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Co-overexpression of self-renewal markers SALL4 and HIWI is correlated with depth of tumor invasion and metastasis in colorectal cancer

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Abstract

Background: SALL4 and HIWI are involved in the maintenance of self-renewal capacity of stem cells. Several scrutinizes have demonstrated that SALL4 and HIWI play a key role in cancer development. However, the correlation between these genes regarding different clinicopathological features of patients with colorectal cancer (CRC) is still unclear.

Methods: The expression of SALL4 and HIWI in different clinicopathological features of 46 CRC patients was analyzed using relative comparative real-time PCR.

Results: mRNA expression levels of SALL4 and HIWI genes were significantly correlated with each other in CRC ($P = 0.013$, Pearson correlation = 0.364). HIWI expression was notably increased in tumors with overexpression of SALL4 in comparison with other samples. This correlation was significant in non-metastatic CRCs compared to the metastatic tumors and in invaded tumors to the serosa (T3/T4) in comparison with non-invaded tumors (T1/T2).

Conclusions: Based on the significant association of SALL4 and HIWI in different indices of CRC poor prognosis, it may be concluded that simultaneous expression of these genes is notably contributed to the growth and development of the disease, and therefore, their co-overexpression may be considered for prognosis of aggressive CRCs.

Keywords: SALL4, HIWI, Colorectal cancer, Oncogene

Background

Colorectal cancer (CRC) is the second most common cancer and the fourth main reason of cancer-related death in the world [1, 2]. CRC is the third and fourth most common cancer in Iranian men and women, respectively [3]. Albeit therapeutic modalities, such as primary surgery and auxiliary treatments, which generally increase patients' survival, specific and targeted therapies are needed to further improve the patients' life quality [4, 5].

The zinc finger transcription factor Sal-like protein 4 (SALL4) maintains pluripotency and self-renewal capacities of embryonic stem cells (ESCs)[6]. It was firstly cloned based on its sequence homology to Drosophila spalt gene [7–9]. The SALL4 expression in adults is closed to the germline and diagnosed within nonspecific spermatogonia [10]. Subsequently, SALL4 expression was reported in different malignancies including germ cell tumors [11, 12], glioma [13], sharp myeloid leukemia [14], as well as breast [15], colorectal [16], lung [17], and liver [18] cancers. Furthermore, SALL4 communicates with the Wnt/β-catenin pathway as one of the major cell signaling pathways [14] to advance cell growth and cancer development [6, 19].

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PIWI-like RNA-mediated gene silencing 1 (PIWIL1), also called HIWI, is an evolutionarily conserved member of the PIWI subfamily of Argonaut proteins involved in stem cell self-renewal, RNA silencing, and regulation of translation. As a critical modulator of the self-renewal capacity, it may play a role in germline and hematopoietic stem cells. [20–23]. HIWI is expressed in tumor tissues, and its overexpression was reported to be associated with poor prognosis in patients with different human malignant tumors, including seminomas [24, 25], esophageal squamous cell carcinoma [26], adenocarcinoma of the pancreas and gastric [27, 28], soft-tissue sarcoma [29], endometrial carcinoma [30] glioma [31], as well as colorectal and lung cancers [32–34]. Further, HIWI was upregulated in cervical cancer and played an important role in oncogenesis [35].

Due to the involvement of SALL4 and HIWI in embryonic and germline stem cells properties, the importance of these genes in CRC tumorigenesis and their correlation with clinicopathological features of the patients is considerable [16]. Since the correlation between SALL4 and HIWI in different pathological states of CRC was not investigated yet, our aim in this study was to assess the linkage between these genes in CRC and their association with each other in different clinicopathological features of the patients.

Methods

Study population

The tumor and adjacent non-tumor tissue specimens of 46 CRC patients were obtained before any therapeutic procedure including preoperative chemotherapy, radiotherapy, and surgery at Omid Oncology Hospital of Mashhad University of Medical Sciences (MUMS), Mashhad, Iran [16, 32]. The whole tissue specimen's collection procedure was approved by the Ethics Committee of MUMS, and all patients formally declared their consent to be enrolled in the study and publication. All specimens were confirmed by postsurgical pathology reports. The clinical data of patients were reviewed according to the TNM classification for colorectal cancer [36].

Quantitative real-time RT-PCR assay

Total RNA was extracted from tumor and non-tumor adjacent tissues of the patients using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) was performed with the Qiagen One-Step RT-PCT kit (Qiagen, Hilden, Germany), and RNA was reverse-transcribed to cDNA using the First-Strand Synthesis kit (Takara, Japan) following the manufacturer's instructions. Quantitative real-time PCR was accomplished using the specific primer sets (Table 1) with SYBR Green Master Mix (Roche, Basel, Switzerland), containing ROX as a reference dye on a Stratagene Mx3000P real-time thermocycler (Stratagene, La Jolla, CA). The used thermal profile included 10 min at 95 ℃ followed by 40 cycles of 15 s at 95 ℃, 30 s at 57 ℃, and 45 s at 72 ℃. For quantification, the samples were normalized against the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA [37]. The Ct values of the GAPDH were analyzed using BestKeeper (http://blooge.cn/RefFinder/) to determine the housekeeping gene stability. Experiments were performed in triplicate. Gene expression was analyzed the using ΔΔCt method. Log 2 fold change > 2″ was considered as overexpression, while log 2 fold change < − 2 was measured as underexpression. The range of log 2 fold change between 2 and − 2 was considered as normal (unchanged) expression.

Statistical analysis

Statistical analysis was performed with SPSS 25 statistical package (SPSS, Chicago, IL). Independent sample t test and ANOVA were used to compare expression levels of each gene between different categorical data. The correlation between SALL4 and HIWI levels of gene expression was assessed by Pearson's correlation. The results were considered to be statistically significant at $p < 0.05$.

Results

Real-time PCR analysis was used to quantify the mRNA expression levels of HIWI and SALL4 on 46 CRCs and adjacent non-tumor tissues. A total of 20 (43.5%) women and 26 (56.5%) men were recruited for this study, with an average age (± standard deviation) of 53.80 (± 14.89). The minimum and maximum of age among patients were 21 and 86 years, respectively. Thirteen tumor samples were taken from proximal part (28.3%) and 33 samples from distal (71.7%) part of colorectal with a mean size (± SD) of 4.85 (± 1.55) cm. Table 2 presents the

Table 1 Primer sequences used for quantitative real-time RT-PCR

	Forward primer sequence	Reverse primer sequence
SALL4	CCAAAGGCAACTTAAAGGTTCAC	CCGTGAAGACCAATGAGATCTC
HIWI	ATGATTGAAGTGGATGACAGAACTG	TACTTGACAACAGACAGACAACTAT
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATATCCACT

Table 2 Correlation of SALL4 and HIWI genes expression with clinicopathological features of the patients

Factors	Number %		HIWI		SALL4		*P* value
			Normal/ Under	Overexpression	Normal/ Under	Overexpression	
Sex	Female	20 (43.5)	14	6	2	18	0.354
	Male	26 (56.5)	16	10	4	22	
Tumor location	Proximal	13 (28.3)	8	5	2	11	0.124
	Distal	33 (71.7)	22	11	4	29	
Grade of differentiation	WD	29 (63)	19	10	4	25	0.004*
	MD	16 (43.8)	10	6	1	15	
	PD	1 (2.2)	1	0	1	0	
Depth of invasion	T1,2	8 (17.4)	3	5	0	8	0.031*
	T3,4	38 (82.6)	27	11	6	32	
Lymph node metastasis	N0	33 (71.7)	23	10	5	28	0.050*
	N1	9 (19.6)	4	5	0	9	
	N2	4 (8.7)	3	1	1	3	
Stage of progression	I/II	34 (73.9)	23	11	2	29	0.362
	III/IV	12 (26.1)	7	5	1	4	

* statistically significant *WD* well differentiated, *MD* moderately differentiated, *PD* poorly differentiated, *T* depth of tumor invasion, *N* node metastasis. "Log 2 fold change > 2" was considered as overexpression, while log 2 fold change < − 2 was measured as underexpression. The range of log 2 fold change between 2 and -2 was considered as normal (unchanged) expression

clinicopathological features of the patients. Figure 1 shows the analysis of the housekeeping stability using the BestKeeper tool.

Correlation between SALL4 and HIWI mRNA expression in CRC

mRNA expression levels of SALL4 and HIWI genes were significantly correlated with each other in CRC. There was a significant direct correlation between the genes in the tumors with co-expression of both genes. As depicted in Fig. 2, HIWI expression was notably increased in tumors with overexpression of SALL4 in comparison with tumors without SALL4 overexpression ($P=0.013$, Pearson correlation = 0.364).

Correlation between expression of the genes and metastasis

As represented in Table 2, there is a significant correlation between SALL4 and HIWI genes in non-metastatic samples ($p=0.05$, Pearson correlation = 0.344). Apperceived that of 33 (71.7%) samples with no metastasis, HIWI and SALL4 were overexpressed in 10 (30.3%) and 28 (84.8) samples, respectively. Of the 13 (28.2%) metastatic tumor samples, SALL4 was overexpressed in the majority of tumors (12 of 13, 92.3%), while overexpression of HIWI was detected in nearly half of the tumors (6 of 13, 46.1%).

Correlation between expression of the genes and depth of tumor invasion

SALL4 and HIWI mRNA expression was significantly correlated with each other in invaded tumors to the serosa indicating T3 and T4 depth of tumor invasion ($P=0.03$, Pearson correlation = 0.352) compared to non-invaded T1 and T2 tumor samples. According to the analyses, from 38 samples with T3 and T4 depth of invasion, 11 (28.9%) samples showed HIWI overexpression, while 32 (84.2%) samples were SALL4 overexpressed.

Correlation between expression of the genes and grade of tumor differentiation

Expression of SALL4 and HIWI genes was significantly correlated with each other in moderately differentiated tumors ($P=0.004$, Pearson correlation = 0.676), compared to either well or poorly differentiated samples. Overexpression of SALL4 and HIWI was detected in 93.75% [15 of 16], and 37.5% [6 of 16] of moderately differentiated samples, respectively.

Discussion

In this study, we showed that SALL4 and HIWI are not only overexpressed in CRC, but also are significantly correlated with each other in different indices of CRC poor prognosis including depth of tumor invasion and lymph node metastasis. This finding may emphasize the

CP data of housekeeping Genes by BEST KEEPER

	hGAPDH
n	29
geo Mean [CP]	19.71
AR Mean [CP]	19.83
min [CP]	16.00
max [CP]	23.00
std dev [+/- CP]	1.83
CV [% CP]	9.25
min [x-fold]	-13.13
max [x-fold]	9.75
std dev [+/- x-fold]	3.56

Pearson correlation coefficient (r) by BEST KEEPER

hGAPDH

Pearson correlation coefficient (r)

BestKeeper vs.	hGAPDH
coeff. of corr. [r]	1.000
p-value	1.000

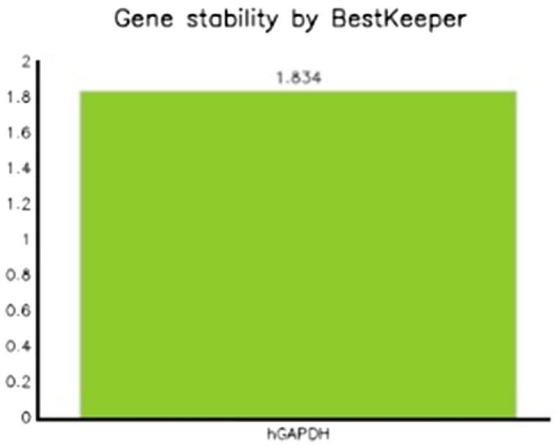

Fig. 1 Analysis of the housekeeping gene GAPDH using BestKeeper tool to determine the stability of the used normalizer

impact of SALL4 and HIWI simultaneous expression in CRC aggressiveness, and extend the role of these self-renewal markers to cancer progression and metastasis.

SALL4 plays a role in embryonic development, and its normal expression in differentiated tissues is restricted to hematopoietic stem cells and germ line [38].Various factors regulate the SALL4 transcription [39, 40]. It has been indicated that SALL4 expression increases cancer risk and mortality [41]. SALL4 has been shown as a determinant of poor prognosis in CRC [16] as well as an essential biomarker for early-stage screening of the patients [42]. SALL4 inhibition restricts CRC oncogenesis through modulating Gli1 expression [43].

It has been shown that cancer stem cell marker SALL4 may be used potentially as a prognostic marker as well as a therapeutic target for ESCC, endometrial and solid cancers [44–46]. It can also be an ideal marker for lung cancer diagnosis [17]. SALL4 is a diagnostic agent for metastasis of the yolk sac tumors and a very sensitive and specific marker for metastatic germ cell tumors [11].

It has been demonstrated that SALL4 overexpression can affect different cell signaling pathways [47]. SALL4 can progress gastric cancer metastasis through upregulation of TGF-β1 and activation of the TGF-β/SMAD signaling pathway [48], Furthermore, SALL4 increases the expression of ZEB1 while represses the expression of E-cadherin, holds cell dispersion in basal-like breast cancer [49].

The growth and propagation of the tumor in the body appear to depend on cancer stem cells [50]. HIWI acts as a transcriptional regulator of apoptosis and cell division in normal and cancerous stem cells [51]. It has been shown that HIWI may be a negative growth regulator [52]. The survival of the male pancreatic cancer patients was associated with different levels of HIWI mRNA expression [51]. Furthermore, it has been found that HIWI plays an oncogenic role in the development of glioma, and its silencing can inhibit the proliferation, migration, and invasion of glioma cells (significantly caused cell cycle to arrest at G0/G1, reduced cell proliferation, and incremented apoptosis) [53]. While HIWI is a poor prognostic factor for patients with soft tissue sarcoma, it has a therapeutic value for lung cancer that can be used as a molecular target for inhibiting lung cancer tumor stem cells [28, 33].

In our study, there was a correlation between overexpression of SALL4 and HIWI in non-metastasized tumors. Since both the genes play role in stem cell phenotype, their expression as well as their correlation with each other at the beginning steps of tumor growth and development may emphasize the importance of SALL4 and HIWI as progressive markers for CRC aggressiveness and metastasis. It has been observed that expression of SALL4 is significantly associated with metastasis of tumor cells in CRC [16]. SALL4 promotes metastasis in gastric cancer by activating the TGF-β / SMAD signaling pathway and inducing EMT [48]. HIWI is also involved in the maintenance and development of germ cells, and its overexpression may cause malignant germline growth [24].

According to the results, overexpression of SALL4 and HIWI was significantly correlated with each other in patients with invaded tumor cells to the serosa indicating the depth of tumor T3/T4. It may highlight the potential of these genes in the progress of tumor invasion and introduce SALL4 and HIWI as markers for CRC invasiveness. It has been reported that SALL4 inhibition

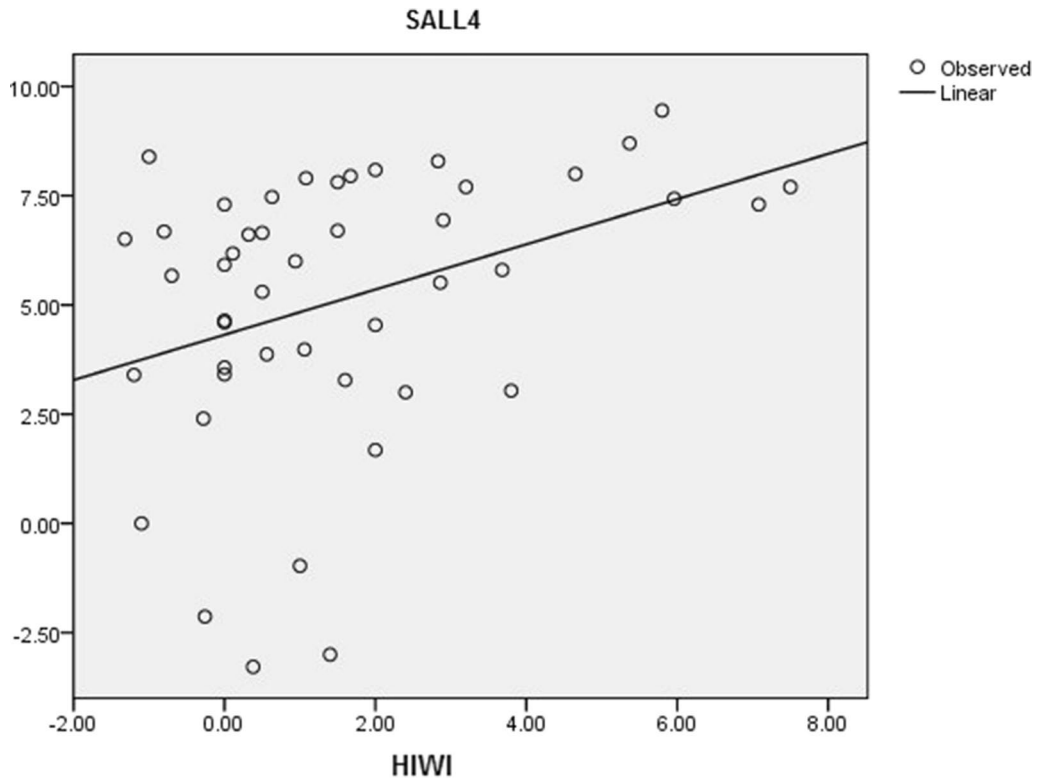

Fig. 2 Regression plot presents the correlation between expression of SALL4 and HIWI genes in CRC ($P=0.013$, Pearson correlation = 0.364). Correlation between SALL4 and HIWI genes as significant in non-metastatic samples ($p=0.05$, Pearson correlation = 0.344). The X- and Y-axes show the level of SALL4 and HIWI gene expression, respectively

significantly limited cellular migration and invasion in colorectal [43] gastric, and esophageal malignancies [44, 48]. Furthermore, SALL4 silencing in endometrial cancer inhibits cell growth and proliferation and induces apoptosis in vitro [45].

SALL4 and HIWI are involved in chemotherapy resistance of different malignancies. It has been detected that the HIWI gene is associated with the overall survival of patients and chemotherapy response in epithelial ovarian cancer [54]. Also, the relationship between HIWI (PIWIL1) and cell signaling pathways can indicate that PIWIL1-expressing tumor cells are important for targeted treatment [55]. In line with this study, it has been reported that SALL4 increases the resistance of endometrial cancer cells to chemotherapy through the regulation of ABCB1 [56]. Furthermore, SALL4 downregulation in breast cancer cells results in a decreased chemotherapy resistance by reducing the expression of ABCG2 [57]. In addition, the amount of SALL4 expression was correlated with tumor grade and with the location of these tumors within the oral squamous cell cancer [58]. A study by Nikki R. Kong and colleagues recognized hundreds of genes that SALL4 straightly regulates [59]. These findings may highlight the importance of SALL4 and HIWI in drug resistance of cancer and based on our results, this function may coordinately perform through significant correlation and concomitant expression of these genes in tumors.

Based on the simultaneous expression pattern of these two genes in CRC and according to their predetermined roles in the self-renewal capacity of stem cells, a linkage between SALL4 and HIWI can be suggested in CRC maintenance. Furthermore, due to the significant association of these genes in different indices of CRC poor prognosis it may be hypothesized that simultaneous expression of the genes is notably contributed to the growth and development of the disease, and therefore, co-overexpression of SALL4 and HIWI genes may be considered for prognosis of aggressive CRC.

Conclusion

We demonstrated the significant correlation between the expression pattern of self-renewal markers SALL4 and HIWI in CRC and presented the impact of their simultaneous expression on the depth of tumor invasion and metastasis in CRC. To the best of our knowledge, this is

the first report regarding the correlation between SALL4 and HIWI in CRC. This finding may extend played role of SALL4 and HIWI from self-renewal to invasive/aggressive behavior of tumor cell and may confirm their potential as therapeutic targets to inhibit CRC progression and metastasis.

Abbreviations
CRC: Colorectal cancer; SALL4: Sal-like protein 4; PIWIL1: PIWI-like RNA-mediated gene silencing 1; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

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Author contributions
MMF designed the concept, conducted and performed the experiments, analyzed the data and edited the manuscript. SS performed the tests and drafted the manuscript. Both authors read and approved the final manuscript.

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Availability of data and materials
All raw data are available in case of request.

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran, and written informed consent was obtained from all recruited patients to participate in the study.

Consent for publication
Written informed consent was obtained from all recruited patients to use the related clinicopathological data in the publications.

Competing Interests
The authors declare that there is no conflict of interest.

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