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Phosphorylated AKT Expression is Associated with *PIK3CA* Mutation, Low Stage and Favorable Outcome in 717 Colorectal Cancers

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Abstract

Background—AKT (AKT1, AKT2, and AKT3) is a downstream effector of phosphatidylinositol-3-kinase (PI3K) and plays crucial roles in protein synthesis, cellular metabolism, survival, and proliferation. The PI3K/AKT pathway is commonly activated in human cancers and recognized as a potential target for anticancer therapy. Nonetheless, clinical, molecular or prognostic features of AKT-activated colon cancer remain uncertain.

Methods—Utilizing a database of 717 colorectal cancers in the Nurses' Health Study and the Health Professionals Follow-up Study, Ser473 phosphorylated AKT (p-AKT) expression was detected in 448 (62%) tumors by immunohistochemistry. Cox proportional hazard model was used to compute mortality hazard ratio (HR), adjusting for clinical and tumoral features, including *PIK3CA*, *KRAS*, *BRAF*, microsatellite instability (MSI), CpG island methylator phenotype (CIMP), LINE-1 methylation, TP53 (p53) and FASN (fatty acid synthase).

Results—Tumor p-AKT expression was associated with *PIK3CA* mutation [odds ratio (OR), 1.77; 95% confidence interval (CI), 1.12-2.80; p=0.015]. p-AKT expression was significantly associated with longer colorectal cancer-specific survival in Kaplan-Meier analysis (log-rank p=0.0005), univariate Cox regression [hazard ratio (HR) 0.62; 95% CI, 0.47-0.82; p=0.0006] and multivariate analysis (adjusted HR 0.75; 95% CI, 0.56-0.99; p=0.048) adjusting for clinical and molecular variables including *PIK3CA*, MSI, CIMP and LINE-1 hypomethylation. p-AKT expression was inversely associated with high stage (III-IV) (adjusted OR, 0.63; 95% CI, 0.45-0.88, p=0.0071).

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No conflict of interest exists.

Conclusions—p-AKT expression in colorectal cancer is associated with low stage and good prognosis. p-AKT may serve as a tissue biomarker to identify patients with superior prognosis and a possible therapeutic target (analogous to estrogen receptor ESR1 in breast cancer).

Keywords

pAKT; PI3K; clinical outcome; personalized medicine; colorectal carcinoma

INTRODUCTION

AKT, a serine/threonine protein kinase, is a major downstream effector of phosphatidylinoside-3-kinase (PI3K).¹⁻³ AKT has three isoforms including AKT1, AKT2 and AKT3, and plays crucial roles in regulating a wide range of cellular processes including protein synthesis, cell survival, proliferation, and metabolism.¹⁻³ In human cancer, the PI3K/AKT pathway is aberrantly activated by growth factor stimulation and subsequent activation of receptor tyrosine kinases, as well as by a mutation, deletion, or amplification of a key pathway component (e.g., *PIK3CA*, *PTEN*, or *AKT1*).¹⁻³ Therefore, this pathway has been recognized as an attractive target for anticancer therapy.⁴⁻⁶ Thus, better understanding of the role of AKT activation in human cancer is increasingly important. However, prognostic significance of AKT activation in human cancers remains inconclusive; phosphorylated AKT (p-AKT) expression (i.e., AKT activation) has been associated with poor prognosis in some types of cancers,⁷⁻⁹ but with favorable prognosis in other types of cancers.¹⁰⁻¹⁴ Studies have shown that p-AKT expression in colorectal cancer is not associated with patient survival;¹⁵⁻¹⁸ however, they are all limited by lack of *PIK3CA* mutation data and/or low statistical power [N<160 in all¹⁶⁻¹⁸ but one study¹⁵]. Given accumulating evidence on vital roles of AKT in cellular metabolism, we hypothesized that AKT-activated cancers and AKT-inactive cancers might behave differently.

To test this hypothesis, we utilized a database of 717 stage I-IV colorectal cancers in two prospective cohort studies, and examined the prognostic role of p-AKT expression. Microsatellite instability (MSI), the CpG island methylator phenotype (CIMP) status and LINE-1 hypomethylation (i.e., global DNA hypomethylation) reflect global genomic and epigenomic aberrations in tumor cells and determine clinical, pathologic, molecular and prognostic characteristics of colorectal cancer.¹⁹ It is widely accepted that activating mutations in *PIK3CA*, *KRAS*, and *BRAF* play vital roles in colorectal carcinogenesis. *TP53* is a crucial tumor suppressor gene in the pathogenesis of colorectal cancer. FASN (fatty acid synthase) is physiologically regulated by energy balance and may interact with the PI3K/AKT pathway.²⁰ Prognostic effect of FASN expression in colon cancer differs according to body mass index.²¹ Since we concurrently assessed these important molecular variables, we could evaluate the independent effect of p-AKT on patient survival after controlling for these molecular events. Our findings of the relationship between p-AKT expression and good outcome suggest that p-AKT may serve as a tissue biomarker to identify patients with better prognosis and a possible therapeutic target (analogous to estrogen receptor ESR1 in breast cancer).

MATERIALS AND METHODS

Study group

We utilized the database of two prospective cohort studies; the Nurses' Health Study (N=121,701 women followed since 1976),²² and the Health Professional Follow-up Study (N=51,529 men followed since 1986).²² We collected paraffin-embedded tissue blocks from hospitals where patients underwent colorectal cancer resections. Tissue sections from all colorectal cancers were reviewed by a pathologist (S.O.). Based on availability of adequate

tissue specimens and follow-up data, a total of 717 colorectal cancers (diagnosed up to 2004) were included. Patients were observed until death or June 30, 2009, whichever came first. Tissue collection and analyses were approved by the Harvard School of Public Health and Brigham and Women's Hospital Institutional Review Boards.

Sequencing of *KRAS*, *BRAF* and *PIK3CA* and microsatellite instability (MSI) analysis

DNA was extracted from tumor. PCR and Pyrosequencing targeted for *KRAS*,²³ *BRAF*,²⁴ and *PIK3CA* were performed.²⁵ MSI-high was defined as $\geq 30\%$ unstable microsatellite markers, and MSI-low/microsatellite stable (MSS) as 0-29% unstable markers.²⁶

Methylation analyses for CpG islands and LINE-1

Bisulfite DNA treatment and real-time PCR (MethyLight) were validated.²⁷ We quantified DNA methylation in 8 CIMP-specific promoters [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*].²⁸⁻³⁰ CIMP-high was defined as the presence of $\geq 6/8$ methylated promoters, CIMP-low as 1/8-5/8 methylated promoters, and CIMP-0 as the absence (0/8) of methylated promoters.^{30, 31} To accurately quantify LINE-1 methylation, we utilized Pyrosequencing.^{32, 33}

Immunohistochemistry

Tissue microarrays (TMAs) were constructed.²² Two 0.6-mm tissue cores each from tumor and normal colonic mucosa were placed in each TMA block. Methods of immunohistochemistry were previously described for TP53 (p53) and FASN.^{21, 26} AKT1 is partially activated through phosphorylation of Thr308 and reaches its maximum activity after phosphorylation of Ser473 in tandem with that of Thr308. Similar phosphorylation sites are present in AKT2 and AKT3. To date, the most successful biomarker for evaluating the status of AKT activation is Ser473 phosphorylated form of AKT.³⁴ In our current study, we utilized the pan-AKT antibody [Rabbit monoclonal anti-Phospho-Akt (Ser473) (736E11), Cell Signaling Technology, Boston, MA], which detects AKT1 only when phosphorylated at Ser 473, and AKT2 and AKT3 only when phosphorylated at equivalent sites, according to previous studies.^{8-10, 15, 17} For phosphorylated-AKT (p-AKT) staining, deparaffinized tissue sections in Antigen Retrieval Citra Solution (Biogenex Laboratories, San Ramon, CA) were treated with microwave in a pressure cooker (25 min). Tissue sections were incubated with 5% normal goat serum (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline (30 min). Primary antibody against p-AKT (1:100 dilution) was applied, and the slides were maintained at 4°C for overnight, followed by rabbit secondary antibody (Vector Laboratories) (60 min), an avidin-biotin complex conjugate (Vector Laboratories) (60 min), diaminobenzidine (5 min) and methyl-green counterstain. Appropriate positive and negative controls were included in each run of immunohistochemistry. In each case, we recorded cytoplasmic p-AKT expression as no expression, weak expression, moderate expression, or strong expression compared to normal colonic epithelial cells (Figure 1). Considering that AKT is downstream of the PI3K pathway,¹⁻³ we used *PIK3CA* mutation frequency data to determine a cutoff for p-AKT positivity. We randomly selected 364 tumors as a training set, leaving the remaining 353 as a validation set. Using the training set, the frequency of *PIK3CA* mutation was as follows: 13% (16/119) in tumors with no expression; 20% (19/94) in tumors with weak expression; 19% (21/110) in tumors with moderate/strong expression. Thus, p-AKT positivity was defined as the presence of weak to strong expression. In the remaining validation set, p-AKT positivity was associated with *PIK3CA* mutation [odds ratio (OR) 2.00; 95% confidence interval (CI), 1.02-3.94; $p=0.040$], confirming the validity of the cutoff for p-AKT positivity, although it might not be the best cutoff. There was no perfect correlation between *PIK3CA* mutation and p-AKT, in part because there were other mechanisms of AKT activation (i.e., *PTEN* loss and/or methylation, *PIK3CA* amplification, and *PIK3RI*

mutation). p-AKT expression might serve as a surrogate of AKT activation by any of these causes.

Each immunohistochemical maker was visually interpreted by one of the investigators (p-AKT by Y.B.; TP53 and FASN by S.O.) unaware of other data. For agreement studies, a random selection of 108-246 cases was examined for each marker by a second pathologist (p-AKT by K.S., TP53 and FASN by K.N.) unaware of other data. The concordance between the two pathologists (all $p < 0.0001$) was 0.81 ($\kappa = 0.59$, $N = 132$) for p-AKT, 0.87 ($\kappa = 0.75$, $N = 108$) for TP53, and 0.93 ($\kappa = 0.57$, $N = 246$) for FASN, indicating good agreement.

Statistical analysis

We used SAS program (Version 9.1, SAS Institute, Cary, NC). All p values were two-sided. When we performed multiple hypothesis testing, a p value for significance was adjusted by Bonferroni correction to $p = 0.0031$. For categorical data, the chi-square test was performed. To assess independent relationship between p-AKT and disease stage (an outcome variable), a multivariate logistic regression analysis was performed, initially including sex, age at diagnosis (continuous), body mass index (BMI, <30 vs. ≥ 30 kg/m²), family history of colorectal cancer in any first degree relative (present vs. absent), tumor location (rectum vs. colon), tumor grade (high vs. low), CIMP (high vs. low/0), MSI (high vs. low/MSS), LINE-1 methylation (continuous), *BRAF*, *KRAS*, *PIK3CA*, TP53, FASN, and p-AKT expression. A backward stepwise elimination with a threshold of $p = 0.20$ was used to select variables in the final model. For cases with missing information in any of categorical variables [BMI (0.1%), tumor grade (0.4%), TP53 (0.6%), FASN (0.8%), CIMP (1.7%), MSI (2.0%), *BRAF* (1.5%), *KRAS* (1.1%), and *PIK3CA* (10%)], we included those cases in a majority category of that missing variable in the initial model. After the selection was done, we assigned separate missing indicator variables to those cases with missing information in any of the categorical covariates in the final model.

For survival analysis, Kaplan-Meier method and log-rank test were used. For analyses of colorectal cancer-specific mortality, deaths as a result of causes other than colorectal cancer were censored. To assess independent effect of p-AKT on mortality, tumor stage (I, IIA, IIB, IIIA, IIIB, IIIC, IV, unknown) was used as a stratifying variable in Cox models using the “strata” option in the SAS “proc phreg” command to avoid residual confounding and overfitting. We constructed a multivariate proportional hazards model to compute a hazard ratio (HR) according to p-AKT status, initially containing sex, age, BMI, family history of colorectal cancer, year of diagnosis, tumor location, tumor grade, TP53, FASN, CIMP, MSI, *BRAF*, *KRAS*, *PIK3CA*, and LINE-1 methylation. A backward stepwise elimination with a threshold of $p = 0.20$ was used to select variables in the final model. The proportionality of hazard assumption was satisfied by evaluating time-dependent variables, which were the cross-product of the p-AKT variable and survival time ($p > 0.31$). An interaction was assessed by including the cross product of p-AKT variable and another variable of interest (without data-missing cases) in a multivariate Cox model, and the Wald test was performed.

RESULTS

p-AKT expression in colorectal cancer

Among 717 colorectal cancers, we observed phosphorylated AKT (p-AKT) expression in 448 tumors (62%) by immunohistochemistry (Figure 1). Although variability of p-AKT staining between tissue cores in a given case was not substantial, we obtained four tissue cores from each case to overcome within-tumor heterogeneity. Table 1 shows p-AKT status in relation to clinical, pathologic and molecular features. Notably, there was no difference in

frequencies of p-AKT positivity between cases prior to 1995 and those in 1995 or after ($p=0.85$), suggesting that the age of tissue block might not substantially influence our results. Consistent with a possible causal link between *PIK3CA* mutation and p-AKT expression, p-AKT expression was significantly associated with *PIK3CA* mutation [odds ratio (OR), 1.77; 95% confidence interval (CI), 1.12-2.80; $p=0.015$]. In addition, p-AKT expression was associated positively with FASN expression ($p=0.0025$), and inversely with stage III-IV disease ($p=0.0022$) and high tumor grade ($p<0.0001$).

In multivariate logistic regression analysis assessing the independent relationship between p-AKT expression and disease stage, p-AKT expression was inversely associated with high disease stage (stage III-IV) (adjusted OR, 0.63; 95% CI, 0.45-0.88; $p=0.0071$) (Table 2).

p-AKT expression and patient survival

During follow-up of 717 patients with survival data (median follow-up time 11.6 years for censored cases), there were 341 deaths, including 210 deaths due to colorectal cancer. In Kaplan-Meier analysis, p-AKT expression was associated with longer colorectal cancer-specific survival (log-rank $p=0.0005$) and overall survival (log-rank $p=0.014$) (Figure 2).

Compared to p-AKT-negative cases, p-AKT-positive cases experienced a significantly lower colorectal cancer-specific mortality in univariate analysis [hazard ratio (HR) 0.62; 95% confidence interval (CI), 0.47-0.82; $p=0.0006$] and multivariate analysis (adjusted HR 0.75; 95% CI, 0.56-0.99; $p=0.048$) (Table 3). The attenuation in the effect of p-AKT expression in the multivariate analysis was principally the result of adjusting for disease stage; when we simply adjusted for disease stage, p-AKT overexpression was associated with HR of 0.72 (95% CI, 0.54-0.94). Similar results, although somewhat attenuated, were observed in analysis for overall mortality (Table 3). Our data suggest that, despite the role of AKT activation in carcinogenesis, p-AKT expression marks a subtype of colorectal cancers with indolent behavior, which is analogous to estrogen receptor expression in breast cancer.

Interaction between p-AKT expression and another variable in survival analyses

We examined the influence of p-AKT positivity on cancer-specific mortality across strata of other potential predictors of survival, including age, sex, year of diagnosis, body mass index, family history of colorectal cancer, tumor location, stage, MSI, CIMP, *KRAS*, *BRAF*, *PIK3CA*, LINE-1 methylation, TP53, and FASN. There was no evidence for significant effect modification by any of the variables examined (all $P_{\text{interaction}} \geq 0.10$). Notably, the effect of p-AKT did not significantly differ between the two independent cohort studies ($P_{\text{interaction}}=0.53$), or between tumors prior to 1995 and those in 1995 or after ($P_{\text{interaction}}=0.15$).

DISCUSSION

We conducted this study to examine the relationship between phosphorylated AKT (p-AKT) expression and patient survival in a large cohort of stage I-IV colorectal cancers. AKT has emerged as a central node in cell signaling pathways, downstream of growth factors, cytokines, and other cellular stimuli.¹⁻³ AKT is aberrantly activated in a wide variety of human cancers and is increasingly important as a promising target for cancer therapy.⁴⁻⁶ We have found that p-AKT expression in colorectal cancer is associated with early stage and favorable prognosis, suggesting that p-AKT may be a biomarker to identify patients with superior outcome and a possible therapeutic target (analogous to estrogen receptor in breast cancer).

Examining tumor factors and clinical outcome is important in cancer research.³⁵⁻³⁹ Studies examining the relation between p-AKT expression and prognosis in human cancers have

yielded variable results.⁷⁻¹² Previous studies on colorectal cancer have suggested no prognostic role of p-AKT expression.¹⁵⁻¹⁸ Notably, in the study with over 1000 colorectal cancers,¹⁵ tumoral p-AKT expression was associated with early nodal stage, but not independently with patient survival. However, that study¹⁵ lacked *PIK3CA* mutation data. A difference in the prognostic data by that study¹⁵ and our current study might be due to a difference in the patient cohorts or the methods to assess p-AKT expression, or simply due to a chance variation between independent studies. In addition, all but one¹⁵ studies¹⁶⁻¹⁸ were limited by small sample sizes ($N < 160$). Our current study ($N = 717$) has shown that p-AKT expression is associated with *PIK3CA* mutation as expected from their causal link, and that our method and cutoff for p-AKT positivity are reasonable for evaluating AKT activation levels in paraffin-embedded tissues. Furthermore, in contrast to the prior studies,¹⁵⁻¹⁸ we assessed the prognostic effect of p-AKT expression independent of *PIK3CA* mutation and other molecular events that have been documented to be critical in colorectal carcinogenesis.

Considering experimental data suggesting that AKT plays critical roles in tumor proliferation, survival, invasion, and angiogenesis,^{1-3, 40} one would expect that p-AKT expression would imply poor prognosis. It is very common to conceive that the presence of oncogene activation (or tumor suppressor inactivation) should imply aggressive tumor behavior. However, this preconception does not always hold true. Colon cancers develop through accumulation of multiple genetic and epigenetic events, and each tumor has its own unique combination of molecular aberrations. Some tumors activate AKT, while others do not. In order to acquire malignant characteristics, those AKT-inactive tumors need to have aberrations which can be an alternative to AKT activation; those aberrations may lead to more aggressive behavior than AKT activation actually does. This is well exemplified by the association between estrogen receptor (ESR1, or ER-alpha) expression in breast cancer and good prognosis. ESR1 is known to contribute to breast cancer development; yet ESR1 expression marks tumors with favorable outcome.⁴¹ This is probably because breast cancer without ESR1 expression might have developed through more detrimental events than ESR1 expression. Another example is MSI in colorectal cancer. MSI is known to cause inactivation of a number of tumor suppressors; yet MSI marks tumors with good prognosis.⁴² The aforementioned preconception that oncogene activation (or tumor suppressor inactivation) should be associated with poor outcome can cause serious publication bias, because reports consistent with this preconception have been regarded favorably during journal's decision process, whereas reports inconsistent with the preconception have been treated unfavorably.⁴³

Another possible explanation for the relationship between AKT activation and good prognosis may be due to "tumor suppressive" roles of AKT.⁴⁴ AKT has blocked cancer cell mortality and invasion through the transcription factor NFAT⁴⁵ or down-regulation of RHO activity.⁴⁶ Another study has reported that AKT1 activation can promote tumorigenesis but suppresses tumor invasion.⁴⁷ The inhibitory effect of AKT activation on cancer cell cycle has been reported.⁴⁸ In addition, it is possible that each AKT isoform may have different functions.^{49, 50} Future studies are necessary to confirm our observations as well as to elucidate biological mechanisms by which AKT activation affects colorectal tumor behavior.

Interestingly, we found that p-AKT expression was independently associated with FASN expression and low tumor grade; the latter association is consistent with a previous study.¹⁵ Inhibition of FASN has resulted in the down-regulation of AKT pathway.²⁰ The PI3K/AKT pathway activation has modulated the expression and/or nuclear maturation of the transcription factor SREBF1, stimulating FASN expression.²⁰ Our finding of the

relationship between p-AKT and FASN expression may be consistent with these experimental data.

There are limitations in this study. For example, data on cancer treatment were unavailable. Nonetheless, it is unlikely that chemotherapy use substantially differed according to AKT status in tumor, since such data were unavailable for treatment decision making. In addition, our multivariate survival analysis adjusted for disease stage as finely as possible (I, IIA, IIB, IIIA, IIIB, IIIC, IV, unknown) on which treatment decision making was mostly based. As another limitation, beyond cause of mortality, data on cancer recurrences were unavailable in these cohort studies. Nonetheless, colorectal cancer-specific survival might be a reasonable surrogate of colorectal cancer-specific outcome.

Immunohistochemical evaluation of p-AKT expression in cancer tissue has been a challenge, and there is no standardized method. Pre-analytical variables such as tissue processing may have considerable impact on antigenicity of p-AKT, which may be substantially influenced by a slight difference in conditions of immunohistochemical procedure. We tried to minimize such an external source of noise in a number of ways. We constructed tissue microarray (TMA) and performed the immunohistochemical procedure in a very similar condition for all specimens. The quality of tissue sections, which depends on age of tissue and time after cutting the TMA blocks, should be considered. In our current study, p-AKT expression was not associated with age of tissue (i.e., year of diagnosis), supporting that p-AKT antigen might not substantially be deteriorated over time. With regard to time after block cutting, as we cut all TMA blocks into sections around the same time, time after block cutting was uniform from case to case. In addition, we obtained four tissue cores from each case to overcome within-tumor heterogeneity. Interpretation of protein expression took into account background noise if any, and we calibrated against such background noise in each case. Furthermore, any pre-analytical variability was largely non-differential (i.e., random) in nature, and thus, might have conservatively biased our results towards the null hypothesis. Because of a large sample size, we were still able to detect the biologically-reasonable relationship between *PIK3CA* mutation and p-AKT expression as well as the relations of p-AKT expression with disease stage and prognosis. The cutoff for p-AKT used in this current study needs to be validated in an independent dataset.

There are advantages in utilizing the database of the two prospective cohort studies, the Nurses' Health Study and the Health Professionals Follow-up Study, to examine prognostic significance of tumor biomarkers. Anthropometric measurements, family history, cancer staging, and other clinical, pathologic, and tumoral molecular data were prospectively collected, blinded to patient outcome. Cohort participants who developed cancer were treated at hospitals throughout the U.S. (in 48 States except for North Dakota and Alaska), and thus more representative colorectal cancers in the general U.S. population than patients in one to a few academic hospitals. There were no demographic difference between cases with tumor tissue analyzed and those without tumor tissue analyzed²². Finally, our rich tumor database enabled us to simultaneously assess pathologic and tumoral molecular correlates and control for confounding by a number of tumoral molecular alterations.

In summary, our large cohort study has shown that p-AKT expression in colorectal cancer is associated with *PIK3CA* mutation, early disease stage and favorable prognosis. The high percentage of p-AKT positive patients in this study certainly supports a vital role of AKT activation in the pathogenesis of colorectal cancer. Our findings suggest a possibility that AKT activation may have a predictive role in a manner analogous to estrogen receptor (ESR1) in breast cancer, identifying a subgroup of patients with better prognosis and serving as a therapeutic target at the same time.

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Figure 1.

Immunohistochemistry for p-AKT expression in colorectal cancer

A. Positive for p-AKT cytoplasmic expression in colon cancer cells (white arrowheads).

B. Negative for p-AKT expression in colon cancer cells (black arrowheads). Stromal cells serve as an internal positive control for p-AKT expression (arrows).

Figure 2.

Kaplan-Meier curves for colorectal cancer-specific survival (upper panel) and for overall survival (lower panel) according to p-AKT status in colorectal cancer. The bottom table indicates the number of patients who were alive and at risk of death at each time point after the diagnosis of colorectal cancer.

Figure 3. Schematic representation of possible relations between AKT activation, disease stage, and patient prognosis.

Table 1

p-AKT expression in colorectal cancer

Clinical or molecular feature	Total N	p-AKT expression		P value
		(-)	(+)	
All cases	717	269	448	
Sex				0.0035
Male	259 (36%)	79 (29%)	180 (40%)	
Female	458 (64%)	190 (71%)	268 (60%)	
Age (years)				0.17
<65	265 (37%)	108 (40%)	157 (35%)	
≥65	452 (63%)	161 (60%)	291 (65%)	
Body mass index (BMI, kg/m ²)				0.30
<25	313 (44%)	126 (47%)	187 (42%)	
25-29	281 (39%)	96 (36%)	185 (41%)	
≥30	122 (17%)	47 (17%)	75 (17%)	
Family history of colorectal cancer in any first degree relative				0.60
(-)	546 (76%)	202 (75%)	344 (77%)	
(+)	171 (24%)	67 (25%)	104 (23%)	
Year of diagnosis				0.85
Prior to 1995	275 (38%)	102 (38%)	173 (39%)	
1995 to 2004	442 (62%)	167 (62%)	275 (61%)	
Tumor location				0.29
Rectum	141 (20%)	61 (23%)	81 (18%)	
Distal colon (splenic flexure to sigmoid)	221 (31%)	80 (30%)	141 (31%)	
Proximal colon (cecum to transverse)	355 (50%)	128 (48%)	227 (51%)	
Stage				0.0022
I	161 (22%)	41 (15%)	120 (27%)	
II	221 (31%)	80 (30%)	141 (31%)	
III	200 (28%)	89 (33%)	111 (25%)	
IV	98 (14%)	44 (16%)	54 (12%)	
Unknown	37 (5.2%)	15 (5.6%)	22 (4.9%)	

Clinical or molecular feature	Total N	p-AKT expression		P value
		(-)	(+)	
Tumor grade				<0.0001
Low	648 (91%)	227 (85%)	421 (94%)	
High	66 (9.2%)	40 (15%)	26 (5.8%)	
MSI status				0.25
MSI-low/MSS	583 (83%)	213 (81%)	370 (84%)	
MSI-high	121 (17%)	51 (19%)	70 (16%)	
CIMP status				0.17
CIMP-0	296 (42%)	105 (40%)	191 (43%)	
CIMP-low	291 (41%)	105 (40%)	186 (42%)	
CIMP-high	118 (17%)	53 (20%)	65 (15%)	
<i>BRAF</i> mutation				0.53
(-)	597 (85%)	222 (83%)	375 (85%)	
(+)	109 (15%)	44 (17%)	65 (15%)	
<i>KRAS</i> mutation				0.61
(-)	441 (62%)	168 (63%)	273 (61%)	
(+)	268 (38%)	97 (37%)	171 (39%)	
<i>PIK3CA</i> mutation				0.015
(-)	534 (83%)	210 (88%)	324 (80%)	
(+)	108 (17%)	29 (12%)	79 (20%)	
LINE-1 methylation level (Mean $\pm$ SD)	61.3 $\pm$ 9.4	61.3 $\pm$ 9.1	61.3 $\pm$ 9.7	0.94
TP53 expression				0.46
(-)	432 (61%)	167 (62%)	265 (60%)	
(+)	281 (39%)	101 (38%)	180 (40%)	
FASN expression				0.0025
(-)	598 (84%)	238 (89%)	360 (81%)	
(+)	113 (16%)	28 (11%)	85 (19%)	

(%) indicates the proportion of cases with a specific clinical, pathologic or molecular feature among p-AKT (+) cases [or p-AKT (-) cases]. CIMP, CpG island methylator phenotype; FASN, fatty acid synthase; MSI, microsatellite instability; MSS, microsatellite stable; p-AKT, phosphorylated AKT; SD, standard deviation.

Table 2

Multivariate logistic regression analysis of the relationship between p-AKT expression and colorectal cancer stage (as an outcome variable)

Variables in the final model for disease stage III-IV (vs. I-II) as an outcome variables	Multivariate OR (95% CI)	P value
p-AKT expression	0.63 (0.45-0.88)	0.0071
Other variables		
MSI-high (vs. MSI-low/MSS)	0.28 (0.16-0.51)	<0.0001
High tumor grade (vs. low tumor grade)	3.51 (1.82-6.77)	0.0002
Age at diagnosis (for a 10-year increase)	0.96 (0.95-0.99)	0.0006
<i>KRAS</i> mutation	1.60 (1.12-2.29)	0.0096
Family history of colorectal cancer	0.62 (0.42-0.92)	0.017
<i>BRAF</i> mutation	1.76 (1.03-3.00)	0.038
TP53 expression	1.66 (1.01-2.76)	0.048

The multivariate logistic regression analysis assessing the relations with cancer stage (as an outcome variable) initially included p-AKT, age, sex, family history of colorectal cancer, body mass index, tumor location, tumor grade, *KRAS*, *BRAF*, *PIK3CA*, LINE-1 methylation, microsatellite instability (MSI), CpG island methylator phenotype, TP53, and FASN. A backward stepwise elimination with threshold of $p=0.20$ was used to select variables in the final model. Variables with $p<0.05$ are listed.

CI, confidence interval; MSI, microsatellite instability; MSS, microsatellite stable; OR, odds ratio; p-AKT, phosphorylated AKT.

p-AKT expression in colorectal cancer and patient mortality

Table 3

Total N	Colorectal cancer-specific mortality				Overall mortality			
	Deaths / person- years	Univariate HR (95% CI)	Stage- matched HR (95% CI)	Multivariate stage-matched HR (95% CI)	Deaths / person- years	Univariate HR (95% CI)	Stage- matched HR (95% CI)	Multivariate stage-matched HR (95% CI)
p-AKT (-) 269 (38%)	97/2056	1 (referent)	1 (referent)	1 (referent)	139/2056	1 (referent)	1 (referent)	1 (referent)
p-AKT (+) 448 (62%)	113/3962	0.62 (0.47-0.82)	0.72 (0.54-0.94)	0.75 (0.56-0.99)	202/3962	0.76 (0.61-0.95)	0.82 (0.66-1.03)	0.78 (0.62-0.98)
P value		0.0006	0.018	0.048		0.014	0.083	0.029

The multivariate, stage-matched conditional Cox regression model included age, year of diagnosis, sex, family history of colorectal cancer, body mass index, tumor location, grade, *KRAS*, *BRAF*, *PIK3CA*, *FASN*, *TP53*, LINE-1 methylation, microsatellite instability, CpG island methylator phenotype and p-AKT. A backward stepwise elimination with threshold of p=0.20 was used to select variables in the final model.

CI, confidence interval; HR, hazard ratio; p-AKT, phosphorylated AKT