Clinical Oncology 22 (2010) 272–280

Contents lists available at ScienceDirect

Clinical Oncology

journal homepage: www.elsevier.com/locate/clon

Original Article

Gene Expression Profiles of Epidermal Growth Factor Receptor, Vascular Endothelial Growth Factor and Hypoxia-inducible Factor-1 with Special Reference to Local Responsiveness to Neoadjuvant Chemoradiotherapy and Disease Recurrence After Rectal Cancer Surgery

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Received 25 March 2009; received in revised form 14 December 2009; accepted 15 December 2009

Abstract

Aims: To establish a causal relationship between the gene expression profiles of angiogenetic molecular markers, including epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1 (HIF-1), in rectal cancer and the local responsiveness to neoadjuvant chemoradiotherapy and subsequent disease recurrence.

Materials and methods: We examined the pre-treatment tumour biopsies ($n = 40$) obtained from patients with rectal adenocarcinoma (clinical International Union Against Cancer stage ll/III) who were scheduled to receive neoadjuvant 5-fluorouracil-based chemoradiotherapy for EGFR, VEGF and HIF-1 expression by quantitative real-time polymerase chain reaction.

Results: Responders (patients with significant tumour regression, i.e. pathological grades 2/3) showed significantly lower VEGF, HIF-1 and EGFR gene expression levels than the non-responders (patients with insignificant tumour regression, i.e. pathological grades 0/1) in the pre-treatment tumour biopsies. The elevated expression level of each gene could predict patients with a low response to chemoradiation. During the median follow-up of all patients (41 months; 95% confidence interval 28–60 months), 6/40 (15%) developed disease recurrence. Although local responsiveness to neoadjuvant chemoradiotherapy was associated with neither local nor systemic disease recurrence, lymph node metastasis and an elevated VEGF gene expression level were independent predictors of systemic disease recurrence. The 3-year disease-free survival rates of the patients with lower VEGF or EGFR expression levels were significantly lower than those of patients with higher VEGF or EGFR expression levels.

Conclusions: Analysing VEGF expression levels in rectal cancer may be of benefit in estimating the effects of neoadjuvant chemoradiotherapy and in predicting systemic recurrence after rectal cancer surgery.

Key words: EGFR; HIF-1; preoperative chemoradiotherapy; rectal cancer; recurrence; tumour regression; VEGF

Introduction

Previous meta-analyses have concluded that compared with surgery alone, a combination of preoperative radiotherapy and surgery significantly improved local control and overall survival in rectal cancer, but only because surgery was not total mesorectum excision and, therefore, substandard [1,2]. However, the exact role of preoperative radiotherapy remains controversial for several reasons, particularly from the point of view of survival benefit, as distant metastasis still remains a significant problem [3,4]. Recently, additional chemotherapy considered to be complementary to radiotherapy has been advocated to resolve this issue. In fact, some studies have shown that in resectable rectal cancer, preoperative chemoradiotherapy not only effectively reduces the rate of local recurrence, but also improves survival [5,6]. The German Rectal Cancer Study Group (CAO/ARO/AIO-94 trial) advocated the concepts of preoperative (neoadjuvant) 5-fluorouracil (5-FU)-based chemoradiotherapy as the recommended

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0936-6555/$36.00
doi:10.1016/j.clon.2010.01.001

therapeutic regimen for rectal cancer, particularly for International Union Against Cancer stage II/III disease [7]. The 5-year cumulative incidence of local cancer recurrence has been reported to be 6% for patients treated with preoperative chemoradiotherapy and 13% for those treated with postoperative chemoradiotherapy [7]. However, despite the local benefits of preoperative treatment, it did not achieve any significant difference in the 5-year overall survival compared with postoperative chemoradiotherapy [7]. It is, therefore, conceivable that predicting both tumour responsiveness to neoadjuvant anti-tumour therapy and subsequent postoperative systemic recurrence may be indispensable for selecting candidates for modifying the neoadjuvant chemoradiotherapeutic regimen as well as the postoperative adjuvant treatment plan in rectal cancer.

Hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor that is stabilised by low oxygen tension, regulates the expression of more than 100 gene products that function to help protect the cells from hypoxic stress [8]. As reviewed elsewhere [9], HIF-1 affects many processes, including glycolysis, mitosis, apoptosis and angiogenesis, which have been shown to influence radioresponsiveness and might, therefore, serve as a link between HIF-1 activity and tumour radiosensitivity. HIF-1 plays a role in this relationship by promoting tumour cells to express cytokines that have radioprotective effects on the neighbouring endothelial cells. These cytokines, including vascular endothelial growth factor (VEGF) and other such factors, send anti-apoptotic signals to tumour vessels, thereby rendering them radioresistant. It has been shown that blocking VEGF results in an increase in the radiosensitivity of the tumour vasculature and, as a result, increases the overall tumour radioresponsiveness [10,11].

Epidermal growth factor receptor (EGFR) is expressed in about 60–80% of colorectal cancers [12]. Moreover, its overexpression is associated not only with a poor prognosis [13,14], but also with radioresistance of tumour cells to single or fractionated radiation exposure [15–17].

The present study was designed to investigate whether the molecular markers, namely VEGF, HIF-1 and EGFR, could predict both rectal tumour response to preoperative chemoradiotherapy and postoperative systemic recurrence and to clarify the clinical potential of these markers in elaborating the therapeutic strategy.

Materials and Methods

Clinical Materials

In total, 40 patients with rectal cancer (clinical International Union Against Cancer stage ll/III) who had been admitted to Mie University Hospital between 2001 and 2006 were enrolled in this study. The age of the patients (31 men and nine women) ranged from 48 to 77 years (mean age 62.2 years). In our institute, since 2001, all patients have been treated with short-course radiotherapy with concurrent chemotherapy because of 5-FU radiosensitisation. They were treated with external irradiation (10 MV photons from a linear accelerator) using a four-field box technique and received 20 Gy in four fractions within 1 week. They also underwent concurrent pharmacokinetic modulating chemotherapy (intravenous infusion of 750 mg/day 5-FU and oral administration of 400 mg/day tegafur-uracil (UFT)) over 1 week [18]. Before irradiation, the rectal tumours were subjected to endoscopic biopsy for histopathological diagnosis. For gene expression analysis, the pre-treatment endoscopic biopsy specimens were snap frozen in liquid nitrogen and were maintained at −80 °C until further use, after obtaining written informed consent. All 40 tumours were diagnosed as adenocarcinomas, and the patients underwent chemoradiotherapy.

All patients underwent standard surgery, including total mesorectum excision, after an interval of about 1 week after the completion of treatment. All patients received 5-FU-based adjuvant chemotherapy after surgery for 6 months to 1 year. All patients were examined during regular follow-up visits, including recording of their clinical history, a physical examination, laboratory investigations, pelvic computed tomography and endoscopy. The patients were followed up on a half-yearly basis for a period of 2–7 years postoperatively until death or until the closing date of the study. There were no cases of regrowth of the tumour within the pelvis, which is considered as local recurrence of the tumour. The median follow-up period was 41 months (95% confidence interval 28–60 months).

RNA Extraction from the Pre-treatment Biopsy Specimens

The pre-treatment endoscopic biopsy specimens were homogenised using a Mixer Mill MM 300 homogeniser (Qiagen, Chatsworth, CA, USA). Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions.

cDNA Synthesis

cDNA was synthesised from a random hexamer primer using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Real-time Quantitative Reverse Transcription Polymerase Chain Reaction

Polymerase chain reaction amplification for quantification of VEGF, HIF-1, EGFR and beta-actin mRNA in the biopsy samples and cell lines was carried out in a LightCycler using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Germany). The primers for beta-actin, VEGF, HIF-1a and EGFR were designed using the primer3 software (Biology Workbench Version 3.2; San Diego Supercomputer Center, University of California, San Diego, CA, USA). The sequences are shown in Table 1. We carried out 40 cycles of amplification under the following conditions: denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and elongation at 72 °C for 20 s. After amplification, the products were subjected to a temperature