Imaging, Diagnosis, Prognosis

Associations of a Polymorphism in the Ornithine Decarboxylase Gene with Colorectal Cancer Survival

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Abstract

Purpose: Activity of ornithine decarboxylase (ODC), the first enzyme in polyamine synthesis, is required for normal growth and is elevated in many cancers, including colorectal cancer. We examined associations of the +316 *ODC1* single nucleotide polymorphism (SNP) with colorectal cancer–specific survival among colorectal cancer cases, and then investigated its functional significance in colon cancer cells.

Experimental Design: The study included 400 incident stage I-III colorectal cancer cases from the population-based University of California Irvine Gene-Environment Study of Familial Colorectal Cancer (diagnosed from 1994 to 1996 with follow-up through March 2008). The primary outcome was colorectal cancer–specific survival dependent on *ODC1* (rs2302615) genotype (GG versus GA/AA). In human colon cancer cell lines, *ODC1* allele-specific binding of E-box transcription factors was determined via Western blotting and chromatin immunoprecipitation assays. *ODC1* allele-specific promoter activity was determined using promoter constructs in combination with vectors expressing either the transcriptional activator c-MYC or the repressor MAD1.

Results: Genotype-specific survival differences were observed among colorectal cancer cases: compared with cases with the *ODC1* GG genotype (hazards ratio, 1; reference) the adjusted colorectal cancer–specific survival hazards ratio was 2.02 (95% confidence interval, 1.17-3.50) for *ODC1* GA/AA cases ($P = 0.012$). In colon cancer cells, the *ODC1* SNP, flanked by two E-boxes, predicts *ODC1* promoter activity. The E-box activator c-MYC and repressors MAD1 and MAD4 preferentially bind to *ODC1* minor A-alleles, compared with major G-alleles, in cultured cells.

Conclusions: These results have implications for conditional regulation of polyamine homeostasis and suggest a model in which the *ODC1* SNP may be protective for colon adenoma recurrence and detrimental for survival after colon cancer diagnosis. (Clin Cancer Res 2009;15(19):6208–16)

Polyamines are small ubiquitous molecules involved in various processes, including transcription, RNA stabilization, ion channel gating, and others (1). Ornithine decarboxylase (ODC), the first enzyme in polyamine synthesis, is essential for normal development and tissue repair in mammals but is downregulated in most adult tissues (2). Multiple abnormalities in the control of polyamine metabolism and transport result in increased polyamine levels that can promote tumorigenesis in several tissues (3). Polyamine metabolism is upregulated in the intestinal epithelial tissues of humans with familial

Translational Relevance

Excess polyamine formation has long been implicated in epithelial carcinogenesis, particularly colorectal carcinogenesis. Recently, a strategy involving inhibition of ornithine decarboxylase (ODC) activity (i.e., the rate-limiting enzyme of polyamine synthesis) has shown remarkable efficacy in preventing the recurrence of colorectal polyps in humans. Epidemiologic and experimental results from the present research show conditional regulation of polyamine homeostasis by genetic polymorphism in *ODC1*, and suggest a model in which the +316 *ODC1* single nucleotide polymorphism may be protective for colon adenoma recurrence and detrimental for survival after diagnosis of nonmetastatic colorectal cancer. This information may be useful for determining colon cancer prognosis. By identifying patients at increased risk of cancer progression/recurrence, early implementation of tertiary prevention management strategies could be instituted. Additionally, this research may help to identify high-risk but otherwise optimally treated locoregional colorectal cancer patients who would benefit from tertiary cancer prevention clinical trials.

adenomatous polyposis (4), a syndrome associated with high risk of colon and other cancers. Familial adenomatous polyposis is caused by mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene, and we have shown that wild-type APC signaling downregulates *ODC1* expression in both human cells (5) and in a mouse model of familial adenomatous polyposis (6). Wild-type APC expression leads to decreased expression of ODC, whereas mutant APC leads to increased expression of ODC. The mechanism of APC-dependent regulation of ODC involves E-box transcription factors, including the transcriptional activator c-MYC and the transcriptional repressor MAD1 (5, 7). c-MYC was shown by others to regulate *ODC1* transcription (8). Several genes involved in polyamine metabolism are essential genes for optimal growth in most organisms, and are downregulated in nonproliferating and/or adult cells and tissues (2). The polyamines influence specific cellular phenotypes, in part, by affecting patterns of gene expression, as reviewed elsewhere (9).

D, L-α-difluoromethylornithine (DFMO), a selective ODC inhibitor, decreases *APC*-dependent intestinal tumorigenesis in mice (6). Oral DFMO administered daily to humans inhibits ODC enzyme activity and polyamine contents in a number of epithelial tissues (10–15). Recently, DFMO in combination with the nonsteroidal anti-inflammatory drug sulindac has been reported to markedly lower the adenoma recurrence rate among individuals with colonic adenomas when compared with placebos in a randomized clinical trial (16). We have associated a common single nucleotide polymorphism (SNP) in the *ODC1* promoter, 316 nucleotides 3′ of the transcription start site, with decreased risk of colon polyp recurrence especially in people who report using aspirin (7), and this latter finding has been confirmed by others (17, 18). This SNP falls between two consensus E-box binding elements. Although the risk of adenoma recurrence based on *ODC1* genotype has been investigated previously, the effects of this SNP on clinical outcomes after colorectal cancer diagnosis are unknown.

In this report, we studied the association of the *ODC1* +316 SNP with colorectal cancer–specific mortality among colorectal cancer cases from a population-based study. Subsequently, using experimental models involving cultured colon cancer cells, we investigated the functional significance of the *ODC1* SNP, as depicted in Fig. 1. Previous work by our group and others has associated the ODC A-allele at +316 to reduced the risk of colon polyp recurrence (7). Ornithine decarboxylase +316 genotype is prognostic for colorectal adenoma recurrence and predicts efficacy of aspirin chemoprevention (18). Visvanathan et al. (19) have reported results apparently at odds with our previous findings in colon carcinogenesis. Visvanathan and colleagues have associated the *ODC1* A-allele at +316 with increased risk of prostate cancer. Our earlier functional studies using promoter reporter methods (7) showed that the repressor MAD1 could suppress the expression of the *ODC1* promoter in an allele-specific manner. However, no direct evidence for binding of E-box transcription factors to the *ODC1* promoter in an allele-specific manner has yet been reported. Consequently, we tested the hypothesis that both transcriptional activators and repressors might bind selectively to the *ODC1* promoter in an allele-specific manner. Such allele-specific binding might explain the apparent discordant associations of this allele with both reduced risk of precancerous lesions but increased risk of invasive cancer, based on differences in the expression of E-box transcriptional activators and repressors. For example, c-MYC is known to be expressed at low levels in normal intestinal epithelium, but at high levels in intestinal polyps, in a mouse model of intestinal carcinogenesis (20).

Materials and Methods

Epidemiologic studies: ODC1 +316 SNP associations with colorectal cancer–specific survival

Study population. We studied incident cases of invasive colorectal cancer with stage I-III disease at presentation enrolled in the University of California, Irvine Gene-Environment Study of Familial Colorectal Cancer (21, 22) during the period 1994-1996 with follow-up through March 2008. The parent study was designed to determine the incidence of non-polyposis colon cancer in a large, population-based cohort of colorectal cancer cases. Participants were identified through the population-based cancer registries of the Cancer Surveillance Program of Orange County/San Diego Imperial Organization for Cancer Control using the April 2008 data file. In the parent study (21), all subjects with colorectal cancer diagnosed at all ages in Orange County, CA, from 1994 to1996 were ascertained. All subjects diagnosed in San Diego and Imperial Counties, CA, at ages <65 y in 1994 and 1995 were also ascertained. Cases were then contacted if they were eligible for the study
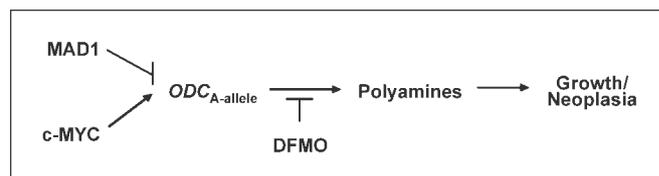

Fig. 1. Schema depicting the proposed differential effects of polyamine regulation by MAD1 and c-MYC on the *ODC1* +316 minor A-allele. Effects of the ODC inhibitor DFMO (difluoromethylornithine) are also shown.

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Table 1. Descriptive analysis for colorectal cancer cases overall and based on *ODC1* genotype

	All CRC cases (n = 400)	ODC1 GG (n = 208)	ODC1 GA/AA (n = 192)	P
Median age in y (range)	56.6 (29-89)	56.8 (29-89)	56.4 (29-82)	0.24
Gender				
Male	230 (58%)	117 (56%)	113 (59%)	0.60
Female	170 (41%)	91 (44%)	79 (41%)	
Ethnicity				
Non-Hispanic Caucasian	351 (88%)	188 (90%)	163 (85%)	0.14
African American	4 (1%)	2 (1%)	2 (1%)	
Caucasian-Hispanic	20 (5%)	11 (5%)	9 (5%)	
Asian	23 (6%)	7 (3%)	16 (8%)	
Other	2 (<1%)	0 (0%)	2 (1%)	
Family history of CRC				
Yes	106 (26%)	56 (27%)	50 (26%)	0.84
No	294 (74%)	152 (73%)	142 (74%)	
Stage at diagnosis				
I	146 (37%)	72 (35%)	74 (39%)	0.49
II	128 (32%)	72 (35%)	56 (29%)	
III	126 (31%)	64 (31%)	62 (32%)	
Colon/Rectum site				
Proximal and transverse	120 (30%)	65 (31%)	55 (29%)	0.90
Descending	22 (6%)	12 (6%)	10 (5%)	
Sigmoid	110 (28%)	55 (26%)	55 (29%)	
Rectosigmoid	57 (14%)	32 (15%)	25 (13%)	
Mid-low rectum	86 (22%)	41 (20%)	45 (23%)	
Colorectum, unspecified	5 (1%)	3 (1%)	2 (1%)	
Histologic subtype				
Adenocarcinoma	364 (91%)	183 (88%)	181 (94%)	0.16
Mucinous adenocarcinoma	26 (7%)	18 (9%)	8 (4%)	
Carcinoma	9 (2%)	6 (3%)	3 (2%)	
NOS	1 (<1%)	1 (<1%)	0 (0%)	
Tumor grade				
I	62 (17%)	27 (14%)	35 (20%)	0.36
II	261 (71%)	140 (73%)	121 (68%)	
III	46 (13%)	24 (13%)	22 (12%)	
Missing data	31	17	14	
Surgical treatment				
Yes	395 (99%)	204 (98%)	191 (99%)	0.21
No	5 (1%)	4 (2%)	1 (1%)	
Radiation therapy				
Yes	63 (16%)	29 (14%)	34 (18%)	0.30
No	337 (84%)	179 (86%)	158 (82%)	
Chemotherapy				
Yes	170 (45%)	84 (44%)	86 (46%)	0.56
No	208 (55%)	109 (56%)	99 (54%)	
Missing data	22	15	7	

Abbreviations: CRC, colorectal cancer; NOS, not otherwise specified.

(alive at the time ascertained and having a contact address) and if their physicians did not deny permission to contact. At the time of study entry, cases signed a consent form allowing for blood draws and the release of medical information. This study was approved by the University of California Irvine Institutional Review Board (#93-257). Clinical and demographic data including vital status and follow-up were obtained through linkage to the regional cancer registry databases as previously described (21–23). Tumor grade was recorded according to standard convention: grade I, well-differentiated; grade II, moderately differentiated; grade III, poorly differentiated; grade IV, undifferentiated. Tumor, node, metastasis (TNM) staging determination was derived from existing American Joint Committee on Cancer (AJCC) codes where available and conversion of the extent of disease codes, as previously reported (24). Family history of cancer in a first-degree relative was ascertained by self-reporting during a telephone interview conducted at enrollment (23, 25). Twenty-two cases with hereditary non-polyposis colon cancer, as defined by Amsterdam criteria, were identified and excluded from the analysis. The median time from colorectal cancer diagnosis until study entry (i.e., date of family history interview) was 18 mo (95% confidence interval, 12-32 mo).

DNA extraction and ODC1 +316 SNP genotyping. DNA was extracted from 2 mL RBC clot samples using the QIAGEN QIAamp DNA Midi or Mini Kits (Qiagen), following the manufacturer's instructions. Genotyping of the *ODC1* (National Center for Biotechnology Information SNP database ID rs2302615) +316 SNP was conducted using oligonucleotide primers designed to amplify a 172-bp fragment containing the polymorphic base at +316 (Applied Biosystems). Allele-specific TaqMan probes were synthesized with different 5′ labels (6-carboxyflourescein or VIC) and the same 3′ quencher dye (6-carboxytetramethylrhodamine; ref. 26). Each PCR reaction (5 μL total) contained 10 ng of participant DNA, 30 pmol of each primer, 12.5 pmol of each TaqMan probe, and 1× TaqMan Universal PCR Master Mix (Applied Biosystems), as previously reported (7, 26).

Statistical analysis – population-based study. An estimated 1:1 ratio of *ODC1* GG genotype to *ODC1* GA/AA genotype was expected based on previous literature (7, 17, 18, 26). Of 481 DNA samples, 440 were

successfully genotyped. A total of 41 cases (8.5%) resulted in an undetermined *ODC1* +316 genotype due to low DNA concentration and/or poor DNA quality, but no clinicopathologic differences were observed between the successfully genotyped and unsuccessfully genotyped cases. The final analytic cohort was restricted to 400 colorectal cancer cases with stage I-III disease. Comparisons of demographic, clinical, and pathologic variables among colon and rectal cases were done using Pearson χ^2 statistic or Fisher's exact test for nominal variables and Student's *t*-test for continuous variables. Colorectal cancer–specific survival was defined as mortality from colorectal cancer itself, and data censoring occurred in the following instances: alive at the end of follow-up, loss to follow-up, or death from any cause other than colorectal cancer. Overall survival was defined as mortality from any cause. Survival curves were constructed for colon and rectal cancer cases using the Kaplan-Meier method and analyzed with the log rank test for univariate analyses. Cox proportional hazards modeling was done for all colorectal cancer cases, colon cancer cases, and rectal cancer cases using time since diagnosis to profile the adjusted risk of overall and colorectal cancer–specific death based on *ODC1* genotype. The effects of *ODC1* genotype on survival time were analyzed in the Cox models with stratification for TNM stage at diagnosis and adjustment for the following covariates: age, gender, ethnicity, family history of colorectal cancer, tumor site within the colon, histologic subtype, treatment with surgery, radiation therapy, and chemotherapy. For covariates with categorical data, dummy coding was assigned (0 or 1) and each category was included in the multivariate models in comparison with the referent group. *ODC1* genotype was analyzed using the dominant model (GG versus GA/AA) with dummy variables, with GA/AA coded as 1 and GG as the referent group. Subset analyses were done using the additive model for *ODC1* either as continuous variable 0, 1, and 2 test for trend, or using two dummy variables, one for GA and one for AA, with GG as the referent group. All analyses were conducted using SAS 9.2 statistical software (SAS Institute). Statistical significance was assumed for a two-tailed $P < 0.05$.

Experimental studies: ODC1 +316 SNP regulation in colon cancer cells

Cell culture. The human colon cancer cell lines HT29 and HCT116 were maintained in McCoy's 5A medium (Invitrogen). All media used were supplemented with 10% fetal bovine serum plus 1% penicillin/

streptomycin solution (Invitrogen). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Genotyping assay. DNA samples from HT29 and HCT116 cells were subjected to a PCR-restriction fragment length polymorphism procedure to detect the polymorphic PstI site. Sequences were amplified by PCR, using the following primers: 5′-TCTGCGCTTCCCCATGGGGCT-3′ and 5′-TTTCCCAACCCTTCG-3′. Each reaction contained 1 μL DNA, 4 pmol of each primer, 12.5 μL 2× PCR PreMixes buffer "G" (EPICENTRE Biotechnologies), and 0.5 unit of Taq DNA polymerase, in a final volume of 25 μL. The expected size of the PCR product was 351 bp. After amplification, 10 to 20 μL of the PCR product were digested with 10 units of PstI in 30 μL for 2 h at 37°C. DNA from HT29 cells (GA), containing the PstI site, yielded two fragments of 156 and 195 bp.

Western blot analysis. Cells were harvested, lysed, and proteins were separated on a 12.5% SDS-PAGE gel. Proteins were transferred by electrophoresis onto a Hybond-C membrane. The membrane was blocked with Blotto A (5% blocking grade dry milk in tris-tween buffered saline solution) and probed using 1:300 dilutions of primary antibodies (Santa Cruz Biotechnology) in Blotto A. Primary antibodies were incubated at 4°C overnight, followed by incubation with an appropriate horseradish peroxidase–tagged secondary antibody (1:1,000 dilution) for 1 h at room temperature. Chemiluminescent detection was conducted using ECL Western Detection reagent (Amersham Biosciences) and exposed on Biomax XAR film (Kodak).

Chromatin immunoprecipitation. Chromatin immunoprecipitation assays were done using a commercial kit, as recommended by the manufacturer (Upstate Biotech). Briefly, cells were treated with 1% formaldehyde to cross-link DNA and proteins, and DNA-protein complexes were disrupted by sonication to lengths between 200 and 1,000 bp. Lysates were diluted 10-fold with immunoprecipitation dilution buffer containing protease inhibitors. Antibodies for c-MYC, MAD1, and MAD4 (Santa Cruz Biotechnology) were used to precipitate chromatin, whereas an additional sample was left as a minus-antibody control. Samples were immunoprecipitated overnight at 4°C with rotation. Immune complexes were obtained by adding 60 μL of salmon sperm DNA/protein A Agarose slurry and incubating for 1 h at 4°C with rotation followed by gentle centrifugation (1,000 rpm, 1 min). Protein A agarose pellets were washed with low-salt buffer, high-salt buffer, LiCl buffer, and TE buffer. Then the complexes were eluted by adding 250 μL elution buffer (0.1 mol/L $NaHCO_3$, 1% SDS) twice, and DNA-protein cross-links were reversed with 0.2 mol/L NaCl by heating at 65°C for 4 h for all samples, including the input DNA and minus-antibody DNA controls. DNA was resuspended in 30 μL of ddH_2O. For visualization of PCR product and its size, standard PCR reactions were carried out. The sequences of *ODC1* primers used for PCR were 5′-CCTGGGCGGCTCTGAGGT-3′ (17mer) and 5′-AGGAAGCGGCGCCT-CAA-3′ (17mer). Quantitative real-time PCR was done using TaqMan gene expression assays kit (Applied Biosystems) on an ABI7700 sequence detection system. Details for the computation of relative binding can be found on the manufacturer's website (http://www.appliedbiosystems.com/).

Transient transfections. Transient transfections were done using LipofectAMINE reagent (Invitrogen) according to the manufacturer's protocol, as detailed in the supplementary file. HCT116 and HT29 cells were transfected with 1 μg of pGL3-*ODC1*/A or pGL3-*ODC1*/G plasmids (7) along with 0.01 μg of Renilla-TK plasmid. The Renilla-TK plasmid was purchased from Promega and used as a transfection efficiency control in all promoter-reporter transfection experiments. For *MYC* experiments, *ODC1* pGL3-plasmids were cotransfected with either pcDNA 3.0 or CMV-*MYC* expression vector (OriGene). For *MAD1* experiments, the *ODC1* plasmids were cotransfected with either pcDNA 3.1 or pcDNA-*MAD1*. For *MYC* and *MAD1* cotransfection, *ODC1* promoter reporter constructs were prepared which contained the first 1.6 Kb of the *ODC1* gene cloned into a pGL3 vector. The constructs included E-box 1 (-485 to -480 bp) intact (wt E-box 1) or deleted (mut E-box 1). Additionally, both variants of the +316 *ODC1* SNP were used, creating a total of four different constructs.
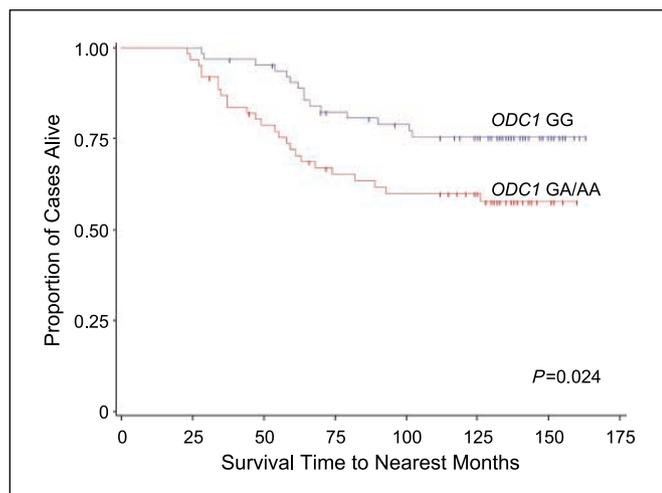
Fig. 2. Kaplan-Meier colorectal cancer–specific survival rate estimates for cases with stage III colorectal cancer, stratified by *ODC1* +316 genotype. Includes cases from the University of California Irvine Gene-Environment Study of Familial Colorectal Cancer diagnosed during the period 1994-1996 with follow-up through March 2008: *ODC1* GG (64 cases, 15 colorectal cancer–specific deaths), *ODC1* GA/AA (62 cases, 25 colorectal cancer–specific deaths).

After 6 h of incubation, cells were supplemented with complete medium containing 20% fetal bovine serum and left to grow overnight. The next day after transfection 20% fetal bovine serum–containing complete medium was replaced with 10% fetal bovine serum–containing medium. Forty-eight hours after transfection, cells were washed with PBS and lysed in Passive Lysis Buffer from the Dual Luciferase Assay kit (Promega). Dual luciferase activities were measured using a Turner Designs TD-20/20 luminometer, as described by the manufacturer, and presented as relative luciferase units. Experiments were done in triplicates and repeated at least twice.

Statistical analysis – experimental studies. For transient transfection experiments, two-sample *t*-tests were used (Microsoft Excel Microsoft Corp.). The effect of *MYC* expression on *ODC1* allele-specific promoter activity was examined in HT29 colon cancer cells using *ODC1* promoter constructs differing by the presence of the first E-box element: (*a*) wild type (wt) E-box1+316 G, (*b*) mutant (mut) E-box1 + 316 G, (*c*) wt E-box1 + 316 A, and (*d*) mut E-box1 + 316 A. For each promoter construct, two-sample *t*-tests were used to compare promoter activity between cells cotransfected with pcDNA3.0 plasmid versus those transfected with the CMV-*MYC* expression vector. Similarly, to examine the effect of *MAD1* expression on *ODC1* allele-specific promoter activity, two-sample *t*-tests were used to compare the effect of promoter activity in promoter constructs cotransfected with pcDNA3.1 plasmid versus those transfected with pcDNA-*MAD1* plasmid. Statistical significance was assumed for a two-tailed $P < 0.05$.

Results

Epidemiologic studies: association of the ODC1 +316 SNP with colorectal cancer–specific survival among colon and rectal cancer cases. A total of 400 stage I-III colorectal cancer cases identified from the University of California Irvine colorectal cancer gene-environment study were used in the case-only analysis. Median follow-up duration was 11 years, 1 month. There were 252 (63%) colon cancer cases, 143 (36%) rectal cancer cases, and 5 (1%) colorectal cancer cases of unspecified location. Clinicopathologic data for colorectal cancer cases are shown in Table 1. Timing of radiation therapy was available for 60 cases (55 rectal and 5 colon cancer cases) of the 63 receiving radiation therapy. Thirteen (21.7%) received neoadjuvant

(i.e., presurgery) radiation, 45 (75.0%) received adjuvant (i.e., postsurgery) radiation, and 2 (3.3%) received combination neoadjuvant and adjuvant radiation therapy. *ODC1* genotype distribution among all colorectal cancer cases was 52% GG, 41% GA, and 7% AA. There were no significant differences in *ODC1* genotype distribution (GG versus GA/AA) by age, gender, ethnicity, family history, stage, site within the colorectum, histology, or tumor grade (Table 1). When analyzed by three genotypes (GG, GA, AA), *ODC1* genotype distribution by ethnicity revealed significant differences: Caucasian (351 cases: 54% GG, 40% GA, 6% AA, minor-A allele frequency = 26%), African American (4 cases: 50% GG, 50% GA, 0% AA, minor-A allele frequency = 25%), Hispanics (20 cases: 55% GG, 45% GA, 0% AA, minor-A allele frequency = 23%), and Asians (23 cases: 30% GG, 44% GA, 26% AA, minor-A allele frequency = 48%; $P = 0.012$). However, within each race *ODC1* genotype distribution was in Hardy-Weinberg equilibrium (Caucasians $P = 0.51$, African Americans $P = 0.51$, Hispanics $P = 0.19$, Asians $P = 0.54$).

Of the 400 stage I-III colorectal cancer cases, 109 (27%) were deceased at the time of analysis. Forty-seven (43%) deaths occurred in cases carrying the *ODC1* GG genotype, compared with 62 (57%) deaths in cases with the GA/AA genotypes. Cause of death was available for 76 of the 109 deceased colorectal cancer cases. Fifty-nine (77%) colorectal cancer cases died as a result of colorectal cancer. A statistically significant improvement in colorectal cancer–specific survival was observed among colorectal cancer cases homozygous for the *ODC1* G-allele (10-year survival, 89%) compared with cases with at least one A-allele (*ODC1* GA/AA; 10-year survival, 81%; $P = 0.011$). Colorectal cancer–specific survival analysis by stage revealed that significantly different survival differences were not observed for AJCC stage I ($P = 0.055$) or II ($P = 0.61$) colorectal cancer. However, among cases with stage III colorectal cancer the *ODC1* GG genotype was associated with improved 10-year colorectal cancer–specific survival at 75% compared with 60% for *ODC1* GA/AA genotype cases ($P = 0.024$; Fig. 2). Among all cases (stage I-III), genotype-specific survival differences were significant for rectal

Table 2. Multivariate overall survival and colorectal cancer–specific survival analysis for colorectal cancer cases based on *ODC1* genotype

	ODC1 Genotype		*P*
	GG	**GA/AA**	
Overall mortality			
Number of events	47	62	
Number at risk	208	192	
Unadjusted HR (95% CI)	1 (reference)	1.57 (1.07-2.29)	0.020
Adjusted HR (95% CI)*	1 (reference)	1.58 (1.07-2.34)	0.021
CRC-specific mortality			
Number of events	22	37	
Number at risk	208	192	
Unadjusted HR (95% CI)	1 (reference)	1.97 (1.16-3.34)	0.012
Adjusted HR (95% CI)*	1 (reference)	2.02 (1.17-3.50)	0.012

Abbreviation: 95% CI, 95% confidence interval.
*Includes stratification for stage (I, II, III) and adjustment for age (y), gender, ethnicity, family history of colorectal cancer, TNM stage at diagnosis, tumor site within the colorectum, histologic subtype, treatment with surgery, radiation therapy, and chemotherapy.
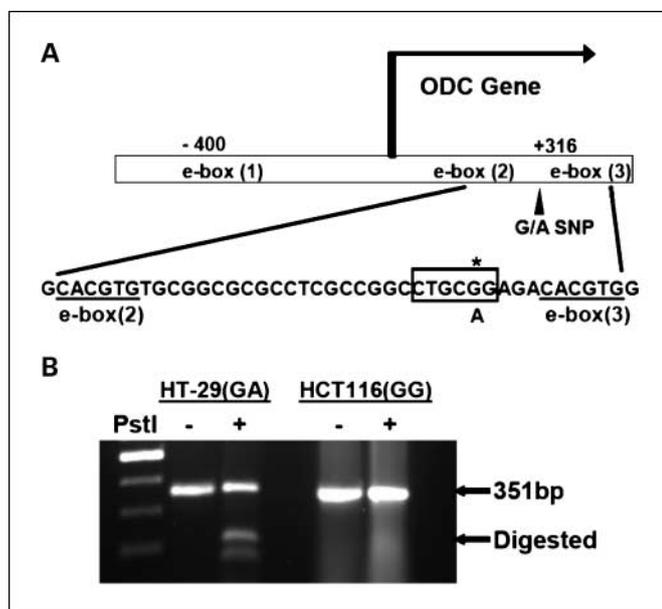
Fig. 3. *A,* location of the *ODC1* promoter SNP. The SNP under investigation in this study is 316 nucleotides 3′ of the *ODC1* transcription start site (*). This SNP resides between two consensus E-boxes as shown by the underlined sequences, and affects a PstI restriction site (box). *B,* restriction fragment length polymorphism analysis of *ODC1* SNP. DNA was obtained from two cell types, and the region surrounding the *ODC1* SNP site was sequenced. Colon-derived HT29 cells were found to be heterozygous GA, whereas HCT116 cells were found to be homozygous GG, at the *ODC1* SNP locus. A 350-bp PCR product of this region was obtained from each cell type and subjected to digestion with PstI. Evidence of an A-allele was indicated by restriction products <350 bp.

(10-year overall survival, 88% for *ODC1* GG versus 75% for *ODC1* GA/AA; *P* = 0.041) but not colon cancer (10-year overall survival, 89% for *ODC1* GG versus 84% for *ODC1* GA/AA; *P* = 0.11).

Among colorectal cancer cases, the colorectal cancer–specific survival estimates based on *ODC1* genotype after stratification for TNM stage at diagnosis and adjustment for age (years), gender, ethnicity, family history of colorectal cancer, tumor site within the colon, histologic subtype, treatment with surgery, radiation therapy, and chemotherapy were a follows: *ODC1* GG hazards ratio (HR), 1 (referent); *ODC1* GA/AA HR, 2.02 (*P* = 0.021; Table 2). In the additive model, adjusted colorectal cancer–specific survival estimates were as follows: *ODC1* GG HR, 1 (referent); *ODC1* GA HR, 1.95; *ODC1* AA HR, 2.25 (*P*-trend = 0.015). Subset multivariate colorectal cancer–specific survival analysis revealed that *ODC1* genotype was a significant prognostic factor among rectal (*ODC1* GA/AA HR, 2.92; 95% confidence interval, 1.22-7.03 using *ODC1* GG as a referent) but not colon cancer cases (*ODC1* GA/AA HR, 1.76; 95% confidence interval, 0.85-3.63 using *ODC1* GG as a referent). No significant associations with *ODC1* genotype and overall survival were detected in subset-adjusted analyses of rectal, or colon cancer cases.

As noted above, the *ODC1* +316 genotype distribution differed across ethnicity. The observed mortality risk, other than by chance, likely reflects differences based on *ODC1* genotype, but the risk may be restricted to a particular ethnic group. Thus multivariate analyses were conducted among Caucasian colon cancer cases, to assess genotype-specific mortality risk within this single ethnic group. Among the 351 Caucasians, there were 52 colorectal cancer–related deaths. Multivariate colorectal

cancer–specific survival analysis revealed that the *ODC1* SNP was an independent predictor of colorectal cancer–specific survival among Caucasian colorectal cancer cases after adjustment for the aforementioned relevant clinical variables. Compared with cases with *ODC1* GG genotype (HR, 1; reference), the colorectal cancer–specific risk of death (HR) was 2.37 (95% CI, 1.33-4.23) for *ODC1* GA/AA genotype.

Experimental studies: ODC1 +316 SNP regulation in colon cancer cells

ODC1 allele-specific binding of E-box transcription factors. We evaluated the functional significance of the +316 *ODC1* SNP, located between two E-boxes (E-box 2 and 3 as depicted in Fig. 3A). Each cell line genotype influences a consensus PstI restriction site in this region. Figure 3B shows that a PCR product made from human colon HT29 cells was partially sensitive to PstI cutting, suggesting that these cells contained at least one *ODC1* A-allele. A PCR product made from human colon HCT116 cells using the same primers was insensitive to PstI action, implying that these cells contained only *ODC1* G-alleles. This result was confirmed by direct DNA sequencing (data not shown).

Expression of specific E-box binding proteins, including the transcriptional activator c-MYC and several transcriptional repressors in HT29 and HCT116 cells (e.g., MAD1 and MAD4), was established by Western blotting (Fig. 4A). Chromatin immunoprecipitation analysis of the region surrounding +316 of the *ODC1* promoter was conducted, using antibodies directed against these proteins. As shown in Fig. 4B, *ODC1* promoter-specific PCR products were synthesized from HT29 DNA obtained after immunoprecipitation of chromatin with antibodies directed against c-MYC, MAD1, or MAD4. PCR products
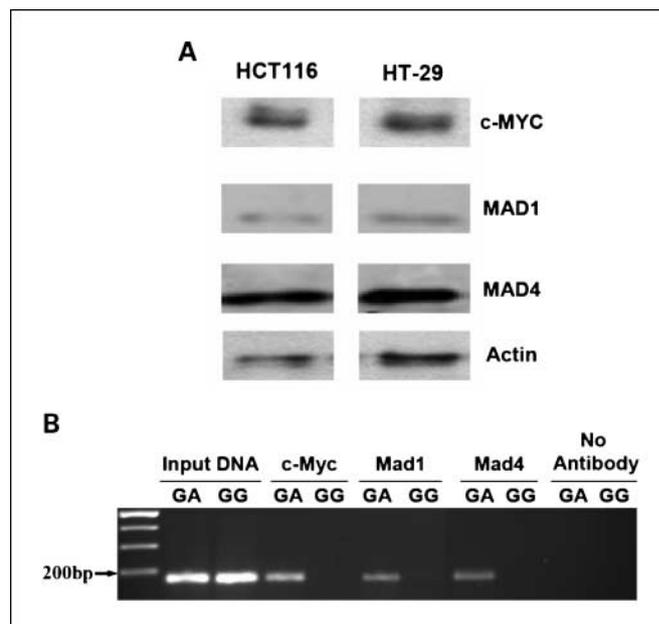

Fig. 4. *A,* E-box protein expression in colon-derived cells. Expression of proteins to be evaluated for binding to the +316 *ODC1* SNP was assessed by Western blot analysis. Extracts of both HT29 and HCT116 cells were evaluated for c-MYC, MAD1, and MAD4; β-actin was used as a loading control. *B,* documentation of allele-specific transcription factor binding by chromatin immunoprecipitation analysis, which was conducted as described in Materials and Methods. HT29 cells were a source of *ODC1* A-alleles, as these cells are heterozygous GA at this site. HCT116 cells were used as a source of *ODC1* G-alleles.

synthesized from HCT116 DNA after similar chromatin immu-noprecipitation were substantially reduced compared with those synthesized from HT29 DNA. Quantification of these results indicated that c-MYC, MAD1, and MAD4 binding to the *ODC1* SNP region were 4 to 14 times greater in HT29 cells, which contained one *ODC1-A* allele, compared with HCT116 cells, which contained only *ODC1-G* alleles (Supplementary Table).

ODC1 allele-specific promoter activity. We tested the hypoth-esis that +316 *ODC1* SNP influenced *ODC1* expression in a manner dependant on the expression of E-box activators and repressors. Transient cotransfection of colon cancer–derived HT29 cells was accomplished with *ODC1* allele-specific pro-moter constructs in combination with vectors expressing either the transcriptional activator *MYC* or the repressor *MAD1* (Fig. 5). The SE bars shown reflect the variability in triplicate measurements within a single representative experiment, which has been replicated. The allele-specific promoter-reporters used in these experiments included all three E-boxes shown in Fig. 3A. As shown in Fig. 5A, *MYC* expression had the greatest stimulatory effect on promoters containing three consensus E-boxes and the *ODC1-A* allele (wt E-box1 +316 A, $P = 0.0014$). Deletion of the upstream E-box reduced promoter activity, but *MYC* expression continued to stimulate this activity (mut E-box1 +316 A, $P = 0.0013$). Substitution of a G for the A at the *ODC1* +316 SNP position reduced the ability of c-MYC to stimulate promoter activity even with an intact 5′ flanking con-sensus E-box. Mutation of the 5′ flanking consensus E-box in combination with the *ODC1-G* allele further reduced promoter activity.

When *MAD1*, rather than *MYC*, was cotransfected with the *ODC1* allele-specific promoter reporters (Fig. 5B), the repressor was only able to reduce the activity of the *ODC1* promoter which contained all three E-boxes and the wild-type +316 A-allele ($P = 0.027$). Deletion of the upstream E-box (mut E-box 1 +316A) significantly reduced the effect of MAD1 on *ODC1* promoter activity. Substitution of G for A at the +316 position rendered promoters containing either two or three E-boxes un-responsive to MAD1 suppression.

Discussion

In our population-based analysis of colorectal cancer cases with >11 years follow-up duration, we observed that the +316 *ODC1* SNP was associated with colorectal cancer–specific survival among colorectal cancer cases, with a greater associa-tion observed among rectal cancer cases. A statistically signifi-cant increased risk of colorectal cancer–specific mortality was observed for *ODC1* GA/AA cases among rectal cancer cases, af-ter stratification for stage and adjustment for age, gender, eth-nicity, family history of colorectal cancer, tumor site, histology, treatment with surgery, radiation therapy, and chemotherapy.

The experimental data presented here provide insights into potential biological mechanisms underlying our clinical obser-vations. In colon cancer epithelial cells, we have shown that the *ODC1* +316 SNP is functionally significant, as evidenced by in-creased binding of E-box transcription factors to promoter ele-ments containing A-alleles, compared with G-alleles. Both the activator c-MYC and the repressor MAD1 show greater effects on promoter activity in reporter elements containing A- versus G-alleles. These results suggest allele-specific regulation of *ODC1* by E-box transcription factors. ODC protein enzyme ac-
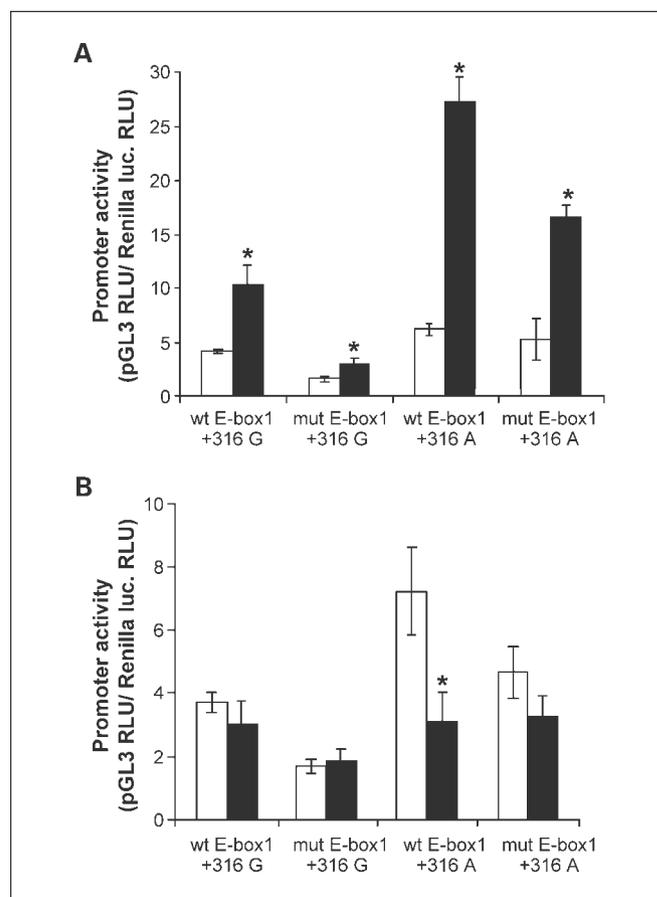

Fig. 5. *A,* effect of *c-MYC* expression on *ODC1* allele-specific promoter activity in HT29 colon-derived cells. Promoter activity was measured after transfection with *ODC1* promoter reporter plasmids co-transfected with pcDNA 3.0 plasmid (□) or CMV-*MYC* expression vector (■). Promoter constructs differ by the presence of the first E-box element, located in -485 to -480 bp ("wt E-box1" for the wild-type sequence or "mut E-box1" for a mutant sequence). The constructs differ also by the *ODC1* +316 SNP ("+316 G" or "+316 A"). *, $P \le 0.013$ for each of the four comparisons relative to promoter activity with pcDNA 3.0 cotransfection. *B,* effect of *MAD1* expression on *ODC1* allele-specific promoter activity in HT29 colon tumor derived cells. Promoter activity was measured after transfection with *ODC1* promoter reporter plasmids cotransfected with pcDNA 3.1 plasmid (□) or with a pcDNA-*MAD*1 plasmid (■). Promoter constructs used were described in the legend for panel *A* of this figure.
*, $P = 0.027$, statistical significance relative to promoter activity with pcDNA 3.1 cotransfection.

tivity is not apparently affected by the *ODC1* +316 SNP geno-type, which we believe influences *ODC1* transcription.

In colon cells, we have shown that conditional expression of wild-type *APC*, a gene expressed in normal colonic mucosa, suppresses *MYC*, and increases *MAD1*, expression (5). Further, we have reported that wild-type *APC* can regulate *ODC1* pro-moter activity in a manner dependent on the +316 SNP (7). Wild-type *APC* is expressed in the apparently normal colonic mucosa of individuals not afflicted with familial adenomatous polyposis, whereas the majority of sporadic colon adenomas show evidence of mutated or deleted *APC* (27). *MYC* is ex-pressed at low levels in normal intestinal mucosa but is in-creased in intestinal adenomas of $APC^{Min/+}$ mice. Conditional knockout of intestinal epithelial *MYC* expression suppresses in-testinal tumorigenesis in $APC^{Min/+}$ mice (20). As described above, previous work by our group (7) and others (18) showed

a protective role for the *ODC1* A-allele, especially in aspirin users, against recurrence of colon polyps in clinical prevention trials. However, in the population-based study presented here, the *ODC1* A-allele was associated with poor survival. This apparent contradiction may be explained by the results shown here, which indicate that both E-box activators and repressors bind the *ODC1* A-allele selectively. If the transformed epithelium begins to express E-box activators (such as c-MYC), then cancer progression may be more likely to occur in individuals with the *ODC1* A-allele genotype. Our results for risk of colon cancer–specific mortality are consistent with those of others showing that risk of prostate cancer may be associated with the *ODC1* A-allele among specific individuals as the result of gene environment interactions (19, 28). Such colon cancer progression could be due to enhanced polyamine synthesis, as has been shown already for prostate cancer (15).

Our finding that a factor, such as the *ODC1* SNP, may have both promoting and inhibiting effects on carcinogenesis is not unique. For example, transforming growth factor-β has diverse roles in carcinogenesis and cancer progression (29–31). Transforming growth factor-β in untransformed cells inhibits cell proliferation and induces apoptosis. Yet, it is overexpressed in all human tumors and is associated with late cancer progression, specifically tumor invasion and metastasis. A single study by Matsubara et al. reporting *ODC1* activity in human colorectal tumors showed that high levels of *ODC1* expression were significantly associated with improved survival (32). This suggests that, although *ODC1* overexpression promotes the formation of human colorectal adenomas, it is possible that in established lesions, *ODC1* overexpression causes enhanced proliferation and is associated with improved response to antiproliferative treatments. However, that study did not include stratification by *ODC1* genotype, so it is not known if these effects are independent of *ODC1* genotype. Population differences between the Matsubara et al. study and ours could account for the differences. For example, the former study included few rectal cancers (5%), whereas our study cohort comprised a substantial proportion of rectal cancer cases (36%). This difference is not trivial, because the observed associations of the *ODC1* SNP with colorectal cancer–specific survival were greater for rectal compared with colon cancer cases. The Matsubara et al. study comprised 63.5% females compared with our study which included 41% females. Furthermore, the distribution by race/ethnicity differs substantially between the two studies. Additionally, chance variation could explain why our findings differ from the prior analysis.

The observed associations of the *ODC1* +316 SNP with colorectal cancer–specific mortality were greatest among rectal cancer cases. Among all colorectal cancer cases, particularly strong effects were observed for Caucasians. Similar to other reports, the *ODC1* +316 SNP allele frequency differs considerably by ethnicity (28). When we limited the survival analysis to Caucasians only (i.e., the only ethnic group with adequate power to test for such analyses), the associations of the *ODC1* +316 SNP were significant and of greater magnitude than the estimates observed for the entire cohort.

The epidemiologic study shares limitations of other population-based analyses, including lack of data on comorbid conditions, performance status, and particular chemotherapeutic regimens utilized. Additionally, the tissue biopsy samples obtained from participants of the University of California Irvine Gene-Environment Study of Familial Colorectal Cancer were paraffin-embedded specimens and therefore could not be used for accurate assessment of tissue polyamine quantification by high-performance liquid chromatography. There is also the potential for selection bias, favoring a relatively healthy group of colorectal cancer survivors, because there was a median 18-month delay from the time of colorectal cancer diagnosis until study enrollment. Other factors affecting polyamine metabolism that were not accounted for in the present study may explain our observations. For example, aspirin activates polyamine acetylation and export, and works with the *ODC1* A-allele to reduce cell and tissue polyamine contents (2, 7, 33). Our experimental findings are limited to the effects of the *ODC1* +316 SNP in two colon cancer cell lines. Thus, validation of our findings is warranted.

In summary, we have observed clinical consequences of the *ODC1* +316 SNP on colorectal cancer–specific mortality among colorectal cancer cases. Additionally, we have further established the functional significance of the *ODC1* +316 SNP in the c-MYC- and MAD1-dependent transcription of this gene in human colon cancer cells. Together, these experimental and epidemiologic findings suggest a role for the *ODC1* SNP in progression of colorectal cancer distinct from its previously reported role in progression to colon adenomas. Provided that our results are validated in future research, these novel findings may contribute to our ability to assess risk of colorectal cancer progression and may direct patient-specific pharmacogenetic management, surveillance monitoring, and inform novel targeted approaches to secondary and tertiary colorectal cancer prevention.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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