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Original Article

Association of TWIST1 mRNA Expression with the Clinicopathological Characteristics of Colorectal Cancer Patients

Mohammad Hossein Sanei [1], **Maryam Katani** [1*], **Maryam Sanei** [2]

[1] Department of Pathology, Isfahan University of Medical Sciences, Isfahan, Iran
[2] Medical Student, Isfahan University of Medical Sciences, Isfahan, Iran

Corresponding Author: Maryam Katani, MD, Botany Department of Pathology, Isfahan University of Medical Sciences, Isfahan, Iran. Tel: +98-9196404694, E-mail: marykatani22@gmail.com

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Abstract

Introduction: TWIST is a key role player in the Epithelial-Mesenchymal Transition (EMT), which is determinative for the tumor progression and metastasis. The main exploratory goal of this study was to access the association of TWIST1 mRNA expression with clinical pathological parameters of Colorectal Cancer (CRC) patients.

Materials and Methods: Seventy formalin-fixed/paraffin-embedded colorectal tumor specimens were evaluated for TWIST1 mRNA expression by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).

Results: The mean Relative Quantification (RQ) of TWIST1 mRNA expression in the CRC patients was 10.5 ± 2.7 No significant association was observed between age ($p = 0.786$), gender ($p = 0.163$), tumor location ($p = 0.300$), tumor size ($p = 0.438$), and the TWIST1 RQ. The mean value of TWIST1 RQ was significantly ($p = 0.040$) higher in the patients with nodal invasion in comparison with the patients without nodal invasion. The subgroup of tumor specimens with T3-4 stages had significantly ($p = 0.024$) higher mean TWIST1 RQ than T1-2 stage specimens. Moreover, the mean value of TWIST1 RQ of the tumors with distant metastasis was significantly ($p = 0.032$) higher than tumors with no metastasis.

Conclusions: Taking together, high TWIST1 mRNA expression was associated with high nodal stage, advanced clinical stage, and distant metastasis. Therefore, TWIST1 can be a biomarker for CRC patients.

Keywords: Colorectal Cancer, TWIST1, Metastasis, Clinicopathological Parameters

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Introduction

It is believed that CRC is one the most common cancer[1-3] and has been identified as the fourth most common cause of cancer deaths, accounting for 9.2% of deaths worldwide.[4,5] CRC is the result of the accumulation of several different genetic changes that cause expression of new features at the malignant cells including metastasis. Metastasis is the deathliest characterization of cancer cells and the last step of tumor progression. More than 90% of the cancer-related deaths are due to metastasis.[6]

The EMT of epithelial cells is defined as the loss of epithelial characteristics and acquisition of mesenchymal phenotypes. EMT is one of the main role-playing pathways for metastasis. Although this complicated transition has been observed in different physiological phenomena including embryonic development,[7,8] wound healing, and organ fibrosis,[9] its ectopic expression can cause tumor progression and induction of invasive or metastatic phenotypes in cancer cells.[10,11] EMT causes loss of tight junctions and apical-basal polarity in tumor cells. The cancer cells down regulate their epithelial markers like E-cadherin and instead exhibit mesenchymal characterizations including N-cadherin.[12,13] Also, the cytoskeletal rearrange its structure for initiation of migrate and metastasis. Different intra- and extra-tumoral stimuli can induce EMT including hypoxia, starvation, inflammatory cytokines, and growth factors.[14,15]

TWIST1 is a transcription factor that exhibits an essential role for EMT. Activation of TWIST can activate many other transcription factors and gene repertoires which are attributed to the EMT. Also, TWIST is involved in chemoresistance,[16] angiogenesis,[17,18] and invasion[19-21] of the cancer cells. Besides, many studies have demonstrated the determinative role of EMT in the progression and metastasis of a vast variety of malignancies including endometrial,[22] gastric,[23] pancreatic,[24] breast,[25] and colorectal cancers.[26] Also, various mutations in the components of Mitogen-Activated Protein Kinase (MAPK) cascades downstream of the epidermal growth factor receptor (EGFR), Notch, PI3K/AKT pathway, transforming growth factor-β (TGF-β), and Wnt signaling pathways have been linked to the development of CRC.[27]

The aim of the present study was to find the association between TWIST mRNA expression and the clinicopathological parameters of the patients including age, gender, tumor size, tumor location, tumor stage, grade, clinical stage, nodal invasion, and distant metastasis.

Materials and Methods

Tissue Samples

This retrospective study was conducted in the Pathology Department of Isfahan University of Medical Sciences, Isfahan, Iran in 2020. The clinicopathological data and Formalin-Fixed/Paraffin-Embedded (FFPE) specimens were collected from patients who underwent colorectal resections between January 2017 and December 2020 at Al-Zahra hospital of Isfahan, Iran. All patients with sporadic cases and familial adenomatous polyposis and clinical criteria for hereditary non-polyposis CRC (Amsterdam criteria) were excluded. Finally, 70 patients were selected as research samples on the mentioned date by convenience sampling method. None of the patients had received preoperative neoadjuvant chemotherapy or radiotherapy. The specimens were histologically verified by two pathologists using hematoxylin and eosin stained slides.

Total RNA Extraction

The RNA isolation kit (Ambion Inc., USA) was employed for RNA extraction from FFPE blocks. In brief, 20 µm sections were cut from the blocks and then, deparaffinized by 20 min incubation in xylene. Subsequently, the samples were centrifuged and washed three times in 100% ethanol. Sequential washing and centrifuging were done and at last, the samples were air-dried and then incubated in a digestion buffer containing proteinase K. The residue was then homogenized and incubated overnight at 55 °C. The RNA extraction buffer was used to purify the RNA. Then, chloroform was added, followed by more incubation and centrifugation. Fresh tubes were prepared to remove the aqueous phase. The RNA was precipitated with an equal volume of isopropanol in the presence of linearized acrylamide. Samples were incubated at -20 °C for about half of an hour, after which RNA was pelleted and washed twice in 75% ethanol, with intervening centrifugation at 4 °C. The isolated pellet was dried and resuspended in 10 µl of RNA storage solution. All of the samples were incubated with DNase to remove genomic DNA, according to the manufacturer. All reagents were from Ambion (Austin, USA).

The RiboGreen RNA quantitation kit (Molecular Probes Inc., Eugene, OR) and fluorometry (Fluoroskan Ascent FL; Thermo Labsystems, Helsinki, Finland) were employed for measuring the amount of RNA in the samples. After appropriate calculations, all samples were diluted to 0.1 µg/µl nuclease-free water.

cDNA Synthesis

An M-MLV Reverse Transcriptase kit (Invitrogen, USA) and random hexamers (Invitrogen, USA) were used to synthesis the complementary DNA (cDNA) according to the manufacturer's recommendations. Briefly, the total RNA (10 µl) was added to the master mix containing 1 µl of 10 mM dNTP mix at a neutral pH, 0.25 µg of random hexamers, and 5 µl of DEPC-treated water in PCR tubes. The reaction mixture was incubated at 65 °C for 5 min, and then quickly chilled on ice. Subsequently, a mixture of 4 µl of 5× first-strand buffer, 2 µl of 0.1 M dithiothreitol (DTT), and 1 µl of M-MLV Reverse Transcriptase (RT) was added to the reaction mixture in PCR tubes, and the cDNA synthesis reaction was performed at 25 °C for 10 min, 37 °C for 50 min, and 70 °C for 15 min.

Primers

The forward, 5'-GCCAGGTACATCGACTTCCTCT-3' and reverse, 5'-TCCATCCTCCAGACCGAGAAGG-3' were used for TWIST1. Also, forward 5'-CACCATTGGCAATGAGC GGTTC-3' and reverse, 5'-AGGTCTTTGCGGATGTCCAC GT-3' primers were used for the beta-actin.

Real-Time PCR

TWIST1 mRNA expression level relative to beta-actin mRNA level was measured by real-time PCR. The cDNA was used as a template for real-time PCR in a reaction mixture containing, the primers, and 2 µl of SYBR Green I kit (Roche, USA). The sample volume reached 20 µl with nuclease-free water. The samples were amplified in the LightCycler System (Roche) and the PCR was performed by an initial denaturation step at 95 °C for 30 sec and then 40 cycles with a 95 °C denaturation immediately followed by annealing temperature for 5 sec and 72 °C extension for 10 sec. After 40 cycles, a melting curve was generated for the final PCR product of all genes investigated by decreasing the temperature to 65 °C for 10 sec followed by a slow increase in temperature to 95 °C. During the slow heating process, the fluorescence was measured at 0.2 °C increments. For more approval, the gel electrophoresis was conducted for selected samples and exhibited bands at the expected length. For calculating the relative abundance of the target gene expression, the $2^{-\Delta\Delta}$Ct method was used. For each cDNA, the target gene mRNA level was normalized to beta-actin mRNA level. The experiments were performed in triplicate.

Statistical Analysis

All statistical analyses were performed using JMP version 11.0 software. Quantitative values of RT-qPCR were presented as means ± standard deviation. The Mann-Whitney U test was used for the quantitative comparison of mRNAs for various clinicopathological parameters. The p-value<0.05 was considered significant has been illustrated as * in the tables.

Results

Patients Characteristics

Seventy CRC specimens were involved in this study. Thirty-two (46%) patients were female and 38 were male. Their ages ranged from 42 to 83 years (Mean: 64 years, Median:

65 years). Most of the tumors were located at the colon (60%). Also, more than half of the tumor specimens (57%) were G2. Nodal invasion was observed in 51% of the patients and 15 (24%) patients exhibited metastasis. In addition, 67% of the specimens' differentiation status was well to moderate and the others were poor to undifferentiated. All the patients' clinicopathological parameters have been presented in Table 1.

Association of Relative Quantification (RQ) of TWIST1 mRNA Expression and the CRC Patients' Clinicopathological Features

The mean RQ of TWIST1 for CRC patients (n = 70) was 10.5 ± 2.7 No significant association was observed between age (p = 0.786), gender (p = 0.163), tumor location (p = 0.300), and size (p = 0.4378) of the patients and the TWIST1 RQ. Besides, when the patients were divided into a well to moderate differentiation subgroup and a poor to undifferentiated subgroup, the two subgroups were not significantly (p = 0.054) different in regards to the mean values of TWIST1 RQ. The TWIST1 RQ was significantly (p = 0.040) higher in the subgroup with nodal invasion (N1-3) in comparison with the without nodal invasion subgroup (N0). The T3-4 stage patients had significantly (p = 0.024) higher mean TWIST1 RQ than T1-2 stage patients. Also, CRC patients at clinical stages III-IV exhibited significantly higher TWIST1 RQ in comparison with the I-II subgroup. Moreover, the mean value of TWIST1 RQ was significantly (p = 0.032) high in patients with distant metastasis. Table 2 summarizes the association of the RQ of TWIST1 mRNA expression and the CRC patients' clinicopathological features.

Table 1. Clinicopathological Parameters of Patients

Clinicopathological Parameters	Patients Number (n = 70)	Proportion (%)
Age		
≤ 65	37	53%
> 65	33	47%
Gender		
Male	38	54%
Female	32	46%
Tumor Location		
Colon	42	60%
Rectum	28	40%
Tumor Size (cm)		
≤ 5	34	49%
> 5	36	51%
Grade		
G1	14	20%
G2	40	57%
G3	16	23%
Tumor Stage		
T1	0	0%
T2	30	43%
T3	36	51%
T4	4	6%
Nodal Stage		
N0	34	49%
N1	22	31%
N2	8	12%
N3	6	8%
Differentiation		
Well to moderate	47	67%
Poor to undifferentiated	23	33%
Clinical Stage		
I-II	21	30%
III-IV	49	70%
Distant Metastasis		
M0	55	76%
M1	15	24%

CRC: Colorectal Cancer; n: Number.

Table 2. Association of Clinicopathological Features and Mean Values of Relative Quantification of TWIST1 mRNA Expression in CRC Patients

Clinicopathological Parameters	TWIST1 (Mean ± SD)	P-value [1]
Age		
≤ 65	10.3 ± 1.4	0.786
> 65	10.8 ± 1.5	
Gender		
Male	11.9 ± 1.8	0.163
Female	8.9 ± 1.2	
Tumor Location		
Colon	9.6 ± 1.3	0.300
Rectum	11.9 ± 1.6	
Tumor Size (cm)		
≤ 5	10.8 ± 1.5	0.438
> 5	11.3 ± 2.4	
Grade		
G1-2	9.2 ± 1.1	0.016 *
G3	15.9 ± 2.1	
Tumor Stage		
T1-2	7.2 ± 1.5	0.005 *
T3-4	13.0 ± 1.3	
Nodal Stage		
N0	8.0 ± 1.4	0.019 *
N1-3	12.9 ± 1.4	
Differentiation		
Well to moderate	12.6 ± 2.5	0.054
Poor to undifferentiated	14.2 ± 2.8	
Clinical Stage		
I-II	7.4 ± 1.6	0.016*
III-IV	12.6 ± 1.3	
Distant Metastasis		
M0	9.4 ± 1.1	0.032 *
M1	15 ± 2.2	

[1]: Mann-Whitney U Test; *: p-Value<0.05; CRC: Colorectal Cancer; n: Number.

Discussion

One of the most determinative phenomena during colon cancer progression is the EMT.[28] EMT causes significant morphologic and characteristic alterations at the cancer cells including loss of cell adhesion, repression of E-cadherin, and increased cell motility. On the other hand, activation of tyrosine kinases, upregulation of N-cadherin, vimentin, fibronectin will dominate the cancer cells. The TWIST transcription factor is a key role player for the induction of EMT changes to the cancer cells.[12,29]

The expression of this transcription factor has exhibited a significant association with cancer cell motility and invasion.[30] *In vitro* and *in vivo* evidence suggest that TWIST expression is related to breast, lung, prostate, liver and pancreas, and other cancers metastasis. Interestingly, transfection of cancer cells to express TWIST can cause a significant increase in their invasion and metastasis.[31]

Also, many clinical studies have demonstrated the function of TWIST in the tumors. TWIST expression is considerably associated with distant metastasis formation and patients' poor prognosis in different cancers.[32-34] Kim et al., suggested

TWIST1 mRNA expression as an important marker of poor prognosis in human CRC. They reported that TWIST1 expression significantly influences the node metastasis in this type of cancer.[35] These observations are consistent with other studies that reported the statistically meaningful relation of TWIST mRNA expression with clinicopathological features, treatment response, and prognosis of colorectal patients.[36-38]

Moreover, TWIST1 mRNA expression assay has been used for different purposes in different cancers. Demir et al., exhibited that TWIST1 expression can predict primary doxorubicin resistance in breast cancer patients.[39] Qiao et al., reported that TWIST expression is associated with larger tumor size, nodal involvement, higher nuclear grade, and positive HER2 status.[40] Taking together, TWIST1 can be an appropriate predicting biomarker in different types of cancer which its higher expression is correlated with poor outcomes.

Conclusion

TWIST is a novel emerging prognostic biomarker in different cancers. In this study, association analysis of the clinicopathological parameters of colorectal cancer patients and RQ of TWIST1 mRNA exhibited high TWIST1 mRNA expression associated with high nodal stage, advanced clinical stage, and distant metastasis.

Authors' Contributions

MHS performed the conception and design of the research performed, prepared tools, and facilities for the field study and wrote the manuscript; MK and MS conducted statistical analysis, drafted and participated in manuscript writing, and revision. All authors read and approved the final manuscript.

Ethical Approval

This study was approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran with code of IR.MUI.REC.1396.3.769.

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Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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