Gene expression profiling revealed overexpression of vesicle amine transport protein-1 (VAT-1) as a potential oncogene in gastric cancer

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Gastric cancer (GC) is one of the most common types of cancer worldwide. Owing to the distinct molecular pathology and the increasing progression rate of GC in Asia, suppression subtractive hybridization (SSH) was used as a high-throughput gene expression analysis method to find genes associated with GC pathogenesis. Total RNA was extracted from the clinical samples, and mRNA was isolated and used in SSH method. The subtracted library was subjected to cloning and the randomly selected clones were sequenced. qRT-PCR was used for expression analysis. The overexpression of vesicle amine transport protein-1 (VAT-1) gene was observed and its expression was analyzed by qRT-PCR in clinical tissue samples. According to the potential oncogenic activity of VAT-1 and its probable surface-occurrence in GC cells, it might be involved in GC pathogenesis and invasion, and it is suggested to be investigated in the diagnosis and therapeutics of GC.

Keywords: Gastric cancer (GC), overexpression, suppression subtractive hybridization (SSH), VAT-1

Introduction

Gastric cancer (GC) is the second most cause of cancer-related deaths in the world. Over recent 50 years, GC incidence has been decreased in the developed countries. While because of unknown reason, it has a growing rate in less developed societies with 70% of GC cases for developing countries. In addition, the molecular pathology of this cancer differs in various parts of the world. Owing to its aggressiveness and late diagnosis at the advance stages, GC has been categorized as a poor prognosis type of cancer. The most common GC type is adenocarcinoma with two following subtypes: the well-differentiated or intestinal type and the poor differentiated or diffuse type. Since the molecular mechanisms leading to GC have not been completely identified, the molecular features of this cancer have not been used for its diagnosis, subtype classification and treatment. Considering the above, it is clear that a precise knowledge about the genetic changes in GC could have a drastic effect on the early diagnosis, treatment and the patient’s survival rate.

There are two types of methodologies to study gene expression in cells. In spite of being high throughput, the first methodology includes microarray and real-time PCR, which requires pre-knowledge about the sequences of the genes in order to design the probes and primers. This limitation makes the pre-selection of the candidate genes in the study inevitable. Therefore novel genes out of this selection could not be analyzed by this methodology. In contrast, the second methodology includes suppression subtractive hybridization (SSH), which enables simultaneous evaluation of differential gene expression in all the genes, between two different conditions, with no need for background information on the genes. Therefore, as a high-throughput method, SSH could be applied to find novel genes involved in cancer molecular biology and new biomarker candidate genes.

In the present study, SSH was used to investigate the adenocarcinoma type of GC in order to provide more information on the genes associated with the molecular mechanisms involved in GC pathogenesis.
and invasion. The identified genes could be studied and applied in the diagnosis and treatment of GC in future.

Materials and Methods

Tissue Collection

For construction of the main library of SSH, tumor and normal tissues were collected from a 64-yr-old male patient who was under gastrectomy. Soon after the resection, target tissues were isolated under microscope by a pathologist. In order to stabilize the RNA, the samples were preserved in RNA later® (Ambion, TX, USA), stored at 4-8°C for 24 h and finally transferred to -70°C. The isolated target, normal and cancerous tissues, were examined by an experienced pathologist using hematoxylin-eosin (H&E) staining for the detection of the tumor type and metastasis.

Further, endoscopic clinical tissue samples were obtained from patients for analysis of the differentially expressed genes by qRT-PCR. The samples were collected from the patients before the chemotherapy. The written consent form was approved by ‘Biologic Sampling Ethics Committee’, Tehran University of Medical Sciences (TUMS), Tehran and obtained from each patient before gastrectomy or endoscopy.

Total RNA Extraction and mRNA Isolation

Total RNA was extracted by an improved form of acid-guanidium-chloroform method using TriPure Isolation Reagent (Roche Applied Sciences, IN, USA) according to the manufacturer’s instructions. The concentration and purity of the extracted RNA were analyzed using Biophotometer (Eppendorf, Hamburg, Germany). Total RNA was visually checked on 1% denatured agarose gel. mRNA was isolated from the extracted total RNA by a method based on base-pairing between polyA residues at the 3’ ends of mRNA and the oligo(dT)25 residues of DynaBeads using the DynaBead® mRNA Isolation Kit (Invitrogen, Oslo, Norway), following the manufacturer’s instructions and used for SSH.

SSH

In SSH (suppression subtractive hybridization) method of cancer research, the subtracted library is constructed from one sample pair of cancerous and normal tissues in both forward and reverse directions, and the expression of the obtained genes are analyzed in clinical tissue samples using methods including quantitative real-time PCR (qR-PCR)6,7. In the present study, SSH was done by the PCR-Select™ cDNA subtraction kit (Clontech, CA, USA) according to the manufacturer’s protocol. Subtraction was performed in both forward and reverse directions. Briefly, after cDNA synthesis using 2 µg mRNA from the normal (driver) and cancerous (tester) gastric tissues, RsaI digestion was carried out to the cDNA. For the reverse subtraction, the tester was used as a driver and the driver as a tester. Tester cDNA was divided into two groups and two distinct adaptors were ligated to these two groups. After two steps of hybridization between tester and driver, using excess amounts of driver cDNA, the differentially expressed sequences were significantly enriched. Finally, using two PCR steps, the differentially expressed sequences were more enriched and any background PCR products were reduced.

Cloning, Sequencing and Analysis of Sequences

After purification by the High Pure PCR Product Purification Kit (Roche Applied Sciences, Mannheim, Germany), PCR products were cloned into pUC19 plasmid vectors and transformed to Escherichia coli NovaBlue competent cells (Novagen, WI, USA). In total, 70 colonies were picked arbitrarily and verified by colony PCR using N1 (5′-TCGAGCGGCGCCGGCGAGGT-3′) and N2R (5′-AGCGTGTCGCGCCGGAGGT-3′) primers. Plasmids from the positive clones were isolated by the High Pure Plasmid Isolation Kit (Roche Applied Sciences, Mannheim, Germany) and sequenced. Similarity searches were done using BLAST (www.ncbi.nlm.nih.gov/blast/blast.cgi) to analyze the sequences.

qRT-PCR

Total RNA was extracted from gastric tissue samples (cancerous & normal) using Tripure Isolation Reagent (Roche Applied Sciences, USA) according to manufacturer’s instructions. First cDNA strand synthesis from 1 µg total RNA was done using Expand Reverse Transcriptase (Roche Applied Sciences, Mannheim, Germany) and oligo (dT)25 primers according to manufacturer’s protocol.

Forward (5′-GAAGCTGCTGCTTGCTTCGCT-3′) and reverse (5′-TCCTTCAGTGCTTGCTTCGCTGTCC-3′) VAT-1 primers were designed on different exons to diminish any possible interruption of DNA contamination. Beta actin (ACTB) gene was selected as an internal control using forward (5′-ATGGCCACGGCTGCTTCGAG-3′) and reverse
(5′-CAGGAGGACATGATCTTGAG-3′) primers. The primers were designed using PrimerSelect software version 7.1.0 (DNastar, WI, USA) and synthesized by GATC Company, Denmark. The qRT-PCR was run on Step-One-Plus apparatus (ABI, USA) using SYBR Premix Ex Taq (TaKaRa, Shiga, Japan). Reactions consisted of 2× master mix 10 µL, forward and reverse primers (10 µM) 1 µL each, ROX dye II 0.4 µL, cDNA 2 µL and DEPC treated water to the final volume of 20 µL. After an initial heating step (95°C for 10 min) for activation of the hot start Taq DNA polymerase, the thermal program was performed for 40 cycles at 95°C 30 sec, 62°C 30 sec, 72°C 30 sec, 80°C 10 sec. Melt curve analysis was done following the PCR step by increasing the temperature from 65°C to 95°C with 0.1°C/sec increments in each fluorescence reading. PCR products were checked on 2% agarose gel for specificity analysis of the primers. The fluorescence was measured at the end of the 80°C step to diminish any possible interruption of the primer dimmers. The efficiency of the reaction was calculated for both VAT-1 and ACTB genes using standard curve, which consisted of 5 concentrations of 10-fold serial dilutions of the prepared cDNA with 100 ng of cDNA as the first quantity. The relative gene expression of VAT-1 in tumor and normal tissues was analyzed by REST software (REST 2008 V2.0.7, Corbett Research, Sydney, Australia) using the Pfaffl method.

Quantitative Real-Time PCR (qRT-PCR)
In the surgical tissue used by SSH, the relative gene expression of VAT-1 in the tumor tissues showed a dramatically overexpression (10 fold, Fig. 2) with compared to normal tissues. The relative gene expression of VAT-1 was primarily checked in some endoscopic tissue samples obtained from patients. The results showed VAT-1 overexpression in endoscopic tissue samples in comparison to normal endoscopic tissues (Fig. 2). The calculated reaction efficiencies for the VAT-1 and ACTB genes were 95.9% and 106.9%, respectively.

Discussion
The present study has focused on the overexpressed genes associated with gastric adenocarcinoma, the most prevalent type of cancer in the developing countries including Iran, and VAT-1 was identified as an overexpressed gene in this type of cancer.

Results
Histological Examination
Histological examination revealed that the diseased tissues had a moderately-differentiated, mucin-producing type of adenocarcinoma, located in the prepyloric area. Local invasion and metastasis to the lymph node were observed in two of the six pre-gastric lymph nodes (Fig. 1).

SSH
Using SSH, two subtractive libraries (100 to 800 bp in size) were made, which resulted in totally 120 clones. Among the significantly overexpressed genes from the forward library, a 153 bp sequence was annotated as vesicle amine transport protein-1 (VAT-1) gene (gene ID 10493) using BLAST algorithm. Since the migration of the cancer cells and metastasis play important roles in GC and according to the demonstrated role of VAT-1 in the invasion of the cancerous cells in glioma cancer, the VAT-1 gene was selected for further analysis in order to check its expression in GC using the qRT-PCR method.

Fig. 1 (a & b)—Histological features of the resected gastric cancerous tissue stained by H&E to determine the type of cancer and metastasis: (a) Tubular structure formation (arrows), which characterizes these cancerous cells as intestinal gastric adenocarcinoma type; & (b) Cross-sectional analysis of a pre-gastric lymph node. The tubular formation of the gastric adenocarcinoma cells and lymph node follicles could be observed. The arrows represent the metastasis of gastric cancer cells to the lymph node.
Fig. 2—Relative gene expression analysis of VAT-1 in the tumor and normal tissue samples by qRT-PCR.

VAT-1 is a calcium regulated, permanently membrane-attached protein, located at synaptic vesicles. It is a member of the medium-chain dehydrogenases/reductases superfamily. Its cytogenetic location is 17q21.31, near the well-known oncogene, BRCA1. And because of this neighborhood, there have been suggestions that it could be associated with susceptibility to breast and ovary cancer. Previous studies have revealed that overexpressions frequently occur at 17q in the GC and the reported overexpressed genes at 17q in the GC are ERBB2, TOP2A, GRB2, AOC3, AP2B1, KRT14, JUP and ITGA3.

Furthermore, VAT-1 is a 42 kDa protein with nucleotide, calcium and zinc ion binding properties, ATPase function and oxidoreductase activity. All of which have raised the speculation about its possible role in signaling cascades. VAT-1 acts in nerve signal transmission, but it is not limited to neuronal cells. It has been demonstrated that VAT-1 has an important role in keratinocyte physiology. As an unknown oncogene, VAT-1 overexpression has been found in some cancers including glioblastomas and prostate cancer. Furthermore, according to previous studies, VAT-1 can be considered as a novel pathogenic factor in BPH associated cell proliferation. It has also been suggested that VAT-1 overexpression could be involved in glioma cell migration and invasion. Considering the role of VAT-1 in cell proliferation, migration and signaling cascades, and its oncogenic activity in glioblastomas and prostate cancer, among the significantly overexpressed genes obtained from the constructed libraries of gastric cancer, VAT-1 gene was selected for further analysis.

Therefore, the VAT-1 gene expression was checked in clinical tissue samples by qRT-PCR. Our results showed that the relative expression (Patient/Normal) of the VAT-1 gene in the main tissue sample used in the construction of the SSH library, was 10±0.36 fold. This fold of induction confirmed the efficiency of the SSH forward library, which also indicated that VAT-1 was significantly overexpressed in GC. VAT-1 overexpression was between 2.5 to 14.9 fold in endoscopic tissue samples. The represented results reflect the importance of VAT-1 overexpression in GC. However, subsequent studies with increased number of tissue samples from different stages of the GC should be performed to reveal the correlation between VAT-1 overexpression and GC stages. These studies would help to clarify the VAT-1 role in cell proliferation and invasion of cancerous cells.

Since VAT-1 overexpression in GC was not reported prior to the present work, the observations and preliminary results of this study lead us to a number of novel hypotheses about VAT-1. First, the experimental results suggest that VAT-1 seems likely to contribute to the pathogenesis of GC. Second, because of its role in cell proliferation and invasion, VAT-1 could be considered as a candidate gene in the treatment of GC. Third, considering that the tumor tissues used in this study were metastatic in nature, we also hypothesize that VAT-1 might as well be involved in cell migration and metastasis of GC. Finally, due to its probable surface-occurrence in GC cells, VAT-1 could be investigated as a biomarker in the diagnosis and targeted drug delivery of GC. Thus, future thorough investigations are required to confirm each of these hypotheses.

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References

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