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Original article

Impact of neoadjuvant therapy on cancer-associated fibroblasts in rectal cancer

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ABSTRACT

Background and purpose: Cancer-associated fibroblasts (CAFs) are increasingly recognised as promoters of tumour progression. It is poorly investigated whether cancer management protocols, such as neoadjuvant radio(chemo)therapy, have an impact on CAFs and, by consequence, on tumour progression. This prompted us to study the impact of neoadjuvant radio(chemo)therapy on the α-SMA/epithelial area ratio in rectal cancer, and the impact of this ratio on recurrence-free survival.

Material and methods: Immunohistochemistry for the CAF marker α-SMA and the proliferation marker Ki67 was performed on sections from 98 rectal cancers of which 62 had undergone neoadjuvant radio(chemo)therapy.

Results: Computer-assisted quantitative analysis showed that the α-SMA/neoplastic epithelial area ratio was higher after neoadjuvant therapy, and that rectal cancers with high α-SMA/epithelial area ratio had low proliferation rates. Interestingly, the α-SMA/epithelial area ratio was an adverse prognostic factor with regard to recurrence-free survival in univariate analysis. In addition, multivariate analysis showed that an α-SMA/epithelial area ratio above 1 provides an independent prognostic value associated with a poor recurrence-free survival.

Conclusion: These results suggest that neoadjuvant treatment has an impact on CAFs in rectal cancer. The correlation of CAFs with decreased recurrence-free survival and abundant experimental data in the literature suggest that under certain circumstances, not yet very well understood, CAFs may favour tumour progression.

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Rectal cancer remains one of the leading causes of cancer mortality. Because of the specific anatomy and biology of low rectal cancer, surgical resection alone is associated with a considerable incidence of locally recurrent disease. In locally advanced disease, preoperative radiotherapy or radiochemotherapy improves local control in patients undergoing total mesorectal excision. A recent study on data from five large European randomised clinical trials for locally advanced rectal cancer revealed, however, different populations: highly responsive patients, good responsive curable patients, and patients with early and late recurrence regardless of pathologic complete response status [1]. Although neoadjuvant therapy reduces local recurrence by more than 50% relative to surgery alone, there is no overall survival benefit [2], even not after the addition of 5-Fluorouracil based adjuvant chemotherapy [3].

Tumours are complex ecosystems consisting of cancer cells embedded in an extracellular matrix scaffold populated by cancer-associated fibroblasts (CAFs), endothelial cells and immune cells [4]. This microenvironment is becoming increasingly recognised to have an important role in either restraining or promoting tumour progression [5]. The non-transformed elements may display relatively few genomic lesions and be more likely to display sustained responses to therapy [6–9]. Indeed, the microenvironment has become a major focus in therapeutic response e.g., by inhibition of tumour vasculature through blockade of endothelial proliferation signals, and exciting clinical studies of antibody

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therapies that target so-called immune checkpoint molecules like CTLA4 and PD-L1 have re-fired longstanding hopes in cancer immunotherapy [10].

A so far underappreciated but equally important compartment of the tumour ecosystem is the CAF, also called myofibroblast. CAFs are activated fibroblast-like cells recruited from local or distant sites that express α-smooth muscle actin (α-SMA) and vimentin whereas quiescent resident fibroblasts do not express α-SMA [11,12]. CAFs produce an extracellular matrix enriched in type III and V collagen, which is considered to be responsible for the firm consistency of many carcinomas [13].

CAFs exert pro-invasive and pro-metastatic activities as evidenced by experimental and clinical observations [14,15]. In colorectal cancer, the number of stromal myofibroblasts [16], vimentin expression [17] and degree of stromal desmoplasia [18–20] have been associated with patient prognosis; the stroma/neoplastic cells ratio has also been identified as an adverse prognostic factor in this cancer type [21–24].

CAFs have been proposed as putative targets for therapy. The issue raised here is slightly different, investigating whether or not neoadjuvant radiochemotherapy may have an impact on the presence of α-SMA positive CAFs and, by consequence, be associated with tumour recurrence.

There is experimental evidence that neoadjuvant radiochemotherapy stimulates the formation and the pro-tumourigenic activity of CAFs [25], though clinical evidence associating such therapy with CAFs and outcome is lacking. We investigated whether the α-SMA positive CAFs/epithelial area ratio increases after radiochemotherapy and whether this increase is associated with recurrence. To investigate this aspect of the tumour ecosystem, we chose locally advanced rectal cancers because most of these receive neoadjuvant irradiation often combined with chemotherapy.

Materials and methods

Patients

We retrospectively analysed tumour samples from consecutive patients operated for rectal adenocarcinoma between 2000 and 2010 at the Erasme University Hospital (Brussels, Belgium) and for whom sufficient residual material and clinical data were available. Sex, age, pTNM status and data on neoadjuvant and adjuvant treatment were obtained by reviewing the medical records of each patient. Recurrence-free survival (RFS) was defined as time between date of surgery and date either of tumour recurrence at imaging or of the latest evaluation without recurrence evidence. Since median follow-up was 53 months, overall survival was not studied. The study was approved by the local ethics board.

Histopathological evaluation of tumour regression grade

Neoadjuvant therapy-induced tumour regression was assessed according to the grading system described by Dworak et al.: Grade 0, no regression; Grade 1, minimal regression (dominant tumour mass with obvious fibrosis and/or vasculopathy); Grade 2, moderate regression (predominantly fibrotic changes with few tumour cells or groups in fibrotic tissue); Grade 3, good regression (very few tumour cells in fibrotic tissue); and Grade 4, total regression (no tumour cells, only fibrotic mass) [26].

Tissue microarray construction

Tissue microarray (TMA) blocks were constructed as described previously [27], with a manual microarrayer (Minicore, Alphelys, France). Six cores of 600 microns diameter each were sampled in 5 areas of the surgical specimen for each patient: in the tumoural front, in the tumoural centre, in the metastatic lymph nodes (if present), in the non-neoplastic rectal mucosa (distal resection margin) and in the non-neoplastic colonic mucosa (proximal resection margin).

Immunohistochemistry and immunostaining evaluation

Immunohistochemical stainings with antibodies against α-SMA (clone αsm-1, 1/100, Menarini, Zaventem, Belgium), a marker for myofibroblasts [28], and Ki67 (clone MIB-1, 1/1000, Dako, Glostrup, Denmark), a proliferation marker, were performed on consecutive 4-μm thick sections. Stainings were performed on the BOND-MAX (Leica, Wetzlar, Germany).

We used the NanoZoomer 2.0-HT slide scanner (Hamamatsu, Louvain-La-Neuve, Belgium) for TMA core image acquisition and the NDP viewer software (Hamamatsu) to visually assess slides and image quality. The stromal and the epithelial compartments were delineated by a pathologist (LV) using the NDP viewer software. The delineated areas were imported in the Visiopharm software package (Visiopharm, Hoersholm, Denmark) in order to quantify the epithelial surface area, the α-SMA positive area and the Ki67 labelling index. The α-SMA/neoplastic epithelial area ratio was calculated for the tumoural front, the tumoural centre and the metastatic lymph nodes; the α-SMA/epithelial area ratio was calculated for the normal colonic and rectal mucosa. The Ki67 labelling index was defined as the percentage of the epithelial nuclei that were Ki67-positive. For each tumour, global scores were calculated by combining the tumoural front and centre. To be included in the statistical analysis, at least two TMA cores were required for the global tumoural ratio, the normal rectal and colonic mucosa.

Statistical analysis

To compare the groups of patients with and without neoadjuvant therapy, we used the non-parametric Mann–Whitney *U* test. The correlation between the α-SMA/epithelial area ratio and the Ki67 labelling index was quantified by the Spearman correlation index. Multivariate survival analyses were performed using standard Cox regression. We first analysed the set of clinicopathological variables presented in Table 1 and then selected those showing a contribution characterized by a p-value <0.10 in the multivariate model. We added the α-SMA/epithelial area ratio with a cut-off value of 1 to this "clinical model" to evidence the independent contribution of this binary variable on recurrence-free survival. We also illustrated this impact using a Kaplan–Meier analysis (and log-rank test).

Results

Final analysis for α-SMA and Ki67 expression could be performed for 98 patients. Sixty-two of them (clinical stage II, III or IV based on the TNM classification established by the International Union Against Cancer) had received neoadjuvant therapy. Clinical and histopathological data are presented in Table 1; types of neoadjuvant and adjuvant therapy are described in Supplementary Table 1. Thirty-six patients did not receive neoadjuvant therapy because the tumour was located above the sacral promontory (n = 17), staged cT1–2N0 (n = 13), subocclusive (n = 2), associated with synchronous metastasis (n = 2) or occurring after radiotherapy for gynaecological cancer (n = 2). In the whole population, 23 patients developed distant metastasis during follow-up; in two of them, local recurrence was synchronously detected.

α-SMA positive myofibroblasts were found in all rectal cancers. Quantitative measurements of the relative proportion of

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