



Investigation of FIH-1 and SOCS3 expression in KRAS mutant and wild-type patients with colorectal cancer

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Abstract Colorectal cancer (CRC) is a multistep process based on the accumulation of somatic mutations in genes such as APC and KRAS. Data on the presence of mutations in KRAS gene in CRC and its relationship with clinicopathological parameters and expression of genes involved in tumor progression are scarce. We unbiasedly examined the KRAS status in samples from 99 patients and its correlation with clinicopathological parameters such as age, sex, tumor loca-

tion, lymph node metastasis, tumor stage, tumor grade, and vascular invasion. Consistent with reports of other researchers, 38.4 % of our samples harbored KRAS mutation in their genomes with preferential mutation in codon 12 (89.4 %). Nevertheless, unlike previous reports, we were not able to correlate KRAS status with clinicopathological parameters ($P > 0.05$) except for vascular invasion. Patients with KRAS mutation have more vascular invasion compared with patient having wild-type KRAS. Next, we investigated the expression of two tumor suppressor genes, factor-inhibiting hypoxia-inducible factor 1 (FIH-1) and suppressor of cytokine signaling (SOCS3), in both KRAS mutant and wild-type groups and looked for any correlation between their expression and clinicopathological parameters. Although the expression of both genes was not regular, none of the clinicopathological parameters were associated with the expressions of FIH-1 and SOCS3 at mRNA level ($P > 0.05$). However, decline in FIH-1 expression at protein level in KRAS mutant group was correlated with stage IV and grade 2 of tumor ($P \leq 0.05$). Our results demonstrated that there is no or low correlation between KRAS status, FIH-1, and SOCS3 expression with epidemiologic and clinicopathological characteristics in CRC.

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Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide with an increase in diagnosis of new patients everyday [1]. CRC is a multistep process based on the accumulation of somatic mutations in genes such as APC and KRAS [2]. It has been shown that mutation in KRAS gene is

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associated with resistance to cetuximab, anti-EGFR therapies, and a lower overall survival [3, 4].

The prevalence of KRAS mutation in CRC patients is 35–40 %, and the majority of these mutations occur in codon 12 and less frequently in codon 13 of KRAS gene [5, 6]. However, there are controversial reports on the association between KRAS mutation and poor CRC outcome. The molecular mechanisms underlying the correlation between KRAS mutations and CRC are not fully understood.

Hypoxia-inducible transcription factor 1 α (HIF-1 α) is a dimeric protein complex which controls angiogenesis, erythropoiesis, and glycolysis regulation of its target genes under hypoxic conditions. As HIF-1 α activates angiogenesis, it allows cancerous cells to survive and proliferate in low oxygen conditions and its inhibition provides new clues for HIF-1 targeting in anti-cancer therapy [7]. Factor-inhibiting hypoxia-inducible factor 1 (FIH-1) binds to HIF-1 and blocks its transactivation function [8]. In addition, FIH-1 binds to von Hippel-Lindau (VHL) tumor suppressor protein, which also functions as a transcriptional corepressor inhibiting HIF-1 α transactivation [8]. FIH-1 protein is widely expressed in human tissues, and since FIH-1 is located at chromosome 10q24, which is often deleted in some cancers, it becomes an important candidate gene to study in cancers [9–12].

There are contradictory reports on overexpression or down-regulation of FIH-1 in cancers [13, 14]. In CRC, it has been shown that the expression of FIH-1 is significantly declined [14, 15].

The suppressors of cytokine signaling (SOCS) are inhibitors of cytokine signaling that function through the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway. Eight SOCS proteins (SOCS-1 to SOCS-7 and CIS) with a similar central SH2 domain and a C-terminal domain have been identified so far [16]. SOCS proteins, however, have different mechanisms for negative regulation of JAK/STAT signaling [17]. Irregularities of the JAK/STAT pathway are associated with cancer [18]. SOCS3 inhibits JAK by binding to the cytokine receptor and JAK, recruiting tyrosine-phosphorylated receptor [19].

The expression of SOCS3 is deregulated in cancers and plays different roles depending on cancer origin. In head, neck, hepatocellular, and lung cancers, its expression is down-regulated by hypermethylation, which causes an enhanced proliferation [18, 20, 21]. In CRC, the expression of SOCS3 has not yet been evaluated.

To better understand the regulation of FIH-1 and SOCS3 genes in colonic adenocarcinoma specimens, we have examined the expressions of FIH-1 and SOCS3 in a large characterized series of CRC tissues with and without mutation in codons 12 and 13 of KRAS gene and investigated their correlation with standard clinicopathological parameters. First, the frequency of KRAS mutation in 99 CRC samples was determined, and 38.4 % of patient harbored KRAS mutation,

which was similar to previous reports [5, 6]. In addition, in agreement with previous reports, most patients with KRAS mutation had mutation in codon 12 (89.4 %) and a few patients in codon 13 (10.5 %). There was no significant difference between KRAS status neither with tumor stage nor its grade ($P > 0.05$), but it was associated with vascular invasion ($P = 0.04$).

In contrast with previous reports, expression of neither FIH-1 nor SOCS3 gene at messenger RNA (mRNA) level was correlated with clinicopathological parameters. Also, no correlation was observed between FIH-1 immunohistochemistry (IHC) scores and clinicopathological parameters such as patient age, sex, stage, and vascular invasion ($P > 0.05$). Only FIH-1 expression was inversely correlated with degree of histological differentiation and tumor grade ($P \leq 0.05$). Our results demonstrated that the expression pattern of FIH-1 or SOCS3 by itself is not a valid prognostic method in CRC.

Materials and methods

Patient samples

A total of 99 fresh colonic adenocarcinoma tissues were obtained from pathology files of Firuzgar Hospital and Mehr Hospital (Tehran, Iran) in 2013 with informed written consent in accordance with Declaration of Helsinki. All tissue samples were obtained at primary resection. None of the patients had undergone prior chemotherapy or radiation therapy. All demographic and clinicopathological data were recorded from corresponding pathology reports [age, gender, location of tumor, tumor grade (well/moderate/poor), depth of invasion, status of lymph nodes, and liver metastases]. All patients were staged according to the American Joint Commission for Cancer staging (AJCC/TNM, the sixth version) system.

DNA extraction

For each case, the percentage of tumor was determined by reviewing H&E stained slides. DNA was extracted from formalin-fixed paraffin-embedded (FFPE) specimens using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. The concentrations were measured using ND-1000 Spectrophotometer (NanoDrop Thermo Scientific, USA).

KRAS mutation analysis by pyrosequencing

The samples were PCR amplified using the KRAS v2.0 kit (Qiagen) according to manufacturer's protocol. Each reaction contained 1 \times PCR buffer, 1.5 mmol/l MgCl₂, 0.2 mmol/l of each dNTP, 5 pmol of forward primer, 5 pmol of reverse primer (biotinylated), 0.8 U of HotStar TaqDNA polymerase

(Qiagen), 10 ng of template DNA, and dH₂O to final volume of 25 µl. Cycling conditions were as follows: 95 °C 15 min, 38× (95 °C 20 s, 53 °C 30 s, and 72 °C 20 s), 72 °C 5 min, 8 °C hold. Following amplification, 10 µl of biotinylated PCR product was immobilized on streptavidin-coated sepharose beads (streptavidin sepharose high performance, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and washed in 70 % EtOH. The purified biotinylated PCR product was released into the PyroMark Q24 (Biotage, SE) with PyroMark Gold reagents (Qiagen) containing 0.3-µmol/l sequencing primer and annealing buffer. The nucleotide dispensation order for codons 12/13 was as follows:

5' ACGACTCAGATCGTAG-3' [22].

RNA extraction and complementary (DNA) synthesis from FFPE specimens

Deparaffinization of microdissected samples was done using 1 ml xylene (Mojalali), was vortexed for 10 s, and centrifuged in 14,000 rpm for 4 min. The supernatant was discarded; the pellet was dipped in 1 ml 100 % ethanol (Merck), was vortexed, and centrifuged as above; and then, the supernatant was incubated in 37 °C. RNA was purified by RNeasy FFPE Kit (50) (Qiagen GmbH) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions.

Relative expression levels of HIF-1α and SOCS3

Real-time PCR for genes of interest was carried out using Light Cycler[®] Real-Time PCR (Roche Life Science) in a final volume of 15 µl using Quanti Nova TM SYBR Green PCR Kit (Qiagen GmbH), 1 µl cDNA (>1 ng) and 0.5 µl (0.2 pmol/µl) of each primer. Cycling conditions were pre-incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 63 °C for 30 s. Real-time PCR reactions were run in duplicate. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The expression of mRNA targets relative to Hypoxanthine

Table 2 Descriptive frequently of KRAS mutation types and patients' sex

Mutation	Sex		Total
	Male	Female	
Gly12Ala	5.6 %	5.0 %	5.3 %
Gly12Asp	33.3 %	45.0 %	39.5 %
Gly12Cys	5.6 %	20 %	13.2 %
Gly12Ser	11.1 %	15.0 %	13.2 %
Gly12Val	27.8 %	10.0 %	18.4 %
Gly13Asp	16.7 %	5.0 %	10.5 %

Phosphoribosyltransferase 1 (HPRT) was determined using the Livak method. Primer sequences for HIF-1α, SOCS3, and HPRT are shown in Table 1.

Immunohistochemistry

Immunohistochemistry (IHC) was performed in Pathology Department of Mehr Hospital. Briefly, 4-mm-thick sections were prepared from formalin-fixed, paraffin-embedded tissue. The sections were dewaxed in xylene and were rehydrated in graduated ethanol solutions. After deparaffinization and rehydration, the sections were retrieved for 10 min with Tris-EDTA in microwave oven. Endogenous peroxidase activity was blocked with 10 % hydrogen peroxide (H₂O₂) for 10 min. The slides were again incubated with primary rabbit monoclonal (EPR3658) antibody to HIF-1α (ab92498, RabMab[®]) diluted at 1:250 for 60 min at 37 °C. After rinsing in buffer, the slides were incubated with the secondary antibodies (EnVision[™] + dual-link system-HRP, Dako). Tissue staining was visualized with a chromogen solution of DAB substrate (Dako). The slides were counterstained with hematoxylin, dehydrated, and mounted. Negative control of phosphate-buffered solution (PBS) was used as the primary antibody for the negative controls. Positive control consisted of samples known to strongly express HIF1AN (human breast carcinoma).

Scoring criteria

HIF-1α immunohistochemical semiquantitation was performed using the H-score. Five-hundred tumor cells from the most cellular microscopic high-power fields (×400) were counted. The H-score is given as the sum of percentage of stained tumor nuclei multiplied by an ordinal value corresponding to the intensity level (0 = none, 1 = weak, 2 = moderate, and 3 = strong). With four intensity levels, the resulting score ranged from 0 (no staining in the tumor) to 300 (diffuse intense staining of the tumor).

Table 1 The primers used for real-time PCR reaction

Gene name	Sequence (5' to 3')
FIH-1α forward	CCGGATCAGTTCGAGTGC
FIH-1α reverse	TTAGGGAACCTCTCGTAGTCG
SOCS3 forward	AGGAGAGCGGCTTCTACTGG
SOCS3 reverse	GACTGGGTCTTGACGCTGAG
HPRT forward	GCTATAAATTCTTTGCTGACCTGCTG
HPRT reverse	AATTACTTTTATGTCCCCTGTTGACTGG

Table 3 Distributions of clinicopathologic characteristics in KRAS and wild-type patients with CRC

Characteristic	Mutant KRAS	Wild-type KRAS	<i>P</i> value
Sex			0.424
Male	57.8 %		
Female	42.2 %		
Tumor grade			0.261
G1	65.8 %	49.2 %	
G2	26.3 %	41 %	
G3	7.9 %	9.8 %	
Tumor stage			0.89
I	7.9 %	8.2 %	
II	26.3 %	32.8 %	
III	44.7 %	42.6 %	
IV	21.1 %	16.4 %	
Tumor localization			0.19
Left colon	12.87 %	28.71 %	
Right colon	24.75 %	31.68 %	
Vascular invasion	50 %	29.5 %	0.4

Statistical analyses

Statistical analyses were performed using SPSS 16.0 software. The association between non-parametric variables was assessed with chi-squared test. Parametric variables were compared using independent sample *t* test. $P < 0.05$ was considered to indicate a statistically significant difference. *T* test was used to evaluate the difference between HIF-1 α and SOCS3 expression scores between KRAS mutant and wild-type groups, and chi-squared analysis was performed for categorical variables. The results were defined as $P < 0.05$ for statistical significance. Statistical significance was evaluated with one-way ANOVA with Dunnett's post hoc test to compare selected groups of data. The $\Delta\Delta C_t$ values were used to determine the statistical significance of differences between

groups for PCR-based studies. Two-way ANOVA with Bonferroni correction was used to compare selected groups of data with respect to time. Scatter plot graphs are $2^{-\Delta C_t}$ values for expressed HIF-1 α and SOCS3 genes and are normalized against HPRT1 gene.

Results

KRAS mutation

Primary samples from 99 CRC patients were analyzed for KRAS mutation using pyrosequencing method. From a total of 99 patients, 61.6 % were KRAS wild-type and 38.4 % were KRAS mutant. It seems that male patients were more likely to possess KRAS mutation than females (57.8 vs. 42.2 %). Codon 12 mutations (89.4 %) were the most common mutation, whereas codon 13 mutations were less common ($n = 4$, 10.5 %). The p.G12D point mutation was the most common mutation of codon 12 (39.5 %), which results in an amino acid substitution at position 12 in KRAS, from glycine (G) to aspartic acid (D). On the other hand, p.G12V mutation (18.4 %) results in an amino acid substitution at position 12 in KRAS, from glycine (G) to valine (V). The p.G12A point mutation was the least frequently observed point mutation in codon 12 (Table 2). None of the samples had mutation in codon 61. The median age of patients was 57 years (27–93 years); 43 patients (43.3 %) were at stage III and 18 patients (18.2 %) were metastatic at the time of diagnosis. KRAS mutation was found in 44.7 % of stage III tumors, and 55 tumors (55.6 %) harboring KRAS mutations were well differentiated (Table 2). Although 57.9 % of mutations were localized in the right colon, the difference did not reach statistical significance, most likely due to the limited number of patients ($P = 0.3$). Patients with KRAS mutations did not exhibit statistically significant differences in terms of gender, tumor location, tumor grade, presence of lymph node metastasis, stage, and

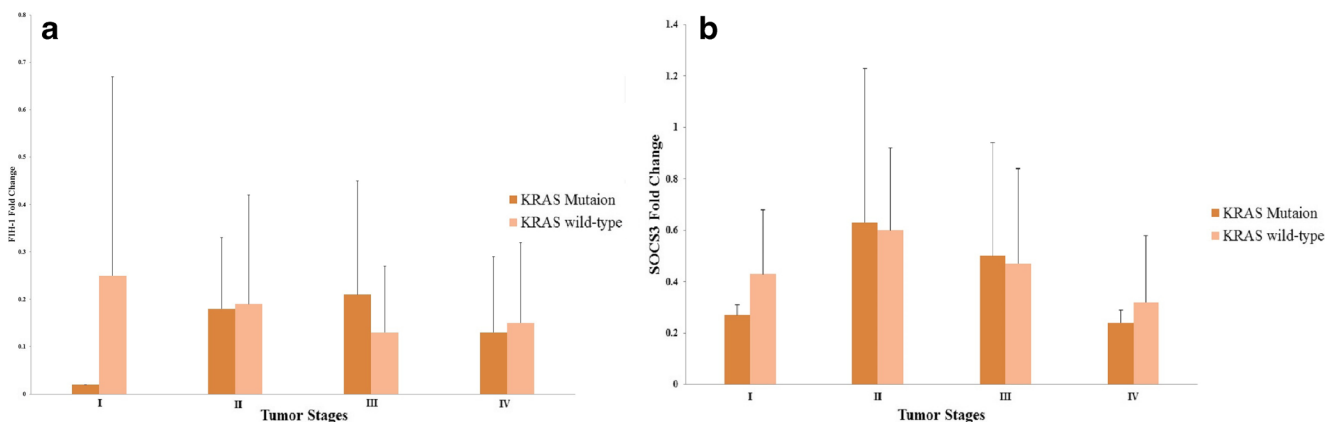


Fig. 1 Expression of HIF-1 (a) and SOCS3 (b) in KRAS mutant and wild-type groups and their association with tumor stages

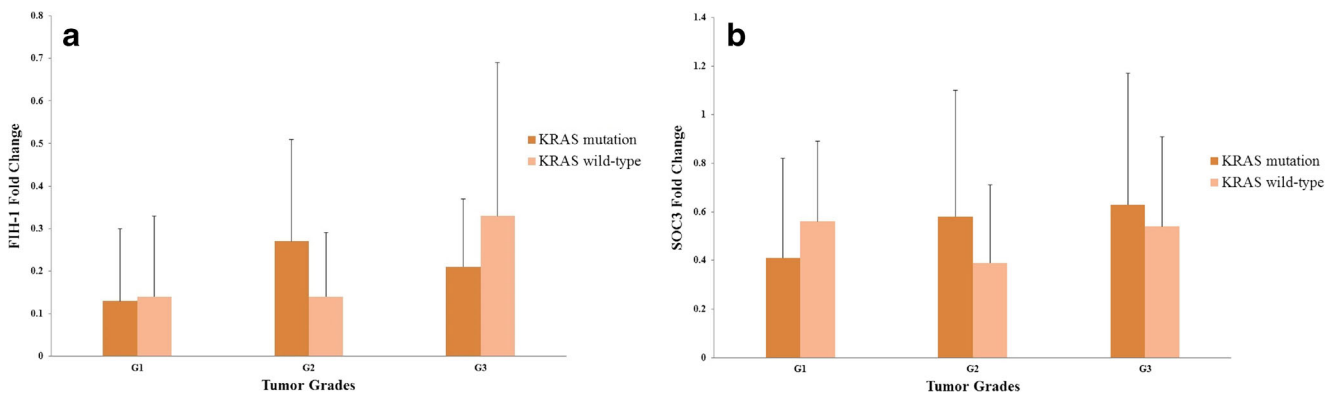


Fig. 2 Expression of FIH-1 (a) and SOCS3 (b) in KRAS mutant and wild-type groups and their association with tumor grades

median tumor diameter. The only difference observed when comparing KRAS status and vascular invasion in tumors was that those harboring mutations had more vascular invasion ($P=0.04$) (Table 3).

Correlation between FIH-1 and SOCS3 expressions at mRNA level and clinicopathological parameters

From a total of 99 patients with CRC, 38.4 % had mutation in KRAS gene and the rest of them had KRAS wild-type gene. There was no significant difference between KRAS status and FIH-1 and SOCS3 expressions at mRNA level ($P>0.05$).

Gene expression analysis was done using real-time PCR for FIH-1 and SOCS3 genes in both mutant and wild-type samples. Albeit, the expressions of FIH-1 and SOCS3 showed deregulation and did not reach statistically significant level ($P>0.05$), perhaps due to limited number of samples in stages I and IV (Fig. 1). In addition, no significant correlation was found between FIH-1 and SOCS3 expressions at mRNA level and tumor grades in KRAS mutant and wild-type groups ($P>0.05$) (Fig. 2). Also, FIH-1 and SOCS3 expressions were not significantly associated with vascular invasion and non-

vascular invasion groups in patients with KRAS mutation and wild-type KRAS ($P>0.05$) (Fig. 3).

Correlation between FIH-1 expression at protein level and clinicopathological parameters

FIH-1 was widely expressed among the analyzed colorectal adenocarcinomas. Ninety-nine percent of tumors stained positive for FIH-1 in the nucleus compartments (Fig. 4). This strong nuclear FIH-1 expression was observed in a large proportion of tumor cells, with a median intensity of 2 and a median percentage of over 80 % of cells. Nearly six percent of tumors were negative for FIH-1. Nuclear FIH-1 expression had a 208 median score of cells. Apart from the tumors of epithelial cells, FIH-1 was widely expressed in non-epithelial elements within the tumors. FIH-1 was expressed in stroma of 78 % of tumors, in the vasculature of 81 % of tumors, and in the infiltrating inflammatory cells in 74 % of tumors.

As shown in Fig. 5a, the pattern of FIH-1 expression at protein level and tumor stages have an inverse relationship, as increase in tumor stage in KRAS mutant group decreases

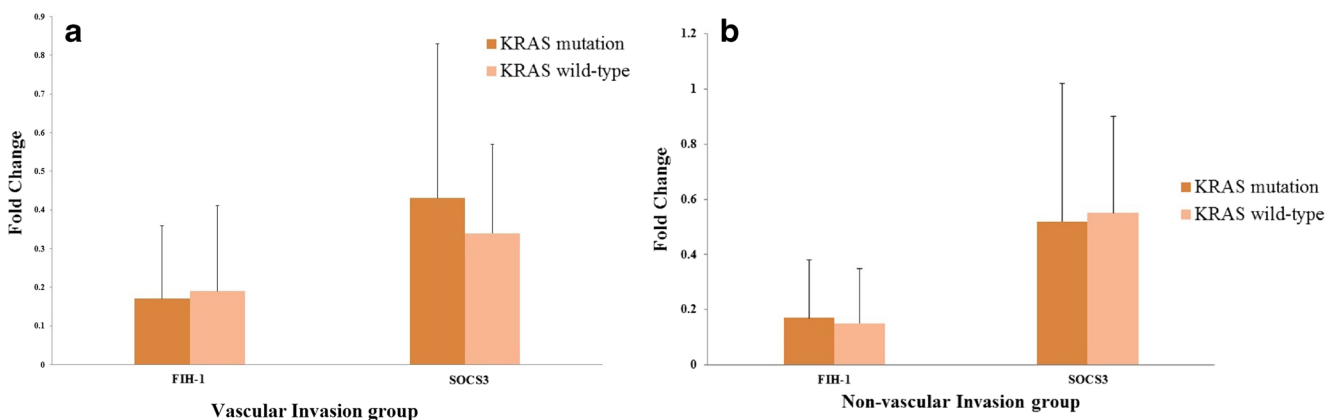
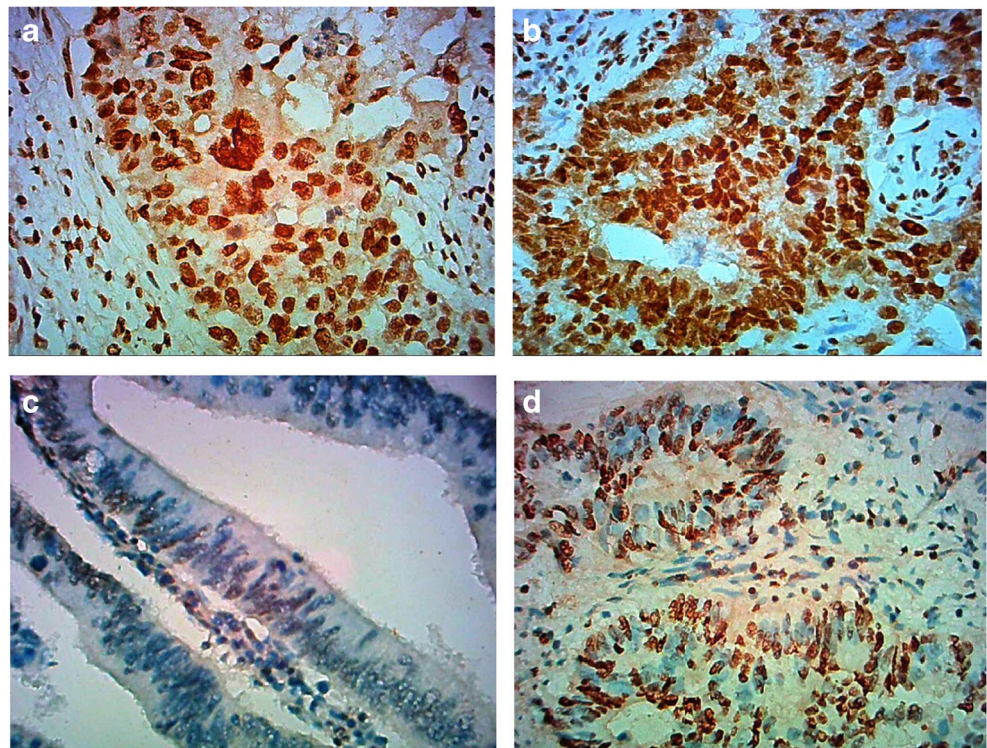


Fig. 3 Expression of FIH-1 and SOCS3 in KRAS mutant and wild-type groups and their relationship in two groups of patients, having vascular invasion (a) and not having vascular invasion (b)

Fig. 4 IHC for FIH-1 protein and strong expression of FIH-1 in wild-type group (a, b) and weak expression in mutant group (c, d)



the expression of FIH-1 compared with wild-type group. However, the correlation of FIH-1 expression and tumor stages in KRAS mutant and wild-type groups were not statistically significant ($P > 0.05$), except for stage IV in which FIH-1 is down-regulated in patients with KRAS mutation ($P = 0.04$).

There was no correlation between the expression of FIH-1 at protein level and tumor grades in KRAS mutant and wild-type groups ($P > 0.05$) except for grade 2, in which the expression of FIH-1 was decreased significantly in KRAS mutant group compared with wild-type group ($P \leq 0.05$) (Fig. 5b). Finally, FIH-1 expression in both KRAS status in vascular and non-vascular invasion groups was not statistically correlated ($P > 0.05$), although it seems to be down-regulated in both groups in patients with KRAS mutation (Fig. 5c).

Discussion

To better understand the regulation of FIH-1 and SOCS3 genes in colonic adenocarcinoma specimens, we have examined the expressions of FIH-1 and SOCS3 in a large characterized series of CRC tissues with and without mutation in codons 12 and 13 of KRAS gene and investigated their correlation with standard clinicopathological parameters.

From a total of 99 patients with CRC, 61.6 % were KRAS wild-type and 38.4 % were KRAS mutant. It appears that male patients are more likely to harbor KRAS mutation than females (57.8 vs. 42.2 %), which is in contrast with a previous report [23] perhaps due to differences in number and patient population. Also, there was no association between KRAS mutation and tumor stage ($P = 0.89$) and tumor grade ($P = 0.26$), which is in contrast with previous reports [23,

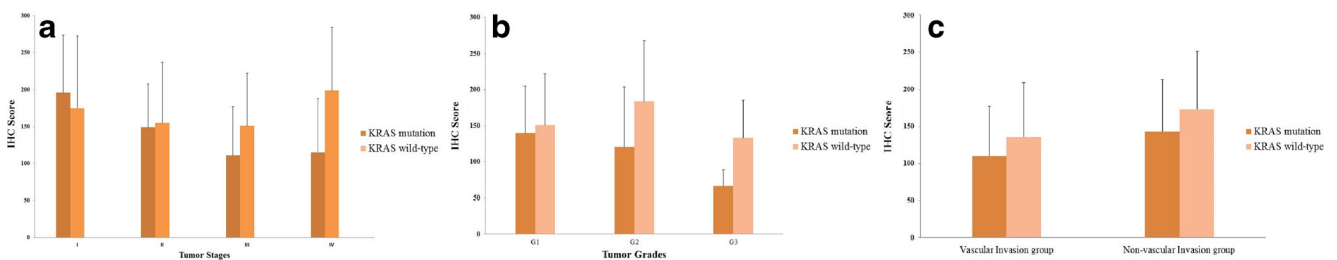


Fig. 5 Expression of FIH-1 expression at protein level in KRAS mutant and wild-type groups using IHC and their correlation with tumor stage (a), tumor grade (b), and vascular invasion (c)

24]. However, 57.9 % of mutations were localized in the right colon but the difference did not reach statistical significance, most likely due to the limited number of patients ($P=0.3$). Moreover, there was no association between KRAS status and gender, presence of lymph node metastasis, and median tumor diameter ($P>0.05$). However, KRAS status was associated with vascular invasion. Vascular invasion was seen in 37.4 % of patients, and it happened more frequently in KRAS mutant group compared with wild-type group (50 vs. 29 %) ($P=0.04$).

Detection of a molecular marker such as a unique gene expression pattern will help us to predict the invasive or migratory potential of a primary tumor to avoid overtreatment or undertreatment of patients. So, there are some reports that show FIH-1 and SOCS3 expressions might be associated with clinicopathological parameters [7, 10, 14, 21, 25]. Hence, we analyzed the expressions of FIH-1 and SOCS3 in 99 CRC cases in search for any correlation between the expression of both genes and clinicopathological parameters such as tumor stage, tumor grade, and vascular invasion in KRAS mutant and wild-type groups. Although the expression of both genes at mRNA level showed deregulation in KRAS mutant group compared to wild-type group, it was not correlated to the tumor stage ($P>0.05$) (Fig. 1). Perhaps this can be due to limitation in patient number or the large number of patients in stage III. In addition, no correlation was found between FIH-1 and SOCS3 expressions at mRNA level with tumor grade or vascular invasion ($P>0.05$) (Figs. 2 and 3). Only at protein level was the down-regulation of FIH-1 in KRAS mutant group associated with stage IV ($P=0.04$) and grade II of tumor ($P=0.05$).

In summary, first we investigated the frequency of KRAS mutation in codons 12 and 13 in 99 CRC samples using pyrosequencing and investigated the correlation between KRAS status and clinicopathological parameters such as sex, age, tumor stage, tumor grade, lymph node metastasis, and vascular invasion. In contrast with previous reports, we did not observe any correlation between KRAS status and clinicopathological parameters ($P>0.05$) except for vascular invasion. It seems that vascular invasion happened in patients harboring KRAS mutation in their genome compared to wild-type (50 vs. 29 %) ($P=0.04$) [23, 24, 26]. Finally, we analyzed the expressions of FIH-1 and SOCS3 using qRT-PCR and correlated the expression results with clinicopathological parameters. There was no association between the expression of both genes at mRNA level and clinicopathological parameters ($P>0.05$). As described above, decreased expression of FIH-1 at protein level is associated with stage IV and grade II of tumor level ($P\leq 0.05$).

This study suggested that there is no or low correlation between KRAS status and epidemiologic and clinicopathological characteristics since in our study, KRAS status was only correlated with vascular invasion. Furthermore, our

results demonstrated that the prediction of cancer prognosis using pattern of FIH-1 and SOCS3 expression is not a reliable approach for prognostic tumor stage, tumor grade, and vascular invasion. However, more research is needed to evaluate our results, for example, using large number of CRC samples. Validation of biomarkers will help to improve management decisions for individual patients based on tumor biology. Notably, this may also be helpful in development of novel therapeutic targets to assist treatment of cancers.

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Compliance with ethical standards

Conflicts of interest None

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