

# Expression of Villin Associated with Epithelial to Mesenchymal Transition in Patients with Gastric Cancer Relating to their Clinical and Morphological Specifications

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## Abstract

**Aim and Background:** Gastric cancer is the fourth most common cancer in the world and the second leading cause of cancer-related deaths. The metastatic invasive cells of tumor tissue are the main cause of mortality. Numerous biological phenomena are involved in organizing the metastatic process. The Epithelial to Mesenchymal Transition is one of the major mechanisms modulating malignant phenotypes by gastric epithelial cells. Specific cell signals are responsible for epithelial or mesenchymal maintenance of the cells in the tissue. These signals are evaluated by measuring the expression of epithelial and mesenchymal biomarkers in that tissue. Villin is an actin-binding protein mainly expressed in the brush border of epithelium which preserves the shape of the cell and its adhesion to the tissue. The aim of the present research is to study the expression of Villin in the cells as a feasible epithelial biomarker in order to evaluate the cross-sectional situation of the cells. **Materials and Methods:** 38 patients with gastric cancer that were admitted to the Cancer Institute of Imam Khomeini in a period of 6 months were chosen randomly. two samples were collected from each individual; one from the tumoral tissue and one from normal margin of the tumorous tissue. These samples were evaluated after obtaining informed consent from the patients. RNA was extracted from the samples and used as template for cDNA synthesis. The Villin expression was then measured through Real-Time PCR and statistical data according to tissue type and different grades were collected. **Results:** The expression of Villin in tumor tissue of the patients with gastric cancer was significantly lower than the normal tissue. **Conclusion:** As it appears decreased expression of Villin can act as an effective factor toward loss of epithelial nature of the cell and occurring Epithelial to Mesenchymal Transition followed by metastasis.

**Keywords:** Villin- EMT- Gastric Cancer- Real-Time PCR

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## Introduction

Cancer is the second leading cause of death globally [1]. Gastric cancer is developed in the lining of the stomach due to uncontrolled division of mucosa cells [2]. Infection with *Helicobacter Pylori* is the major cause responsible for more than 60% of developed gastric cancers [3-4]. In spite of decreasing incidence and mortality over last decades, gastric cancer is still the fourth most common cancer and the second related to cancer deaths worldwide.

Gastric cancer occurs two times as often in males as in females [5]. Older people are mostly affected by gastric cancer. Average age of diagnosis is 69. The probability of developing stomach cancer in lifetime is about 1 in 111 which changes by risk factors including sexuality, common bacterial infections, smoking and obesity [6].

The progression of cancer is commonly evaluated by two parameters called Grade and Stage [7]. In order to

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grade gastric cancer, doctors attribute numbers 1 to 4 to the tumor tissue in which lesser numbers mean more differentiation and less desire of spreading through the neighboring tissues [8]. Whereas, staging a cancer means the spreading distance of tumor which is attributed to numbers from 0 to 4. Lesser numbers mean that the tumor has not spread distant to the neighboring tissues yet [9].

Gastric cancer is often diagnosed when it has metastasized and this is the reason of its poor prognosis [10]. During metastasis, some tumor cells are detached from their primary site and achieve the ability to penetrate to vessels and migrate through the bloodstream in a process called lymphatic or hematogenous spread. Then these abnormal cells could exit from the vessels and re-penetrate to another tissue and begin proliferating there forming a new tumor. This mechanism is thought to be the major reason of cancer-related deaths [11]. Numeral biological phenomena are involved in organizing metastasis. One of the major mechanisms is Epithelial to Mesenchymal Transition (EMT). EMT is an evolutionarily conserved process which occurs during organogenesis and morphogenesis for shaping embryos [12-13]. In addition, this mechanism occurs during wound healing process where cells gain the ability to migrate across the wound bed to restore the epidermal barrier [14-15]. EMT could be organized in abnormal cancerous epithelial cells and lead to malignant phenotypes [16].

Cytoskeleton is an intracellular network consisting of actin, microtubules and inter-mediate filaments which is present in cytoplasm of eukaryotes [17-18]. The cytoskeleton is a contractive network and involves in many different cell signaling pathways such as cell division and migration by reforming the cell [19]. Villin is an actin-binding protein which associates with actin in order to form brush border of epithelium. Villin counts as an epithelial marker and its downregulation would cause the cell to undergo epithelial to mesenchymal transition followed by metastasis [20].

With duo attention to wide incidence of gastric cancer and also involvement of Villin which is being affected in EMT phenomenon leading to metastasis, the present research is conducted to measure alterations of Villin expression as an epithelial marker.

## Materials and Methods

In a cross-sectional study, 76 samples belonging to 38 patients with gastric cancer who were admitted to Cancer Institute of Imam Khomeini Hospital in a period of 6 months beginning from September 2016 were randomly chosen. Samples were saved in Iran National Tumor Bank (INTB). All of these samples were confirmed by a pathologist in order to evaluate tumor grade. Samples were entered the study after obtaining informed consent from the patients. Two samples were collected from each individual: one from the tumoral tissue and one from normal margin of the tumorous tissue. Clinical characteristics of patients are shown in Table 1.

First total RNA was extracted from tumorous and normal tissues using TriPure (Roche – Germany)

and the purity of extracted RNA was quantified with spectrophotometer (NanoDrop 1000 Thermo Fisher Scientific – USA). Extracted RNA was used as sample for cDNA synthesis using PrimeScript (Takara Bio – USA). The integrity of synthesized cDNA was confirmed by PCR with GAPDH primer and electrophoresis. Villin reference sequence was obtained from GenBank database (NCBI – USA) and was used as template in order to design forward and reverse Villin primers by Primer 3 software. designed primers had appropriate  $T_m$  and GC ratio, avoided secondary structures, cross homology. The same protocol was done for GAPDH as the housekeeping gene as well (Table 2). Primers were synthesized by Takapou Zist company (Iran).

Then the synthesized primers were mixed with SYBR Green Quantitative RT-PCR Kit (Merck – Germany) and Real-Time PCR (Rotor-Gene Q, QIAGEN – USA) was run for 40 cycles and the expression of Villin was quantified during replication. Real-Time PCR setup parameters are shown in Table 3.

REST software (developed by QIAGEN – USA) was used to process yielded data from Real-Time PCR and then Cycle Thresholds ( $C_t$ ),  $\Delta C_t$ ,  $\Delta\Delta C_t$  and Fold Change ( $2^{-\Delta\Delta C_t}$ ) were calculated. The data were processed in SPSS software using 2-tailed Independent T-test ( $P \leq 0.05$ ) and significance of correlation between tumorous and normal tissues and different tumoral grades was evaluated.

## Results

Demographic data of patients including sexuality, tumor size and tumor grade are shown in Table 4. There was no statistically significant correlation between Villin expression changes and sexuality.

The comparison between samples showed that Villin expression was upregulated in 8 and downregulated in 28 patients. The rest 2 patients didn't show significant changes. Also, there was statistically significant correlation ( $P=0.001$ ) between tumoral and normal tissues of the patients (Figure 1). Comparison of Villin expression between tumor grade groups showed no significant correlation (Figure 2).

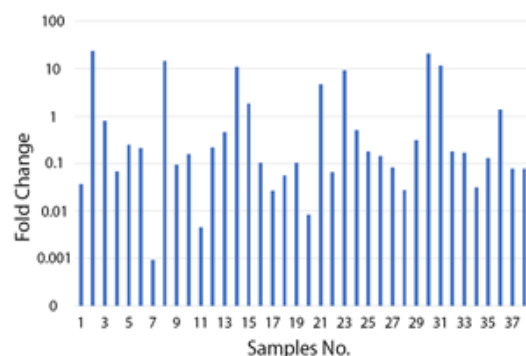


Figure 1. Villin Expression in Patients

Table 1. Clinical Characteristics of Patients

No.	Sex	Tumor Size (cm)	Grade
1	Female	10	I
2	Male	4	I
3	Male	13	III
4	Male	9	III
5	Male	7	II
6	Male	5	II
7	Male	3	III
8	Male	3	I
9	Male	4	I
10	Male	5.5	III
11	Male	3	II
12	Male	2.1	II
13	Male	3	II
14	Female	3.5	III
15	Male	14	III
16	Male	6	III
17	Male	5	II
18	Male	4.5	II
19	Female	8	II
20	Male	10	III
21	Female	6	I
22	Female	2	II
23	Male	2	II
24	Male	1.5	IV
25	Male	3	X
26	Male	3.5	III
27	Male	7.5	III
28	Male	11	III
29	Male	10	III
30	Male	2.5	III
31	Male	5	II
32	Male	16	X
33	Male	8	II
34	Male	4	III
35	Female	5.5	X
36	Male	9	II
37	Male	N/A	X
38	Male	N/A	X

## Discussion

In most cancers, primary tumor is not fetal itself but its metastasis to distant tissues and uncontrolled proliferation. Metastasis and cell invasion are regulated by numeral

Table 2. Primer Sequences

Target Gene	Forward Primer	Reverse Primer
Villin	5'- GGCCAGCCAAGATGAAATTA -3'	5'- CTCAAAGGCCTTGGTGTGTG -3'
GAPDH	5'- GAAGGTGAAGGTCGGAGTCA -3'	5'- AATGAAGGGGTCATTGATGG -3'

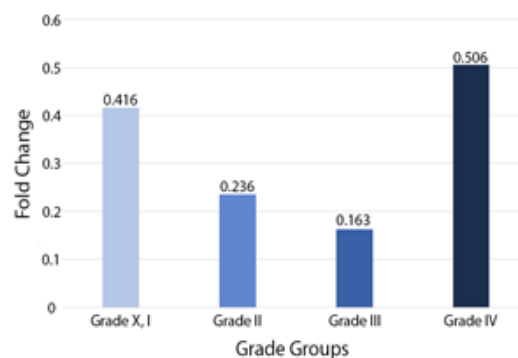


Figure 2. Mean Comparison of Villin Expression between Tumor Grade Groups

phenomena. Actin filaments rearrangement followed by Epithelial to Mesenchymal Transition is a major biological mechanism of metastasis. Villin is an actin binding protein which associates with numeral cell mechanisms and facilitates growth, differentiation, division and motility of the cell [21]. In fact, Villin molecules play an important role in adhesion of the cell to each other and the basal layer and avoid cell migration [22-23]. The present research is a relative quantification study and the results may differ than other studies.

According to similar studies, Villin protein is expressed in all intestinal cells and the expression level increases parallel to differentiation [24-25]. In addition, in another research studying on a cancerous intestinal cell line with

Table 3. Real-Time PCR Setup Parameters

Step	T (°C)	Duration (min)
Primary Preparation	95	10
Step 1: Denaturation	95	0.25
Step 2: Annealing	55	0.5
Step 3: Extension	72	0.5

Table 4. Patients' clinical Characteristics Analysis

Specifications	Number	Frequency (%)
Sex		
Female	6	15.8
Male	32	84.2
Tumor Size		
< 3 cm	7	18.4
≥ 3 cm	31	81.6
Tumor Grade		
X, I	10	26.3
II	13	34.2
III	14	36.8
IV	1	2.7

unstable microsatellites, it showed poorly differentiated cells followed by significantly decreased Villin expression [26]. Generally, it appears that more differentiation results in significantly higher survival rate [27]. Overall, Epithelial to Mesenchymal Transition is one of crucial mechanisms during morphogenesis and transformation of primary tumors into invasive malignancies [28] and evaluation of Villin expression changes can act as a beneficial clinical biomarker in diagnosis, prognosis and treatment [29].

In conclusion, in the present study, we found a statistically significant decreased amount of Villin protein in cancerous tissue versus normal tissue which suggests that Villin expression changes can be a good biomarker to evaluate cross-sectional situation of the tissue and a predicting factor in Epithelial to Mesenchymal Transition followed by metastasis.

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