SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 18: 6572-6582, 2019

Differences in the early stage gene expression profiles of lung adenocarcinoma and lung squamous cell carcinoma

NITIN VENUGOPAL[1*], JUSTIN YEH[1*], SAI KARTHIK KODEBOYINA[1], TAE JIN LEE[1], SHRUTI SHARMA[1,2], NIKHIL PATEL[3] and ASHOK SHARMA[1,4]

[1]Center for Biotechnology and Genomic Medicine; Departments of [2]Ophthalmology, [3]Pathology and [4]Population Health Sciences, Medical College of Georgia, Augusta University, Augusta, GA 30912, USA

Received February 27, 2019; Accepted August 6, 2019

DOI: 10.3892/ol.2019.11013

Abstract. The discovery of lung carcinoma subtype-specific gene expression changes has the potential to elucidate the molecular differences and provide personalized therapeutic targets for these pathologies. The aim of the present study was to characterize the genetic profiles of the early stages (IA/IB) of two non-small cell lung cancer subtypes, adenocarcinoma (AD) and squamous cell carcinoma (SC). RNA-Seq gene expression data from The Cancer Genome Atlas was analyzed to compare the gene expression differences between AD and SC. The gene sets specific to each subtype were further analyzed to identify the enriched Gene Ontology terms, Kyoto Encyclopedia of Genes and Genomes pathways and biological functions. The results demonstrated that a unique set of genes (145 upregulated and 27 downregulated) was altered in AD, but not in SC; another set of genes (146 upregulated and 103 downregulated) was significantly altered in SC, but not in AD. Genes highly upregulated specifically in AD included albumin (1,732-fold), protein lin-28 homolog A, which is a positive regulator of cyclin-dependent kinase 2 (150-fold) and gastric lipase (81-fold). Genes highly upregulated specifically in SC included amelotin (618-fold), alcohol dehydrogenase 7 (57-fold), aclerosteosis (55-fold) and claudin-22 (54-fold). Several cancer/testis antigen family genes were notably upregulated in SC, but not in AD, whereas mucins were upregulated only in AD. Functional pathway analysis demonstrated that the dysregulation of genes associated with retinoid X receptors was common in AD and SC, genes associated with 'lipid metabolism' and 'drug metabolism' were dysregulated only in SC, whereas genes associated with 'molecular transport' and 'cellular growth and proliferation' were significantly enriched in AD specifically. These results reveal fundamental differences in the gene expression profiles of early-stage AD and SC. In addition, the present study identified molecular pathways that are uniquely associated with the pathogenesis of these subtypes.

Correspondence to: Dr Ashok Sharma, Center for Biotechnology and Genomic Medicine, Medical College of Georgia, Augusta University, 1120 15th Street, CA4094, Augusta, GA 30912, USA
E-mail: assharma@augusta.edu

*Contributed equally

Key words: non-small cell lung cancer, lung adenocarcinoma, lung squamous cell carcinoma, The Cancer Genome Atlas

Introduction

Non-small cell lung cancers (NSCLC), which are classified into adenocarcinomas (AD) and squamous cell carcinomas (SC), account for ~85% of primary lung cancer cases and are responsible for ~25% of cancer deaths in the United States (1-4). Previous studies have identified key differences between these histological subtypes at the molecular level, and have demonstrated the potential of these differences as diagnostic biomarkers and predictors of overall survival (5-7). For example, the mammary serine protease inhibitor maspin has been demonstrated to be highly expressed in SC, but not in AD (5). In addition, thyroid transcription factor 1 has been effectively used as an immunohistochemical marker to differentiate AD from SC (7). Several studies have examined gene expression profiles in lung cancer, including studies differentiating AD and SC (8-12). Shi *et al* (9) identified 2,961 microRNA sequences that may regulate differentially expressed genes (DEGs) in both NSCLC and small cell lung cancer across all clinical stages. Lu *et al* (10) studied DEGs in NSCLC subtypes across all stages, identifying a set of upregulated and downregulated genes in AD and SC but had a limited sample size. A total of 1,127 DEGs in NSCLC were identified by Grigoroiu *et al* (12), however they focused specially on stage IIIA disease and did not differentiate between AD and SC. Thus, the number of studies focusing on gene expression profiles specifically at the early stages (IA and IB) of NSCLC is low. Therefore, the present study aimed to provide a unique perspective by identifying gene expression changes specific to the early stages of AD and SC. Gene expression profiling of early-stage lung cancer may have great value in identifying potential molecular targets for the early detection and treatment of NSCLC.

The 5-year survival rate of patients with NSCLC who start treatment during stage IA of the disease is ~92%; however, the 5-year survival rate is 60% for stage IIA, 36% for stage IIIA

and <10% for stage IVA (13). Thus, diagnosis and treatment at the early stages are crucial for improving the survival rates of patients with NSCLC. Genomic profiles of early-stage NSCLC may be particularly advantageous with the advent of next generation sequencing panels that allow rapid identification of personalized therapies for cancer by analyzing genetic variants in tissue biopsies (14). This technology has been demonstrated to provide clinical benefits in NSCLC and is routinely used to identify common mutations in lung cancer, such as *KRAS* and epidermal growth factor receptor (15,16). The identification of novel genes and pathways uniquely expressed in early stages of AD and SC may provide more specific elements for evolving personalized therapies, such as specific drug targets or as a component of a panel for a prognostic screening test.

The aim of the present study was to identify the unique signatures of SC and AD, by comparing the gene expression levels in each carcinoma to fully characterize the genetic profiles of each subtype. These unique gene sets may improve our understanding of the molecular basis of each NSCLC subtype and may provide more specific targets for personalized therapy.

Materials and methods

The cancer genome atlas (TCGA) datasets. TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) is a landmark dataset, which comprises the molecular characterization of over 20,000 samples spanning 33 different cancer types, publicly available to the research community. TCGA gene expression RNA-Seq data was downloaded from Xenabrowser (http://xenabrowser.net). Data for the early stages (IA and IB) (AJCC 7th Edition TNM Staging System; https://cancerstaging.org) of AD and SC, as well as those for adjacent normal tissues, were selected. Gene expression levels were compared between the cancer and normal lung tissue samples to identify DEGs in each subtype.

Statistical analysis. All statistical analyses were performed using the R language and environment for statistical computing (R version 3.2.2; R Foundation for Statistical Computing; www.r-project.org). The *edgeR* package (https://bioconductor.org/packages/release/bioc/html/edgeR.html) was used to perform differential expression analysis of all genes with count per million (CPM)>1 in at ≥2 samples, and two separate differential gene expression analyses were performed for each cancer type (AD and SC). To identify DEGs in each subtype, the gene expression data for the early stages of each carcinoma were compared with those for the adjacent normal tissues. The Benjamini and Hochberg's method (17) was used to control the false discovery rate. The level of change in gene expression was expressed as the mean fold-change (FC) between the cancerous and adjacent normal tissues. To identify highly significantly upregulated genes, a filter of |FC|≥ 4 and adjusted P<0.001 was used. The FCs of downregulated genes were transformed with a negative reciprocal, as the negative reciprocal FC of a downregulated gene has a negative sign, but retains the fold difference information, which is similar to logFC. For example, FC of 0.5 is the same as 2-fold downregulation (-2 fold-change). To identify the genes uniquely differentially expressed in either subtype, DEGs were assigned to the following categories: i) Genes upregulated in AD (FC_{AD}>4; P_{AD}<0.001), but not in SC; ii) genes upregulated in SC (FC_{SC}>4; P_{SC}<0.001), but not in AD (Table I); iii) genes downregulated in AD (FC_{AD}<-4; P_{AD}<0.001), but not in SC; and iv) genes downregulated in SC (FC_{SC}<-4; P_{AD}<0.001), but not in AD (Table II).

Functional analysis of DEGs. An Ingenuity Pathway Analysis (IPA) software tool (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) was used to determine the underlying mechanisms, functions, pathways and associations between the gene sets identified during DEG analysis. Molecular and cellular functions and canonical pathways were identified using IPA to distinguish the complex biology underlying the pathogenesis of the two lung cancer subtypes. Upstream regulator analysis using IPA was performed to discover the upstream transcriptional factors regulating changes in the expression of the identified genes. The analysis is based on the expected effects between transcriptional regulators and their targets stored in the Ingenuity database. The analysis provides a P-value and an activation z-score based on the number of known targets present in the DEGs for each transcriptional regulator. Overall, this analysis is part of IPA core analysis examining the mechanisms, functions and pathways associated with a given set of genes.

Results

Identification of DEGs. RNA-Seq gene expression data from TCGA were analyzed to compare the gene expression changes in the early stages of two NSCLC subtypes. A total of 145 genes were upregulated specifically in AD, and 146 genes were upregulated specifically in SC. Among the downregulated genes, 27 were downregulated specifically in AD, whereas 103 were downregulated specifically in SC. Venn diagrams representing the number of upregulated and downregulated genes in the two NSCLC subtypes are presented in Fig. 1A and B. A heat map was constructed to identify the expression patterns of these unique gene sets in each subtype (Fig. 1C). The patients were stratified by smoking status to determine whether the DEGs were associated with smoking; the results demonstrated that early-stage differences unique to each subtype were not associated with smoking (data not shown).

Genes differentially expressed in AD. A total of 145 genes were highly upregulated specifically in AD (FC_{AD}>4; P_{AD}<0.001) with no significant upregulation in SC. The genes with the highest FC uniquely upregulated in each subtype are presented in Table I. In addition, 27 genes were significantly downregulated specifically in AD (FC_{AD}<-4; P_{AD}<0.001) with no significant downregulation in SC (Table II). Highly upregulated genes specific to AD included albumin (*ALB*; AD, FC=1,732.04; SC, FC=-1.48), protein lin-28 homolog A (*LIN28A*; AD, FC=150.39; SC, FC=1.25), gastric lipase (*LIPF*; AD, FC=81.24; SC, FC=2.69), transmembrane 4 L six family member 4 (*TM4SF4*; AD, FC=68.74; SC, FC=-2.73)

Table I. Upregulated genes in AD and SC.

A, Genes upregulated specifically in AD

Gene	Gene name	AD FC	AD P-value	SC FC	SC P-value
ALB	Albumin	1,732.04	$8.37x10^{-21a}$	-1.48	0.205
LIN28A	Protein lin-28 homolog A	150.39	$1.18x10^{-8a}$	1.25	0.482
LIPF	Lipase, gastric	81.24	$5.48x10^{-7a}$	2.69	0.057
TM4SF4	Transmembrane 4 l six family member 4	68.74	$4.11x10^{-18a}$	-2.73	<0.001[a]
AGXT2L1	Alanine-glyoxylate aminotransferase 2-like 1	66.16	$4.11x10^{-11a}$	-1.89	0.087
ACMSD	Aminocarboxymuconate semialdehyde decarboxylase	50.76	$1.05x10^{-17a}$	1.60	0.314
PLUNC	Palate, lung and nasal epithelium carcinoma associated	38.01	$4.59x10^{-13a}$	1.57	0.299
CDHR2	Cadherin-related family member 2	34.47	$9.39x10^{-11a}$	1.49	0.135
CNGA3	Cyclic nucleotide gated channel α 3	33.38	$3.92x10^{-21a}$	-1.63	0.020
SLC14A2	Solute carrier family 14 (urea transporter), member 2	32.14	$8.58x10^{-14a}$	-1.02	0.894
TMEM229A	Transmembrane protein 229A	26.86	$1.86x10^{-12a}$	-1.47	0.125
GLTPD2	Glycolipid transfer protein domain containing 2.	25.59	$4.10x10^{-13a}$	1.23	0.671
COL25A1	Collagen, type xxv, α 1	24.22	$2.69x10^{-15a}$	1.08	0.797
GKN1	Gastrokine 1	23.84	$4.54x10^{-5a}$	-2.85	<0.001[a]
NPTX1	Neuronal pentraxin I	21.88	$3.19x10^{-18a}$	1.17	0.448
FAM177B	Family with sequence similarity 177, member b	20.74	$1.13x10^{-21a}$	1.09	0.797
LOC643763	Hypothetical protein loc643763	19.57	$6.23x10^{-12a}$	1.92	0.077
LOC145837	Hypothetical protein loc145837	19.20	$1.96x10^{-20a}$	1.47	0.128
ZP2	Zona pellucida glycoprotein 2 (sperm receptor)	18.62	$8.39x10^{-6a}$	-2.63	<0.001[a]
TFPI2	Tissue factor pathway inhibitor 2	17.86	$2.40x10^{-17a}$	-1.01	0.930
LGALS4	Lectin, galactoside-binding, soluble, 4 (galectin 4)	17.76	$6.23x10^{-12a}$	-2.71	<0.001[a]
KNG1	Kininogen 1	17.56	$1.99x10^{-7a}$	1.83	0.097
TTR	Transthyretin (prealbumin, amyloidosis type I)	17.14	$3.31x10^{-7a}$	2.86	0.054
PHGR1	Proline, histidine, and glysine-rich protein 1	17.13	$4.30x10^{-6a}$	2.38	0.076
MUC21	Mucin 21, cell surfaceassociated	14.87	$1.96x10^{-20a}$	-2.79	<0.001[a]
MUC5B	Mucin 5b, oligomeric mucus/gel-forming	14.01	$4.66x10^{-14a}$	-1.13	0.640
FGA	Fibrinogen α chain	12.68	$2.45x10^{-10a}$	-1.92	0.020
UPK3A	Uroplakin 3a	12.53	$2.47x10^{-20a}$	1.28	0.239
SLC1A7	Solute carrier family 1 (glutamate transporter), member 7	12.38	$1.11x10^{-14a}$	-3.80	<0.001[a]
RGS7	Regulator of g-protein signaling 7	12.38	$6.30x10^{-12a}$	1.56	0.208

B, Genes upregulated specifically in SC

Gene	Gene name	AD FC	AD P-value	SC FC	SC P-value
AMTN	Amelotin	1.70	0.137	618.16	$4.66x10^{-25a}$
ADH7	Alcohol dehydrogenase 7 (class iv), mu or sigma polypeptide	1.52	0.277	57.12	$3.30x10^{-23a}$
SOST	Sclerostin	-1.81	0.027	54.92	$3.90x10^{-18a}$
CLDN22	Claudin 22	2.46	0.059	54.24	$5.77x10^{-6a}$
SOX10	SRY (sex determining region y)-box 10	1.76	0.050	39.80	$1.92x10^{-10a}$
C12orf54	Chromosome 12 open reading frame 54	1.72	0.053	39.51	$2.34x10^{-34a}$
GPR149	G protein-coupled receptor 149	2.42	0.116	38.62	$7.30x10^{-13a}$
SCGN	Secretagogin, ef-hand calcium binding protein	1.96	0.172	37.66	$2.92x10^{-5a}$
SLC35D3	Solute carrier family 35, member d3	-1.20	0.456	37.43	$3.85x10^{-13a}$
CT45A3	Cancer/testis antigen family 45, member a3	3.01	0.151	35.61	$2.05x10^{-5a}$
ADAM23	Adam metallopeptidase domain 23	1.01	1.000	34.46	$1.71x10^{-29a}$
ST8SIA3	St8 α-n-acetyl-neuraminide α-2,8-sialyltransferase 3	3.17	0.059	33.69	$7.23x10^{-5a}$
HOXD10	Homeobox d10	1.53	0.120	32.56	$3.08x10^{-36a}$

Table I. Continued.

Gene	Gene name	AD FC	AD P-value	SC FC	SC P-value
LMO1	Lim domain only 1 (rhombotin 1)	1.59	0.072	31.08	$1.71x10^{-24a}$
ODZ2	Odz, odd oz/ten-m homolog 2	1.00	0.972	30.69	$2.36x10^{-30a}$
CLDN19	Claudin 19	-1.62	0.070	30.28	$1.95x10^{-10a}$
FOXN1	Forkhead box n1	1.19	0.339	29.93	$6.61x10^{-35a}$
APOA1	Apolipoprotein a-i	1.81	0.064	29.75	$8.79x10^{-9a}$
HS3ST4	Heparan sulfate (glucosamine) 3-o-sulfotransferase 4	1.74	0.097	29.69	$3.25x10^{-11a}$
PAX1	Paired box 1	1.63	0.225	28.10	$7.10x10^{-11a}$
OLFM3	Olfactomedin 3	2.73	0.050	26.58	$2.93x10^{-9a}$
FAM181B	Family with sequence similarity 181, member b	1.11	0.668	25.19	$1.25x10^{-39a}$
CRNN	Cornulin	2.06	0.129	24.94	$1.37x10^{-8a}$
TP53AIP1	Tumor protein p53 regulated apoptosis inducing protein 1	-1.02	0.916	23.03	$8.00x10^{-25a}$
TCHHL1	Trichohyalin-like 1	2.34	0.242	22.08	$1.38x10^{-17a}$
SERPINB2	Serpin peptidase inhibitor, clade b (ovalbumin), member 2	-1.06	0.802	21.55	$8.28x10^{-21a}$
QRFPR	Pyroglutamylated rfamide peptide receptor	-2.30	0.003	21.09	$1.94x10^{-10a}$
TGM3	Transglutaminase 3	-1.59	0.039	20.25	$2.65x10^{-13a}$
CT45A1	Cancer/testis antigen family 45, member a1	1.74	0.302	20.10	$5.63x10^{-7a}$
CT45A4	Cancer/testis antigen family 45, member a4	3.55	0.079	19.29	$1.5x10^{-4a}$

[a]P<0.001. AD, lung adenocarcinoma; SC, squamous cell carcinoma; FC, fold change.

and alanine-glyoxylate aminotransferase 2-like 1 (*AGXT2L1*; AD, FC=66.16; SC, FC=-1.89). The *LIN28A* gene is a cell cycle regulator, the role of which has been identified in a number of human cancers (18,19), but not in NSCLC. AD also demonstrated ~15-fold upregulation in the mucin (*MUC*) family of genes, which may be associated with the secretory nature of the tumor. The top downregulated genes specific to AD included adenylate cyclase 8 (AD, FC=-11.65; SC, FC=2.13), sclerostin domain-containing 1 (*SOSTDC1*; AD, FC=-10.96; SC, FC=-1.01), solute carrier organic anion transporter family member 1A2 (AD, FC=-10.09; SC, FC=-1.29), cholinergic receptor nicotinic α2 subunit (AD, FC=-10.08; SC, FC=-1.43) and odontogenic ameloblast-associated (*ODAM*; AD, FC=-9.92; SC, FC=3.22).

Genes differentially expressed in SC. A total of 146 genes were highly upregulated in SC (FC_{SC}>4; P_{SC}<0.001) with no significant upregulation in AD (Table I). In addition, 103 genes were significantly downregulated (FC_{SC}<-4; P_{SC}<0.001) in SC with no downregulation in AD (Table II). The top upregulated genes unique to SC were amelotin (*AMTN*; SC, FC=618.16; AD, FC=1.70), alcohol dehydrogenase 7 (*ADH7*; SC, FC=57.12; AD, FC=1.52), sclerostin (*SOST*; SC, FC=54.92; AD, FC=-1.81), claudin 22 (*CLDN22*; SC, FC=54.24; AD, FC=2.46) and SRY-box 10 (SC, FC=39.80; AD, FC=1.76). In addition, early-stage SC exhibited unique upregulation of several members of the cancer/testis antigen (*CTA*) family of genes. The top downregulated genes specific to SC were myosin heavy chain 1 (SC, FC=-16.35; AD, FC=-1.33), progastricsin (SC, FC=-16.06; AD, FC=3.89), chitinase acidic (SC, FC-15.74; AD, FC=1.25), surfactant-associated 2 (SC, FC=-14.51; AD, FC=1.15) and apolipoprotein H (SC, FC=-13.95; AD, FC=1.82).

Analysis of molecular pathways in AD. The IPA tool was used to generate an interaction network for genes specifically differentially regulated in AD and SC, based on known interactions (Fig. 2). The genes uniquely dysregulated in AD were enriched in a number of molecular and cellular functions, including 'molecular transport', 'cell-to-cell signaling and interaction', 'amino acid metabolism' and 'cellular growth and proliferation'. A number of the dysregulated genes specific to AD were also involved in the canonical farnesoid X receptor (*NR1H4*)/retinoid X receptor (RXR) activation and liver X receptor (LXR)/RXR activation pathways. The roles of these regulators in NSCLCs have not been previously reported. A number of upstream regulators of these genes were identified, including hepatocyte nuclear factor 4 α (*HNF4A*), which regulated 26 genes, *HNF1A*, which regulates 22 genes, transcription activator BRG1 (*SMARCA4*), which regulates 14 genes, and Forkhead Box A2 (*FOXA2*), which regulated 10 genes (Table III). The *HNF* family of genes and *FOXA2* have been independently associated with AD as positive and negative regulators of growth, respectively (20,21).

Analysis of molecular pathways in SC. The genes unique to early-stage SC were enriched in 'xenobiotic metabolism', 'lipid metabolism', 'vitamin and mineral metabolism', 'drug metabolism' and 'free radical scavenging'. These results suggested that impaired lipid metabolism is specific to SC.

Table II. Downregulated genes in AD and SC.

A, Genes downregulated specifically in AD

Gene	Gene name	AD FC	AD P-value	SC FC	SC P-value
ADCY8	Adenylate cyclase 8 (brain)	-11.65	$8.60x10^{-24a}$	2.13	0.073
SOSTDC1	Sclerostin domain containing 1	-10.96	$2.73x10^{-39a}$	-1.01	0.937
SLCO1A2	Solute carrier organic anion transporter family, member	-10.09	$1.71x10^{-26a}$	-1.29	0.146
CHRNA2	Cholinergic receptor, nicotinic, α 2 (neuronal)	-10.08	$1.20x10^{-43a}$	-1.43	0.292
ODAM	Odontogenic, ameloblast associated	-9.92	$2.10x10^{-33a}$	3.22	0.009
KRT79	Keratin 79	-8.53	$3.65x10^{-33a}$	-1.06	0.788
SYN2	Synapsin ii	-7.58	$5.50x10^{-37a}$	-1.05	0.790
S100A12	S100 calcium binding protein a12	-6.92	$5.97x10^{-45a}$	-1.26	0.310
TGM1	Transglutaminase 1	-6.87	$1.19x10^{-78a}$	2.44	<0.001[a]
ANXA8L2	Annexin a8-like 2	-6.84	$2.81x10^{-29a}$	1.78	<0.001[a]
SLITRK2	Slit and ntrk-like family, member 2	-6.63	$3.63x10^{-36a}$	-1.09	0.730
DCC	Deleted in colorectal carcinoma	-6.26	$1.78x10^{-40a}$	1.10	0.773
FGFBP2	Fibroblast growth factor binding protein 2	-5.78	$1.31x10^{-24a}$	5.56	<0.001[a]
VIT	Vitrin	-5.56	$3.27x10^{-28a}$	5.59	<0.001[a]
LPPR5	Lipid phosphate phosphatase-related protein type 5	-5.55	$9.97x10^{-19a}$	-1.34	0.311
VWC2	Von willebrand factor c domain containing 2	-4.62	$4.26x10^{-31a}$	2.15	0.008
NOS1	Nitric oxide synthase 1 (neuronal)	-4.50	$1.75x10^{-11a}$	1.11	0.769
HSPB3	Heat shock 27 kda protein 3	-4.47	$3.32x10^{-26a}$	1.73	0.021
SEMA6D	Semaphoring 6D	-4.28	$1.70x10^{-33a}$	-1.14	0.404
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	-4.21	$1.10x10^{-18a}$	9.48	<0.001[a]
SLC27A6	Solute carrier family 27 (fatty acid transporter), member 6	-4.12	$4.37x10^{-10a}$	-1.34	0.297
CHRNA4	Cholinergic receptor, nicotinic, α 4	-4.11	$5.82x10^{-16a}$	2.21	0.013
EDN3	Endothelin 3	-4.09	$3.04x10^{-9a}$	3.96	0.014
NDRG4	Ndrg family member 4	-4.08	$6.96x10^{-34a}$	1.73	0.002
KRT4	Keratin 4	-4.03	$2.93x10^{-11a}$	2.55	0.006
FEZ1	Fasciculation and elongation protein zeta 1 (zygin i)	-4.02	$3.18x10^{-77a}$	-1.19	0.141
ANXA8	Annexin a8	-4.01	$1.57x10^{-12a}$	3.94	<0.001[a]

B, Genes downregulated specifically in SC

Gene	Gene name	AD FC	AD P-value	SC FC	SC P-value
MYH1	Myosin, heavy chain 1, skeletal muscle, adult	-1.33	0.336	-16.35	$8.37x10^{-21a}$
PGC	Progastricsin (pepsinogen c)	3.89	<0.001[a]	-16.06	$1.18x10^{-8a}$
CHIA	Chitinase, acidic	1.25	0.420	-15.74	$5.48x10^{-7a}$
SFTA2	Surfactant associated 2	1.15	0.361	-14.51	$4.11x10^{-18a}$
APOH	Apolipoprotein h (β-2-glycoprotein i)	1.82	0.035	-13.95	$4.11x10^{-11a}$
CAPN9	Calpain 9	-1.22	0.316	-12.42	$1.05x10^{-17a}$
DPCR1	Diffuse panbronchiolitis critical region 1	4.44	<0.001[a]	-12.26	$4.59x10^{-13a}$
FOLR1	Folate receptor 1 (adult)	-1.33	0.076	-11.98	$9.39x10^{-11a}$
SFTA3	Surfactant-associated 3	-1.20	0.184	-11.69	$3.92x10^{-21a}$
C16orf89	Chromosome 16 open reading frame 89	1.19	0.394	-11.54	$8.58x10^{-14a}$
HNF1B	Hnf1 homeobox b	1.23	0.114	-11.46	$1.86x10^{-12a}$
SLC10A2	Solute carrier family 10 member 2	1.95	0.158	-10.75	$4.10x10^{-13a}$
NAPSA	Napsin a aspartic peptidase	-1.15	0.382	-10.39	$2.69x10^{-15a}$
CYP2B7P1	Cytochrome p450, family 2, subfamily b, polypeptide 7 pseudogene 1	1.23	0.316	-9.97	$4.54x10^{-5a}$

Table II. Continued.

Gene	Gene name	AD FC	AD P-value	SC FC	SC P-value
MIA2	Melanoma inhibitory activity 2	-1.05	0.783	-9.17	$3.19x10^{-18a}$
CCL14.CCL15	C-C motif chemokine 14	-1.91	0.053	-9.13	$1.13x10^{-21a}$
C4BPA	Complement component 4 binding protein, α	-1.38	0.075	-8.55	$6.23x10^{-12a}$
TDRD10	Tudor domain containing 10	-1.01	0.931	-8.50	$1.96x10^{-20a}$
AQP7	Aquaporin 7	2.16	<0.001[a]	-8.20	$8.39x10^{-6a}$
FMO5	Flavin containing monooxygenase 5	1.54	0.008	-7.99	$2.40x10^{-17a}$
NKX2.1	Homeobox protein Nkx-2.1	1.26	0.067	-7.72	$6.23x10^{-12a}$
SLC26A9	Solute carrier family 26, member 9	1.27	0.249	-7.64	$1.99x10^{-7a}$
SCGB3A1	Secretoglobin, family 3a, member 1	1.77	0.062	-7.60	$3.31x10^{-7a}$

[a]P<0.001. AD, lung adenocarcinoma; SC, squamous cell carcinoma; FC, fold-change.

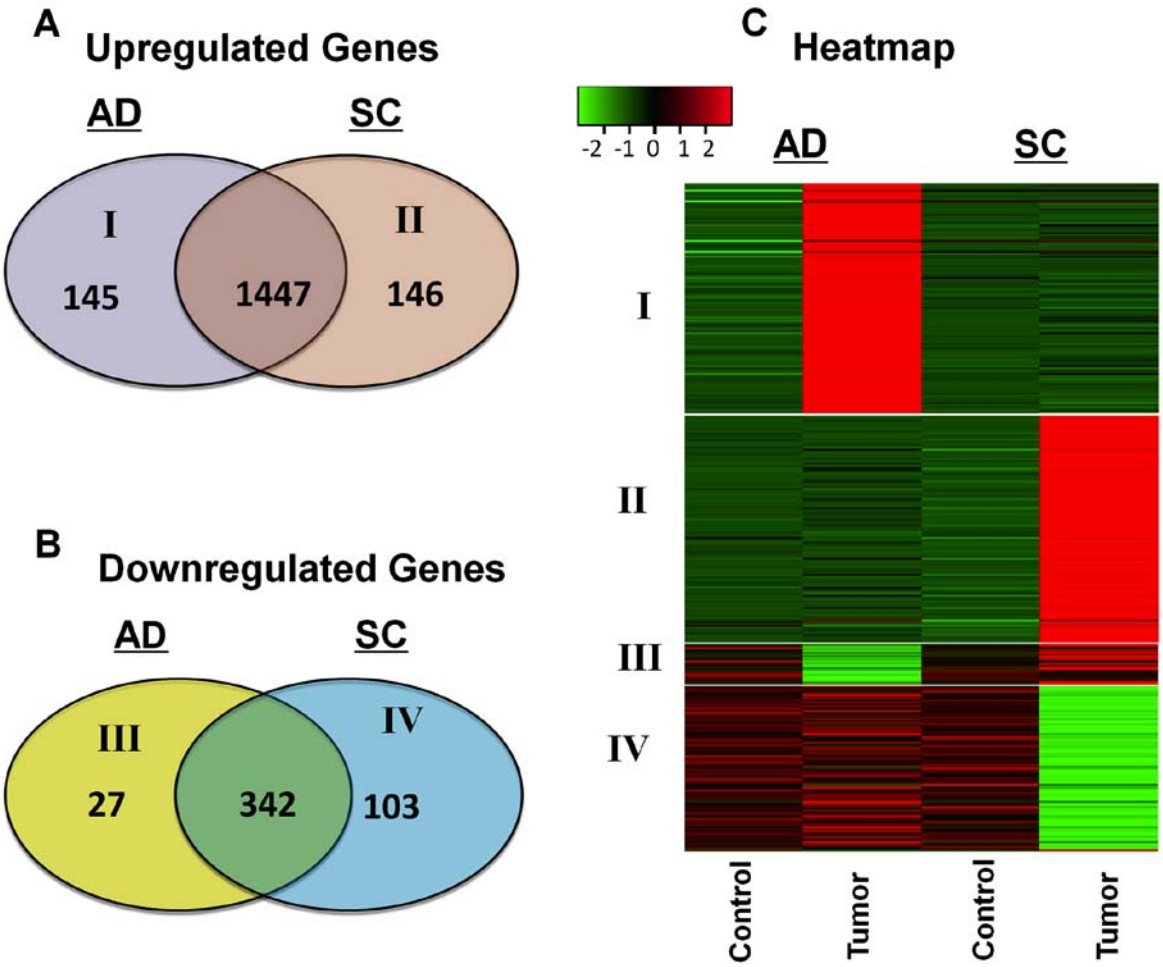

Figure 1. Differential gene expression in AD and SC. (A and B) Venn diagrams of the total number of genes commonly and uniquely (A) upregulated and (B) downregulated in the two cancer types. (C) Heat map demonstrating the clustering of unique gene sets of each subtype into four distinct groups. Gene expression magnitude is represented by the color ranging from high expression (red) to low expression (green). Groups I and II represent genes upregulated specifically in AD and SC, respectively while groups III and V represent genes downregulated specifically in AD and SC, respectively. AD, lung adenocarcinoma; SC, lung squamous cell carcinoma.

The canonical pathways 'LPS/IL-1 mediated inhibition of RXR function', 'xenobiotic metabolism signaling' and 'aryl hydrocarbon receptor signaling' were among those specifically dysregulated in SC. Peroxisome proliferator-activated receptor-γ (*PPARG*), which regulates 15 genes, *c-Jun*, which regulates 15 genes, and *RXR* α (*RXRA*), which regulates 14 genes, were among the upstream regulators of the differentially regulated genes (Table IV).

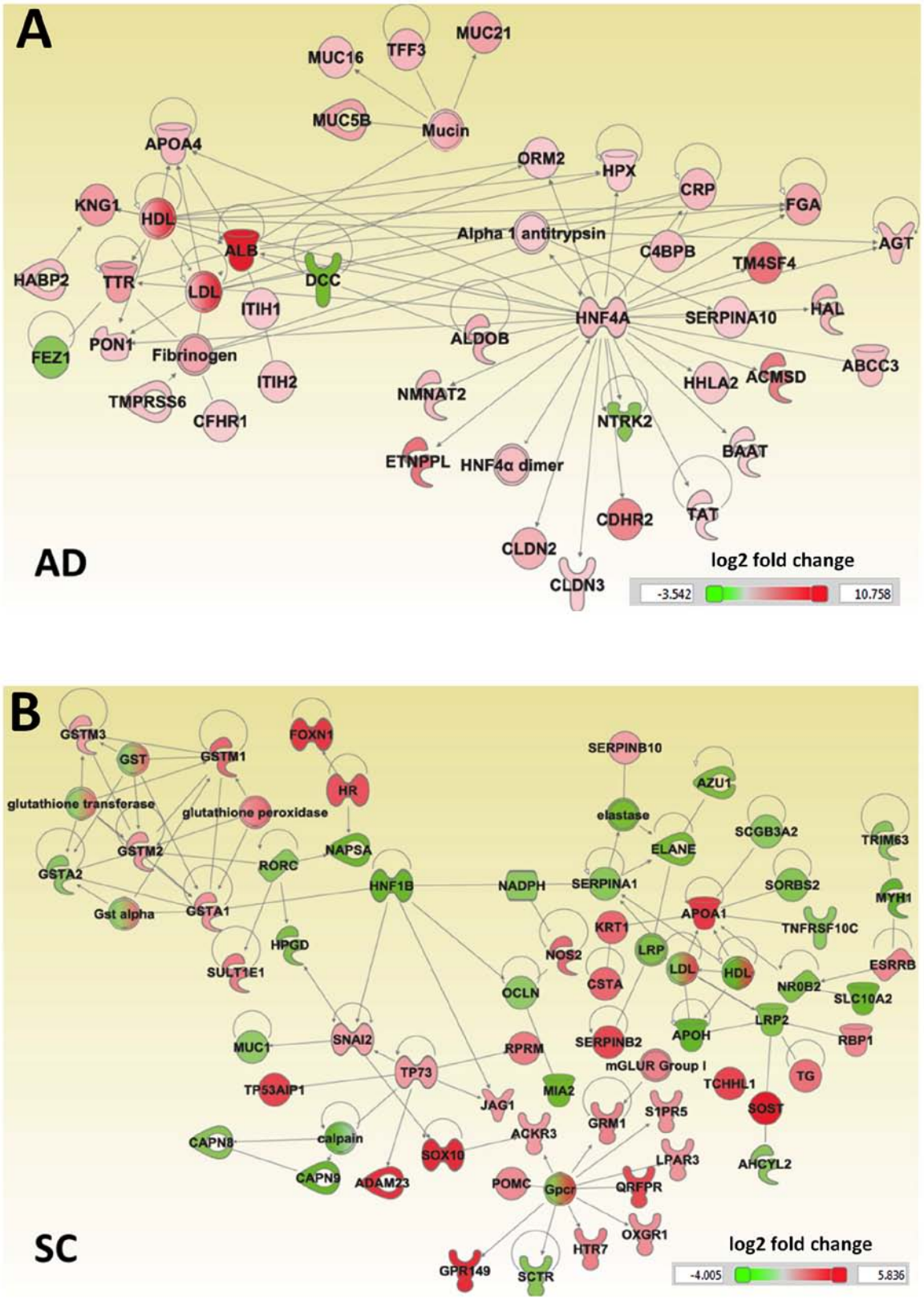

Figure 2. Interaction networks of genes uniquely altered in AD and SC. (A and B) Ingenuity Pathway Analysis tool was used to generate interaction networks using (A) AD- and (B) SC-specific genes. Each gene is represented as a node, and edges represent interactions between nodes. Red nodes indicate upregulated genes, and green nodes indicate downregulated genes. AD, lung adenocarcinoma; SC, lung squamous cell carcinoma.

Discussion

Lung carcinomas account for >25% of cancer-associated mortalities worldwide, and the majority of primary lung cancers are NSCLC histological subtypes, including AD and SC (3). Personalized treatment for these cancers requires a complete and detailed understanding of the distinct molecular mechanisms that contribute to tumorigenesis, especially in

Table III. Functional annotation terms of genes enriched in lung adenocarcinoma.

Gene Ontology term	Count	P-value
Molecular and cellular functions		
Small molecule biochemistry	50	3.0×10^{-5}
Molecular transport	43	1.6×10^{-5}
Cell-to-cell signaling and interaction	42	2.1×10^{-5}
Amino acid metabolism	18	3.0×10^{-5}
Cellular growth and proliferation	16	2.1×10^{-5}
Canonical pathways		
FXR/RXR activation	13	1.1×10^{-11}
LXR/RXR activation	9	3.2×10^{-7}
Acute phase response signaling	9	7.3×10^{-6}
eNOS signaling	6	1.9×10^{-3}
Coagulation system	3	2.2×10^{-3}
Upstream regulators		
Hepatocyte nuclear factor 4-α	26	7.7×10^{-3}
Hepatocyte nuclear factor 1-α	22	2.6×10^{-12}
Transcription activator BRG1	14	2.0×10^{-4}
Forkhead Box protein A2	10	1.9×10^{-6}
Peroxisome proliferator-activated receptor α	10	2.1×10^{-3}
LIM/homeobox protein Lhx1	8	1.2×10^{-6}
Forkhead Box protein A1	8	1.8×10^{-5}
Bile acid receptor	7	1.6×10^{-4}
PR domain zinc finger protein 1	7	1.6×10^{-3}
Myoblast determination protein 1	6	2.6×10^{-3}
Mothers against decapentaplegic homolog 3	6	7.4×10^{-3}

Table IV. Functional annotation terms enriched in lung squamous cell carcinoma.

Gene Ontology term	Count	P-value
Molecular and cellular functions		
Small molecule biochemistry	58	7.9×10^{-8}
Lipid metabolism	45	7.9×10^{-8}
Vitamin and mineral metabolism	26	7.9×10^{-8}
Drug metabolism	15	3.8×10^{-6}
Free radical scavenging	10	1.1×10^{-5}
Canonical pathways		
LPS/IL-1 mediated inhibition of RXR function	14	4.6×10^{-8}
Xenobiotic metabolism signaling	12	3.5×10^{-5}
Aryl hydrocarbon receptor signaling	10	1.6×10^{-6}
PXR/RXR activation	6	5.8×10^{-5}
Glutathione-mediated detoxification	5	5.0×10^{-5}
Upstream regulators		
Peroxisome proliferator-activated receptor γ	15	5.8×10^{-5}
Transcription factor AP-1	15	5.4×10^{-5}
Retinoic acid receptor RXR-α	14	5.0×10^{-7}
Estrogen receptor β	13	7.2×10^{-5}
Tumor protein 63	13	1.4×10^{-4}
Retinoic acid receptor α	12	8.9×10^{-5}
Histone deacetylase 1	11	5.0×10^{-5}
Homeobox protein Nkx-2.1	10	1.4×10^{-5}
Zinc finger protein GLI2	7	7.9×10^{-5}
LIM/homeobox protein Lhx1	7	9.0×10^{-5}

early stages when survival rates are >90%. In the present study, a total of 172 genes with differential expression (145 upregulated and 27 downregulated) specific to AD, and another 249 genes (146 upregulated and 103 downregulated) specific to SC were identified.

The present study demonstrated that early-stage AD exhibited a 150-fold upregulation of the oncogene *LIN28A*, which is involved in cell cycle progression through the regulation of cyclin-dependent kinase 2 in lung, breast, ovarian, colon, liver and pancreatic cancer (22). In addition, *LIN28* has been demonstrated to confer resistance to radiotherapy in lung carcinoma cell lines (23) and has been explored for its potential role in breast cancer therapy (24). The results of the present study revealed that the activity of this oncogene was unique to the AD subtype of NSCLC, whereas no significant changes in *LIN28* expression levels were observed in SC. These results suggested that *LIN28A* may be a novel therapeutic target for AD. The palate, lung and nasal epithelium carcinoma-associated gene, which has a documented association with respiratory tumors with a glandular phenotype (25), was upregulated 38-fold in AD in the present study. Members of the mucin family of genes, mucin 21 cell surface-associated and mucin 5b oligomeric mucus/gel-forming, were identified to be upregulated ~15-fold in AD, and are likely to be involved in the excessive secretion of mucus by neoplastic cells in AD (26). Mucin peptides incorporated into liposomal vaccines are associated with extended survival times in patients with lung cancer (27). Downregulation of several tumor suppressor genes including *SOSTDC1*, *ODAM*, deleted in colorectal carcinoma, fasciculation and elongation proteins ζ 1 and annexin A8 was observed in AD, but not in SC. These genes are associated with a variety of cancers, including lung, breast, colon and prostate cancer (28-31).

In the present study, early-stage SC exhibited specific upregulation of *CTA* family 45 members A1-4. Auto-antibodies against the genes of this family have been demonstrated to serve as biomarkers for NSCLC with low sensitivity and high specificity (32), and a RNAseq catalog of 90 cancer testis antigens were established by Djureinovic *et al* (33). The results of the present study also demonstrated that the expression of another member of the *CTA* family, X antigen family member 2, was downregulated in SC; this gene has previously been identified as a tumor suppressor in metastatic melanoma and Ewing sarcoma (34). In addition, SC exhibited downregulation of several tumor suppressor genes, including melanoma inhibitory activity 2, which is involved in hepatocellular carcinoma (30), and secretoglobin family 3a member 1, which serves a role in testicular germ cell tumors (35,36).

In the present study, 'cellular function' and 'lipid metabolism' were associated with the genes dysregulated specifically in early-stage SC. Alterations in lipid metabolism have been previously implicated in human cancers, particularly oral squamous cell carcinoma, in which increased lipid metabolism is associated with invasiveness (37). Previous studies have reported that impaired lipid metabolism in NSCLC results in the loss of malignant potential (38-40). The results of the present study suggested that abnormal lipid metabolism may be specific to SC. AD exhibited significant upregulation of *LIPF*; however, no evidence of dysregulated lipid metabolism in AD was observed at a functional level. By contrast, AD exhibited alterations in 'molecular transport', 'cell-to-cell signaling and interaction' and 'amino acid metabolism'.

Only SC exhibited strong enrichment of the 'drug metabolism' cellular function in the present study. Previous studies have suggested that high activity levels of cytochrome P450 isotypes, particularly cytochrome P450 family 1 (*CYP1*) subfamily B member 1 (*CYP1B1*), serve a role in carcinogenesis and drug resistance in human cancers, including NSCLC, and may serve as therapeutic targets or prognostic indicators (41,42). In addition, 5,7-dimethoxyflavone and resveratrol have been used to inhibit CYP1 family protein expression Hep G2 human hepatoma and MCF-10a non-tumorigenic human mammary epithelial cell lines, respectively (43,44). The results of the present study revealed that the 'PXR/RXR', 'xenobiotic metabolism signaling', 'aryl hydrocarbon receptor signaling' and 'glutathione-mediated detoxification' canonical pathways were also altered in early-stage SC. Previous studies have demonstrated that PXR serves a role in xenobiotic metabolism in human malignancies, such as colon, breast and gynecologic cancers (45). The involvement of *PXR* in NSCLC has not been previously reported. The aryl hydrocarbon transcription factor is also involved in cytochrome metabolism and activates the CYP1B1, CYP1A1 and CYP1A2 isotypes (46). The results of the present study suggest that the dysregulation of genes associated with drug metabolism may be specific to SC, and that the role of these catabolic enzymes may be evident in early-stage cancer. These results also identify several potential mechanisms of chemotherapy resistance in SC.

The canonical pathways 'FXR/RXR activation' and 'LXR/RXR activation' were altered in early-stage AD, and the *NR1H4* gene was identified as an upstream regulator of AD in the present study. Previous studies of *FXR/RXR* in human cancers have demonstrated that it is activated in breast and esophageal cancers, but can be downregulated in hepatobiliary cancers (47-49). Loss of *LXR/RXR* is involved in the growth and progression of prostatic carcinomas, and LXR agonists have emerged as a novel therapy for prostate cancer (50). The canonical pathways 'LPS/IL-1 mediated inhibition of RXR function' and 'PXR/RXR activation' were altered in SC in the present study. *PXR/RXR* is involved in the metabolism of xenobiotics and has been demonstrated to be involved in multiple types of human cancer, including colon, breast and gynecological cancers (45). Previous studies have demonstrated the use of retinoid receptor expression as a prognostic indicator in stage I NSCLC, but the role of specific retinoid receptors has not been explored (51,52). The involvement of *FXR/RXR*, *LXR/RXR* and *PXR/RXR* in NSCLC subtypes is a novel finding of the present study.

The results of the present study demonstrated that *HNF4A* and *HNF1A* were upstream regulators of the genes specifically dysregulated in AD. A previous study has identified the use of *HNF4A* as a biomarker for AD, and another study identified *HNF4G* to be involved in the AKT signaling pathway in lung cancer (20,53). The present results suggest that *HNF* may be an upstream driver of tumorigenesis. In addition, *SMARCA4* was identified as another upstream regulator in AD. Upregulation of this gene in AD is associated with poor prognosis and a poor response to platinum-based chemotherapy (54,55). Analysis of the upstream regulators in the present study also identified *FOXA2* and *FOXA1* as specific regulators of AD; the *FOXA2* gene product has been demonstrated to prevent lung tumor growth and metastasis by preventing epithelial-mesenchymal transition (21).

In the present study *PPARG*, *c-JUN* and *RXRA* were the most significant upstream regulators of the genes specifically differentially regulated in early-stage SC. The role of *PPARG* in lung cancer is unclear, although *PPARG* has been studied in the context of pulmonary fibrosis, where it was demonstrated to repress myofibroblast differentiation (56). However, upregulation of *PPARG* repressed tumor growth in pancreatic and colorectal cancer (57,58), and *PPARG* inhibitors have been used to induce anti-estrogen susceptibility in mammary tumors (59). The role of the *c-JUN* regulator in NSCLC may be related to the dysregulation of retinoid signaling by the inhibition of *RXRA*, which is another upstream regulator of the genes altered in SC (60). Based on prior studies (61,62), *c-JUN* may be activated and *RXRA* may be consequently inhibited in SC. Anti-tumor activity has been achieved through c-JUN protein inhibition using a bisphenazine anticancer drug (63). Several other upstream regulators were identified in the present study, such as estrogen receptor 2 and tumor protein p63, which have been previously demonstrated to serve roles in NSCLC (64,65).

The major limitation of the present study was the lack of experimental validation of the findings using *in vivo* or *in vitro* experiments. However, to minimize false positives, a very stringent cut-off was used to select the DEGs. In addition, the large sample set provided high statistical power to discover the differences with high confidence.

In conclusion, the present study revealed early-stage differences in the gene expression profiles of AD and SC. Unique sets of genes altered in each subtype were identified; for example, *ALB*, *LIN28A*, *LIPF*, *TM4SF4*, *AGXT2L1* and *ACMSD* genes were upregulated >50-fold in AD, but were not significantly upregulated in SD. Similarly, *AMTN*, *ADH7*, *SOST* and *CLDN22* were upregulated >50-fold in SC, but not in AD. Several *CTA* family genes were highly upregulated in SC, but not in AD, whereas several mucins were upregulated only in AD. In addition, 'lipid metabolism' and 'drug metabolism' pathways were associated with genes dysregulated specifically in SC, whereas 'molecular transport' and 'cellular growth and proliferation' were significantly enriched only in AD. The results of the present study provided gene expression alterations specific to each subtype, which may help to identify the molecular mechanisms underlying the pathogenesis of these subtypes. These findings also provide targets for future studies investigating novel diagnostic methods and personalized therapeutic approaches for AD and SC.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Institutional Start-Up Package to AS from the Medical College of Georgia at Augusta University, Augusta, GA, USA.

Availability of data and materials

The datasets analyzed for this study are available from The Cancer Genome Atlas (https://cancergenome.nih.gov).

Authors' contributions

NV, JY and AS wrote the manuscript and created the figures and tables. TJL and SKK performed data analysis. NV, JY, SS, AS and NP contributed to the data interpretation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study utilizes a publicly available data set from TCGA (https://cancergenome.nih.gov/abouttcga) and was granted an exemption from requiring ethics approval by the Institutional Review Boards at Augusta University Augusta GA USA.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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