p21 Expression in Colon Cancer and Modifying Effects of Patient Age and Body Mass Index on Prognosis

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Abstract

p21 (Cyclin-dependent kinase inhibitor-1A, *CDKN1A* or CIP1) plays a role in regulating cell cycle, and its expression is lost in most colorectal cancers. p21 Is related with energy balance status, cellular senescence, and stem cell aging. Thus, the influence of p21 loss on tumor behavior and clinical outcome may be modified by patient age and body mass index (BMI). Using 647 colon cancers in two independent prospective cohorts, p21 loss was observed in 509 (79%) tumors by immunohistochemistry. Cox proportional hazard models computed hazard ratio (HR) for death, adjusted for potential confounders, including p53, cyclin D1, *KRAS*, *BRAF*, *PIK3CA*, LINE-1 hypomethylation, CpG island methylator phenotype (CIMP), and microsatellite instability (MSI). p21 Loss was independently associated with low colon cancer–specific mortality [HR, 0.58; 95% confidence interval (95% CI), 0.38-0.89; adjusted for the covariates including MSI, CIMP, and LINE-1 methylation]. The prognostic effect of p21 loss differed significantly by age at diagnosis ($P_{interaction} < 0.0001$) and BMI ($P_{interaction} = 0.002$). The adjusted HR for cancer-specific mortality (p21 loss versus p21 expression) was 4.09 (95% CI, 1.13-14.9) among patients <60 year old and 0.37 (95% CI, 0.24-0.59) among patients ≥60 year old. The adverse prognostic effect of obesity was limited to p21-expressing cases (adjusted HR, 5.85; 95% CI, 2.28-15.0; BMI, ≥30 versus <30 kg/m^2), but no such effect was observed among p21-lost cases. In conclusion, p21 loss in colon cancer is associated with longer survival among patients ≥60 year old, whereas it is associated with shorter survival among patients <60 year old. Patient BMI also differentially influences prognosis according to p21 CDKN1A status. Our data suggest host-tumor interactions influencing tumor aggressiveness. (Cancer Epidemiol Biomarkers Prev 2009;18(9):2513–21)

Introduction

Cell cycle progression involves sequential activation and inactivation of cyclin-dependent kinases (1). p21 (*CDKN1A* or CIP1) plays a key role in regulating the cell cycle (1). Energy restriction up-regulates p21 through activation of p53 by AMP kinase (2-4). In fact, energy balance status has been linked to cellular proliferation and carcinogenesis (3). Functions of p21 seem to be multifaceted, and p21 has a proapoptotic role (5) as well as an antiapoptotic role (6). p21 May facilitate tumor invasion and metastasis possibly through p21-activated kinase-1 (*PAK1*; refs. 7-9). In addition, p21 has been related with cellular senescence and aging of stem cells (10-12). Thus, it is plausible that the influence of p21 loss on tumor behavior may be modified by cellular energy balance and patient age.

Previous data on p21 loss and clinical outcome in colon cancer have not been conclusive. Although p21 loss has been associated with poor prognosis in a few studies (13-15), most studies showed no independent prognostic value of p21 (16-25). p21 Loss in colon cancer is inversely associated with microsatellite instability (MSI) (26-28), the CpG island methylator phenotype (CIMP) (27), and *BRAF* mutation (27), and MSI, CIMP, and *BRAF* mutation have been related with clinical outcome (29-31). However, none of the previous studies (13-23) have considered potential confounding effect of CIMP, MSI, and *BRAF*. In addition, no previous study has examined potential modifying effect of patient age or body mass index (BMI).

We therefore examined the effect of tumoral p21 loss, patient age, and BMI on patient survival in 647 patients with stages I to IV colon cancer identified through two independent prospective cohort studies. Because we concurrently assessed related molecular variables, including p53, cyclin D1, *KRAS*, *BRAF*, *PIK3CA*, MSI, and CIMP, we could evaluate the effect of p21 loss independent of these potential confounders (27, 29-32, 39). In addition, we had sufficient power to examine potential modifying effects of patient age and BMI on p21 loss and mortality.

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Materials and Methods

Study Population. We used the databases of two independent prospective cohort studies: the Nurses' Health Study (N = 121,701 women followed since 1976; ref. 33) and the Health Professionals Follow-up Study (N = 51,529 men followed since 1986; ref. 33). Thus, we could validate our findings in one cohort by the other cohort by examining whether there was a significant interaction between the p21 and cohort variables. Upon each biennial questionnaire, participants reported whether they had a diagnosis of colorectal cancer in themselves or any of their first-degree relatives. Study physicians, while blinded to exposure data, reviewed all records related to colorectal cancer and recorded tumor-node-metastasis stage and tumor location. We calculated BMI (kilogram per square meter), using self-reported height from the baseline questionnaire and weight from the biennial questionnaire that immediately preceded the diagnosis of colon cancer. In validation studies in both cohorts, self-reported anthropometric measures were well correlated with measurements by trained technicians ($r > 0.96$). We collected paraffin-embedded tissue blocks from hospitals where colon cancer patients underwent tumor resections (33). Tissue sections from all colon cancer cases were reviewed by a pathologist (S. Ogino). Tumor grade was categorized as high (≤50% glandular area) or low (>50% glandular area). We excluded patients who were preoperatively treated with chemotherapy or radiation. Up to 2002, there were 1,834 incident colorectal cancer patients who were eligible for survival analysis, including 1,378 colon cancer patients.

Among them, tumor tissue materials were available in 705 cases, and p21 data were available in 647 cases. Thus, we included a total of 647 stage I to IV colon cancer cases. Between cases with available p21 data and those with unavailable tissue or p21 data, there was no significant difference in survival time (adjusted hazard ratio, 1.02; 95% CI, 0.88-1.18 for overall mortality; p21 available versus unavailable), or tumor characteristics (tumor location, stage; $P > 0.17$). We have previously analyzed p21 expression in these tumors (27); however, clinical outcome data were unavailable at that time, and we have not examined the interactive effect of p21 loss, patient age, and BMI on patient survival. Patients were followed until death or June 2006, whichever came first. Ascertainment of deaths included reporting by the family or postal authorities. In addition, the names of nonresponders were searched in the National Death Index. More than 98% of deaths in the cohorts were identified by these methods. The cause of death was assigned by physicians blinded to information on lifestyle exposures and molecular changes in colon cancer. Written informed consent was obtained from all study subjects. This study was approved by the Human Subjects Committees at Brigham and Women's Hospital and the Harvard School of Public Health.

Sequencing of *KRAS*, *BRAF*, and *PIK3CA*, and Microsatellite Instability (MSI) Analysis. DNA from paraffin-embedded tissue was extracted, and PCR and Pyrosequencing targeted for *KRAS* codons 12 and 13 (34), *BRAF* codon 600 (35), and *PIK3CA* exons 9 and 20 were done (36). MSI status was determined using D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67 and D18S487 (37). MSI-high was defined as the presence of instability in ≥30% of the markers; MSI-low, as the presence of instability in 1% to 29% of the markers; and microsatellite stability, as no unstable marker.

Real-Time PCR for CpG Island Methylation and Pyrosequencing to Measure LINE-1 Methylation. Sodium bisulfite treatment on DNA and subsequent real-time PCR (MethyLight) assays were validated and done as previously described (38). We quantified promoter methylation in eight CpG island methylator phenotype (CIMP)–specific genes (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*; refs. 31, 37, 40). CIMP-high was defined as six to eight or more methylated promoters using the eight-marker CIMP panel and CIMP-low/0 was defined as zero to five methylated promoters, according to the previously established criteria (31). To accurately quantify relatively high LINE-1 methylation levels, we used Pyrosequencing as previously described (41).

Immunohistochemistry for p21, p53, and Cyclin D1. Tissue microarrays were constructed (42), and immunohistochemistry for p53 (43) and cyclin D1 was done as previously described (44). Immunohistochemistry for p21 (Fig. 1) was done as previously described (27, 44). Whole tissue sections (instead of tissue microarrays) were used for p21 immunohistochemistry. p21 Loss was defined as no to weak staining in tumor cells or <20% of tumor cells with moderate or strong staining. This cut point was based on the frequency of p53 expression (that is, moderate/strong p53 staining in ≥50% tumor cells) in colorectal cancer groups categorized by p21 status. The frequency of p53 positivity was 50% (278 of 556) in tumors with p21 expression in 0% to 9% of cells, 39% (44 of 114) in tumors with 10% to 19% p21-expressing cells, 29% (19 of 66) in tumors with 20% to 29% p21-expressing cells, 19% (7 of 36) in tumors with 30% to 39% p21-expressing cells, and 17% (9 of 53) in tumors with ≥40% p21-expressing cells. Appropriate positive and negative controls were included in each run of immunohistochemistry. All immunohistochemically stained slides for each marker were interpreted by one of the investigators (p21 and p53 by S. Ogino; cyclin D1 by K. Nosho) unaware of other data. A random sample of 118 to 179 tumors were re-examined for p21, cyclin D1, or p53 by a second observer (p21 and cyclin D1 by K. Shima; p53 by K. Nosho) unaware of other data. The concordance between the two observers was 0.83 ($\kappa = 0.62$; $P < 0.0001$; $n = 179$) for p21, 0.83 ($\kappa = 0.64$; $P < 0.0001$; $n = 160$) for cyclin D1, and 0.87 ($\kappa = 0.75$; $P < 0.0001$; $n = 118$) for p53, indicating substantial agreement.

Statistical Analysis. All analyses used SAS version 9.1 (SAS Institute), and all Ps were two sided. The χ^2 test was used to examine an association between categorical variables. The t test assuming unequal variances was done to compare mean age and mean LINE-1 methylation level. The Kaplan-Meier method was used to describe the distribution of colon cancer–specific and overall survival time, and the log-rank test was used to test a deviation from the null hypothesis. For analyses of colon cancer–specific mortality, death as a result of colon cancer was the primary end point, and deaths as a result of other causes were censored.

To evaluate independent effect of p21 status on mortality, we used stage-matched (stratified) Cox proportional

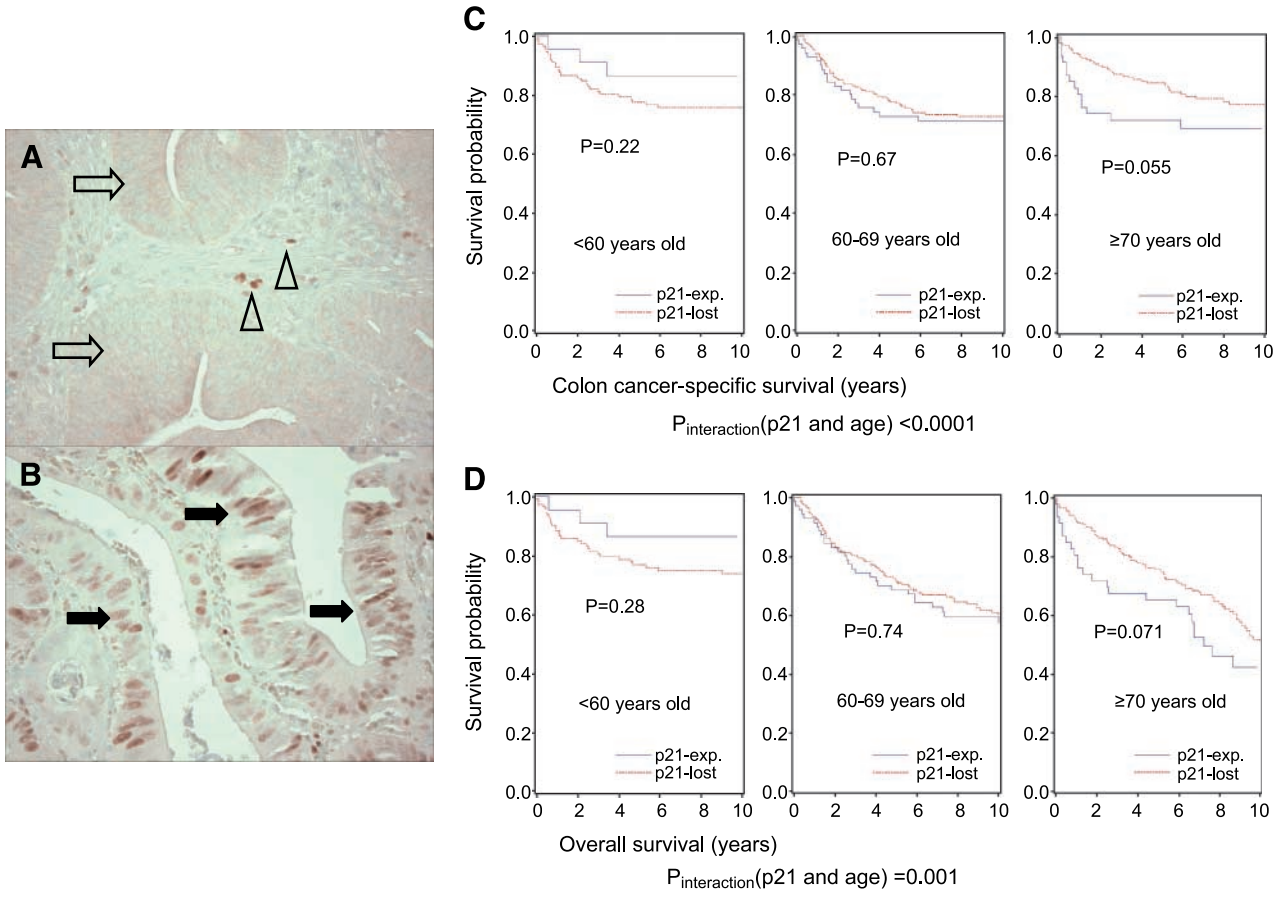

Figure 1. p21 Expression in colon cancer cells and patient survival. **A.** Loss of p21 expression in colon cancer cells (*empty arrows*). Normal cells serve as internal positive controls (*empty arrowheads*). **B.** p21 Expression in colon cancer cells (*arrows*). **C** and **D.** Kaplan-Meier curves for colon cancer–specific (**C**) and overall (**D**) survival of colon cancer patients according to p21 status, stratified by age. p21-exp., p21 Expressing.

hazard models and calculated hazard ratio (HR) of death, adjusted for sex, age at diagnosis (continuous), year of diagnosis (continuous), BMI (<30 versus ≥30 kg/m^2), family history of colorectal cancer in any first-degree relative (present versus absent), tumor location (proximal versus distal), grade (high versus low), MSI (high versus low/microsatellite stability), CIMP (high versus low/0), LINE-1 methylation (continuous), *KRAS*, *BRAF*, *PIK3CA*, p53, and cyclin D1 (positive versus negative). Tumor stage (I, IIA, IIB, IIIA, IIIB, IIIC, IV, missing) was used as a matching (stratifying) variable (using the "strata" option in SAS "proc phreg" command, without using any degree of freedom) to avoid residual confounding and overfitting. The proportionality of hazards assumption was verified by evaluating time-dependent variables, which were the cross-product of the p21 variable and survival time ($P = 0.08$ for colon cancer–specific mortality; $P = 0.13$ for overall mortality). When there was missing information on tumor location (1.2% missing), BMI (3.9%), tumor grade (0.5%), MSI (2.6%), p53 (0.5%), *PIK3CA* (13.7%), *BRAF* (4.5%), or *KRAS* (2.2%), we included those cases in the majority category of the given variable to minimize the number of indicator variables and avoid overfitting. We confirmed that excluding cases with a missing variable did not substantially alter results (data not shown). We also did stepwise backward elimination, with $P = 0.20$ as a cutoff, using tumor stage as a matching variable. As a result, LINE-1, MSI, cyclin D1, BRAF, tumor grade, and tumor location remained in the model. Adjusted hazard ratio for colon cancer–specific mortality in p21-lost cases (versus p21-expressing cases) was as follows: 4.78 (95% CI, 1.34-17.1) for age <60 y; 0.63 (95% CI, 0.29-1.38) for age 60 to 64 y; 0.48 (95% CI, 0.22-1.07) for age 65 to 69 y; and 0.25 (95% CI, 0.13-0.49) for age ≥70 y. Thus, the results were similar to those by the model containing all variables.

An interaction was assessed by the Wald test on the cross-product of the p21 variable and another variable of interest (without data-missing cases) in a multivariate Cox model. *P*s for interaction were interpreted conservatively, given multiple hypothesis testing. To assess an interaction between p21 and age, we used age as an ordinal categorical variable (<60 versus 60-69 versus ≥70 y old) or a continuous variable.

Results

Loss of p21 Expression in Colon Cancer and Patient Survival. Among 647 patients with stage I to IV colon cancer, loss of p21 was observed in 509 (79%) tumors by

Table 1. Clinical and molecular characteristics according to p21 status in colon cancer

Clinical or molecular feature	All cases	p21		*P*
		Expressed	Lost	
Total *n*	647	138	509	
Sex				0.25
Male (HPFS)	276 (43%)	53 (38%)	223 (44%)	
Female (NHS)	371 (57%)	85 (62%)	286 (56%)	
Mean age ± SD	66.5 ± 8.2	67.0 ± 7.9	66.4 ± 8.3	0.43
BMI, kg/m^2				0.39
<30	513 (82%)	113 (85%)	400 (82%)	
≥30	109 (18%)	20 (15%)	89 (18%)	
Family history of colorectal cancer in any first-degree relative				0.73
Absent	485 (75%)	105 (76%)	380 (75%)	
Present	162 (25%)	33 (24%)	129 (25%)	
Year of diagnosis				0.51
Before 1990	100 (15%)	18 (13%)	82 (16%)	
1990-1999	467 (72%)	105 (76%)	362 (71%)	
2000-2002	80 (12%)	15 (11%)	65 (13%)	
Tumor location				<0.0001
Proximal (cecum to transverse)	375 (59%)	101 (74%)	274 (55%)	
Distal (splenic flexure to sigmoid)	264 (41%)	36 (26%)	228 (45%)	
AJCC tumor stage				0.94
I	134 (21%)	29 (21%)	105 (21%)	
IIA	203 (31%)	46 (33%)	157 (31%)	
IIB	20 (3.1%)	6 (4.3%)	14 (2.8%)	
IIIA	21 (3.2%)	5 (3.6%)	16 (3.1%)	
IIIB	84 (13%)	15 (11%)	69 (14%)	
IIIC	55 (8.5%)	10 (7.2%)	45 (8.8%)	
IV	87 (13%)	19 (14%)	68 (13%)	
Unknown	43 (6.6%)	8 (5.8%)	35 (6.9%)	
Tumor grade				<0.0001
Low	571 (89%)	109 (79%)	462 (91%)	
High	73 (11%)	29 (21%)	44 (8.7%)	
MSI				<0.0001
MSI low/MSS	512 (81%)	83 (62%)	429 (86%)	
MSI high	118 (19%)	51 (38%)	67 (14%)	
CIMP				<0.0001
CIMP low/0	523 (81%)	77 (56%)	446 (88%)	
CIMP high	124 (19%)	61 (44%)	63 (12%)	
Mean LINE-1 methylation (%) ± SD	61.0 ± 9.6	63.6 ± 8.9	60.3 ± 9.6	0.0002
p53 expression				<0.0001
(-)	397 (62%)	108 (78%)	289 (57%)	
(+)	247 (38%)	30 (22%)	217 (43%)	
Cyclin D1 expression				<0.0001
(-)	323 (50%)	45 (33%)	278 (55%)	
(+)	324 (50%)	93 (67%)	231 (45%)	
BRAF mutation				<0.0001
(-)	517 (84%)	84 (64%)	433 (89%)	
(+)	101 (16%)	47 (36%)	54 (11%)	
KRAS mutation				0.28
(-)	400 (63%)	90 (67%)	310 (62%)	
(+)	233 (37%)	44 (33%)	189 (38%)	
PIK3CA mutation				0.0077
(-)	462 (83%)	88 (75%)	374 (85%)	
(+)	96 (17%)	30 (25%)	66 (15%)	

NOTE: (%) Indicates the proportion of tumors with a specific clinical or molecular feature in p21-lost (or p21 expressing) tumors.
Abbreviations: AJCC, American Joint Commission on Cancer; CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; LINE-1, long interspersed nucleotide element-1; MSI, microsatellite instability; MSS, microsatellite stable; NHS, Nurses' Health Study.

immunohistochemistry. We assessed clinical and molecular characteristics of colon cancers, according to tumoral p21 status (Table 1). p21 Loss was significantly associated with distal location ($P < 0.0001$), p53 expression ($P < 0.0001$), and inversely with MSI-high; ($P < 0.0001$), CIMP-high; ($P < 0.0001$), *BRAF* mutation ($P < 0.0001$), and *PIK3CA* mutation ($P = 0.0077$).

During follow-up, there were 279 deaths, including 162 colon cancer–specific deaths. We assessed the influence of p21 loss on patient survival. Five-year colon cancer–specific survival among patients with p21-lost tumors (80%) was not significantly different from those with p21-expressing tumors (75%; log rank $P = 0.37$). In univariate Cox regression analysis, patients with p21-lost tumors experienced a nonsignificant decrease in cancer-specific mortality (hazard ratio, HR 0.85; 95% CI, 0.59-1.22) compared with patients with p21-expressing tumors (Table 2). In the multivariate Cox model adjusting for potential predictors of patient outcome, p21 loss was associated with a significantly lower colon cancer–specific mortality (adjusted HR, 0.58; 95% CI, 0.38-0.89; $P = 0.013$), and overall mortality (adjusted HR, 0.71; 95% CI, 0.51-0.98; $P = 0.035$). The decrease in the HR for p21-lost tumors (versus p21-expressing tumors) in the multivariate

analysis was mainly the result of adjusting for tumor stage and LINE-1 methylation; when we simply adjusted for tumor stage and LINE-1, the HR for colon cancer–specific mortality in p21-lost tumors was 0.67 (95% CI, 0.45-0.99). No other major confounder was present.

Modifying Effect of Age on the Relation Between p21 Loss and Mortality. Considering the importance of p21 in cellular senescence, we assessed whether patient age modified the influence of p21 loss on patient outcome. We found a significant modifying effect of age on the relation between p21 loss and patient mortality ($P_{interaction}$ < 0.0001 for colon cancer–specific mortality and $P_{interaction}$ = 0.001 for overall mortality). Among patients <60 years of age, p21 loss was associated with a higher cancer-specific mortality (multivariate HR, 4.09; 95% CI, 1.13-14.9) when compared with patients with intact p21 expression (Table 3). In contrast, among patients ≥60 year old, p21 loss conferred a significantly low cancer-specific mortality (multivariate HR, 0.37; 95% CI, 0.24-0.59; P < 0.0001; p21-lost versus p21-expressing tumors). Moreover, the beneficial effect of p21 loss on survival was stronger with increasing patient age (multivariate HR for cancer-specific mortality changing from 0.61 among 60- to 64-year-old patients to 0.38 among 65- to 69-year-old patients to 0.21 among ≥70-year-old patients; Table 3). A similar interaction between patient age and p21 loss was observed for overall mortality. In Kaplan-Meier method, the differential effect of p21 loss on patient survival according to age category was also evident (Fig. 1).

To eliminate potential effect of hereditary nonpolyposis colorectal cancer (HNPCC) status, we identified 19 possible or suspected HNPCC cases [that is, MSI-high CIMP-low/0 tumors (none of which turned out to be BRAF mutated) with any of the following: (*a*) positive family history of colorectal cancer in at least one first-degree relative; (*b*) loss of MLH1 without evidence of MLH1 methylation; (*c*) loss of PMS2 without evidence of MLH1 loss; (*d*) loss of MSH2 and/or MSH6]. After we excluded these 19 cases, multivariate Cox regression analysis showed following adjusted HR for colon cancer–specific mortality in p21-lost cases (versus p21-expressing cases): 4.69 (95% CI, 1.31-16.9) for age <60 years, 0.58 (95% CI, 0.26-1.30) for ages 60 to 64 years, 0.47 (95% CI, 0.21-1.04) for ages 65 to 69 years, and 0.24 (95% CI, 0.12-0.48) for age ≥70 years ($P_{interaction}$ < 0.0001). These results were similar to Table 3.

Modifying Effect of BMI on the Relation Between p21 Loss and Mortality. Considering a role of the p53 to p21 pathway in the link between energy restriction and cell cycle arrest (3), we assessed a potential interaction between tumoral p21 and patient BMI. We found a significant modifying effect of BMI on the relation between p21 loss and patient mortality ($P_{interaction}$ = 0.002). Among patients with BMI ≥30 kg/m^2, tumoral p21 loss was associated with a greater reduction in cancer-specific mortality (multivariate HR, 0.13; 95% CI, 0.05-0.36; p21-lost versus p21-expressing tumors) than among patients with BMI <30 kg/m^2 (multivariate HR, 0.75; 95% CI, 0.46-1.22; p21-lost versus p21-expressing tumors; Fig. 2). A similar modifying effect of BMI was obtained in analysis of overall mortality (data not shown).

Prognostic Effect of Obesity in Strata of Tumoral p21 Status. In light of the significant interaction ($P_{interaction}$ = 0.002) between p21 and BMI in survival analysis, we assessed the effect of obesity (that is, BMI ≥30 kg/m^2) in strata of p21 status (Table 4). Notably, adverse effect of obesity on patient survival was principally limited to patients with p21-expressing tumors. The adjusted HR (obese cases versus nonobese cases) for colon cancer–specific and overall mortality was 5.85 (95% CI, 2.28-15.0) and 3.40 (95% CI, 1.65-6.98), respectively, whereas obesity had no influence on patient survival among patients with p21-lost tumors.

Stratified Analysis of p21 Loss and Mortality. We further examined the influence of p21 loss on colon cancer–specific mortality across strata of other potential effect modifiers, including sex (cohort), family history of colon cancer, tumor location, stage, grade, and status of MSI, CIMP, LINE-1 methylation, *KRAS*, *BRAF*, p53, and cyclin D1 (Fig. 2). There was no evidence of significant modifying effect by any of the variables (except for age and BMI) on the p21-mortality relation. Notably, the effect of p21 loss did not significantly differ between the two independent cohort studies ($P_{interaction}$ = 0.70).

Discussion

In this study, we examined the prognostic significance of tumoral p21 loss in stage I to IV colon cancer, especially in relation to patient age and BMI. We found that tumoral p21 loss was associated with longer survival, independent of patient characteristics and other related molecular variables, including p53, cyclin D1, *KRAS*, *BRAF*, *PIK3CA*, MSI, and CIMP. In addition, we found substantial

Table 2. Loss of p21 in colon cancer and patient mortality

	Total *n*	Colon cancer–specific mortality				Overall mortality			
		Deaths/person-years	Univariate HR (95% CI)	HR adjusted for stage and LINE-1 (95% CI)	Multivariate HR (95% CI)	Deaths/person-years	Univariate HR (95% CI)	HR adjusted for stage and LINE-1 (95% CI)	Multivariate HR (95% CI)
p21-Expressing cases	138 (21%)	38/1,103	1 (reference)	1 (reference)	1 (reference)	63/1,103	1 (reference)	1 (reference)	1 (reference)
p21-Lost cases	509 (79%)	124/4,193	0.85 (0.59-1.22)	0.67 (0.45-0.99)	0.58 (0.38-0.89)	216/4,193	0.90 (0.68-1.19)	0.81 (0.60-1.08)	0.71 (0.51-0.98)
P			0.37	0.047	0.013		0.46	0.072	0.035

NOTE: The multivariate stage-matched (stratified) Cox regression model included age, year of diagnosis, sex, family history of colorectal cancer, BMI, tumor location, grade, *KRAS*, *BRAF*, *PIK3CA*, p53, cyclin D1, long interspersed nucleotide element-1 methylation, microsatellite instability, and CpG island methylator phenotype.
Abbreviation: HR, hazard ratio.

Table 3. Mortality of patients with p21-lost colon cancer (compared with p21-expressing tumor) in strata of age category

Age category	Colon cancer–specific mortality (p21-lost cases vs p21-expressing cases as a reference)			Overall mortality (p21-lost cases vs p21-expressing cases as a reference)		
	No. of deaths/cases (p21-lost vs p21-expressing)	Stage-matched HR (95% CI)	Multivariate HR (95% CI)	No. of deaths/cases p21-lost vs p21 expressing)	Stage-matched HR (95% CI)	Multivariate HR (95% CI)
<60 y old	29/112 vs 3/22	3.55 (1.06-11.9)	4.09 (1.13-14.9)	38/112 vs 5/22	2.46 (0.93-6.46)	2.62 (0.96-7.18)
60-64 y old	28/92 vs 10/30	0.75 (0.35-1.60)	0.61 (0.27-1.36)	38/92 vs 13/30	0.86 (0.45-1.64)	0.77 (0.39-1.52)
65-69 y old	28/121 vs 10/40	0.60 (0.28-1.27)	0.38 (0.17-0.86)	53/121 vs 19/40	0.73 (0.42-1.27)	0.60 (0.34-1.07)
≥70 y old	39/184 vs 15/49	0.37 (0.20-0.70)	0.21 (0.10-0.43)	88/184 vs 26/49	0.52 (0.33-0.82)	0.41 (0.25-0.67)
P for interaction (p21 and age)*		0.0006	<0.0001		0.004	0.001
≥60 y old total	95/397 vs 35/116	0.55 (0.37-0.83)	0.37 (0.24-0.59)	179/397 vs 58/116	0.71 (0.52-0.97)	0.57 (0.40-0.79)
P		0.004	<0.0001		0.029	0.001

NOTE: The multivariate stage-matched (stratified) Cox model included the p21 variable stratified by age category, year of diagnosis, sex, family history of colorectal cancer, BMI, tumor location, grade, *KRAS, BRAF, PIK3CA*, p53, cyclin D1, long interspersed nucleotide element-1 methylation, microsatellite instability, and CpG island methylator phenotype.
*Age is used as an ordinal variable (<60 versus 60-69 versus ≥70 years old).

modifying effect of patient's age on the relation between p21 loss and clinical outcome. Specifically, for patients >60 years, tumoral p21 loss was associated with progressively superior cancer-specific survival with increasing age. In contrast, among patients <60 years, p21 loss was associated with a worse cancer-specific mortality. We also found that the adverse effect of obesity on clinical outcome was principally limited to patients with p21-expressing tumors, but no adverse effect of obesity was apparent among patients with p21-lost tumors. Our results support the role of tumoral p21 status and its interaction with patient age and BMI (that is, tumor-host interactions) in determining clinical outcome of colon cancer patients.

The function of p21 seems to be multifaceted and can be tumor suppressive or oncogenic (5, 6). p21 (*CDKN1A*) is a well-known cyclin-dependent kinase inhibitor induced by wild-type p53 and plays an important role in blocking cell cycle progression (1). On the other hand, p21 may facilitate tumor invasion through *PAK1* in melanoma cells (8). PAK1 activation has also been associated with greater metastatic potential in colorectal cancer (7, 9). Thus, it is possible that p21 loss can be a marker for aggressive tumor in a subset of people (<60 year old in this study) and a maker for less aggressive tumor in a different subset of people (≥65 year old in this study) with a different context of host microenvironment and tumor development.

Examining molecular changes or risk factors is important in colon cancer research (45-51). Although previous studies have examined the relationship between tumoral p21 loss and clinical outcome in colon cancer (13-25), those studies have yielded inconsistent results. A few studies have shown that p21 loss has been associated with poor prognosis (13-15), whereas most studies have shown no independent prognostic value of p21 (16-25). There have been no studies that reported beneficial prognostic influence of p21 loss possibly by publication bias, which is because of the preconception that p21 loss in colon cancer must be detrimental for patients. However, as discussed above, p21 loss may be associated with indolent tumors because p21 may facilitate tumor progression through PAK1 (7-9). A study has reported strong inverse associations of p21 loss in colorectal cancer with CIMP, MSI, and *BRAF* mutation (27), and all of the latter have been related with clinical outcome (29-31). Thus, CIMP, MSI, and *BRAF* are potential confounders in analysis of p21 loss and clinical outcome. However, none of the previous studies (13-25) have examined these potential confounders. Moreover, most previous studies were limited by small sample sizes ($N < 250$), except for the two large studies ($N > 450$; refs. 16, 24). In our current study, we controlled for potential confounding by the relevant molecular features (CIMP, MSI, *BRAF, KRAS*, LINE-1 methylation, p53, and cyclin D1). In addition, to ensure statistical power, we used a large number ($N = 647$) of stage I to IV colon cancers identified through the two independent prospective cohort studies.

The modifying effect of age on the relation between p21 loss and patient mortality is intriguing. In nonneoplastic state, function of p21 has been related with cellular senescence and aging of stem cells (10-12). Thus, it is plausible that the influence of functional p21 loss on tumor behavior may be modified by age of patients and the state of stem cells that gave rise to tumor. This modifying effect may be mediated by other factors such as telomere length. It is possible that stem cells that give rise to tumor in older individuals may have substantially different molecular features from stem cells that give rise to tumor in younger individuals. One can speculate that cancerous stem cells that have been close to senescence or in the state of senescence (in old individuals) may be more susceptible to apoptotic signal when cell cycle is not blocked by p21. In contrast, in cancerous stem cells in young individuals, the adverse effect of cell cycle progression by p21 loss may have more direct influence on tumor behavior. Further studies are necessary to examine the exact mechanism of the tumor-host interaction between age (host) and p21 loss (tumor) in determining tumor aggressiveness.

Another possible tumor-host interaction between obesity (BMI $\geq$ 30 kg/m^2) and p21 status warrants discussion. Cellular proliferation, senescence, and apoptosis have been known to be influenced by cellular energy balance status. Obesity and physical inactivity have been consistently shown to be risk factors for colon cancer development and mortality (52). We have recently discovered potential host-tumor interactions (affecting colon cancer behavior) between obesity and fatty acid synthase (53), stathmin STMN1 (54), or p27 (55). With

regard to energy balance and p21, experimental studies have shown that energy restriction up-regulates p21 through phosphorylation and activation of p53 by AMP kinase (2-4), suggesting a link between energy balance and cellular p21 regulation. Thus, it is possible that there is an interaction between patient BMI and tumoral p21 status in determining biological behavior of colon cancer. Our data support the hypothesis that excess energy balance can make much stronger impact on tumor behavior if tumor cells can up-regulate p21 to arrest cell cycle than if tumor cells have lost the ability to up-regulate p21. This hypothesis needs to be tested by additional studies.

There are advantages in using the database of the two independent prospective cohort studies, the Nurses' Health Study and Health Professionals Follow-up Study, to examine tumor-host interactions. Clinical information was prospectively collected and entered into the database blinded to patient diagnosis, tumoral molecular features, and outcome. Data were updated every 2 years. Cohort participants who developed colon cancer were treated at hospitals throughout the United States. Tumor specimen procurement rate has been ~60%, and there were no demographic difference between cases with tumor tissue analyzed and those without tumor tissue analyzed (33). However, a limitation of this study is that data on cancer treatment were limited. Nonetheless, it is unlikely that chemotherapy use differed according to tumoral p21 status because such data were unavailable to treating physicians. In addition, beyond cause of mortality, data on cancer recurrences were not available. Nonetheless, given the median survival for metastatic colon cancer was ~10 to 12 months during much of the period of this study, colon cancer–specific survival

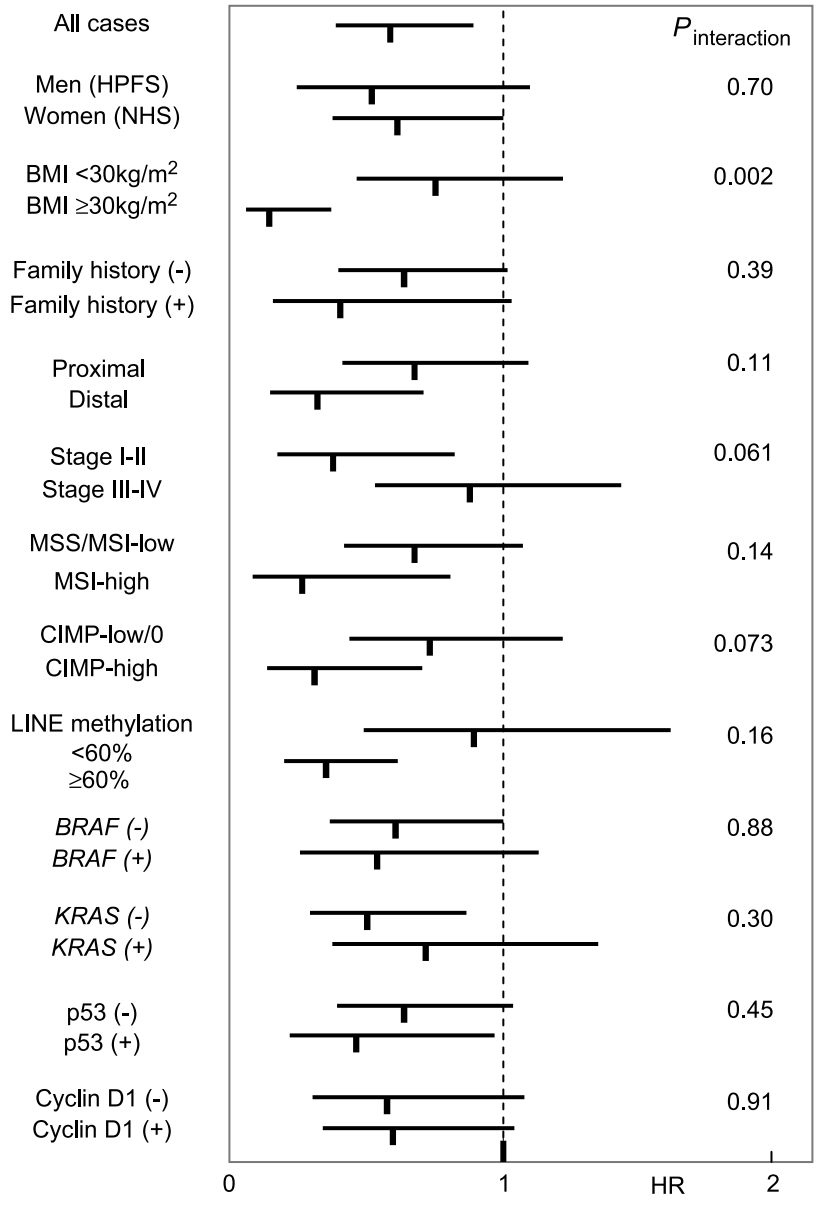

Figure 2. p21 Loss in colon cancer and colon cancer–specific mortality in various strata. Adjusted hazard ratio (with 95% CI) for colon cancer–specific mortality in p21-lost cases (versus p21-expressing cases) is shown. Not all strata examined are shown. CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable; NHS, Nurses' Health Study.

Table 4. Colon cancer mortality in obese patients (compared with nonobese patients) in strata of p21 status

	Colon cancer–specific mortality			Overall mortality		
	No. of deaths/cases	Stage-matched HR (95% CI)	Multivariate HR (95% CI)	No. of deaths/cases	Stage-matched HR (95% CI)	Multivariate HR (95% CI)
p21-Expressing cases						
BMI < 30 kg/m^2	28/113	1 (reference)	1 (reference)	48/113	1 (reference)	1 (reference)
BMI ≥ 30 kg/m^2	8/20	7.24 (3.09-17.0)	5.85 (2.28-15.0)	12/20	3.96 (2.03-7.74)	3.40 (1.65-6.98)
p21-Lost cases						
BMI < 30 kg/m^2	99/400	1 (reference)	1 (reference)	177/400	1 (reference)	1 (reference)
BMI ≥ 30 kg/m^2	18/89	1.08 (0.64-1.80)	1.05 (0.60-1.83)	30/89	0.89 (0.60-1.33)	0.94 (0.62-1.43)
P for interaction (p21 and BMI)		0.0002	0.002		0.0002	0.002

NOTE: The multivariate, stage-matched (stratified) Cox model included the p21 variable stratified by BMI, age, year of diagnosis, sex, family history of colorectal cancer, tumor location, grade, *KRAS, BRAF, PIK3CA*, p53, cyclin D1, long interspersed nucleotide element-1 methylation, microsatellite instability, and CpG island methylator phenotype.

should be a reasonable surrogate for cancer-specific outcomes.

There are currently no standardized methods to assess p21 loss in colon cancer. Nonuniform methods to evaluate tumoral p21 may contribute to the inconsistent results in the previous studies. Nonetheless, our method yielded highly significant associations between p21 loss and other related molecular variables (including p53, MSI, CIMP, and *BRAF* mutation; see Table 1). Moreover, any random misclassification of tumors in terms of p21 expression would drive our results toward the null hypothesis.

In summary, tumoral p21 loss is associated with a low colon cancer–specific mortality among patients ≥60 year old, whereas it is associated with inferior survival among patients <60 year old. It is possible that this differential association may reflect a difference in senescence and aging of stem cells between young and old patients. In addition, the adverse effect of obesity on clinical outcome was observed only among patients with p21-expressing tumors, implying a tumor-host interaction between energy balance and regulatory machinery of the cell cycle. Future studies are needed to confirm these findings as well as to elucidate exact mechanisms by which p21 loss interacts with host factors and affects tumor behavior.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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