



# Identification of novel genes involved in gastric carcinogenesis by suppression subtractive hybridization

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## Abstract

Gastric cancer (GC) is one of the most common and life-threatening types of malignancies. Identification of the differentially expressed genes in GC is one of the best approaches for establishing new diagnostic and therapeutic targets. Furthermore, these investigations could advance our knowledge about molecular biology and the carcinogenesis of this cancer. To screen for the overexpressed genes in gastric adenocarcinoma, we performed suppression subtractive hybridization (SSH) on gastric adenocarcinoma tissue and the corresponding normal gastric tissue, and eight genes were found to be overexpressed in the tumor compared with those of the normal tissue. The genes were ribosomal protein L18A, RNase H2 subunit B, SEC13, eukaryotic translation initiation factor 4A1, tetraspanin 8, cytochrome c oxidase subunit 2, NADH dehydrogenase subunit 4, and mitochondrially encoded ATP synthase 6. The common functions among the identified genes include involvement in protein synthesis, involvement in genomic stability maintenance, metastasis, metabolic improvement, cell signaling pathways, and chemoresistance. Our results provide new insights into the molecular biology of GC and drug discovery: each of the identified genes could be further investigated as targets for prognosis evaluation, diagnosis, treatment, evaluation of the response to new anticancer drugs, and determination of the molecular pathogenesis of GC.

## Keywords

Gene expression profiling, suppression subtractive hybridization, overexpression, gastric cancer, oncogene

## Introduction

Being the second cause of cancer-related deaths, gastric cancer (GC) continues to be one of the most common and life-threatening types of malignancies throughout the world.<sup>1</sup> GC rate in the Middle East differs from very high in Iran to very low in Egypt.<sup>2,3</sup> Owing to its aggressiveness and late diagnosis at the advanced stage, GC has been considered a poor prognosis cancer type.<sup>4</sup> The most prevalent type of GC is adenocarcinoma with two subtypes: the well-differentiated or intestinal type and the poorly differentiated or diffuse type. These two types of GC have distinct molecular features.<sup>5</sup> There are many reports on the genetic and epigenetic alterations in GC, which include alterations in tumor suppressor genes (RUNX3), oncogenes (c-met), cell cycle regulators (cyclin D1), DNA repair genes (hMLH1), and

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signaling molecules (TGFB1/2).<sup>6–8</sup> A recent genome expression analysis performed in Iranian population has reported four isoforms of humanin (HN1, HN3, HN6, and HN10) as the overexpressed genes in GC.<sup>9</sup> Identification of the differentially expressed genes in GC is one of the best approaches for establishing new diagnostic and therapeutic targets. Furthermore, these investigations could advance our knowledge about molecular biology and the carcinogenesis of GC.

There are two methodologies to survey gene expression in cancer cells. In the first methodology, including Northern blot and polymerase chain reaction (PCR), a limited number of specific genes could be studied. The second methodology, which includes microarrays and subtractive hybridization, enables simultaneous evaluation of differential gene expression in all genes, between two different conditions, as described in our study between normal and cancerous tissues. The drawback associated with microarrays is their probe-based analysis technique that necessitates the pre-knowledge of the gene sequences or the identity to be coded on the chip: it means that novel genes could not be found in this method. However, suppression subtractive hybridization (SSH) needs no prior knowledge of the gene sequences: therefore, genes with novel functions in cancer cells could be identified with this method.<sup>10</sup> Systems biology is a new integrative approach used in cancer biology research that concentrates on complex interactions within biological systems with a whole view. Transcriptomics, metabolomics, proteomics, and high-throughput techniques are used to collect data for the construction and validation of models in systems biology.<sup>11,12</sup>

There are many studies on gene alterations that have occurred in GC, but the data provided by these investigations are not enough to elucidate the molecular pathogenesis of GC. Along with these studies, toward establishing more data about the gene alterations in GC and finding targets for drug discovery, we performed SSH on gastric adenocarcinoma tissue and the corresponding normal gastric tissue to investigate the gene overexpression in GC.

## Methods

### Sample collection

Human gastric tissue samples (normal and tumor) were collected from a 64-year-old male patient who underwent operation for gastric adenocarcinoma at

the Arad Hospital of Iran in October 2010. RNAlater<sup>®</sup> (Ambion, Austin, TX, USA) was used to stabilize the RNA. In order to determine the tumor type and metastasis, the discarded tissue samples were examined by an experienced pathologist using hematoxylin–eosin (H&E) staining. The request for acquisition of the gastric tissues was approved by the Biologic Sampling Ethics Committee, Tehran University of Medical Sciences (TUMS), and a written consent form was obtained from the patient before surgery.

### Total RNA extraction

Total RNA extraction was performed by acid–guanidium–chloroform method (Chomczynski and Sacchi, 1987) using TriPure Isolation Reagent (Roche Applied Sciences, IN, USA) according to the manufacturer's protocol. RNA concentration and purity were determined using a biophotometer (Eppendorf, Germany). A260 was used to determine the RNA concentration and the A260/A280 ratio was used to assess the RNA purity (the acceptable values for the A260/A280 ratio were considered to be 1.9–2.1). In addition, RNA was visually detected by staining the 18S and 28S RNAs on gel electrophoresis using ethidium bromide.

### mRNA isolation

mRNA isolation was done using the DynaBead<sup>®</sup> mRNA Isolation kit (Invitrogen, CA, USA) from the extracted total RNA following the manufacturer's protocol. First of all, equilibration of the DynaBeads oligo(dT)<sub>25</sub> was performed with 100 µl of binding buffer (100 mM Tris–hydrochloric acid (Tris–HCl), 500 mM lithium chloride (LiCl), 10 mM ethylenediaminetetraacetic acid (EDTA), 1% lithium dodecyl sulphate (LiDS), and 5 mM dithiothreitol (DTT)). Total RNA was diluted with the binding buffer and mixed with the equilibrated DynaBeads. This mixture was then incubated at 37°C for 5 min to allow the hybridization between oligo(dT)<sub>25</sub> and the mRNA poly A<sup>+</sup> tail. The bead/mRNA complex was washed using 200 µl of washing buffers A (10 mM Tris–HCl, 0.15 M LiCl, 1 mM EDTA, and 0.1% LiDS) and B (10 mM Tris–HCl, 0.15 M LiCl, and 1 mM EDTA). Finally, the elution buffer (10 mM Tris–HCl) was used to elute mRNA from the beads. Agarose gel electrophoresis (1%) was used to detect the isolated mRNA.

**Table 1.** Primers for SSH and PCR analysis of the recombinant clones.

Primer name	Primer sequence 5' to 3'	Primer length	Annealing temperature
N1	TCGAGCGGCCGCCGGGCAGGT	22	68°C
N2R	AGCGTGGTCGCGGCCGAGGT	20	
PCR primer 1	CTAATACGACTCACTATAGGGC	22	66°C
$\beta$ -actin-F	ATGGCCACGGCTGCTTCCAGC	21	60°C
$\beta$ -actin-R	CAGGAGGAGCAATGATCTTGA	21	

SSH: suppression subtractive hybridization; PCR: polymerase chain reaction.

### Subtracted library construction using SSH

The subtracted library was constructed using the SSH method using the PCR-Select™ cDNA subtraction kit (Clontech, CA, USA) according to the manufacturer's protocols. To find the overexpressed genes in GC, the normal gastric tissue was used as the driver and the corresponding tumor tissue was used as the tester. The tester and driver cDNAs were synthesized using 2 µg of the mRNA isolated from the two types of tissues being compared (normal and tumor gastric tissues), and they were purified using the phenol–chloroform extraction method. The purified tester and driver cDNAs were each digested with Rsa I restriction enzyme to yield shorter, blunt-ended molecules. The Rsa I-digested cDNA was further purified using the phenol–chloroform extraction method. Two populations of the tester were prepared using adaptor 1 and adaptor 2R, which were independently ligated to the tester cDNA. Two steps of hybridization were performed between the tester and excess amounts of the driver: the hybridization step equalized and enriched the differentially expressed sequences. In the first hybridization step, the denaturation temperature was set at 98°C (1.5 min) for both driver and tester populations, and the hybridization temperature was set at 68°C (8 h). For the second hybridization step, the two first hybridization mixtures were mixed, fresh denatured driver cDNA (98°C for 1.5 min) was added to this mixture and the hybridization temperature was set at 68°C overnight. The entire populations of molecules were then subjected to primary suppression PCR, which exponentially amplified only the desired differentially expressed sequences. Finally, the secondary PCR amplification was performed using nested primers to further reduce any background PCR products and enrich for differentially expressed sequences. Table 1 represents the sequences of the primers used for subtracted library construction.

### Analysis of subtraction efficiency

Real-time PCR was used to estimate the efficiency of subtraction by comparing the abundance of a non-differentially expressed gene (a housekeeping gene:  $\beta$ -actin) before and after subtraction. Table 1 represents the sequences of the  $\beta$ -actin forward and reverse primers. Reactions consisted of 10 µl SYBR Premix Ex Taq (Takara, Japan), 1 µl cDNA, 0.8 µl each forward and reverse primers (10 µM), 0.4 µl ROX dye, and DEPC-treated water to a final volume of 20 µl. The thermal program for the reaction was set at 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, and 1 min at 60°C. Melting curve analysis was done by increasing the temperature from 65°C to 95°C in 0.1°C/s increments for each fluorescence reading, using the Step-One-Plus Apparatus (Applied Biosystems, CA, USA). The relative expression of the  $\beta$ -actin gene in the subtracted and non-subtracted samples was used in the calculation of the subtraction efficiency.

### Identification of the differentially expressed sequences

The constructed library was purified with the PCR Product Purification kit (Roche Applied Sciences, IN, USA). The purified products were then cloned into pUC19 plasmid vectors and transformed into *Escherichia coli* NovaBlue competent cells (Novagen, WI, USA). The primary verification of the randomly selected positive colonies was performed by colony PCR using N1 and N2R primers (Table 1). Confirmed positive clones were subjected to plasmid isolation by the High Pure Plasmid Isolation kit (Roche Applied Sciences, IN, USA) and the purified plasmids were then used for single direction DNA sequencing with the BigDye Terminator Version 3.1 Sequencing kit and a 3730xl Automated Sequencer (Applied Biosystems, CA, USA). Identification of the differentially expressed sequences was performed by

**Table 2.** Analysis of RNA concentration and purity.

Sample	Concentration ( $\mu\text{g}/\mu\text{l}$ )	A260/A280
<b>Total RNA</b>		
Gastric normal tissue	4.25	1.96
Gastric tumor tissue	1.1425	1.7
<b>mRNA</b>		
Gastric normal tissue	1	1.9
Gastric tumor tissue	0.9	1.8

similarity searches with a Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

## Results

### *Pathologic characteristics of the tissue*

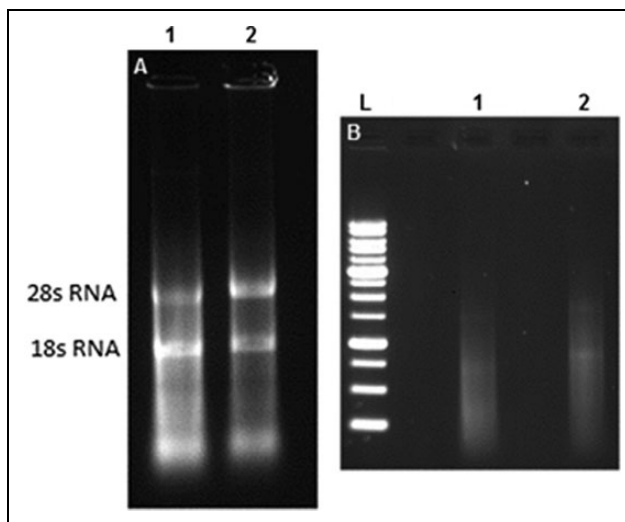
Histological results revealed that the tumor was a moderately differentiated, mucin-producing type of gastric adenocarcinoma located in the prepyloric area. Local invasion to the lymph node was observed in two of the six perigastric lymph nodes.

### *Total RNA extraction and mRNA isolation*

A260 was used to determine the RNA concentration and the A260/A280 ratio was used to assess the RNA purity (Table 2). Since the gastric tumor tissue shows high levels of mucin, the yield of RNA isolation from this tissue was less than the normal one, and the purity of the RNA was less than the acceptable value. RNA was then visually detected on 1% agarose gel (Figure 1).

### *Subtracted library construction by SSH*

Tester and driver cDNAs were synthesized from the isolated mRNA of the normal and tumor tissues and they were analyzed with 1% agarose gel electrophoresis. Figure 2(a) shows the results of cDNA synthesis (before and after purification). Figure 2(b) shows the cDNA before and after restriction digestion which indicates that Rsa I digestion was performed on the cDNA and generated shorter sequences. Figure 2(c) shows the products of the primary and secondary PCR amplifications, indicating that we successfully constructed a putative subtracted cDNA library of GC cells representing the overexpressed genes in this cancer. The library was between 100 to 1200 bp in size.



**Figure 1.** RNA preparation. (a) Total RNA extracted from normal (lane 1) and tumor (lane 2) gastric tissues. (b) mRNA isolated from the total RNA from normal (lane 1) and tumor (lane 2) gastric tissues.

### *Analysis of subtraction efficiency*

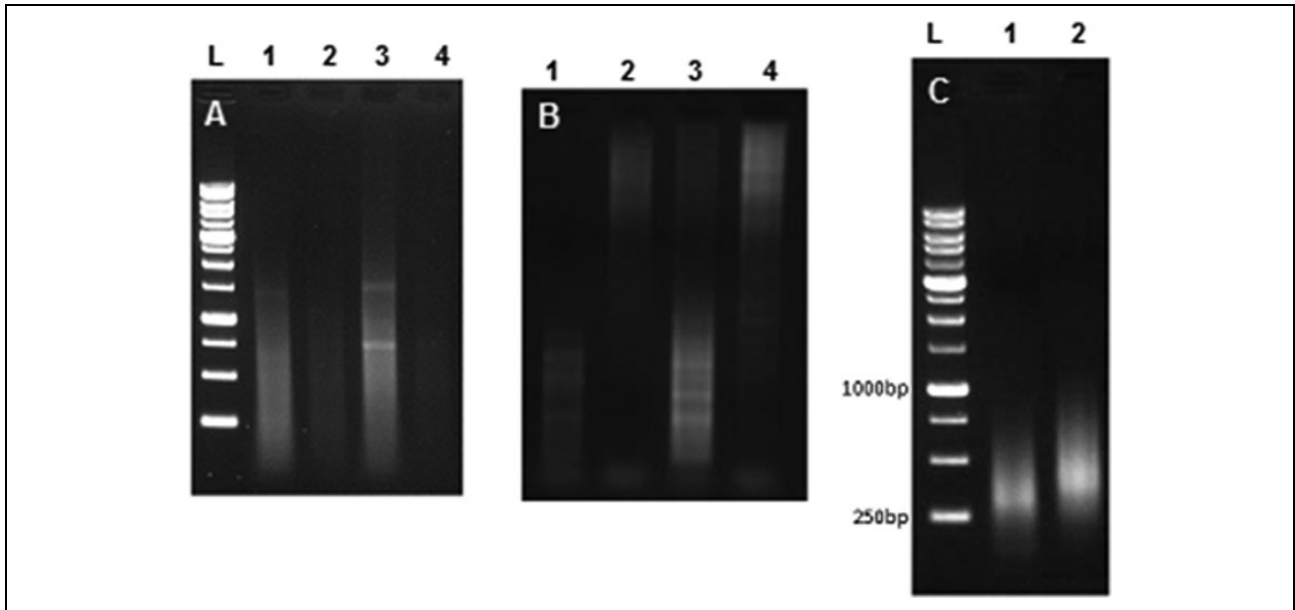
We evaluated the subtraction efficiency by comparing the abundance of a housekeeping gene,  $\beta$ -actin before and after subtraction with real-time PCR analysis. The results indicated that  $\beta$ -actin had an 8.9-fold reduction in the subtracted library, compared with the non-subtracted library, demonstrating that the differentially expressed genes were enriched in the subtracted library.

### *Identification of the differentially expressed sequences*

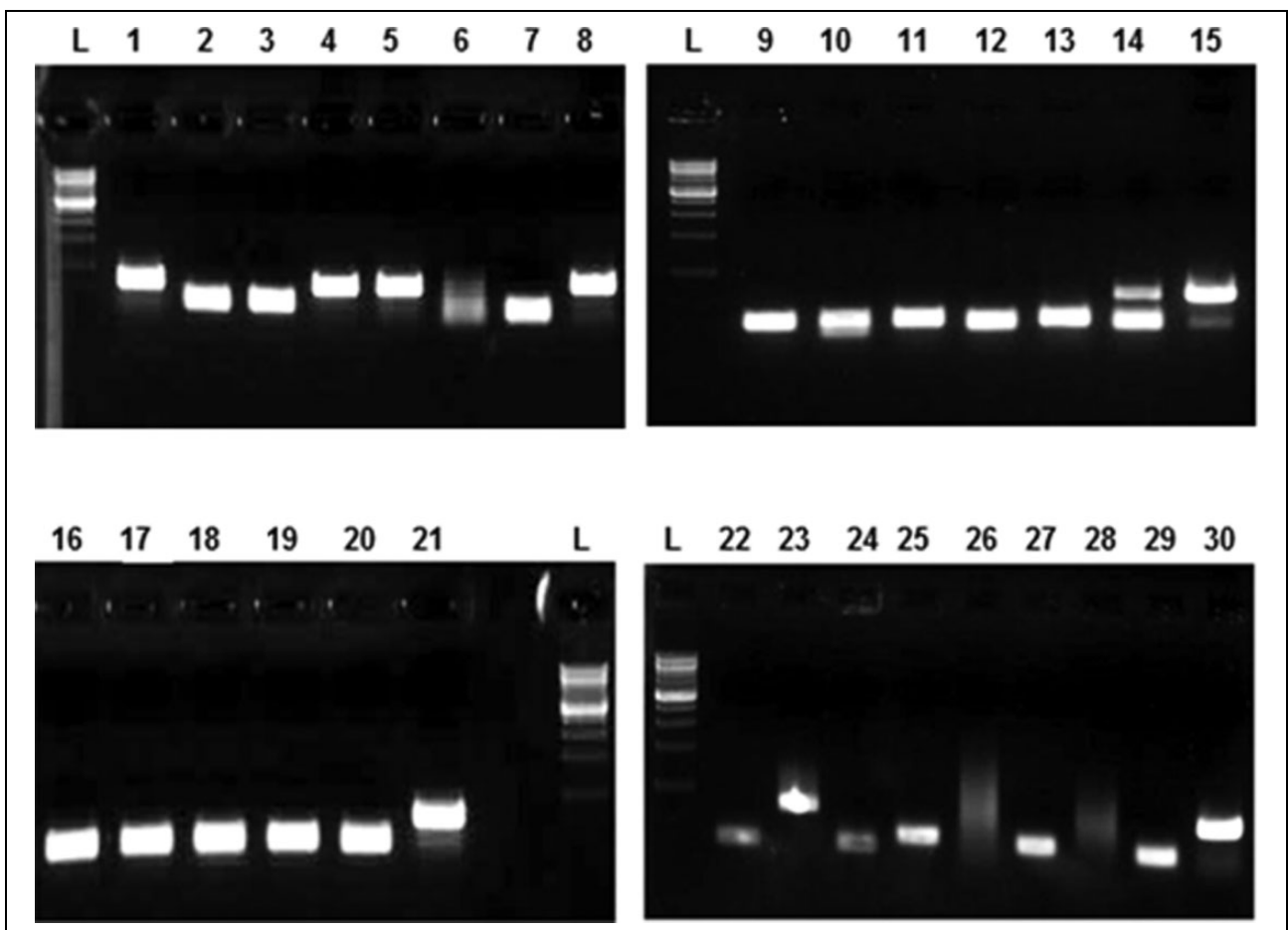
We used the SSH method to examine the differential gene expression using human gastric adenocarcinoma as the tester and the adjacent normal gastric tissue as the driver, with specific enrichment for the overexpressed sequences in the adenocarcinoma tissue. The constructed subtractive library was cloned into the pUC19 plasmid vectors and transformed into Nova-Blue cells. In total, 70 subtractive clones were obtained and randomly picked for subsequent colony PCR (Figure 3), sequencing and identification by BLAST. Table 3 represents the identified genes in this study as the overexpressed genes in GC.

## Discussion

One of the goals of the gene expression profiling studies is to unravel the precise roles of the genes under



**Figure 2.** The analysis on the subtracted library construction. (a) Analysis of ds cDNA synthesis. L: DNA ladder, 1: driver cDNA before purification, 2: driver cDNA after purification, 3: tester cDNA before purification, and 4: tester cDNA after purification. (b) Analysis of *Rsa* I digestion. 1: tester cDNA after digestion, 2: tester cDNA before digestion, 3: driver cDNA after digestion, and 4: driver cDNA before digestion. (c) PCR amplification. L: DNA ladder, 2: primary PCR amplification, and 3: secondary PCR amplification. PCR: polymerase chain reaction.



**Figure 3.** PCR analysis of the recombinant clones. Lanes 1–30: PCR products from different clones; lanes 26 and 28 were false positive clones. L: DNA ladder. PCR: polymerase chain reaction.

**Table 3.** Representative overexpressed genes in GC.

Gene name	Gene ID	Cytogenetic location	Description	Functions
RPL18A	6142	19p13	60S ribosomal protein L18a	Translation and potential extraribosomal function
TSPAN8	7103	12q14.1–q21.1	Tetraspanin 8	Cell signaling, cell migration, cell invasion, and angiogenesis
COII	4513	MT	Cytochrome c oxidase subunit 2 (COII)	Metabolic effect
ND4	4538	MT	NADH dehydrogenase, subunit 4	
ATP6	4508	MT	Mitochondrially encoded ATP synthase 6	
EIF4A1	1973	17p13	Eukaryotic translation initiation factor 4A1	Translation and mediating the transforming effects
RNASEH2B	79621	13q14.3	Ribonuclease H2, subunit B; Aicardi-Goutieres syndrome 2 protein; RNase H2 subunit B	RNA degradation in DNA/RNA hybrids (replication, transcription, etc.) and genomic stability
SEC13	6396	3p25-p24	Protein SEC13 homologue	Nuclear transport, COP-II coated vesicle biogenesis, mitotic progression, and genomic instability

GC: gastric cancer.

normal and disease conditions to increase the knowledge about the disease and improve the strategies used in diagnosis, treatment, and prevention of the disease. This study was focused on the overexpressed genes in gastric adenocarcinoma as the most prevalent type of cancer in Iran, and we used SSH as a high throughput gene expression analysis method to identify the differentially overexpressed genes. Eight genes were identified, each of which could be further investigated as targets for prognosis evaluation, diagnosis, treatment, and determination of the molecular pathogenesis of GC. Furthermore, the biomarkers determined by such studies can be applied in a system's pharmacology framework to build predictive models of signaling networks that control cell death and survival in cancer cells.<sup>13</sup>

TSPAN8 or CO-029, a tumor associated antigen, belongs to the tetraspanin superfamily that directly regulates all of the cellular events related to tumor cell migration and metastasis.<sup>14–19</sup> The involvement of TSPAN8 in cancer has been previously reported in colorectal, gastric, esophageal, pancreatic, and liver cancer analyses.<sup>20–23</sup> The expression level of TSPAN8 is associated with the poor prognosis of gastrointestinal cancer patients. TSPAN8 overexpression in GC could be related to its role in cell migration and metastasis: TSPAN8 facilitates metastasis by promoting angiogenesis. Furthermore, due to its existence in the blood, TSPAN8 could be used to evaluate the patient response to new anticancer drugs.<sup>24</sup>

RPL18A is one of the 60S ribosomal proteins (r-proteins). Many r-proteins serve as RNA chaperones. Furthermore, they could organize the interactions between the ribosome and mRNA/translation factors.<sup>25</sup> The gene expression alterations of many r-proteins have been reported in many types of cancer which could be due to two reasons: (1) changes in r-proteins interrupt their roles in protein synthesis, which could be the result or the trigger of tumorigenesis and (2) r-proteins directly participate in the tumorigenesis by their extraribosomal roles. RPL18A overexpression has been reported in colorectal cancer.<sup>26</sup> Although different extraribosomal functions have been reported for many r-proteins, but that of RPL18A remains to be elucidated.<sup>27</sup>

COII, ND4 and ATP6 are mitochondrial genes involved in oxidative phosphorylation (OXPHOS) biochemical cascade: OXPHOS produces more than 90% of the cellular ATP. The fact that ATP decreases in all cell death mechanisms indicates that energy metabolism might have an important role in tumor cell survival especially under stress conditions including chemotherapy. Previous studies reported mutation and deletion of mitochondrial genes including ND1, ND5, D-loop, COXI, and ATP6 in GC and this is the first report on the overexpression of ND4, COII, and ATP6 in this type of cancer.<sup>28–35</sup>

RNASEH2B encodes the subunit B of the RNase H2 enzyme which degrades RNA in the DNA/RNA hybrids: these hybrids are formed during different

processes in the cell including replication, transcription, and telomere elongation. Therefore, RNase H2 is necessary for the maintenance of the genome integrity and stability and is involved in DNA repair. DNA/RNA hybrids have short time half-life but their pathologic accumulation leads to genomic instability which correlates with the susceptibility to cancer.<sup>36–39</sup> This is the first report on RNASEH2B overexpression in GC.

EIF4A1 is an RNA helicase and a subunit of the eIF4F complex which is involved in cap recognition and is required for mRNA binding to the ribosome. The transforming potential of other members of the EIF4F complex has been demonstrated and it was suggested that EIF4A1 has a mediating role in the transforming effect of other members of the complex.<sup>40–42</sup> EIF4A1 overexpression has been reported in melanoma and hepatocellular carcinoma.<sup>43,44</sup>

SEC13 is a constituent of the endoplasmic reticulum and the nuclear pore complex (NPC). According to their functions in the entry/exit control of the molecules to the nucleus, NPCs have been suggested to be involved in drug resistance. SEC13 involves in transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling cascade due to its function in the entry of the small mothers against decapentaplegic (SMAD) into the nucleus. Since the TGF- $\beta$  signaling pathway is related to cancer, SEC13 might also be associated with oncogenesis through the TGF- $\beta$ -dependent signal transduction cascade. In addition, SEC13 is required for the maintenance of genomic stability during mitosis. Its overexpression has been reported in breast cancer cells.<sup>45–49</sup>

Common functions were observed among the identified genes in this study including their involvement in protein synthesis (EIF4A1 and RPL18A), maintenance of genomic stability (SEC13 and RNA-SEH2B), metastasis (TSPAN8), metabolic improvement (CO-II, ND4 and ATP6), cell signaling pathways (TSPAN8 and SEC13), and chemoresistance (SEC13 and mitochondrial genes). However, as a complementary survey, the study of the precise roles of these eight identified genes in different stages of GC pathogenesis is suggested, which might lead us to find suitable targets for diagnosis and/or treatment of this cancer. Furthermore, due to their potential role in drug resistance, mitochondrial genes and SEC13 could be investigated as targets to alleviate chemoresistance, which is one of the main concerns in the treatment of GC.

Although our results suggest the possibility of the relevance between these new overexpressed genes and gastric carcinogenesis, little is known about the relation

of these genes to GC. Furthermore, long-term follow-up data for GC patients with a large sample size is needed for evaluating these genes as a diagnostic or prognostic marker. If the prognostic or predictive value of these genes is confirmed, they could be considered as factors to determine the treatment modality for gastric carcinoma. An investigation on targeted manipulation of expressions of these genes may provide the possibility of new treatment modalities.

In summary, we screened the difference in gene expression between gastric carcinoma and normal gastric tissues using the SSH method. Our results showed that eight genes including ND4, COII, ATP6, RPL18A, RNASEH2B, EIF4A1, TSPAN8, and SEC13 were involved in carcinogenesis. Their role in gastric carcinogenesis and their diagnostic and prognostic significances remain to be revealed.

### Conflict of Interest

The authors declared no conflicts of interest.

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