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Increased IFRD1 expression in human colon cancers predicts reduced patient survival

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Abstract

Background: Colon cancer (CRC) is the third most common cancer worldwide. CRC develops through combinations of genetic and epigenetic changes. However, there is marked heterogeneity in the “driver gene” mutational profiles within and among colon cancers from individual patients, and these are not sufficient to explain differences in colon cancer behavior and treatment response. Global modulation of the tumor landscape may play a role in cancer behavior. Interferon-related developmental regulator 1 (IFRD1) is a transcriptional co-regulator that modulates expression of large gene cassettes and plays a role in gut epithelial proliferation following massive intestinal resection.

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Aims: We address the hypothesis that increased IFRD1 expression in colon cancers is associated with poorer patient survival.

Methods: Tumor and normal tissue from colon cancer patient cohorts from the United States, Spain and China were used for this study. Cancers were scored for intensity of IFRD1 immunostaining. The primary clinical outcome was overall survival defined as time from diagnosis to death due to cancer. Kaplan-Meier method and Log-rank analysis were used to assess the association between IFRD1 expression and survival.

Results: Almost all (98.7%) colon cancers showed readily detectable IFRD1 expression, with immunoreactivity primarily in the tumor cytoplasm. High IFRD1 colon cancer expression was significantly associated with decreased 5 year patient survival. Patients in the American cohort with high IFRD1 expression had a poorer prognosis.

Conclusions: We have demonstrated that high IFRD1 protein expression in colon cancer is associated with poorer patient prognosis, suggesting a potential role for IFRD1 in modulating tumor behavior.

Keywords

Human colon cancer; IFRD1; transcriptional co-regulator; tumor landscape

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the world, and the global burden is expected to increase due to the growth and aging of the population. Despite advances in diagnosis and therapy, CRC remains the third most common cancer related cause of death in the United States among men and women [1] and the overall 5-year survival rate for patients with colorectal cancer is 65% [1]. Colon cancer is a biologically heterogeneous disease that develops via distinct pathways involving combinations of genetic and epigenetic changes. Defining tumor subtypes based upon pathway-driven alterations has the potential to improve prognostication and guide targeted therapy [2]. However, it has become increasingly clear that there is marked heterogeneity in the “driver gene” mutational profiles within and among colon cancers from individual patients. Some of these mutations are also found in non-tumor tissue [3], and are not sufficient to explain differences in colon cancer behavior and tumor response among patients [4]. Changes in the tumor landscape which may involve global modulation of gene expression have been suggested to play a role in these processes [4].

Interferon-related developmental regulator 1 (IFRD1, aka mouse Tis7, PC4) is a transcriptional co-regulator with a putative role in regulating intestinal lipid metabolism and epithelial cell proliferation [5]. Expression of IFRD1 is increased in injury states in multiple organ systems, such as after massive intestinal resection [6] and after nerve [7] and muscle [8, 9] injury. Analysis of the intracellular localization of IFRD1 in cultured cells demonstrates cytoplasmic or nuclear localization, depending on the cellular differentiation state [10]. This suggests that IFRD1, an immediate early gene, may function in the cytoplasm as a sensor of cellular stimuli and, in the nucleus, as a transcriptional modifier. In the nucleus, IFRD1 has been shown to interact with the SIN3 protein complex, scaffold

histone deacetylases [11], and regulate the expression of large gene cassettes in epithelial cells, myoblasts, hematopoietic cells, and neurons [12]. Review of the expression patterns of IFRD1 in 79 human tissues revealed that it is ubiquitous but particularly abundant in colorectal adenocarcinoma as well as whole blood, testis, olfactory bulb, pancreas and other highly secretory tissues [13]. In large scale genomic/proteomic colon cancer analyses (including TCGA/Protein Atlas), IFRD1 expression is increased in multiple cancers [14, 15]. We have shown that IFRD1 expression is increased up to eightfold in the repairing small intestine following gut resection, and it is associated with a marked increase in gut epithelial cell proliferation [16]. Conversely, we showed that loss of IFRD1 inhibited the crypt cell proliferative adaptive response after massive intestinal resection [6].

Herein we aimed to explore the role of IFRD1 in human colon cancer pathogenesis. Specifically, we address the hypothesis that, given IFRD1's role in driving stress-induced proliferative response, increased IFRD1 expression in colon cancers would be associated with reduced survival. IFRD1 expression patterns were analyzed by immunohistochemical analysis of 378 human colon cancers and normal adjacent colon epithelium. We used a large, multinational, ethnically and racially diverse patient cohort to investigate how IFRD1 expression correlates with tumor stage, patient clinical demographics and overall survival.

METHODS

Subjects

Formalin-fixed paraffin-embedded tissue (FFPE) from colon cancer and normal colonic mucosa from patients of three institutions from the United States, Spain and China were used for this analysis. The American cohort of colon cancer patients (n=72) were randomly selected from a subset of the Barnes-Jewish Hospital Oncology Data Service cancer registry. These studies were approved by the Human Studies Committee (IRB) of Washington University School of Medicine. These patients were initially treated between 01/01/1999 and 06/30/2003 at the Washington University School of Medicine Siteman Cancer Center, a tertiary care institution affiliated with Barnes-Jewish Hospital in St. Louis, Missouri. The data in the cancer registry includes demographic, clinical, and survival data in accordance with the American College of Surgeons Commission on Cancer guidelines. These patients underwent surgical resection. Pathological and surgical data was extracted from the medical chart. The Spanish cohort consisted of 227 consecutive patients with colon cancer obtained from Hospital Clinic Pathology Department files, University of Barcelona, Barcelona, Spain. Sections from tumor microarrays were reviewed after IFRD1 staining and specimens from n=105 cases were of sufficient quality for accurate scoring. All cases were anonymized and the study was approved by the Hospital's Institutional Review Board and Ethics Committee. Patients were diagnosed between 1998 and 2005 and were subjected to curative-intent surgical resection. For the Chinese cohort, all cases were anonymized (n= 201 cases on tumor microarrays were of sufficient quality for accurate IFRD1 scoring) and the study was approved by the China Medical University First Hospital Institutional Review Board and Ethics Committee. The Chinese patient cohort was initially treated at the China Medical University first hospital. Resection specimens were obtained from patients treated between 2005 and 2009. TNM stage was determined for all patients.

Immunohistochemistry

Formalin-fixed paraffin embedded colon cancer specimens were obtained for tissue processing. Tumor and adjacent normal tissue sections or tissue microarray (TMA) sections containing representative cores from tumor and normal mucosa were used for immunohistochemistry (IHC) staining. Monoclonal antibody anti-Tis7/IFRD1 (Sigma-Aldrich, St. Louis, MO; 1:500) was incubated overnight on deparaffinized tissue or TMA sections after decloaking with Diva antigen retrieval buffer (Biocare, Concord, CA). Antigen-antibody complexes were detected using biotinylated secondary antibody and streptavidin-horseradish peroxidase.

Three trained, independent, blinded observers graded the intensity of immunostaining of the tumors and normal adjacent mucosa on a 4-category scale of 0-3 (Figure 1). The histological scoring system and individual specimen scoring were supervised by a pathologist (JCM). Adjacent uninvolved colonic mucosa that appeared histologically normal was graded separately using the same scale of 0-3. A score of 0 was assigned if there was no to minimal IFRD1 immunoreactivity, with light, scattered staining in occasional nuclei in normal mucosa or in well differentiated tumors, a score of 1+ for light staining throughout the tumor or more intense but scattered staining, a score of 2+ for uniform staining of the entire tumor with medium brown intensity, and a score of 3+ for uniform staining of the entire tumor with intense dark brown staining.

Two tumor sections from each patient were selected based on tissue quality and were used to score tumor staining. Tumor microarray patient samples were not scored if tissue integrity and quality were deemed poor by the observers. The final score was the mean of the scores from the individual observers.

Statistical Analysis

The association between IFRD1 expression with other demographic/clinical characteristics was assessed using Chi-square Nonparametric test. The primary clinical outcome was overall survival (OS) which was defined as the time from diagnosis to death due to cancer, and survivors were censored at the date of last contact. Kaplan-Meier method and Log-rank analysis were used to assess the association between IFRD1 expression and OS, while adjusting potential confounding effects of other demographic/clinical characteristics. Cox regression analysis was used to determine the independent effect of each variable on patient survival. All analyses were two-sided and significance was set at a p-value of 0.05. Statistical analyses were performed using SPSS 19.0.

RESULTS

Patient Characteristics

Colon cancer specimens from 378 patients from the United States (n=72), Spain (n=105) and China (n=201) were analyzed by immunohistochemical staining for IFRD1 expression. The average age for the entire patient cohort was 66 years (Table 1). There were 194 males and 184 females. Of 378 colon cancers, 6% were TNM stage I, 45% TNM stage II, 45% TNM stage III and 4% TNM stage IV.

The final IFRD1 staining score was the mean of the staining intensity scores of three independent observers as defined in the Methods. IFRD1 immunoreactivity scores were based on the intensity of stain (Figure 1A-1D). Overall, 36.2% of the tumors had a score of 0-1 (low) and 63.7% had a score of 2-3 (high) (Table 2).

IFRD1 expression is increased in colon adenocarcinomas compared to normal colon epithelium.

Normal colonic mucosa demonstrated no or minimal IFRD1 immunoreactivity (Figure 1E). When present in normal mucosa, staining was low in intensity, scattered and localized in the nuclei of crypt cells with minimal cytoplasmic immunoreactivity (Figure 1E inset). In contrast, almost all (373/378, 98.7%) of the colorectal cancers showed evidence of readily detectable IFRD1 expression (Figure 1B-D). Immunoreactivity was localized predominantly in the tumor cytoplasm (Figure 1B, C), with nuclear staining detectable in tumors high intensity 3+ score. (Figure 1D). Tumor clusters at the infiltrating margin exhibit more intense immunoreactivity (Figure 1F).

A majority of the patients were 60 years or older at the time of diagnosis (69.8%, 264/378, Table 2). There was a significant association between age and IFRD1 immunostaining intensity, comparing patients <60 or ≥60 and tumors with low vs. high expression ($p = 0.013$). There was no significant association of low vs. high IFRD1 expression levels with gender (Table 2). We also found a significant relationship between tumor location, i.e., right vs. left colon, and IFRD1 expression levels (Table 3, $p=0.036$). Seventy percent of right sided tumors had high IFRD1 expression compared to 59% of left sided tumors. TNM stage and IFRD1 expression showed no association (Table 3).

Increased IFRD1 expression in colon cancers is associated with reduced five year patient survival.

High IFRD1 colon cancer expression (score 2-3) correlated with decreased 5 year patient survival (Figure 2, $p=0.025$). Subgroup analysis showed that patients in the American cohort with high IFRD1 colon cancer expression had a poorer prognosis and reduced 5 year survival compared to patients in the Chinese or Spanish cohorts ($p<0.001$; Fig. 3). There was a significant difference among the three cohorts with high IFRD1 expressing tumors for age ($p=0.011$, Table 4) and gender ($p=0.007$; Table 4). Chinese and Spanish cohorts showed a male predominance for high IFRD1 expressing tumors but the American cohort had more females. There were also differences among the cohorts with respect to tumor location, but not TNM stage (Table 5). There were more right-sided tumors in the Chinese cohort, more left-sided tumors in the Spanish cohort and a relatively equal distribution of right and left sided tumors in the American cohort ($p=0.001$; Table 5).

Although high vs. low IFRD1 expression in colon cancers correlated with reduced patient survival (Figure 2), multiple regression analysis showed that simply the presence or absence of IFRD1 expression in tumors was not related to patient survival (Table 6). As expected, TNM stage ($p<0.001$; Table 6) independently predicted patient survival. In addition, country of origin ($p<0.001$; Table 6) independently predicted patient survival.

DISCUSSION

In the present study we show that expression of the transcriptional co-regulator IFRD1 is increased in colon carcinomas compared to normal colon mucosa. Univariate analysis showed that patients with high IFRD1-expressing colon cancers have a reduced 5 year survival compared to patients with low IFRD1-expressing tumors. IFRD1 expression also correlated significantly with tumor location; 70% of right sided colon cancers exhibited high IFRD1 expression compared to 58% of left sided tumors. The prognosis of right sided colon cancer is significantly worse than for left sided cancers [17] thus the reduced survival associated with high IFRD1 expression and the higher percentage of right compared to left colon tumors with high IFRD1 expression suggest a role for IFRD1 as a modulator of tumor aggressiveness.

IFRD1 is a transcriptional co-regulator that interacts with the SIN3-histone deacetylase complex which then binds to DNA promoter sites and regulates global gene transcription [11] [12]. Depending on the cell type and context, IFRD1 may act as a transcriptional co-repressor or coactivator [12] to regulate a variety of cellular processes include cell proliferation and differentiation [8]. IFRD1 exhibits a low basal level of expression in multiple organs and cell types in normal homeostasis; in contrast, its expression is highly regulated in a wide variety of tissue injury models, suggesting a conserved role for IFRD1 in the cellular response to injury and stress [6, 9, 18]. For example, in the intestine, we have shown that IFRD1 plays a role in regulating the adaptive increase in gut epithelial cell proliferation that occurs in response to resection-induced injury [6]. Following removal of 50% of the murine small intestine, the residual gut exhibits an adaptive response characterized by increased crypt cell proliferation resulting in increased crypt depth and villus length, beginning at 48-72h post resection. *Ifird1*^{-/-} mice show a blunted adaptive response to bowel resection. *Ifird1*^{-/-} mice have decreased crypt cell proliferation early after massive gut resection compared to wild types.

IFRD1 expression is regulated by growth factors including nerve growth factor, fibroblast growth factor and epidermal growth factor [16] and glucagon-like peptide 2 [16]. IFRD1 also has effects on immune cell function and on tumor immune surveillance and plays a complex role in NF-κB signaling [19]. IFRD1 has been shown to regulate viral immune evasion mechanisms in human papilloma virus-induced keratinocytes with similar effects in cervical cancer cell lines, via suppression of immune driven RelA-associated NF-κB cytokine expression mediated by the EGFR [20]; in this model, IFRD1 acts downstream of the EGFR to deacetylate NFκB/RelA. In patients with cystic fibrosis who are homozygous for the F4508 deletion mutation, IFRD1 was identified as a modifier of lung disease via effects on neutrophil effector function [21]; in this study, IFRD1 deficiency was associated with decreased NF-κB p65 transactivation, mediated by effects on NF-κB induced transcription via HDAC. In contrast, IFRD1 is a repressor of NF-κB transcriptional activity in myoblasts via recruitment of HDAC3 in a murine model of muscle regeneration following injury [19]. Finally, IFRD1 deficiency increased p65 acetylation via inhibition of histone deacetylase-dependent deacetylation in bone marrow macrophages, repressing NF-κB dependent transcription of NFATc1 [22]. Thus depending on the cellular context and lineage, IFRD1 may increase or decrease NF-κB dependent transcriptional activity [22]. In sum, the

observed worse survival for patients with high IFRD1 expressing colon cancers may result from alterations in multiple pathways, including direct effects on tumor cell proliferation [16], facilitating tumor immune surveillance evasion [20] or by changes in immune cell function [21]. In this study, we observed that there was a lack of association between TNM stage and IFRD1 expression. We have previously shown that IFRD1 regulates metabolic processes including small intestinal enterocytic lipid metabolism [6] [23]. Thus, we hypothesize that IFRD1 may regulate the metabolic status of cancer cells, which in turn may alter sensitivity to adjuvant chemotherapy. Therefore while we show that IFRD1 may influence prognosis, expression may not be correlated with TNM stage.

We observed a worse prognosis for American patients with high IFRD1 expression compared to Spanish or Chinese cohorts. Although we were unable to identify a specific causal factor, our cohort has a high percentage of African Americans (55%), who exhibit a marked disparity in outcomes in colon cancer [24, 25]. Black vs. white disparity in mortality is increased in each stage of disease but appear driven in large part by differences in late stage disease [26]. Due to the limited number of African American patients in our entire study cohort, we cannot determine whether increased IFRD1 expression in colon cancers is also significantly associated with African American populations; this will be the subject of future investigation in a larger cohort of American patients.

In summary, we have identified novel IFRD1 expression patterns in colon cancer which suggest a role for IFRD1 in increasing tumor aggressiveness and contributing to a worse prognosis. The precise mechanisms by which IFRD1 exerts its effects are unknown; the adverse effect on survival associated with high IFRD1 expression suggests that understanding these mechanisms may provide novel targets for colon cancer therapy.

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Figure 1: IFRD1 immunohistochemical staining in colon carcinomas

Tumors were analyzed for IFRD1 expression using an anti-IFRD1 monoclonal antibody. IFRD1 immunostaining intensity was quantified by scoring on a scale of 0-3. (A) Tumors with a score of 0 had no to minimal IFRD1 immunoreactivity. Scattered light staining can be observed in the nuclei of well-differentiated tumors. (B) Immunohistochemical score of 1 showed light staining throughout the tumor or more intense, but scattered staining. (C) Uniform staining of the entire tumor with medium intensity (score 2). (D) Uniform staining of the entire tumor with intense brown staining, often associated with increased nuclear staining (score 3). (E) Comparison of tumor and normal mucosal staining demonstrates that IFRD1 staining is readily detectable in colon cancer, but adjacent, uninvolved mucosa shows no or minimal IFRD1 immunoreactivity. When IFRD1 staining was detected in the uninvolved mucosa, it was low in intensity and localized in the nuclei of crypt cells with minimal cytoplasmic immunoreactivity (Figure 1E inset). (F) Tumor clusters at the invasive margin show more intense staining.

Figure 2: Reduced 5 year survival in patients with high IFRD1 expressing colon cancers
Censored patients with high IFRD1 expression in tumors demonstrate significantly poorer survival at 5 years post diagnosis compared to patients with tumors with low IFRD1 expression (*p= 0.025).

Figure 3: American patients with high IFRD1 expressing tumors have reduced 5 year survival compared to Chinese and Spanish patients

Five year survival analysis of all patients with high IFRD1 expressing tumors from American, Chinese and Spanish cohorts. American patients with high IFRD1 expressing tumors had reduced 5 year survival compared to Chinese and Spanish patients with high IFRD1 expressing tumors *** $p<0.001$).

Table 1:

Patient demographics.

Patient Demographics	
Age range of patients	25-84
Mean age of patients	66
Gender	
Males	194
Females	184
Tumor Location	
right	146
left	202
other (transverse or flexures)	30
TNM Stage	
Stage I	23
Stage II	169
Stage III	171
Stage IV	15

Table 2.
Patient age and gender distribution in low compared to high IFRD1 expressing colon cancers.

IFRD1 immunostaining intensity was scored for each cancer on a scale of 0–3 (n = 378). Colon cancers were then classified as expressing low IFRD1 levels (score of 0–1, n = 137) or high IFRD1 levels (score of 2–3, n = 241).

	Low IFRD1 (n=137)		High IFRD1 (n=241)	P-value
Age	<60	52	62	0.013*
	60	85	179	
Gender	male	79	115	0.063
	female	58	126	

Table 3.
Clinical characteristics of low vs. high IFRD1 expressing colon cancers.

IFRD1 immunostaining intensity was scored for each cancer on a scale of 0-3 (n=378). Colon cancers were then classified as expressing low IFRD1 levels (score of 0-1, n=137) or high IFRD1 levels (score of 2-3, n= 241).

		Low IFRD1 (n=137)	High IFRD1 (n=241)	P-value
Tumor Location	right	44	102	0.036*
	left	83	119	
TNM Stage	Stage I	7	16	0.660
	Stage II	65	104	
	Stage III & IV	65	121	

Table 4.**Demographics of high IFRD1 expressing cancers by country of origin.**

Tumor samples with a score of 2 or 3 were analyzed for relationship to age and gender based on country of origin.

	IFRD1 High American (n=68)	IFRD1 High Chinese (n=65)	IFRD1 High Spanish (n=108)	P-value
Age	<60	32	35	0.011*
	60	33	73	
Gender	male	33	61	0.007*
	female	32	47	

Table 5.
Clinical analysis of colon cancer patient cohort samples by country of origin.

Tumor samples with high IFRD1 expression (scored 2 or 3) were analyzed for tumor location and TNM stage based on country of origin.

		IFRD1 High American (n=68)	IFRD1 High Chinese (n=65)	IFRD1 High Spanish (n=108)	P-value
Tumor Location	right	32	45	32	0.001*
	left	36	20	76	
TNM Stage	Stage I	16	14	22	0.856
	Stage II	21	25	35	
	Stage III & IV	31	26	51	

Table 6.

Multiple regression analysis of patient variables.

Patient variables were analyzed for their independent impact on colon cancer prognosis using Cox's proportional regression model.

Cox's Proportional Hazard Regression Model				
	95% CI for RR			
	P-value	RR	Lower	Upper
Age	0.808	1.046	0.728	1.502
Gender	0.825	1.041	0.732	1.479
Location	0.768	0.968	0.783	1.198
TNM Stage	0.000	2.138	1.67	2.737
Country of Origin	0.000	0.491	0.391	0.615
IFRD1 expression	0.819	0.954	0.637	1.428