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ORIGINAL RESEARCH

Regulatory Fluctuation of WNT16 Gene Expression Is Associated with Human Gastric Adenocarcinoma

Seyedeh Elham Norollahi[1] · Majid Alipour[2] · Ali Rashidy-Pour[3,4] · Ali Akbar Samadani[1,3] · Laleh Vahedi Larijani[5]

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Abstract
Introduction Gastric cancer is one of the most serious and lethal kinds of cancer in the world. It is a multi-step, multi-factor, and elaborated process that is associated to gene abnormal expression. This study intended to investigate the *WNT16* gene's expression in human gastric tumor and the margin tissues of the stomach (normal tissues).
Methods Correspondingly, 40 samples (20 tumoral tissues and 20 non tumoral or margins tissues) were investigated in Imam Khomeini Hospital in Sari City, Mazandaran Province, Iran. In this way, real-time PCR, Taqman assay was employed to evaluate the upregulation and downregulation of this gene in both tissues in triplicate form. The *GAPDH* gene was selected as housekeeping gene.
Results Conspicuously, the results have shown a remarkable modification in tumoral tissues, and the gene expression increased significantly in tumoral tissue.
Conclusions Conclusively, the upregulation of *WNTt16* gene expression in tumoral tissues was impressive and the *P* value was 0.005 and the SE range was 0.064–142.154.

✉ Ali Akbar Samadani
a_a_hormoz@yahoo.com

[1] Faculty of Medicine, Cancer Research Center, Semnan University of Medical Sciences, Semnan, Iran

[2] Department of Biology, Islamic Azad University of Babol, Babol, Iran

[3] Faculty of Medicine, Research Center of Physiology, Semnan University of Medical Sciences, Semnan, Iran

[4] Faculty of Medicine, Department of Physiology, Semnan University of Medical Sciences, Semnan, Iran

[5] Faculty of Medicine, Department of Pathology, Mazandaran University of Medical Sciences, Sari, Iran

Keywords *WNT16* · Gene expression · Gastric cancer · Fluctuation

Introduction

Gastric cancer is the fourth prevalent and is the second cause of mortality in the world particularly in East Asia and it is divided into two main types: intestinal metaplasia and diffuse [1]. Approximately 90% of gastric tumors are related to adenocarcinoma tissues, and the pathology and disease evolution may differentiate among different populations, tumor location and position, histological subtypes of adenocarcinoma, and other factors [2].

Infection with *Helicobacter pylori* bacteria is a common cause of gastric cancer which is the most common cause. In this way, there are many evidences in order to confirm the carcinogenesis process as a result of improper gene expression, genetic and epigenetic alteration in oncogenes, tumor suppressor genes, DNA mismatch repair, and cell cycle regulator [3]. Besides the modifications of epigenetic factors like DNA methylation, histone modification, histone phosphorylation, histone acetylation, and others factors, the fluctuations of gene expression are shown as a main factor in tumorogenesis and carcinogenesis [4].

Gene expression studies have indicated the correlation of molecular basis of gastric cancer [5, 6]. Relatively, the normalization of expression data of many different genes without validation and confirmation may undermine the final results [7, 8].

Tumor suppressor genes have a key role in preventing of tumorogenesis by controlling the cell division of DNA repairing and inducing the apoptosis in suitable time. Correspondingly, molecular genetics researches in order to find the notable, and now genes can lead to a new strategy

for prognosis, prevention, and treatment. Remarkably, there are many important genes like *WNTs*(*19* sub classes), *SOX*, *CDXI/2*, *SALL4*, *OCT3/4*, *NOTCH*, *NANONG*, *KLF4/5*, *KLFI*, *FEMS EGF*, and *BMP* which are involved in this serious disease, and their fluctuations may lead to many kinds of disorders especially different cancers [9–11]. These genes have different molecular pathways and functioning. One of these impressive genes is *WNT* 16 which belonged to *WNT* pathway [12, 13].

Recent studies have shown that the stimulation of *WNT16* gene expression in normal cells is responsible in the development and resistance to chemotherapy in carcinoma cells. This gene is involved in carcinogenesis and also in many evolutionary processes like regulation and patterning of cell during embryogenesis [14, 15]. An understanding of pathobiology of gastric cancer is essential to diagnose cancer biomarkers, which may contribute in early determination and also in the progression of novel targets therapies methods and, therefore, help to decrease the mortality or morbidity rates. The aim of this research was to investigate the expression of *WNT16* genes fluctuation (upregulation and also downregulation) in gastric adenocarcinoma, both tumoral and non tumoral tissues using real-time PCR, Taqman assay with special primers on 20 people who were involved with gastric cancer. Hypothetically, we believe that the fluctuation of this gene can be employed as a remarkable note in gastric cancer studies.

Methods

Sampling

The research was accomplished on 40 samples (20 normal and 20 tumoral tissues) with gastric cancer which was confirmed by the pathology department and also agreement by patients in Imam Khomeini Hospital (Sari, Iran). Tissues were histologically verified to include at least a minimum of 70 to 80% of neoplastic cells for gastric cancer samples.

Among a total of 20 gastric adenocarcinoma patients (20 normal and 20 tumoral tissues), there were 15 men and 5 women. The histopathological qualifications of tissues are shown in Table 1.

Table 1 The histopathological qualifications of tissues both in normal and tumoral tissues

Histopathological qualifications of normal and tumoral tissues of the gastric cancer patients		
Sex	Tumor stage (number):	Metastasis (number):
Female = 5	Stage 1: 2	Positive: 16
Male = 15	Stage 2: 6	Negative: 4
	Stage 3: 11	
	Stage 4: 1	

Tissue Samples

The tumoral and normal tissues (margins tissues) were provided directly in surgery room after total gastrectomy for all samples.

In this way, DEPC (diethylpyrocarbonate) was employed to clean and treat all surgical instruments during sampling (tumoral and non tumoral tissues) in order to avoid RNAs enzyme. After sampling operation, all specimens were transferred to liquid nitrogen for deep freezing. Eventually, tissue samples were stored at − 80 °C for long preservation and investigation.

Gene Expression Analysis

RNA was isolated by using a commercial reagent, Trizol. (Invitrogen cat no 15596-025, USA.) Approximately, 80–120 mg of tissue was crushed in order to powder them by a mortar and pestle in the presence of liquid nitrogen, and 40–70 mg of powdered tissue was employed for RNA isolation according to the manufacture's protocol. The quality of extracted RNA was checked by electrophoresis on agarose gel 2% in order to detect and observe the ribosomal RNA bands, and the quantity of extracted RNA was measured by a nanodrop instrument (TC100, USA).

Relatively, cDNA was done in the presence of 1 pg total RNA, 4 μL 5X reaction buffer, 10 mM each of dNTPs, and 1 μL (200 U/μL) by QuantiTect Reverse Transcription Kit (cat no 20S313, USA) in a final volume of 20 μL, by 60 min incubation at 44 °C. Real-time PCR was accomplished on Exicycler q6, Bioneer, USA by using a universal reverse primer and Universal Taqman-specific probe, as well as mRNA-specific forward primer. The expression levels of *WNT16* were normalized against *GAPDH*, RNA as control. The 20 μL PCR included 1 μ μL RT product, 0.25 mM universal-specific probe, 0.5 mM each forward and reverse primers. The PCR reagents were all from Qiagen HotStarTaq reagent set (Qiagen, cat no 203205). In this way, the mixtures were incubated at 95 °C for 5 min, followed by 42 cycles of 90 °C for 40 s, and 62 °C for 1 min. All reactions were performed in triplicate. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The sequence of forward and reverse primers in conjunction with Universal Taqman-specific probe (Table 2).

Statistical Analyses

Gene expression was analyzed was by an Exicycler set. Correspondingly, the significant difference was statistically

Table 2 Sequences of primers and specific probe for *WNT16* gene expression analysis

Forward *WNT16*	5′AGATGGAACTGCATGATCACC3′
Reverse	5′ CAAGGTGGTGTCACAGGAAC 3′
Probe	5′ Fam-GTCTCTTTGGTGCCGCTGCTCAG-Tamra 3′

interpreted by paired Student's *t* test. A value of $P < 0.05$ was considered as statistically significant.

Analyses were accomplished using commercially available statistical software (SPSS *Statistics* software, version 19, Chicago).

Results

In order to calculate and study the expression levels of 20 tumoral and 20 non tumoral tissues for *WNT16 gene*, we determined the levels using a scatter plot (Fig. 1). The fluctuations and related results showed the upregulation situation in tumoral tissues in comparison to non tumoral tissues (*P* value was 0.005 and the SE range was 0.064–142.154). In this way, the quantitative gene expression of *WNT16* employing real-time PCR with relative quantification analysis using relative expression software tool appears in Fig. 2.

Relatively, the final findings and result reports of *WNT16* gene expression in both tumoral and non tumoral tissues according to its *P* value and SE amounts are shown in Table 3.

Relative Expression Report

Assay Parameters

Discussion

Gastric cancer is one of the most serious kinds of cancers in the world and due to its multi-factorial function, has a high mortality rate. Remarkably, there are many different molecular pathways like *WNT*, *NOTCH*, and *SHH* with eclectic molecular

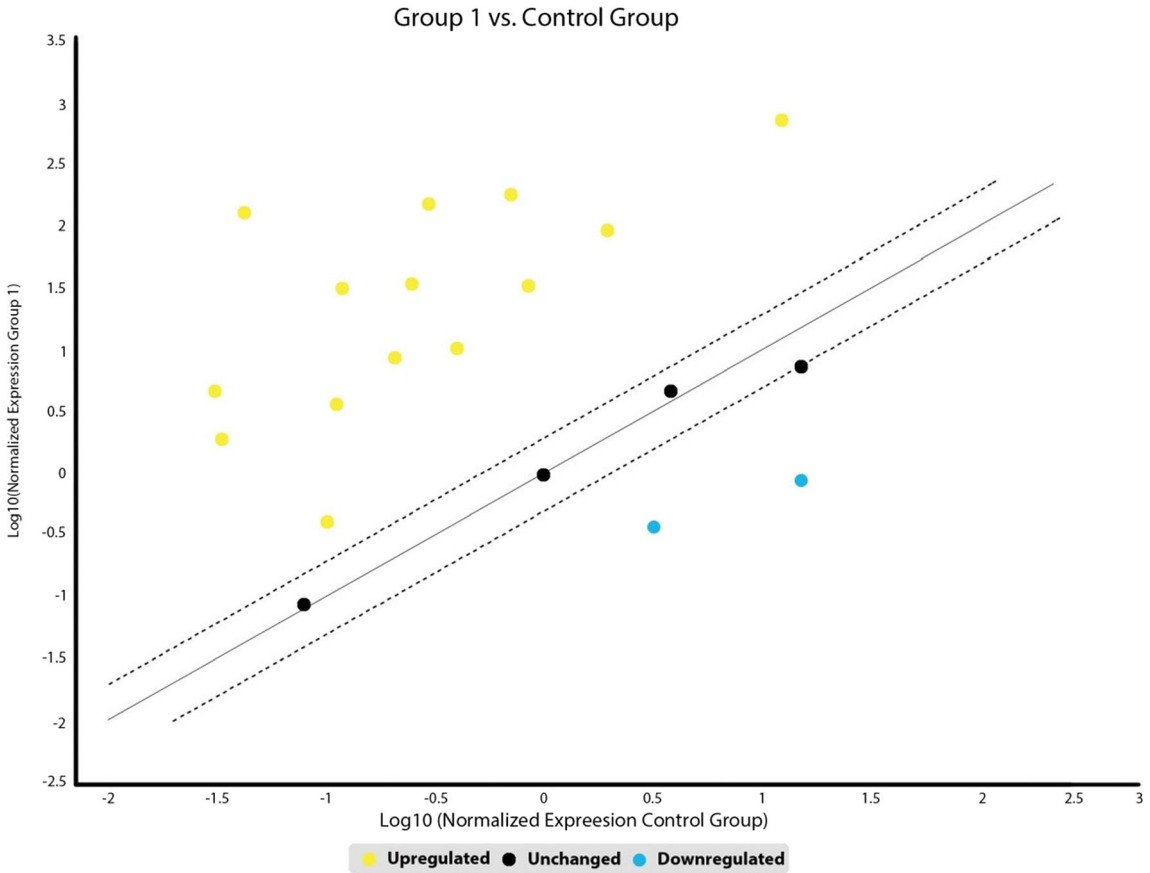

Fig. 1 Scatter plot analysis of *WNT16* gene expression profiling for both tumoral and non tumoral tissues. This diagram illustrates the fluctuations of gene expression as upregulated, unchanged, or downregulated conditions in *WNT16* gene

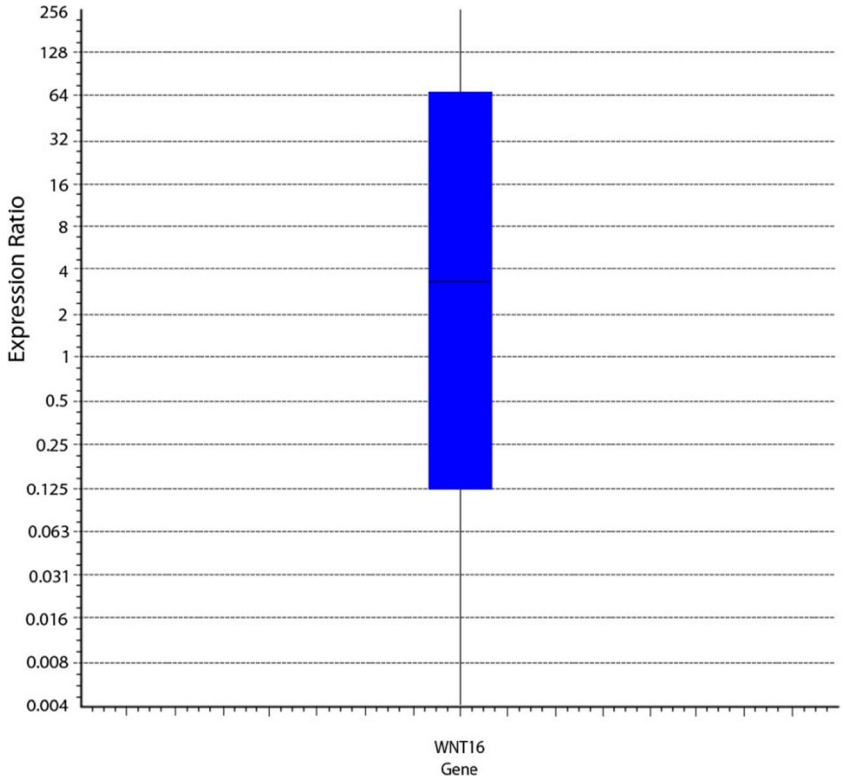

Fig. 2 Quantitative gene expression of *WNT16* employing real-time PCR with relative quantification analysis using relative expression software tool. In this way, fold change (*y* axis) indicates the relative expression of the *WNT16* in comparison to the control group (equals 1 by definition) normalized by *GAPDH* reference gene expression

mechanism, and modification of these genes which are involved in these signaling may lead to carcinogenesis or tumorogenesis. In this way, *WNT16* gene expression which is included in *WNT* pathway is involved in carcinogenesis [16].

Conspicuously, the *WNT* gene family comprises of fundamentally associated genes that encode secreted signaling proteins in the cellular processes. These proteins have been indicated in carcinogenesis and in many cellular and developmental processes, containing regulation and organizing of cell fate and modeling during embryogenesis. *WNT16* gene is a member of the *WNT* gene family and it includes two transcript variants which are suggested to be the final yields of discrete promoters and considerably not to be splice variants from a single promoter in this mechanism [19]. Notably, they are dissimilarly expressed in normal tissues, which variant 2 is expressed at impressive grade only in the pancreas, whereas variant 1 is expressed more ubiquitously with the maximum levels in the spleen, brain, kidney, heart placenta, and slightly in the digestive system. Correspondingly, many studies have confirmed the basic and main role of *WNT16* gene in bone disease [17, 18].

Relatively, another research that is entitled "Treatment-induced secretion of *WNT16B* promotes tumor growth and acquired resistance to chemotherapy" analyzed its function in cell proliferation and tumorogenesis.

In this regard, malignant tumors including both neoplastic and stromal fibroblasts, which can affect the tumor's function to cytotoxic therapy, were investigated. It is shown that the increased expression of WNT family

Table 3 Final findings and result reports of *WNT16* gene expression in both tumoral and non tumoral tissues according to its *P* value and SE amounts

Parameter	Value						
Iterations	1000						
Gene	Type	Reaction efficiency	Expression	Std. error	95% C.I.	P(H1)	Result
GAPDH	REF	1.0	1.000				
WNT16	TRG	1.0	2.523	0.064–142.154	0.002–3027.613	0.005	UP

Interpretation of final results is as follows: *WNT16* is upregulated in sample group (in comparison to control group) by a mean factor of 2.523 (S.E. range is 0.064–142.154). *WNT16* sample group is different to control group. P (H1) = 0.005

P (H1) probability of alternate hypothesis that difference between sample and control groups is due only to chance, *TRG* target, *REF* reference

member wingless-type MMTV integration of WNT16B by the tumor microenvironment in response to cytotoxic damage and signals through the canonical WNT pathway in order to progress the tumor growth and chemotherapy resistance [20]. These findings in this mechanism by which cytotoxic therapies given in cyclical doses can be used certainly in later treatment resistance and may employ in future medicine specially in research and development of new therapeutic aims like DNA biology. Correspondingly, there are certain similarities between our work's results in comparison with this research. Considerably, we can say that this gene has a noteworthy upregulation in carcinogenesis and tumorogenesis.

Finally, the effect of good quality and quantity of extracted nucleic acids is really so essential and important in molecular studies particularly in gene expression [21, 22]. Impressively, we confirm that the results of this project that investigated the regulatory fluctuation of *WNT16* gene expression in human gastric adenocarcinoma can be employed as a biomarker in this cancer, and also, supplementary studies are recommended. Remarkably, this study concerns the molecular analysis of *WNT16* gene in adenocarcinoma cancer. This is the first time such molecular analysis in this region is performed in gastric cancer and we believe that our data provide a basis for a deeper comprehension of molecular mechanisms underlying gastric cancer progression.

Conclusion

It is concluded that the expression of *WNT16* gene was increased in tumoral tissues significantly. Meaningly, the results have shown a remarkable modification in tumoral tissues than non tumoral tissues alongside with a considerable gene expression in tumoral tissue.

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Authors' contributions SEN collected data, specimens and done molecular and technical experiments. LVL as a pathologist confirmed, controlled, and managed all the specimens and also all patients' histopathological qualifications. MA, A R-P, and AAS accomplished the data quality control, investigated and optimized the informatics database, accomplished the statistical analyses and data processing, wrote the paper and evaluated it, did some other scientific activities including study design, project and protocol development, data analysis, and edited the article. All authors revised the manuscript carefully and gave their assistance in order to progress the article. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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