after surgery [17]. The proportion of patients with rectal cancer (54%) was similar to that in the previous study. Samples with > 50% tumour cells displaying strong membrane labelling were assigned to the high IGF1R group (28% of the cases) were found to be associated with a significantly lower recurrence rate. These observations are in agreement with our current findings suggesting that low IGF1R expression is associated with a poor prognosis, irrespective the adjuvant chemotherapy regimen, 5FU alone or with oxaliplatin.

In keeping with our observations, a favourable prognostic value of IGF1R protein expression was further reported in various series of patients with mCRC [10,16]. In a cohort of 85 chemo-refractory mCRC patients exposed to cetuximab-based therapy, the survival of patients with a high IGF1R protein expression (representing 74.3% of patients) was found to be significantly longer than that of patients with a low expression, although no significant differences were observed in times to progression or response rates [10]. The notion that a high IGF1R protein expression level may be a favourable prognostic factor was substantiated in another series of 73 patients with mCRC treated with a cetuximab-containing regimen in which IGF1R protein overexpression was found to be significantly associated with a prolonged overall survival, without affecting the progression-free survival or overall response rate [16]. The prognostic value of IGF1R has also been investigated at the mRNA level in a cohort of 110 patients with chemo-refractory mCRC receiving cetuximab monotherapy for advanced disease [15]. A higher IGF1R mRNA level was found in nearly two-thirds of the patients and to be significantly associated with a disease-control rate benefit and a longer progression-free survival in patients with non-mutated *KRAS* tumours.

Nevertheless, other studies have reported divergent results or a lack of any association between IGF1R expression and clinical outcome [11,19,21]. In a study performed on 91 patients receiving fluoropyrimidine-based chemotherapy for recurrent or residual tumours IGF1R overexpression, defined as membrane expression in ≥ 50% tumour cells, was observed in half of the cases and was found to be associated with a shorter overall survival, serving as an independent biomarker for a poor prognosis [21]. The prognostic value of IGF1R protein expression has also been investigated in a series of 538 patients with mCRC receiving irinotecan and cetuximab [19]. The authors concluded that there was no association between IGF1R overexpression, observed in nearly half of the tumours, and progression-free survival [19]. A more recent study, performed on 470 mCRCs, focused on nuclear expression of the phosphorylated form of the receptor, pIGF1R, and reported that nuclear pIGF1R staining detected in one-third of the patients was significantly correlated with a poor overall survival, without impacting the time to progression [11]. As of yet, only a single study investigated IGF1R expression at both the protein and mRNA levels in CC [14]. In their series of 121 patients with CC at various stages, higher protein and mRNA IGF1R expression levels were both found to be associated with the degree of differentiation, the presence of lymph node or distant metastases and with lymphatic invasion. Associations between these clinicopathological features and IGF1R expression were statistically found to be more robust when considering protein expression. Unfortunately, the authors did not report whether IGF1R protein expression was correlated with mRNA expression. In our series of 151 patients with stage III CC, IGF1R protein expression was detected by immunohistochemistry in 149 samples. The mRNA levels were determined in a subgroup of 69 patients. We found that only the protein expression level was associated with disease-free survival and that there was no correlation between IGF1R protein and mRNA levels, illustrating the complexity of the mechanisms involved in the regulation of receptor tyrosine kinase protein expression. Another explanation for the discrepancies noted between studies may reside in the widely variable scoring systems used for IGF1R protein expression. Most studies consider the percentage of cells expressing IGF1R exclusively, but differ in the thresholds used [11,14,17,18,20,21], or take into account the intensity only, provided that there is a minimal percentage of cells expressing IGF1R, starting from no more than 1% [19]. Other studies use a semi-quantitative complex composite scoring system based on both the

percentage of positive cells and the staining intensity [10,16, 19,22]. Although comparing the robustness of the various scoring systems, based on number of positive cells, staining intensities or both, seems hazardous due to the variability in criteria, none showed a clearly stronger predictive value. In addition, the use of various antibodies and antigen retrieval methods, as well as the fact that some studies focused on membrane IGF1R expression only [17] or on nuclear activated phosphorylated IGF1R staining [11], may have contributed to the discordant observations. In our study, we opted for a simple scoring of IGF1R protein expression, based on staining intensity of tumour cells only, without distinguishing membrane from cytoplasmic or nuclear location, so that it might be easier to translate into routine investigation, being more reproducible and robust between laboratories. Of note, when expressed at high levels, IGF1R was mainly localized in the cytoplasm, with some tumours also displaying membrane expression. Yet another explanation for discrepancies among studies may be that patients with CRC, especially those with metastatic disease, may have been treated according to different regimens [10,11,16,19], and/or preselected on the basis of *KRAS*^{WT} exon 2 [19], leading to unreliable comparisons. In order to avoid this issue, our study was conducted on a series of patients with stage III CC receiving 5FU or FOLFOX as adjuvant therapy. The main limitations of our study are its retrospective nature and the relatively small sizes of the subgroups of patients whose tumours expressed low levels of IGF1R or IGF-II.

In stage III CC, the deficient MMR/MSI phenotype is known to serve as a predictive marker of lack of efficacy of 5FU adjuvant chemotherapy [29,32,33], and a good prognostic factor for patients treated with FOLFOX adjuvant chemotherapy [30,34,35]. In our study, no significant association between MMR status and DFS was observed, most likely because the number of patients treated with 5FU was comparable to those receiving FOLFOX, thus balancing the opposite prognostic impact of deficient MMR/MSI on these two groups of patients (the HR for MSI *vs* MSS were 2.07 and 0.71 in the 5FU and FOLFOX adjuvant groups, respectively; these differences were not statistically significant). The prognostic impact of *RAS/BRAF* mutations in adjuvant settings has been investigated in several studies [36-39]. Recent analyses of large cohorts of patients with stage III CC receiving FOLFOX-based adjuvant chemotherapy revealed that *KRAS* exon 2 and *BRAF*^{V600E} mutations were associated with a shorter DFS compared to those without *KRAS* exon 2

or *BRAF*^{V600E} mutations (double wild type tumours), especially for patients with proficient MMR/ MSS tumours [37, 39]. In our study, neither *KRAS* exon 2 nor *BRAF*^{V600E} mutations were found to be associated with a shorter DFS (Table 2). Among the subgroup of proficient MMR/MSS cases, a trend towards a shorter DFS was observed for those with *KRAS* exon 2 mutated tumours compared to those with double wild type tumours, but the difference was not statistically significant (HR 0.79; p = 0.54, data not shown). Analysing the prognostic impact of *BRAF* mutational status among proficient MMR/MSS cases was not possible in our cohort due to the limited number of patients with *BRAF*^{V600E} mutated tumours in this subgroup. Since the IGF signalling pathway and *RAS* mutations both affect the metabolism of cancer cells, one may assume that IGF1R expression levels and *KRAS/BRAF* mutations are confounding. However, we failed to find any association between IGF1R or IGF-II protein expression and the presence of mutations in *KRAS* or *BRAF*. Moreover, no interaction between IGF1R expression and *KRAS* mutations could be ascertained (data not shown). Interactions between IGF1R expression and *BRAF* mutations could not be tested because there was only a single tumour with a *BRAF* mutation in the IGF1R low expression group.

Interestingly, we found that a high IGF1R protein expression served as a favourable prognostic biomarker irrespective adjuvant chemotherapy. Although seemingly paradoxical, such an association has been reported in several cancers, including mCRC [17]. To explain their results, the latter authors proposed that high IGF1R protein expression could reflect an absence of ligands, which in turn could lead to weak receptor signalling. Conversely, they proposed that a low IGF1R content may indicate that the receptor is highly active and subsequently down-regulated upon ligand binding, thus stimulating tumour growth leading to a poor prognosis. In favour of this hypothesis, an inverse correlation between IGF1R expression and IGF1 levels has been reported [40,41]. We investigated IGF-II protein expression because it is a major IGF1R ligand known to act as an autocrine/paracrine growth factor in CRC [42] and to serve as a powerful biomarker to predict liver metastasis in patients with CRC [43]. In our series, we found that the IGF1R protein expression levels were not related to those of IGF-II, an observation that does not support the former hypothesis. It would be interesting to investigate whether IGF1R activation may be driven by IGF1, another IGF1R ligand. According to their second hypothesis, IGF1R protein expression may also define relatively well-differentiated tumours that need IGF to proliferate [17]. In our series of patients, the percentage of tumours exhibiting a high IGF1R expression was higher in well and

moderately differentiated tumours (86.4%), compared to poorly differentiated tumours (75%). Although this difference was not statistically significant, possibly due to lack of power, it underscores this latter hypothesis.

In conclusion, our data indicate that a low IGF1R protein expression in stage III CC, based on immunohistochemistry, may predict a poor outcome. This notion warrants confirmation in a larger prospective series of patients.

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Authors' contributions AZ, FP and YLB designed the study. MS, PV and JFF provided tumour samples. AZ, YP, BG, JT and CL took care of the patients. JH undertook the statistical analysis. AZ, CC, MS, HB, CDM, FM, CG, JFF, YLB and FP contributed to the results and their interpretation. AZ and FP supervised the acquisition of the data. AZ and FP wrote the article, which was critically read by JH, CDM and YLB. All authors approved the final version of the manuscript, including the authorship list.

Compliance with ethical standards

This study has been approved by the “Comité de Protection des Personnes - Ile-de-France VI” ethics committee and has been performed in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest

All authors declare to have no conflict of interest.

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Figure captions

Fig. 1 Examples of tumours exhibiting different IGF1R protein expression levels. IGF1R intensity was scored as 1 for faint labelling (**a**), 2 for clearly positive cells (**b**) and 3 for strong signals (**c**)

Fig. 2 Association between IGF1R and IGF-II protein expression levels and disease-free survival (DFS) in patients with stage III colon cancer. Kaplan-Meier curves of DFS of stage III patients according to IGF1R (**a**) and IGF-II (**b**) protein expression levels determined by IHC. Hazard ratios (HR) and their 95% confidence interval [95%CI] were estimated using Cox regression

Fig. 3 Association between IGF1R mRNA expression levels and disease-free survival (DFS) in patients with stage III colon cancer and correlation between IGF1R mRNA and protein levels. IGF1R mRNA expression was estimated by qRT-PCR relative to HPRT expression in tumour samples obtained from patients with stage III colon cancer treated by FOLFOX (**a**). To compare DFS curves between the groups with high and low IGF1R expression, the median IGF1R level was used as cut-off value. Relative IGF1R mRNA expression was analysed as a function of its protein level defined by IHC in counterpart tumour samples (**b**)

Fig. 4 Association between IGF2 mRNA expression levels and disease-free survival (DFS) in patients with stage III colon cancer and correlation between IGF2 mRNA and IGF-II protein levels. IGF2 mRNA expression was estimated by qRT-PCR relative to HPRT expression in tumour samples obtained from patients with stage III colon cancer treated by FOLFOX (**a**). To compare DFS curves between the groups with high and low IGF2 mRNA expression, the median IGF2 level was used as cut-off value. Relative IGF2 mRNA expression was analysed as a function of its protein level defined by IHC in counterpart tumour samples (**b**)

Electronic Supplementary Fig. 1 Association between IGF1R protein expression level and disease-free survival (DFS) in patients with stage III colon cancer regarding

chemotherapeutic regimen. Kaplan-Meier curves of DFS of patients with stage III colon cancer treated with 5FU (**a**) or FOLFOX (**b**), according to IGFIR protein expression level determined by IHC, as described in the Materials and methods section

Electronic Supplementary Fig. 2 Association between IGF-II protein expression level and disease-free survival (DFS) in patients with stage III colon cancer regarding chemotherapeutic regimen. Kaplan-Meier curves of DFS of patients with stage III colon cancer treated with 5FU (**a**) or FOLFOX (**b**), according to IGF-II protein expression level determined by IHC, as described in the Materials and methods section

Table 1.

Clinicopathological characteristics of patients with stage III colon cancer according to IGF1R and IGF-II tumour protein expression

	All patients n = 151	IGF1R^{low} n = 24	IGF1R^{high} n = 125	*P*	IGF-II^{low} n = 13	IGF-II^{high} n = 121	*P*
Age at diagnosis (years)							
Median	66.57	68.38	65.22		65.22	66.85	
Min-max	30.79-82.80	36.76 - 82.80	30.79 - 82.03		46.01-76.03	30.79-82.80	
Gender							
Male	69 (45.70)	10 (41.67)	57 (45.60)	0.72	7 (53.85)	53 (43.80)	0.49
Female	82 (54.30)	14 (58.33)	68 (54.40)		6 (46.15)	68 (56.20)	
Tumour location							
Proximal	51 (33.77)	7 (29.17)	43 (34.40)	0.62	5 (38.46)	43 (35.54)	1.00
Distal	100 (66.23)	17 (70.83)	82 (65.60)		8 (61.54)	78 (64.46)	
Differentiation grade							
Well/Moderate	128 (84.77)	18 (75.00)	108 (86.40)	0.21	11 (84.62)	103 (85.12)	1.00
Poor	23 (15.23)	6 (25.00)	17 (13.60)		2 (15.38)	18 (14.88)	
Stage							
III A / III B (Tx, N1)	94 (62.25)	11 (45.83)	82 (65.60)	0.07	7 (53.85)	78 (64.46)	0.55
III C (Tx, N2)	57 (37.75)	13 (54.17)	43 (34.40)		6 (46.15)	43 (35.54)	
Bowel perforation / obstruction							
No	131 (86.75)	19 (79.17)	110 (88.00)	0.33	10 (76.92)	107 (88.43)	0.22
Yes	20 (13.25)	5 (20.83)	15 (12.00)		3 (23.08)	14 (11.57)	
MMR status							
dMMR	19 (12.58)	4 (16.67)	15 (12.00)	0.51	1 (7.69)	16 (13.22)	1.00
pMMR	132 (87.42)	20 (83.33)	110 (88.0)		12 (92.31)	105 (86.78)	
***KRAS*/*BRAF* status**							
KRAS exon 2 mutated	40 (27.21)	6 (27.27)	34 (27.64)		4 (30.77)	33 (27.97)	
BRAF^{V600E} mutated	12 (8.16)	1 (4.55)	11 (8.94)	0.78	2 (15.38)	9 (7.63)	0.42
KRAS/*BRAF* double wild type	95 (64.63)	15 (68.18)	78 (63.41)		7 (53.85)	76 (64.41)	
Adjuvant chemotherapy							
5FU	72 (47.68)	13 (54.17)	59 (47.20)	0.53	9 (69.23)	56 (46.28)	0.15
FOLFOX	79 (52.32)	11 (45.83)	66 (52.80)		4 (30.77)	65 (53.72)	
Events (death/recurrence)	42 (27.81)	14 (58.33)	28 (22.40)	**0.0003**	3 (23.08)	35 (28.93)	0.76
Follow-up in months							
Median	58.4	65.26	57.83	0.49*	56.48	59.87	0.38*
(95% CI)	(54.57-61.74)	(41.74-103.13)	(53.65-61.38)		(15.07-68.36)	(55.53-66.12)	

Abbreviations: MMR, Mismatch Repair ; dMMR, deficient MMR ; pMMR, proficient MMR ; *KRAS*/*BRAF* double wild type, tumours devoid of *KRAS* exon 2 and *BRAF*^{V600E} mutations. The IGF1R and IGF-II protein expression in tumour was assessed by immunohistochemistry. Intensity was scored from 1 to 3 as follow: 1 for faint labelling (requiring to vary the microscope settings to be detected), 2 for clearly positive cells and 3 for very strong signals. For the statistics analyses, 0 and 1 staining were considered as "low", while 2 and 3 were classified as "high". *logrank *P* value.

Table 2.
Univariate and multivariate analyses of IGF1R and IGF-II tumour protein expression for DFS in patients with stage III colon cancer

		Univariate model			Multivariate models					
					Association between IGF1R and DFS			Association between IGF-II and DFS		
		n (events)	HR (CI 95%)	P value	n (events)	HR (CI 95%)	P value	n (events)	HR (CI 95%)	P value
					149 (52)			132 (38)		
IGF1R	Low vs high	149 (42)	3.32 (1.75-6.31)	**0.0003**		2.73 (1.40-5.31)	**0.0031**		2.61 (1.30-5.25)	**0.0070**
IGF-II	Low vs high	134 (38)	0.91 (0.28-2.96)	0.8732					0.94 (0.29-3.07)	0.9206
Age at diagnosis (years)	≥70 vs <70	151 (42)	1.73 (0.93-3.21)	0.0818		1.91 (1.03-3.56)	**0.0414**		2.03 (1.06-3.88)	**0.0330**
Gender	Female vs male	151 (42)	0.92 (0.50-1.68)	0.7797						
Tumour location	Proximal vs distal	151 (42)	0.97 (0.51-1.84)	0.9204						
Differentiation grade	Poor vs well/moderate	151 (42)	1.84 (0.88-3.84)	0.1069						
Stage	C vs A/B	151 (42)	2.29 (1.24-4.20)	**0.0078**		2.02 (1.07-3.80)	**0.0293**		2.55 (1.31-4.97)	**0.0059**
Bowel perforation/obstruction	Yes vs no	151 (42)	1.56 (0.69-3.51)	0.2864						
MMR status	pMMR vs dMMR	151 (42)	0.66 (0.30-1.50)	0.3231						
***KRAS/BRAF* status**		147 (39)		0.9763						
***KRAS* exon 2**	$KRAS^{mut}$ vs double wild type		1.08 (0.53-2.19)	0.8417						
***BRAF*^{V600E}**	$BRAF^{mut}$ vs double wild type		0.97 (0.29-3.21)	0.9584						
Adjuvant chemotherapy	FOLFOX vs 5FU	151 (42)	0.63 (0.34-1.17)	0.1454						

Abbreviations: MMR, Mismatch Repair ; dMMR, deficient MMR ; pMMR, proficient MMR ; double wild type, tumours devoid of KRAS exon 2 and $BRAF^{V600E}$ mutations

Multivariate Cox model was performed on variables potentially predictive of the risk of events in univariate analysis (threshold, 0.10).

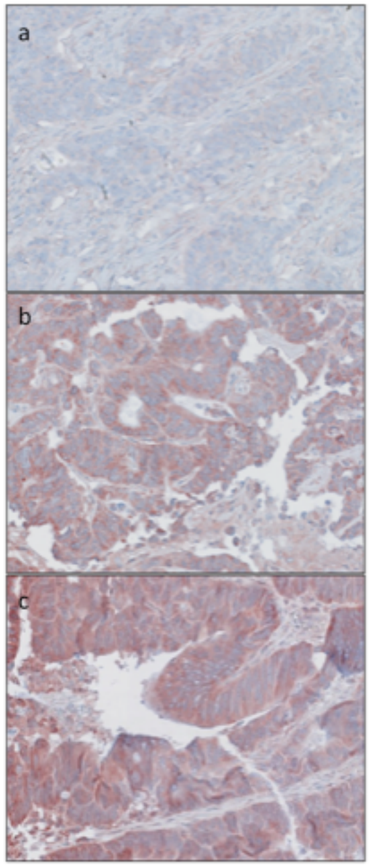


Fig. 1

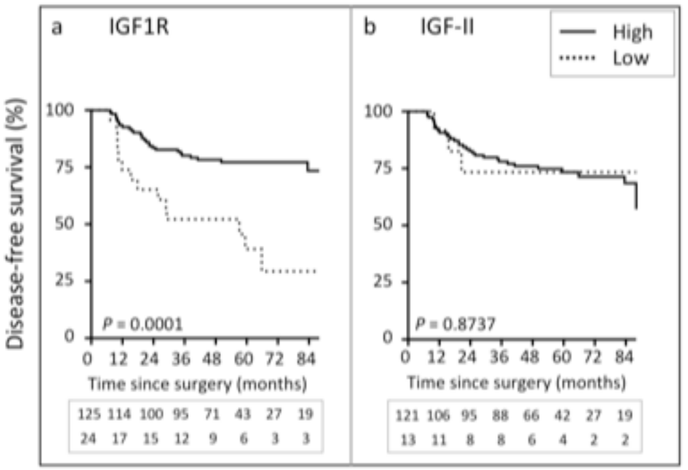

a
IGF1R
b
IGF-II
High
Low
Disease-free survival (%)
100
75
50
25
0
P = 0.0001
P = 0.8737
0 12 24 36 48 60 72 84
Time since surgery (months)
125 114 100 95 71 43 27 19
24 17 15 12 9 6 3 3
121 106 95 88 66 42 27 19
13 11 8 8 6 4 2 2

Fig. 2

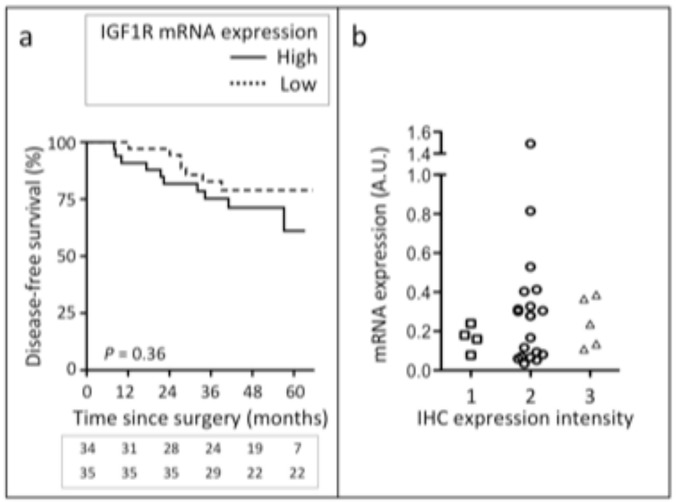

a
IGF1R mRNA expression
High
Low
Disease-free survival (%)
100
75
50
25
0
P = 0.36
0 12 24 36 48 60
Time since surgery (months)
34 31 28 24 19 7
35 35 35 29 22 22
b
mRNA expression (A.U.)
1.6
1.4
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3
IHC expression intensity

Fig. 3

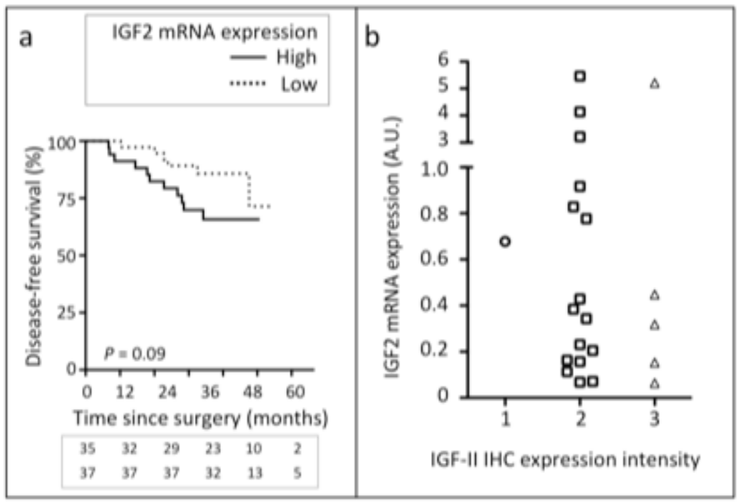
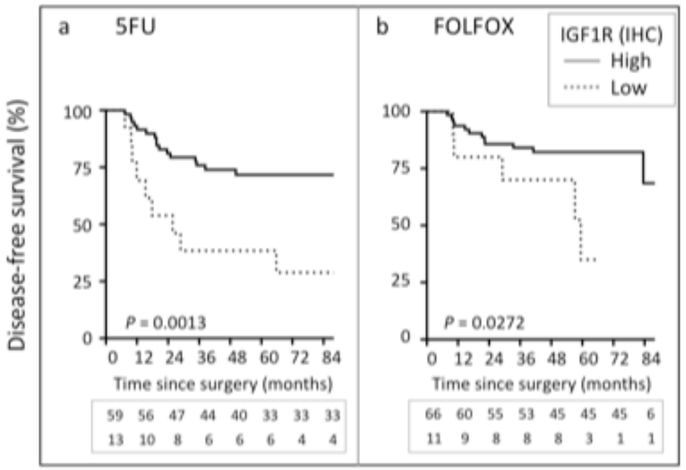
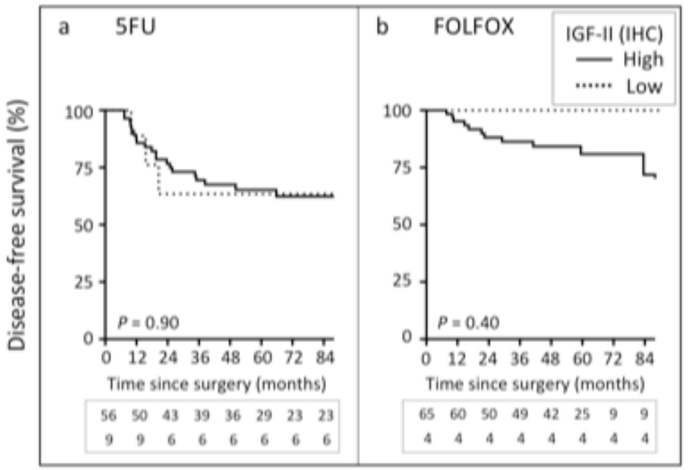

a
IGF2 mRNA expression
High
Low
Disease-free survival (%)
100
75
50
25
0
P = 0.09
0 12 24 36 48 60
Time since surgery (months)
35 32 29 23 10 2
37 37 37 32 13 5
b
IGF2 mRNA expression (A.U.)
6
5
4
3
1.0
0.8
0.6
0.4
0.2
0
1 2 3
IGF-II IHC expression intensity

Fig. 4



Supplementary Fig. 1



Supplementary Fig. 2