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MEIS1 knockdown upregulates WNT signaling pathway genes in esophageal squamous cell carcinoma

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Abstract

Background The transcription factor MEIS1 belongs to the 3-amino acid loop extension (TALE) family of homeodomain proteins which plays various functions in normal and tumor cell progression. The canonical WNT/β-catenin pathway governs a plethora of biological processes including cell proliferation, differentiation, and tumor development. In the present study, the effect of MEIS1 gene silencing was assessed on WNT pathway genes in esophageal squamous cell carcinoma (ESCC) cells.

Materials and methods Along with the packaging plasmids, the pLKO.1-MEIS1 plasmid was cotransfected into HEK293T to generate lentiviral particles, followed by transduction of a semi-confluent KYSE-30 cell culture. After total RNA extraction and cDNA synthesis, comparative real-time PCR was applied to assess the efficiency of MEIS1 knockdown and the expression of genes related to the WNT signaling pathway.

Results The results revealed effective downregulation of MEIS1 in KYSE-30 cells. Interestingly, MEIS1 silencing led to a substantial overexpression of WNT pathway key components while the expression of negative regulators of this pathway was substantially decreased.

Conclusions Our data suggest that MEIS1 gene probably induces WNT/β-catenin pathway deactivation in ESCC cells. Consequently, the inverse correlation of MEIS1 expression and WNT signaling pathway activation may introduce a new molecular linkage through ESCC progression and aggressiveness.

Keywords Esophageal squamous cell carcinoma, MEIS1, WNT/β-catenin pathway

Introduction

Esophageal cancer (EC) is the 8th most frequent malignancy and the 6th leading cause of cancer-related death in the world [1]. Geographical factors, ethnicity and local culture play a role in the EC prevalence rate in diverse regions. According to histopathology appearance, EC is classified into two main subtypes including esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC is an aggressive malignancy, with a high prevalence and poor prognosis. The five-year survival rate of ESCC is ranged from 10 to 25%, despite the substantial attempts in the development of preventive

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procedures, diagnostic technologies, and treatment modalities made for ESCC [1, 2]. Since early diagnosis is critical in patient's survival, illumination of molecular mechanisms involved in ESCC progression and metastasis is essential to present new prognostic and therapeutic targets.

Myeloid ecotropic viral integration site 1 (MEIS1), as a homeobox protein, belongs to the TALE (Tree Amino acid Loop Extension) family of transcription factors. MEIS1 plays crucial roles in cell growth, differentiation and vascular development during vertebrate embryogenesis [3]. It is implicated in the development of several peripheral organs and functions as a transcriptional factor in different signaling pathways regulating pluripotency of stem cells and mesodermal commitment pathway [4]. MEIS1 acts as a negative regulator in non-small-cell lung cancer, prostate cancer, colon cancer and ESCC [5]. It may persuade cell cycle arrest and inhibit cell proliferation [3].

The development and homeostasis of multicellular organisms mostly depends on cell-to-cell communication to establish cell fate [6]. The WNT pathway plays a remarkable role in the regulation of multiple biological processes including cell proliferation/migration/fate during embryogenesis, stem cell development/differentiation, as well as tumorigenesis. The extra-cellular WNT ligands stimulate intra-cellular signal transduction via the canonical (WNT/β-catenin dependent) and the non-canonical (β-catenin-independent) pathways [6]. The canonical WNT signaling has a well-established role in cancers and regulates the transcription of target genes by its central component β-catenin [6].

WNT/β-catenin signaling pathway contains ligands, receptors (Frizzled), co-receptors (LRP5/6), β-catenin, β-catenin destruction complex (APC, GSK3β, CK1α, AXIN), and transcription factors (TCF/LEF) [6, 7]. This pathway is activated by binding of WNT ligand to receptors/co-receptors complex. The β-catenin destruction complex is subsequently inhibited by triggering recruitment of Dishevelled (DVL/Dsh) to the plasma membrane [8]. As a consequence, DVL assembles a signalosome that engages the Axin degradasome to inhibit the enzymatic activity of GSK3β within this complex. This allows β-catenin to accumulate, migrate to the nucleus and bind to transcription factors TCF/LEF. These transcription factors regulate the expression of downstream target genes, such as cyclin D1 (CCND1) and c-MYC [9]. In the absence of WNT ligands, WNT signaling is inactive and the levels of β-catenin are kept low, while the destruction complex is active and results in β-catenin phosphorylation, ubiquitination, and eventually its degradation in the proteasome [10]. The crosstalk between WNT signaling and other pathways has been reported in ESCC [11].

Although the downregulation of MEIS1 and deregulation of WNT/β-catenin signaling pathway have been revealed in ESCC, their probable correlation in this malignancy is still unclear. Therefore, we aimed in the present study to examine the impact of MEIS1 gene silencing on WNT pathway genes in ESCC cell line KYSE-30 to disclose a plausible new way relating the contributions of MEIS1 and WNT/β-catenin pathway in ESCC development.

Materials and methods

Cell lines and culture conditions

Embryonal kidney cells HEK293T and human ESCC cells KYSE-30 were supplied from the cell bank of Pasteur Institute of Iran (Tehran, Iran), and cultivated in DMEM and RPMI-1640 medium, respectively,. supplemented with heat-inactivated fetal bovine serum (10%), glutamine (10 mM), and penicillin-streptomycin (100 U/mL, and 100 mg/mL). Cells were grown at 37 °C in a 95% humidified atmosphere with 5% CO2 [5].

Knockdown of MEIS1 gene expression in KYSE30 cells

The pLKO.1-MEIS1-puro lentiviral shRNA expression vector was acquired from Sigma-Aldrich (St. Louis, MO). The pLKO.1 plasmid DNA contains the green fluorescent protein (GFP) gene under the control of the cytomegalovirus (CMV) promoter. This vector encodes a validated shRNA sequence which targets human MEIS1. Lentiviral second-generation packaging plasmids pMD2.G and psPAX2 were obtained from Addgene (plasmids 12259 and 12260, respectively, Cambridge MA). Beside the packaging plasmids, the pLKO.1-MEIS1 plasmid was cotransfected into HEK293T cells to generate lentiviral particles. 21 µg of each pLKO.1-MEIS1 and psPAX2 along with 10 µg of pMD2.G were transiently cotransfected into HEK293T cells according to the standard calcium phosphate method for producing lentiviral particles. The supernatant including viral particles was harvested via ultracentrifugation 48 h after transfection, filtered through 0.45-µm filter (Orange, Belgium), and was pelleted using ultracentrifugation (Beckman-Coulter ultracentrifuge XL-100 K, USA) at 70,000×g, 4 °C for 1 h and resuspended in fresh medium. For transduction, 0.5-1 × 106 KYSE-30 cells were seeded in a 6-well plate the day before transduction. On the day of infection, the culture media were replaced with fresh ones containing the lentiviruses for an additional 4–5 days. The infected cells were selected via puromycin after 48 h since infection [5]. By using inverted fluorescence microscopy, the transduced KYSE-30 cells with recombinant lentiviral particles of GFP (control) and GFP-shMESI1 were analyzed. MEIS1 mRNA knockdown quantification was verified by qRT-PCR.

RNA extraction, cDNA synthesis and relative comparative real-time PCR analysis

Total RNA was isolated from GFP and GFP-shMESI1 transduced KYSE-30 cells using TRIZOL reagent pursuant to the manufacturer's instructions (Invitrogen). Since DNase I treatment significantly reduces the RNA yield, the majority of used primer sets in this study were designed as exon-exon junction primers. For non-exon-exon junction primers, DNaseI treatment was applied on extracted RNA before cDNA synthesis to avoid genomic DNA contamination. The cDNA was synthesized from isolated total RNA using the first-strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA). To assess MEIS1 knockdown, comparative real-time PCR amplification was carried out using SYBR green PCR Master Mix (SYBR Green, AMPLIQON, Denmark), in a Stratagene Mx-3000P real-time thermocycler (Stratagene, La Jolla, CA). ROX was used as reference dye. The thermal cycling conditions were 95 °C for 12 min, 35 cycles of 15 s at 95 °C, 56 °C for 30 s, and 72 °C for 30 s. Furthermore, comparative real-time PCR was conducted to assess the expression of the involved genes in WNT signaling pathway using specific primer sets presented in Table 1. The whole experiments were conducted in technical triplicate. Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was employed as a reference gene to normalize the data. The qRT-PCR results were analyzed using ΔΔct method and log 2 fold change was considered for a better understanding of the changes of gene expression. Log 2 fold change more than 1 was considered as overexpression, while less than −1 was considered as underexpression. The range between +1 and −1 was interpreted as no change or normal expression.

Statistical analysis

Data analyses were conducted using the SPSS 19.9 statistical package (SPSS, Chicago, IL, USA). The association between gene expressions was evaluated applying the chi-squared test and Pearson's correlation. The *P*-value less than 0.05 was considered statistically significant. The results of gene expression analysis were assessed using ΔΔct method and presented as log2 fold change ± SD.

Results

MEIS1 knockdown in ESCC cell line KYSE-30

To examine the impact of MEIS1 gene expression on WNT pathway genes, MEIS1 knockdown was performed in KYSE-30 cells using lentiviral particles. To confirm MEIS1 silencing, the MEIS1 expression was assessed in GFP-hMEIS1 in comparison with GFP-control KYSE-30 transduced cells (> 95% positive). The fluorescent microscopy images of transduced GFP-shMESI1 and GFP control KYSE30 cells are shown in Fig. 1. Furthermore, underexpression of MEIS1 was confirmed at mRNA level in KYSE-30 cells. As shown in Fig. 2, MEIS1 knockdown was confirme in MEIS1-silenced cells compared to control.

MEIS1 knockdown upregulates WNT pathway genes expression

To confirm whether or not MEIS1 knockdown intercedes the expression of WNT pathway genes, the expression of main genes implicated in WNT pathway was analyzed in MEIS1-silenced cells using qRT-PCR. Interestingly, MEIS1 silencing caused a significant overexpression of WNT pathway key components including FZD1 (receptor), LRP5 (co-receptor), DVL1 (cytoplasmic middle molecule), LEF-1 and TCF7 (transcription factors) with log 2 fold changes nearly 2.12, 2.97, 3.46, 1.54 and 1.95, respectively (Fig. 2). In contrast, the expression of negative regulators of WNT pathway including APC/GSK3β (the main components of the β-catenin destruction complex) and FBXW7 were significantly decreased with log 2 fold changes approximately −3.29, -1.1 and −1.68, respectively. These results are depicted as box plot in Fig. 2.

Discussion

MEIS1 is a member of TALE transcription factors family which is implicated in cell proliferation, differentiation and vertebrate embryogenesis [6]. MEIS1 function, as an either oncogene or tumor suppressor gene, depends on the origin and context of the cell [12]. It has been proposed that MEIS1 may act as a cell cycle regulator which prevent cell proliferation and induce cell cycle arrest [3]. An inverse correlation is reported between MEIS1

Table 1 Primer sequences used in relative comparative real-time PCR

Gene	Forward primer	Reverse primer	Amplicon size
MEIS1	ATGACACGGCATCTACTCGTTC	T GTCCAAGCCATCACCTTGCT	105
FZD1	CTCCTACCTCAACTACCACTTC	CACTGACCAAATGCCAATCC	136
LRP5	CGGCAGAAGGTGGTGGAG	CAGCGAGTGTGGAAGAAAGG	212
DVL1	TGGTCTCCTGGCTGGTCCTG	TCCGTGCCTGTCTCGTTGTC	184
APC	AGACTGGTATTACGCTCAACTTC	CTGGCTATTCTTCGCTGTGC	195
GSK3β	ACTTCACCACTCAAGAACTGTCAAG	TGTCCACGGTCTCCAGTATTAGC	139
FBXW7	CAGCAACAGCAACTCAGACAAC	CCTCCTCCTCATCCTCCTCATC	226
LEF1	GACAAGCACAAACCTCTCAG	TTATTTGATGTTCTCGGGATGG	202
TCF7	GGACAACTACGGGAAGAAGAAG	TAGAGCACTGTCATCGGAAGG	138

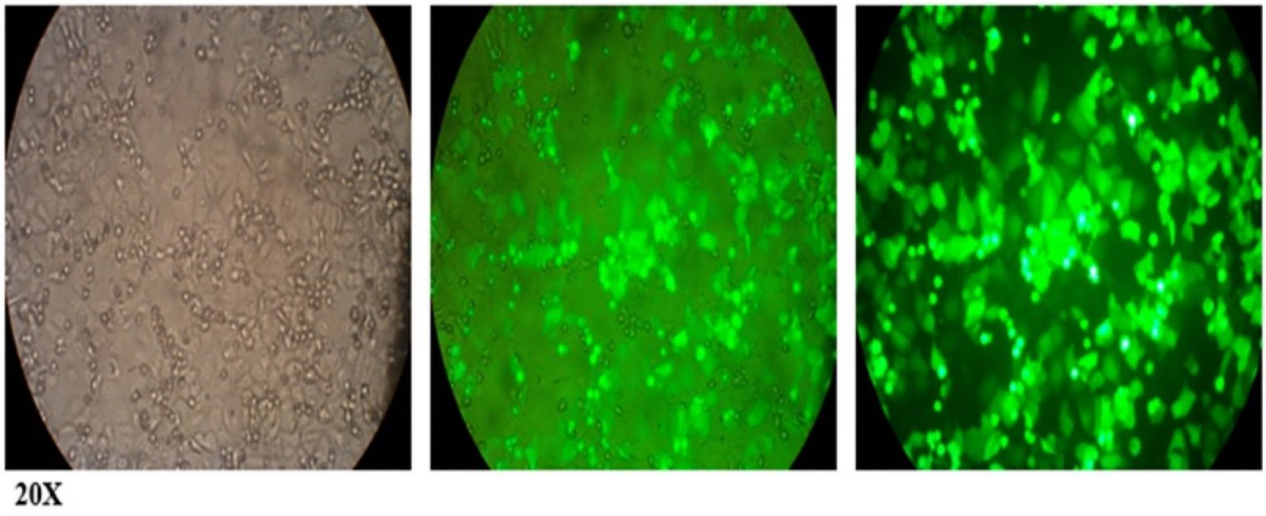

Fig. 1 MEIS1 gene expression knockdown in KYSE-30 cells. Invert microscopy, merge, and fluorescent microscopy images of GFP-hMEIS1 KYSE-30 cells after day 3 of transduction

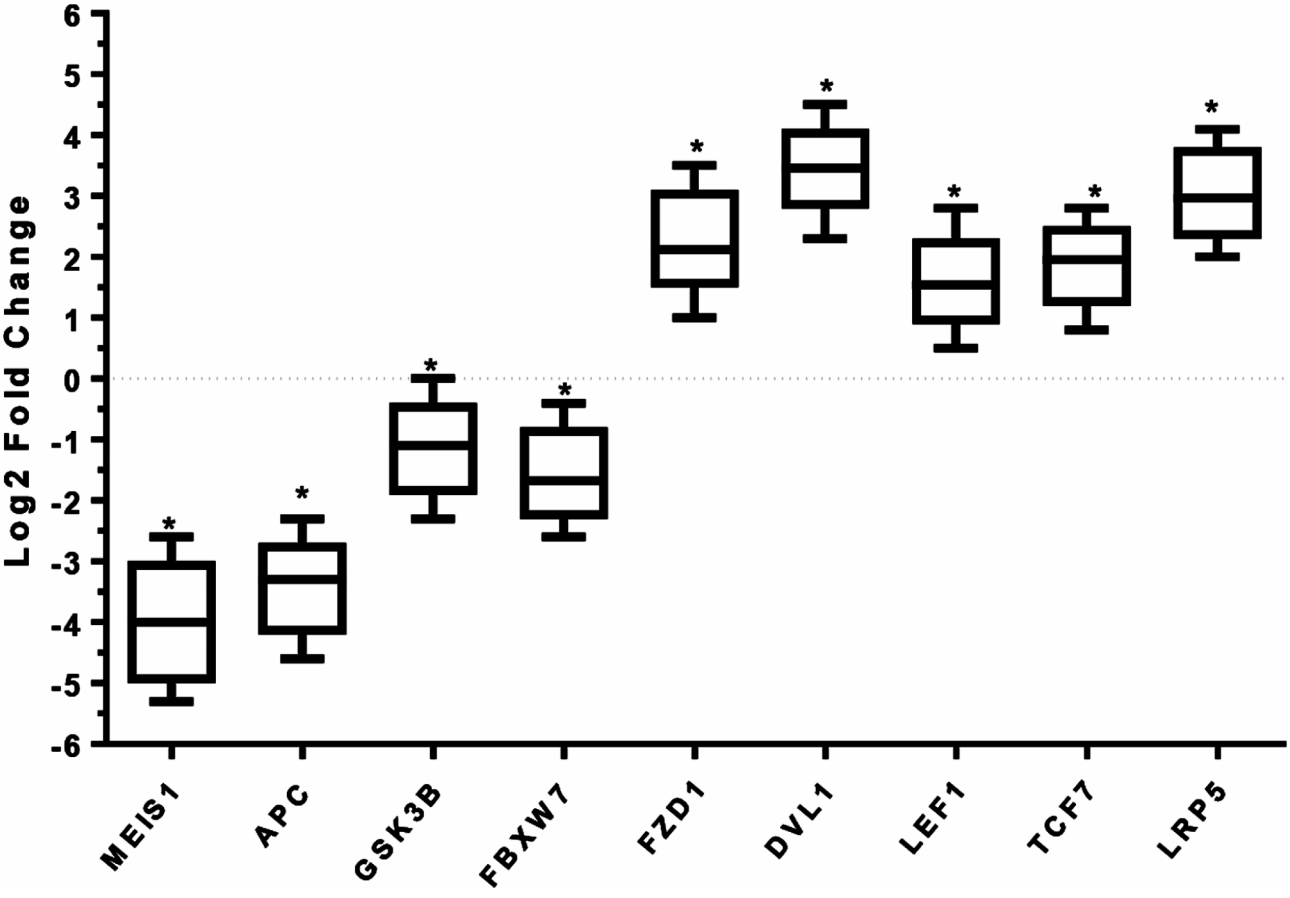

Fig. 2 Lentiviral transduction enforced remarkable MEIS1 underexpression in KYSE-30 nearly −4 folds compared to control. MEIS1 knockdown up-regulates the main components of WNT signaling pathway including DVL1, LRP5, LEF1/TCF7 and FZD1 expression and down regulates the expression of negative regulators of the pathway including APC/GSK3β and FBXW in KYSE-30 cells. The results are assessed using ΔΔct method and presented as log2 fold change ± SD

expression and indices of ESCC poor prognosis including metastasis and tumor staging [5]. In the present study, MEIS1 gene silencing was performed using retroviral system and the expression of WNT pathway genes was analyzed to explore probable regulatory role of MEIS1 on WNT pathway in ESCC cell line KYSE-30. Interestingly, an inverse correlation between MEIS1 expression and WNT/β-catenin pathway was elucidated for the first time in ESCC. We showed that MEIS1 knockdown can upregulate the involved genes in WNT signaling pathway in ESCC KYSE-30 cells.

The 'canonical' WNT/β-catenin pathway is strongly involved in embryonic development and adult homeostasis, and deregulation of this pathway is mostly linked to several disease states such as cancer. In multiple tumor types, activation of the WNT/β-catenin pathway is related to increased proliferation, tumorigenesis and diminished patient survival [13–15]. Deregulation of WNT/β-catenin pathway is reported in a variety of malignancies including gastrointestinal, lung, cervical, mammary, prostate, and colorectal cancers, pancreatic ductal adenocarcinoma, melanoma, as well as tumors of the central nervous system [13, 16]. In addition, several studies have illustrated the significant role of WNT canonical signaling pathway components in ESCC tumorigenesis [15–17].

In the WNT pathway activate state, DVL1 links WNT/FZD1/LRPs engagement to the deactivation and disintegration of the β-catenin destruction complex which results in β-catenin accumulation and nuclear translocation [18]. Then, in the nucleus β-catenin interacts with transcription factors, LEF-1/TCF7, to regulate the expression of pathway target genes such as c-MYC and cyclin D1. As a consequence, FZD1 and LRP5/6 can effectively enhance the expression level of active form of β-catenin in the presence of WNT ligand by cooperating with DVL1 protein [19, 20]. Frizzled (FZD) family are demonstrated to have critical functions in the development and progression of human cancers [20]. Upregulation of the FZD1 receptor was found in different cancers including colon and breast malignancies, as well as squamous cell/adenosquamous carcinoma of the gallbladder which likely indicate the effect of its overexpression in WNT/β-catenin pathway activation [21]. Furthermore, upregulation of LRP5 was found crucial for β-catenin-dependent tumorigenesis and results in activation of WNT/β-catenin pathway [22, 23]. Dishevelled1 (DVL1) is also a significant downstream component of canonical WNT pathway. It regulates production of cell polarity, specification of cell fate and embryonic induction by activation of WNT signaling pathway [10, 24]. Previous studies have revealed that upregulation of DVL1 is involved in different types of cancer such as cervical squamous cell carcinomas, breast cancer, non small cell lung cancer and is also remarkably correlated with liver metastasis [24, 25].

LEF-1/TCF7 transcription factors interact with β-catenin to mediate a nuclear response to WNT signals and transcribe target genes in the nucleus [26, 27]. It has been demonstrated that activation of β-catenin TCF/LEF signaling pathway mediated CTGF oncogenic activity, a transcriptional target of β-catenin/WNT pathway, which promotes tumorigenicity through overexpression of CTGF in ESCC [28]. Collectively, these evidences confirm activation of WNT signaling pathway following overexpression of the key components of the pathway in different cell types. In this study, overexpression of the key components of WNT signaling pathway was observed following MEIS1 gene silencing in KYSE-30 cells. Therefore, it can be extrapolated that MEIS1 silencing may activate WNT signaling in the ESCC cell line.

In contrast to active state, β-catenin in the cytoplasm is sequestered into β-catenin destruction complex in the absence of WNT ligand. APC is one of the main components of the β-catenin destruction complex in WNT pathway which prevents proliferation, facilitates apoptosis, suppresses invasion and tumor progression by negatively regulating canonical WNT signaling [13]. Hypermethylation of the APC was remarkably related to decreased survival of ESCC patients [17]. Another β-catenin component destruction complex is glycogen synthase kinase 3β (GSK3β) which plays substantial roles in regulating cell proliferation, differentiation, and apoptosis [29]. GSK3β acts as a negative regulator in WNT pathway by downregulating β-catenin levels [30]. FBXW7 (F-box and WD-40 domain protein 7) is also a WNT pathway inhibitor which acts as a tumor suppressor gene by inducing degradation of positive regulators of the cell cycle. FBXW7 deactivates WNT pathway via targeting several major oncogenic proteins such as c-MYC and β-catenin [31]. Low FBXW7 expression is suggested to be associated with tumor progression and local invasiveness in ESCC [32]. These evidences confirm that down regulation of involved proteins in β-catenin destruction complex is a usual event when WNT signaling pathway is activated in the cell. By silencing MEIS1 expression in KYSE-30 cells in the present study, underexpression of WNT pathway negative regulators including APC/GSK3β and FBXW7 was observed, supporting activation of WNT signaling pathway in ESCC cell line.

Consistent with these findings, our results showed a significant potential association between MEIS1 and WNT/β-catenin pathway in ESCC. Interestingly, after MEIS1 knockdown in KYSE-30 cells, overexpression of receptor (FZD1), co-receptor (LRP5), cytoplasmic middle molecule (DVL1) and transcription factors (LEF-1/TCF7) was found while underexpression of negative regulators (APC, GSK3β, FBXW7) of WNT/β-catenin

pathway was observed. However, the exact molecular linkage between MEIS1 and WNT/β-catenin signaling pathway genes should be further verified by prospective analysis and more comprehensive follow-up.

In conclusion, the indirect regulatory role of MEIS1 on WNT signaling pathway genes was revealed in this study. We showed that MEIS1 knockdown can significantly increase the expression of the main components of WNT signaling pathway in KYSE-30 cells, while the negative regulators of the pathway experienced a significant decreased expression. To the best of our knowledge, this is the first report describing regulatory role of MEIS1 on WNT signaling pathway activation in ESCC cell line KYSE-30.

Abbreviations

EC	Esophageal cancer
ESCC	esophageal squamous cell carcinoma
EAC	esophageal adenocarcinoma
MEIS1	Myeloid ecotropic viral integration site 1
DVL/Dsh	Dishevelled
FZD	Frizzled
GSK3β	glycogen synthase kinase 3β
FBXW7	F-box and WD-40 domain protein 7
ccRCC	clear cell renal cell carcinoma

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Author contributions
NH and MMF performed the experiments and drafted the manuscript. MMF designed the study, analyzed data, and had a critical scientific revision on the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication
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Competing interests
The authors declare no competing interests.

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