Identification of target genes of cediranib in alveolar soft part sarcoma using a gene microarray

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Abstract. The aim of the present study was to identify the target genes of cediranib and the associated signaling pathways in alveolar soft part sarcoma (ASPS). A microarray dataset (GSE32569) was obtained from the Gene Expression Omnibus database. The R software package was used for data normalization and screening of differentially expressed genes (DEGs). The Database for Annotation, Visualization and Integrated Discovery was used to perform Gene Ontology analysis. Gene Set Enrichment Analysis was performed to obtain the up- and downregulated pathways in ASPS. The Distant Regulatory Elements of co-regulated genes database was used to identify the transcription factors (TFs) that were enriched in the signaling pathways. A protein-protein interaction (PPI) network was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins database and was visualized using Cytoscape software. A total of 71 DEGs, including 59 upregulated genes and 12 downregulated genes, were identified. Gene sets associated with ASPS were enriched primarily in four signaling pathways: The phenylalanine metabolism pathway, the mitogen-activated protein kinase (MAPK) signaling pathway, the taste transduction pathway and the intestinal immune network for the production of immunoglobulin A. Furthermore, 107 TFs were identified to be enriched in the MAPK signaling pathway. Certain genes, including those coding for Fms-like tyrosine kinase 1, kinase insert domain receptor, E-selectin and platelet-derived growth factor receptor D, that were associated with other genes in the PPI network, were identified. The present study identified certain potential target genes and the associated signaling pathways of cediranib action in ASPS, which may be helpful in understanding the efficacy of cediranib and the development of new targets for cediranib.

Introduction

Alveolar soft part sarcoma (ASPS) is a rare type of highly vascular tumor, which accounts for between 0.5 and 1.0% of all soft tissue sarcomas (1). It predominantly affects adolescents and young adults between 15 and 35 years of age, and most commonly occurs in the head and neck (2,3). ASPS consists of numerous epithelioid tumor cells which are arranged in a pseudoalveolar growth pattern or nests (4). ASPS is an indolent painless disease that exhibits increased metastatic rates by the time of diagnosis (5). The marked fatality rate, and poor 5-year and median survival rate (6) observed in patients with ASPS make it a high-risk disease. The pathogenesis of ASPS remains unclear and ASPS is resistant to the standard cytotoxic chemotherapy regimens that are typically used in the treatment of soft tissue sarcomas (7), therefore, complete excision of the primary tumor is the therapy of choice (8). Radiation therapy may accompany limited surgery to provide marked palliation for patients (9); however, radiation therapy does not provide any survival advantage to patients (8). Therefore, there is no effective systemic treatment for patients with unresectable metastatic disease.

Previously, research has been conducted on molecularly targeted treatments for curing systemic cancer (10). ASPS is characterized by a tumor-specific translocation of der(17)

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t(X;17)(p11;q25) (11). The translocation may lead to fusion of the transcription factor 3 (*TFE3*) gene at Xp11 with the alveolar soft part sarcoma locus (*ASPL*) gene at 17q25, which codes for an ASPL-TFE3 fusion protein that appears to act as an aberrant transcription factor, inducing unregulated transcription of TFE3-regulated genes (11). As a transcriptional target of ASPL-TFE3, c-Met receptor (MET) may contribute to the malignant progression in ASPS (12). In addition, tyrosine kinase inhibitors (TKIs) may be used due to the marked levels of activated receptor tyrosine kinases (RTKs), including platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor and MET family members in ASPS (13). This alteration in medical management has led to the identification of novel pharmaceutical targets.

Cediranib (AZD-2171) is an orally bioavailable potent small-molecule inhibitor which consists of three vascular endothelial growth factor receptor (VEGFR) tyrosine kinases: VEGFR-1, -2 and -3 (14). It is able to mediate angiogenesis and lymphangiogenesis by inhibiting the development of new blood vessels, and has recently been identified to exhibit antitumor activity (15). Cediranib has been safely applied in clinical practice, whether as a single agent or in combination with other agents, in patients with progressed cancer (16-18). Kummar *et al* (15) reported that cediranib exhibited marked single-agent activity when used to treat metastatic ASPS. Therefore, in order to elucidate the underlying molecular mechanism for the treatment of ASPS with cediranib, expression profiles were evaluated utilizing a focused ASPS tissue microarray which was downloaded from the Gene Expression Omnibus (GEO) and evaluated using Gene Set Enrichment Analysis (GSEA). Using this unique bioresource, the differentially expressed genes (DEGs), signaling pathways and protein-protein interaction (PPI) networks that are involved in the development of ASPS were identified.

Materials and methods

Microarray data. Gene expression profile GSE32569 was downloaded from the GEO database (www.ncbi.nlm.nih.gov/geo). A total of 6 samples that were treated with cediranib (case group) for between 3 and 5 days, and 6 samples without any treatment (control group) were included in the dataset. The dataset was based on the GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Inc., Santa Clara, CA, USA; www.affymetrix.com/catalog/131455/AFFY/Human+Genome+U133+Plus+2.0+Array).

Data normalization and screening of DEGs. The Affy package (version 1.52.0; bioconductor.org/packages/release/bioc/html/affy.html) in R software (version 3.1.3; www.r-project.org/) was used for the normalization of the raw CEL data. DEGs in case groups compared with control groups were screened using the limma package (version 3.30.7; bioconductor.org/packages/release/bioc/html/limma.html) in R software with the thresholds of P<0.05 and |log(fold change)|>1.

Functional enrichment analysis. GO analysis was conducted based on the Database for Annotation, Visualization and Integrated Discovery (DAVID; david.abcc.ncifcrf.gov). Functionally enriched terms with P<0.05 were considered to be statistically significant. GSEA is a powerful microarray data analysis approach for functional enrichment of gene sets (19). It is a computational method that is able to evaluate microarray data at the level of gene sets, which contains predefined biological knowledge from published information about biochemical pathways or coexpression in previous experiments (20). GSEA is especially useful when gene expression alterations in a given microarray data set are minimal or moderate. Due to the relatively small sample size in the present study, GSEA was suitable for analyzing the microarray data to obtain the predominant signaling pathways. The number of genes analyzed in the Kyoto Encyclopedia of Genes and Genomes pathway was between 15 and 500, and P<0.05 was set as the threshold.

The Distant Regulatory Elements of co-regulated genes (DiRE) database (dire.dcode.org/details.php) was used to enrich transcription factors (TFs) in each pathway obtained from GSEA analysis. DiRE is based on the Enhancer Identification method, to determine the chromosomal location and functional characteristics of distant regulatory elements in higher eukaryotic genomes. DiRE was also able to score the association of individual TFs with the biological function shared by the group of input genes.

PPI network of DEGs. In order to achieve an improved understanding of interactions of DEGs, a PPI network was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (www.string-db.org), which is primarily known and used to predict protein interactions. PPIs contain direct and indirect connections derived from four sources, including prior knowledge, high-throughput experiments, genomes and coexpression. The visualization of the PPI network was performed using Cytoscape software (version 3.4.0; www.cytoscape.org). The visualized PPI network was able to intuitively present the organization of the interactions of DEGs. Furthermore, the total number of edges connected to a node (defined as the degree) was calculated using Cytoscape.

Results

Data normalization and screening of DEGs. Microarray data were preprocessed to obtain gene expression profiles. The expression profiling data prior to and following normalization were compared with only limited system deviation existing among samples (Fig. 1A and B). A total of 6 controls were clustered with 6 samples treated with cediranib from the result of the clustering analysis. A total of 71 DEGs were identified between cediranib-treated samples and controls, including 59 upregulated and 12 downregulated genes (Table I). A hierarchical clustering analysis heat-map is presented in Fig. 1C, with red representing downregulated DEGs and blue representing upregulated DEGs.

Functional enrichment analysis. A total of 64 GO terms were identified using DAVID. The 10 most enriched GO terms are presented in Table II. A total of four predominant signaling pathways, including the phenylalanine metabolism pathway,

Table I. A total of 71 differentially expressed genes that were identified between pre-treated and post-treated with cediranib for between 3 and 5 days.

Gene	P-value	Log(fold change)
KCNE3	0.000005	-1.919640
ANGPT2	0.000008	-2.396400
TM4SF18	0.000011	-1.561610
CALCRL	0.000026	-1.376910
NETO2	0.000032	-1.546550
GPR4	0.000052	-1.008890
ESM1	0.000076	-3.129660
TNFRSF4	0.000082	-1.148190
ITGA8	0.000095	-1.693490
FLT1	0.000119	-2.289080
SERPINI1	0.000134	-1.854420
ZEB1	0.000175	-1.179100
SEMA3F	0.000211	-1.178100
KDR	0.000538	-1.245470
GABRD	0.000648	-1.209740
KCNJ2	0.000764	-1.785310
ADAMTS5	0.000787	-1.325420
LOC653602	0.000800	-1.609370
FAM19A5	0.001229	-1.278160
ACKR3	0.001235	-1.718300
FOLH1	0.001352	-2.054950
PLXNA2	0.001447	-1.165320
PLVAP	0.001538	-1.436030
ADAMTS9	0.001662	-1.174250
EFNB2	0.002143	-1.194150
PRDM1	0.002486	-1.176870
RBP7	0.002693	-1.036200
CCL2	0.002933	1.738888
HECW2	0.003132	-1.301010
CXorf36	0.003219	-1.168170
FOLH1B	0.003407	-1.698340
SOX11	0.003442	-1.681560
SELE	0.003804	1.598531
BNIP3	0.004939	1.310759
CDH13	0.004995	-1.376530
LBH	0.005046	-1.112670
RGS5	0.005448	-1.598050
TRIL	0.006434	-1.290740
MECOM	0.006666	-1.006220
C3orf70	0.006700	-1.168890
TAGLN	0.008038	1.182453
P2RY8	0.008253	-1.000790
PNKD	0.009432	1.002066
FAM84A	0.009696	-1.104130
BTNL9	0.010359	-1.134410
APOLD1	0.010814	-1.208060
NPNT	0.010933	-1.444800
IL1RN	0.011180	1.857269
SLC16A14	0.011959	-1.107210
EDNRB	0.014824	-1.235820

Table I. Continued.

Gene	P-value	Log(fold change)
CA4	0.021125	-1.112460
PTP4A3	0.022276	-1.042590
PLCL1	0.023654	-1.168560
STC1	0.024547	-1.287580
CYP26B1	0.024733	1.383835
MIR210HG	0.025097	1.387346
GUCY1A2	0.026047	-1.060530
SPP1	0.026251	1.563289
COL21A1	0.027842	-1.098360
PDGFRA	0.029188	1.513710
S1PR3	0.030017	-1.043710
RGCC	0.031406	-1.129620
AK4	0.031932	1.445851
CA2	0.032294	-1.361530
PDGFD	0.032935	-1.075380
P2RY14	0.036130	-1.002250
MFSD6	0.037175	-1.206520
LOC100288985	0.043962	-1.748150
HEY1	0.044615	-1.228280
CD200	0.045409	-1.060250
FCGR2B	0.046718	1.896813

the mitogen-activated protein kinase (MAPK) signaling pathway, the taste transduction pathway and the intestinal immune network for the production of immunoglobulin A were identified using GSEA analysis (Table III). These four cancer-associated signaling pathways were analyzed using DiRE, and TFs enriched in each pathway were identified. The majority of enriched TFs were components of the MAPK signaling pathway, with 107 identified (Fig. 2).

PPI network of DEGs. Identified DEGs of ASPS were imported into the STRING database to construct a PPI network. As a result, a total of 65 nodes were demonstrated to be involved in network construction and 28 interactive genes were established in the network. The visualization of the PPI network is presented in Fig. 3. Key genes, including Fms-like tyrosine kinase 1 (*FLT1*), kinase insert domain receptor (*KDR*), E-selectin (*SELE*) and PDGFR D (*PDGFD*), possessed degrees of 7, 6, 6 and 3, which were markedly more compared with those of other genes.

Discussion

ASPS is a rare disease that accounts for ~1% of all soft tissue sarcomas (21). The clinical course is relatively indolent, and exhibits a marked frequency of metastasis and poor prognosis (22). ASPS is typically highly vascular, and research has demonstrated that 18 angiogenesis-associated genes were upregulated (23). A number of complementary trials have demonstrated that an anti-angiogenic approach may be the preferred choice in the treatment of ASPS (24,25). Therefore, the anti-angiogenic activity of cediranib and

Table II. Most enriched GO terms for differentially expressed genes.

Category	GO ID	GO name	Number of genes	P-value
CC	GO:0044421	Extracellular region part	14	7.23×10^{-5}
BP	GO:0016477	Cell migration	8	8.32×10^{-5}
CC	GO:0005576	Extracellular region	20	1.50×10^{-4}
BP	GO:0048870	Cell motility	8	1.61×10^{-4}
BP	GO:0051674	Localization of cell	8	1.61×10^{-4}
BP	GO:0001944	Vasculature development	7	3.83×10^{-4}
MF	GO:0005021	Vascular endothelial growth factor receptor activity	3	4.01×10^{-4}
CC	GO:0005887	Integral to plasma membrane	14	6.00×10^{-4}
BP	GO:0042127	Regulation of cell proliferation	11	7.13×10^{-4}
CC	GO:0031226	Intrinsic to plasma membrane	14	7.43×10^{-4}

GO, gene ontology; ID, identifier; CC, cellular component; BP, biological process; MF, molecular function.

Table III. Predominant signaling pathways identified using Gene Set Enrichment Analysis.

Pathway name	ES	NES	Nominal P-value
KEGG_PHENYLALANINE_METABOLISM	0.78	1.22	0.002
KEGG_MAPK_SIGNALING_PATHWAY	0.28	1.31	0.012
KEGG_TASTE_TRANSDUCTION	0.39	1.46	0.028
KEGG_INTESTINAL_IMMUNE_NETWORK_FOR_IGA_PRODUCTION	0.53	1.5	0.038

ES, enrichment score; NES, normalized enrichment score; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; IgA, immunoglobulin A.

other TKIs, including sunitinib (26), make them promising drugs for ASPS. Although cediranib was able to suppress the growth of blood vessels by inhibiting the tyrosine kinase activity, the associated pathways and key genes of the underlying biological processes remain unclear. Therefore, the present study identified four pathways and four key genes associated with the underlying molecular mechanism of ASPS following treatment with cediranib using microarray analysis.

The MAPK signaling pathway containing 107 enriched TFs identified in the present study may be the most important pathway that is affected by cediranib. The MAPK signaling pathway is involved in a variety of cellular functions, including cell proliferation, differentiation and migration (27). The MAPK signaling pathway is a downstream signaling cascade that may be activated by mutated RTKs in cancer cells or kinase activity of oncogenes, resulting in tumorigenesis (28). Interfering with the mutated tyrosine kinase activity or over-expressing oncogenes, or inhibiting several cancer-associated signaling pathways or cancer angiogenesis, ultimately led to tumor shrinkage and cancer cell death (29). The inhibition of the MAPK signaling pathway served a key role in mediating the antitumor effects of TKIs (30). The suppression of tumor growth by the TKI cediranib in the treatment of ASPS may occur through the MAPK signaling pathway. The three other signaling pathways identified exhibited fewer enriched TFs

and demonstrated limited association with ASPS; further validation of these signaling pathways is required.

Key genes were identified in the PPI network including *FLT1*, *KDR*, *SELE* and *PDGFD*. *FLT1* and *KDR* are tyrosine kinases also known as VEGFR-1 and VEGFR-2, and are associated with angiogenesis and vascular proliferation. Angiogenesis is associated with various physiological and pathophysiological processes. The growth of tumors requires abundant blood vessels to provide adequate oxygen. Overexpression of vascular endothelial growth factor (VEGF) may predict cancer recurrence, metastasis and decreased survival (31). However, vascular proliferation and angiogenesis are regulated by VEGF specifically acting on the vascular endothelium via the endothelial cell receptors *FLT1* and *KDR* (32). Keyt *et al* (33) reported that *FLT1* and *KDR* may serve to dimerize RTKs, resulting in endothelial mitogenesis, proliferation, and initiation of angiogenesis and vasculogenesis. Another study demonstrated that overexpression of *FLT1* and *KDR* were associated with local disease recurrence and metastases of colorectal tumor (34). High expression of phosphorylated *KDR* was associated with increased tumor diameter and poor histological differentiation (35). Therefore, cediranib, as a VEGF TKI, was able to block the formation of new blood vessels and exhibit anti-tumor activity in therapy of ASPS (16). *In vitro*, cediranib inhibited VEGF-stimulated proliferation and *KDR* phosphorylation, and inhibits *FLT1*-associated
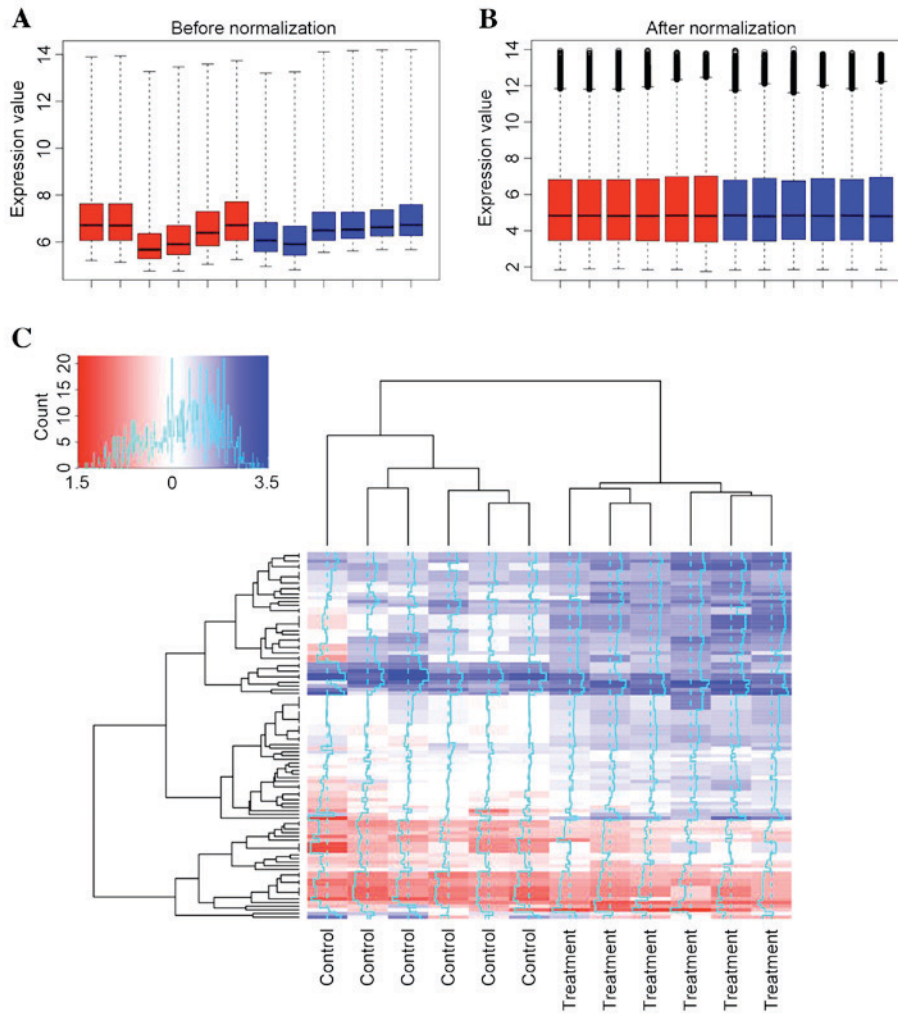
Figure 1. Microarray data normalization and heat-map of DEGs. Microarray data, (A) prior to normalization and (B) following normalization. Red represents pre-treatment with cediranib and blue represents post-treatment with cediranib, for between 3 and 5 days. (C) Hierarchical clustering analysis heat-map of DEGs. Red represents downregulated genes and blue represents upregulated genes. DEG, differentially expressed gene.
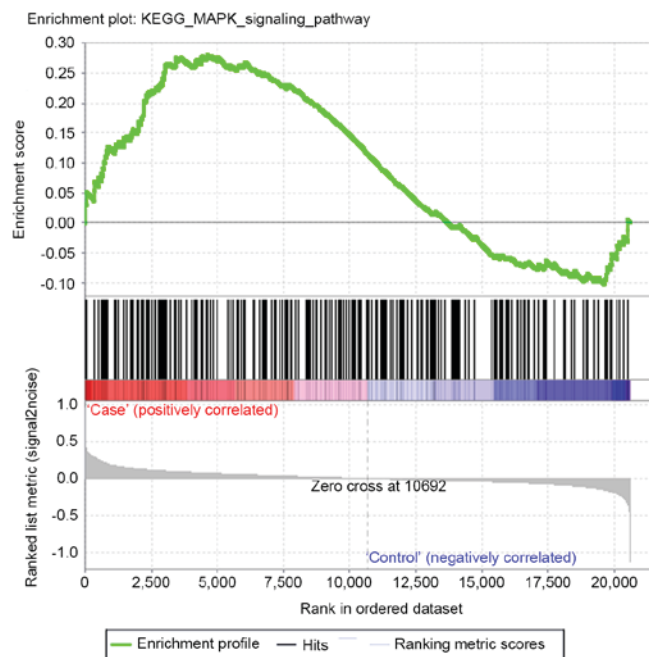

Figure 2. Enrichment scores of the MAPK signaling pathway. 'Case' represents the case groups. 'Control' represents the control groups. KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase.
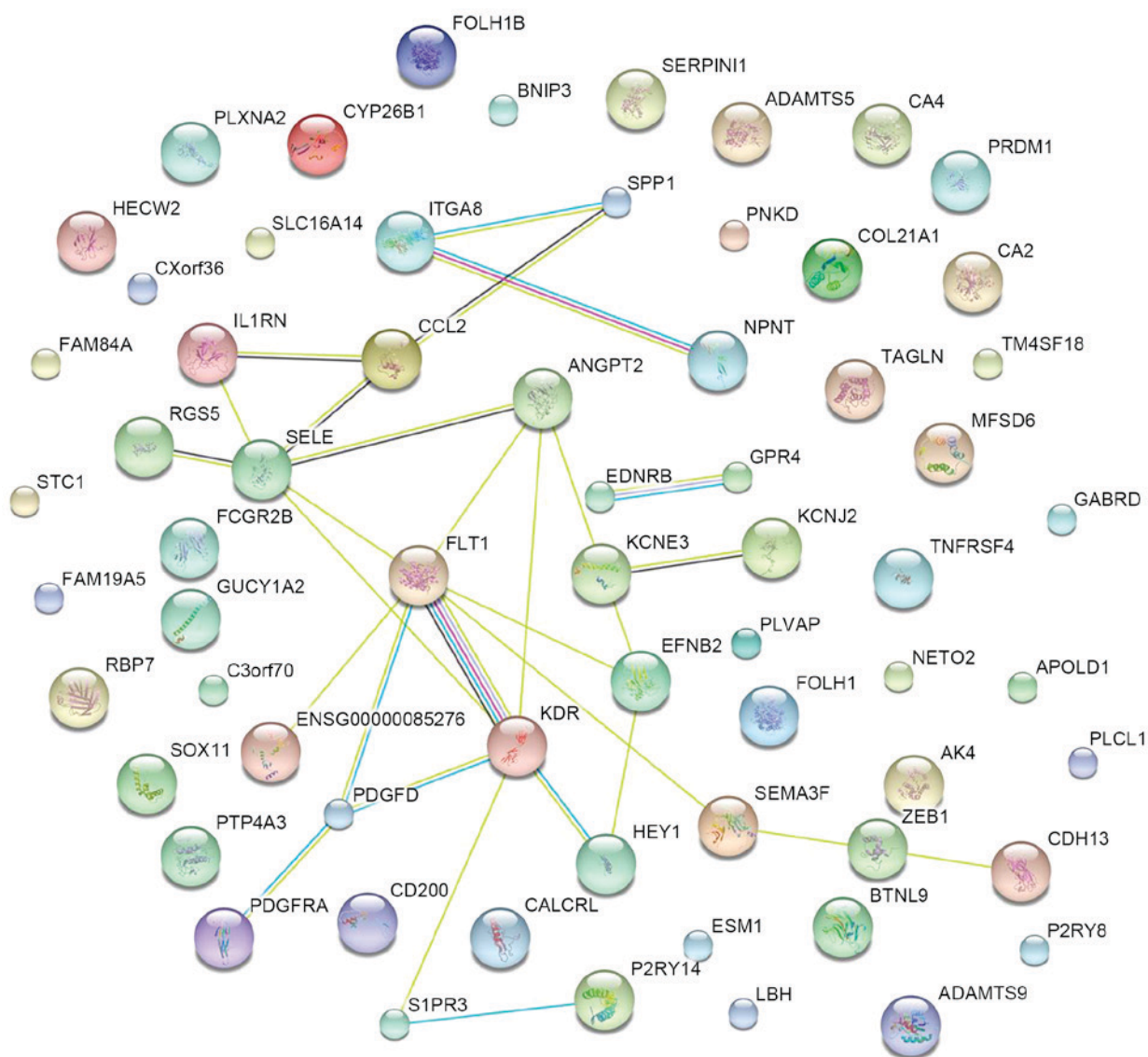
Figure 3. Protein-protein interaction network of DEGs. Spheres represent DEGs and lines represent direct interactions between DEGs. DEG, differentially expressed gene.

kinase (36). *In vivo*, cediranib exhibited broad-spectrum activity in human tumor models to suppress tubule sprouting and inhibit VEGF-induced angiogenesis (37,38).

Cediranib, an inhibitor of *FLT1* and *KDR*, has also been demonstrated to be able suppress platelet-derived growth factor (PDGF) (39). This is because the PDGF receptors (PDGFRs) are structurally and functionally similar to the VEGFRs (33). *PDGFD* belongs to the PDGF family and is a growth factor that is able to regulate a number of cellular processes, including cell proliferation, transformation, invasion and angiogenesis (40). A number of TKIs have been used for targeted anti-cancer therapeutic agents to block PDGFRs. For instance, imatinib (STI-571) effectively inhibited PDGFR, and thus cell growth and potential invasion in human breast cancer cell lines (41). Cediranib reduced intraosseous growth of prostate tumors and exhibited a marked inhibition of tumor-associated bone response in patients with overexpression of *PDGFD* (42). Furthermore, cediranib exhibited marked potency to inhibit the PDGFR-associated kinases PDGFR-α and PDGFR-β, similar to VEGFR tyrosine kinases (43). These

results suggested that inactivation of *PDGFD* or PDGFR is a novel approach to cancer therapy.

Another high degree gene obtained from the PPI network was *SELE*, primarily because it exhibited affinity with high-frequency ASPS metastasis. In humans, *SELE* encodes E-selectin, a member of the selectin family of cell adhesion molecules (44), and is expressed only on endothelial cells and activated by cytokines (45). E-selectin is not stored in cells, and has to be transcribed, translated and transported to the cell surface. The functions of E-selectin are primarily associated with inflammation and cancer metastasis. During inflammation, E-selectin serves an important function in recruiting leukocytes to the site of injury and damaged cells induces the overexpression of E-selectin on endothelial cells of nearby blood vessels (46). As the inflammatory response progresses, chemokines released by injured tissue enter the blood vessels and begin translocating to the tissue (46). Furthermore, E-selectin mediates the adhesion of tumor cells to endothelial cells, by binding to E-selectin ligands on the tumor cells, and E-selectin ligands also serve a role in cancer metastasis (44).

Tumor cells are able to infiltrate the inflammatory system by interacting with selectins. Therefore, this interaction leads to metastatic dissemination of cancer.

GSEA, used in the present study to enrich signaling pathways, has many advantages compared with traditional methods (47,48). GSEA makes it simple to identify pathways and processes for annotation of a large-scale experiment (49). Other tools used pathway or ontological information to analyze gene expression (50,51), whereas, with GSEA, rather than focusing on identifying individual genes between two samples and high scoring genes, researchers are able to focus on more interpretable and more reproducible gene sets. Other features, including promoting the signal-to-noise ratio, detecting minor changes in individual genes, and defining gene subsets, either sensitive or robust, lead to GSEA having wider application (20).

The MAPK signaling pathway and four key genes obtained using GSEA were demonstrated to be associated with the pathogenesis of ASPS. However, a limitation of the present study is that the sample size is limited. This is associated with the low incidence of ASPS. Therefore, these data require further experimental validation. In future studies, the key genes associated with ASPS require investigation using a preclinical model to reveal the underlying molecular mechanisms of cediranib in ASPS treatment and provide novel insight into cediranib as a therapy for ASPS.

Acknowledgements

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