Molecular cloning and characterization of a novel esophageal cancer related gene

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Abstract. We previously identified four novel cDNA fragments related to human esophageal cancer. One of the fragments was named esophageal cancer related gene 2 (ECRG2). We report here the molecular cloning, sequencing, and expression of the ECRG2 gene. The ECRG2 cDNA comprises a 258 bp nucleotide sequence which encodes for 85 amino acids with a predicted molecular weight of 9.2 kDa. Analysis of the protein sequence reveals the presence at the N terminus of a signal peptide followed by 56 amino acids with a significant degree of sequence similarity with the conserved Kazal domain which characterizes the serine protease inhibitor family. Pulse-chase experiments showed that ECRG2 protein was detected in both cell lysates and culture medium, indicating that the ECRG2 protein was extracellularly secreted after the post-translational cleavage. *In vitro* uPA/plasmin activity analysis showed the secreted ECRG2 protein inhibited the uPA/plasmin activity, indicating that ECRG2 may be a novel serine protease inhibitor. Northern blot analysis revealed the presence of the major band corresponding to a size of 569 kb throughout the fetal skin, thymus, esophagus, brain, lung, heart, stomach, liver, spleen, colon, kidney, testis, muscle, cholecyst tissues and adult esophageal mucosa, brain, thyroid tissue and mouth epithelia. However, ECRG2 gene was significantly down-regulated in primary esophageal cancer tissues. Taken together, these results indicate that ECRG2 is a novel member of the Kazal-type serine protease inhibitor family and may function as a tumor suppressor gene regulating the protease cascades during carcinogenesis and migration/invasion of esophageal cancer.

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Abbreviations: bp, base pair; cDNA, complimentary DNA; RT-PCR, reverse-transcription polymerase chain replication; kDa, kilodalton; ORF, open reading frame; RACE, rapid amplification of cDNA ENDs; UTRs, untranslated regions; EST, Expressed Sequence Tag; EC, human esophageal cancer; ESCC, esophageal squamous cell carcinoma

Key words: esophageal cancer related gene 2, SPINK7, serine protease inhibitor, tumor suppressor gene, esophageal cancer

Introduction

Esophageal cancer (EC) is a frequently fatal cancer that is common in some geographic regions of the world (1). The Tai-Hang Mountain region of China has been well recognized as the highest incidence area for EC in the world (2). With a standardized incidence rate in excess of 100/100,000 person-years, esophageal cancer is the second leading cause of cancer death in this region (3). EC has the poorest prognosis among the malignant tumors of the digestive tract. Despite advances in multimodality therapy, the overall 5-year survival rates for EC still remain poor (4). The occurrence and development of EC is a multifactor and multistage process involving the activation of the oncogenes and loss-of-function of tumor suppressor genes (5). Several gene alterations such as mutations and deletions of Rb, p53, p16, APC, MCC and DCC or chromosomal abnormalities such as amplification of C-myc, Cyclin D, epidermal growth factor receptor gene and int-2 gene have been reported in the development of EC (6-8). Although recent reports have documented alterations of a few oncogenes and tumor suppressor genes in the EC (9,10), the molecular events associated with the initiation and progression of EC remain poorly understood. To better understand the role of genetics in the etiology of EC and to identify potential susceptibility genes, we previously compared normal esophageal epithelia and primary squamous cell carcinomas tissues from high incidence families in Lin-Xian County using differentially displayed PCR (DD-PCR) and identified 18 differentially expressed genes [13 expressed in normal esophageal epithelia but not in EC (NEG) and 5 expressed in EC but not in normal esophageal epithelia (MEG)]. Four of the NEGs are not homologous to the known sequence in the public database of GenBank and named Esophagus Cancer-

Related Gene 1-4 (ECRG1-4) (11). ECRG2 was chosen for further study.

In the present study, we isolated and characterized full-length cDNA of ECRG2 gene, described the major structural characteristics of ECRG2 protein, and compared ECRG2 mRNA levels in various tissues with emphasis on esophageal cancers. Based on our current results, we propose that ECRG2 is a novel member of serine protease inhibitor family and may function as a tumor suppressor gene during the development of EC.

Materials and methods

Primary tumors and normal tissue samples. A total of 84 esophageal tissue specimens, comprising of 51 esophageal squamous cell carcinoma and 33 adjacent tissues (with no evidence of tumor), were obtained from patients undergoing surgery for esophageal cancer in Lin-Xian County, Henan province, China. Seven normal esophageal epithelia samples were donated from a healthy man who died in an accident. In addition, liver cancer, colon cancer, lung cancer and the corresponding adjacent tissues were collected from patients undergoing surgery for cancer in Cancer Institute, CAMS, Beijing, China. Fourteen kinds of total RNA of human fetus (brain, lung, thymus, liver, esophagus, testis, stomach, heart, muscle, skin, colon, spleen, kidney and cholecyst) were purchased from Clontech (CA, USA).

Molecular cloning of ECRG2 gene through RACE. Total RNA was initially extracted from normal esophageal epithelia using the TRIzol reagent (Gibco). The 5'RACE and 3'RACE cDNA libraries were synthesized using 20 μg of total RNA as template following the manufacture's protocol (Clontech, CA, USA). The RACE-PCR reactions were performed using GSPs (Table I) as gene-specific primers. All reactions were performed using the following cycling conditions: 94˚C for 3 min followed by 35 cycles of 94˚C for 45 sec, 56˚C for 30 sec, and 72˚C for 30 sec. PCR was carried out in 96-well plates and in duplicate. The obtained PCR fragments were ligated to pGEM-T-Easy vector (Promega, USA), transformed into *E. coli* JM109 competent cells and plated on appropriate indicator LB amp$^+$-dishes. Three independent clones for each fragment were selected and sequenced.

Northern blot analysis. Total RNA was extracted using the MirVana RNA Isolation kit (Ambion), and subsequent poly(A) RNA was isolated using the poly(A) purist kit (Ambion). Northern blots were carried out using standard techniques. Briefly, 10 mg of poly(A) RNA was resolved on 1% agarose-formaldehyde denaturing gels, transferred to Hybond-N membranes (GE Healthcare) by capillary action using 10xSSC (1.5 M NaCl, 0.15 M sodium citrate at pH 7.0). A cDNA fragment corresponding to the coding region sequence of ECRG2 labeled with ^{32}P-dCTP was used to probe the membrane. Hybridization was carried out overnight at 68˚C in 2xPIPES buffer (0.8 M NaCl, 20 mM PIPES, 50% deionized formamide, 0.5% SDS, 100 μg/ml salmon sperm DNA, pH 6.5). The blot was washed at 50˚C with 2xSSC solution (0.3 M NaCl, 0.03 M sodium citrate) 3 times and then 0.1xSSC solution. After washing, the blot was exposed to X-ray film for 3 days at -80˚C. RNA integrity and equal loading were confirmed via hybridization with ß-actin probe.

Table I. Primers utilized for the molecular cloning and isolation of the ECRG2 gene.

Name	Oligonucleotide sequence (5'→3')	Length
GSP1-1	GCTGGTCTGTCAGTGACC	18
GSP2-1	TTCTCCATTCAGTCAAGATTACT	23
GSP-1	ATTACTCTGAATCTGGCTCTGT	22
GSP1-2	TACAGAGCAGAAGGAGAC	18
GSP2-2	TGCTGGAAATGGCTGTGGTG	20
GSP-2	GCTGCTCTGAGATTGTCACTAC	22
GSP1-3	GACCTGCAACAGAAATAG	18
GSP2-3	CCACTGTACAGAGCAGAAGG	20
GSP-3	GGCTGTGGTGTAGAAGCTGCTC	22

uPA/plasmin activity. Reaction mixture (140 μl) was added to a 96-well plate. The reaction mixture was composed of 1 mg/100 ml plasminogen and 0.5 mM plasmin substrate D-Val-Phe-Lys-p-nitroanilide 200 (Chromogenix, Viale Monza, Milano, Italy). Ten microliters of serum-free conditioned medium was added to the mixture and incubated at 37˚C. Absorption (A405) was measured after 0.5 h.

Results

Molecular cloning and characterization of ECRG2 cDNA. To reveal carcinogenesis mechanism and genetic susceptibility of esophageal cancer (EC), we previously performed mRNA differential display analysis (DD-PCR) to search for new genes related to human esophageal cancer from 3 normal esophageal epithelia and 2 primary squamous cell carcinomas collected from high incidence family in Lin-Xian County, China. A total of 18 differential fragments were identified. Thirteen of which were expressed in normal esophageal epithelia but not in EC (assigned as normal esophageal gene, NEG), while 5 of which were expressed in EC but not in normal esophageal epithelia (assigned as mutated esophageal gene, MEG). As a result of the sequencing and Blast search, 4 fragments were not homologous to the known sequence in the public NCBI databases and named esophageal cancer related gene 1 to 4 (11). In this study, we focus on the ECRG2 gene, also named SPINK7 (serine peptidase inhibitor, Kazal type 7).

To obtain the full length cDNA sequence of ECRG2 and characterize the 5'/3'-UTR, we performed 5'- and 3'-RACE approach. As shown in Fig. 1, the entire cDNA of the ECRG2 gene was 569 bp (CDS: 58-315), including a coding nucleotide sequence of 258 bp, a 5' untranslated region of 57 bp and a 3' noncoding region of 255 bp (http://atlasgeneticsoncology.org/Genes/SPINK7ID40396ch5q33.html). The CDS is complete since there is an in-frame stop codon at -42 bp in the 5' UTR. A predicted ATG translation initiation site, an in frame stop codon, and a standard AATAAA polyadenylation signal were identified (Fig. 1). The complete cDNA sequence of ECRG2 gene was deposited in GeneBank with accession number AF268198.

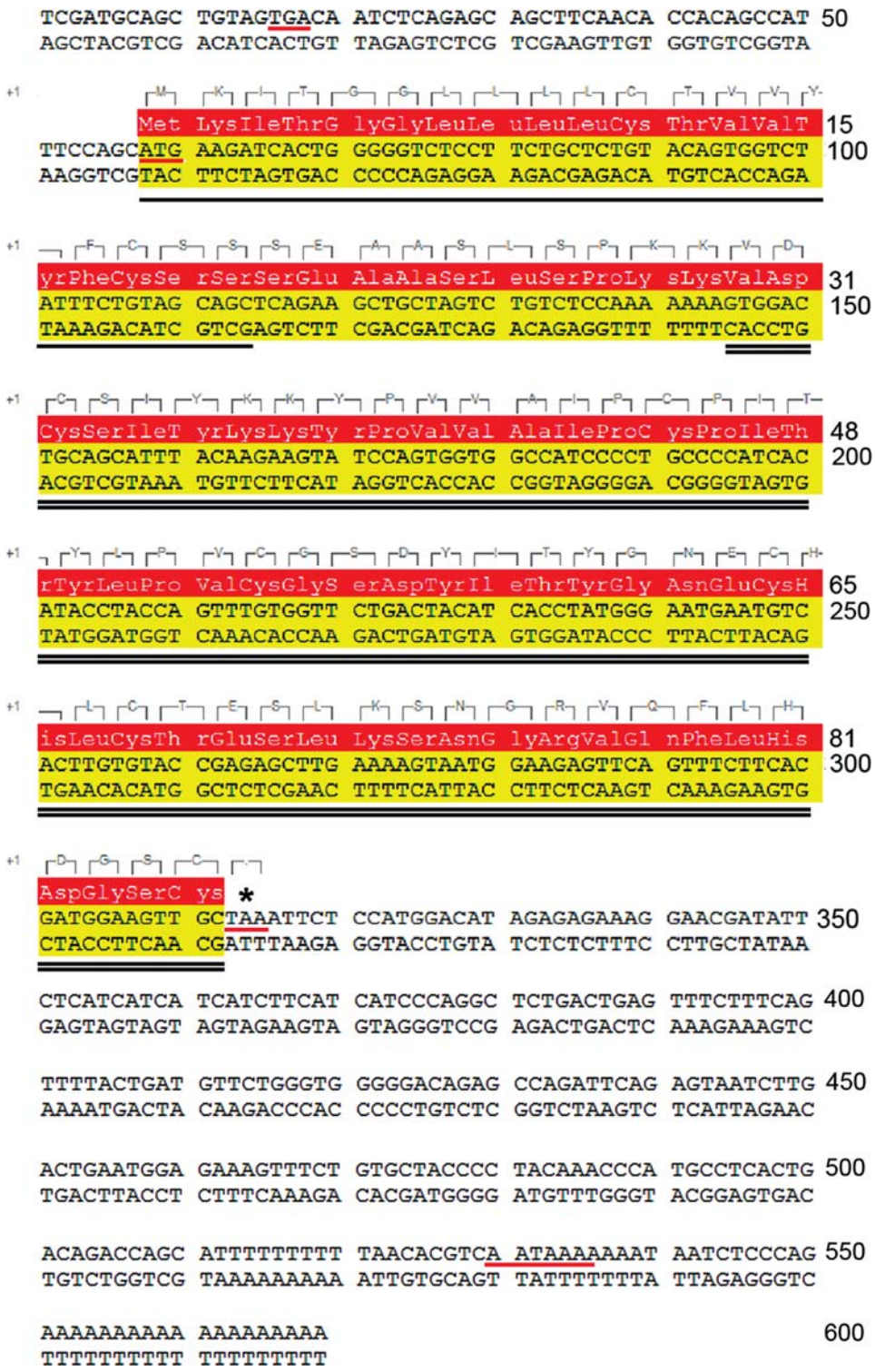

Figure 1. DNA and deduced amino acid sequences of the ECRG2 gene. The yellow and red columns indicate the nucleotide and the deduced amino acid sequence of the ECRG2 gene, respectively. Numbers to the right of the sequence correspond to amino acids (upper) and nucleotides (lower). The in-frame stop codon TGA, the ATG translation initiation codon, the stop codon TAA and the polyadenylation signal are underlined in red. The translation termination codon is also denoted by an asterisk. The putative signal peptide predicted using the signal P program (underlined in black) and the conserved Kazal-type serine protease inhibitor domain (double underlined in black) are also shown. The GenBank accession number of this sequence is AF268198.

Chromosomal localization and genomic organization of ECRG2 gene. The chromosomal localization of ECRG2 was determined by aligning the ECRG2 cDNA sequence against chromosome DNA. The sequence of ECRG2 gene exactly matches the Homo sapiens chromosome 5 genomic contig: NT_029289, which corresponds to 5q32 (Fig. 2A and B). ECRG2 has 4 exons separated by 3 introns and spans across a genomic region of ~3.49 kb (from 147691990 bp to 147695482 bp) (http://www.ensembl.org/Homo_sapiens/Location/View?db=core;g=ENSG00000145879;r=5:147691986-147695479 Fig. 2C). All exon-intron boundaries comply with the GT/AG rule (Table II).

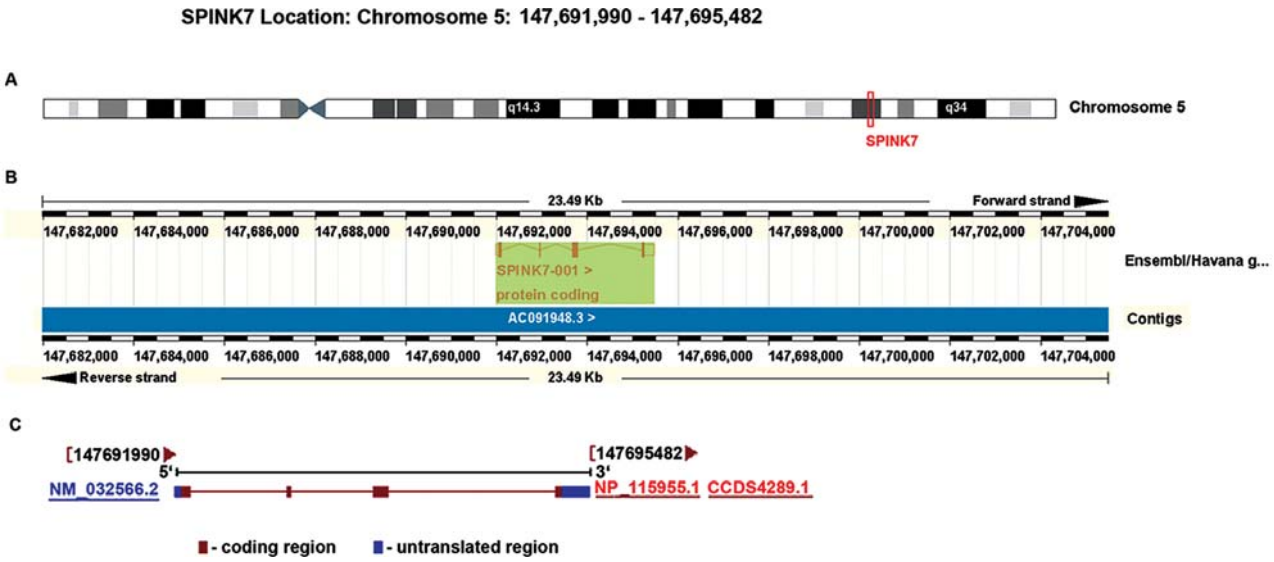

Figure 2. Genomic structure of the ECRG2 gene. (A) ECRG2 is localized at 5q32 in chromosome. (B) The ECRG2 gene starts at 147691990 and ends at 147695482 at chromosome 5. (C) Exon-intron structure of the ECRG2 gene. ECRG2 gene contains 4 exons separated by 3 introns and spans across a genomic region of ~3.49 kb.

Table II. Exon-intron boundaries of ECRG2 gene.

Exon no.	Exon size	Splice acceptor	Intron size	Splice donor	Exon no.
1	118 bp	cagctcaggtgagtac	825 bp	ctttttcagaagctgct	2
2	26 bp	aaaaaaagtaagtatg	696 bp	atgacacaggtggactg	3
3	125 bp	cagagcttgtgagtacc	1,410 bp	ttcccttaggaaaaga	4
4	300 bp	Ggaagttgctaa3'UTR			

Characterization of ECRG2 protein. The ECRG2 protein has an open reading frame encoding a polypeptide of 85 amino acids (http://www.uniprot.org/uniprot/P58062). The predicted molecular weight of the ECRG2 was calculated to be 9.2 kDa and the isoelectronic point (pI) was calculated to be 7.52. As shown in Fig. 3B, a potential signal peptide of 1-19 amino acids was predicted at the N-terminus of the ECRG2 protein using the Signal-P program (http://atlasgeneticsoncology.org/Genes/SPINK7ID40396ch5q33.html). Blasting the protein sequence deduced from ORF against the NCBI databases predicted ECRG2 to be a novel member of serine protease inhibitor family as the C-terminal 30-85 of the protein contains a typical x(8)-C-x(12)-C-x(7)-C-x(6)-Y-x(3)-C-x(2)-C-x(17)-C conserve region, coding a Kazal type serine protease inhibitors (Kazal) domain (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?INPUT_TYPE=live&SEQUENCE=AAK27795.1&log$=seqview_list_cdsearch, Fig. 3A and B). Moreover, several Kazal-type inhibitor genes were located in clusterin at chromosome 5q31-33, including the serine protease inhibitor 1 (SPINK1), the serine protease inhibitor 5 (SPINK5), the serine protease inhibitor 6 (SPINK6), and the serine protease inhibitor 9 (SPINK9) (http://www.ensembl.org/Homo_sapiens/Location/View?g=ENSG00000145879;r=5:147691986-147695479, Fig. 3C).

Expression of ECRG2 in EC109 cells. Sequence analysis discloses that the open reading frame of ECRG2 gene encodes 85 amino acids with a signal peptide at the N-terminus. To examine whether pcDNA3.1-Flag-ECRG2 harboring cells secrete the ECRG2 protein as predicted from the amino acids sequence, pulse-chase experiment was performed. Although the first 19 residues of ECRG2 protein is a signal peptide to mediate targeting to the endoplasmic reticulum for secretion, pulse-chase experiment showed that ECRG2 was transferred to both intracellular and extracellular. ECRG2 was detected at an apparent molecular weight of ~10 kDa in EC109/pcDNA3.1-Flag-ECRG2 cells. A smaller cleaved form of ECRG2 (~7 kDa) was additionally detected in culture medium (Fig. 4A), indicating the post-translational cleavage of ECRG2 protein. Meanwhile, the inhibition of serine protease activity of ECRG2 protein was monitored *in* a *vitro* serine protease activity analysis by using a specific chromogenic substrate. Serum-free conditioned medium from EC109/pcDNA3.1-Flag-ECRG2 cells reduced proteolysis of the plasmin substrate D-Val-Phe-Lys-p-nitroanilide, as indicated by a decrease in absorbance (0.536±0.021) at 405 nm compared with control (2.238±0.024) (Fig. 4B). This result confirms that expression of ECRG2 contributes to the inhibition of activity of a specific serine protease, uPA.

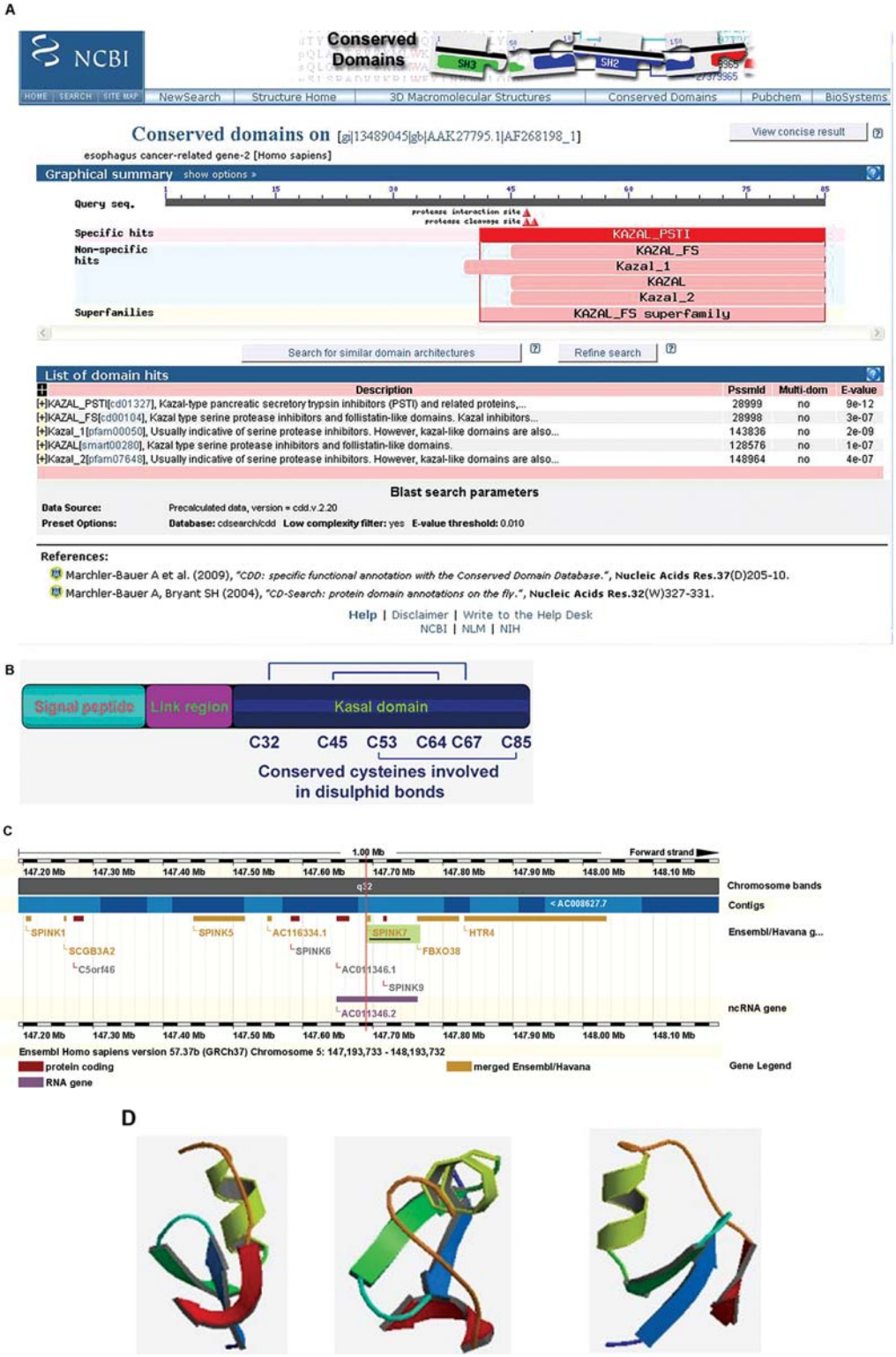

Figure 3. Characterization of ECRG2 protein. (A) Alignment of the deduced amino acid sequence of ECRG2 gene against the NCBI Conserved Domains database. Classical structure of Kazal motif in the Kazal protease inhibitors is shown in red/pink. (B) Predicted structure of the deduced ECRG2 protein. Potential signal peptide (1-19), predicted chain (20-85), Kazal-like domain (30-85). The N-terminal 1-19 is the single peptide of the protein. The C-terminal 30-85 of the protein contains a typical x(8)-C-x(6)-C-x(7)-C-x(6)-Y-x(3)-C-x(2,3)-C-x(17)-C conserve region, coding a Kazal type serine protease inhibitors (Kazal) domain. (C) The ECRG2 gene (SPINK7) is mapped on chromosome 5q32 where several Kazal-type inhibitor genes are located in clusterin, including SPINK1, TATI, SPINK5, SPINK6, SPINK9. (D) ModBase Predicted Comparative 3D Structure on the ECRG2 putative protein. Three different views are shown (front, top and side).

Tissue distribution of ECRG2 mRNA. To confirm tissue specific expressions of the ECRG2 gene at the transcriptional level, total RNAs were prepared from human fetus tissues (esophagus, brain, liver, cholecyst, colon, heart, lung, muscle, kidney, stomach, spleen, skin, testis and thymus) as described in Materials and methods and analyzed by Northern blotting

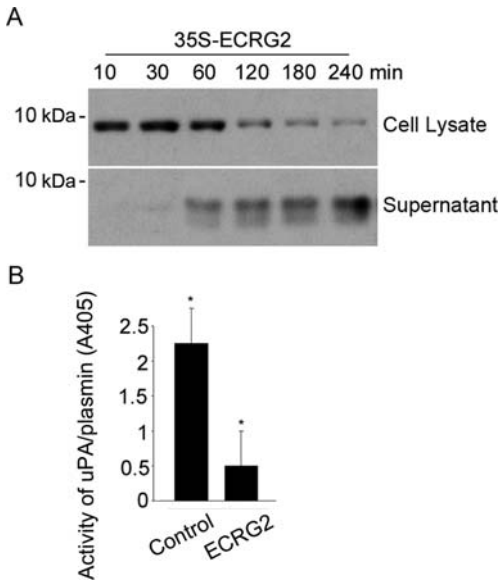

Figure 4. (A) Expression of ECRG2 gene in EC109 cells by Pulse-chase experiment. EC109 cells were transient transfected with pcDNA3.1-Flag-ECRG2 construct, pulsed with [^{35}S]methionine for 5 min and chased for different times. [^{35}S]methionine-ECRG2 were detected by autoradiograph. (B) Detection of uPA/plasmin activity by proteolytic chromogenic substrate analysis. Conditioned media collected from EC109 cells transfected with or without ECRG2 plasmid was concentrated, and uPA/plasmin activity was analyzed as previous described. Data are shown as mean ± SD of three independent experiments.

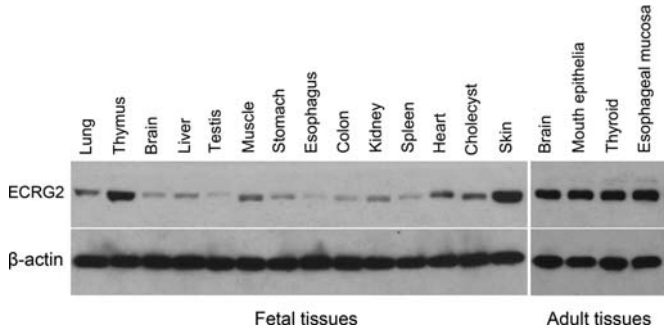

Figure 5. Expression pattern of ECRG2 gene in fetal tissues. Qualitative transcript profiles of ECRG2 across fourteen fetal tissues and four adult tissues by Northern blot analysis. The ECRG2 was highly expressed in fetal skin and thymus compared to other 12 kinds of fetal tissues. ß-actin was used as the loading control.

using the labeled ECRG2 fragment as probe. The amount of total RNAs loaded were quantified using a ß-actin probe of 1.8 kb size known to be expressed constitutively in all tissues and compared with 28S ribosomal RNA. Northern blot results showed that ECRG2 mRNA was highly expressed in adult esophageal mucosa, brain, thyroid, mouth epithelia and fetal skin, thymus while lowly expressed in the fetal esophagus, brain, lung, heart, stomach, liver, spleen, colon, kidney, testis, muscle, cholecyst tissues (Fig. 5).

ECRG2 mRNA expression in tumors. We also examined ECRG2 transcripts in primary esophagus cancer, liver cancer, colon cancer and lung cancer (Fig. 6). RT-PCR results demonstrated that ECRG2 was expressed in esophagus cancer, liver cancer, colon cancer and lung cancer as well as the corresponding adjacent tissues. But the expression ratio was significantly different. Expression ratio of ECRG2 in 7 normal adult esophageal epithelia, 33 esophageal cancer adjacent tissues and 51 esophageal cancer tissues was 100, 52 and 21%, respectively. The expression of ECRG2 was dramatically down-regulated in esophageal cancer. However, the expression ratios of ECRG2 were similar between the adjacent and tumor tissues in the other three kinds of cancer (Table III and Fig. 6A). Furthermore, Northern blot analysis confirmed the presence of the ECRG2 transcript in the normal esophagus tissue and

Table III. RT-PCR analysis of ECRG2 expression in human tumors.

Tissue	No. of samples	Positive expression no.	Expression ratio (%)
Esophagus			
Normal epithelia	7	7	100
Adjacent tissue	33	17	52
Carcinoma tissue	51	14	21
Liver			
Adjacent tissue	22	11	50
Carcinoma tissue	22	10	45
Colon			
Adjacent tissue	17	4	24
Carcinoma tissue	18	4	22
Lung			
Adjacent tissue	9	5	55
Carcinoma tissue	9	4	44

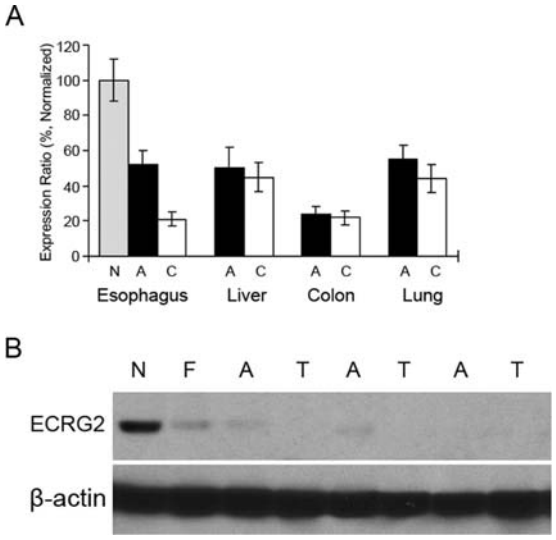

Figure 6. RT-PCR and Northern blot analysis of ECRG2 gene in human tumors. (A) Screening the ECRG2 transcripts in primary tumors using RT-PCR. (B) Confirmation of ECRG2 expression in esophageal cancer tissues by Northern blot analysis. N, normal epithelium; F, normal fetal esophagus epithelium; A, esophagus cancer adjacent tissue; T, esophagus cancer tissue.

absence in the adjacent and EC tumor tissues (Fig. 6B). Together, both RT-PCR and Northern blot results showed that ECRG2 gene was expressed in normal esophagus, liver, colon, and lung tissues, but was down-regulated in the adjacent and cancerous tissues, especially in esophageal cancer.

Discussion

Four novel fragments related to human esophageal cancer have previously been isolated and identified from three normal esophageal epithelia and two primary squamous cell carcinomas collected from high incidence family in Linxian county, Henan province, China and named esophageal cancer related gene 1-4 (ECRG1-4) (11). In the present study, we cloned, sequenced and characterized one fragment named ECRG2.

We initially performed RACE-PCR experiments using the RACE cDNA library from normal esophageal epithelia as a primary template to identify the full-length nucleotide sequence of the ECRG2 cDNA. Our results showed that the ECRG2 full-length cDNA clone comprised of 569 bp and it was characterized by a coding region of 258 bp, and 5'- and 3'-UTR regions 57 bp and 255 bp, respectively (GenBank Accession Number AF268198, Fig. 1). ECRG2 gene contains four exons and three introns which span 3.49 kb on the chromosome 5q32 (Fig. 2). The molecular weight of the encoding protein contains 85 amino acids is about 9.23 kDa. N-terminal analysis of the ECRG2 protein showed that the first 1-19 amino acids could be a signal peptide (Fig. 3B). This signal peptide may be cleaved off during its passage through the inner membrane by endogenous signal peptidase, as observed for the other secreted protein (12). As expected, pulse-chase experiment showed that ECRG2 was detected in both cellular and culture media, and secretion of ECRG2 is accompanied by cleavage into a lower molecular weight protein. This result confirmed that ECRG2 protein was secreted after cleavage of the signal peptide (Fig. 4A).

The sequence of the ECRG2 gene did not reveal remarkable similarity to the known sequence in the homology analysis with the public database of GenBank. However, the deduced amino acid sequence showed 97% homology to a tumor associated Kazal-type serine protease inhibitor peptide (US patent 5851987) (Fig. 3A). As shown in Fig. 3B, ECRG2 gene contains a typical Kazal serine protease inhibitor conserved domain at its C-terminal and three kinase phosphorylation sites (protein kinase C, Casein kinase II and Tyrosine kinase). The Kazal serine protease inhibitor family includes pancreatic secretary trypsin inhibitor, avian ovomucoid, acrosin inhibitor, and elastase inhibitor (13). These proteins contain 1 to 7 Kazal-type inhibitor repeats (14). The presence of typical Kazal domain in ECRG2 indicates that ECRG2 may be a novel member of serine protease inhibitor family and function as a serine protease inhibitor. To confirm this, we performed *in vitro* uPA/plasmin activity analysis. The results from Fig. 4B suggest that the ECRG2 gene inhibited the uPA/plasmin activity and had a protease inhibitory function. uPA is a specific serine protease to active serine protease plasmin. uPA-mediated proteolysis is of great importance during the process of tumor cell invasion, metastasis and angiogenesis (15). Plasmin is a broad-specificity protease, which degrades several ECM components, such as fibronectin, laminin and collagen (16). Our results indicated that ECRG2 gene, as a novel Kazal-type serine protease inhibitor, may be associated with deregulated protease cascades contributing to carcinogenesis and migration/invasion of esophageal cancer.

Frequent deletion of chromosome arm 5q has been reported in human EC (17). Several serine protease inhibitors were located at chromosome 5q31-33, including the serine protease inhibitor 1 (SPINK1, also named pancreatic secretary trypsin inhibitor PSTI) (18), tumor-associated trypsin inhibitor (TATI) (19), serine protease inhibitor 5 (SPINK5) (20), serine protease inhibitor 6 (SPINK6), serine protease inhibitor 9 (SPINK9) (21) (Fig. 3C). PSTI was reported to prevent premature activation of pancreatic proteases, decrease the rate of mucus digestion by luminal proteases and involve in both the earlier and late phases of the healing response following injury (20). SPINK5 encodes the lymph-epithelial Kazal-type related inhibitor (LEKTI) which is a human 15-domain serine protease inhibitor, initially described in thymus and mucous epithelia as the precursor of two proteolytic fragments (22). LEKTI has been reported as an important factor for the anti-inflammatory and/or antimicrobial protection of mucous epithelia. Mutations in SPINK5 cause netherton syndrome-a severe autosomal recessive skin disorder (20). Gion *et al* has reported that TATI participated in the carcinogenesis and metastasis of EC. They also found the expression of TATI were dramatically down-regulated in 71 primary squamous cell esophageal tissues compared with normal esophageal mucosa (23). ECRG2, also named SPINK7 (serine peptidase inhibitor, Kazal type 7), was mapped on chromosome 5q32 where several Kazal-type inhibitor genes were located in clusterin. We hypothesize that ECRG2 gene, as a novel serine protease inhibitor, may play important roles during the development of EC. To address this question, we initially investigated the expression of ECRG2 in fetal tissues

and different types of cancer. As shown in Fig. 5, ECRG2 transcript was detected in 14 kinds of fetus tissues including esophagus and well expressed in the normal epithelium of upper-digestive tract (mouth and esophagus) in adult. ECRG2 expression was various in the primary cancer tissues of esophagus, lung, liver and colon (Fig. 6). The ECRG2 expression was significantly down-regulated in esophagus cancer. Expression profile of ECRG2 gene in 7 normal esophageal epithelia, 51 esophageal cancer and 33 tumor adjacent tissues were 100, 21 and 52% respectively. About 79% of ECRG2 gene was absent in the esophageal cancer (Table III). The facts that ECRG2 gene was highly expressed in the adult normal esophageal tissue, low expressed in the fetal esophageal tissue and esophageal cancer indicate that ECRG2 may be a specific gene for carcinogenesis of esophagus. Although the mechanism of down-regulation of ECRG2 gene in EC remains unknown, the loss of expression pattern suggests that ECRG2 might be a candidate tumor suppressor gene.

Taken together, our results suggest that ECRG2 gene is a novel EC associated Kazal-type serine protease inhibitor and may function as a tumor suppressor gene. The down-regulation of ECRG2 may be associated with deregulated protease cascades contributing to tumor migration/invasion. Further studies on the function analysis of ECRG2 gene could provide an insight into the role of ECRG2 gene in the carcinogenesis of esophageal cancer.

In conclusion, ECRG2 is a novel member of Kazal-type serine protease inhibitor family and may function as a tumor suppressor gene regulating the protease cascades during carcinogenesis and migration/invasion of esophageal cancer.

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