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The levels of telomerase gene expression in gastric lavage fluid

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KEYWORDS

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A B S T R A C T

Achieving a noninvasive method for early detection of gastric cancer with high sensitivity has been always considered by researchers. In previous studies, high level of telomerase gene expression in tissue samples obtained from endoscopy of patients has been proven repeatedly as a tumor marker, but it was lacking clinical application due to the need for endoscopy to achieve tissue samples. The aim of this study was to determine the levels of telomerase gene expression in gastric lavage fluid obtained from healthy subjects and patients with gastric cancer. In a descriptive cross-sectional study conducted at the Department of surgery on patients with gastric cancer during 2012-2013, gene expression level of telomerase in gastric lavage fluid obtained from healthy subjects and patients with gastric cancer was examined. In this study, 30 patients with primary gastric adenocarcinoma and 50 healthy individual were selected randomly and entered the study. The level of telomerase gene expression in gastric lavage fluid was high for all 30 patients with gastric cancer and it was up to 6.653 times more than healthy people (the efficacy of 1.226 to 33.980). Considering that all of the people had high levels of gene expression in stage II-IV, Real-time PCR is regarded to have high sensitivity to investigate the level of activity of telomerase gene to detect cancerous tissue in the advanced stages of the disease. This method has no statistical value to be generalized to the whole society, since it lacks participation of people with early stages of disease (stage-I).

Introduction

Gastric cancer with high prevalence is an important cause of mortality caused by malignancies in our society (1-2). In examining the predictive factors in five-year survival of patients who had undergone surgery because of gastric cancer, early stage had high predictive significance (3-4). Unfortunately, a high percentage of patients with gastric cancer refer to medical centers with non-specific symptoms in the early stages of the disease. It seems that achieving a method with high sensitivity and

specificity as well as noninvasive plays a significant role in screening these patients with proper guidance of medical staff and reduces diagnostic and therapeutic expenses. It is regarded a big step in order to help these patients and to prevent common costly and invasive diagnostic interventions (including radiological assessment of stomach with barium swallowing and endoscopy and histological biopsy) in most patients.

In this regard, several studies have been done on human protein as a tumor marker for gastric cancer; although, the findings were not satisfactory.

During the past decade, with the advances on molecular genetics and the use of gene replication methods in the laboratory environment, a lot of attention has been paid to the levels of telomerase gene expression as a tumor marker with high sensitivity and specificity in the diagnosis and follow-up of treatment of different cancers (5).

This gene which is responsible for immortality of cancer cells is accessible with small values in the the body's tissues and the high level of expression of this gene is in correlation with presence of dysplastic tissue in the sample. It has been proven for gastric adenocarcinoma in previous studies (6-9), but it lacks clinical value practically due to the need for endoscopy to achieve tissue samples and the excellence of histological investigations rather than molecular findings in tissue samples. In this study it is tried to check the level of telomerase gene expression in gastric lavage samples compared to healthy subjects and remove restriction of need to tissue samples and subsequently endoscopy for the detection. Perhaps, this result in achieving an appropriate and non-invasive method with better screening of these patients in the early stages of disease which is an effective step in order to help patients and reduce diagnostic and therapeutic expenses.

The aim of this study was to compare the levels of telomerase gene expression in normal subjects and patients with cancer.

Materials and Methods

Sampling: The gastric lavage was collected before any incision or biopsy; Gastric cavity was washed with 100 ml of normal saline solution (20 ° C) following the nasogastric tube placement in both groups of patients and the healthy subjects (control group). The liquid was stored in the standard, sterilized containers to prevent RNA lysis after suctioning and eventually it was sent to the lab for examination as soon as possible.

The inclusion criteria for patients

The risk for the gastric cancer (adenocarcinoma) and the consent for participation in a research study.

The inclusion criteria for healthy subjects

No risk for gastric cancer, normal Gastroendoscopy experience in the last two months and the consent for participation in a research study.

The exclusion criteria for patients

History of cancer elsewhere in the body, history of chemotherapy or treatment with drugs that affect the cell cycle, previous radiotherapy and other pathologic reports for cancer other than primary gastric adenocarcinoma (just in case).

RNA extraction from tissue samples

RNA was extracted from the samples according to the RNX-plus protocol:

- 2 mL of the homogenized sample was extracted via the centrifugation at a speed of 1000 rpm , at 25 º C for 10 minutes;
- 1cc of RNX-plus was added to the extracted sample, at room temperature. The solution was stirred for 5 to 10 seconds then incubated for 5 minutes at room temperature;
- 200 micrograms of chloroform was added. The solution was stirred for 15 seconds and then incubated at 4 ° C for 5 minutes;
- Samples obtained was centrifuged with a speed of 12000 rpm, at 4 ° C for 15 min
- Aqueous phase was transferred to a new tube (1.5 cc) without RNA following the completion of centrifugation and then isopropanol was added to an equal volume (1.5 ml);
- After incubation, the solution was maintained on ice for 15 min and then it was centrifuged with a speed of 12000 rpm, at 4 ° C for 15 min
- The suspension phase was poured out of the bottle then 1 cc of 75% ethanol was added and gently stirred and the solution was centrifuged with a speed of 7500 rpm, for 8 min
- The poured suspension phase was dried at the room temperature then the separation was accomplished in 60°C water bath by use of the 50 micrograms of DEPC-treated-water.

How to check the level of gene expression

Beta-actin gene was used as a house-keeper gene in both patients and controls, and the intensity of telomerase gene expression was calculated in comparison with the intensity of Beta-actin gene expression in each sample.

After determining the delta CT in 50 healthy subjects, the level of telomerase gene activity in the control group was determined by real-time-PCR method, which was considered to be equal to the average obtained from healthy subjects.

Delta CT was calculated in 30 patients by using real-time-PCR. The enzyme activity level was quantitatively assessed by use of the device software compared to the baseline activity in patients then the distribution curve was plotted.

Evaluation of disease stages

The stage of disease was preoperatively determined by CT scan, based on TNM system then it was correct with the help of pathological stage in the cases undergoing surgery.

Ethical considerations

The purpose of the study and methodology were understandably and briefly explained for the patients who were supposed to be registered in the study. All information was presented to patients about the purpose of examination and the risks and the written consent was obtained before deciding to participate or not to participate. All costs include the cost of materials and the studies were paid by the researcher in order to eliminate the patients' costs.

Results and Discussion

The 30 patients with gastric cancer and 50 healthy subjects as the control group have been studied in this research. It should be noted that the 24 case patients and 13 control patients were male.

The mean age of patients and controls was 61 and 49 years old respectively. There were

7 patients in stage-IV, 22 patients in stage-III and a patient in stage-II. The surgical procedure was performed on the stage II & III patients and the postoperative pathologic findings were compatible with the clinical stage. No postoperative pathologic findings were obtained for 7 patients who were in stage-IV since the surgical procedure was not performed. The 30 patients with dyspepsia and 20 patients with upper GI bleeding were evaluated by endoscopy among the 50 controls who had participated in the study.

Telomere-telomerase gene was designed after obtaining the samples and extraction of RNA. The Beta-actin (ß – actin) gene was used as house-keeping gene in order to examine the samples. Delta CT was calculated as follows for each sample sent to the laboratory.

CT derived from the ß – actin intensity activity ÷ CT derived from the telomerase intensity activity = Delta CT. Given that gene expression has strongly normal distribution in normal, so it could be referred to the Delta CT of healthy subjects. Consequently, the average delta CT calculated in healthy subjects could be considered as the basic level (number one)

Figure I: The horizontal axis represents the delta CT in healthy subjects and the vertical axis represents the number of subjects.

Considering the normal distribution of delta CT in healthy subjects, the Delta CT average calculated in healthy subjects could be considered as a reference standard to determine the changes in patients' CT Delta.

Also the intensity of gene expression was measured as the delta CT by using the ß - actin house keeping gene in samples obtained from 30 patients.

CT derived from the ß - actin intensity activity ÷ CT derived from the telomerase intensity activity = Delta CT

Figure II: indicates the intensity of telomerase gene amplification compared to ß - actin (horizontal axis) in the patients group (vertical axis).

As is clear from the graph, the intensity of expressed genes has a wide range and most patients were in the range of 4 to 7 times.

The telomerase gene expression in patients was variable compared with the average of healthy subjects which was between 1.226 and 33.980 times.

The diagram designed by the statistical software represents the intensity of telomerase gene expression in patients compared with the average of healthy subjects. This study was conducted with high accuracy (reaction efficiency = 0.97).

Conclusion

Gastric cancer, or uncontrollable growth of malignant cells in the stomach, is regarded as an important reason of mortality caused by malignancies worldwide. According to the statistics provided by WHO, about one million people die due to this disease worldwide every year. According to presented statistics, it is thought that gastric cancer is the most prevalent cancer in men and the third prevalent type of cancer in women. According to presented statistics in Iran, 26.1 per 100000 men and 11.1 per 10000 women suffer from this disease and this is considered from high prevalence areas based on global stats(1-2).

The initial signs and symptoms of gastric cancer are obscure and similar to benign gastrointestinal discomforts, so early detection by doctors in patients has been impossible without taking advantage of the invasive procedures such as endoscopy(3-4).

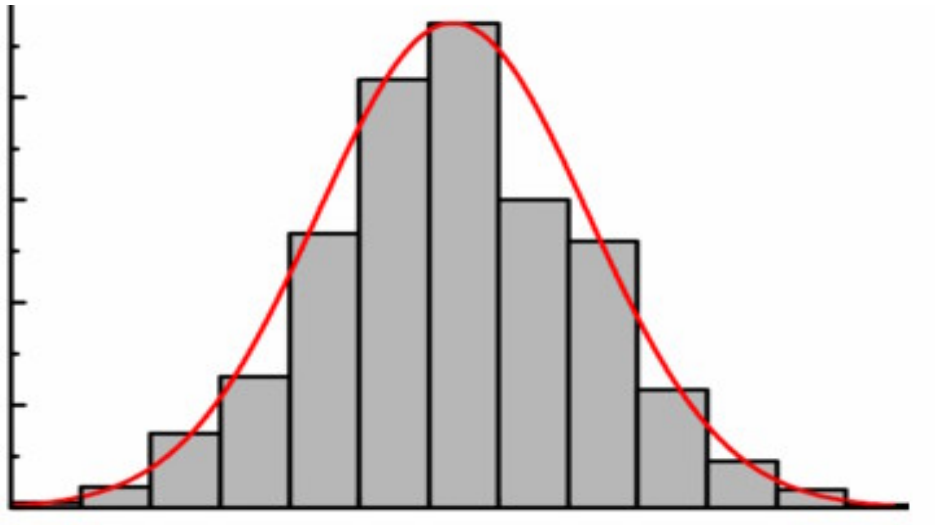

Figure.I The horizontal axis represents the delta CT in healthy subjects and the vertical axis represents the number of subjects.

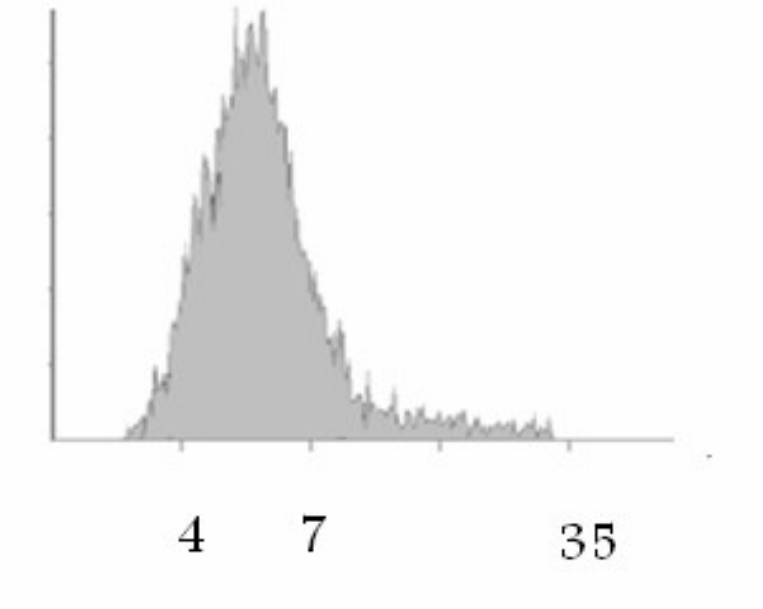

Figure.II The intensity of telomerase gene amplification compared to ß - actin

On the other hand, among the numerous prognostic factors which are known effective in patient's survival, stage of disease is more important. Therefore, to achieve a non-invasive screening for a suspicious individual has high importance for the reduction of financial and human burden and helps improving patients with early detection of prognostic patients.
In this regard, several studies have been done on human proteins as tumor markers of gastric cancer, while the findings were not satisfactory.

During the past decade, with the improvements on molecular genetics and ge ne replication methods in the laboratory, considerable attentions have been paid to the telomerase gene expression level as tumor marker with high sensitivity and specificity for the diagnosis and follow up of different types of cancers (5).
Physical terminal sequence of non-linear chromosomes of mammals has been formed from a non-coding sequence of TTAGGG repeats under the name of Telomere.

Despite that Telomere is a double-stranded DNA, it has a short sequence rich of T and A (14 to 16 nucleotide) in the end region joining an interior area and forms a structural Telomere called T-loop that will be consolidated with creating a connection with the proteins which are bind to the Telomere.

Telomere has critical importance as a protective shield to protect the genome of eukaryotic cells and excessive reduction of the length of the Telomere leads to loss of the ability of this structure to function and eventually causing cell death. This would lead to the ageing and finally destruction of the cell.

Although it has not took a long time to identify telomerase gene and its role in the immortality of cancer cells, several studies were designed and implemented to evaluate the diagnostic and prognostic value of this gene in different types of cancers (10-15). It is considered by researchers in the case of gastric cancer as well.

During the three-year life of the human knowledge of telomerase, several articles published in this field in different journals(16). In a nutshell, we referred to a few cases:

In the investigation of the importance of telomerase gene expression in the diagnosis of gastric cancer by Dr. Ahn MJ et al. they expressed that the sensitivity of this method is 88% for the diagnosis of gastric cancer (6).
Dr. Hiyama et al examined the activity of telomerase gene in 66 patients with gastric cancer and high activity of the telomerase gene was observed only in 85% of cases (16).

Zhan WH et al studied the role of telomerase gene expression in patients with gastric cancer and sensitivity of 88% has been mentioned for detection of cancer in advanced stages and 50% at early stage for this method (17). The common weakness of all of these studies is emphasizing the level of gene expression in tissue specimens which are provided via endoscopy. More conclusive results obtaining from histological analysis of tissue samples has been limited virtually utilization of PCR methods to research studies

In a qualitative evaluation of telomerase gene expression in samples which are obtained from gastric lavage in the method of endoscopy in healthy subjects and patients with gastric ulcer,

which is performed by Wong SC et al, it was expressed that gene expression has not been reported in all healthy individuals; report on 24 out of 25 patients with cancer and 7 out of 25 patients with gastric ulcers has been reported positive and sensitivity of gastric cancer detection is equal to 80% and PPV% is 74 for this method (9). Li-ming et al, in a qualitative evaluation of telomerase gene expression in samples which are obtained from gastric lavage and lavage of the peritoneal membrane in healthy subjects and patients with gastric ulcer expressed that there exist significant differences between the two groups in terms of gene expression of telomerase in gastric lavage fluid and peritoneal membrane lavage (18). With the clarification that the non-tissue samples of gastric lavage are equivalent to tissue samples in matters of sensitivity in the study of telomerase gene activity, a number of studies were done to determine the existence of this gene in gastric lavage fluid.

The common weakness of these studies was to achieve high sensitivity and NPV and PPV, especially in the early stages of the disease.

During the past decade with the advances of molecular genetics, the possibility of gene expression evaluation with Real-time PCR was created and the ability of studying and comparing the severity of expression of these genes has been possible quantitatively; therefore this study was designed and carried out accordingly.

Unfortunately, as was pointed out at the beginning of the study, the majority of patients with gastric cancer are referred to hospital in advanced stages of disease which is clearly noticeable in this study causing weakening of the study and the lack of the presence of patients with early gastric cancer destroyed the capability of generalization of comments and findings to the whole society.

According to the findings of this study it is observed that the intensity of Telomerase gene expression in the patient, especially in advanced stages, increased dramatically compared to healthy people, but generalizing this feature to the whole community only on the basis of the findings of the study is quite unreasonable (including those with early-stage and advanced- stage and healthy individuals) and it needs further investigations with more samples including patients with stage I and stage II disease.

One of the subsidiary objectives of this study was to evaluate the prognostic value of gene expression levels in lavage fluid for diagnosis of patients with stage IV and avoiding the unnecessary laparatomies. To answer this question that whether the stage of the disease is associated with gene expression or not and to what extent is the ability to predict stage of patients from gene expression level, results of two groups of patients with stage 3 and 4 were compared. There was no significant difference between the stage III and stage IV. These results were not the same as findings from two studies conducted on peritoneal membrane lavage which had reported significant differences. However, surveys on gastric lavage fluid have not yet been published so far.

The confounding factor in the hypothesis of this study is probably not considering type of tissue subgroup of the cancer and mitotic severity (cellular grade).

Suggestions

Reduction of the human and financial burden on patients. A method alternative to histological studies. Innovation of intelligent catheter for continuous screening of patients.

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