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Expression analysis of PTEN and CDKN1C/p57kip2 in cancerous tissues from iranian patients with gastric cancer

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Summary. *Background:* Since gastric cancer (GC) is the third prevalence cause of cancer-related death, early diagnosis can improve survival rate. Some studies indicated that loss of tumor suppressor gene (TSG) is a key event in gastric carcinoma. Based on epigenetic alteration each population is valuable to evaluate. So this study investigated the expression rate of phosphatase and tensin homolog (PTEN) and a cyclin-dependent kinase inhibitor of G1 cyclin complexes (CDKN1C) in a population in Iran. *Methods:* 64 gastric samples (32 tumoral gastric tissues and 32 healthy adjacent tissues) were collected from patients referred to Imam Khomeini Hospital Cancer Institute during 2008-2011. Total RNA was extracted, cDNA was synthesized, and then expression level of PTEN and CDKN1C was detected by Real time-PCR. *Results:* Our results displayed PTEN and CDKN1C expression significantly decreased in cancerous tissues compared to healthy adjacent tissues ($P<0.05$). In the case of PTEN, $\Delta\Delta CT$ was calculated 3.04 that showed 8.2 times expression reduction in tumorous tissues. Also, the $\Delta\Delta CT$ of CDKN1C was 2.6 which represents 6.1 times expression reduction in tumorous samples. Furthermore, there is an association between PTEN and CDKN1C expression and vascular invasion. However, the study of parameters such as age, tumor size, sex, ethnicity, and stage were not significantly associated with decreased expression of these TSGs. *Conclusion:* A significant expression reduction of both TSGs in tumoral tissues compared with healthy adjacent tissues suggest that these genes have an important role in gastric cancer incidence and future researches may reveal their advantage in treatment and diagnosis.

Key words: stomach neoplasms, gene expression, PTEN phosphohydrolase, cyclin-dependent kinase inhibitor p57, real-time PCR

Introduction

Gastric cancer is known as fifth common cancer and third common cause of cancer-related death around the world (1). According to the latest statistics released by WHO in 2012 most incidence of this cancer was reported in Korea, Japan, China, Russia and Iran, and also the least cases were related to Western developed countries (2). Based on the accomplished study by Enayatrad and his colleagues on registered cases during the years 2003-2009, gastric cancer in Iran has an upward trend, particularly in the Western and North-Western regions (3).

On the other hand, mainly due to late diagnosis of this disease in advanced stages, the prevalence of gastric cancer is different from its mortality and gastric cancer-related death (4). About half of patients with gastric cancer diagnosed in the advanced stages and less than 30% of this patients had 5 years survival (5).

Like others environmentally induced cancers, the risk of gastric cancer is gradually increasing as the age increases, while between the ages of 55-80 years reach-

es a steady state depending on their associations with risk factors. Overall, gastric cancer incidence in men is two times more than women (6). It is important to note the fact that there are main differences between gastric cancer risk and different ethnicity in the same geographical regions (7).

Gastric cancer is a heterogeneous group of tumors with differences in pathogenesis, morphological characteristics and diverse molecular content (8).

Genetic and epigenetic alterations of TSGs are effective factors for gastric cancer incidence (9) which could inactivate or reduce the expression of these genes (10). While several investigations studied the role of TSGs in cancers, however, their roles in gastric cancer are still not entirely clear (11). PTEN and CDKN1C are TSGs and there are various reports about their hypermethylation, lack of hypermethylation, mutation and loss of Heterogeneity in some malignancy including gastric cancer which is indicative of the diversity of epigenetic alterations in different societies (12-14).

Since gastric cancer is usually asymptomatic until it reaches an advanced stage and also most of the early symptoms are common in other gastric diseases, thus most countries with high prevalence of gastric cancer have planned for a screening of this cancer (15, 16).

In this regard, several tumor markers have been identified which are in associated with gastric cancer (17). But low sensitivity and specificity of these markers have been prevented their clinical usage (16). On the one hand, genetic diversity of each population is the major determinant of susceptibility to different diseases including cancers, therefore extensive studies in a different population are necessary (18).

According to the above, genetic and epigenetic alterations can lead to a different expression of genes in each society. On the other hand, such studies on PTEN and CDKN1C expression in Iranian population have not been done yet.

Also, regarding to the conflicting results of previous studies and lack of definitive conclusions about the value of these genes as biomarkers for early diagnosis of gastric cancer, we investigated the expression level of PTEN and CDKN1C on the 32 early stage tumorous samples compared to 32 healthy adjacent samples of the same patients using Real time-PCR technique.

Methods

1. Samples

This case-control study measured the expression rate of PTEN and CDKN1C on 64 gastric samples (32 tumorous tissues and 32 healthy adjacent tissues of the same patients were used as the control group) of GC patients in the age group of 31 to 83 years, who did not receive any treatment previous to the study. The samples were collected from patients with gastrointestinal complaint that referred to Imam Khomeini Hospital Cancer Institute during 2008-2011.

Since the purpose of this study was early detection of gastric cancer, samples that were in their first or second stages with no advanced metastasis. The cancer stage was determined based on TNM staging system (19) by taking a biopsy during endoscopy and confirmed by a pathologist. Based on this staging system there is a Tumor invasion to lamina propria or muscularis mucosae and metastasis to 1 or 2 regional lymph nodes in stage 1, but there is more than 7 regional lymph nodes metastasis and tumor invades up to serosa in stage 2 (19). Samples were collected based on ethical principles and an informed consent obtained from all patients (previously taken by the staff of Cancer Institute of Imam Khomeini Hospital, Tehran, Iran). The study was approved by the Medical Ethics Committee of the cancer Institute. In addition, the healthy adjacent tissues of the same patients were located farther than 5 cm from the tumor and there were no tumorous cells (based on pathologist report) were used as control group.

2. RNA extraction

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Concisely, each sample was homogenized in 1 ml TRIzol reagent then 0.2 ml of chloroform was added for phase differentiation. Following centrifugation at 12,000 × g for 15 minutes at 4°C, RNA was left entirely in the aqueous phase and precipitated with 0.5 ml isopropanol. The RNA pellet was washed with 75% ethanol and resuspended in diethyl pyrocarbonate-treated water. The RNA quan-

tity and quality respectively measured with NanoDrop spectrophotometer (Bio-TeK, USA) and agarose gel electrophoresis stained with SYBR Safe dye (Invitrogen, USA). Finally extracted RNAs were stored at -80°C until cDNA synthesis.

3. cDNA synthesis

cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas Co., Canada). According to the manufacturer's instructions all reagents included: 5 µg total RNA, 1 µg Random Hexamer Primer, Up to 12 µl nuclease-free water, 4 µl Reaction Buffer (5X), 1 µl RiboLock RNase Inhibitor (20 U/µL), 2 µl dNTP Mix (10 mM), 1 µl RevertAid M-MuLV RT (200 U/µL), were mixed gently and centrifuge briefly and incubated for 5 min at 25˚C followed by 60 min at 42˚C finally reaction terminated by heating at 70˚C for 5 min.

4. Real-time PCR amplification

Real time quantitative PCR with SYBR® Premix Ex Taq™ kit (Cat No: RR820L, Takara, Japan) was performed in the total volume of 20 µl containing 10 µl SYBR Premix Ex Taq II, 7 µl nuclease-free water (CinnaGen, Iran), 1 µl cDNA and 1 µl of each primer. All primers were designed with Allele ID 7 software and listed in Table 1. Each sample was processed in duplicate by using Bio-Rad thermal cycler (Bio-Rad, USA) with the following cycling conditions: 95˚C for 30 s, 40 cycles of 95˚C for 5 s and 49-53˚C for 30 s (annealing), and 72˚C for 30 s, and finally 72˚C for 15 s. Also controls were done with no template for each gene and 18srRNA was used as internal reference gene. Analysis of real time -PCR results performed with a ΔΔCT method in order to this aim average of cycle threshold (CT) values for each target gene and reference gene was calculated. Then ΔCt ($CT_{\text{target gene}} - CT_{\text{18srRNA}}$) of tumoral tissues acquired and by compare with ΔCt of healthy adjacent tissues ΔΔCT [$\Delta\Delta CT = (CT_{\text{target gene}} - CT_{\text{18srRNA}})_{\text{tumoral tissues}} - (CT_{\text{target gene}} - CT_{\text{18srRNA}})_{\text{healthy adjacent tissues}}$] calculated and by $2^{-\Delta\Delta CT}$ fold change computed (20, 21).

5. Statistical analysis

Differences in mean between both target genes expression in the sample of tumorous tissue and healthy adjacent tissue and also the relation of both genes expression with demographical and clinicopathological characteristics (ages, genders, and stages) of each patient were analyzed using Paired sample t-test and Independent sample t-test respectively. To determine the relationship between gene expression and different ethnicity of studied group one-way ANOVA test was used. Statistical analysis was accomplished with SPSS version 10 for Windows (SPSS Inc., Chicago, IL, USA). The differences were assumed to be significant when the p-value is less than 0.05.

Results

Investigation of relationship between both genes expression in gastric tumor samples and demographical and clinicopathological characteristics indicated that there were no significant differences in both genes expression rate and ethnicity, gender, age (patients 59 ≤ years old vs. patients 59 > years old), tumor size (>5 cm vs. ≤5 cm) and stage. On the other hand, there was a significant association between PTEN/CDKN1C ex-

Table 1. PCR primers designed with Allele ID 7 software.

Gene name	PTEN	CDKN1C
Association No.	Variant1:NM_000314.6 Variant2:NM_001304718.1	Variant3: NM_001122631 Variant2: NM_001122630 Variant1: NM_000076
Forward primer	**5′**AGTCCAGAGCCATTTCCATC **3′**	**5′**CCACATCTGGTTATTGACAAG **3′**
Reverse primer	**5′**GATAAATATAGGTCAAGTCTAAGTCG **3′**	**5′**ATAAGAGAGACAGCGAAAGC **3′**

pression rate and vascular invasion (P <0.05). A summary of this results is given in Table 2.

PTEN Expression in cancerous and healthy adjacent tissues

The expression rate of PTEN decreased in 88.2% cases (twenty-six samples out of thirty-two) and increased in 18.7% cases (six samples). This gene expression shows a significant difference between tumorous and healthy adjacent tissues groups. The expression fold change of PTEN was -8.2, which means that the expression rate of PTEN decreased in the tumorous tissues (Table 3).

CDKN1C Expression in cancerous and healthy adjacent tissues

The expression rate of CDKN1C decreased in 78.2% cases (25/32) and increased in 21.8% cases. Statistical analysis indicated that CDKN1C expression was significantly different between tumorous and healthy adjacent tissues groups (p<0.00) and calculated fold change was -6.1 which indicated that the expression rate of CDKN1C decreased 6.1-fold in the tumorous tissues in compare with healthy adjacent tissues. Also, it is important to say the expression changes in 18srRNA was not significant and this reveals the accuracy of 18srRNA as a reference gene (Table 3).

Discussion

The purpose of this study was to investigate the alterations of the expression level of two important genes in gastric tumor tissues and healthy adjacent tissues as a control. Results of the present study indicated that the expression level of PTEN / CDKN1C was respectively decreased 8.2/6.1 times in gastric tumor samples compared to healthy adjacent samples (p<0.05).

A review of the studies on the role of PTEN and CDKN1C confirm the present results and suggests a significant down-regulations or inactivation in vari-

Table 2. Summary of demographical and clinicopathological characteristics of samples and PTEN/CDKN1C expression.

Parameters		NO (%)	PTEN ΔCT ± SD	P-Value	CDKN1C ΔCT ± SD	P-Value
Gender	Males	26 (81)	10.53 ± 2.16	0.343	11.66 ± 1.89	0.257
	Females	6 (19)	11.07 ± 0.86		12.12 ± 1.59	
Age (years)	≥ 59	24 (75)	11.49 ± 2.82	0.289	12.34 ± 2.36	0.349
	< 59	8 (25)	10.30 ± 1.61		11.54 ± 1.55	
Tumor size	> 5cm	26 (81)	10.21 ± 2.01	0.786	11.44 ± 1.53	0.551
	≤ 5cm	6 (19)	10.79 ± 1.99		11.86 ± 1.89	
*TNM stage	I	10 (31)	10.2 ± 2	0.649	11.62 ± 1.75	0.353
	II	22 (69)	9.8 ± 2.6		10.82 ± 2.40	
Vascular invasion	Yes	19 (59.4)	11.8 ± 1.9	<0.001	12.90 ± 1.53	<0.001*
	No	13 (40.6)	8.7 ± 1.9		9.81 ± 1.69	

TNM: tumor, lymph nodes, metastasis stage

Table 3. Comparison of PTEN and CDKN1C expression in cancerous and healthy adjacent tissues. (P < 0.05 was considered to show a significant difference)

Variables (ΔCT)	N	Tumorous Tissues Mean ± SD	Healthy Adjacent Tissues Mean ± SD	Paired t-test P-value
PTEN	32	9.9 ± 2.4	6.9 ± 2.7	<0.001
CDKN1C	32	11 ± 2.2	8.4 ± 2.5	<0.001

ous malignancies, including brain cancer, endometrial cancer, breast cancer, ovarian cancer, prostate cancer, bladder cancer, liver cancer and oral squamous cell cancer, but there were little information about these genes expressions in gastric cancers (11, 12, 22-29). For example, the study done by Wen *et al.* on 144 gastric cancer patients revealed that the expression level of PTEN was decreased and also decreased expression of E-cadherin, simultaneously with the overexpression of PI3K, AKT, MMP-2, MMP-9, and NF-κBp65, participated in accelerated progress of gastric cancer (30). The study conducted by Shin *et al.* on 30 gastric tumor-normal pairs and 8 gastric cancer cell lines have shown that no mutation was detected in CDKN1C but the expression level of this gene was decreased significantly in gastric cancer cell lines compared to normal cells. As a result, they concluded that inactivation of this gene expression probably involved in gastric cancer tumorigenesis (31).

Although many evidence suggests that PTEN and CDKN1C are involved in the cell proliferation and tumor progression as tumor suppressors (32, 33) which seem to be decreased expression but some studies have reported different results (12, 34, 35). For example, Sato *et al.* observed similar levels of PTEN expression in all gastric cancer cell lines and primary tumors and concluded that PTEN doesn't take part in gastric carcinogenesis as a tumor suppressor agent (12). Also, a study done by Kai Sun *et al.* on the expression level of CDKN1C and MiR-221 in colorectal carcinoma revealed that the expression level of CDKN1C was not significantly different between tumorous and adjacent non-tumorous tissues. However, the expression level of CDKN1C protein in tumorous tissues was obviously decreased and this changes attributed to post-transcriptional regulation (35).

Altogether, genetic and epigenetic factors have a different effect on genes expression in each population and this is the main value of such studies.

Findings of the present study showed that there were no relationship between PTEN/CDKN1C expression and demographical and clinicopathological features except one case. Both genes expressions were decreased significantly when the vascular invasion occurred in compare to the expression level in samples without vascular invasion ($p<0.05$).

Our results in this cases are inconsistent and contrast with some other studies for example Yang *et al.* revealed that there was no detectable correlation of PTEN and phosphorylated PTEN expression with pathological features from gastric cancer patients (36). Lin Yang *et al.* stated that the expression level of PTEN protein was reduced as well as gastric cancer progression and recommend PTEN as a good prognostic biomarker for gastric cancer (32). Moreover, Nan KJ *et al.* in a study on hepatocellular carcinoma understood that the low expression of CDKN1C is in association with the low or medium differentiation of tumor cells, high stage, and poor prognosis but there was no significant correlation between metastasis, tumor size, and age (28). Another study accomplished by Fan *et al.* represented that reduced level of CDKN1C expression in the oral carcinoma was remarkably in a relationship with advanced stage, metastasis and tumor size, and they nominated this gene as a good prognostic biomarker in oral carcinoma (29).

Although, we have to consider that the obtained results can be attributed to our small sample sizes. However, a post hoc power analysis indicated that limited sample size leads to a medium affect size in case of the type 5%. However, more studies with a larger cohort are necessary to approve these associations.

Conclusion

The present study approved that the expression level of PTEN and CDKN1C significantly decreased in Iranian studied group. Based on this study and some previous studies these tumor suppressor genes could be considered as an early stage biomarkers for primary diagnosis of gastric cancer. Also, the practical value of these biomarkers could be confirmed by further comprehensive studies on more accessible samples such as blood.

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