Bioinformatics analysis of key biomarkers and pathways in KSHV infected endothelial cells

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Abstract
Kaposi sarcoma (KS) is an endothelial tumor etiologically related to Kaposi sarcoma herpesvirus (KSHV) infection. The aim of our study was to screen out candidate genes of KSHV infected endothelial cells and to elucidate the underlying molecular mechanisms by bioinformatics methods. Microarray datasets GSE16354 and GSE22522 were downloaded from Gene Expression Omnibus (GEO) database, the differentially expressed genes (DEGs) between endothelial cells and KSHV infected endothelial cells were identified. And then, functional enrichment analyses of gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis were performed. After that, Search Tool for the Retrieval of Interacting Genes (STRING) was used to investigate the potential protein–protein interaction (PPI) network between DEGs, Cytoscape software was used to visualize the interaction network of DEGs and to screen out the hub genes. A total of 113 DEGs and 11 hub genes were identified from the 2 datasets. GO enrichment analysis revealed that most of the DEGs were enriched in regulation of cell proliferation, extracellular region part and sequence-specific DNA binding; KEGG pathway enrichment analysis displayed that DEGs were mostly enriched in cell cycle, Jak-STAT signaling pathway, pathways in cancer, and Insulin signaling pathway. In conclusion, the present study identified a host of DEGs and hub genes in KSHV infected endothelial cells which may serve as potential key biomarkers and therapeutic targets, helping us to have a better understanding of the molecular mechanism of KS.

Abbreviations: ANLN = anillin actin binding protein, ASPM = abnormal spindle microtubule assembly, BECs = blood endothelial cells, BINGO = biological networks gene oncology, BP = biological processes, CC = cellular components, CCNA2 = CyclinA2, CCNB1 = Cyclin B1, CCNB2 = Cyclin B2, CDC20 = Cell Division Cycle 20, CDKN3 = Cyclin Dependent Kinase Inhibitor 3, DEGs = differentially expressed genes, DEPDC1 = DEP domain containing 1, GEO = gene expression omnibus, GO = gene ontology, GSK3B = glycogen synthase kinase-3B, HPV = human papilloma virus, KEGG = Kyoto encyclopedia of genes and genomes, KIF20A = kinesin family member 20A, KS = Kaposi sarcoma, KSHV = Kaposi sarcoma herpesvirus, LANA = latency-associated nuclear antigen, LECs = lymphatic endothelial cells, MCD = multi-centric Castleman disease, MCODE = molecular complex detection, MF = molecular function, NUF2 = NUF2 Component Of NDC80 Kinetochore Complex, PEL = primary effusion lymphoma, PPI = protein–protein interaction, SAGE = serial analysis of gene expression, SCs = spindle cells, STRING = search tool for the retrieval of interacting genes, TOP2A = DNA Topoisomerase II Alpha, vFLIP = viral FLICE inhibitory protein, vGPCR = KSHV G protein-coupled receptor.

Keywords: bioinformatics, biomarker, differentially expressed genes, Kaposi sarcoma, KSHV

1. Introduction
Kaposi sarcoma herpesvirus (KSHV), taxonomical name human gamma herpesvirus 8 (HHV-8) was first discovered in 1994 by Chang.[1] It is a linear double-stranded DNA virus belongs to Gamma Herpesviridae subfamily.[2] KSHV was identified as a class I carcinogen by International Agency for Research on Cancer (IARC) in 2009 for its linkage with tumorigenesis of several cancer types.[3,4] During the last score years after KSHV was first discovered, a great deal of experimental and epidemiological evidence has been made to demonstrate that KSHV is the etiological agent of three tumor types including Kaposi sarcoma (KS), Primary Effusion Lymphoma (PEL), and Multi-centric Castleman Disease (MCD).

Observations indicated that the continued presence of KSHV is needed during the development of KS, the main cell type of KS lesions which were called Spindle cells (SCs). However, whether the original precursor of SCs was lymphatic endothelial cells (LEC) or blood endothelial cells (BECs) is yet an unanswered question. Several studies have indicated that SCs may be closely related to LECs more than BECs.[5,6] LECs can be more successfully infected by KSHV and keep a higher viral copy number than BECs. In addition, the overall gene expression profile analysis showed that Kaposi sarcoma is more similar to...
LEC, than to that of BECs. But the existence of KSHV-infected BECs during KS development cannot be ignored, some studies hypothesize that KSHV infects both BECs and LECs at the early stages of KS development. And then, KSHV-infected LECs become predominant in advanced KS lesions.

Dishearteningly, after almost 25 years of discovery of KSHV, Mechanisms underlying the development of KS remains unclear. In recent years, microarray technology and bioinformatic analysis enabled us to investigate the molecular mechanism(s) and identify tumor-related genes in a new way. Thus, the purpose of this work is to have a better understanding of the exact mechanism(s) of KSHV-associated cancer and to identify potential novel diagnostic and prognostic markers or therapeutic targets by bioinformatic analysis.

2. Materials and methods

2.1. Microarray data

We searched Gene Expression Omnibus database[9] (http://www.ncbi.nlm.nih.gov/geo/) to obtain microarray data by using searching strategy: ((KSHV) OR Kaposi sarcoma herpesvirus) AND endothelial cells. There were 201 results in PubMed GEO Datasets. After we have read all these results, We found only 2 gene expression Datasets of GSE16354[10] and GSE22522[11] can be used for further analyzing by synthesis. The platforms of both datasets were all based on GPL570, [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. There were 24 samples in GSE16354, 6 blood vessel endothelial cells (BECs) control samples compared with KSHV 72 hours post infection of blood vessel endothelial cells (BEC); and 6 LECs control samples compared with KSHV 72 hours post infection of LECs. GSE22522 contained 6 samples including 3 LECs controls and 3 KSHV infected LECs.

2.2. Identification of DEGs

The candidate differentially expressed genes between LECs (BECs) and KSHV infected LECs (BECs) were screened using GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r). GEO2R is an online web tool for users to identify DEGs between experimental samples and control samples. [logFC] (fold change) > 1.0, and adjusted P values < .01 were considered statistically significant. We used the online software Venn diagram (http://bioinfogp.cnb.csic.es/tools/ venny/) to obtain the overlapping genes in 3 different experiments.

2.3. GO and KEGG pathway enrichment analysis for DEGs

The DAVID database[12] (https://david.ncifcrf.gov/) (version 6.7) was used for GO annotation and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis, it is an online analysis tool for grouping a list of related genes into certain organized classes or related networks. KEGG database is a database that systematically analyzes the metabolic pathways of gene products and their functions in cells, it helps to study genes and their expression as a whole network. The GO database provides a standardized description of gene products from the perspectives of function, biological pathways involved and location in cells, namely, simple annotation of gene products. [14]

2.4. PPI network construction and module analyzing

The Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org/) was used to identify the interactions between known proteins and predicted proteins. We set confidence score >0.4 as the cut-off criterion. Then, we used Cytoscape software[13] (version 3.6.1) to conduct the protein–protein interaction network visualization. The APP Molecular Complex Detection (MCODE)[16] (version 1.5.1) was used to identify the most significant module in the networks. The selection criteria were chosen as follows: MCODE scores >5, node score cut-off=0.2, degree cut-off=2, Max depth=100 and k-score=2. After that, we used DAVID to perform GO and KEGG analyses for the most significant module.

2.5. Central gene screening and analysis

We identified the hub genes using degrees ≥10. Biological Networks gene Oncology tool (BiNGO)[17] (version 3.0.3) is an open-source Java tool which used for analyzing the GO terms of hub genes, p<.01 was regarded as statistically significant. Database Oncomine (P < .01, fold change ≥2) (https://www.oncomine.org) and Serial Analysis of Gene Expression (SAGE) (https://cgap.nci.nih.gov/SAGE) were used to analyze the expression profiles of TOP2A and DEPDC1.

3. Results

3.1. Identification of DEGs in KSHV infected endothelial cells

A total of 2363 DEGs (593 in GSE16354 BECs, 677 in GSE16354 LECs, 1093 in GSE22522 LECs) were identified, among them, there were 113 overlapping DEGs (Fig. 1A) including 61 upregulated genes and 33 down regulated genes, while 19 of the DEGs have inconsistent gene regulatory trends between different databases.

3.2. KEGG and GO enrichment analysis of DEGs

We used DAVID to analyze the biological processes (BP), cellular components (CC), molecular functions (MFs) and KEGG pathways of the 113 overlapping DEGs. GO enrichments showed that BP mainly enriched in regulation of cell proliferation, positive regulation of developmental process, and negative regulation of cell proliferation; CC was significantly enriched in extracellular region part, extracellular space, and extracellular signaling pathway, Pathways in cancer, and Insulin signaling pathway (Table 1). KEGG pathway enrichment displayed that Cell cycle, Jak-STAT signaling pathway, Pathways in cancer, and Insulin signaling pathway (Table 1).

3.3. PPI network construction and hub gene analyzing

We download the PPI network of the 113 overlap genes from STRING, and then we used Cytoscape to construct the whole network (Fig. 1B). The most significant module of the whole network was obtained with 11 nodes and 55 edges (Fig. 1C). A total of 11 hub genes were identified from the whole network. The results of GO and KEGG enrichments of hub genes were listed in Table 2, the Biological Networks of 11 hub genes using BiNGO was shown in Figure 2. The full name and functional role of hub genes are shown in Table 3. Among these hub genes, the results of MCODE analysis showed that DEP Domain Containing 1 (DEPDC1) was the only seed gene of this cluster, and DNA Topoisomerase II Alpha (TOP2A) is a protein coding gene which
**Table 1**

GO and KEGG pathway enrichment analysis of DEGs in KSHV infected endothelial cells.

| Term | Description | Count | P value |
|------|-------------|-------|---------|
| GO: 0042127 | regulation of cell proliferation | 18 | 1.04E-05 |
| GO: 0051094 | positive regulation of developmental process | 11 | 1.28E-05 |
| GO:0008285 | negative regulation of cell proliferation | 12 | 2.14E-05 |
| GO: 0044421 | extracellular region part | 20 | 2.28E-06 |
| GO:0005576 | extracellular region | 15 | 4.40E-05 |
| GO:0043965 | sequence-specific DNA binding | 11 | 0.002731 |
| GO:0003707 | steroid hormone receptor activity | 4 | 0.002927 |
| GO:0008144 | drug binding | 4 | 0.004906 |
| hsa04110 | Cell cycle | 5 | 0.018948 |
| hsa04630 | Jak-STAT signaling pathway | 5 | 0.037855 |
| hsa05200 | Pathways in cancer | 7 | 0.05062 |
| hsa04910 | Insulin signaling pathway | 4 | 0.090406 |

DEGs = differentially expressed genes, GO = gene ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes, KSHV = Kaposi sarcoma herpesvirus.

**Table 2**

GO and KEGG pathway enrichment analysis of hub genes in KSHV infected endothelial cells.

| Term | Description | Count | P value |
|------|-------------|-------|---------|
| GO:0000278 | mitotic cell cycle | 8 | 1.21E-09 |
| GO:0022403 | cell cycle phase | 8 | 2.65E-09 |
| GO:0007067 | mitosis | 7 | 3.44E-09 |
| GO:0015630 | microtubule cytoskeleton | 6 | 1.57E-05 |
| GO:0005856 | cytoskeleton | 7 | 9.90E-05 |
| GO:0004421 | extracellular region part | 20 | 2.28E-06 |
| GO:0005615 | extracellular space | 15 | 4.40E-05 |
| GO:0005576 | extracellular region | 24 | 0.001048 |
| GO:0043565 | sequence-specific DNA binding | 11 | 0.002731 |
| GO:0003707 | steroid hormone receptor activity | 4 | 0.002927 |
| GO:0008144 | drug binding | 4 | 0.004906 |
| hsa04110 | Cell cycle | 4 | 1.45E-05 |
| hsa04914 | Progesterone-mediated oocyte maturation | 3 | 8.39E-04 |
| hsa04114 | Oocyte meiosis | 3 | 0.01372 |
| hsa04115 | p53 signaling pathway | 2 | 0.039592 |

DEGs = differentially expressed genes, GO = gene ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes, KSHV = Kaposi sarcoma herpesvirus.

Figure 1. Venn diagram, PPI network and the most significant module of DEGs. (A) DEGs were selected with \(|\log FC| \geq 1\) and \(P\) value < 0.01 among the mRNA expression profiling sets GSE16354 and GSE22522. The 2 datasets showed an overlap of 113 genes. (B) The PPI network of DEGs was performed using Cytoscape. (C) The most significant module was obtained from PPI network with 11 nodes and 55 edges.
is widely recognized having strong associations with a lot of different cancer types. Hence, we selected DEPDC1 and TOP2A to conduct further analysis using online database Oncomine and SAGE. The results are shown in Figures 3 and 4. Data of Oncomine showed that DEPDC1 and TOP2A were significantly upregulated when compared with normal tissues in various cancer types. And the results of SAGE analysis displayed that the expression profile of DEPDC1 and TOP2A were higher in different cancer tissues compared with matched normal tissues.

4. Discussion

KSHV is a tumor-producing virus which is believed to have the largest number of potential oncogenes. Its oncogenicity derived from inducing and encoding a lot of cellular and viral oncogenes after infecting different human cell types.\[18] One of the most studied disease associated with KSHV is KS which is believed an endothelial cell origin tumor. Our study focuses on the bioinformatic analysis to identify DEGs between LECs (BECs) and KSHV infected LECs (BECs).

In our present work, we conducted the analysis by extracting data from the 2 datasets, a total of 113 DEGs were obtained. 61 upregulated genes and 33 down regulated genes. What puzzled us was that 19 of the DEGs have inconsistent gene regulatory trends between different databases. One possible explanation for this phenomenon is that KSHV infected BECs and KSHV infected LECs may play different roles at different stages of KS.\[8] Among the 113 DEGs, 11 hub genes were identified by using Cytoscape software. The GO enrichment results of hub genes revealed that they were mostly enriched in mitotic cell cycle, cell cycle phase, and mitosis, and KEGG enrichment results displayed that they were mainly enriched in Cell cycle, Oocyte meiosis, p53 signaling pathway. Evidences in previous studies showed that these GO terms and KEGG pathways may play important roles in the occurrence and developments of tumors.\[19–21]

All these 11 hub genes might serve as key biomarkers in the process of KSHV induced tumor development. Among them, we show great interest in DEPDC1, CCNB1, CCNB2, and TOP2A. For as the results of MCODE analysis, DEPDC1 is the only seed gene of this hub gene cluster. More importantly, DEPDC1, CCNB1, and CCNB2 has been proved to have a significant impact on NF-kB signaling pathway, and NF-kB signaling pathway is widely recognized as having a strong correlation of angiogenesis. As for TOP2A, we did a survey on the databases of PubMed and GeneCards. TOP2A has the biggest number of articles among the 11 hub genes when searching PubMed, and it has been proved to have strong correlations with lots of cancer types. The detail information of these hub genes is as following.

DEPDC1 is a Protein Coding gene which was first reported aberrantly overexpressed and plays important roles in bladder cancer.\[22] After that, DEPDC1 was found to be involved in the process of tumorigenesis in many other cancer types.\[23,24] As is known to all, Cancer is commonly regarded as a cell cycle dysregulation disease. In Mi et al’s study,\[25] DEPDC1 was found have a higher expression in the mitotic phase during the cell cycle,

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**Table 3**

Functional roles of 11 hub genes.

| NO. | Gene symbol | Full name | Function |
|-----|-------------|-----------|----------|
| 1   | KIF20A      | Kinesin Family Member 20A | KIF20A is involved in mitosis. |
| 2   | DEPDC1      | DEP Domain Containing 1 | May be involved in transcriptional regulation as a transcriptional corepressor. |
| 3   | NUF2        | NDC80 Kinetochore Complex Component | Acts as a component of the essential kinetochore-associated NDC80 complex, which is required for chromosome segregation and spindle checkpoint activity. |
| 4   | ASPM        | Abnormal Spindle Microtubule Assembly | This gene is essential for normal mitotic spindle function in embryonic neuroblasts |
| 5   | CDC20       | Cell Division Cycle 20 | This gene is involved Cell Cycle, Mitotic and Class I MHC mediated antigen processing and presentation. |
| 6   | CCNA2       | Cyclin A2 | Cyclin which controls both the G1/S and the G2/M transition phases of the cell cycle. |
| 7   | TOP2A       | DNA Topoisomerase II Alpha | This gene encodes a DNA topoisomerase, Diseases associated with TOP2A include Female Breast Cancer and Malignant Peripheral Nerve Sheath Tumor. |
| 8   | ANLN        | Anillin Actin Binding Protein | This gene encodes an actin-binding protein that plays a role in cell growth and migration, and in cytokinesis. |
| 9   | CCNB1       | Cyclin B1 | The protein encoded by this gene is a regulatory protein involved in mitosis. |
| 10  | CDKN3       | Cyclin Dependent Kinase Inhibitor 3 | This gene was reported to be deleted, mutated, or overexpressed in several kinds of cancers. |
| 11  | CCNB2       | Cyclin B2 | Essential for the control of the cell cycle at the G2/M (mitosis) transition, Diseases associated with CCNB2 include Anauxetic Dysplasia 1 and Breast Cancer. |
and siRNA-mediated knockdown of DEPDC1 would make an effect of mitotic arrest and defects. Moreover, the downregulated DEPDC1 would make an upregulated of A20 – a protein which negatively regulate the NF-κB signaling pathway as well as another two NF-κB regulated cell cycle genes of CCNB1 (Cyclin B1) and CCNB2 (Cyclin B2), and the activity of NF-κB pathway is believed to have a significant correlation with cell cycle regulation.[26] CCNB1 and CCNB2 are two hub genes we have screened out in our present work. They are also cell cycle-related genes regulated by NF-κB signaling pathway.[27] More importantly, NF-κB is a known signaling pathway activated by KSHV to facilitate host cell proliferation and angiogenesis.[28] A lot of KSHV-encoded proteins including viral FLICE inhibitory protein (vFLIP), KSHV G protein-coupled receptor (vGPCR), KS-associated herpesvirus K1, together with viral miRNAs activate NF-κB signaling pathway by decreasing the expression level of IκBa.[29–31] Thus, DEPDC1 might be a target gene of KSHV in the process of infecting host cells, Further studies are needed to test this hypothesis.

Evidences suggested that persistent activation of Wnt signaling could also confer risk to cancer.[32,33] TOP2A and ASPM (Abnormal Spindle Microtubule Assembly) were found to be involved in regulating Wnt signaling pathway. In Pei et al’s study,[34] TOP2A together with β-catenin worked as co-activator to promote the process of epithelial-mesenchymal transition in pancreatic cancer cells via activating Wnt signaling pathway. ASPM is another novel Wnt co-activator of Wnt signaling pathway in prostate cancer. Evidence from Pai et al’s study[35] indicated that the ASPM expression level is upregulated in prostate cancer samples and the downregulation of ASPM could inhibit the biological behaviors such as proliferation, invasion, and colony formation in prostate cancer cells. In addition, A gastric cancer research highlighted that ANLN (Anillin Actin Binding Protein) expression was significantly associated with Wnt/β-catenin signaling pathway.[36] Wnt Signal Transduction Pathways also plays a key role in KSHV-related Tumors, latency-associated nuclear antigen (LANA) is an KSHV encoded protein that plays a vital role during the process of infection. The
interaction of glycogen synthase kinase-3β (GSK-3β) and LANA could make a dysregulation of β-catenin and thus regulating Wnt signaling pathway. Therefore, TOP2A, ASPM, and ANLN might be possible functional targets in the formation and development of KS and other KSHV-related tumors.

As for other hub genes, such as Cyclin Dependent Kinase Inhibitor 3 (CDKN3), CCNA2 (Cyclin A2), CDC20 (Cell Division Cycle 20), NUF2 (NUF2, NDC80 Kinetochore Complex Component), Kinesin Family Member 20A (KIF20A), the literature retrieval results indicated that their relationship between KSHV-induced tumors and KSHV regulated pathways has not been widely reported. However, there are plenty of studies on other tumor types, elevated CDKN3 expression level has been reported in various types of human cancer, including lung cancer, cervical cancer, and gastric cancer. In addition, CDKN3 seems to be regulated by another virus human papilloma virus (HPV) the same way KSHV to host genes.

Taken together, our analysis identified the crucial genes which may serve as key diagnostic biomarkers and therapeutic targets for KS and other KSHV-induced tumors by comprehensive bioinformatics analysis. However, Molecular and cell biology studies are required to validate the functional role of these genes.

5. Conclusion

In conclusion, the present study identified a host of DEGs and hub genes in KSHV infected endothelial cells which may serve as potential key biomarkers and therapeutic targets, helping us to have a better understanding of the molecular mechanism of KS. Further studies are strongly encouraged in the future to validate these biomarkers.

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