In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Nilesh V. Patel Date

Krüppel-Like Factor 4 and Colon Cancer

By

Nilesh Patel
Master of Science

Clinical Research

_____

Vincent Yang, MD PhD
Advisor

_____

John Boring, MD
Committee Member

_____

Tom Zeigler, MD
Committee Member

_____

John McGowan, MD
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the Graduate School

_____

Date

Krüppel-Like Factor 4 and Colon Cancer

By

Nilesh Patel

AB, University of Chicago, 2005

Advisor: Vincent Yang, MD PhD

An abstract of

A thesis submitted to the Faculty of the Graduate School of Emory University

in partial fulfillment of the requirements for the degree of Master of Science

in Clinical Research

2010

ABSTRACT

Krüppel-Like Factor 4 and Colon Cancer
By Nilesh Patel

***Purpose*:** The zinc finger transcription factor Krüppel-Like Factor 4 (KLF4) has critical roles in both normal intestinal development and intestinal carcinogenesis. Various studies have investigated its role in *wnt* signaling, cell cycle progression, apoptosis, and cell to cell adhesion. To further elucidate its role, we have correlated the expression pattern of KLF4 with various clinical outcomes.

Experimental Design: We employed a tissue microarray consisting of 367 independent colon cancer sections to investigate KLF4 in human tissues. Univariate KLF4 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.

***Results*:** Cancer tissues had significantly less KLF4 expression overall in comparison to non-cancer tissues ($p<0.0001$). Using logistic regression, a trend, though not significant, was noted such that higher stages of cancer had decreased odds of KLF4 expression. In univariate survival and recurrent analysis, KLF4 was a significant predictor of survival and recurrence. This relationship, was not, however, independent of other covariates.

***Conclusions*:** KLF4 expression is significantly down-regulated in intestinal tumors, but loss of KLF4 is not an independent predictor of survival or recurrence.

Krüppel-Like Factor 4 and Colon Cancer

By

Nilesh Patel

AB, University of Chicago, 2005

Advisor: Vincent Yang, MD PhD

A thesis submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Master of Science in Clinical Research

2010

ACKNOWLEDGEMENTS

I would like to express my gratitude for the many individuals who have helped me complete the study and research involved in this project. Foremost, I wish to sincerely thank Dr. Vincent Yang and the members of his lab, Mandayam Nandan, Amr Ghaleb, James Du, Agnieszka Bialkowska, Engda Hagos, Beth McConnell, and Bing Yu, for their patience, guidance, and support that allowed me to carry out this research. I am also grateful to the faculty and Executive Committee of the Master of Science in Clinical Research for their dedicated teaching throughout the program. I also would like to thank the Atlanta Clinical and Translational Science Institute, the National Institutes of Health, and the National Cancer Institute's Cancer Diagnosis Program for the support of this project.

TABLE OF CONTENTS

Introduction ……..... 1

Background ……..... 3

Methods ……..... 8

Results ……..... 12

Discussion ……..... 15

Tables and Figures ……..... 18

Table 1a: Characteristics of participants…….. 18
Table 1b: General characteristics of tumors included in the tissue microarray…..... 19
Table 2a: Univariate measures of covariates among cancer patients……..... 20
Table 2b: Univariate measures of covariates among stage I cancer patients……..... 20
Table 2c: Univariate measures of covariates among stage II cancer patients…… 21
Table 2d: Univariate measures of covariates among stage III cancer patients…..... 21
Table 2e: Univariate measures of covariates among stage IV cancer patients……..... 22
Table 2f: Assessment of interaction of covariates in determining the odds ratio for KLF4 given age (>70/<70)……..……..... 23
Table 2g: Assessment of interaction of covariates in determining the odds ratio for KLF4 given race (white/non-white)……..... 23
Table 2h: Assessment of interaction of covariates in determining the odds ratio for KLF4 given gender (male/female)…… 23
Table 3a: Multivariate analysis of KLF4 using a binary logistic model including all available covariates...……...…… 24
Table 3b: Multivariate analysis of KLF4 using a binary logistic model including all possible two way interaction terms. ...…….. 25
Figure 1: Representative samples of KLF4 staining……..... 26
Figure 2: Overall Kaplan-Meier survival curve with all patients……..... 27
Figure 3a: Kaplan-Meier survival curve among stage I patients..…… 28
Figure 3b: Kaplan-Meier survival curve among stage II patients…… 29
Figure 3c: Kaplan-Meier survival curve among stage III patients……..... 30
Figure 3d: Kaplan-Meier survival curve among stage IV patients…….. 31
Figure 4: Overall Kaplan-Meier disease-free survival curve with all patients…… 32
Figure 5a: Kaplan-Meier diseases free-survival curve among stage I patients…… 33
Figure 5b: Kaplan-Meier diseases free-survival curve among stage II patients…….. 34
Figure 5c: Kaplan-Meier diseases free-survival curve among stage III patients…… 35
Table 4a: Cox proportional hazards model for overall survival, without adjustment…..... 36
Table 4b: Final extended Cox model for overall survival stratified by stage…….. 36
Table 5a: Cox proportional hazards model for disease free-survival, without adjustment…….. 37
Table 5b: Final extended Cox model for disease free-survival……...…… 37

References ……..... 38

INTRODUCTION

Despite substantial advances into early diagnosis and treatment of colon cancer, it remains a disease with both a high morbidity and mortality. Over a hundred thousand new cases of colon cancer were estimated last year, making it the third most common cancer diagnosed in men and women (1). It remains the second most common cause of cancer related death in the United States despite improving mortality attributed to increased screening for resectable, early stage cancer and recent improvements in treatment for later stage cancer. Studies have implicated dysregulation of multiple tumor suppressors and oncogenes as catalysts of carcionogenesis. Nonetheless, the utility of research in colorectal cancer depends on the ability to conduct research relative to potential applications of this data, translate this mechanistic data into the clinical arena, and evaluate markers independent of already known clinicopathologic predictors of disease.

Biomarker research in colorectal cancer is becoming increasingly popular for a variety of clinical and research applications. Fairly 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 controversy surrounding the role of chemotherapuetics in American Joint Committee on Cancer (AJCC) stage II cancer provides one 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 (2). 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 (3). 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 (2). 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 (3). 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. S-phase fractions and vascular endothelial growth factor expression are being evaluated in this regard (4-5).

Kruppel-like factor 4 (KLF4) stands as a prime candidate for further translational research. A dearth of mechanistic data has determined its importance at various stages of normal intestinal development as well as its possible role in carcinogenesis. In order to determine the use as a biomarker, we have correlated KLF4 with survival, recurrence, and stage both alone and in the context of possible confounding clinicopathologic variables. We paid particular attention to the role of KLF4 in stage II and stage III cancer.

In order to accomplish this, we used tissue microarray (TMA) technologies. Recent advances have allowed for successful attachment, storage, and immunostaining of multiple tissue sections a few millimeters on a few TMAs. This allows each slide to contain dozens if not hundreds of samples, in which to apply standardized and rapid screening process to.

BACKGROUND

Krüppel-like factors (KLFs) are a family of evolutionarily conserved zinc finger transcription factors named for their homology with Krüppel, a *Drosophilia melanogaster* protein. Krüppel itself is important in the development of continuguous embryonal segments of *Drosophilia*, wherein deletion of the gene leads to absence of the thoracic and anterior abdominal segments, or the "crippled" phenotype (6). As a family, KLFs are involved in a diverse array of fundamental biologic processes. All KLFs bind a similar "CACCC elements," an affinity they share with the Sp1family of zinc finger proteins, though the two families are structurally distinguished by additional conserved residues between each zinc finger (7). Within the KLF family itself, the functions of at least seventeen identified factors are distinguished from one another by unique amino termini and the context of the tissue in which they are expressed (7). Together, they have been linked to cellular development, differentiation, proliferation, and apoptosis in a variety of tissues. Of particular note within the intestinal epithelium is expression of Krüppel-like factor 4 (KLF4), which has been shown to inhibit proliferation and promote differentiation.

Roles of KLF4 in Intestinal Homeostasis

The colonic epithelium is organized such that proliferating cells reside at the base of a crypt and migrate toward the lumen as they differentiate. Consistent with its role, KLF4 is highly expressed in the postmitotic terminally differentiated epithelial cells at the luminal surface (9). Its role in normal intestinal development lies, in part, to its interactions with the *wnt* signaling pathway. The *wnt* singalling pathway is an essential regulator of embryogenesis, where in stimulation of the pathway leads to activation of

genes responsible for progression through the cell cycle. The regulation of this pathway is dependent on APC, a member of a complex of proteins that inhibits the *wnt* signaling pathway. KLF4 expression parallels that of APC, and, in the HT29 cell line, activation of APC leads to induction of KLF4 (11). Furthermore, KLF4 directly interacts with the downstream mediator of *wnt* signaling, beta-catenin, in order to inhibit signaling (8).

In effect, KLF4 serves to prohibit growth if fully expressed by negatively regulating the *wnt* pathway. This growth prohibiting effect of KLF4 can be readily seen in the NIH3T3 cell line, which when actively proliferating have a low KLF4 expression. The same line markedly increases KLF4 expression when growth is arrested by serum starvation of contact inhibition (13).

Another member of the Kruppel family, Kruppel like factor 5 (KLF5) serves a contrasting function. In fact, KLF5 directly inhibits the transcription of KLF4 by competing for the same promoter with which KLF4 positively feeds back on itself (15). As histiologic proof of this distinction, KLF5 is expressed in actively dividing cells that the base of intestinal crypts. It is also a positively regulated target of the *wnt* signaling pathway.

Gene microarrays have further confirmed that KLF4 activates many negative regulators of the cell cycle (9). The primary mechanism of KLF4's inhibition of the cell cycle is thought to be through $p21^{Cip1/Waf1}$, which is elevated when KLF4 is induced in the RKO cell line and subsequent to DNA damage in cell cultures (10-11). $p21^{Cip1/Waf1}$ is known to suppress both G1 to S and G2 to M cell cycle transition checkpoints (16).

Furthermore, KLF4 directly inhibits cell cycle promoters cyclin D1 and cyclin B1 (12-13).

During normal embryogenesis, the role of KLF4 is evident. KLF4 expression is acquired just prior to birth and is a late step in epidermal differentiation. It is vital in the formation of the lipid barrier that protects animals against infection and dehydration. KLF4 knockout mice have a defective barrier function of skin leading to a loss of body fluids and rapid postnatal lethality (14). Specifically in the intestinal epithelium, Laminin-1 a basement membrane component, and alkaline phosphatase, a marker of differentiated enterocytes, are known transcriptional targets of KLF4 (15). In vivo, KLF4 Is likely responsible for goblet cell differentiation, as evidenced by KLF4 knockout mice having 90 percent depletion in goblet cells in the colon and only patchy expression of goblet cell marker MUC2 (16).

Another role for KLF4 is the regulation of apoptosis. Interestingly, in colorectal cancer cells and mouse embryo fibroblasts, gamma irradiated cells only undergo apoptosis if KLF4 is absent (17). If KLF4 is present, the degree of apoptosis is significantly decreased. KLF4 accomplishes this regulation of apoptosis via its inhibition of the *Bax* promoter or by directly acting on the p53 promoter (18-20). Restoration of KLF4 in MDA-MB-134 cells using KLF4 siRNA results in restoration of p53 levels and an abrupt increase in apoptosis (19).

Further supporting KLF4's role in differentiation is its function in inducement of pluripotent stem cells from mouse fibroblasts with *Oct3*/4, *Sox2*, and *c-Myc* (20). One hypothesis postulates that c-*Myc* and KLF4 together are responsible for increased

proliferative capacity on potential pluripotent cells (21). However, this role of KLF4 remains controversial, with a recent study that substituted Nanog and LIN28 for c-*Myc* and KLF4 still produced pluripotent cells (22). Thus, KLF4 may only act as a catalyst for stem cell formation rather than having a required role. Nonetheless, given its significance in development and its relationships with known growth regulators, KLF4 has become increasingly important in cancer research.

Roles of KLF4 in Cancer: An Ideal Tumor Suppressor and a Surprising Oncogene

Given KLF4's role in regulating the cell cycle, it is logical to hypothesize a role of KLF4 as a tumor suppressor. In fact, decreased KLF expression has been reported in stomach, esophagus, and bladder cancer animal models (23,24,25). In a small number of case studies, KLF4 transcription and expression patterns in human tissues have been shown to be reduced in human esophageal squamous cell carcinoma, lung cancer, and adult T-cell leukemia (26-27, 30).

The $APC^{min/+}$ mouse develops numerous intestinal adenomas early in life, and is widely used as a model of intestinal tumorigenesis. In these adenomas, KLF4 is downregulated, with the degree of downregulation being proportional to the adenoma's size (28). This is consistent with the fact that KLF4 parallels expression of APC in normal tissue. As the APC protein is truncated in $APC^{min/+}$, the Wnt pathway, and subsequently β-catenin, is left unregulated. Interestingly, KLF4 can interact with β-catenin to inhibit tumor growth in tumor xenografts (11). Furthermore, $APC^{min/+}$ crossed with $KLF4^{+/-}$ mice result in significantly more adenomas than $APC^{min/+}$ $KLF4^{+/+}$ mice (29). Notably, this phenotype was similar to that $APC^{min/+}/TCF1^{-/-}$ mice. TCF1 also inhibits

Wnt/β-catenin signaling, suggesting KLF4 expression during tumorogenesis may be restoration Wnt signaling (29).

Surprisingly, contradictory evidence supporting an oncogenic role of KLF4 has been reported in oropharyngeal cancers and mammary carcinomas (30-31). KLF4 has also been identified as a factor responsible for transforming E1A-immortalized rat kidney epithelial cells, which can produce tumors in xenografted mice (36). This oncogenic potential of KLF4 may lie in its ability to inhibit apoptosis via suppression of *p53* and downregulation of the *Bax* promoter. The depletion of KLF4 from breast cancer cells, indeed, restores *p53* function and thereby *p53* mediated apoptosis (21). This oncogenic ability thus requires a milieu in which KLF4 is induced but its $p21^{Cip1/WAF1}$ dependent tumor suppressor activity is suppressed. In the Ras^{v12} oncogenic mutants, where $p21^{Cip1/WAF1}$ expression is inhibited, KLF4 anti-apoptotic function through suppression of *p53* predominates, resulting in repression of apoptosis (21). This contrasting role of KLF4 raises a number of hypotheses as to what role KLF4 may play in carcinogenesis within the gastrointestinal tract.

METHODS

Hypotheses

We have addressed two general hypotheses with this study. The first concerns the research question of how KLF4 is expressed in human tissues. Our null hypothesis in this case is that the expression of KLF4 at various stages of cancer is equivalent to that of normal tissue. The second concerns the clinical concerns of KLF4. Our null in this case states that KLF4 positive patients do not differ from KLF4 negative patients in terms of overall survival and recurrence. We have used several univariate and multivariate models to address these hypotheses.

Study Design

A retrospective case control study was conducted to evaluate the association between KLF4 and cancer while adjusting for a number of covariates. In order to assess a large number of colon cancer cores across various stages of cancer, a tissue microarray will be utilized. These tissue microarrays were assembled by taking 0.6 mm in diameter core needle biopsies of various tissues and embedding them into a single paraffin block. Sections of this block were then mounted onto slides for immunohistochemistry. As a result, hundreds of specimens were available to evaluate, all of which were processed at one time using identical conditions. Each slide was processed in duplicate, as two-fold redundancy permits accurate analysis of protein expression (32).

The paraffin fixed colon tissue microarray used was constructed between 1989 and 1996 by the National Cancer Institute's Cancer Diagnosis Program. The microarray was assembled using cores 367 colon tumors (49 stage 1, 122 stage 2, 144 stage 3, and 52

stage 4), 37 cores from adenomatous polyps, 34 cores of normal colon tissue matched to tumor sections, and an additional 40 normal colon sections from individuals with diverticulosis. Of the colon tumor cores, 5 year follow up data was complete on 96 stage 2 tumors (26 recurred) and 125 stage 3 tumors (65 recurred). Of all stage 2 and stage 3 tumors, 45 were censored before 5 year follow up was complete. Colon cancer cell-line and non-colon tissue cores were also embedded on the microarrays for internal control of staining. Of all patients, 418 were Caucasian, 12 were African American, and 11 identified with another race, and race was unknown from 37 individuals. 224 subjects were male and 253 were female (gender of one individual was unknown). Mean age was 68.62 (standard deviation 12.48, median age 70). Additional covariate data was 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 2 and stage 3 cancer independently. The cases were chose to detect a difference in the prevalence rate of 0.35 within stage 2 or stage 3 tumors that recurred and those that did not within the 5 year follow up period. In order to detect differences in a binary outcome marker across various stages of disease, enough stage 1 and stage 4 tumors were also included so over 80% power was available to detect a 0.30 difference in prevalence rate of KLF4 (33).

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 minutes. The sections were then incubated for 1 hour in blocking buffer (2% nonfat dry milk, 0.001% Tween 20, and 10% normal horse serum in PBS). Vector Laboratories avidin/biotin blocking kit was used in conjunction with blocking buffer as directed by manufacturer to reduce background and nonspecific secondary antibody binding. Sections were stained with KLF4 (goat anti-human KLF4 from R and D systems) at a dilution of 1:1000 in blocking buffer for 1 hour. 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).

Analysis

Images were graded by an investigator 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). Intensity of 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.

Disease free survival was defined by time between diagnosis of colorectal cancer and recurrence of disease. Overall survival was defined as time from diagnosis to death of patients. 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

General Characteristics of Study Population

General characteristics of participants enrolled in the study are reported in Table 1a. Gender and age (Mean 69.69, Standard deviation 12.00) were fairly evenly distributed among non-cancer participants, and the majority of participants were white (93.24%). Among participants with cancer, the majority was again white (95.10%) with generally even distributions of gender and age (Mean 64.46, Standard deviation 14.17). Table 1b shows the characteristics of tumor sections between AJCC stages. Lymph nodes were not examined in 7 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 5 stage IV patients, margins could not be assessed.

Univariate Associations of KLF4

Cancer tissues had significantly less KLF4 expression overall in comparison to non-cancer tissues ($p<0.0001$). In order to assess the relationship of certain covariates with KLF4, a univariate analysis was first undertaken. Table 2a show the frequencies of KLF4 positive and negative tumors within subsets of interest. The proportions of KLF4 positive tumors were significantly different among men and women ($p=0.0432$). Proportions of KLF4 positive tumors were also significantly different among stage I ($p=0.0341$) and stage III ($p=0.0438$) tumors. However, no significant difference was noted among age or race groups or among stage II and stage IV tumors. Tables 2b through 2e evaluate frequencies among individual stages of cancer. The sole significant

result is a difference in proportions among males and females in stage II cancer patients (p=0.0190).

Tables 2f through 2h are attempts to ascertain multiplicative interaction between covariates in determining odds ratios for KLF4. Table 2d evaluates the whether the odds ratio for age (greater than 70/less than 70) is dependent on any of the other covariates included in the analysis. Table 2e similarly evaluates the odds ratio for race (white/non-white), and table 2f evaluates the odds ratio for gender (male/female) with respect to other included covariates. In all cases, the Breslow-Day Test did not yield significant results.

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 (Table 3b) and, in concert with results from the univariate analysis, none were found to significantly contribute to the model. As such, the final model (Table 3a) accounts for age at diagnosis, gender, race, and stage as possible predictors for KLF4 status. Among all possible predictors, only stage III, as compared to stage I in the odds ratio, is significant (p=0.0211). However, a trend, though not significant, showing a decreased odds ratio with reference to stage I at higher stages of disease 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). Figures 3a-3d show stage specific survival curves, of which none demonstrate independent differences in survival between individuals with KLF4 positive and KLF4 negative tumors. Kaplan-Meier curves were also constructed to determine difference in recurrence between KLF4 positive and KLF4 negative tumors. Figure 4 shows overall time to recurrence, or disease free survival, is greater in KLF4 positive patients (p=0.0001). Stage specific curves, Figures 5a-5c, show only a significant difference among stage III tumors (p=0.0046), where in KLF4 positive tumors have significantly improved disease free survival.

A Cox model for survival is represented in Tables 4a and 4b. Table 4a represents an unadjusted Cox model with all available covariates: gender race, stage, and KLF4 status. Table 4b represents the final model, extended for age and gender, and stratified by stage, as all three of these variables did not fulfill the proportional hazards assumption of the Cox model. In addition to KLF4 status, gender and age were included as possible confounders as well as interaction terms between gender and age, age and stage, and gender and stage. In this final model, KLF4 is no longer a significant predictor of overall survival (p=0.0627). A Cox model was also constructed for recurrence, with an unadjusted model represented in Table 5a. A model extended for gender, age, and KLF4 status with a gender and age interaction term is represented in Table 5b. In this case, KLF4 is again not a significant predictor of time to recurrence (p=0.2869).

DISCUSSION

Krüppel-like factors, and most notably KLF4, are without doubt important regulators of the intestinal homeostasis and tumorognesis (8). Nonetheless, the particular role of KLF4 in the colonic epithelium and how its expression may correlate clinical outcomes has not been without controversy. Using tissue microarrays, we have demonstrated that KLF4 expression is significantly down-regulated in intestinal tumors, but loss of KLF4 is not an independent predictor of survival or recurrence.

We first demonstrated that KLF4 is grossly reduced in histological intestinal tumor sections as compared to normal colonic sections. This is consistent with the brunt of data from our lab and others that suggests that KLF4 is a putative tumor suppressor. In a set of 30 colorectal cancer sections, KLF4 mRNA transcripts were reduced by 50% compared to matched normal tissue.[34] This reduction paralleled the reduction in $p21^{Waf1/Cip1}$, suggesting that the reduction in the latter may be a direct consequence of loss of KLF4 expression. Furthermore, chromosomal loci flanking KLF4 have a loss of heteroxygosity and the 5' UTR in a subset of tumor samples was hypermethelated, suggesting possible mechanisms of KLF4 loss (39). This study furthered such evidence by demonstrating KLF4 protein itself is reduced grossly in tumor sections via immunohistochemistry.

In order to evaluate which clinical covariates are associated with expression of KLF4, we performed a univaraite analysis as well as building a multivariate logistic model. In the univariate analysis, only gender was associated with KLF4 status. In the multivariate model, a trend was noted, though not significant, in which higher stages of

diseases had decreased odds of KLF4 expression with reference to stage I tumors. This suggests a possible role of KLF4 as a marker of prognosis in future studies, if it were in fact associated with tumor aggression. This would be consistent with observations from the RKO colon cancer cell line, in which hypermethylation and hemizygous deletion of KLF4 contribute to aggression of the cell line. In fact, re-expression of KLF4 reduces potential tumorogenecity in vitro and in vivo (35-36). 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.

By Kaplan-Meir analysis, KLF4 is a significant predictor of overall survival. 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 (37). In our Kaplan-Meier analysis, we were not able to find significant survival benefits in any particular stage of disease. However, given our much larger sample size, we were able to demonstrate a significant benefit in survival due to KLF4. Inclusion of age, gender, and AJCC stage as well as appropriate extension and interaction terms do not lead to the same conclusions. Thus, KLF4 is not an independent predictor of survival. We were the first to also model recurrence data on the population with reference to KLF4. Here again, we found significant unadjusted results, but did not have significance in the multivariate model.

For this investigation, a tissue microarray was utilized. Perhaps the greatest controversy surrounding tissue microarrays stem from the fact that they reduce analysis of tissues from the whole section to a 0.6 millimeter disk. Camp and colleagues addressed this in a previous study using breast carcinomas that shows that in over 95% of cases,

analysis of the microarray is consistent with results for whole-tissue analysis (38). Furthermore, the utilization of microarrays in our study provided enough cases in order to evaluate both survival and recurrence in patients who were first recruited in 1989, since paraffin embedded disks can retain antigenicity for over 60 years (38). It also provides a consistent set with which to replicate the data or investigate further biomarkers.

Our conclusions serve to corroborate the data that suggests KLF4 has a role in tumor suppression, but do not definitively yield KLF4 as a novel prognostic biomarker. Technical drawbacks are unlikely to have influenced the results of our analysis as both the immunohistochemistry technique and the antibody used have been previously published as valid tools to investigate KLF4 (26, 35). However, both are concerns that plague immunohistochemical studies. Also, the retrospective nature of our investigation may raise caution in interpreting the data. For these reasons, we believe the data should be reproduced in prospective studies with techniques more suitable for clinical laboratories for assessment as to whether KLF4 could be an effective clinical tool.

TABLES AND FIGURES

Table 1a: **Characteristics of participants.**

	Status	
	Normal	**Cancer**
Race		
White	69 (93.24%)	349 (95.10%)
Black	3 (4.05%)	9 (2.45%)
Other	2 (2.7%)	0 (0%)
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%)

Race, gender, and age distributions participants without and with cancer. 9 cancer patients had an unknown race. 1 non-cancer patient had an unknown gender. 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 1b: **General characteristics of tumors included in the tissue microarray.**

	AJCC Summary Stage			
	1	**2**	**3**	**4**
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, NOS	0	0	0	1
Blood/Lymphatic Vessel Invasion				
Intramural	1	1	12	4
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:

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

T refers to tumor depth, N to number of nodes, and M to number of metastases. NOS is an abbreviation for " Not Otherwise Specified".

Table 2a: Univariate measures of covariates among cancer patients.

Variable	KLF4 Negative		KLF4 Positive		Missing	χ^2 Statistic	p-value
	Number	% of KLF4-	Number	% of KLF4+			
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

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 2b: **Univariate measures of covariates among stage I cancer patients.**

Variable	KLF4 Negative		KLF4 Positive		Missing	χ^2 Statistic	p-value
	Number	% of KLF4-	Number	% of KLF4+			
Age							
>70 years	15	55.56	13	59.09			
<70 years	12	44.44	9	40.91	0	0.0619	0.8036
Race							
White	25	92.59	22	100.00			
Non-White	2	7.41	0	0	0	*	0.4949
Gender							
Male	14	51.85	13	59.09			
Female	13	48.15	9	40.91	0	0.2568	0.6123

Frequency of KLF4 positive and negative tumors among stage I tumors under specific covariates. *A Fisher's Exact Test was used if assumptions for a Chi-Squared Test were not fulfilled.

Table 2c: Univariate measures of covariates among stage II cancer patients.

Variable	KLF4 Negative		KLF4 Positive		Missing	χ^2 Statistic	p-value
	Number	% of KLF4-	Number	% of KLF4+			
Age							
>70 years	45	57.69	22	50.00			
<70 years	33	42.31	22	50.00	0	0.6723	0.4122
Race							
White	76	97.44	41	93.18			
Non-White	2	2.56	3	6.82	0	*	0.3500
Gender							
Male	36	46.15	30	68.18			
Female	42	53.85	14	31.82	0	5.4970	0.0190

Frequency of KLF4 positive and negative tumors among stage II tumors under specific covariates. *A Fisher's Exact Test was used if assumptions for a Chi-Squared Test were not fulfilled.

Table 2d: Univariate measures of covariates among stage III cancer patients.

Variable	KLF4 Negative		KLF4 Positive		Missing	χ^2 Statistic	p-value
	Number	% of KLF4-	Number	% of KLF4+			
Age							
>70 years	53	49.53	22	59.46			
<70 years	54	50.47	15	40.54	0	1.0856	0.2972
Race							
White	101	64.59	36	97.30			
Non-White	6	5.61	1	2.70	0	*	0.6777
Gender							
Male	50	46.73	20	54.05			
Female	57	53.27	17	45.95	0	0.5903	0.4422

Frequency of KLF4 positive and negative tumors among stage III tumors under specific covariates. *A Fisher's Exact Test was used if assumptions for a Chi-Squared Test were not fulfilled.

Table 2e: Univariate measures of covariates among stage IV cancer patients.

Variable	KLF4 Negative		KLF4 Positive		Missing	χ^2 Statistic	p-value
	Number	% of KLF4-	Number	% of KLF4+			
Age							
>70 years	15	40.54	8	61.54			
<70 years	22	59.46	5	38.46	2	1.7076	0.1913
Race							
White	34	91.89	12	92.31			
Non-White	3	8.11	1	7.69	2	*	1.000
Gender							
Male	22	59.46	7	53.85			
Female	15	40.54	6	46.15	2	0.1244	0.7243

Frequency of KLF4 positive and negative tumors among stage IV tumors under specific covariates. *A Fisher's Exact Test was used if assumptions for a Chi-Squared Test were not fulfilled.

Table 2f: Assessment of interaction of covariates in determining the odds ratio for KLF4 given age (>70/<70).

Controlled Variable	Stratum OR (Variable=1)	Stratum OR (Variable=0)	MH Odds Ratio*	95% Confidence Interval on MH OR	Breslow-Day Test Statistic	Breslow-Day Test p - value
Race	0.9963	2.2222	1.0355	0.7048, 1.5156	0.7352	0.3912
Gender	1.8032	0.9435	1.0170	0.6923, 1.4938	0.1227	0.7261
Stage I	1.1556	1.0134	1.0285	0.7022, 1.5065	0.0452	0.8316
Stage II	0.7333	1.1888	1.0451	0.7132, 1.5316	1.1965	0.2740
Stage III	1.4943	0.9056	1.3084	0.7013, 1.5377	1.2284	0.2677
Stage IV	2.3467	0.9289	1.0107	0.6888, 1.4831	1.8324	0.1758

Stratum odds ratios given controlled variables, Mantel-Haenszel adjusted odds ratios, and Breslow Day Tests on the odds ratio for KLF4 given age (>70/<70). Race =1 for white, =0 for non-white. Gender =1 for males, = 0 for females. Variable=0 if any other stage for stage specific odds ratios.

Table 2g: Assessment of interaction of covariates in determining the odds ratio for KLF4 given race (white/non-white).

Variable	Stratum OR (Variable=1)	Stratum OR (Variable=0)	MH Odds Ratio*	95% Confidence Interval on MH OR	Breslow-Day Test Statistic	Breslow-Day Test p - value
Age	0.5520	1.2312	0.9514	0.4061, 2.2289	0.7352	0.3912
Gender	0.7533	1.8347	1.0007	0.4241, 2.3615	0.8318	0.3617
Stage I	*	0.7924	0.9558	0.4108, 2.2238	1.9488	0.1627
Stage II	0.3596	1.3063	0.9828	0.4238, 2.2794	1.5774	0.2091
Stage III	2.1386	0.7951	0.9872	0.4064, 2.3979	0.6945	0.4046
Stage IV	1.0588	0.8911	0.9121	0.3865, 2.1524	0.0179	0.8937

Stratum odds ratios given controlled variables, Mantel-Haenszel adjusted odds ratios, and Breslow Day Tests on the odds ratio for KLF4 given race (white/non-white). Age =1 for participants older than 70, =0 for those younger. Gender =1 for males, = 0 for females. Variable=0 if any other stage for stage specific odds ratios. *Unable to calculate the stratum specific odds ratio for stage I=1 given a zero cell.

Table 2h: Assessment of interaction of covariates in determining the odds ratio for KLF4 given gender (male/female).

Variable	Stratum OR (Variable=1)	Stratum OR (Variable=0)	MH Odds Ratio*	95% Confidence Interval on MH OR	Breslow-Day Test Statistic	Breslow-Day Test p - value
Age	1.5657	1.3638	1.4640	0.9954, 2.1533	0.1227	0.7621
Race	1.4076	3.4286	1.4651	0.9956, 2.1540	0.8318	0.3618
Stage I	1.3413	1.4809	1.4642	0.9963, 2.1518	0.0258	0.8723
Stage II	2.5000	1.2275	1.4739	1.0022, 2.1677	2.4379	0.1184
Stage III	1.3412	1.4336	1.4072	0.9471, 2.0909	0.0219	0.8824
Stage IV	0.7955	1.6000	1.4987	1.0173, 2.2078	1.0649	0.3021

Stratum odds ratios given controlled variables, Mantel-Haenszel adjusted odds ratios, and Breslow Day Tests on the odds ratio for KLF4 given gender (male/female). Age =1 for participants older than 70, =0 for those younger. Race =1 for white, = 0 for non-white. Variable=0 if any other stage for stage specific odds ratios.

Table 3a: 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)

The first logistic model run. No selection strategies were run and interaction was not evaluated. Age at diagnosis was included as continuous with other variables set as follows:

Gender: 1=male, 2=female

Race: 1= caucasian, 0= non-caucasian

Stage II-IV run with Stage I set as reference.

Table 3b: Multivariate analysis of KLF4 using a binary logistic model including all possible two way interaction terms.

Variable	Estimated Coefficient	Estimated Standard Error	Wald Chi Squared Statistic	p- value
Age at diagnosis	-53.4383	283.0	0.0357	0.8502
Gender	0.1794	0.1311	1.8737	0.1711
Race	14.6823	95.0966	0.0238	0.8773
Stage II	50.9667	283.0	0.0324	0.8571
Stage III	15.2556	209.0	0.0053	0.9418
Stage IV	14.0696	209.1	0.0045	0.9463
Age*Gender	13.8479	209.1	0.0044	0.9472
Age*Race	-0.0113	0.0208	0.2977	0.5853
Age*Stage II	-0.1556	0.1218	1.6334	0.2012
Age*Stage III	-0.0238	0.0309	0.5901	0.4424
Age*Stage IV	0.0244	0.0314	0.6044	0.4369
Gender*Race	0.0137	0.0410	0.1117	0.7383
Gender*Stage II	-13.4490	95.0789	0.0200	0.8875
Gender*Stage III	0.4077	0.7274	0.3142	0.5751
Gender*Stage IV	-0.2318	0.7203	0.1035	0.7476
Race*Stage II	-0.9291	0.9234	1.0124	0.3143
Race*Stage III	-14.7120	209.0	0.0050	0.9439
Race*Stage IV	-16.3263	209.0	0.0061	0.9377

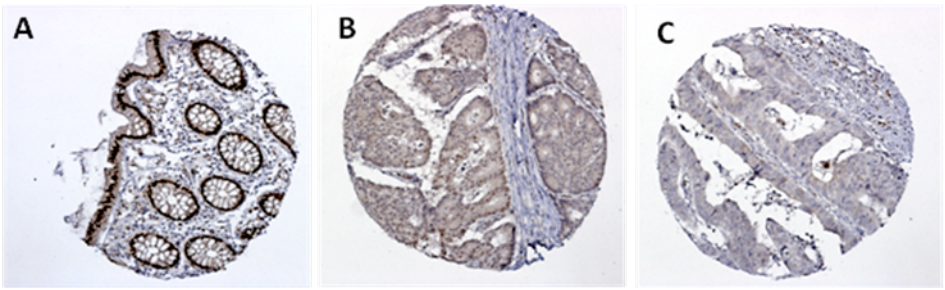
A model with all possible interaction terms, validity of this model was questionable. No interaction terms were included in final model. Validity of fit of this full model was questionable. At each step, one interaction term was dropped. After dropping one variable each was tested to see if a previously dropped variable could be returned to the model, which in no case was true. As such, all interaction terms were dropped in the following order: Race*Stage, Gender*Race, Gender*Stage, Age*Stage, and, finally, Age*Race. Age at diagnosis was included as continuous with other variables set as follows:

Gender: 1=male, 2=female

Race: 1= caucasian, 0= non-caucasian

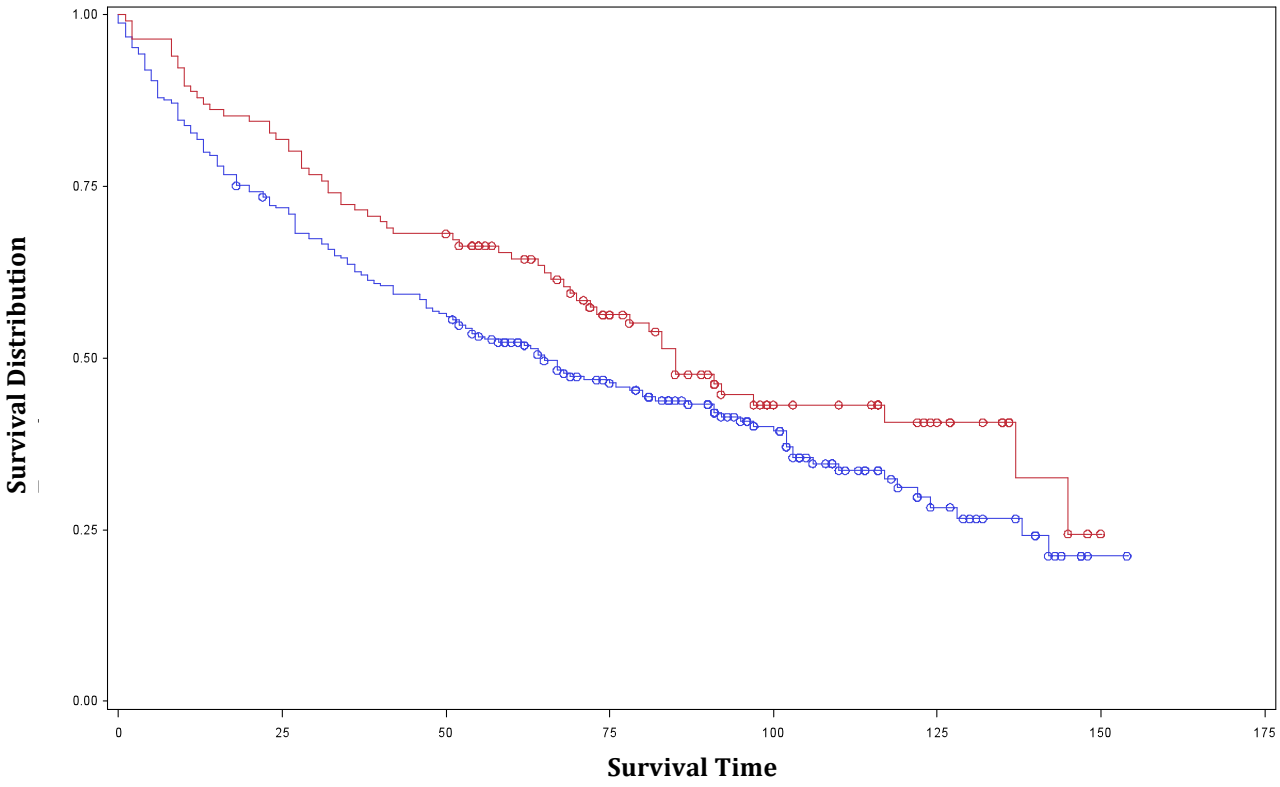
Stage II-IV run with Stage I set as reference.

Figure 1: Representative samples of KLF4 staining.



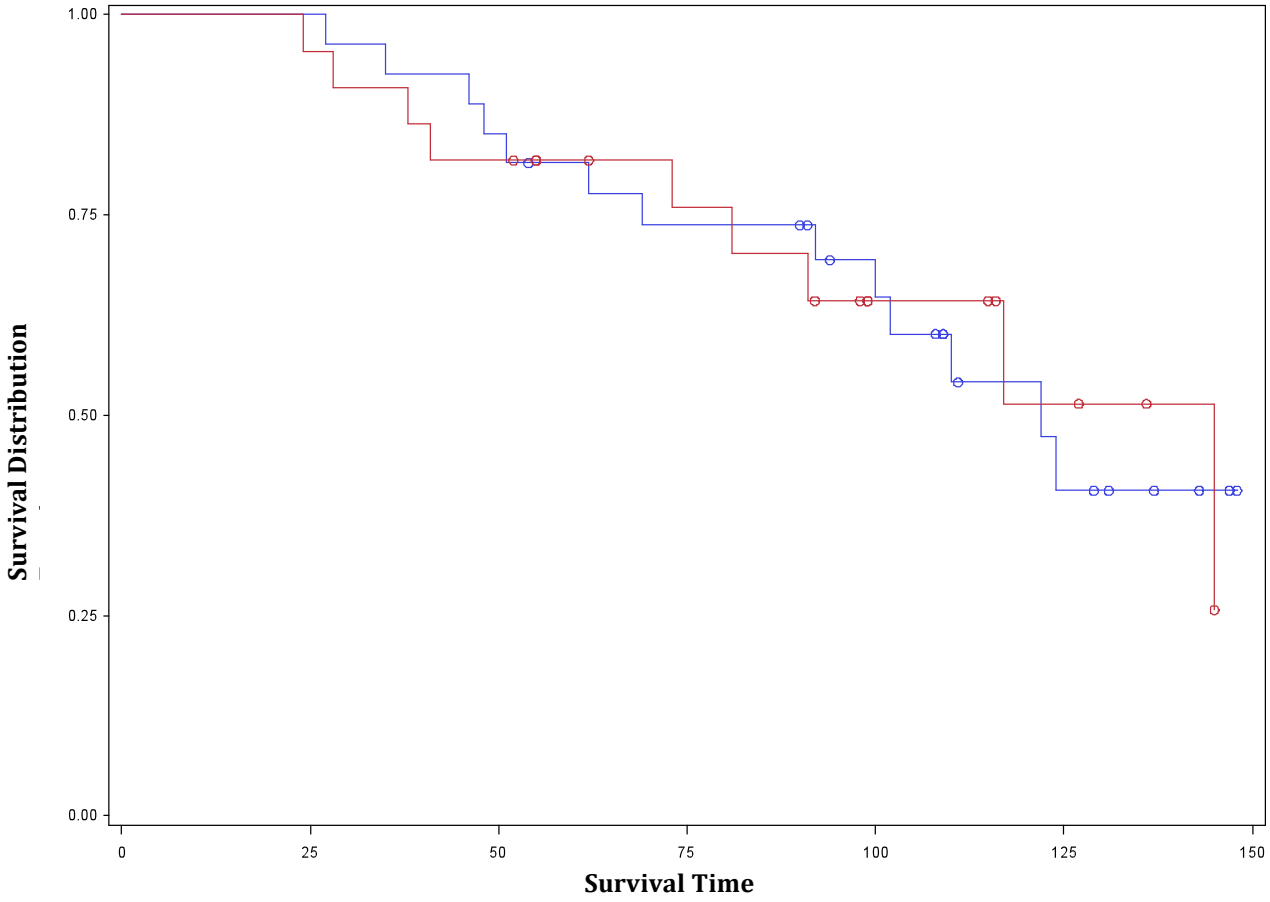
A representative example of KLF4 staining in the tissue microarray of (A) normal colon, (B) colon cancer with positive KLF4 staining, and (C) colon cancer with negative KLF4 staining.

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



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

Figure 3a: **Kaplan-Meier survival curve among stage I patients.**

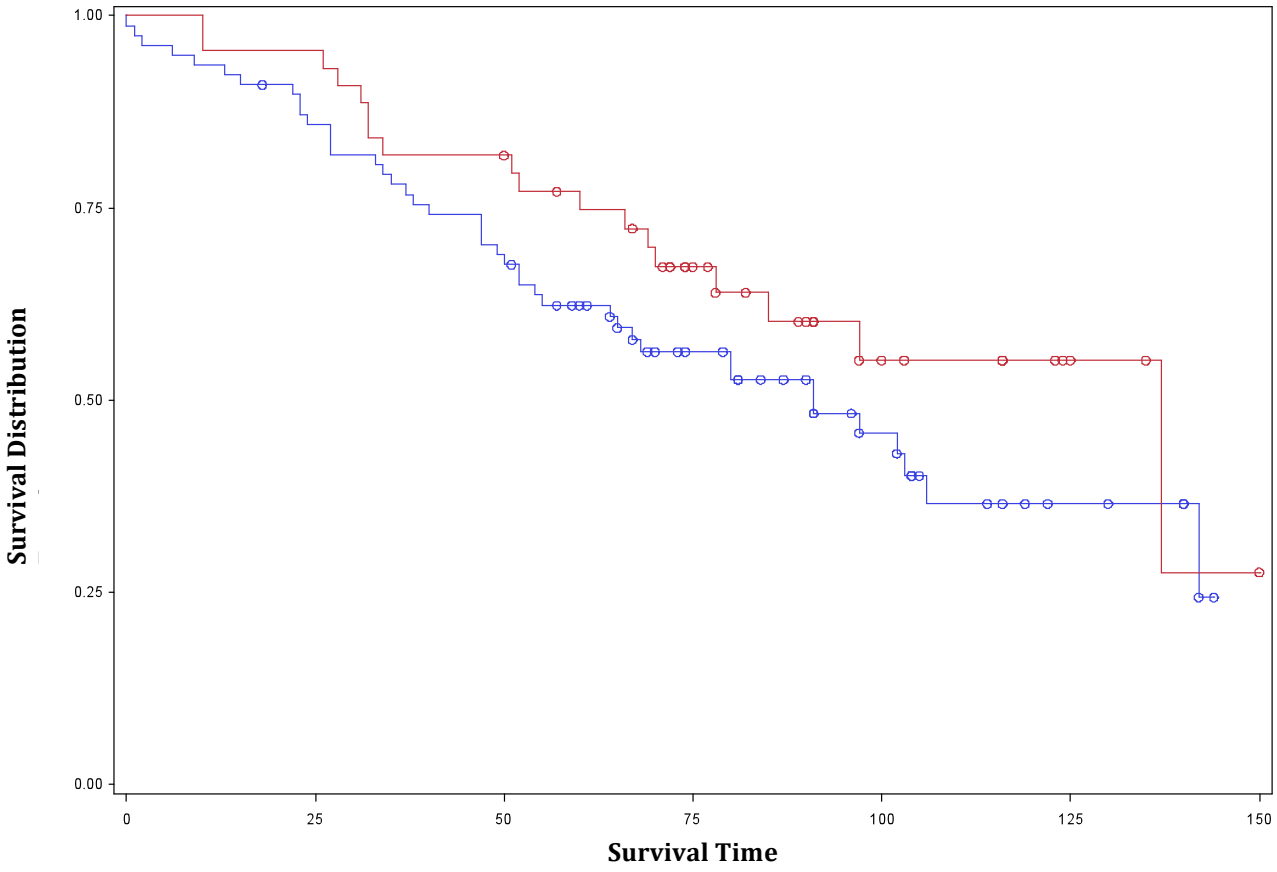


Kaplan-Meier curve for stage I patients. The log rank statistic for the curve was 0.0147 with 1 degree of freedom, yielding an insignificant p-value of 0.9035.

KLF4 negative — Censored KLF4 negative participant

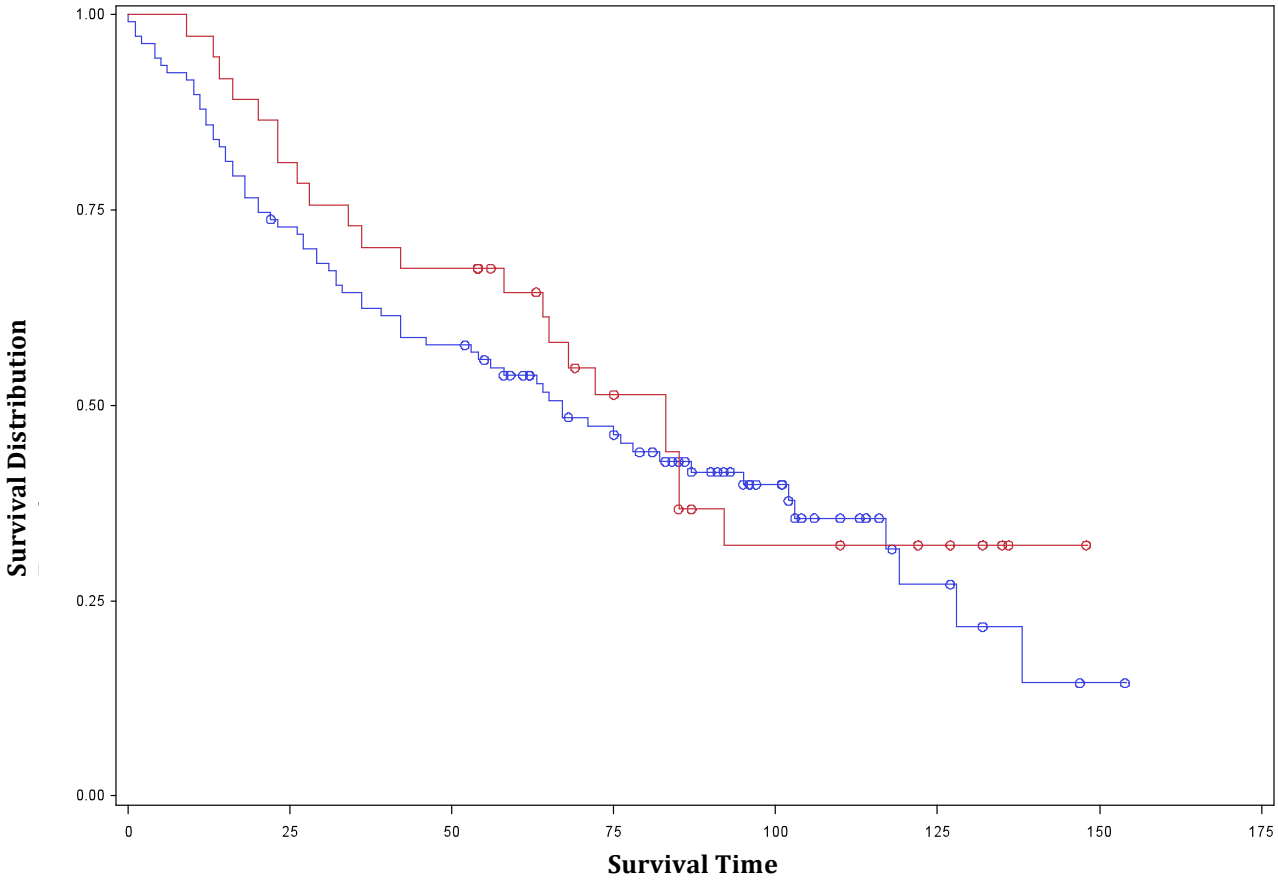
KLF4 positive — Censored KLF4 positive participant

Figure 3b: Kaplan-Meier survival curve among stage II patients.



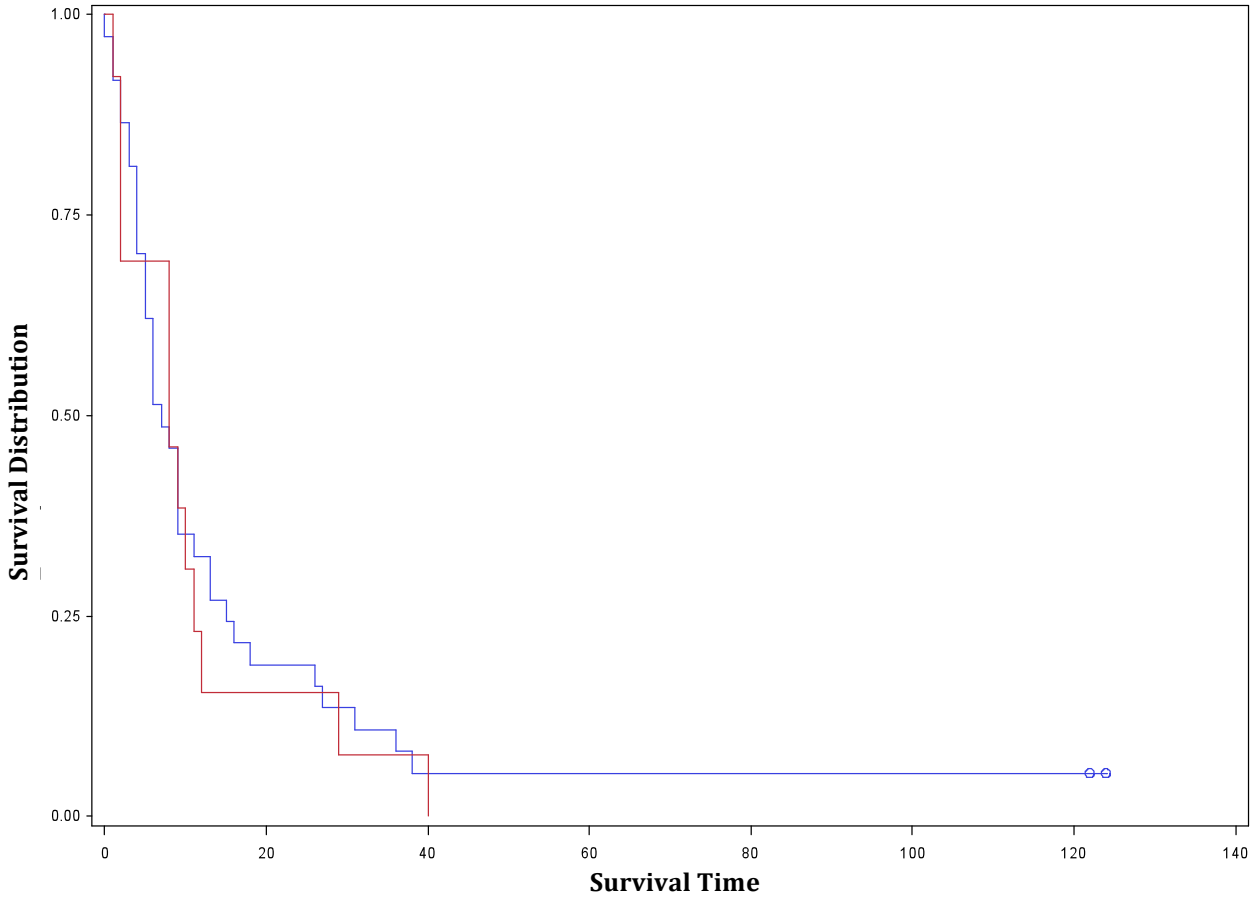
Kaplan-Meier curve for stage II available patients. The log rank statistic for the curve was 2.0629 with 1 degree of freedom, yielding an insignificant p-value of 0.1509.

Figure 3c: Kaplan-Meier survival curve among stage III patients.



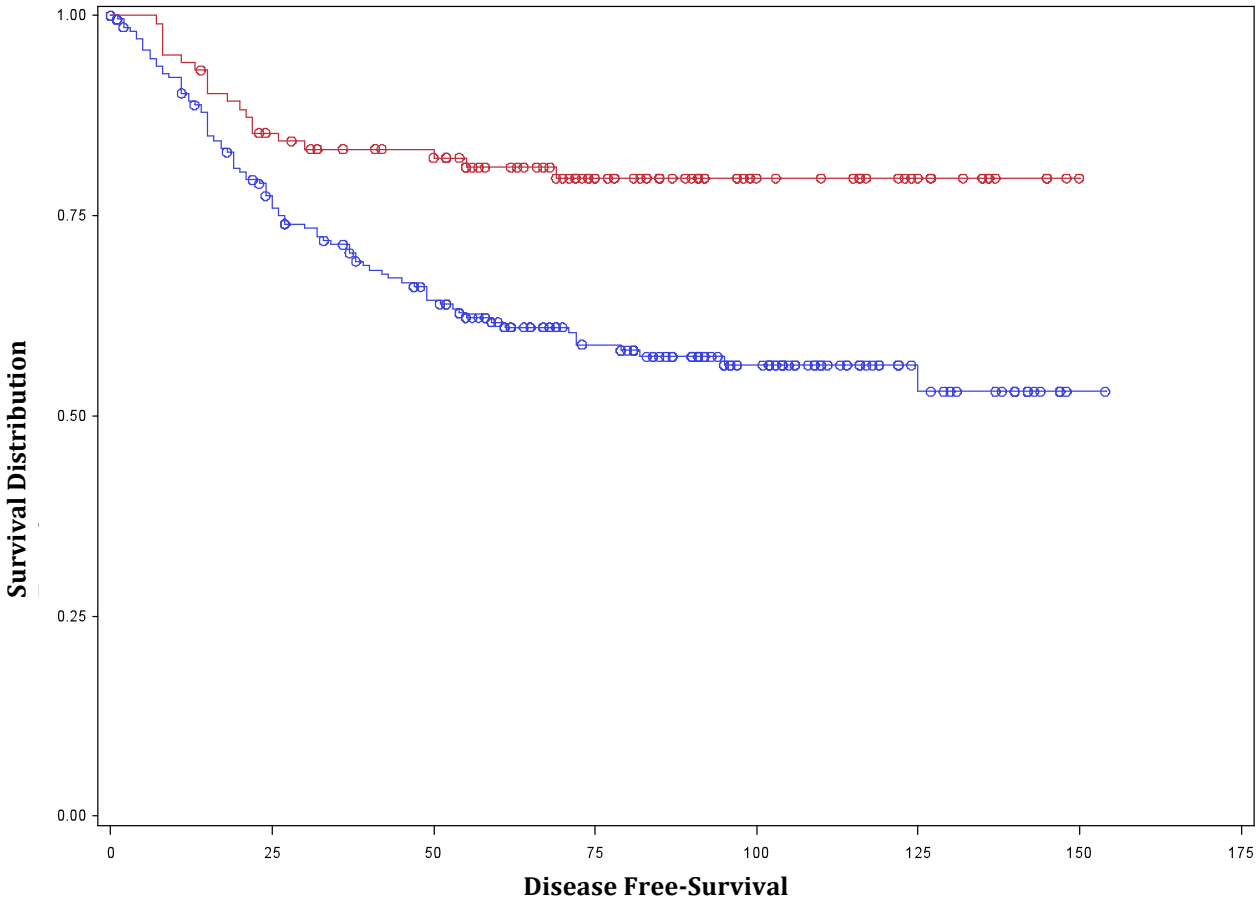
Kaplan-Meier curve for stage III patients. The log rank statistic for the curve was 0.5295 with 1 degree of freedom, yielding an insignificant p-value of 0.4668.

Figure 3d: **Kaplan-Meier survival curve among stage IV patients.**



Kaplan-Meier curve for stage IV patients. The log rank statistic for the curve was 1.8910 with 1 degree of freedom, yielding an insignificant p-value of 0.1691.

Figure 4: Overall Kaplan-Meier disease-free survival curve with all patients.

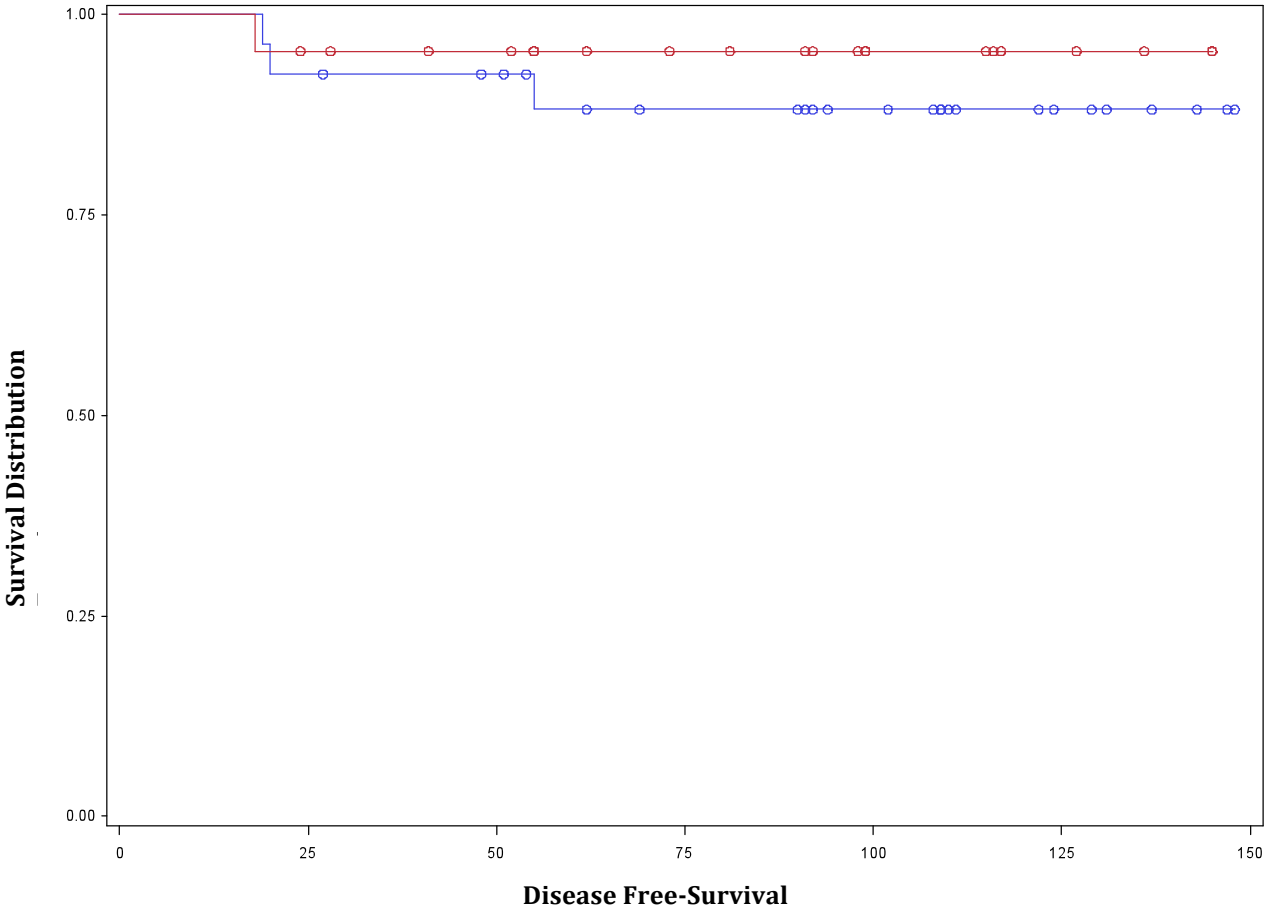


Kaplan-Meier disease-free curve for all available patients. The log rank statistic for the curve was 14.9437 with 1 degree of freedom, yielding a significant p-value of 0.0001.

KLF4 negative — Censored KLF4 negative participant

KLF4 positive — Censored KLF4 positive participant

Figure 5a: Kaplan-Meier diseases free-survival curve among stage I patients.



Kaplan-Meier disease-free curve for stage I patients. The log rank statistic for the curve was 0.5479 with 1 degree of freedom, yielding an insignificant p-value of 0.5479.

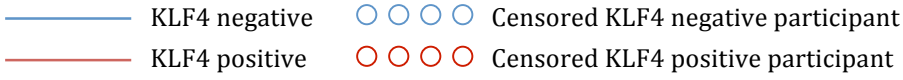
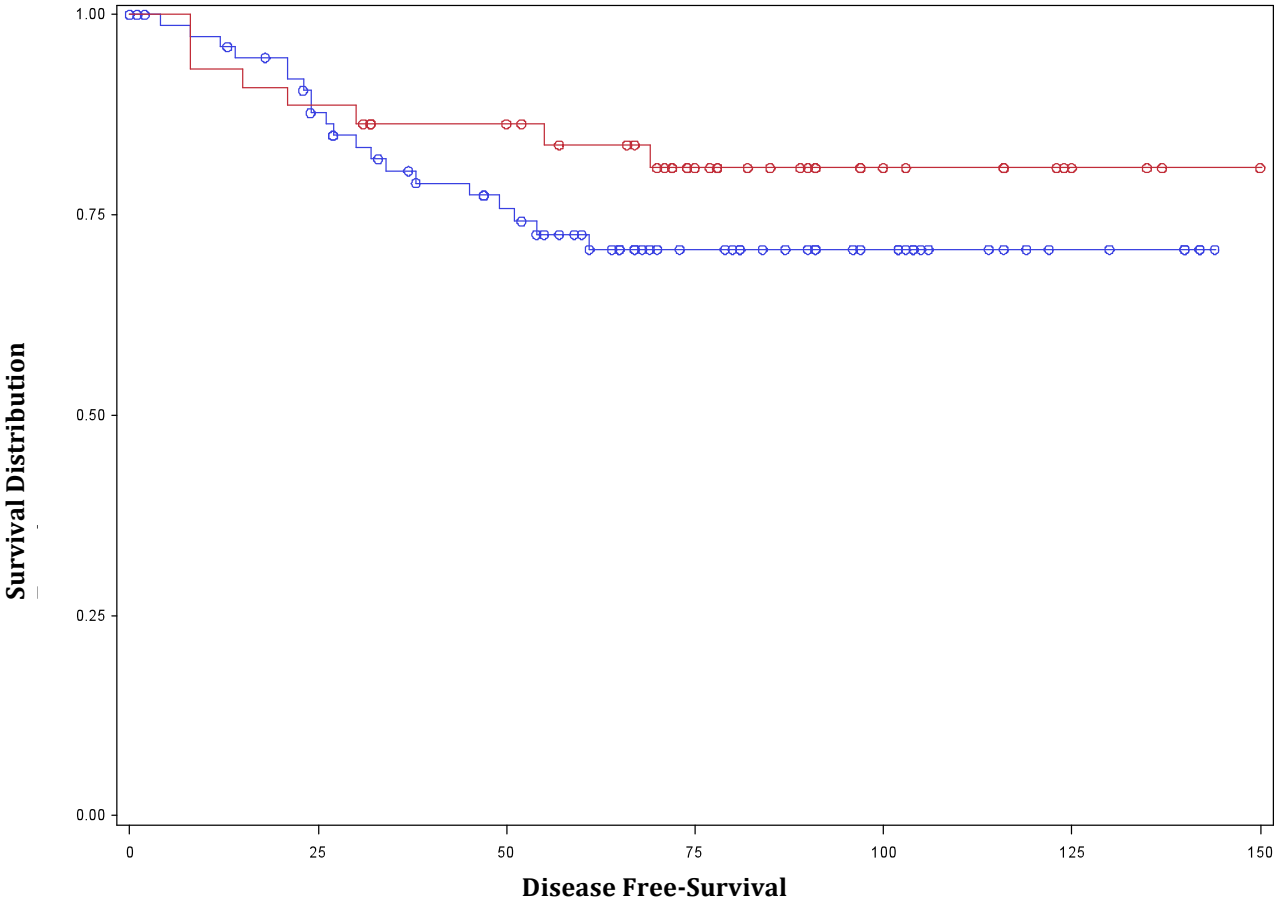


Figure 5b: Kaplan-Meier diseases free-survival curve among stage II patients.

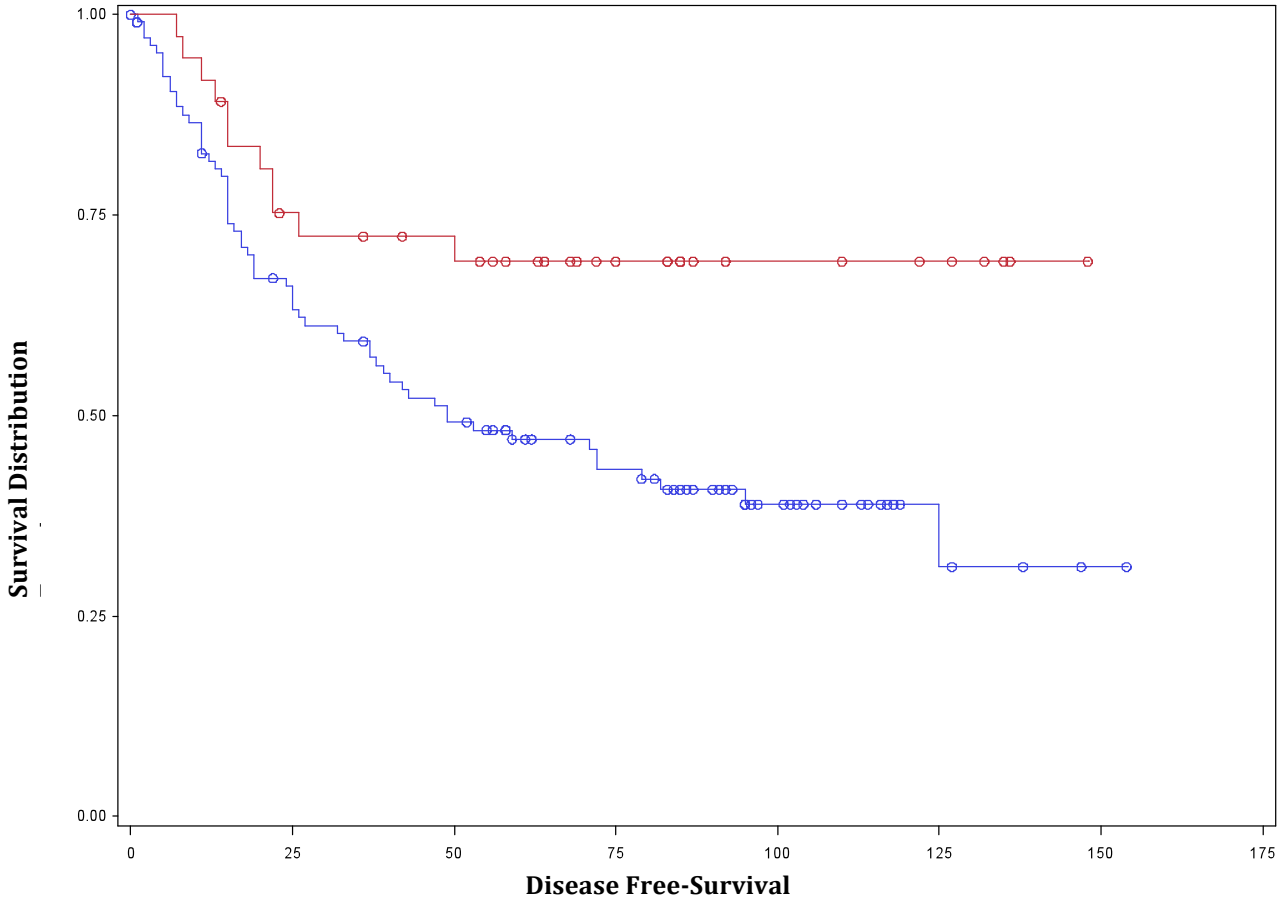


Kaplan-Meier disease-free curve for stage II patients. The log rank statistic for the curve was 1.5990 with 1 degree of freedom, yielding an insignificant p-value of 0.2060.

KLF4 negative — Censored KLF4 negative participant

KLF4 positive — Censored KLF4 positive participant

Figure 5c: Kaplan-Meier diseases free-survival curve among stage III patients.



Kaplan-Meier disease-free curve for stage III patients. The log rank statistic for the curve was 6.5876 with 1 degree of freedom, yielding a significant p-value of 0. 0103.

KLF4 negative — Censored KLF4 negative participant

KLF4 positive — Censored KLF4 positive participant

Table 4a: Cox proportional hazards model for overall survival, without adjustment.

Parameter	Estimate	Estimated Standard Error	Chi-Squared Statistic	p-value	Hazard Ratio	Hazard Ratio Confidence Interval
Age at diagnosis	0.04172	0.00678	37.8457	<.0001	1.043	(1.029, 1.057)
Gender	-0.06184	0.13817	0.2003	0.6545	0.940	(0.717, 1.232)
Race	-0.47455	0.31951	2.2059	0.1375	0.622	(0.333, 1.164)
Stage II*	0.46366	0.25141	3.4011	0.0652	1.590	(0.971, 2.602)
Stage III*	0.83931	0.24108	12.1205	0.0005	2.315	(1.443, 3.713)
Stage IV*	2.79962	0.27561	103.1856	<.0001	16.438	(9.578, 28.213)
KLF4	-0.14739	0.15342	0.9230	0.3367	0.863	(0.639, 1.166)

A first Cox model including confounders. A Cox model without any confounding terms, but extended for KLF4 to meet the proportional hazards assumption, showed KLF4 to be significant in predicting survival with a chi-squared statistic of 82.4065 ($p<0.0001$). No selection strategies were run and neither interaction nor extensions of the model were evaluated. Age at diagnosis was included as continuous with other variables set as follows:

Gender: 1=male, 0=female
Race: 1= caucasian, 0= non-caucasian
Stage II-IV run with Stage I set as reference
KLF4: 1= positive, 0=negative.

Table 4b: Final extended Cox model for overall survival stratified by stage.

Parameter	Estimate	Estimated Standard Error	Chi-Squared Statistic	p-value
Age at diagnosis	0.40314	0.05225	59.5226	<.0001
Gender	9.58952	1.64312	34.0607	<.0001
KLF4	-0.33771	0.18141	3.4656	0.0627
DxAge*TimeToDeath	-0.00268	0.0002155	155.1136	<.0001
Gender*TimeToDeath	-0.05594	0.00727	59.1937	<.0001
DxAge*Gender	-0.06125	0.01456	17.7028	<.0001
DxAge*stage2	-0.12003	0.04418	7.3802	0.0066
DxAge*stage3	-0.17730	0.04421	16.0862	<.0001
DxAge*stage4	-0.25510	0.04828	27.9242	<.0001
Gender*stage2	-1.90484	0.74014	6.6235	0.0101
Gender*stage3	-3.13608	0.77693	16.2932	<.0001
Gender*stage4	-4.93535	0.93389	27.9284	<.0001

The final Cox model. Significant extensions of the model were included to satisfy the proportional hazards assumption. Furthermore, significant interaction terms were included in the model. At each step, one interaction term was dropped. After dropping one variable each was tested to see if a previously dropped variable could be returned to the model. Age at diagnosis (DxAge) was included as continuous with other variables set as follows:

Gender: 1=male, 0=female
Race: 1= caucasian, 0= non-caucasian
Stage II-IV run with Stage I set as reference
KLF4: 1= positive, 0=negative.

Table 5a: Cox proportional hazards model for disease free-survival, without adjustment.

Parameter	Estimate	Estimated Standard Error	Chi-Squared Statistic	p-value	Hazard Ratio	Hazard Ratio Confidence Interval
Gender	0.01323	0.19725	0.0045	0.9465	1.013	(0.688, 1.492)
Race	0.08602	0.52640	0.0267	0.8702	1.090	(0.388, 3.058)
Age at diagnosis	0.00862	0.00852	1.0227	0.3119	1.009	(0.992, 1.026)
Stage 2	1.16741	0.53560	4.7507	0.0293	3.214	(1.125, 9.181)
Stage 3	2.09040	0.51615	16.4024	<.0001	8.088	(2.941, 22.243)
KLF4	-0.71878	0.25141	8.1739	0.0042	0.487	(0.298, 0.798)

A first Cox model for recurrence including confounders. A Cox model without any confounding terms, but extended for KLF4 to meet the proportional hazards assumption, showed KLF4 to be significant in predicting survival with a chi-squared statistic of 49.6695 ($p<0.0001$). No selection strategies were run and neither interaction nor extensions of the model were evaluated. Age at diagnosis was included as continuous with other variables set as follows:

Gender: 1=male, 0=female
Race: 1= caucasian, 0= non-caucasian
Stage II-IV run with Stage I set as reference
KLF4: 1= positive, 0=negative.

Table 5b: Final extended Cox model for disease free-survival

Parameter	Estimate	Estimated Standard Error	Chi-Squared Statistic	p-value
Gender	4.79825	1.75676	7.4601	0.0063
Age at Diagnosis	0.19832	0.03585	30.6078	<.0001
KLF4	0.51719	0.48568	1.1340	0.2869
KLF4*TimeToRecur	-0.05616	0.01948	8.3155	0.0039
Gender*TimeToRecur	-0.06481	0.01197	29.2938	<.0001
DxAge*TimeToRecur	-0.00512	0.0005366	91.1539	<.0001
Gender*DxAge	-0.04456	0.02221	4.0262	0.0448

The final Cox model for recurrence. Significant extensions of the model were included to satisfy the proportional hazards assumption. Furthermore, significant interaction terms were included in the model. At each step, one interaction term was dropped. After dropping one variable each was tested to see if a previously dropped variable could be returned to the model. Age at diagnosis (DxAge) was included as continuous with other variables set as follows:

Gender: 1=male, 0=female
Race: 1= caucasian, 0= non-caucasian
Stage II-IV run with Stage I set as reference
KLF4: 1= positive, 0=negative.

REFERENCES

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics. 2008. CA Cancer J Clin. 2008; 58(2): 71-96.
2. Wolpin BM, Meyerhardt JA, Mamon HJ, Mayer RJ. Adjuvant treatment of colorectal cancer. CA Cancer J Clin. 2007; 57(3):168-185.
3. Benson AB 3rd, Schrag D, Somerfield MR, Cohen AM, Figueredo AT, Flynn PJ, Krzyzanowska MK, Maroun J, McAllister P, Van Cutsem E, Brouwers M, Charette M, Haller DG. American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. J Clin Oncol. 2004; 22(16):3408-3419.
4. Cascinu S, Ligi M, Graziano F, Del Ferro E, Valentini M, Grianti C, Bartolucci M, Catalano G. S-phase fraction can predict event free survival in patients with pT2-T3N0M0 colorectal carcinoma: implications for adjuvant chemotherapy. Cancer. 1998; 83(6):1081-1085.
5. Cascinu S, Staccioli MP, Gasparini G, Giordani P, Catalano V, Ghiselli R, Rossi C, Baldelli AM, Graziano F, Saba V, Muretto P, Catalano G. Expression of vascular endothelial growth factor can predict event-free survival in stage II colon cancer. Clin Cancer Res. 2000; 6(7):2803-2807.
6. Preiss A, Rosenberg UB, Kienlin A, Seifert E, Jäckle H. Molecular genetics of Krüppel, a gene required for the segmentation of the Drosophila embryo. Nature. 1985; 313(5997): 27-32.
7. Bieker JJ. Krüppel-like factors: three fingers in many pies. J Biol Chem. 2001; 276(37): 34355-34358.
8. Zhang W et al. "Novel Cross Talk of Krüppel-Like Factor 4 and ß-Catenin Regulates Normal Intestinal Homeostasis and Tumor Repression" Mol Cell Biol. 2006; 26(6): 2055–2064.
9. Chen X, Whitney EM, Gao SY, Yang VW. Transcriptional profiling of Krüppel-like factor 4 reveals a function in cell cycle regulation and epithelial differentiation. J Mol Biol. 2003; 326(3): 665-677.
10. Chen X, Johns DC, Geiman DE, Marban E, Dant DT, Hamlin G, Sun R, Yang VW. Krüppel-like factor 4 (gut-enriched Krüppel-like factor) inhibits cell proliferation by blocking G1/S progression of the cell cycle. J Biol Chem. 2001; 276(32): 30423-30428.
11. Zhang W, Geiman DE, Shields JM, Dang DT, Mahatan CS, Kaestner KH, Biggs JR, Kraft AS, Yang VW. The gut-enriched Kruppel-like factor (Kruppel-like factor 4) mediates the transactivating effect of p53 on the p21WAF1/Cip1 promoter. J Biol Chem. 2000; 275(24): 18391-18398.
12. Shie JL, Chen ZY, Fu M, Pestell RG, Tseng CC. Gut-enriched Krüppel-like factor represses cyclin D1 promoter activity through Sp1 motif. Nucleic Acids Res. 2000; 28(15): 2969-2976.
13. Yoon HS, Yang VW. Requirement of Krüppel-like factor 4 in preventing entry into mitosis following DNA damage. J Biol Chem. 2004; 279(6): 5035-2041.
14. Segre JA, Bauer C, Fuchs E. Klf4 is a transcription factor required for establishing the barrier function of the skin. Nat Genet. 1999; 22(4): 356-360.
15. Hinnebusch BF, Siddique A, Henderson JW, Malo MS, Zhang W, Athaide CP, Abedrapo MA, Chen X, Yang VW, Hodin RA. Enterocyte differentiation marker

intestinal alkaline phosphatase is a target gene of the gut-enriched Krüppel-like factor. Am J Physiol Gastrointest Liver Physiol. 2004; 286(1): G23-G30.
16. Katz JP, Perreault N, Goldstein BG, Lee CS, Labosky PA, Yang VW, Kaestner KH. The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. Development. 2002; 129(11): 2619-2628.
17. Ghaleb AM, Katz JP, Kaestner KH, Du JX, Yang VW. Krüppel-like factor 4 exhibits antiapoptotic activity following gamma-radiation-induced DNA damage. Oncogene. 2007; 26(16): 2365-2373.
18. Rowland BD, Bernards R, Peeper DS. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. Nat Cell Biol. 2005; 7(11): 1074-1082.
19. Rowland BD, Peeper DS. KLF4, p21 and context-dependent opposing forces in cancer. Nat Rev Cancer. 2006; 6(1): 11-23.
20. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126(4): 663-676.
21. Yamanaka S. Induction of pluripotent stem cells from mouse fibroblasts by four transcription factors. Cell Prolif. 2008; 41(Suppl 1): 51-56.
22. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thompson JA. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007; 318(5858): 1917-1920.
23. Wang N, Liu ZH, Ding F, Wang XQ, Zhou CN, Wu M. Down-regulation of gut-enriched Kruppel-like factor expression in esophageal cancer. World J Gastroenterol. 2002; 8(6): 966-970.
24. Katz JP, Perreault N, Goldstein BG, Actman L, McNally SR, Silberg DG, Furth EE, Kaestner KH. Loss of Klf4 in mice causes altered proliferation and differentiation and precancerous changes in the adult stomach. Gastroenterology. 2005; 128(4): 935-945.
25. Ohnishi S, Ohnami S, Laub F, Aoki K, Suzuki K, Kanai Y, Haga K, Asaka M, Ramirez F, Yoshida T. Downregulation and growth inhibitory effect of epithelial-type Krüppel-like transcription factor KLF4, but not KLF5, in bladder cancer. Biochem Biophys Res Commun. 2003; 308(2): 251-256.
26. Bianchi F, Hu J, Pelosi G, Cirincione R, Ferguson M, Ratcliffe C, Di Fiore PP, Gatter K, Pezzella F, Pastorino U. Lung cancers detected by screening with spiral computed tomography have a malignant phenotype when analyzed by cDNA microarray. Clin Cancer Res. 2004; 10(18 Pt 1): 6023-6028.
27. Yasunaga J, Taniguchi Y, Nosaka K, Yoshida M, Satou Y, Sakai T, Mitsuya H, Matsuoka M. Identification of aberrantly methylated genes in association with adult T-cell leukemia. Cancer Res. 2004; 64(17): 6002-9.
28. Dang DT, Bachman KE, Mahatan CS, Dang LH, Giardiello FM, Yang VW. Decreased expression of the gut-enriched Krüppel-like factor gene in intestinal adenomas of multiple intestinal neoplasia mice and in colonic adenomas of familial adenomatous polyposis patients. FEBS Lett. 2000; 476(3): 203-207.

29. Ghaleb AM, McConnell BB, Nandan MO, Katz JP, Kaestner KH, Yang VW. Haploinsufficiency of Krüppel-like factor 4 promotes adenomatous polyposis coli dependent intestinal tumorigenesis. Cancer Res. 2007; 67(15): 7147-7154.
30. Foster KW, Ren S, Louro ID, Lobo-Ruppert SM, McKie-Bell P, Grizzle W, Hayes MR, Broker TR, Chow LT, Ruppert JM. Oncogene expression cloning by retroviral transduction of adenovirus E1A-immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLF. Cell Growth Differ. 1999; 10(6): 423-34.
31. Foster KW, Frost AR, McKie-Bell P, Lin CY, Engler JA, Grizzle WE, Ruppert JM. Increase of GKLF messenger RNA and protein expression during progression of breast cancer. Cancer Res. 2000; 60(22): 6488-6495.
32. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. Lab Invest. 2000; 80(12):1943-1949.
33. Guidance for Statistical Analysis of Biomarker Data Generated From the NCI Colon Tissue Microarray. National Cancer Institutes. Last Revision Feb. 24, 2008.
34. Zhao W, Hisamuddin IM, Nandan MO, Babbin BA, Lamb NE, Yang VW. "Identification of Krüppel-like factor 4 as a potential tumor suppressor gene in colorectal cancer." Oncogene. 2004; 23(2): 395-402.
35. Dang DT, Mahatan CS, Dang LH, Agboola IA and Yang VW. "Expression of the gut-enriched Krüppel-like factor (Krüppel-like factor 4) gene in the human colon cancer cell line RKO is dependent on CDX2." Oncogene. 2001; 20(35): 4884–4890.
36. Dang DT, Chen X, Feng J, Torbenson M, Dang LH, Yang VW. "Overexpression of Krüppel-like factor 4 in the human colon cancer cell line RKO leads to reduced tumorigenecity." Oncogene. 2003; 22(22):3424-30.
37. Xu J, Lü B, Xu F, Gu H, Fang Y, Huang Q, Lai M. "Dynamic down-regulation of Krüppel-like factor 4 in colorectal adenoma-carcinoma sequence." J Cancer Res Clin Oncol. 2008; 134(8): 891-8.