TFAP2E–DKK4 and Chemoresistance in Colorectal Cancer

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BACKGROUND
Chemotherapy for advanced colorectal cancer leads to improved survival; however, predictors of response to systemic treatment are not available. Genomic and epigenetic alterations of the gene encoding transcription factor AP-2 epsilon (TFAP2E) are common in human cancers. The gene encoding dickkopf homolog 4 protein (DKK4) is a potential downstream target of TFAP2E and has been implicated in chemoresistance. We aimed to further evaluate the role of TFAP2E and DKK4 as predictors of the response of colorectal cancer to chemotherapy.

METHODS
We analyzed the expression, methylation, and function of TFAP2E in colorectal-cancer cell lines in vitro and in patients with colorectal cancer. We examined an initial cohort of 74 patients, followed by four cohorts of patients (total, 220) undergoing chemotherapy or chemoradiation.

RESULTS
TFAP2E was hypermethylated in 38 of 74 patients (51%) in the initial cohort. Hypermethylation was associated with decreased expression of TFAP2E in primary and metastatic colorectal-cancer specimens and cell lines. Colorectal-cancer cell lines overexpressing DKK4 showed increased chemoresistance to fluorouracil but not irinotecan or oxaliplatin. In the four other patient cohorts, TFAP2E hypermethylation was significantly associated with nonresponse to chemotherapy (P<0.001). Conversely, the probability of response among patients with hypomethylation was approximately six times that in the entire population (overall estimated risk ratio, 5.74; 95% confidence interval, 3.36 to 9.79). Epigenetic alterations of TFAP2E were independent of mutations in key regulatory cancer genes, microsatellite instability, and other genes that affect fluorouracil metabolism.

CONCLUSIONS
TFAP2E hypermethylation is associated with clinical nonresponsiveness to chemotherapy in colorectal cancer. Functional assays confirm that TFAP2E-dependent resistance is mediated through DKK4. In patients who have colorectal cancer with TFAP2E hypermethylation, targeting of DKK4 may be an option to overcome TFAP2E-mediated drug resistance. (Funded by Deutsche Forschungsgemeinschaft and others.)
THE TREATMENT OPTIONS AND PROGNOSIS for patients with advanced colorectal cancer have improved through the development of novel drugs. However, studies of the molecular biology of cancer initiation and progression have so far provided scant knowledge of the molecular mechanisms contributing to chemotherapy resistance.

Epigenetic alterations underlying the pathogenesis of colorectal cancer have been reported by several groups. These alterations include hypomethylation and hypermethylation of DNA as well as histone modifications, all of which have a profound effect on transcriptional gene regulation. The role of these molecular alterations in response prediction and treatment resistance are far less well known.

The AP-2 transcription factor family has five members and plays an important role in both developmental biology and cancer biology. The gene encoding transcription factor AP-2 epsilon (TFAP2E) is located on chromosome 1p34, a region deleted in several cancers, including colorectal cancer. TFAP2E has two cytosine–phosphate–guanine (CpG) islands, underscoring the potential for regulation of gene expression by means of CpG hypermethylation. In this study, we analyzed the role of TFAP2E in the biologic characteristics of colorectal cancer.

METHODS

PATIENTS AND TISSUE SAMPLES

We obtained samples from patients undergoing surgery or chemotherapy at the University Hospitals in Munich, Mannheim, Bochum, Berlin, Kiel, and Dresden (all in Germany). The response of colon cancer to chemotherapy was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria (version 1.1) (see the Supplementary Appendix, available with the full text of this article at NEJM.org); the response of rectal cancer to chemoradiation was assessed on the basis of histologic characteristics, as previously described. Written informed consent was obtained from all patients before enrollment in the clinical trial (study protocol, in German, available at NEJM.org). Analysis of tissue samples from cancer patients was approved by the human subjects committee of the Technische Universität München.

CELL CULTURE

The colon-cancer cell lines LoVo and DLD-1 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen; the lines SW480, HT-29, HCT-116, and Caco-2 were obtained from the American Type Culture Collection. Cells were cultured in standard medium and treated with aza-2-deoxycytidine or chemotherapeutic agents as described in the Supplementary Appendix.

DNA ANALYSIS

Genomic DNA samples were analyzed with the use of the MethyLight technique or methylation-sensitive high-resolution melting as previously described, after being treated according to the manufacturer’s instructions (Qiagen). The extent of methylation at a specific locus was measured as the ratio of (methylation of the gene + methylation of beta-actin) for the sample to that for Ssxl-treated genomic DNA, multiplied by 100. This formula determines the percentage of the reference material that is methylated. Cutoff values permitting the clearest discrimination between non-neoplastic mucosal samples and tumor samples were determined by means of receiver-operating-characteristic analysis.

EXPRESSION MICROARRAY AND VERIFICATION OF TARGET CANDIDATES

The clones pTFAP2E-pTarget and pTarget SW480 were used in global-expression analysis with the use of a microarray (Human Gene 1.0 ST Expression Array, Affymetrix), according to the manufacturer’s protocol. Genes with a change in expression by more than a factor of 3 were verified by means of quantitative reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay. Data obtained from the microarray have been deposited at the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, accession number 15902739) (see the Supplementary Appendix).

REPORTER AND EXPRESSION VECTORS

The full-length TFAP2E coding sequence was amplified from SW480 cells and cloned, with and without the FLAG epitope (forming pTFAP2E and pTFAP2eFlag, respectively), into the pTarget vector (Promega) according to the manufacturer’s instructions. The promoter sequence of the gene encoding dickkopf homolog 4 protein (DKK4) was cloned into the pGL3 basic vector (Promega): pGL3-DKK4-1kb (with a 1-kb insert starting 1 kb upstream...
of the transcription start site). The pRL-TK reporter plasmid (Promega) was used as an internal control reporter vector. Full-length DKK4-CDS plasmid (pcDNA3-Dkk4) and the DKK4-promoter plasmid pGL3-DKK4-2kb (a pGL3 basic vector with a 2-kb insert starting 2 kb upstream of the transcription start site) were also used.

**GENERATING CLONES WITH STABLE TFAP2E OVEREXPRESSION**

SW480 clones stably overexpressing TFAP2E, and control clones using an empty pTarget vector, were obtained after transfection with pTFAP2E and pTFAP2EFlag by means of a transfection reagent (Lipofectamine 2000, Invitrogen) according to the manufacturer’s instructions. Stable transfectants were selected through G418 treatment (Invitrogen) for 2 weeks. Single colonies were selected and grown further in selective medium. TFAP2E expression was assessed by means of quantitative RT-PCR.

**TRANSIENT TRANSFECTION AND LUCIFERASE ASSAYS**

SW480, CACO-2, and HT-29 cells were transfected with either a pGL3-DKK4-1kb or pGL3-DKK4-2kb vector and pRL-TK as a transfection control (renilla luciferase). Transfections were carried out with the use of a transfection reagent (Satisfaction, Agilent). After 3 days, cells were harvested, and firefly and renilla luciferase activities were measured by means of a luminometer (Luciferase Dual Reporter Assay, Promega). All experiments were repeated independently in triplicate, and all cells were seeded in triplicate on the 96-well plates.

**CHROMATIN IMMUNOPRECIPITATION**

Human colorectal-cancer cells were subjected to chromatin immunoprecipitation with the use of an anti-FLAG antibody (Sigma) according to a standard protocol (Upstate Biotechnology) followed by genomic real-time quantitative PCR analysis of the eluted DNA fragments. Primers were designed to interrogate the promoter regions 1 to 2 kb from the transcriptional start site and flanking the predicted AP-2 binding sites. Agarose beads coupled to either anti-FLAG antibody or control IgG were used, and specific and quantitative amplification of genomic fragments was performed. Cycle-threshold values of immunoprecipitated DNA were normalized to cycle-threshold values of input DNA and were presented as the change factor relative to the IgG control value.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Polyclonal antiserum samples were generated against human TFAP2E. Rabbits were immunized with the sequence-specific peptide NH₂-HTYSAMERPDGLGAAAGARC-CONH₂. The peptide was custom-synthesized, and the monospecific IgG fraction was affinity-purified against the immobilizing peptide (Pineda). Specificity was tested by means of Western blotting and dot-blot analyses, as well as immunohistochemical investigations of formalin-fixed and paraffin-embedded sections of primary or metastatic colorectal-cancer specimens. Immunostaining was carried out according to standard protocols (see the Supplementary Appendix).

**MICROSATELLITE INSTABILITY AND ANALYSIS OF GENE MUTATIONS**

Microsatellite instability was analyzed according to the recommendation of the National Institutes of Health by means of PCR amplification of five microsatellite markers: BAT25, BAT26, D2S123, DSS346, and D17S250. Biochip arrays (Randox Laboratory) were used for mutation analysis of the relevant colorectal-cancer gene mutations.

**STATISTICAL ANALYSIS**

Receiver-operating-characteristic analysis was performed to determine the optimal cutoff value for the percentage of methylated reference material. Levels of at least 30% were considered to indicate hypermethylation, assigned a value of 1, whereas levels below 30% were considered to indicate hypomethylation, assigned a value of 0. A detailed description of additional statistical analyses is given in the Supplementary Appendix.

**RESULTS**

**FREQUENCY OF TFAP2E HYPERMETHYLATION**

Genomic DNA was obtained from samples of primary colorectal cancer and adjacent mucosa (all snap-frozen) from the 74 patients in the initial cohort and was analyzed for TFAP2E hypermethylation. DNA from 38 of the 74 patients (51%) was classified as hypermethylated. None of the common clinicopathological characteristics of patients with colorectal cancer — including primary tumor site, histologic grade of differentiation or stage of cancer, age, or sex — correlated with TFAP2E methylation status (Table 1).
TFAP2E and Colorectal Cancer

Tumor messenger RNA (mRNA) from 28 of the 74 patients in the initial cohort was analyzed for TFAP2E expression by means of RT-PCR. Twelve patients showed TFAP2E hypermethylation and decreased TFAP2E mRNA expression, whereas 3 showed hypomethylation and strong mRNA expression. The remaining 13 patients showed mRNA expression and hypermethylation or no mRNA expression and hypomethylation. Furthermore, TFAP2E expression was assessed in a group of patients who had tissue samples from matched primary cancers and metastases. Immunohistochemical evaluation revealed expression of TFAP2E in the cell nuclei; cells from both the primary cancers and metastases showed TFAP2E immunoreactivity. No difference in expression patterns was noted in synchronous and metachronous lesions. Overall, there was a trend toward increased expression in cancers with TFAP2E hypomethylation, but significance was not reached, because of the limited number of samples (Fig. 1, and the Supplementary Appendix).

DKK4 Targeted by TFAP2E

For the identification of potential downstream targets of TFAP2E, SW480-pTFAP2E and SW480-pTarget cell clones were subjected to microarray analysis. The verification of these results by means of quantitative real-time PCR confirmed DKK4 as the target gene, which was significantly down-regulated (on average, to 17% of normal expression [range, 33 to 11]) in all stable SW480-pTFAP2E clones, as compared with empty SW480-pTarget vector controls. DKK4 mRNA levels were then analyzed in nontransfected colorectal-cancer cell lines. SW480 and CACO-2 showed strong DKK4 expression that was lost on reexpression of TFAP2E through aza-cytidine treatment (see the Supplementary Appendix).

Table 1. Characteristics of Patients with Colorectal Cancer Screened for TFAP2E Methylation (Initial Cohort).* 

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (N = 74)</th>
<th>TFAP2E Hypermethylation (N = 38)</th>
<th>TFAP2E Hypomethylation (N = 36)</th>
<th>P Value</th>
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<tr>
<td>Age — yr</td>
<td>67.2±9.7</td>
<td>69.6±9.2</td>
<td>64.8±10.2</td>
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<tr>
<td>Sex — no. (%)</td>
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<tr>
<td>Male</td>
<td>43 (58)</td>
<td>23 (61)</td>
<td>20 (56)</td>
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</tr>
<tr>
<td>Female</td>
<td>31 (42)</td>
<td>15 (39)</td>
<td>16 (44)</td>
<td></td>
</tr>
<tr>
<td>Grade of differentiation — no./total no. (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>G0 or G1</td>
<td>59/64 (92)</td>
<td>30/34 (88)</td>
<td>29/30 (97)</td>
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<tr>
<td>G2 or G3</td>
<td>5/64 (8)</td>
<td>4/34 (12)</td>
<td>1/30 (3)</td>
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<td>Localization — no./total no. (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
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<td>Rectum</td>
<td>19/64 (30)</td>
<td>8/34 (24)</td>
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<td>Sigmoid colon</td>
<td>19/64 (30)</td>
<td>9/34 (26)</td>
<td>10/30 (33)</td>
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<td>Colon</td>
<td>26/64 (41)</td>
<td>17/34 (50)</td>
<td>9/30 (30)</td>
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<td>Staging — no./total no. (%)</td>
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<td>T1 or T2</td>
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<td>11/34 (32)</td>
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<td>T3 or T4</td>
<td>47/64 (73)</td>
<td>23/34 (68)</td>
<td>24/30 (80)</td>
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<td>Lymph nodes involved — no./total no. (%)</td>
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<td>N0</td>
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<td>N1 or N2</td>
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<td>Metastasis — no./total no. (%)</td>
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<td></td>
<td>&gt;0.99</td>
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<td>M0</td>
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<td>31/34 (91)</td>
<td>28/30 (93)</td>
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<tr>
<td>M1</td>
<td>5/64 (8)</td>
<td>3/34 (9)</td>
<td>2/30 (7)</td>
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* Plus–minus values are means ±SD.
Effect of TFAP2E Expression on Fluorouracil Resistance

Since DKK4 has been implicated in responsiveness to chemotherapy, and our findings showed that DKK4 was a downstream target of TFAP2E, the role of TFAP2E and DKK4 in mediating drug resistance in colorectal-cancer cells was assessed in vitro. SW480 cells were transiently transfected with pTFAP2E or pcDNA3-Dkk4 and treated with oxaliplatin, irinotecan, or fluorouracil. The number of surviving cells was measured by means of a 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium

Figure 1. Immunohistochemical Evaluation of TFAP2E Expression.

Immunostaining with a polyclonal anti-TFAP2E antibody showed strong, homogeneous staining in the primary tumor (Panel A) and corresponding liver metastasis (Panels B and C) or weak or no staining in the primary colon cancer (Panel D) and corresponding liver metastasis (Panels E and F). Hepatocytes from the nonneoplastic liver tissue did not express TFAP2E (Panels C and F, shown at half the magnification of the other panels; arrows). Hematoxylin was used as a counterstain.
bromide (MTT) assay. All experiments were performed in triplicate. After 2 and 3 days of exposure to fluorouracil, SW480-pTFAP2E–transfected cells showed a significant decrease in survival (of about 20% of control values) and SW480 pcDNA3-Dkk4–transfected cells showed a significant increase in survival (of about 10% of control values), respectively (P<0.01 by the Kruskal–Wallis test) (Fig. 2A). Cells transfected with both pTFAP2E and pcDNA3-Dkk4 had intermediate responsiveness. These results were confirmed in SW480-pTFAP2E clones and SW480-pTarget vector controls treated with fluorouracil (P<0.01 by the Mann–Whitney U test) (Fig. 2B). Similar effects of overexpressed TFAP2E and DKK4 on cell growth were observed in HT29 and DLD1 cells treated with increasing concentrations of fluorouracil (see the Supplementary Appendix).

**TFAP2E METHYLATION AND CHEMOTHERAPY RESISTANCE**

We assessed TFAP2E methylation in four cohorts of patients with colorectal cancer undergoing chemotherapy or chemoradiation with a fluorouracil-based regimen, to analyze chemoresponse in vivo (Table 2). Cohort I (Bochum) included 76 patients who were enrolled in a prospective trial comparing the oral fluoropyrimidine capcitabine and oxaliplatin (CAPOX) with intravenous fluorouracil and oxaliplatin (FUFOX) in patients with metastatic colorectal cancer.15 Of the 76 patients, 74 had samples of enough DNA for methylation analysis as well as clinical-response data. Cohort II (Dresden) consisted of 44 samples from patients with metastatic colorectal cancers undergoing fluorouracil–based polychemotherapy (folinic acid [leucovorin], fluorouracil, and irinotecan [FOLFIRI] or folinic acid, fluorouracil, and oxaliplatin [FOLFOX]). Of the 44 patients, 43 had sufficient DNA for methylation analysis; of these, 39 had clinical-response data but 3 stopped chemotherapy owing to intolerable toxic effects and were excluded from the analysis, leaving 36 patients with data. Cohort III (Mannheim) comprised 50 patients with rectal cancer undergoing chemoradiation with fluorouracil in combination with irinotecan and cetuximab. A total of 49 samples yielded DNA, but matching clinical data were available for only 42. Cohort IV (Munich) consisted of 70 patients with primary rectal cancer who underwent fluorouracil-based chemoradiation (i.e., intravenous fluorouracil and 45.0 Gy of radiation). Sufficient DNA samples were available for 69 patients, 68 of whom had clinical-response data.

Table 2 shows the frequencies of TFAP2E hypermethylation and response to treatment for each cohort. All four cohorts showed a negative association of methylation and treatment response (i.e., higher response rates among patients with hypomethylated cancers), indicated by a significant difference in response rates between patients with hypomethylation and patients with hypermethylation. A substantial effect size was estimated for the pooled cohort data (Fig. 3A): the treatment-response ratio for hypomethyl-
ation versus hypermethylation was 5.29 (95% confidence interval [CI], 2.87 to 9.76). In patients with metastatic colorectal cancer, tumor response was assessed on the basis of RECIST; the treatment-response ratio was 7.38 (95% CI, 2.86 to 19.03). In patients with rectal cancers who underwent chemoradiation, treatment response was evaluated according to histologic response; the response ratio was 5.10 (95% CI, 2.67 to 9.74). When the cohorts were pooled according to response-evaluation criteria (histologic assessment or RECIST), the overall estimated effect was strong, with a response ratio of 5.74 (95% CI, 3.36 to 9.79) (Fig. 3B).

**DISCUSSION**

The AP-2 transcription factor family consists of five members — proteins encoded by the transcription factor AP-2 alpha, beta, gamma, delta, and epsilon genes TFAP2A, TFAP2B, TFAP2C, TFAP2D, and TFAP2E, respectively. These proteins can bind to keratin promoters and act as heterodimers or homodimers. Expression of AP-2 proteins has been reported in various tissues, notably the breast for TFAP2C and skin for TFAP2E. TFAP2A has been shown to be hypermethylated and acts as a tumor suppressor in various cancers. TFAP2B seems to play a role in diabetes. TFAP2C acts as a tumor suppressor in breast cancer and may predict tamoxifen resistance through its relation to human epidermal growth factor receptor type 2 (HER2). TFAP2D and TFAP2E have also been shown to be hypermethylated in prostate and colorectal cancer.

We report that the TFAP2E gene is frequently hypermethylated in cancers from patients with colorectal cancer, and in these cancers, TFAP2E
mRNA levels are frequently down-regulated. We also observed decreased protein expression in cancers with increased TFAP2E methylation; however, probably owing to the limited number of cases in which both expression and methylation could be analyzed, this association was not significant. Nonetheless, the inverse relationship between TFAP2E hypermethylation and TFAP2E expression in our patients supports the findings from our in vitro experiments in which TFAP2E expression was induced by azacytidine in colorectal-cancer cells with low or no TFAP2E expression.

To characterize the downstream targets of TFAP2E, we performed a microarray analysis of TFAP2E-overexpressing cells and found DKK4 to be a potential target gene that was significantly down-regulated by way of TFAP2E. Further studies confirmed repression of DKK4 promoter activity through TFAP2E and binding of TFAP2E to the DKK4 promoter in vitro. DKK4 is a member of the dickkopf family, comprising various antagonists of WNT signaling by binding to the WNT coreceptor low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6). DKK4 overexpression has been observed in the colon mucosa of patients with colitis. However, the precise role of DKK4 in the colon mucosa and its contribution to carcinogenesis are so far unknown. Our assessment of DKK4 expression in human colorectal cancer specimens and cell lines revealed an inverse expression pattern of TFAP2E and DKK4. Despite the complexity of the human cancer tissues, which clearly limits the interpretation of expression data, we confirmed this inverse expression pattern in a subgroup of patients with colorectal cancer as well.

Over the past decade, DKK4 has been implicated in fluorouracil resistance in colorectal-cancer cell lines. Our in vitro data from cell lines treated with fluorouracil confirmed this observation. DKK4 overexpression led to increased fluorouracil chemoresistance in colorectal-cancer cell lines, whereas the introduction of TFAP2E was associated with increased sensitivity to fluorouracil treatment. This in vitro observation was further supported by the analysis of biopsy specimens from patients with colon or rectal cancer undergoing fluorouracil-based chemotherapy or chemoradiation. Tissue samples from patients undergoing treatment with fluorouracil resistance were analyzed for TFAP2E and DKK4 expression, and a significant inverse relationship was observed.

A

<table>
<thead>
<tr>
<th>Center</th>
<th>No. of Patients</th>
<th>Risk Ratio (95% CI)</th>
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<tbody>
<tr>
<td>Bochum</td>
<td>74</td>
<td>4.07 (1.40–11.82)</td>
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<tr>
<td>Dresden</td>
<td>36</td>
<td>15.43 (3.27–72.90)</td>
</tr>
<tr>
<td>Mannheim</td>
<td>42</td>
<td>3.30 (1.53–7.13)</td>
</tr>
<tr>
<td>Munich</td>
<td>68</td>
<td>8.10 (2.73–24.06)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5.29 (2.87–9.76)</strong></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Method of Response Evaluation</th>
<th>No. of Patients</th>
<th>Risk Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
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<td>RECIST</td>
<td>110</td>
<td>7.38 (2.86–19.03)</td>
</tr>
<tr>
<td>Histology</td>
<td>110</td>
<td>5.10 (2.67–9.74)</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>5.74 (3.36–9.79)</strong></td>
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</tr>
</tbody>
</table>

Figure 3. Treatment Responses in Four Patient Cohorts and Association with TFAP2E Methylation.

Effects on treatment response owing to hypomethylation are shown according to center (Panel A) as well as response-evaluation technique (Panel B).
uracil only were not available, since most patients with colorectal cancer undergo combination chemotherapy. Nonetheless, we found a strong association between TFAP2E methylation and a lack of fluorouracil-based chemotherapy response in the tumor in four independent cohorts of patients with colorectal cancer. Random-effects model analysis of the pooled cohort data revealed that the probability of response to treatment was six times as high among patients with hypomethylation as among patients with hypermethylation. The correlation was observed in primary rectal cancers and metastatic colorectal cancers, independent of whether the treatment was fluorouracil-based chemotherapy or chemoradiation. Also, assessment of response in these cohorts, with the use of either standard RECIST or histologic-response criteria, did not influence this strong association, indicating that TFAP2E methylation may be valuable for response prediction in either setting. Since treatment strategies differed across the four cohorts of patients with colorectal cancer, alterations of TFAP2E–DKK4 not only may be associated with fluorouracil resistance but also could present a more global chemotherapy-resistance marker.

The identification and validation of potential genetic and molecular alterations underlying the pathogenesis of cancers, and the role of such alterations as targets or response predictors in various cancers, has been the focus of several research groups. The molecular mechanisms underlying TFAP2E–DKK4-mediated chemoresistance are still unknown. Our studies did not reveal an association with genes encoding fluorouracil-catalyzing enzymes or genes underlying microsatellite instability or WNT signaling (see the Supplementary Appendix). Further studies will be necessary to understand the precise molecular changes leading to chemoresistance.

Overall, our data indicate that fluorouracil-based chemotherapy is largely ineffective in patients with colorectal cancer with TFAP2E hypermethylation. Specific targeting of DKK4 in these individuals may therefore be an option for overcoming TFAP2E-mediated chemoresistance.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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REFERENCES


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