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ORIGINAL RESEARCH

The landscape of alternative splicing in cervical squamous cell carcinoma

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Background: Alternative splicing (AS) is a key regulatory mechanism in protein synthesis and proteome diversity. In this study, we identified alternative splicing events in four pairs of cervical squamous cell carcinoma (CSCC) and adjacent nontumor tissues using RNA sequencing.

Methods: The transcripts of the four paired samples were thoroughly analyzed by RNA sequencing. SpliceMap software was used to detect the splicing junctions. *Kyoto Encyclopedia of Genes and Genomes* pathway analysis was conducted to detect the alternative spliced genes-related signal pathways. The alternative spliced genes were validated by reverse transcription-polymerase chain reaction (RT-PCR).

Results: There were 35 common alternative spliced genes in the four CSCC samples; they were novel and CSCC specific. Sixteen pathways were significantly enriched ($P<0.05$). One novel 5′AS site in the *KLHDC7B* gene, encoding kelch domain-containing 7B, and an exon-skipping site in the *SYCP2* gene, encoding synaptonemal complex 2, were validated by RT-PCR. The *KLHDC7B* gene with 5′AS was found in 67.5% (27/40) of CSCC samples and was significantly related with cellular differentiation and tumor size. The exon-skipping site of the *SYCP2* gene was found in 35.0% (14/40) of CSCC samples and was significantly related with depth of cervical invasion.

Conclusion: The *KLHDC7B* and the *SYCP2* genes with alternative spliced events might be involved in the development and progression of CSCC and could be used as biomarkers in the diagnosis and prognosis of CSCC.

Keywords: cervical squamous carcinomas, alternative splicing events, RNA sequencing

Introduction

Alternative splicing (AS) is a biological process by which different exons are joined together to generate a series of mRNA isoforms from a single primary transcript. Nearly 90% of human multiple-exon genes are alternatively spliced, and AS is a common mechanism for generating both different transcription products and protein diversity in higher eukaryotic cells.[1] The roles of AS in human diseases, especially in cancer, have been widely studied.[2] Tumor formation might be due to the imbalanced expressions of either the splicing variants or the incorrect isoforms.[3] Many oncogenes and tumor suppressor genes, such as *BRCA1/2*[4] and *p53*,[5] are alternatively spliced in cancer cells. The cancer-specific isoforms induce the phenotypic transformation of cancer cells.[6,7] Transcript sequencing has indicated that the gene mutations associated with cancer-specific AS events could be potentially used as valuable biomarkers in the diagnosis and therapy of cancer.[8]

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer deaths among women around the world.[9] Cervical cancer comprises 80% of squamous cell carcinoma (CSCC).[10] The etiology of cervical cancer is absolutely related to persistent infection by human papillomavirus (HPV).[11]

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The carcinogenesis due to HPV depends on the activities of viral oncoproteins E5, E6, and E7, which inhibit various cellular targets, including the tumor suppressor proteins *p53*, pRb, *p21*, and *p27*, as well as disrupting critical cellular processes, including cell cycle, apoptosis, and malignant transformation of cervical basal cells.[12] In high-risk HPV types, transcription is initiated at the early promoter located in the E6 open reading frame (ORF) and the late promoter in the E7 ORF of HPV. All viral genes are transcribed to many polycistronic RNAs with two or more ORFs, which then undergo further processing, including AS and polyadenylation.[13] For HPV16, at least 13 different mRNAs with the capacity to encode capsid proteins are produced by AS.[14]

In this study, we detected the CSCC-specific AS events by comparing the global transcriptional changes of CSCC to that of the adjacent nontumor tissues (ATN) through RNA sequencing. This study aims to advance our understanding of CSCC.

Patients and methods

Tissue specimens

Forty paired fresh-frozen tissue samples (CSCC and ATN) were collected from patients receiving radical hysterectomy for CSCC during the period of January 2012 to August 2013 (Peking Union Medical College Hospital, People's Republic of China). Diagnosis of all cases was histologically confirmed by two independent pathologists, and all tumor tissues were assessed by hematoxylin–eosin (HE) staining, and only those tissues with percentage of tumor cells more than 90% were used. Four paired samples were randomly selected for RNA sequencing from among these cases. Informed consent from each patient was obtained. The procedures have been approved by the ethics review committee of Peking Union Medical College Hospital and are in accordance with the Helsinki Declaration of 1975.

Raw read filtering

The complementary DNA (cDNA) library of the four paired samples was constructed and sequenced. The raw RNA-sequencing data were filtered according to the following criteria: 1) reads containing sequencing adaptors were removed; 2) nucleotides with a quality score <20 were removed; 3) reads with more than 8% nitrogenous bases were removed. All subsequent analyses were based on clean reads.[15]

Detection of AS

SpliceMap was used to detect splicing junctions and different types of AS events, including exon skipping, mutually exclusive exons, intron retention, 5′AS, and 3′AS in CSCC and ATN tissues.[16] The read was separated into segments. Each segment was mapped to the human genome with Bowtie software.[17] Then all of the segments were pieced together to determine the locations of exons and possible junctions. We filtered the splice junctions originally detected according to two criteria: quality of the alignment and coverage of splice junction. The AS events presented only in the CSCC or ATN samples were detected.

Kyoto Encyclopedia of Genes and Genomes pathway analysis

The unique lists of CSCC-specific AS genes were submitted to the Web-based functional annotation tool, which is known as the Database for Annotation, Visualization and Integrated Discovery v6.7.[18] The false discovery rate (FDR) was set at 5%, and the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) pathway analysis was conducted for functional annotation categories.

Validation of AS genes by reverse transcriptase–polymerase chain reaction

Total RNA was extracted from frozen CSCC and ATN samples, and cDNA was synthesized from 5 μg of RNA (Invitrogen). To validate AS genes, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using AS gene-specific PCR primers in CSCC and ATN tissues, with cycling parameters: 95°C for 10 minutes; followed by 35 cycles each of 95°C for 45 seconds, annealing at 55°C or 60°C for 45 seconds, and extension at 72°C for 45 seconds; and a final extension at 72°C for 10 minutes. Electrophoresis on 1.5% agarose gels was carried out for PCR products. The primers were as follows: *KLHDC7B* (kelch domain-containing 7B) sense primer: 5′-AGGTGAGGCTCAGACAAGA-3′, antisense primer: 5′-GAGATGGTGGGAGAATGG-3′; *SYCP2* (synaptonemal complex 2) sense primer: 5′-GATTACGGTGTCAGGAGG-3′, antisense primer: 5′-CTGGGAGATAAGTCAAGG-3′; *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) sense primer: 5′-GTCAAGGCTGAGAACGGGAA-3′, antisense primer: 5′-AAATGAGCCCCAGCCTTCTC-3′. *GAPDH* was used as the reference gene.

Correlation between AS genes and the clinicopathologic characteristics of CSCC patients was tested by the chi-square test. Fisher's exact text was used when theoretical frequency was <5.0. Statistical significance was assumed as $P<0.05$. Statistical analysis was performed using the SPSS 13.0 statistical software.

Table 1 Newly detected AS events in CSCC and ATN samples

Sample	AS events in sample transcripts	AS events in reference transcripts	Newly detected AS events
LHR-CSCC	147,341	129,879	17,462
LHR-ATN	142,583	125,422	17,161
LYM-CSCC	166,558	141,457	25,101
LYM-ATN	194,893	154,596	40,297
ZTF-CSCC	122,182	113,148	9,034
ZTF-ATN	153,975	134,074	19,901
WSN-CSCC	170,865	144,940	25,925
WSN-ATN	116,545	103,266	13,279

Abbreviations: AS, alternative splicing; CSCC, cervical squamous cell carcinoma; ATN, adjacent nontumor tissues.

Results

Characterization of RNA sequences

Four paired samples were collected for RNA sequencing. We obtained 2.0 million, 2.6 million, 0.8 million, and 2.2 million paired reads from the four CSCC samples, respectively. Similarly, 2.8 million, 5.4 million, 2.4 million, and 1.6 million paired reads were obtained from the ATN samples accordingly. The mapped ratio ranged from 63.1% to 73.2%.

Detection of CSCC-specific AS genes

SpliceMap was used to detect splice junctions. We compared the CSCC transcripts and ATN transcripts with the reference genome (Table 1). There were 17,462, 25,101, 9,034, and 25,925 newly detected AS events in the CSCC tissues and 17,161, 25,101, 19,901, and 13,279 newly detected AS events in the ATN tissues from the four paired samples. We screened out the AS events with more than one mapped reads; thus, 307, 555, 86, and 603 specific AS events were present in the four CSCC tissues, respectively. There were 35 common AS genes among the four CSCC tissues. One novel junction with a 5′AS site in the *KLHDC7B* gene was supported by 1, 3, 18, and 22 reads in the four CSCC tissues, and an exon-skipping site in the *SYCP2* gene was supported by 2, 3, 12, and 13 reads in the four CSCC tissues (Table S1).

KEGG pathway analysis

KEGG pathway analysis was used to identify AS gene-related significantly enriched pathways. In total, 16 pathways were significantly enriched ($P<0.05$), including metabolic pathways, endocytosis, and the Ras signaling pathway, and all of these pathways were specific for CSCC (Table 2).

Validation of AS genes

KLHDC7B and *SYCP2* genes were chosen as the candidate genes according to the number of mapped reads in the four pairs of samples. One novel junction with a 5′AS site in the *KLHDC7B* gene (Figure 1) and an exon-skipping site in the *SYCP2* gene were found (Figure 2). The AS events in the *KLHDC7B* (Figure 3A) and the *SYCP2* genes (Figure 3B) were CSCC specific and were validated by RT-PCR. In total, the *KLHDC7B* gene with 5′AS was found in 67.5% (27/40) of CSCC samples and was positively related with cellular differentiation and tumor size (Table 3). The exon-skipping site of the *SYCP2* gene was found in 35.0% (14/40) of CSCC samples and was positively related with the depth of cervical invasion (Table 3).

Discussion

AS is crucial in normal development programs and its dysregulation is related to tumorigenesis.[19,20] AS events are involved in cell cycle, metabolism, tumor suppression, and various cell signaling pathways.[21] The cancer-specific AS events can lead to the activation of oncogenes and cancer-related

Table 2 Signal pathways related with CSCC-specific AS events ($P<0.05$)

KEGG entry	Signal pathway	Gene number
Ko05144	Malaria	6
Ko04270	Vascular smooth muscle contraction	7
Ko04962	Vasopressin-regulated water reabsorption	4
Ko01100	Metabolic pathways	23
Ko05152	Tuberculosis	12
Ko04670	Leukocyte transendothelial migration	5
Ko04145	Phagosome	6
Ko05016	Huntington's disease	8
Ko04014	Ras signaling pathway	10
Ko04144	Endocytosis	12
Ko04514	Cell adhesion molecules	4
Ko04120	Ubiquitin-mediated proteolysis	6
Ko04142	Lysosome	5
Ko04530	Tight junction	6
Ko00563	GPI-anchor biosynthesis	5
Ko05146	Amebiasis	4

Abbreviations: AS, alternative splicing; CSCC, cervical squamous cell carcinoma; GPI, glycosylphosphatidylinositol; KEGG, *Kyoto Encyclopedia of Genes and Genomes*.

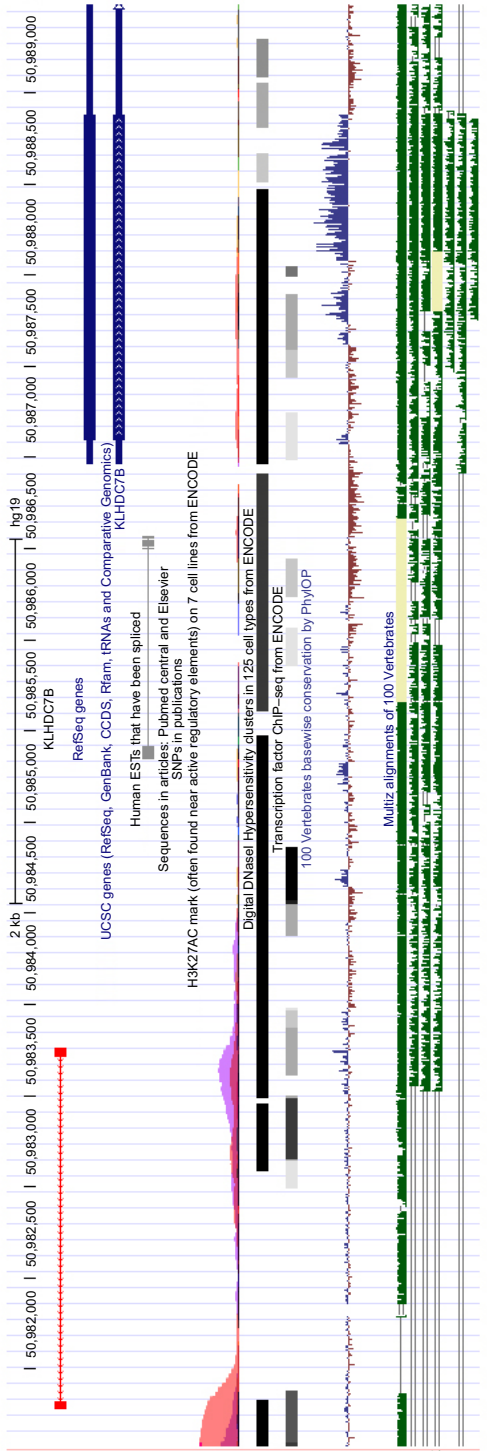

Figure 1 Analysis of novel AS junctions in *KLHDC7B* gene on the UCSC genome browser of the human genome built via custom track.
Notes: The novel splice junctions of the genes are indicated at the top followed by UCSC genes and mammalian conservation of the corresponding regions. The red labels indicate the 5′AS junction of the *KLHDC7B* gene.
Abbreviations: AS, alternative splicing; EST, expressed sequence tags; ChIP, chromatin immunoprecipitation; *KLHDC7B*, kelch domain-containing 7B; SNPs, single-nucleotide polymorphisms; UCSC, University of California, Santa Cruz.

Figure 2 Analysis of novel AS junction in *SYCP2* gene on UCSC genome browser of the human genome built via custom track.
Notes: The novel splice junctions of genes are indicated at the top followed by UCSC genes and mammalian conservation of the corresponding regions. The red labels indicated the exon-skipping splice junction of the *SYCP2* gene.
Abbreviations: AS, alternative splicing; EST, expressed sequence tags; ChIP, chromatin immunoprecipitation; SNPs, single-nucleotide polymorphisms; *SYCP2*, synaptonemal complex 2; UCSC, University of California, Santa Cruz.

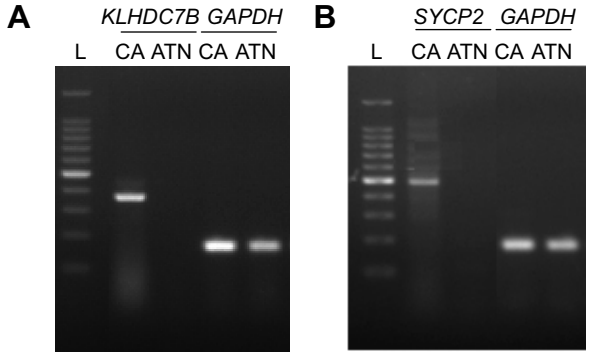

Figure 3 The AS events of the *KLHDC7B* and *SYCP2* genes were validated by RT-PCR.
Notes: (**A**) The 5′AS junction of *KLHDC7B* gene was CSCC specific. (**B**) The exon skipping of *SYCP2* gene was CSCC specific. *GAPDH* was used as the reference gene. L: O'RangeRuler 100-bp DNA ladder.
Abbreviations: AS, alternative splicing; ATN, adjacent nontumor tissues; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *KLHDC7B*, kelch domain-containing 7B; RT-PCR, reverse transcriptase-polymerase chain reaction; *SYCP2*, synaptonemal complex 2; CA, CSCC; CSCC, cervical squamous cell carcinoma.

pathways, as well as inactivation of tumor suppressors.[22] In this study, a total of 35 novel CSCC-specific AS genes and 16 significant pathways were identified.

Metabolic pathways, endocytosis, and the Ras signaling pathway were the three most important pathways detected by KEGG pathway analysis. It has been reported that metabolic alternations contribute to the development of cancer.[23] The major metabolic pathways, such as glycolysis and oxidative phosphorylation, are altered in cancer cells to meet the bioenergetic and biosynthetic demands associated with tumor growth.[24,25] The process of endocytosis and endocytic proteins are involved in the regulation of cell cycle, mitosis, and apoptosis in cancer cells.[26,27] The AS isoforms of Ras were able to activate the MAP kinase signaling pathway and to induce tumor formation in nude mice.[28] The Ras/Raf/MAPK cascade can be activated by the epidermal growth factor receptor (EGFR/ErbB1), a member of the ErbB receptor tyrosine kinase family, which is frequently mutated and overexpressed in different human cancers, including glioma, non-small cell lung carcinoma, ovarian carcinoma, and prostate carcinoma.[29] This research demonstrated that the AS genes in these signal pathways might participate in the progress of CSCC.

Table 3 Correlation between AS genes and the clinicopathologic characteristics of CSCC patients

Variable feature	n	*KLHDC7B*		*P*-value	*SYCP2*		*P*-value
		+	−		+	−	
Age							
≥40	24	17	7	0.304	6	18	0.104
<40	16	10	6		8	8	
Differentiation							
Good	17	10	7	0.012	5	12	0.058
Moderate	16	12	4		7	9	
Poor	7	5	2		2	5	
Tumor size (cm)							
≥4.0	18	16	2	0.016	9	9	0.072
<4.0	22	11	11		5	17	
Lymphatic metastasis							
Yes	8	5	3	0.736	3	5	0.868
No	32	22	10		11	21	
Cervical invasion							
≥1/2	12	8	4	0.94	8	4	0.006
<1/2	28	19	9		6	22	
FIGO staging							
Ia	6	4	2	0.518	1	5	0.495
Ib	27	17	10		11	16	
IIa	7	6	1		2	5	

Note: Fisher's exact text was used when theoretical frequency was <5.
Abbreviations: AS, alternative splicing; CSCC, cervical squamous cell carcinoma; FIGO, International Federation of Gynecology and Obstetrics; *KLHDC7B*, kelch domain-containing 7B; *SYCP2*, synaptonemal complex 2.

On the basis of the RNA-sequencing analysis, we confirmed the AS events in *KLHDC7B* and *SYCP2* genes in CSCC tissues by RT-PCR for the first time. The *KLHDC7B* mRNA is increased under several biological conditions mainly due to infection. *KLHDC7B* expression was increased during acute HCV infection and was induced by interferon gamma, TNF-α, and IL-4. The *KLHDC7B* gene regulates and facilitates HCV replication in hepatocytes.[30,31] In the study of Kim et al the *KLHDC7B* gene containing a kelch domain was identified as a candidate novel epigenetic marker that was hypermethylated and upregulated in breast cancer; the methylation level of the 14CpG sites at the promoter region of the gene was higher in cancer tissues and cultured breast cell lines.[32] *KLHDC7B* was upregulated in vulvar intraepithelial neoplasia due to HPV infection.[33] We speculated that the spliced *KLHDC7B* gene might bind to HPV oncoproteins to promote CSCC progression.

SYCP2 is a proteinaceous structure that links homologous chromosomes during the prophase of meiosis. The protein encoded by this gene is a major component of the synaptonemal complex and may bind DNA at scaffold attachment regions. It had been reported that *SYCP2* was upregulated in Caski and SiHa cells and is associated with invasive cervical cancer.[34,35]

The expression of the *KLHDC7B* gene with 5′AS was positively related with cellular differentiation and tumor size. The *SYCP2* gene with exon skipping was positively related with depth of cervical invasion. The AS events in *KLHDC7B* and *SYCP2* genes might generate new transcripts or regulatory proteins to promote the progress of CSCC, and *KLHDC7B* and *SYCP2* genes with the novel AS events could

be used as potential biomarkers in diagnosis and therapy of CSCC patients. However, the mechanisms of action of the two genes with AS events in the carcinogenesis of CSCC need further investigation.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 The alternative splicing junctions of the four paired CSCC and ATN tissues

Junction	Strand	Official gene name	LHR	LYM	ZTF	WSN
chr9:100767385–100773605	+	ANP32B	(1/0)	(2/0)	(1/0)	(3/0)
chr1:111985966–111986338	–	WDR77	(1/0)	(2/0)	(1/0)	(2/0)
chr1:156015471–156018341	–	UBQLN4	(1/0)	(1/0)	(1/0)	(1/0)
chr1:156563314–156563723	+	APOA1BP	(1/0)	(2/0)	(1/0)	(2/0)
chr1:160959034–160967990	+	F11r	(1/0)	(8/0)	(2/0)	(3/0)
chr1:1770627–1784640	–	GNB1	(1/0)	(2/0)	(1/0)	(5/0)
chr1:241799325–241803233	–	OPN3	(2/0)	(1/0)	(1/0)	(6/0)
chr10:103769156–103771529	–	c10orf76	(2/0)	(1/0)	(2/0)	(1/0)
chr10:134143939–134145035	–	LRRC27	(1/0)	(1/0)	(2/0)	(2/0)
chr12:100574444–100589409	–	microRNA 1827	(1/0)	(1/0)	(2/0)	(1/0)
chr13:103436812–103440233	–	Kdelc1	(1/0)	(2/0)	(1/0)	(1/0)
chr13:77703315–77713380	–	MYCBP2	(1/0)	(2/0)	(1/0)	(4/0)
chr13:79889075–79893005	–	RBM26	(2/0)	(1/0)	(1/0)	(1/0)
chr14:103589770–103592696	+	TNFAIP2	(3/0)	(4/0)	(2/0)	(11/0)
chr15:30939867–30951018	+	ARHGAP11B	(1/0)	(2/0)	(1/0)	(2/0)
chr15:68489916–68521889	–	calml4	(1/0)	(1/0)	(1/0)	(1/0)
chr15:93444004–93467600	+	CHD2	(2/0)	(2/0)	(1/0)	(2/0)
chr16:55463342–55478074	–	MMP2	(1/0)	(1/0)	(2/0)	(3/0)
chr19:13228895–13246063	+	NACC1	(1/0)	(1/0)	(1/0)	(1/0)
chr19:48653417–48657178	–	lig1	(1/3)	(2/0)	(1/0)	(1/0)
chr20:32224464–32228199	+	CBFA2T2	(2/0)	(2/0)	(1/0)	(1/0)
chr20:57484584–57485055	+	Gnas	(1/0)	(1/0)	(1/0)	(1/0)
chr20:58450474–58453112	–	sycp2	(13/0)	(12/0)	(2/0)	(3/0)
chr22:24179991–24180800	–	Derl3	(2/0)	(1/0)	(1/0)	(4/0)
chr22:50981278–50983265	–	KLHDC7B	(3/0)	(18/0)	(1/0)	(22/0)
chr4:119459113–119461423	+	CEP170P1	(0/2)	(0/3)	(0/2)	(0/5)
chr4:184255479–184255672	–	CLDN24	(1/0)	(2/0)	(1/0)	(1/0)
chr5:33466262–33467025	+	Tars	(1/0)	(4/0)	(2/0)	(1/0)
chr6:52128956–52129242	–	MCM3	(4/0)	(3/0)	(2/0)	(3/0)
chr7:6066538–6068295	–	Eif2ak1	(1/0)	(1/0)	(1/0)	(1/0)
chr8:145666203–145667469	–	TONSL	(1/0)	(1/0)	(1/0)	(1/0)
chr8:74872003–74884326	–	TCEB1	(2/0)	(1/0)	(2/0)	(1/1)
chr9:5436644–5438262	–	PLGRKT	(1/0)	(1/0)	(2/0)	(1/0)
chr9:99086401–99122485	–	SLC35D2	(1/0)	(1/0)	(1/0)	(1/0)

Abbreviations: ATN, adjacent nontumor tissues; CSCC, cervical squamous cell carcinoma.