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Phase I/II Trial of Bevacizumab and Radiotherapy for Locally Advanced Inoperable Colorectal Cancer: Vasculature-Independent Radiosensitizing Effect of Bevacizumab

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Abstract

Purpose: Anti–vascular endothelial growth factor therapy enhances the activity of radiotherapy in experimental models, and bevacizumab has therapeutic activity in patients with metastatic colorectal cancer.

Experimental Design: Twenty-two patients with locally advanced inoperable colorectal carcinomas (LA/I-CRC) were treated with conformal hypofractionated (3.4 Gy/fraction × 15) split-course accelerated radiotherapy (biological equivalent dose, 67.2 Gy) supported with amifostine, capecitabine (600 mg/m^2 daily, 5 days/week), and bevacizumab (5 mg/kg every 2 weeks, five cycles). Biopsies from nine patients, performed before and 1 week after bevacizumab administration, were analyzed for changes in mRNA expression with Illumina gene arrays.

Results: No serious grade 3 chemotherapy-related side effects were recorded. There was low acute toxicity, with moist perineal desquamation noted in 2 of 22 patients, diarrhea grade 2 to 3 in 5 of 22 patients, and severe proctalgia in 2 of 22 patients. One patient died from Fournier's gangrene before treatment completion. Within a median follow-up of 18 months, two patients with preradiotheraphy direct involvement of adjacent organs expressed recto-vaginal/perineal fistula. Out of 19 evaluable cases, 13 (68.5%) showed complete response and 4 showed (21.1%) partial response. Fourteen patients are alive with no evidence of loco-regional relapse. In the gene array analysis, 30 known genes associated with transcription factors, DNA repair, and proliferation were downregulated by bevacizumab. DUSP1 gene was the most consistently downregulated transcript.

Conclusions: The combination of radiotherapy with bevacizumab is feasible and results in a high rate of durable complete responses in patients with LA/I-CRC. Radiosensitization may occur through a direct effect on tumor cells followed by a wide scale suppression of transcription factors and genes involved in DNA repair and proliferation. (Clin Cancer Res 2009;15(22):7069–76)

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Locally advanced inoperable colorectal cancer is a major therapeutic challenge. Radiochemotherapy offers good palliation with prolonged survival and tumor control in a small fraction of patients (1–3). The anti–vascular endothelial growth factor (VEGF) monoclonal antibody (bevacizumab) has been approved for the treatment of metastatic colorectal cancer in combination with chemotherapy (4). The blockage of angiogenesis during radiotherapy and/or chemotherapy has significantly improved the efficacy of the regimens in experimental models (5, 6). It has been suggested that anti-VEGF therapy results in destruction of immature nonfunctional vessels leading to a vascular "normalization" and a better blood flow provided by the remnant mature vessels (7), which may improve drug distribution and reduce tumor hypoxia.

We report here the encouraging results of a phase I/II trial combining radiotherapy with bevacizumab (Avastin) for locally advanced inoperable/recurrent colorectal tumors. Moreover,

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Translational Relevance

Combining antiangiogenesis agents with radiotherapy relies on a strong biological rational, but clinical experience is limited. In this study, the combination of anti–vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab with radiotherapy showed an acceptable tolerance profile and high complete response rates that persisted through time. These data may help in the design of prospective trials on anti-VEGF therapy in combination with preoperative or adjuvant radiochemotherapy for rectal cancer. The finding that bevacizumab directly repressed transcription factors and DNA repair enzymes in cancer cells suggests that the VEGF/receptor autocrine pathway is an important regulator of cancer cell biology and may be a major, vascular-independent, target of bevacizumab. Predictive markers of response to bevacizumab should be, therefore, sought not only in tumor vascular features but also in the cancer cell. Dependence of cancer cells on DNA or mitochondrial DNA repair activity may define responsiveness of tumors to anti-VEGF therapy especially when combined with DNA-damaging agents.

using gene arrays, we provide evidence that bevacizumab, aside from its vascular effect, may have a direct effect on cancer cells themselves.

Materials and Methods

From October 2007 to January 2008, 22 patients with locally advanced inoperable colorectal cancer were recruited in a prospective phase I/II study aiming to investigate (*a*) the feasibility and efficacy of the combination of bevacizumab (at the standard schedule approved for colorectal cancer) with a previously established accelerated chemoradiotherapy scheme used for radical intent (8) and (*b*) the effect of bevacizumab on tumor gene expression profile. The study had been approved by the local scientific and ethics committees, and written informed consent was obtained from all patients.

The patient and disease characteristics are shown in Table 1. Twenty of 22 cases had large tumor with invasion into adjacent organs and/or recurrent nonresectable disease and 2/22 had T3/N+ tumors but were inoperable for medical reasons. Such patients are regularly considered for palliative radiotherapy and chemotherapy but, in the current study, were recruited for radical/curative intent. Following the aggressive scheme and the high radiotherapy dose applied as per protocol (67 Gy), no surgery was allowed even in cases with complete remission due to the high rate of postoperative complications expected. Patients with previous exposure to radiotherapy at the site of intended therapy were excluded. Also excluded were patients with WBC of <2500/μL and platelets of <120.000/μL. Patients with hemoglobin of <8.5 g/mL were transfused, whereas patient with hemoglobin of 8.5 to 9.5 g/mL received recombinant human erythropoietin, a policy that continued also during therapy so that hemoglobin levels were >10 g/dL before entering the study and throughout the radiotherapy period. Pregnant women or patients with major heart, liver, renal, psychiatric disease, or hematologic malignancies were also excluded. The follow-up of patients ranges from 10 to 36 mo (median, 18 mo).

Radiotherapy details. An 18-MV linear accelerator endowed with a multileaf collimator was used for the irradiation of patients. For tumor located within the pelvis, radiotherapy was given to the whole pelvic region including the tumoral mass, the common and external/internal iliac node area with the lower margin reaching the outer margin of the anus. A four-field conformal technique based on computed tomography (CT) simulation was used (anteroposterior and lateral fields) to deliver a daily fraction of 3.4 Gy (5 fractions per week) for 10 consecutive fractions. After a 1-wk break, a new CT simulation was done and a new six-field conformal planning was carried out to deliver five booster fractions of 3.4 Gy confined to the gross tumor mass with 1 to 2 cm margins. In this way, the total dose to the tumor reached 51 Gy with 3.4-Gy fractions in 4 wk. For tumors located to the cecum and ascending colon, conformal radiotherapy directed to the tumor with at least 2-cm margins around the CT-detectable mass was given, using the same fractionation and overall dose.

The time-corrected normalized total dose was calculated as follows (9, 10): $\text{normalizedtotaldose}_{(T)} = D\,[(\alpha/\beta + d)/(a/b + 2)] + \lambda(Tc–To)$, where D is the total physical dose, d is the dose per fraction, α/β is the tissue-specific ratio, Tc is the number of days required for the delivery of the normalized total dose using a conventionally fractionated scheme, To is the number of days required for the delivery of the current scheme, and λ is the estimated daily dose consumed to compensate for rapid tumor repopulation. Suwinski et al. (11) recently suggested a 5 Gy value for colorectal cancer. Here, we assumed a value of 4 Gy for normal and 5 Gy for cancer tissues. For cancer cells, an acceleration of 2 wk and a λ value of 0.4 Gy was considered, although higher λ values may apply for large tumors. For normal tissues, a λ

Table 1. Patient, disease, and pre-RT treatment characteristics

No. patients	22
Gender	
F	7
M	15
Age	
Median	71
Range	48-85
PS	
0	12
1	7
2	3
Tumor location	
Rectum	9
Rectosigmoid	5
Sigmoid	1
Ascending colon	1
Cecum	1
Perineal	1
Presacral/sacral area	4
Histology	
Adenocarcinoma	22
Grade	
1/2	14
3	8
Duke's stage	
C2g*	2
D1	13
Recurrent†	7
Surgery before RT	
Palliative colostomy	5
None	17
Previous chemotherapy	
Yes	7
No	15

*Inoperable rectal cancer for medical reasons.
†Recurrent large mass to the sacral area after abdomino-perineal resection.

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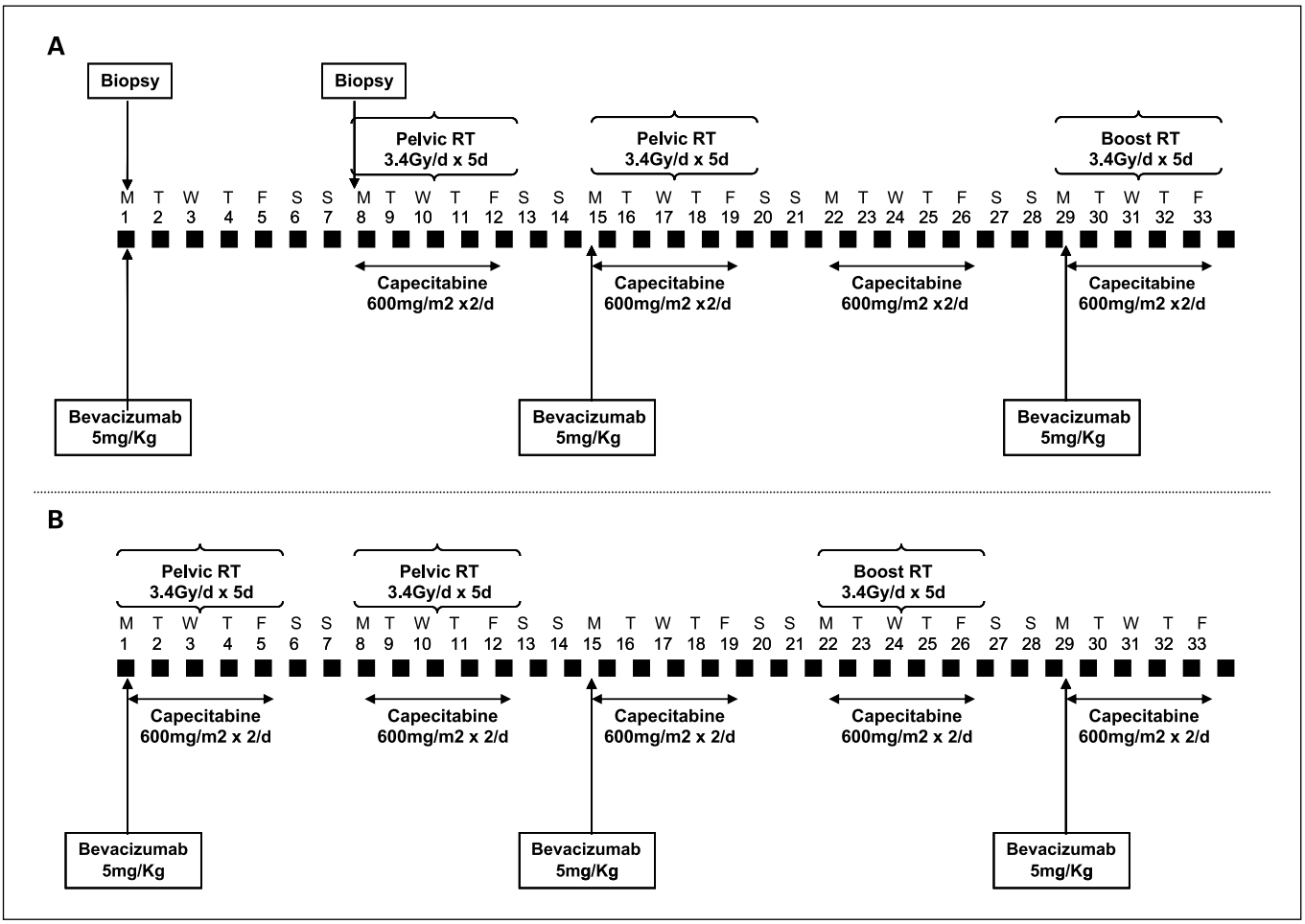

Fig. 1. Therapeutic schedule followed in 8 patients who underwent biopsy (*A*) and 14 patients who did not (*B*). RT, radiotherapy.

value of 0.2 Gy was adopted. Using these assumptions, the biological dose (equivalent to standard fractionation) to normal pelvic tissues was 45.3 Gy and to the tumor 67.2 Gy.

Cytoprotection. Ondasetron (8 mg) was administered p.o., 30 to 60 min before amifostine injection as antiemetic policy. Amifostine (1,000 mg) was diluted in 5 mL water for injection and was injected in two sites (usually at the right and left shoulder), the patient being at a sitting position. The higher dose of amifostine (1,000 mg instead of 350-500 mg used in other studies) applied in the protocol was chosen to better protect tissues against the large fractions of radiotherapy in the HypoARC scheme. The dose of 1,000 mg was reached gradually (1st day, 500 mg; 2nd day, 750 mg; and 3rd day 1,000 mg) using a previously published algorithm (12).

Chemotherapy. Chemotherapy with capecitabine at a dose of 600 mg/m^2 twice a day p.o. for 5 d per week started together with the first radiotherapy fraction (8). Bevacizumab was given i.v. at a dose of 5 mg/kg every 2 wk, starting on the first day of radiotherapy. Bevacizumab was given for four consecutive biweekly cycles. Two weeks after the end of radiotherapy, the dose of capecitabine was increased to 800 mg/m^2 twice daily, 5 d per week. Chemotherapy with capecitabine alone continued for 4 mo.

Biopsies. In eight cases, the first cycle of bevacizumab was given 1 wk before the administration of radiotherapy and capecitabine. In this way, endoscopical biopsies of the tumor were done before and 7 d after bevacizumab. Cancer tissue samples were stored in mRNA-later (Ambion Biosystems) for subsequent gene analysis. As the endoscopical biopsies are too small to allow slicing and histologic appraisal of the existing cancer tissue, multiple biopsies were obtained to minimize the chance for lack of cancer cells in the material stored for gene analysis. An additional four pairs of biopsies from patents with radiologically American Joint Committee on Cancer stage T3/N+ (CT or magnetic resonance imaging–detectable enlarged nodes and extramural invasion) rectal cancer receiving the same schedule of bevacizumab before preoperative radiotherapy were also analyzed together with the eight samples from inoperable tumors. However, only 9 of 12 pairs of samples (before and after bevacizumb) fulfilled the tissue quality criteria for gene analysis (7 pairs from the herein analyzed cases).

Figure 1 shows schematically the therapeutic schedule in 8 patients who underwent biopsy and 14 patients who did not.

Follow-up of patients. Radiation toxicity was recorded daily during the radiotherapy phase and weekly thereafter for the first month. The National Cancer Institute Common Toxicity Criteria Version 2 scale[3] was used to assess chemotherapy and acute radiation toxicity.

Hematologic variables and clinical status were recorded every 2 wk during the chemotherapy period. Patients were followed thereafter every 3 mo with clinical examination. CT scan of the chest, abdomen, and pelvis was done at 2 mo and every 6 mo thereafter.

Statistical analysis of clinical data. The statistical analysis and graphical presentation of survival curves was done using the GraphPad Prism 4.0 version package (GraphPad).[4] Survival curves were plotted using the method of Kaplan and Meier.

[3] http://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcv20_4-30-992.pdf

[4] http://www.graphpad.com

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Table 2. Early toxicity assessed with the National Cancer Institute Common Toxicity Criteria version 2 scale

	No. patients, 22 (%)
Radiation dermatitis	
0. None	14 (63.6)
1. Faint erythema/dry desquamation	7 (31.8)
2. Brisk erythema/patchy moist desquamation	0 (0)
3. Confluent moist desquamation	0 (0)
4. Skin necrosis	1 (4.6)
Perineal toxicity	
0. None	10 (45.5)
1. Dry desquamation	9 (40.9)
2. Patchy moist desquamation	2 (9)
3. Confluent moist desquamation	1 (4.6)
Diarrhea	
0. None	8 (36.4)
1. <4 stools/d	8 (36.4)
2. 4-6 stools	4 (18)
3. >6 stools or dehydration	1 (4.6)
4. Intensive care demanded	1 (4.6)
Abdominal Pain	
0. None	12 (54.5)
1. Mild	9 (40.9)
2. Moderate	0 (0)
3. Severe	1 (4.6)
Proctalgia	
1. None	10 (45.5)
2. Mild pain	7 (32)
3. Moderate pain	2 (9)
4. Severe pain	2 (9)
5. Disabling	1 (4.6)
Bladder toxicity	
1. None	21 (95.5)
2. Increased frequency up ×2	1 (4.5)
3. Frequency >×2 but <hourly	0 (0)
4. Requirement of catheter	0 (0)

RNA extraction—microarray analysis. Tumor biopsies were homogenized in Tri reagent (Sigma) using an IKA Ultra Turrax T25 disperser. The RNA was extracted using chloroform and precipitated with isopropanol. The resuspended pellet was DNase treated (Promega) and cleaned again using RNeasy spin columns (Qiagen). The RNA quantity was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and RNA quality was checked using RNA6000 Nano Assay on Agilent bioanalyzer 2100 (Agilent Technologies).

Commercially available high-density oligonucleotide, from Illumina Genome-Wide Expression BeadChips (Human WG-6 v3, Illumina), was used with 48687 probes representing 46,713 human transcripts. In brief, 500 ng of total RNAs were reverse transcribed to synthesize first- and second-strand complementary DNA, purified on spin columns and *in vitro* transcription to synthesize biotin-labeled complementary RNA. A total of 1,500 ng of biotin-labeled complementary RNA was hybridized HumanWG-6 Expression BeadChip (Illumina, Inc.) at 55°C for 18 h. The hybridized BeadChip was washed and labeled with streptavidin-Cy3 according to the manufacture protocols. Chips were scanned with Illumina BeadScan, and the scanned image was imported into BeadStudio 3.2.6 (Illumina Inc) for background correction. Further microarray analysis was done using R[5] and Bioconductor[6] resources. Data were rescaled to eliminate negative values after background correction, normalized using quantile normalization, and a paired SAMR analysis was done on the normalized $\log_2$ data to examine changes in gene expression before and after Avastin treatment (13).

Results

Cytoprotection. Using the reported individualization algorithm, 12 of 22 patients received 1,000 mg of amifostine before each radiotherapy fraction, 3 of 22 patients received 750 mg, and 5 of 22 patients received 500 mg. At this individualized dose level, all patients completed therapy either without symptoms or with mild nausea and/or fatigue. Two of 22 patients did not receive amifostine as they could not tolerate the dose of 500 mg (intolerable nausea/vomiting and asthenia).

Chemotherapy tolerance. No hypersensitivity reactions were observed following administration of bevacizumab. Two of 22 patients interrupted bevacizumab after the second cycle due to hypertension. One patient received two cycles of bevacizumab and treatment was interrupted due to grade 3 diarrhea. One patient died from Fournier's gangrene syndrome after the second cycle of bevacizumab. The remaining 18 patients received three or four cycles of bevacizumab (6 and 12 patients, respectively).

[5] http://www.r-project.org/
[6] http://www.bioconductor.org/

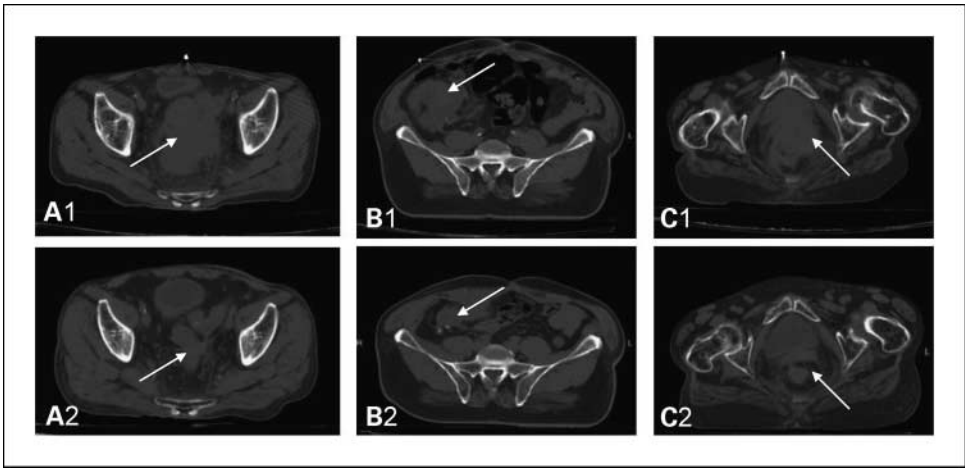

Fig. 2. Complete response of large tumor masses shown on CT scans done 2 mo after therapy. Arrows, the tumor mass and its disappearance. *A*, the patient is alive and without any evidence of disease 24 mo after therapy. *B*, the patient relapsed locally 12 mo after therapy with no evidence of distant metastasis. *C*, the patient is alive 24 mo after the end of therapy with no evidence of local or distant relapse.

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Capecitabine at the dose of 600 mg/m^2 twice a day was well tolerated without any hematologic toxicity. One patient interrupted capecitabine due to grade 2 diarrhea and abdominal cramps observed since the first day of administration of the drug. Similarly, capecitabine at the dose of 800 mg/m^2 twice a day administered after the radio-chemoterapy phase showed an excellent tolerance without any hematologic or other toxicity.

Pelvic toxicity. Table 2 reports the acute toxicity. Overall, the regimen was well tolerated with most patients experiencing grade 0/1 toxicity. Moist perineal desquamation was noted in 2 of 22 patients. Diarrhea grade 2/3 appeared in 5 of 22 patients and severe proctalgia requiring narcotics in 2 of 22. Bladder toxicity was negligible. One patient with a large tumor infiltrating the perineal area died from Fournier's gangrene syndrome after the 2nd week of therapy, as described in the previous section. Colorectal bleeding did not occur in any of the patients. One patient with a large tumor of the cecum developed perforation of the affected colon area 1 week after the end of therapy, which was successfully surgically treated.

In terms of late radiation sequel, within a median follow-up of 18 months, one patient developed recto-perineal fistula. In this case, there was an initial direct extension of the tumor to the perineum and the fistula appeared together with local recurrence (after complete response), suggesting that it was disease related, as opposed to a radiotherapy complication. Another patient with a sigmoid tumor infiltrating the bladder and vagina, developed a recto-vaginal fistula after complete response of the tumor. An additional patient complained of persistent abdominal cramps although still in complete remission for 12 months since radiotherapy. None of the remaining patients developed any other late sequel.

Local control and survival. The response rate to therapy was assessed with CT scan and, whenever feasible, endoscopy of 2 months after radiotherapy completion. One patient was lost to follow-up before the first assessment of tumor response and two died before this interval (one due to irrelevant cause and one due to Fournier's gangrene syndrome). From the remaining 19 patients, 13 (68.5%) showed complete response, 4 (21.1%) partial response, 1 (5.2%) minimal response, and 1 (5.2%) stable disease. Figure 2 shows three cases with large tumor mass and complete response after therapy.

Out of 19 patients, 1 died from distant metastasis and local disease 6 months after radiotherapy. All the remaining 17 are alive, 14 of them with no evidence of loco-regional relapse. Figure 3 shows the Kaplan Meier curves of local and distant relapse interval and of the overall survival.

Array analysis of changes in mRNA expression induced by bevacizumab. Samples were available from nine patients, and initial analysis showed high correlation of overall expression between samples, indicating reproducibility of the assay. No specific batch effect was observed and all arrays passed BeadStudio QC checks. When comparing the pre- and post-bevacizumab samples, a paired SAMR analysis revealed small absolute changes in gene expression but some significant changes could be detected with false discovery rate of <0.05. Specifically, 75 transcripts were found to be downregulated after bevasizumab treatment of which 30 are known genes (Table 3). Of particular interest is that there were no significantly upregulated genes after treatment. The downregulated transcripts generally fell into the category associated with

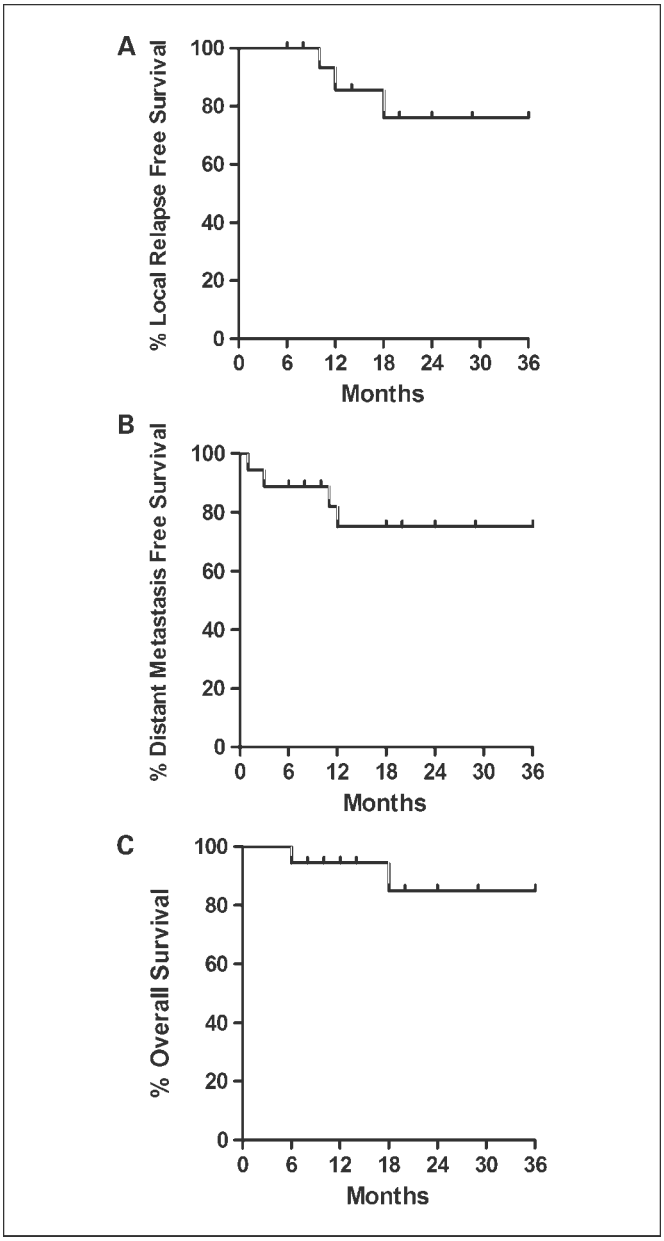

Fig. 3. Kaplan-Meier curves: (*A*) local relapse-free survival, (*B*) distant metastasis-free survival, and (*C*) overall disease-specific survival.

transcription factors and proliferation, for example reduction in several histones, basal transcription factors, and fos. A Gene Ontology analysis of the gene changes carried out using Genecodis[7] showed significant (false discovery rate, <0.05) overrepresentation of nuclear proteins that have DNA binding as their molecular function, including several transcription factors (Supplementary Table S1 and Fig. S1; Table 3]. However, the most consistently and significantly downregulated transcript was dual specificity phosphophatase 1 (DUSP1), although the level of downregulation varied between patients (Fig. 4). CDC14B, a proline-directed phosphatase shown to be a critical upstream regulator of G_2 arrest in response to DNA damage, was

[7] http://genecodis.dacya.ucm.es/

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also markedly reduced. The magnitude of the change was not associated with treatment response, but the sample size is too small for any stratification or multivariate analysis. The second samples were too small to confirm these results by PCR or immunochemistry.

Of interest, the expression of hypoxia target genes, such as lactate dehydrogenase, VEGF, and carbonic anhydrase 9 were not affected. Furthermore, the expression of endothelial markers such as CD31, CD34, VEcadherin, endoglin, and other makers of tumor vasculature remained unaltered.

Discussion

VEGF is a potent angiogenic factor in colorectal cancer (reviewed in ref. 14). Taking into account the clinically proven efficacy of the anti-VEGF monoclonal antibody bevacizumab (4, 15) and the evidence of tumor vascular "normalization" induced by the antibody (7, 16), we initiated this phase I/II trial to assess the toxicity and efficacy of radiotherapy combined with bevacizumab in locally advanced inoperable colorectal cancer. There is strong experimental evidence that VEGF increases during radiotherapy and that blockage of VEGF results in potent radiosensitization (5, 6).

The combination of accelerated hypofractionated radiotherapy with capecitabine and bevacizumab, supported with high-dose amifostine, showed an acceptable overall tolerance, given the large tumor burden and invasive disease in patients recruited in the study. Diarrhea and proctalgia were the main acute, still easily manageable, side effects. Among severe acute complications, we report one case with intestinal perforation that was effectively treated with surgery and one death from Fournier's gangrene syndrome, but it is not clear whether this was a bevacizumab-related or a radiotherapy-related effect (17). The late side effects of the regimen were minimal with

Table 3. Heathmap of fold changes in gene expression post- vs pre-Avastin in 9 patients for 30 known genes, which significantly (false discovery rate, <0.05) downregulated in a SAMR analysis

	3	2	1	7	9	6	8	4	5	
NM_004417.2	0.88	0.64	0.74	0.66	0.69	0.83	0.51	0.39	0.40	DUSP1
NM_198403.2	0.90	0.84	0.92	0.90	0.88	0.92	0.95	0.97	0.94	MMD2
NM_005252.2	0.79	0.90	0.84	0.92	0.68	0.52	0.89	0.26	0.32	FOS
NM_001004704.1	0.91	0.94	0.91	0.98	0.85	0.92	0.94	0.97	0.90	OR4C6
NM_021058.3	0.98	0.81	0.83	0.82	0.76	0.84	1.05	0.99	0.85	HIST1H2BJ
NM_130791.1	0.96	0.86	1.00	0.87	0.97	0.96	0.87	0.89	0.88	WWOX
NM_003539.3	0.86	0.87	1.01	0.94	0.86	0.91	0.97	0.91	0.95	HIST1H4D
NM_016328.1	0.88	0.97	1.01	0.83	0.88	0.90	0.95	0.95	0.87	GTF2IRD1
NM_003671.2	1.90	0.56	1.06	0.42	0.38	0.58	0.74	0.44	0.28	CDC14B
NM_147161.2	0.74	1.08	0.81	0.68	0.84	0.81	1.14	0.81	0.73	ACOT11
NM_003517.2	1.01	0.63	0.74	0.57	0.84	0.81	1.15	0.78	0.35	HIST2H2AC
NM_172111.1	0.89	1.00	0.96	0.88	0.80	0.90	0.96	0.96	0.90	EYA2
NM_002109.3	0.89	0.98	0.76	0.80	0.93	0.74	1.08	0.86	0.90	HARS
NM_005319.3	0.51	0.61	0.70	0.42	0.53	0.71	0.91	1.82	0.62	HIST1H1C
NM_003538.3	0.94	0.84	1.01	0.91	0.90	0.90	0.86	1.02	0.85	HIST1H4A
NM_031865.1	0.94	1.00	0.90	0.86	0.89	0.96	0.97	0.94	0.90	PCDHA13
NM_016819.2	0.95	0.97	0.97	0.96	0.93	0.90	0.95	0.94	0.96	OGG1
NM_013290.3	0.87	0.85	1.00	0.87	0.99	0.90	0.96	0.94	0.92	TBPIP
NR_001565.1	0.90	0.92	0.98	0.90	0.94	0.85	0.90	1.03	0.90	MGC10997
NM_005025.2	0.82	0.93	0.67	1.02	0.57	0.89	0.84	0.86	1.00	SERPINI1
NM_024894.1	0.82	1.01	0.93	1.01	0.80	0.84	0.89	0.88	0.97	NOL10
NM_001003894.1	0.98	0.94	0.94	0.89	0.93	0.93	0.92	0.99	0.94	CDY1B
NM_178476.1	0.89	0.95	0.96	0.95	0.95	0.93	0.96	0.94	0.97	C20orf62
XM_934974.1	0.93	0.95	0.86	0.90	0.97	1.00	0.96	0.91	0.93	NBPF1
NM_003518.3	0.83	0.97	0.92	0.81	0.84	0.83	1.06	0.66	0.96	HIST1H2BG
NM_000212.2	0.90	0.96	0.94	0.82	0.97	0.86	0.88	1.05	0.86	ITGB3
NM_152550.2	0.68	1.04	0.86	0.79	0.99	0.86	1.06	0.77	0.82	SH3RF2
NM_004126.2	0.86	0.69	0.81	0.63	0.81	1.08	0.81	1.16	0.81	GNG11
NM_020771.2	0.92	0.95	0.89	0.92	0.98	0.78	0.90	1.02	0.84	HACE1
NM_138575.1	0.91	0.84	0.98	0.97	0.86	0.93	0.91	1.02	0.91	PGAM5

NOTE: Numbers across the top refer to the patient tissue sample number. Darker shades stress the cases higher fold gene expression decrease. The Gene Ontology analysis of molecular function by Genecodis is in Supplementary Table S1.

Abbreviations: DUSP1, dual-specificity phosphatase 1 (MAP kinase phosphatase-1); MMD2, monocyte to macrophage differentiation factor 2; FOS, human homologue of transforming gene of Finkel-Biskis-Jinkins murin osteosarcoma; OR4C6, olfactory receptor family 4 subfamily C member 6; HIST1H2BJ, histone cluster 1 H2bj; WWOX, WW domain containing oxidoreductase; HIST1H4D, histone cluster 1 H4d; GTF2IRD1, GTF2I repeat domain containing 1; CDC14B, cell division cycle 14 homologue B; ACOT11, acyl-CoA thoesterase 11; HIST2H2AC, histone cluster 2 H2ac; EYA2, eyes absent homologue 2; HARS, Histidyl-tRNA synthetase; HIST1H1C, histone cluster 1 H1c; HIST1H4A, histone cluster 1 H4a; PCDHA13, protocadherin α 13; OGG1, 8-oxoguanine DNA glycosylase 1; TBPIP, TBP-1 interacting protein; MGC10997, pseudogene MGC10997; SERPINI1, Serpin peptidase inhibitor, class I (neuroserpin), member 1; NOL10, nucleolar protein 10; CDY1B, chromodomain protein; C20orf62, chromosome 20 open reading frame 62; NBPF1, neuroblastoma breakpoint family member 1; HIST1H2BG, histone cluster 1 H2bg; ITGB3, integrin β 3; SH3RF2, putative E3 ubiqutin-protein ligase; GNG11, Guanine nucleotide binding protein γ 11; HACE1, E3 ubiquitin ligase; PGAM5, phsophoglycerat mutase family member 5.

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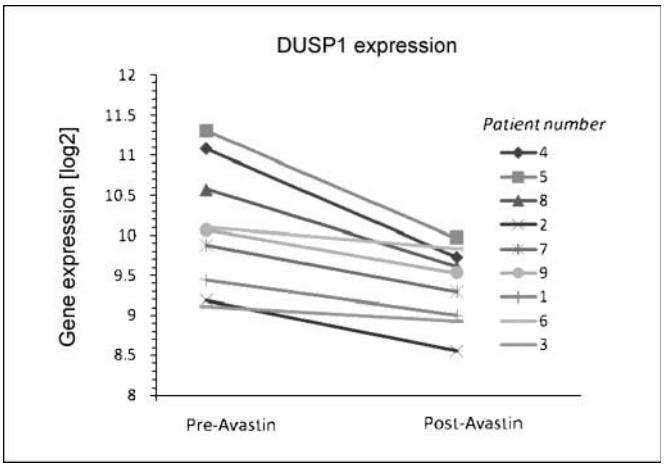

Fig. 4. Downregulation of DUSP1 in nine rectal tumors 7 d after administration of bevacizumab.

one case of rectoperineal fistula and one rectovaginal fistula. Both cases, however, had large tumor with extensive infiltration of the perineum and vagina, respectively, so that the observed fistula may in fact be a result of compete tumor remission instead of a late radiation sequel.

The efficacy of the regimen was high as complete response was documented in 68.5% of cases and, within a median of 18 months of follow-up, 14 of 20 evaluable patients are alive with no evidence of disease. These results compared favorably to a previously reported cohort of 10 patients receiving the same radio-chemotherapy regimen without bevacizumab, where the complete response rate was 40% and the median survival was 9 months (8). These encouraging results are indicative of an important bevacizumab-induced radio-sensitization of rectal tumors, which deserves further investigation in randomized trials. Obtaining biopsies before and 7 days after administration of bevacizumab alone, we had the opportunity to examine potential pathways underlying the interaction of VEGF blockade with radiation. We had initially expected that blocking excessive angiogenesis and vascular normalization, as suggested in previous studies (7, 15), may had an effect on the expression of well-known hypoxia target genes, such as lactate dehydrogenase, VEGF, and carbonic anhydrase 9. Furthermore, reduction of the overall vascularity would be expected to reduce expression of endothelial markers such as CD31, CD34, VE-cadherin, endoglin, and other markers of tumor vasculature. However, none of these changes were detected.

By contrast, our results suggest a direct effect of bevasizumab on specific pathways of the cancer cells themselves, suggestive of a vascular-independent activity on the VEGF blockade. Recent studies have indicated a direct role VEGF in colon cancer cell biology, as both FLK1 (VEGFR1) and the phosphorylated activated form of the VEGFR2 are expressed in primary and metastatic colorectal carcinomas and associated with increased vascularization and poor prognoses (18, 19). In colon cancer cell lines, FLK1 is expressed and mediates migration caused by VEGF (20, 21). Proliferation of colon cancer cell lines can be blocked by VEGFR2 blockade (22, 23), and VEGF acting via VEGFR2 may induce HIF in colon cancer cell lines (24). So, in the short time frame that we were trying to assess *in vivo* biology of VEGF receptor blockade before systemic therapies or radiation therapy, the indications are that many genes involved in cell cycle proliferation, particularly histones, and fos were downregulated. Although this would support the direct effect of VEGFR blockade on tumor cells, there are caveats. Thus, the vascular effects of bevacizumab may also have affected the cancer cell biology and reduced proliferation. It is also possible that areas of biopsy did not match the previously most vascular areas.

For further consideration is the vascular normalization reported by Jain et al. (16), whereby vessels become normalized for a short period of time and this may reduce the hypoxia stimulus to tumor growth. In support of a reduction in hypoxia was our observation that the hypoxia inducible gene DUSP1 (or mitogen-activated protein kinase/mitogen-activated protein kinase phosphatase 1, MKP1; ref. 25) was downregulated, and was the most downregulated gene we found. However, there were no other genes regulated by hypoxia that we found downregulated and it is likely that suppression is more related to the reduction in proliferation, as the mitogen-activated protein kinase is a basic pathway controlling cell growth, differentiation, and apoptosis (26).

Of interest, a number of enzymes involved in DNA repair, such as DNA glycosylases and ligases, including mitochondrial DNA-repair enzymes [such as OGG1 (ref. 27)], were significantly downregulated, which may be of importance in the sensitization of colon cells to radiotherapy, compatible with the high rate of complete and durable responses found in the current study. Moreover, recent studies suggest that DUSP1 activity abrogation increases radiosensitivity through increased extracellular signal-regulated kinase 1/2 activity, so that downregulation of DUSP1 may directly sensitize cancer cells to radiotherapy (28). The similarity of the sequential biopsies was substantial in terms of the array analysis and the numbers are too small to classify patients by degree of change in gene expression, which we had hoped to be able to do if there had been bigger differences. The clear downregulation of the proline-directed phosphatase CDC14B may also be relevant to radiosensitization. CDC14B is a key regulator of G_2-M transition checkpoint in response to DNA damage in G_2 (29, 30), so that deregulation of CDC14B by bevacizumab may allow entry into mitosis cells with nonrepaired DNA, making them vulnerable to fractionated irradiation.

In conclusion, combination of radiotherapy with bevacizumab is a promising therapeutic approach for locally advanced and recurrent colorectal cancer, providing high complete response rates and long lasting disease-free interval. Despite the aggressiveness of the regimen, severe acute (one fatal) and late complications were noted in <10% of patients, respectively. Our gene array data also shows the reproducibility of our methodology and stress significant changes in proliferation and DNA repair ability induced by VEGFR blockade alone, which could be mediated directly on colon cancer cells and provides a major mechanism for further investigation of the variability in patients and their response to the antiangiogenic reagents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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