Original article

The Significance of ES274071 Up–Regulated in Human Colorectal Adenocarcinoma

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ABSTRACT **Objective:** To study the structure and function of the differently expressed EST sequences from suppression subtractive hybridization combining with cDNA microarray in human colorectal adenocarcinoma. **Method:** From the cDNA subtractive library combining with cDNA microarray, collecting differentially expressed sequence tag ES274071, and utilizing the bioinformatics to extend EST sequence for getting full-length cDNA sequence. Then designing one pair of primer on the basis of full-length cDNA, and amplifying the ORF by RT-PCR technique, and separating the PCR products on 1.0% agarose gel at last. **Result:** ES274071 was up-regulated expressing in colorectal adenocarcinoma tissue, but in normal colorectal tissue it was lower expression. **Conclusion:** ES274071 is up-regulated; we can conclude that ES274071 is related with colorectal adenocarcinoma pathogenesis.

KeyWords: Bioinformatics; Expression sequence; Tag contig; Colorectal adenocarcinoma

Colorectal adenocarcinoma is one of common malignant tumors, which is the second in the digestive tract malignant tumors. The initiation and development of colorectal adenocarcinoma is a multistep processes that involves multi-gene change. It will be benefit to the early diagnosis, effective treatment and prevention that molecular mechanism of human colorectal adenocarcinoma continues to be researched. With the accomplishment of human genome program, Genome research has entered into a new phase of gene abstraction and data analysis. Analyses gene function by means of bioinformatics has become a new strategy. This study aims to study the genes related to genesis of human colorectal adenocarcinoma. On the basis of the EST sequence (accession number ES274071) of human colorectal adenocarcinoma (1), and bioinformatics was utilized to extend EST sequence in order to acquire full-length cDNA and validating express by RT-PCR. Furthermore, it is being predicted that the gene's chromosome location, tissue distribution and protein function.

Materials and methods

Tissue

With the consent of the patients, we collected fresh colorectal adenocarcinoma tissue (the diagnosis of colorectal adenocarcinoma were confirmed by pathology) and adjacent normal colorectal tissue (more than 5cm from the edge of the tumor, no cancer cell has found by pathological examination) in West China Hospital of Sichuan University .We totally collected 9 cases.

Primers

We designed primers in the open reading frame of full-length cDNA sequence of ES274071 by software Primer Premier 5.0.We used GAPDH as internal reference primer. Primers were synthesized by Bao Sheng Wu Company. Primers were shown in Table 1.

Reagent

TRIzol Reagent was purchased from Invitrogen Company; RevertAid First Strand cDNA Synthesis Kit and PCR Reagent was purchased from Fermentas Company.

Obtaining full–length cDNA

Draw one differential expressed EST fragment from the cDNA subtractive library (Accession number ES274071). The first step is to choose this EST as seed sequence and to find its matching sequence. According to American Genetics Research Institute, if two ESTs' alkali bases overlap beyond 40bp and share 95% similarity in their overlapping zone,they are matching sequences (2).The

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second step is to extend this EST as long as possible through the extension method of matching sequences blast cycling. That is, assembling these matching sequences together to form a longer EST and then using the new EST to conduct blast retrieval to find more matching sequences. Repeat such process until no more matching sequences can be found. Thus, a contig is obtained.

Predict protein function

We analyze the obtained full-length cDNA sequence by softwares of bioinformatics and databases on network (1). Search with the ORF Finder program: (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) offered by NCBI to carry on the open read-frame ORF identification of contig sequence (2). Directly use genome sequence to do genetic electron location in order to acquire chromosome location (3). Take the assembled full-length cDNA sequence as a probe and use the virtual Northern program (http://www.ncbi.nlm.nih.gov/SAGE/) provided by CGAP in NCBI as a tool. Doing expression quantum analysis by drawing genetic representative tag form the SAGE library (4). The prediction and analysis of the protein structural domain and functional site were performed through Prosite software (http://www.expasy.Org/prosite/) (3).

RNA isolation

Total RNA was isolated through Invitrogen system of RNA isolation according to the manufacturer's instructions.

RT-PCR

A reaction mixture containing 1ul Oligo dT18 primer, total RNA and DEPC-treated water, 12 uL in all. The reaction eppendorf was incubated at 65℃ for 5min and chilled on ice. Then added 4ul 5× reaction buffer, 1ul RiboLock Rnase inhibitor, 2uL dNTP (10mmol/L) and 1uL RevertAid M-Mulv Reverse Transcriptase. The mixture was incubated at 42℃ for 60min. The reaction was terminated by heating at 70℃ for 5 min and then the mixture was kept at -20℃.

PCR amplification system is 25uL in all, including 1uL first strand cDNA, 1uL Forward primer, 1uL Reverse primer, 12.5uL MIX, 9.5uL deionized water. The condition of reaction was shown in Table 2.

Results

Full-length obtaining

ES274071 contains 420bp, we got its 2936bp full-length by BLASTn, its landing number is NM_003011, ES274071 locates from 1596bp to 2015bp.

Analysis of function prediction

Table 1
Primers

Name	Oligo	Sequence	Production length
ES274071	Forward primer	5' -CTCAACTCCAACCACGACG -3'	505bp
	Reverse primer	5' -TCTTCCTGCTGGCTTTATTCT -3'	
GAPDH	Forward primer	5' -GACCTGACCTGCCGTCTA -3'	148bp
	Reverse primer	5' -AGGAGTGGGTGTCGCTGT -3'	

Table 2
Condition of PCR reaction

Name	PCR CYCLE
GAPDH	94℃ 4min; 94℃ 30 sec,54℃ 30sec, 72℃ 1min, 25 cycles; 72℃ 5min
ES274071	94℃ 4min; 94℃ 30 sec,53.2℃ 30sec, 72℃ 1min, 30 cycles; 72℃ 5min

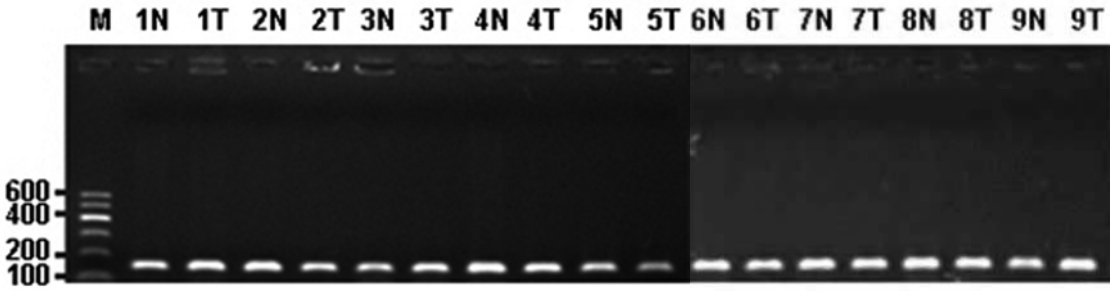

Fig.1 RT–PCR results of GAPDH. 1N is the normal tissue of the first specimen, 1T is the colorectal adenocarcinoma tissue of the first specimen, followed by analogy. Marker is Marker 1.

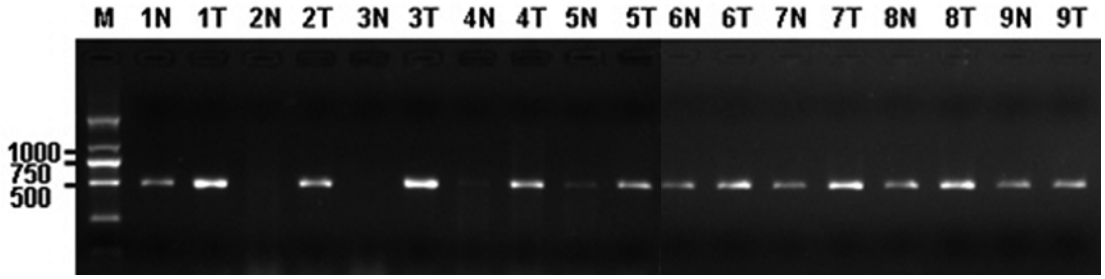

Fig.2 RT–PCR results of ES274071. 1N is the normal tissue of the first specimen, 1T is the colorectal adenocarcinoma tissue of the first specimen, followed by analogy. Marker is Marker 1.

ES274071's longest ORF sequences of the full-length is present in 354-1187bp, encodes 277 amino acid residues, encodes SET gene. The gene locates on human chromosome 9q34. Gene expression analysis shows that it distributes in a variety of tissues. It distributes in both of the lesion and normal brain tissue, especially in the cortex of oligodendroglioma. It distributes more in undifferentiated astrocytes tumor than normal astrocytes and normal spinal cord. It also distributes in neural tube cells and glioblastoma. Moreover, it distributes much in ovarian cancer, especially the serous adenocarcinoma. Breast cancer also has the volume of distribution, especially in the ductal carcinoma. At the same time, it also distributes in metastases of breast cancer, particularly in the lung metastasis.

RT–PCR results

Using the same volume of cDNA to amplify GAPDH, seting PCR reaction cycle as 25 times, making the products amplified in the exponential growth phase. Then using Q1 software to analysis the gray value of electrophoresis strip of GAPDH to adjust the volume of cDNA samples, making all the cDNA samples have the same amount when ES274071 amplified. The PCR products were separated on 1.0% agarose gel. The result of electrophoresis was shown in figure 1 and 2.

Using Q1 software to analysis the electrophoresis strip of ES274071, the Gray value of the electrophoresis strip was shown in Table 4, then having a T test by SPSS13.0 to analysis the Gray value of the electrophoresis strip, the result shows that T =- 2.202, P = 0.043,it has statistical significance.

Discussion

The relationship between the SET and leukemia

Genomic and cDNA cloning found that SET was fused to Can gene. Von Lindern M showed that SET-can fusion gene was found in a case of acute undifferentiated leukemia by Southern and Northern (RNA) blotting, and some researchers Cloned chimeric set-can cDNA, Cloned the 3' end of the 2-kb set mRNA, Sequenced determination and analysied set-can mRNA in situ hybridization (4). Adachi,Y identified SET as a 39-kDa protein by immunoprecipitation with rabbit antiserum against each of three synthetic peptides predicted from the open reading frame of the set gene. They proposed that SET played a key role in the mechanism of leukemogenesis in acute undifferentiated leukemia, perhaps by activating CAN in nuclei and stimulating the transformation potential of SET-CAN (5). To clarify a role(s) of SET-CAN in leukemo-

genesis, Saito S developed transgenic mice expressing SET-CAN under the control of the Gata1 gene hematopoietic regulatory domain that was active in distinct sets of hematopoietic cells. Their results demonstrated that SET-CAN blocked the hematopoietic differentiation program--one of the characteristics of acute myeloid leukemia (6). Mei Li indicated that some nuclear protein was fused to nucleoporin in acute nonlymphocytic leukemia, they found that SET can inhibit the activity Of PP2A, and the damage of PP2A's control may result in acute nonlymphocytic leukemia (7). Paolo Neviani found that in the cell line of BeR/ABL+ in chronic granulocytic leukemia, SET had more expression, it can inhibit PP2A (8).

The relationship between SET and other tumor

Ouellet V observed a significant correlation between expression of all SET complex proteins (SET Complex included SET, PP2A, APE, NM23 and HMGB) and the ovarian cancer differentiation, 4 out of 5 members of the SET complex, were highly expressed in invasive grade 3 tumors. When combining all tumors, overexpression of Nm23 ($p = 0.04$), Set ($p = 0.004$) and Ape1 ($p = 0.004$) was associated with the clinical stage of the disease. No marker by itself was associated with prognosis. They suggested that a strategy that sums the activities of different partners within a pathway may be more appropriate in designing nomograms for patient stratification (9). Nagata,K. purified TAF-I as 39- and 41-kDa polypeptides from HeLa cells. Nucleotide sequence analysis revealed that the 39-kDa polypeptide corresponded the protein encoded by the set gene. A particular feature of TAF-I proteins was the presence of a long acidic tail in the C-terminal region, which was thought to be an essential part of the SET-CAN fusion protein (10).

The research about probable mechanism of SET

SET is an inhibitor of PP2A (proteinphosphataseZA) and PP2A is a phosphorylase, tumor suppressor proteins, it can regulate cell proliferation, growth and differentiation. Kandilci showed that SET was a target of chromosome translocation, it encoded a common kind of phosphoprotein, and it controlled many function, such as the Chromatin remodeling, transcription, cell cycle regulation of apoptosis. Adachi, Y. investigated in detail the in vivo phosphorylation of SET. Phosphorylation of SET occurred in all human cell lines examined in vivo, primarily on serine residues. Endoproteinase Glu-C digestion of phosphorylated. SET yielded two phosphopeptides. By radiosequencing, they identified the in vivo phosphorylation sites of SET as Ser9 and Ser24. The surrounding sequences of Ser9 and Ser24 contained an apparent consensus site sequence for protein kinase C (11). Rossana Trotta showed that SET expression was essential for suppressing PP2A phosphatase activity that would otherwise limit NK cell antitumoral and/or antiinl ammatory functions by impairing NK cell production of IFN- 1 (12). Samanta, A.K showed that Jak2 inhibition deactivated Lyn kinase through the SET-PP2A-SHP1 pathway, causing apoptosis in drug-resistant cells from chronic myelogenous leukemia patients, and indicated that Lyn was downstream of Jak2, and Jak2 maintained activating Lyn kinase in CML through the SET-PP2A-Shp1 pathway (13). In Mukhopadhyay A's study, they found that direct interaction of I2PP2A with ceramide played important biological roles via the regulation of PP2A activity and signaling, which in turn controlling ceramide-mediated degradation of c-Myc and antiproliferation (14). Hunt TA's data indicated that interaction between WNV capsid and I (2) (PP2A) resulted in increased PP2A activity. Given the central role of this phosphatase in cellular physiology, capsid/I (2) (PP2A) interactions may yet prove to be important for viral pathogenesis (15). Trotta R found that SET expression was essential for suppressing PP2A phosphatase activity that would otherwise limit NK cell antitumoral and/or antiinflammatory functions by impairing NK cell production of IFN-gamma (16).

Further more, studies demonstrated that the SET oncoprotein was capable of binding to cyclin B. Li Y showed that TSPY and TSPX indeed bound competitively to cyclin B at their SET/NAP domains in vitro and in vivo. Epigenetic dysregulation of TSPY in incompatible germ or somatic cells could promote cell proliferation and predispose susceptible cells to tumorigenesis (17). Liu Z showed that asparagine endopeptidase (AEP) was activated under acidic condition, cut SET, an inhibitor of DNase, and triggered DNA damage in brain, which was inhibited by PIKE-L. SET, a substrate of caspases, was cleaved by acidic cytosolic extract independent of caspase activation. Fractionation of the acidic cellular extract yielded AEP that was required for SET cleavage. They found that kainate provoked AEP activation and SET cleavaged at N175, triggering DNA nicking in wild-type, but not AEP null, mice. PIKE-L strongly bound SET and prevented its degradation by AEP, leading to resistance of neuronal cell death. Moreover, AEP also mediated stroke-provoked SET cleavage and cell death in brain. Thus, AEP might be one of the proteinases activated by acidosis triggering neuronal injury during neuroexcitotoxicity or ischemia (18). Qu D indicated that SET was imported into the nucleus through its association with impalpha3/impbeta, and that localization of SET was important in regulation of neuronal death (19). Gamble MJ showed that SET, DEK and PARP1 constituted a network governing access to chromatin by the transcription machinery (20).

Other information about SET

Senda M showed an example of crystal-quality improvewith the cDNA sequence of SET. Both PHAPI and PHAPII had an extended highly acidic C-terminal region. Based on their primary structure they speculated that PHAPI and PHAPII were involved in the generation of intracellular signaling events that lead to regulation of transcriptional activity after binding of a ligand to HLA class II molecules (22).

The significance of up–regulated expression set in human colorectal adenoca rcinoma

In recent years, there have been many researches on the SET gene and its function in leukemia and ovary cancer has been identified, but there is no report about the role in the occurrence and development of colorectal adenocarcinoma. At the same time, there is no patient experiment about leukemia. By the human colorectal adenocarcinoma samples, we have studied the expression of SET in the colorectal carcinoma. Although we have much follow-up work to be fully aware of the gene, but this experiment lays the foundation for us to understand set function in onset of human colorectal adenocarcinoma.

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