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Expression of the Tumor Suppressor Krüppel-Like Factor 4 as a Prognostic Predictor for Colon Cancer

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Abstract

Background—The zinc finger transcription factor Krüppel-like factor 4 (KLF4) regulates numerous physiologic processes including proliferation, differentiation, and development. Studies also showed that KLF4 is involved in tumorigenesis and somatic cell reprogramming. Here we aimed to assess whether KLF4 is a prognostic indicator for colon cancer.

Methods—Levels of KLF4 were measured by immunohistochemical analysis of a tissue microarray containing 367 independent colon cancer sections. Univariate data analysis was performed in addition to construction of multivariate models with several clinicopathologic factors to evaluate KLF4 as an independent predictor of survival and cancer recurrence (disease-free survival).

Results—Colon cancer tissues had significantly overall lower KLF4 levels compared to non-cancer tissues ($P < 0.0001$). Using logistic regression, a trend was noted for decreased odds of KLF4 expression in higher stages of tumors. In both univariate and multivariate analyses, KLF4 was a significant predictor of survival and recurrence.

Conclusions—KLF4 expression is significantly down-regulated in colon cancer and loss of KLF4 is an independent predictor of survival and recurrence.

Impact—These findings suggest that KLF4 may serve as a prognostic biomarker for colon cancer.

Keywords

KLF4; Colon Cancer; Tissue microarray; Survival; Recurrence

INTRODUCTION

Despite substantial advances in the early diagnosis and treatment of colorectal cancer, it remains a disease with both high morbidity and mortality. Approximately 150,000 new cases of colon cancer were diagnosed last year in the United States, making it the fourth most common cancer diagnosed in men and women (1). It is also the second most common cause of cancer-related death despite a recent reduction in mortality due to increased screening and improvements in treatment for late-stage cancer (2). Studies have identified mutations in tumor suppressor genes and oncogenes that result in dysregulation of numerous pathways leading to colorectal carcinogenesis (3,4). Nonetheless, future research in colorectal cancer depends on

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the ability to conduct research relative to potential applications of available data, translate the mechanistic data into the clinical arena, and evaluate markers independent of established clinicopathologic predictors of the disease.

Biomarker research in colorectal cancer is becoming increasingly popular for a variety of clinical and research applications. Precise biomarkers may be useful as a surrogate endpoint in preliminary studies, for the stratification of patients in clinical trials, and in the refinement of disease prognosis. The debate surrounding the role of chemotherapuetics in American Joint Committee on Cancer (AJCC) stage II colon cancer provides an example of such an application. Within the past decade, a number of new systemic treatments for colon cancer, including oral fluropyrimidines, oxaliplatin, and irinotecan, have been shown to improve overall and disease-free survival in stage III cancer patients (5). Unlike patients with third stage cancer, current recommendations by the American Society of Clinical Oncology (ASCO) do not promote the routine use of chemotherapeutics in stage II cancer (6). Though no clinical trial has proven overall benefit of adjuvant therapy in stage II, it is hypothesized that up to twenty percent of patients with a risk of recurrence similar to that of stage III disease, would have benefited from chemotherapuetics (5). Currently, the ASCO suggests that patients with inadequately sampled nodes, T4 lesions, perforation, or poorly differentiated histology, should at least be considered for such treatment at the discretion of the treating physician and patient (6). However, no studies have evaluated the benefit of such a recommendation or whether the suggested criteria correctly predict recurrence risk. Biomarkers may prove especially useful in further stratifying stage II patients into those who have a high risk of recurrence and those who do not—the former group being more likely to benefit from chemotherapuetics. Several biomarkers such as S-phase fractions and vascular endothelial growth factor expression have been evaluated in this regard (7,8).

Krüppel-like factors (KLFs) are a family of evolutionarily conserved mammalian zinc finger transcription factors named for their homology with Krüppel, a *Drosophilia melanogaster* protein (9). KLFs are involved in a diverse array of fundamental biological processes including proliferation, differentiation, development, and apoptosis (10–12). Among the KLF family, the Krüppel-like factor 4 (KLF4; also called gut-enriched Krüppel-like factor or GKLF) was one of the first identified (13,14). In addition to regulating many important physiologic processes, KLF4 has been shown to play a role in pathological conditions such as cancer and inflammation (15–20). More recently, KLF4 was shown to play a crucial role in the reprogramming of somatic cells into induced pluripotent stem cells (21–25).

Expression of KLF4 is enriched in epithelial tissues including the gut (13,14). In the intestine, KLF4 is highly expressed in the postmitotic, terminally differentiated epithelial cells at the luminal surface (26,27). *In vitro*, KLF4 inhibits proliferation by blocking cell cycle progression at the G1/S boundary (28). In addition, KLF4 mediates the cell cycle checkpoint functions of the tumor suppressor p53 following DNA damage (29–31). Moreover, loss of expression of KLF4 due to several reasons including loss of heterozygosity, promoter hypermethylation, and loss-of-function mutations has been documented in a small set of colorectal cancer specimens (32). This tumor suppressive effect of KLF4 is supported by *in vivo* evidence in which mice heterozygous for *Klf4* manifest increased tumor burden when bred to the Apc^{Min} mice that are genetically predisposed to intestinal adenoma formation (17). From a mechanistic perspective, KLF4 inhibits Wnt signaling, a key pathway in colorectal carcinogenesis, by inhibiting the activity of β-catenin, a mediator of Wnt signaling (33).

Because of its putative tumor suppressor function, KLF4 may serve as a prognostic indicator for colon cancer. Here we measured KLF4 expression levels in a large cohort of primary colon cancer specimens in an attempt to correlate its expression with clinical parameters including survival and recurrence.

MATERIALS AND METHODS

Study Design

A retrospective case control study was conducted to evaluate the association between KLF4 and colon cancer while adjusting for a number of covariates. Tissue microarrays bearing a large number of colon cancer across various stages were analyzed by immunohistochemistry for KLF4. Each tissue section was processed in duplicate, as two-fold redundancy permits accurate analysis of protein expression (34).

The paraffin-fixed colon tissue microarray was constructed between 1989 and 1996 by the National Cancer Institute's Cancer Diagnosis Program of the National Cancer Institute (35). The microarray was assembled using 367 cores of colon cancer (49 stage I, 122 stage II, 144 stage III, and 52 stage IV), 37 cores of adenomatous polyps, 34 cores of normal colon tissues matched to tumor sections, and an additional 40 normal colon sections from individuals with diverticulosis. Of the colon cancer cases, five-year follow up data were complete on 96 stage II tumors (26 recurred) and 125 stage III tumors (65 recurred). Of all stage II and stage III tumors, 45 were censored before the five-year follow up was complete. Non-colon tissue cores were embedded on the microarrays for internal control of staining. In addition, cores from colon cancer cell lines were embedded as additional internal control. These cell lines were characterized and authenticated by the National Cancer Institute under ATCC guidelines (35). Of all patients, 418 were Caucasian, 12 were African American, and 11 identified with another race. Two hundred and seven subjects were male and 233 were female (gender of one individual was unknown). Mean age was 68.62 (standard deviation 12.48). Additional covariate data were collected on tumor depth, nodal status, metastasis, histology, location, and degree of dysplasia as assessed by an independent pathologist.

Selection of patients across various stages was done to ensure enough power to detect differences in recurrence within stage II and stage III cancer independently. The cases were chosen to detect a difference in the prevalence rate of 0.35 within stage II or stage III tumors that recurred and those that did not within the five year follow up period. In order to detect differences in a binary outcome marker across various stages of disease, enough stage I and stage IV tumors were also included so over 80% power was available to detect a 0.30 difference in prevalence rate of KLF4 (see Guidance for Statistical Analysis of Biomarker Data Generated from the NCI Colon Tissue Microarray; ref. 36).

Immunohistochemistry

The microarrays were treated with xylene for deparaffinization and rehydrated with ethanol. Endogenous peroxidase activity was blunted with 10% H_2O_2 in methanol. Antigen retrieval was performed using 10 mmol/L citrate buffer (pH=6.0) at 120°C for 15 m. The sections were then incubated for 1 h in blocking buffer (2% nonfat dry milk, 0.001% Tween 20, and 10% normal horse serum in phosphate-buffered saline). Vector Laboratories avidin/biotin blocking kit was used in conjunction with blocking buffer as directed by the manufacturer to reduce background and nonspecific secondary antibody binding. Sections were stained with KLF4 (goat anti-human KLF4; R and D Systems) at a dilution of 1:1,000 in blocking buffer for 1 h. Detection of primary antibody and color development was done using Biocare Medical Betazoid DAB development kit. Sections were counterstained with Mayer's hematoxylin (Invitrogen), dehydrated, and coverslipped. Images were acquired with an Axioskop 2 plus microscope (Zeiss) with an AxioCam MRc5 CCD camera (Zeiss).

Data Analysis

Images were graded by two investigators blinded to tissue stage as assessed by an independent pathologist and other covariate information. Tissues were graded either negative (< 10%

staining) or positive (≥ 10% staining) in a manner similar to previous reports (37). Intensity of nuclear KLF staining was compared between histopathological stages using the χ^2 test or Fisher's Exact Test where appropriate. A binary logistic model was created in order to assess the role of stage, age at diagnosis, race, and gender in determining odds ratios for KLF4. All two-way interaction terms were evaluated using a Wald test for inclusion into the model. At each step of modeling, the most insignificant interaction term was dropped, and the model was then evaluated. After a covariate was dropped, assessment as to whether previously dropped covariates could be reentered into the model was done. Once all covariates were evaluated, the final model was constructed.

Overall survival was defined as time from diagnosis to death of patients. Disease-free survival was defined by time between diagnosis of colon cancer and recurrence of disease. The association between KLF4 expression and survival was assessed by Kaplan-Meier survival analysis. Differences between curves were assessed using a log-rank test. In order to evaluate KLF4 expression as an independent prognostic factor for overall and disease-free survival, a Cox regression model was applied and hazard ratios were estimated. In a similar fashion to logistic model building, all possible two-way interaction terms were evaluated after adjustment of the model to fulfill the proportional hazards assumption. $P < 0.05$ was considered indicative of statistical significance. The statistical software package SAS 9.2 was used for statistical analysis and graphics.

RESULTS

Characteristics of the Study Population

The general characteristics of subjects enrolled in the study are shown in Table 1. The majority of participants were white for both cancer and non-cancer cases. Gender and age were relatively evenly distributed in the two groups of subjects. Table 2 shows the characteristics of tumor sections among AJCC stages. Lymph nodes were not examined in seven stage IV patients and status was unknown in one stage IV patient. Distal margins were involved in one stage I patient, otherwise no enrolled patients had either proximal or distal margin involvement. In five stage IV patients, margins could not be assessed.

Univariate Associations of KLF4

Figure 1 shows a representative example of KLF4 immunostaining of the colon cancer tissue microarray. To assess the relationship of certain covariates with KLF4, a univariate analysis was first undertaken (Table 3). Cancer tissues had significantly less overall KLF4 expression in comparison to non-cancer tissues ($P < 0.0001$). The proportions of KLF4-positive tumors were significantly different among men and women ($P = 0.0432$). Proportions of KLF4-positive and KLF4-negative tumors were also significantly different among stage I ($P = 0.0341$) and stage III ($P = 0.0438$) tumors. In contrast, no significant difference was noted among age or race groups or among stage II and stage IV tumors.

Multivariate Associations of KLF4

A multivariate logistic model was created in order to assess the relationship between covariates independent of possible confounders and KLF4. All possible two-way interaction terms were tested, and, in concert with results from the univariate analysis, none were found to significantly contribute to the model. As such, the final model accounts for age at diagnosis, gender, race, and stage as possible predictors for KLF4 status (Table 4). Among all possible predictors, only stage III, as compared to stage I in the odds ratio, is statistically significant ($P = 0.0211$). However, a trend, though not significant, showing a decreased odds ratio at higher stages with reference to stage I cancer is evident.

Survival Analyses

A Kaplan-Meier curve representing univariate survival analysis is shown in Figure 2. For all included participants, overall survival was significantly better for individuals that retained KLF4 expression as compared to those that did not ($P = 0.0437$). Stage-specific survival curves demonstrate no independent differences in survival between individuals with KLF4-positive and KLF4-negative tumors (not shown). Kaplan-Meier curves were also constructed to determine difference in recurrence between KLF4-positive and KLF4-negative tumors. Figure 3 shows overall time to recurrence, or disease-free survival, is significantly greater in KLF4-positive patients ($P = 0.0001$). Stage-specific curves (not shown) show only a significant difference among stage III tumors ($P = 0.0046$), where in KLF4-positive tumors have significantly improved disease-free survival.

Finally, a Cox model for survival was created that included stage, age, race and gender, and all possible two-way interactions among the factors as possible confounders in addition to KLF4 status. In this model, KLF4 is a significant predicator for both overall survival ($P = 0.0427$) and disease-free survival ($P = 0.0486$).

DISCUSSION

Krüppel-like factors, and notably KLF4, are important regulators of the intestinal epithelial cell homeostasis and tumorigenesis (27,38). However, the clinical utility of KLF4 as a prognostic marker in colon cancer has not been established. In this study using tissue microarrays from a large cohort of colon cancer cases, we convincingly demonstrated that KLF4 expression level is significantly down-regulated in cancer and that loss of KLF4 is an independent predictor of survival and disease recurrence.

We first demonstrated that KLF4 is grossly reduced in histological colon cancer sections as compared to normal colonic sections. This is consistent with the brunt of data from our lab and others that suggests KLF4 is a putative tumor suppressor (17,32,39). One study showed that in a set of 30 colorectal cancer sections, *KLF4* mRNA transcripts were reduced by 50% compared to matched normal tissue (32). This reduction paralleled the reduction in $p21^{Waf1/Cip1}$, a cell cycle inhibitor gene, suggesting that the reduction in the latter may be a direct consequence of loss of *KLF4* expression (32). This is loss of KLF4 expression is thought in part due to loss of heterozygosity of the *KLF4* loci and promoter hypermethylation (32). In the current study, we provided corroborative evidence that the level of KLF4 protein is significantly reduced in colon cancer specimens as detected by immunohistochemistry. This result is consistent with that of a similar study showing loss of KLF4 protein expression in colorectal cancer (39), although in contrast to our study, that study failed to demonstrate a statistically significant correlation between KLF4 expression and several clinicopathologic parameters including stage and lymph node metastasis. The reason behind this difference is unclear although it could be due to the different sample size, statistical methodology or criteria by which KLF4 positivity was assessed between the two studies.

In addition to colon cancer, decreased KLF4 expression has been observed in cancer of the stomach, esophagus, bladder, lung, and T cell leukemia (40–44). In addition, KLF4 is reduced in precancerous lesions, such as adenomas of the colon (45,46). In a model of familial adenomatous polyposis syndrome, the Apc^{Min} mouse, expression of KLF4 is lower in intestinal adenomas when compared to normal-appearing mucosal tissues (45). Conversely, the burden of intestinal adenomas is significantly increased when Apc^{Min} mice are bred with mice heterozygous for the *Klf4* alleles (17). These results are consistent with a tumor suppressive function of KLF4, at least in the intestine. However, whether KLF4 serves as a prognostic marker in colon cancer has not been determined.

In order to evaluate which clinical covariates are associated with expression of KLF4, we performed a univariate analysis as well as building a multivariate logistic model. In the univariate analysis, gender, but not age or race, was associated with KLF4 status in that men had higher proportions of KLF4-postivite tumors than women (Table 3). A similar trend, although not statistically significant, was noted for gender in a multivariate analysis taking into consideration all available covariates (Table 4). The reason for the potential association of KLF4 expression with gender is unclear. KLF4 expression has previously been associated with prognosis of early-stage breast cancer but not with the status of either estrogen or progesterone receptors (35). The estrogen and progesterone status in the colon cancer of the current study was not assessed.

Also in the multivariate model, a trend was noted in which higher stages of diseases had decreased odds of KLF4 expression relative to stage I colon cancer (Table 4). This suggests a possible role of KLF4 as a marker of stage prognosis in future studies, if it were in fact associated with tumor progression. This would be consistent with observations from the RKO colon cancer cell line, in which KLF4 loss due to hemizygous deletion of KLF4 contributes to aggression, which is suppressed with re-expression of KLF4 (47). Perhaps the true potential of KLF4 as a prognostic marker may be seen when combined with other biomarkers of disease, providing a refined model with which to evaluate patients.

The most important aspect of the current study is that KLF4 represents a significant predictor of overall survival and disease-free survival by Kaplan-Meir analysis (Figures 2 and 3, respectively). This is different from the only previously published study on KLF4 as a prognostic factor, wherein a trend, though not significant, was noted for improved, unadjusted survival only among stage III cancer patients (20). In our Kaplan-Meier analysis, we were not able to find significant survival benefits in any particular stage of disease with the exception of disease-free survival in stage III disease. However, given the much larger combined sample size, we were able to demonstrate a significant benefit in survival due to KLF4. The association of KLF4 expression with disease-free survival of stage III cancer patients is consistent with the trend noted in the previous study (20) and is consistent with the results of multivariate analysis showing a significant association of KLF4 expression with stage III relative to stage I disease (Table 4). Although speculative, these data suggest that KLF4 has a particularly important function in tumor suppression during the transition from local to metastatic colon cancer. Most importantly, inclusion of age, gender, race, and stage, as well as appropriate extension and interaction terms indicated that KLF4 status is a significant predicator for survival and recurrence. Therefore, we conclude that KLF4 is an independent predictor of survival in colon cancer.

Our data served to further corroborate previous studies indicating that KLF4 has a role in tumor suppression in colon cancer. Importantly, our study established that KLF4 as a novel prognostic marker for colon cancer survival, particularly in stage III disease and suggests that KLF4 could become a potential clinical marker for risk stratification and response to therapy. However, due to the retrospective nature of this investigation, reproduction of the results in prospective studies, ideally with techniques more suitable for clinical laboratories, is probably warranted to assess whether KLF4 could be an effective clinical tool in colon cancer.

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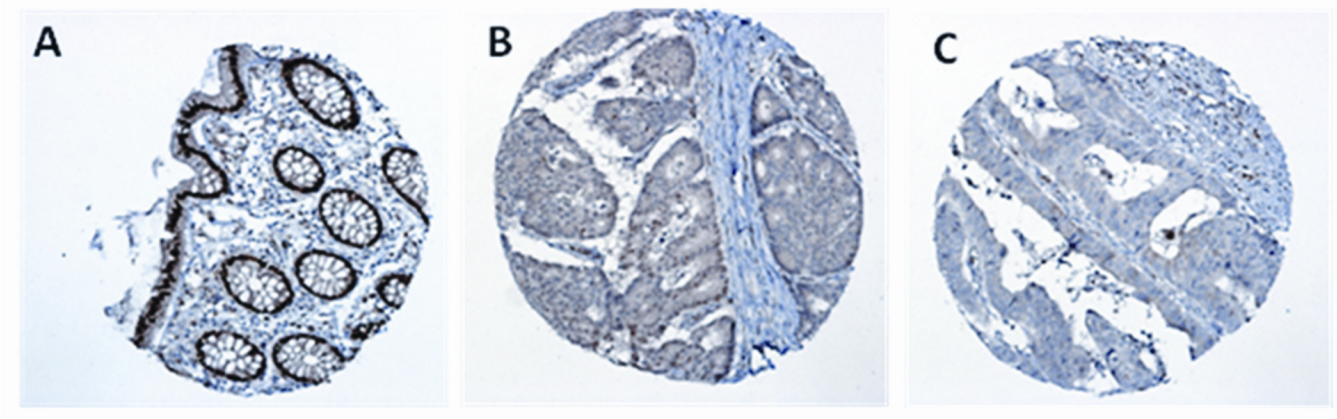

Figure 1. Representative samples of KLF4 staining

Shown is a representative example of KLF4 staining in the tissue microarray of (A) normal colon, (B) colon cancer with positive KLF4 staining, and (C) colon cancer with negative KLF4 staining.

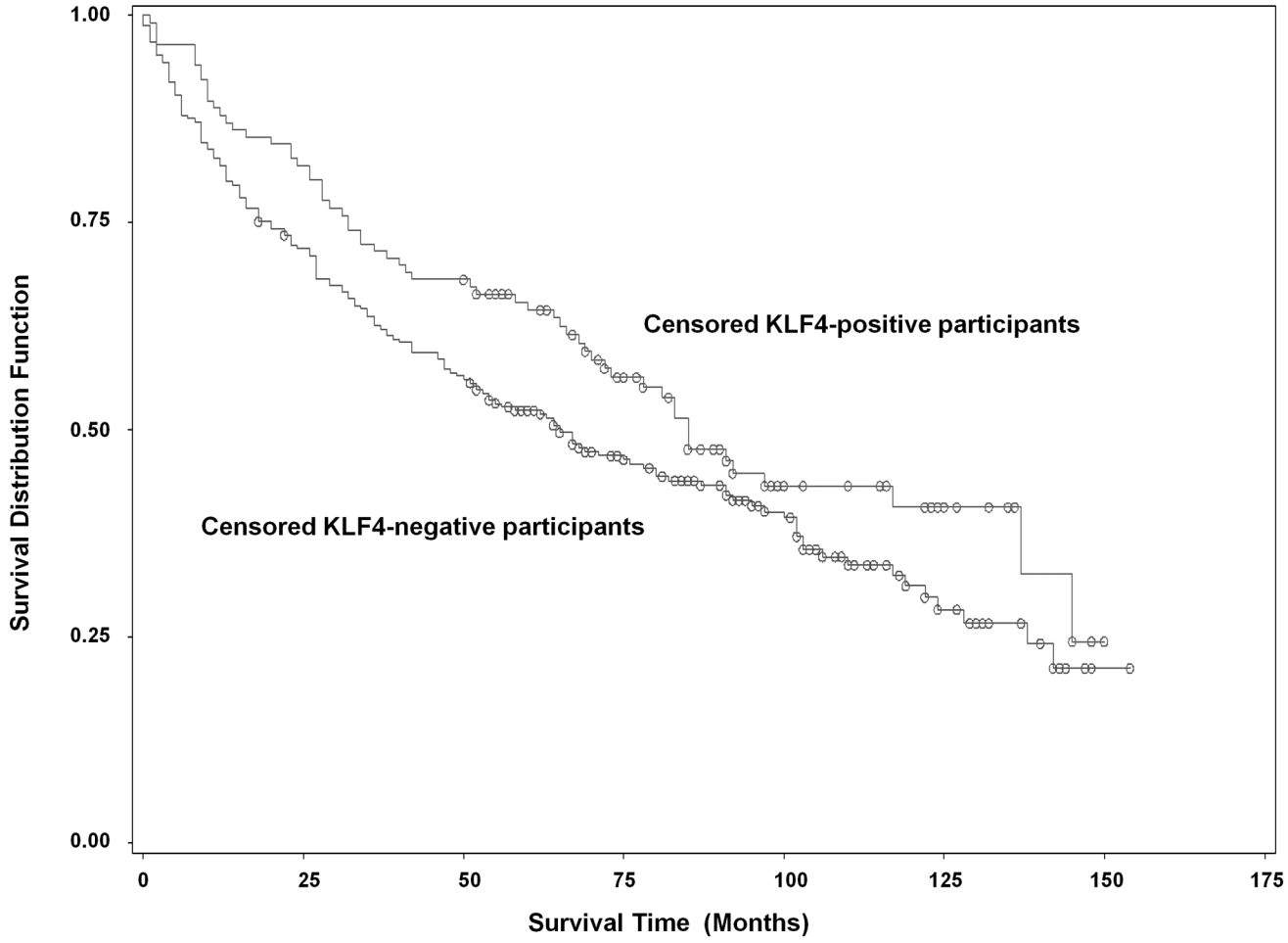

Figure 2. Overall Kaplan-Meier survival curve with all patients

Shown is the Kaplan-Meier curve for all available patients. The log rank statistic for the curve was 4.0697 with 1 degree of freedom, yielding a significant *P* value of 0.0437.

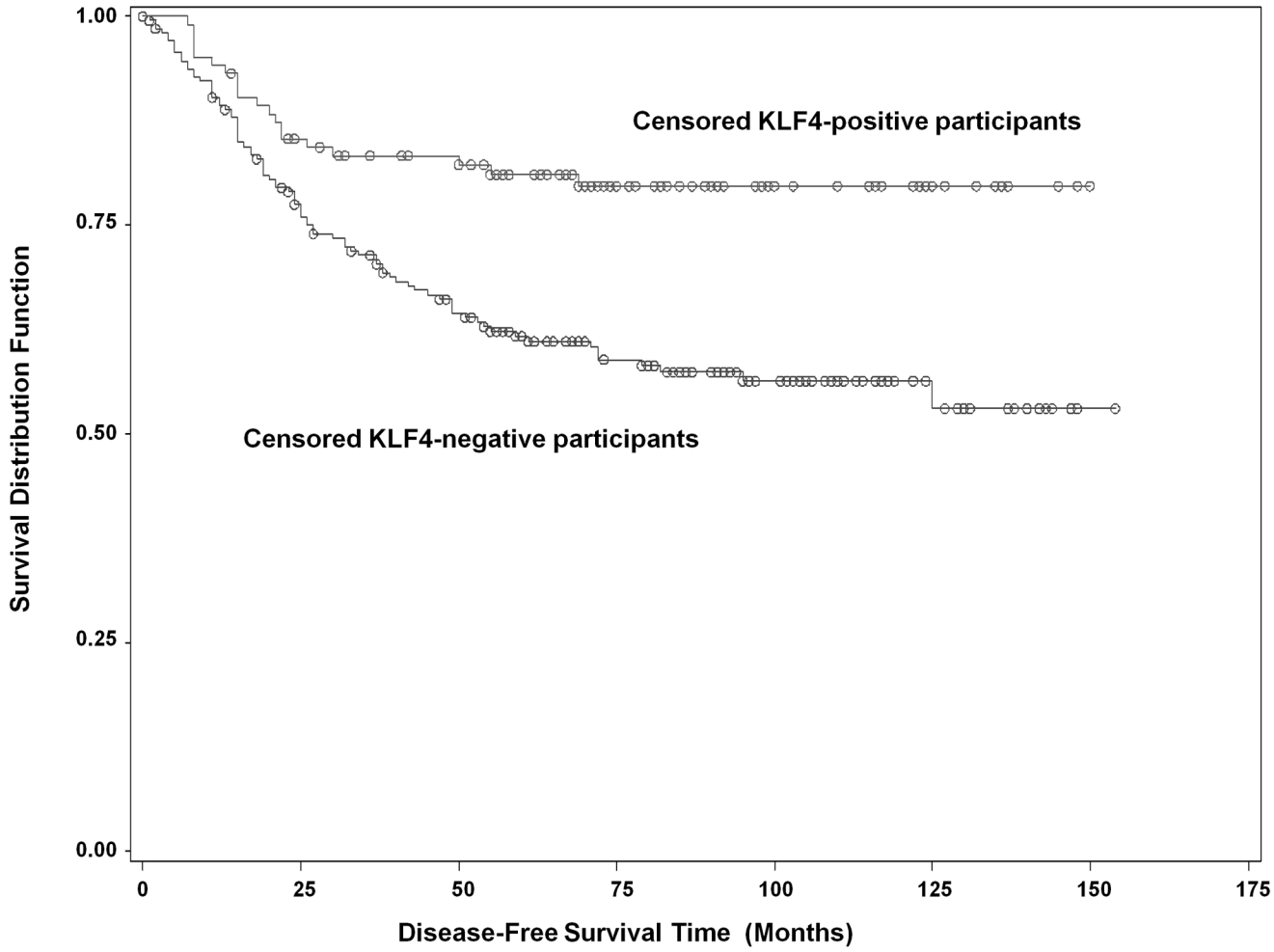

Figure 3. Overall Kaplan-Meier disease-free survival curve with all patients

Show is the Kaplan-Meier disease-free curve for all available patients. The log rank statistic for the curve was 14.9437 with 1 degree of freedom, yielding a significant *P* value of 0.0001.

Table 1

Characteristics of study subjects.

	Status	
	Normal	**Cancer**
Race		
White	69 (93.24%)	349 (95.10%)
Black	3 (4.05%)	9 (2.45%)
Other	2 (2.7%)	9 (2.45%)
Gender		
Male	32 (43.24%)	175 (47.68%)
Female	41 (55.41%)	192 (52.32%)
Age*		
<70 years old	43 (58.11%)	173 (47.14%)
>70 years old	31 (41.89%)	194 (52.86%)

* When taken as a continuous variable, mean age among the non-cancer participants was 64.46 with a standard deviation of 14.17. Mean age among cancer participants was 69.69 with a standard deviation of 12.00.

Table 2

Characteristics of colon cancers included in the tissue microarray.

	AJCC Summary Stage			
	I	**II**	**III**	**IV**
Nodes Positive				
< 1	49	122	0	5
1–3	0	0	85	9
≥ 3	0	0	59	30
No nodes examined	0	0	0	7
Unknown	0	0	0	1
Nodes Examined				
< 8	21	28	29	11
8–12	10	31	36	13
12–16	9	31	33	11
> 16	9	32	45	16
No nodes examined	0	0	0	7
Unknown	0	0	1	1
Proximal Margin Involvement				
Involved	0	0	0	0
Uninvolved	49	122	144	47
Cannot be assessed	0	0	0	5
Distal Margin Involvement				
Involved	1	0	0	0
Uninvolved	48	122	144	47
Cannot be assessed	0	0	0	5
Location				
Ascending Colon	4	19	21	7
Hepatic Flexure	2	8	10	2
Transverse Colon	3	18	10	4
Splenic Flexure	1	5	7	1
Descending Colon	0	7	4	0
Rectosigmoid Junction	0	2	2	1
Cecum	11	28	32	14
Appendix	0	0	0	2
Sigmoid Colon	28	35	58	20
Colon, Not Specified	0	0	0	1
Blood/Lymphatic Vessel Invasion				
Intramural	1	1	12	4

	AJCC Summary Stage			
	I	II	III	IV
Extramural	0	4	13	2
Absent	48	116	119	42

Characteristics of tumors included in the tissue microarray are noted as distinguished by American Joint Committee on Cancer (AJCC) summary stage. Definitions of stages with reference to TMN and Duke's stages are as follows (T refers to tumor depth, N to number of nodes, and M to number of metastases):

Stage I: T1–T2, N0, M0; Dukes A

Stage II: T3–T4, N0, M0; Dukes B

Stage III: Any T, N1–N2, M0; Dukes C

Stage IV: Any T, Any N, M1; Dukes C

Table 3

Univariate measures of covariates among colon cancer patients.

Variable	KLF4-Negative		KLF4-Positive		Missing	χ^2 Statistic	*P* Value
	Number	%	Number	%			
Age							
>70 years	128	48.59	65	56.03			
<70 years	121	51.41	51	43.97	2	0.6805	0.4094
Race							
White	236	94.78	111	95.69			
Non-White	13	5.22	5	4.31	2	0.1399	0.7083
Gender							
Male	122	49	70	60.34			
Female	127	51	46	39.66	2	4.0800	0.0432
Stage							
Stage I	27	10.84	22	18.97	2	4.4917	0.0341
Stage II	78	31.33	44	37.93	2	1.5518	0.2129
Stage III	107	42.97	37	31.90	2	4.0636	0.0438
Stage IV	37	14.86	13	11.21	2	0.8930	0.3447

The table shows the frequency of KLF4-positive and -negative tumors under specific covariates. The univariate association between cancer and normal tissues for KLF4 expression resulted in a *P* value of < 0.0001 (χ^2 statistic of 279.4290).

Table 4

Multivariate analysis of KLF4 using a binary logistic model including all available covariates.

Variable	Estimated Coefficient	Estimated Standard Error	Wald Chi Squared Statistic	*P* Value	Estimated Odds Ratio	Confidence Interval on Odds Ratio
Age at diagnosis	0.00847	0.00982	0.7456	0.3879	1.009	(0.989, 1.028)
Gender	0.4384	0.2328	3.5458	0.0597	1.550	(0.982, 2.447)
Race	0.1780	0.5569	0.1021	0.7493	1.195	(0.401, 3.559)
Stage II*	−0.3522	0.3466	1.0325	0.3096	0.703	(0.356, 1.387)
Stage III*	−0.8049	0.3490	5.3187	0.0211	0.447	(0.226, 0.886)
Stage IV*	−0.8162	0.4376	3.4791	0.0621	0.442	(0.188, 1.042)

*Stage II–IV run with Stage I set as reference.