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Differential gene expression identified in Uigur women cervical squamous cell carcinoma by suppression subtractive hybridization

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Cervical cancer is one of the most common gynecological cancers worldwide. Over the past decade, much progress has been made in understanding the genetic changes associated with the development and progression of cervical cancer. However, the precise mechanisms of cervical carcinogenesis in Uigur women remain unclear. To screen differential gene expression in squamous cell carcinoma (SCC) of the cervix in Uigur women, suppressive subtractive hybridization (SSH) was performed on the cervical squamous cell carcinoma and corresponding normal cervical tissues of a Uigur patient. Thus we were be able to find the genes that are related with cervical tumors of Uigur women. A total of 300 samples were subject to DNA sequencing analysis and 46 genes were found to express differentially in tumors compared with normal tissues. Of the 46 genes, 24 genes were up-regulated whereas 22 genes were down-regulated in cervical tumors. The expression profiles of 5 of the 46 genes were further confirmed in 15 other Uigur patients by semi-quantitative reverse-transcription polymerase chain reaction. Our results revealed that ACADVL, CEBPB, IFITM1 and DNAJC9 are involved in cervical carcinogenesis.

Keywords: Uigur women squamous cell carcinoma, suppression subtractive hybridization, gene expression, cervical tumor.

Cervical cancer is the most common gynecological malignancy worldwide and is one of the most common cancers diagnosed in women [1]. It is important to determine the mechanisms that are involved in cervical carcinogenesis. Over the past decade, much progress has been made in understanding the genetic changes associated with development and progression of cervical cancer. For example, it is now clear that the H-ras, N-ras and LAMP3 genes are activated in cervical tumors, whereas KAI1 and galectin-3 genes are inactivated [2, 3, 4, 5]. In addition, it was found that the retinoic acid receptor beta gene is down-regulated in cervical tumors, whereas a gene for telomerase reverse transcriptase is up-regulated [6, 7]. We found that the promoter of RASSF1A gene was methylated in squamous cervical carcinoma of Uigur women [8]. The precise mechanisms of cervical carcinogenesis in Uigur women, however, remains unclear and it is likely that more genes are involved in this type of cervical carcinogenesis.

Using a combination of suppressive subtractive hybridization (SSH) and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), we investigated the genes that were differentially expressed between this Uigur cervical tumor tissues and their corresponding normal cervical tissues. We chose two up-regulated genes and three down-regulated genes in tumor cells that were involved in apoptosis or proliferation of cells, as detected by SSH analysis. These five differentially expressed genes were investigated in 15 cervical tumor tissue samples and normal cervical tissues. The differences in the expression of these genes provide clues to further understand the specificity of cervical carcinogenesis in Uigur women.

Materials and methods

Clinical sample collection. Sixteen patients with cervical squamous cell carcinoma were surgically treated in the First Affiliated Hospital and the Third Affiliated Hospital of the Medical College of Shihezi University, Xinjiang, China. None of these patients received chemotherapy or radiotherapy before the cervical tissues were obtained. All histological diagnoses were confirmed by experienced pathologists. Approval was obtained from the ethics committees of the First Affiliated Hospital and the Third Affiliated Hospital, Medical College of Shihezi University. The tumor tissues and their paired mor-

Table 1 Sixteen Uigur cervical SCC with the clinic stage and HPV16, HPV18 infection

Number	Age	Clinic stage	HPV16 infection	HPV18 infection
1	31	Ia	Yes	No
2	34	Ib	No	Yes
3	44	Ia	No	No
4	47	IIa	Yes	No
5	35	IIa	Yes	No
6	49	IIa	No	Yes
7	51	IIb	Yes	No
8	39	Ib	Yes	Yes
9	54	Ia	No	No
10	59	IIa	Yes	No
11	62	Ib	No	No
12	44	Ia	Yes	No
13	35	Ia	Yes	No
14	43	Ia	No	Yes
15	58	Ib	Yes	No
16*	49	IIa	Yes	No

* for SSH sample

phologically normal cervical tissues or adjacent cervical tissue were placed in liquid nitrogen immediately after resection from the patients and stored in liquid nitrogen until use.

Sample preparation. The 16 pairs of tumor tissue and normal cervical tissue that were surgically removed were immediately examined by a gynecologic pathologist (Table 1). Two or three pieces (5×5 mm) of tumor tissue from the periphery of the tumor mass were immediately stored in liquid nitrogen until use. The normal cervical tissues were sampled approximately 1.5 cm away from the primary lesion and were confirmed by examination of the H&E slides of the tissue samples. The 16 pairs of cervical tumor tissue samples and corresponding normal cervical tissue samples were transported to laboratory immediately after surgery for RNA extraction. All tumor samples were determined to contain at least 70% tumor cellularity by H&E staining.

One sample of cervical tumor tissue and its corresponding normal tissue sample were used to construct the SSH library; the other 15 pairs of cervical tumor tissue samples and their corresponding normal tissue samples were used in semi-quantitative RT-PCR.

RNA preparation and construction of tissue-specific libraries by SSH. Total RNAs were isolated from cervical tumor samples and normal cervical tissue samples with TRIZOL reagent (Invitrogen, USA) following the manufacturer's instruction. Total RNAs were dissolved in nuclease-free water. The mRNAs were purified with the Oligotex mRNA purification system (Qiagen, Germany) according to the manufacturer's instruction.

Two SSH experiments were performed in the forward (cervical tumor mRNA as tester and corresponding normal tissue mRNA as driver) and reverse (corresponding normal tissue mRNA as tester and cervical tumor mRNA as driver) directions to identify the mRNA species that were up-regulated or down-regulated in cervical tumors. The Clontech PCR Select cDNA Subtraction Kit (Clontech, USA) was used according to the manufacturer's instruction. The tester and driver cDNAs were digested with the restriction enzyme Rsa1. The tester cDNA was then divided into two portions and each was ligated with a different cDNA adaptor, which resulted in two populations of tester-adaptor cDNA. An excess of driver cDNA was added to each sample of tester cDNA for the first hybridization. The mixed tester-driver samples were heat-denatured and allowed to re-anneal. The two primary hybridization samples were then mixed with new driver cDNA, allowed to hybridize, and then subjected to PCR amplification. Products from the secondary PCRs were inserted into pMD19-T vectors (Takara Biotechnology, Japan) and then analyzed by DNA sequencing. We confirmed the differential expression of the subtracted clones with the PCR-select Differential Screening Kit (Clontech, USA) according to the manufacturer's instruction.

Sequencing and homology search. Plasmids used in DNA sequencing were purified with the Qiaprep Mini Plasmid Kit (Qiagen), according to the manufacturer's instruction. Cycle sequencing was performed in a 10-μl reaction mix using PRISM BigDye terminator mix (Applied Biosystems, Shanghai, China) according to the manufacturer's instruction. The sequences generated were searched for homologous sequences in the National Centre for Biotechnology Information (NCBI) database with the program BLASTn.

Semi-quantitative reverse transcription polymerase chain reaction analysis. Total RNA was extracted from 15 pairs of cervical tumor samples and adjacent normal cervical tissue samples, and then reverse transcribed to cDNA using Super-Script II reverse transcriptase (Invitrogen, USA) in a 20-ul reaction. PCR assays were performed in 25-μl reactions; each reaction contained 100 μM of each deoxynucleotide triphosphate, 2 mM of MgCl2, 10 pmol of each primer

Table 2 Primers used in semi-quantitative RT-PCR experiments

Gene name	Forward primer	Reverse primer	PCR-fragment size(bp)
CD44	5'-cattcaaatccggaagtgct-3'	5'-gttgccaaaccactgttcct-3'	228
DNAJC9	5'-atgagcagggaacagtggac-3'	5'-gcaaagcacagactccatga-3'	215
ACADVL	5'-atggtggaggagaccacttg-3'	5'-aaagagcaggatgcctttga-3'	198
CEBPβ	5'-gcacagcgacgagtacaaga-3'	5'-agctgctccaccttcttctg-3'	153
IFITM1	5'-atgtcgtctggtccctgttc-3'	5'-gtcatgaggatgcccagaat-3'	184
GAPDH	5'-gccaaaagggtcatcatctc-3'	5'-gtagaggcagggatgatgttc-3'	287

Table 3 Genes identified as differentially and highly expressed in Uigur cervical tumors compared with normal Uigur cervical tissue samples

Gene symbol	Gene name	Accession No.	Chromosomal localization
RPL26	Ribosomal protein L26	NM_000987	17p13
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1	NM_001402	6q14.1
FYTTD1	Forty-two-three domain containing 1 transcript variant 2	NM_001011537	3q29
BAG1	BCL2-associated athanogene	NM_004323	9p12
DNAJC9	DnaJ (Hsp40) homolog, subfamily C, member 9	NM_015190	10q22.2
CD44	CD44(Indian blood group) transcript variant 2	NM_001001389	11p13
SETD5	SET domain containing 5	XM_944280	3
NBPF1	Neuroblastoma breakpoint family, member 1	NM_017940	1p36.13
SPRR3	Small proline-rich protein 3	NM_005416	1q21-q22
KRT8	Keratin 8	NM 002273	12q13
PIGT	Phosphatidylinositol glycan anchor biosynthesis, class T	NM_015937	20q12
FGF1	Fibroblast growth factor 1	NM_000800	5q31
ANXA8	Annexin A8	NM_001040084	10q11.22
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	NM_033360	12p12.1
TMPO	Thymopoietin	NM_003276	12q22
RANBP2	RAN binding protein 2	NM_006267	2q12.3
ATF5	Activating transcription factor 5	NM_012068	19q13.3
PTK6	PTK6 protein tyrosine kinase 6	NM_005975	20q13.3
EEF1A2	Eukaryotic translation elongation factor 1 alpha 2	NM_001958	20q13.3
GDF15	Growth differentiation factor 15	NM_004864	19p13.11
EFNB1	Ephrin-B1	NM_004429	Xq12
ID3	Inhibitor of DNA binding 3	NM_002167	1p36.13-p36.12
AKT1	V-akt murine thymoma viral oncogene homolog 1	NM_001014432	14q32.32
CYC1	Cytochrome c-1	NM_001916	8q24.3

and 0.5 U Taq polymerase (Takara Biotechnology, Japan). The PCR conditions were: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; followed by 5 minutes at 72°C. GAPDH was used as an internal control in each PCR reaction. We selected five genes that were involved in apoptosis or proliferation of cells to examine the expression levels of mRNA. The PCR primers for individual genes were designed and analyzed using the program Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi; Table 2). All primers were synthesized by Sangon Ltd (Shanghai, China). PCR products were analyzed by 1.5% agarose-gel electrophoresis. The gel images were obtained with a CCD camera (Bio-Rad Laboratories, USA); the band intensity was calculated using Quantity One 4.1 applied software. Each pair of tumor and normal tissue samples was normalized to the expression level of GAPDH in the same sample.

Results

The two SSH cDNA libraries were generated from a cervical tumor tissue sample and a normal cervical tissue sample of one Uigur patient. We sequenced 150 clones from the up-regulated library and 150 clones from the down-regulated library. The sequences were aligned against the GenBank database and any redundant or false-positive sequences were excluded. A total of 46 non-redundant transcripts were found, of which 24 were genes known to be expressed at high levels in cervical tumors and 22 were genes known to be expressed at low levels in cervical tumors. BLAST analysis found genes in GenBank that had more than 95% homology to the cDNAs we sequenced (Tables 3 and 4).

We chose five of the 46 differentially expressed genes for validation by semi-quantitative RT-PCR in 15 pairs of Uigur women cervical tumor tissues and the their cervical normal

Table 4 Genes identified as differentially expressed at low levels in Uigur cervical tumors compared with normal Uigur cervical tissue samples

Gene symbol	Gene name	Accession No.	Chromosomal localization
ACADVL	Acyl-Coenzyme A dehydrogenase, very long chain	NM_001033859	17p13-p11
CEBPβ	CCAAT/enhancer binding protein(C/EBP), beta	NM_005194	20q13.1
FABP4	Fatty acid binding protein 4	NM_001442	8q21
RPL31	Ribosomal protein L31	NM_000993	2q11.2
RPS20	Ribosomal protein S20	NM_001023	8q12
IFITM1	Interferon induced transmembrane protein 1	NM_003641	11p15.5
TMEM70	Transmembrane protein 70	NM_017866	8q21.11
IGLL1	Immunoglobulin lambda-like polypeptide 1	NM_152855	22q11.23
RPL3	Ribosomal protein L3	NM 000967	22q13
RPL30	Ribosomal protein L30	NM_000989	8q22
RPL39	Ribosomal protein L39	NM_001000	Xq22-q24
LOC644082	Similar to ribosomal protein L18a	XR_016460	3
RHCG	Rh family, C glycoprotein	NM_016321	15q25
IGFBP5	Insulin-like growth factor binding protein 5	NM_000599	2q33-q36
MNDA	Myeloid cell nuclear differentiation antigen	NM_002432	1q22
CEBPα	CCAAT/enhancer binding protein (C/EBP), alpha	NM_004364	19q13.1
TPP2	Tripeptidyl peptidase II	NM_003291	13q32-q33
CDH3	Cadherin 3	NM_001793	16q22.1
DKK3	Dickkopf homolog 3	NM_015881	11p15.2
PAX1	Paired box 1	NM_006192	20p11.2
CLCA2	Ca^{2+}-activated chloride channel-2	NM_006536	1p31-p22
RAMP2	Receptor activity modifying protein 2	NM_005854	17q12-q21.1

tissue samples. The low rates of false-positive results indicated that the two SSH libraries were effective for analysis of the five differential expression genes. We found that two genes (DNAJC9 and CD44) were up-regulated in cervical tumor tissues. In addition, the expression of three genes (ACADVL, CEBPB and IFITM1) was repressed in cervical tumor tissues (Figure 1).

Discussion

It was reported that KRT8 gene was over-expressed in HPV positive cervical carcinoma [9]. This is similar to what we found in this study by SSH. Using microarray method, Grigsby et al. found that PTPRM and LAPTM4B gene were up-regulated in cervical cancer and ELF4 and DOCK2 genes

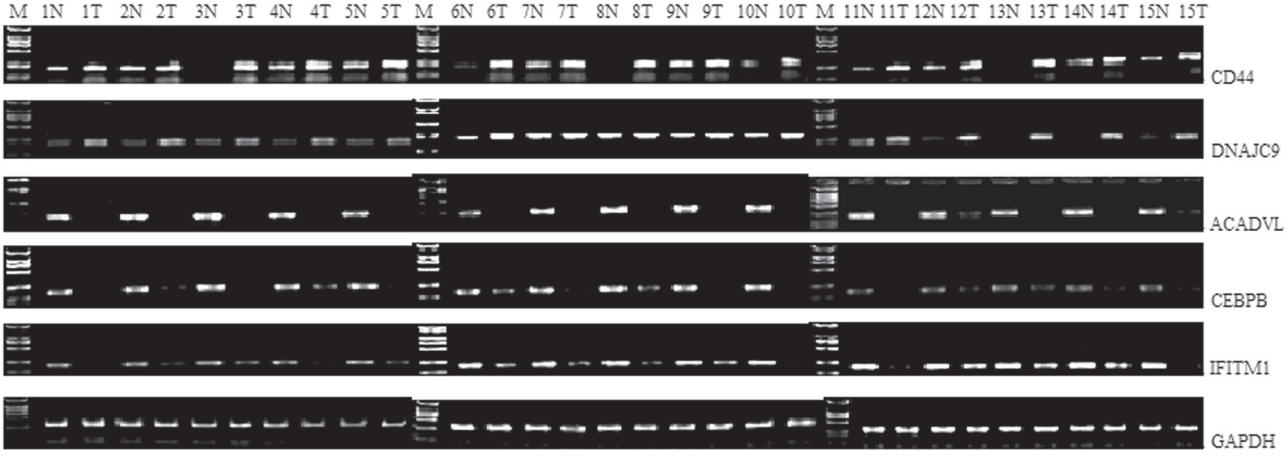

Figure 1 Gene expression levels were analyzed by semi-quantitative RT-PCR. Five genes (CD44, DNAJC9, ACADVL, CEBPB and IFITM1) were selected for RT-PCR from 15 pairs of matched cervical Uigur SCC tumor tissues and normal tissue samples. N, normal cervical tissues; T, tumor tissues; M, TakaRa Biotechnology DL2000 DNA Marker. Total RNA was isolated and RT-PCR was performed as described in the text. The molecular size of the resulting PCR products were shown in Table 1. RT-PCR with a GAPDH primer was performed as a control. Images obtained were processed for quantitative analysis using the Quantity One 4.1 applied software. The relative quantities of the specific PCR products were determined by densitometry and standardized to the GAPDH level in the same sample.

were down-regulated in cervical cancer [10]. In addition, SDHB gene was up-regulated and IFN-γ gene was down-regulated in lymph node metastasis of cervical carcinoma by microarray [11].

Our results showed that 24 genes were up-regulated whereas 22 genes were down-regulated in cervical tumors of Uigur women. These differentially expressed genes encode ribosomal proteins (RPL31, RPL26), a metabolism enzyme (ACADVL), a protein responsible for cell-cell interactions (KRT8), a protein responsible for cell adhesion and migration (CD44), a transcription factor (CEBPβ) and a translation elongation factor (EEF1A1).

To validate the differentially expressed genes identified by the SSH method, two up-regulated genes and three down-regulated genes were selected and further screened by semi-quantitative RT-PCR analysis. Among the five genes tested, the up-regulation of DNAJC9 and CD44 and down-regulation of ACADVL, CEBPB and IFITM1 were consistent with the expression profiles obtained from SSH analysis. The finding that the CD44 gene is up-regulated in cervical tumors and is rarely expressed in normal tissues is in agreement with the results of previous research [12]. However, to our knowledge, the expression profiles of the ACADVL, CEBPB, IFITM1 and DNAJC9 genes in cervical tumors of Uigur women have not been reported previously.

Of the up-regulated genes, DNAJC9 is a member of the Hsp40 gene family. Hsp40 contains a DnaJ domain; Hsp40 and GrpE protein are co-chaperones that assist Hsp70. Hsp70 proteins are universally conserved ATP-dependent molecular chaperones that help proteins to adopt and maintain their native conformations [13]. Many proteins contain DnaJ domains that can participate in endocytosis and protect cells from various stresses. A DnaJ/Hsp40 homolog has been found to be up-regulated in human leukemia Molt-4 cells treated with non-thermal low-intensity-pulsed ultrasound [14]. The DNAJC9 gene may be up-regulated to protect cells from apoptosis in these cervical tumors.

Of the three down-regulated genes, ACADVL is a member of the family of multimeric enzymes that catalyze the alpha, beta-dehydrogenation of acyl-CoA esters in fatty-acid beta-oxidation and amino-acid catabolism [15]. The ACADVL gene has recently been reported to be significantly down-regulated in adrenocortical carcinomas [16]. It is possible that the ACADVL gene has a role in development of cervical tumors in Uigur women.

The CEBPB gene is known to be down-regulated in hepatoblastoma cells. Expression of CEBPB proteins may have an important role in the genesis of hepatoblastoma cells, probably by inducing different stages of arrest of differentiation [17]. It is likely that the CEBPB gene is involved in differentiation of these cervical tumors.

Interferon-induced trans-membrane protein 1 (IFITM1) has a key role in the anti-proliferative action of IFN-gamma and cell-adhesion signals. Suppression of IFITM1 has been shown to block the anti-proliferative effect of IFN-gamma, accelerate the cell growth rate and confer tumorigenicity to a non-malignant hepatocyte cell line in nude mice [18]. IFITM1 is a component of a multimeric complex involved in the transduction of anti-proliferative signals. It has been reported that IFITM1 expression is significantly different between patients classed as being at high or low risk of chronic myeloid leukemia. In addition, higher levels of IFITM1 expression are associated with improved survival [19]. It is likely that low-level expression of the IFITM1 gene is associated with a high proliferative status of Uigur women cervical tumors.

In summary, we identified 46 genes that were differentially expressed between cervical tumor tissues and normal cervical tissues in Uigur patients. The expression profiles of 5 of these 46 genes were further confirmed in 15 other Uigur cervical tumor patients by semi-quantitative reverse-transcription polymerase chain reaction. The CD44 and KRT8 genes have previously been reported to be associated with cervical tumors, yet their roles in cervical tumors need to be further investigated. However, four other genes shown to be differentially expressed by the semi-quantitative RT-PCR analysis have not been previously associated with cervical tumors. Further characterization of these differentially expressed genes will help us to understand the role of genes responsible for the development of Uigur women cervical tumors.

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