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Identification of candidate genes associated with bone metastasis in non-small-cell lung cancer based on epithelial-mesenchymal transition

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Abstract:	Bone metastasis in non-small-cell lung cancer (NSCLC) is a complex and multi-stage process that is a major reason for poor survival of patients. Epithelial-mesenchymal transition (EMT), a developed program in tumor progression, has been extensively shown to promote tumor metastasis, including bone metastasis, in NSCLC. Nevertheless, how EMT influence bone metastasis remains unknown. In this study, we downloaded two gene expression profiles—an EMT model and a bone metastasis model—to identify differentially expressed genes (DEGs). Thereafter, we performed Gene Ontology (GO) analysis and pathway analysis based on DEGs to gain a better understanding of the potential molecular mechanisms. In addition, we identified seven up-regulated and 10 down-regulated DEGs, which appeared in both the EMT and bone metastasis model. Subsequently, the protein-protein interaction(PPI) network was constructed to visualize potential interactions. These analyses and candidate genes may provide new evidence for EMT-induced metastasis and could help to identify new predictive biomarkers and therapeutic targets.
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Dear editor,

We submit our manuscript entitled "**Identification of candidate genes associated with bone metastasis in NSCLC cells**" to ***Clinica Chimica Acta.***

In this work, we evaluated the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of DEGs using profiling datasets of epithelial-mesenchymal transition and bone metastasis projects respectively. Finally, 17 genes were identified as candidate genes between epithelial-mesenchymal transition and bone metastasis in non-small-cell lung cancer, and protein-protein interaction was constructed to predict the potential interactions.

We hope our manuscript can be reviewed for publication in ***Clinica Chimica Acta***. No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication.

We deeply appreciate your consideration of our manuscript, and if you have any queries, please don't hesitate to contact me at the address below. Thank you!

Yours sincerely,

Junshan Ruan

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Highlights

- Epithelial-mesenchymal transition (EMT), has been extensively shown to promote tumor metastasis, including bone metastasis, in NSCLC.
- Downloaded two gene expression profiles—an EMT model and a bone metastasis model—to identify differentially expressed genes (DEGs).
- Performed Gene Ontology (GO) analysis and pathway analysis based on DEGs to gain a better understanding of the potential molecular mechanisms.
- Identified seven up-regulated and 10 down-regulated DEGs, which appeared in both the EMT and bone metastasis model.
- Provide new evidence for EMT-induced metastasis and could help to identify new predictive biomarkers and therapeutic targets.

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Introduction

Lung cancer is one of the most malignant types of cancers, which by the time of detection is already in the metastatic stage. Non-small-cell lung cancer（NSCLC）is accounted for 80% lung cancers, and it can form metastasize through both blood vessels and lymphatic system. Its target organs include the liver, kidney, brain, and bone; however, of which bone metastasis is one of the earliest and most frequent of the rest (1). A study has shown that the percentage of NSCLC patients with bone metastasis has reached to 57.5%, leading to suppression of the bone marrow, pathological fracture and a quality of life (2). Bone metastasis is a multistep process whose underlying mechanisms remain unclear, limiting the diagnostic and therapeutic strategies(3). Thus investigating the molecular mechanism of bone metastasis is an effective method to find diagnostic approaches and predicted therapeutic targets in NSCLC.

Epithelial-mesenchymal transition (EMT) is a regulatory process in tumor metastasis, whereby epithelial tumor cells lose their polarity and change their epithelial phenotype to a mesenchymal phenotype, resulting in an increased migration and invasion ability (4). Loss of cell junctions is the first step in tumor dissemination, and it is the basic phenomenon occurring in EMT. Transforming growth factor (TGF-β), hepatocyte growth factor (HGF), fibroblast growth factor (FGF) have all been accounted for EMT process; among these, TGF-β is the most typical and widely known inducer that can change cell morphology and remove cell junctions easily. TGF-β also can be released from bone during bone destruction, contributing to the vicious cycle driving the development of metastatic osteolysis (5, 6). A recent study regarded EMT as the carcinogenesis factor in tumor progression and bone metastasis in NSCLC (7). Accumulating evidence has demonstrated that multiple genes and cellular pathways participate in the development of EMT-induced tumor metastasis, but the underlying

crosstalk between EMT and bone metastasis of NSCLC is unclear.

With the development of microarray experiments, a number of biological markers have been explored, which have critical roles in the field of medical science. These biomarkers are widely utilized in clinical practice as drug targets for anti-tumor therapy, as tools for molecular diagnosis and prognosis, and classification of tumors (8). Gene expression profiling studies have long been performed to explore the molecular mechanisms of tumor progression, but independent expression profiling is not sufficient to establish the validity of the evidence. Bioinformatics is a subject that has been expending rapidly in recent years. Bioinformatic analysis can effectively integrate big data produced by microarry experiments and find promising biomarkers by identifying differentially expressed genes (DEGs) (9).

In the current study, we combined two expression profiles, GSE10096 and GSE49644, to identify the candidate genes associated with bone metastasis in NSCLC, in relation to EMT. In addition, up-regulated and down-regulated DEGs were further analyzed to identify overlapping genes that may account for the two progression of tumors. Finally, seven genes were found in the up-regulated gene group and 10 genes were found in the down-regulated gene group. These candidate genes may provide an insight into the interactions between EMT and bone metastasis in NSCLC.

Material and methods

Gene expression profile data. The gene expression profiling data from GSE10096 (submitted by Vicent et al. (10)) and GSE49644 (submitted by Sun et al. (11)) were downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database. GSE10096 expression analysis was performed using 13 microarrays, corresponding to four samples of non-metastatic cell lines (control) and nine samples of three different, highly metastatic cell lines with three biological replicates for each. GSE49644 was designed to model EMT by culturing NSCLC cells in the presence of TGF-β for 3 weeks.

Identification of DEGs. Raw data analysis was carried out using the Affy package in R

language (version 3.3.2). The limma package in R/Bioconductor (https://bioconductor.org/packages/release/bioc/html/limma.html) was used to screen for DEGs. We converted different gene or probe IDs to Entrez IDs and used a classical t-test to identifyDEGs with |log2FC| ≥1 and P<0.05. The top ten up-regulated and down-regulated genes in GSE10096 and GSE49644 are indicated in Table 1.

Gene Ontology (GO) and pathway enrichment analysis of DEGs. GO analysis is the framework for the model of biology. GO defines classes used to describe gene function and relationships between these concepts. To explore the functions and process levels in identified DEGs, we mapped our DEGs list to the online database DAVID (https://david.ncifcrf.gov/). GO enrichment and pathway analysis were performed in DAVID, and P<0.05 was considered statistically significant.

Identification of candidate genes. A Venn diagram was used to identify potential regulatory genes that were up-regulated or down-regulated simultaneously during EMT and bone metastasis in NSCLC. Overlapping genes in these groups were regarded as candidate genes that may function as key regulators in EMT and bone metastasis of NSCLC.

Establishment of the protein-protein interaction (PPI) network. The potential interactions between proteins were investigated using the online database STRING (version 10.0; http://www.string-db.org/). In this study, only the interactions containing at least one DEG were filtered out, with the criterion of a combined score of >0.4, which were then utilized for constructing the PPI network using Cytoscape (version 3.4.0; http://cytoscape.org/) software.

Results

Identification of DEGs. GSE10096 expression analysis was performed using 13 microarrays, corresponding to four control samples of non-metastatic cell lines and nine samples of three different, highly metastatic cell lines with three biological replicates

for each. We analyzed DEGs between non-metastatic cell lines and highly metastatic cell lines using R language. This analysis aided the identification of 304 DEGs, of which 132 genes were up-regulated, and 172 genes were down-regulated. GSE49644 was designed to model EMT by culturing NSCLC cells in the presence of TGF-β for 3 weeks. The results revealed a total of 901 DEGs, which included 376 up-regulated and 525 down-regulated genes. The 10 most significantly up- or down-regulated DEGs in these two analyses are displayed in Table 1.

GO enrichment and pathway analysis. GO category and KEGG pathway enrichment analysis were performed using online database DAVID. GO analysis of GSE10096 (Table 2) showed that the DEGs were significantly enriched in response to inorganic substance (GO:0010035), insulin-like growth factor binding (GO:0005520), and regulation of cell growth (GO:0001558). The main pathways identified were transcriptional misregulation in cancers (hsa:05202), adherens junction (hsa:04520), and focal adhesion (hsa:04510), indicating a potential crosstalk with the EMT process (Fig. 1A).

GO analysis of GSE49644 (Table 3)showed that the DEGs were significantly enriched in extracellular matrix organization (GO:0030198), proteinaceous extracellular matrix (GO:0005578), and extracellular region (GO:0005576). As shown in Fig. 1B, The major pathways include focal adhesion (hsa04510), ECM-receptor interaction (hsa04512), and Proteoglycans in cancer (hsa05205).

Identification of candidate genes. By analyzing 304 DEGs in the bone metastasis profile (GSE10096) and 902 DEGs in the EMT profile (GSE 49644), we identified seven genes were highly expressed and 10 genes were significantly down-regulated in both the metastasis and EMT models (Fig. 2A)

These seven up-regulated hub genes include serpin peptidase inhibitor clade E member 1 (SERPINE1); sushi Domain Containing 5(SUSD5); cholinergic receptor nicotinic alpha 9 (CHRNA9); ADAM metallopeptidase domain 19 (ADAM19), Paired Box 6 (PAX6); carbonic anhydrase II (CA2); and transcription factor 4(TCF4). The 10

down-regulated genes include caveolin-1(CAV1); dickkopf WNT signaling pathway inhibitor 1(DKK1); prostaglandin E Receptor 4(PTGER4); mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK3); basic leucine zipper ATF-Like transcription factor 3 (BATF3); keratin 8 (KRT8); FAT atypical cadherin 4 (FAT4); WNT1 inducible signaling pathway protein 2 (WISP2); secreted and transmembrane 1 (SECTM1); and insulin like growth factor binding protein 3 (IGFBP3), as shown in Fig. 2A. These candidate genes may help us understand in depth, the molecular mechanisms of interaction between EMT and bone metastasis in NSCLC and act as potential prognostic indicators in bone metastasis in NSCLC.

PPI network. The online analytical database STRING was used to screen and predict the potential interacting genes among these 17 overlapping genes. The PPI network was built with the criterion of a combined score of >0.4 and maximum of 30 interactors. The result showed that there are 234 pairs with a score >0.4, which represent a potential protein interaction. Then, this data was further visualized by using the Cytoscape software (Fig. 2B). Through the PPI network, we can also find some vital molecule like SRC, JUN, EGFR and HSBP1. SUSD5 and CHRNA9 were not included in this network because they were not contributing to present max 30 protein interactors.

Discussion

Bone metastasis is a common type of NSCLC metastasis that, accompanied by a series of skeletal-related events, leads to a significant reduction in the quality of life. Bone metastasis in NSCLC is unpredictable and its complicated mechanism suggests the participation of several regulators. Previous research has demonstrated that EMT can endow tumor cells with migration and invasion capabilities, which play a critical role in tumor metastasis. To investigate the underlining relationship between EMT and bone metastasis in NSCLC, we extracted data from the exprssion proflies GSE10096 and GSE49644 to conduct bioinformatic analysis. In the present study, we have identified 132 up-regulated and 172 down-regulated DEGs in GSE10096 based on a comparison

with a non-metastatic NSCLC model and highly metastatic bone metastasis model. A related experiment was designed to establish an EMT model using NSCLC cells incubated in the presence of TGF-β for 3 weeks, whereas 376 up-regulated and 525 down-regulated DEGs were identified, upon comparison with the control NSCLC cells.

To understand the DEGs in bone metastasis model and EMT model in depth, GO and KEGG pathway analysis were performed using DEGs. The GO term analysis in GSE10096— the bone metastasis project— mainly suggested an enhancement in differential expression in response to inorganic substance, insulin-like growth factor binding and regulation of cell growth. The enriched pathway includes transcriptional misregulation in cancers, adherens junction, and focal adhesion, of which the last two pathways suggested that EMT might be one of the most important regulators in bone metastasis. All of these GO terms and pathways were consistent with the existing knowledge on metastasis in NSCLC progression. Enrichment of GO and KEGG pathway in GSE49644, the EMT model, were related to cell adhesion, cell proliferation, cell morphology change and ECM-receptor interaction. Thses findings have were widely characterized in other studies.

Further exploration was conducted to identify candidate genes associated with bone metastasis in NSCLC based on EMT. By analysing overlapping up-regulated genes amongst the 132 up-regulated genes in bone metastasis model and 376 up-regulated genes in EMT model, we identified ADAM19, SUSD5, TCF4, SERPINE1, PAX6, CHRNA9 and CA2 as candidate genes for their potential functions in bone metastasis activation. The ADAM19 gene belongs to the ADAM family, which is characterized by disintegrin and metalloproteinase domains (12). These proteins can be activated and released when tumor cells come in contact with the extracellular matrix, suggesting the ADAMs active affect the disseminated of metastatic disease. ADAM 8, ADAM10 and ADAM17 were observed to be involved in EGFR cell signaling, contributing to the enhanced tumor cell invasion and osteolytic metastasis (3, 13, 14). Therefore, whether ADAM19 is involved in cascade signaling of bone metastasis is the focus of our future research. SUSD5 is a protein-coding gene and GO annotations related to this gene include hyaluronic acid binding. The TCF4 gene encodes transcription factor 4, a basic

helix-loop-helix transcription factor. Our findings were consistent with previous research on GSE10096, which demonstrated that a combination of SUSD5/TCF4/PRKD3 up-regulation generates a dramatic increase in the tumor burden (10). CA2 is one of the several isozymes of carbonic anhydrase. Sclerostin can induce the expression of CA2 and subsequently regulate the release of bone mineral, inducing an increased osteocyte lacunar area (15). SERPINE1, PAX6 and CHRN9 were also been characterized as downstream molecules of tumor metastasis and participate in multiple biological process (16-18). Thus, these seven up-regulated genes represent promising candidates for regulation of bone metastasis and therapeutic intervention in NSCLC patients.

Some tumor suppressor genes are often inhibited during tumor progression. These genes have hardly been studied in relation to bone metastases in NSCLC. To explore the potential functional molecules, we constructed an overlapping gene list and identified 10 down-regulated genes both in GSE10096 and GSE49644. PTGER4 is a member of the G-protein-coupled receptor family. Knockout studies in mice suggest that this receptor may be involved in the development of osteoporosis, indicating its crucial role in protecting the integrity of bone cells and marrow (19). The down-regulation of PTGER4 may result in encroaching of bone marrow by tumor cells. The protein encoded by BATF3 may play a role in repression of interleukin-2 and matrix metalloproteinase-1 (MMP1) transcription (20, 21). Thus inhibition of BATF3 may cause the overexpression of MMPs, leading to ECM remodeling and cell invasion. DKK1 is a secretory protein with two cysteine-rich regions and is involved in embryonic development through its inhibition of the WNT signaling pathway. Elevated levels of DKK1 in bone marrow plasma and peripheral blood are associated with the presence of osteolytic bone lesions in patients (22, 23). CAV1 is an important component of focal adhesion, and its expression is often down-regulated in many types of tumor tissue. Our research also showed that CAV1 was down-regulated both in the bone metastasis model and EMT model, indicating that it may function as a tumor suppressor. MAPKAPK3 was shown to play a critical role in tumor progression

according to previous study (24), while WISP2, KRT8, FAT4, SECTM1 and IGFBP3 may function as direct or indirect tumor suppressor in bone metastasis and EMT.

Protein-protein interaction is an important part of cell signaling, cellular components and cell fate determination. Hence, the establishment of PPI network has been widely used to explore the process of disease and drug design (25). In this study, we performed PPI network using these candidate genes and almost all the overlapping genes were presenting great potential interactions, except SUSD5 and CHRNA9. Through the PPI network, we can also find some vital molecule like SRC, JUN, EGFR and HSBP1, which confirmed that our candidate genes were involved in important cellular process.

In conclusion, the identification of these candidate genes may provide new insight into the underlining molecular crosstalk between EMT and bone metastasis. However, it should be noted that this study has examined the questions through a bioinformatic analysis rather than an experimental verification. Despite their limited character in the previous study, we will mainly focus on their potential function in vivo and in vitro, which may guide a knowledge of bone metastasis in NSCLC and EMT. Further investigation of these candidate genes through clinical, statistical, and biological experiments could confirm their possible molecular function. Moreover, the predictive biomarkers and therapeutic targets of bone metastasis in NSCLC can be identified and validated in the future.

Conflicts of Interst

The authors declare no conflict of interest.

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Figure 1. Pathway enrichment analysis of the DEGs. (A) identification of main pathways in GSE10096. (B) identification of main pathways in GSE49644. DEGs, differentially-expressed genes. DEGs, differentially expressed genes.

Figure 2. Identification of candidate genes and prediction of the PPI networks**. (A)** Graphical representation of computational analysis using the DEGs of bone metastasis (GSE10096) and EMT model (GSE49644). **(B)** PPI networks of the overlapping DEGs. Red nodes represent up-regulated genes, blue nodes represent down-regulated genes and the lines between two nodes denote the interactions between them. DEGs, differentially expressed genes. PPI, protein-protein interaction.

Table(s)

Table 1 The10 most significantly up- or down-regulated DEGs in GSE10096 and GSE49644

GSE10096				GSE49644			
Gene ID	Gene symbol	Status	Pvalue	Gene ID	Gene symbol	Status	Pvalue
8470	SORBS2	up-regulated	4.02E-06	3488	IGFBP5	up-regulated	7.16E-11
54502	RBM47	up-regulated	1.13E-05	122786	FRMD6	up-regulated	7.16E-11
3613	IMPA2	up-regulated	2.14E-05	79444	BIRC7	up-regulated	1.04E-10
6320	CLEC11A	up-regulated	6.52E-05	1277	COL1A1	up-regulated	1.69E-10
7103	TSPAN8	up-regulated	0.00018227	5919	RARRES2	up-regulated	2.67E-10
26032	SUSD5	up-regulated	0.00024552	2841	GPR1	up-regulated	3.53E-10
6275	S100A4	up-regulated	0.00025461	90625	ERVH48-1	up-regulated	5.20E-10
63910	SLC17A9	up-regulated	0.00035414	6442	SGCA	up-regulated	7.87E-10
26256	CABYR	up-regulated	0.0004058	7057	THBS1	up-regulated	1.29E-09
9516	LITAF	up-regulated	0.00049732	239	ALOX12	up-regulated	2.34E-09
3486	IGFBP3	down-regulated	2.62E-05	4316	MMP7	down-regulated	2.00E-20
8644	AKR1C3	down-regulated	9.87E-05	90288	EFCAB12	down-regulated	8.87E-18
1122	CHML	down-regulated	0.00013109	10551	AGR2	down-regulated	9.36E-15
54707	GPN2	down-regulated	0.0002045	5874	RAB27B	down-regulated	4.87E-14
256227	STEAP1B	down-regulated	0.00022823	200879	LIPH	down-regulated	1.50E-13
6398	SECTM1	down-regulated	0.00043	5166	PDK4	down-regulated	1.70E-13
7162	TPBG	down-regulated	0.00057881	2069	EREG	down-regulated	1.77E-13
79633	FAT4	down-regulated	0.00059901	84331	FAM195A	down-regulated	2.32E-13
10769	PLK2	down-regulated	0.00077691	1356	CP	down-regulated	3.46E-13
1969	EPHA2	down-regulated	0.000811	4645	MYO5B	down-regulated	6.90E-13

Table 2 The enriched GO categories of DEGs in GSE10096

Category	Term	Count	%	PValue
GOTERM_BP_DIRECT	GO:0010035~response to inorganic substance	14	4.844291	6.93E-05
GOTERM_MF_DIRECT	GO:0005520~insulin-like growth factor binding	6	2.076125	7.21E-05
GOTERM_BP_DIRECT	GO:0001558~regulation of cell growth	13	4.498270	1.66E-04
GOTERM_BP_DIRECT	GO:0048514~blood vessel morphogenesis	13	4.498270	3.61E-04
GOTERM_BP_DIRECT	GO:0051262~protein tetramerization	6	2.076125	5.71E-04
GOTERM_MF_DIRECT	GO:0019838~growth factor binding	9	3.114187	6.13E-04
GOTERM_CC_DIRECT	GO:0042995~cell projection	24	8.304498	8.60E-04
GOTERM_BP_DIRECT	GO:0040008~regulation of growth	16	5.536332	0.00105325
GOTERM_BP_DIRECT	GO:0006916~anti-apoptosis	12	4.152249	0.00106417
GOTERM_BP_DIRECT	GO:0007242~intracellular signaling cascade	38	13.148789	0.00123807

Table 3 The enriched GO categories of DEGs in GSE49644

Category	Term	Count	%	PValue
GOTERM_CC_DIRECT	GO:0031012~extracellular matrix	34	9.418283	3.02E-17
GOTERM_CC_DIRECT	GO:0005578~proteinaceous extracellular matrix	32	8.864266	1.01E-16
GOTERM_CC_DIRECT	GO:0005576~extracellular region	74	20.498610	4.62E-14
GOTERM_BP_DIRECT	GO:0030198~extracellular matrix organization	25	6.925208	8.74E-14
GOTERM_BP_DIRECT	GO:0030574~collagen catabolic process	15	4.155125	4.68E-12
GOTERM_CC_DIRECT	GO:0005615~extracellular space	62	17.174520	9.33E-12
GOTERM_CC_DIRECT	GO:0005788~endoplasmic reticulum lumen	22	6.094183	3.33E-11
GOTERM_BP_DIRECT	GO:0007155~cell adhesion	29	8.033241	1.28E-08
GOTERM_BP_DIRECT	GO:0001525~angiogenesis	20	5.540166	1.73E-08
GOTERM_CC_DIRECT	GO:0005581~collagen trimer	13	3.601108	8.28E-08

Figure(s)

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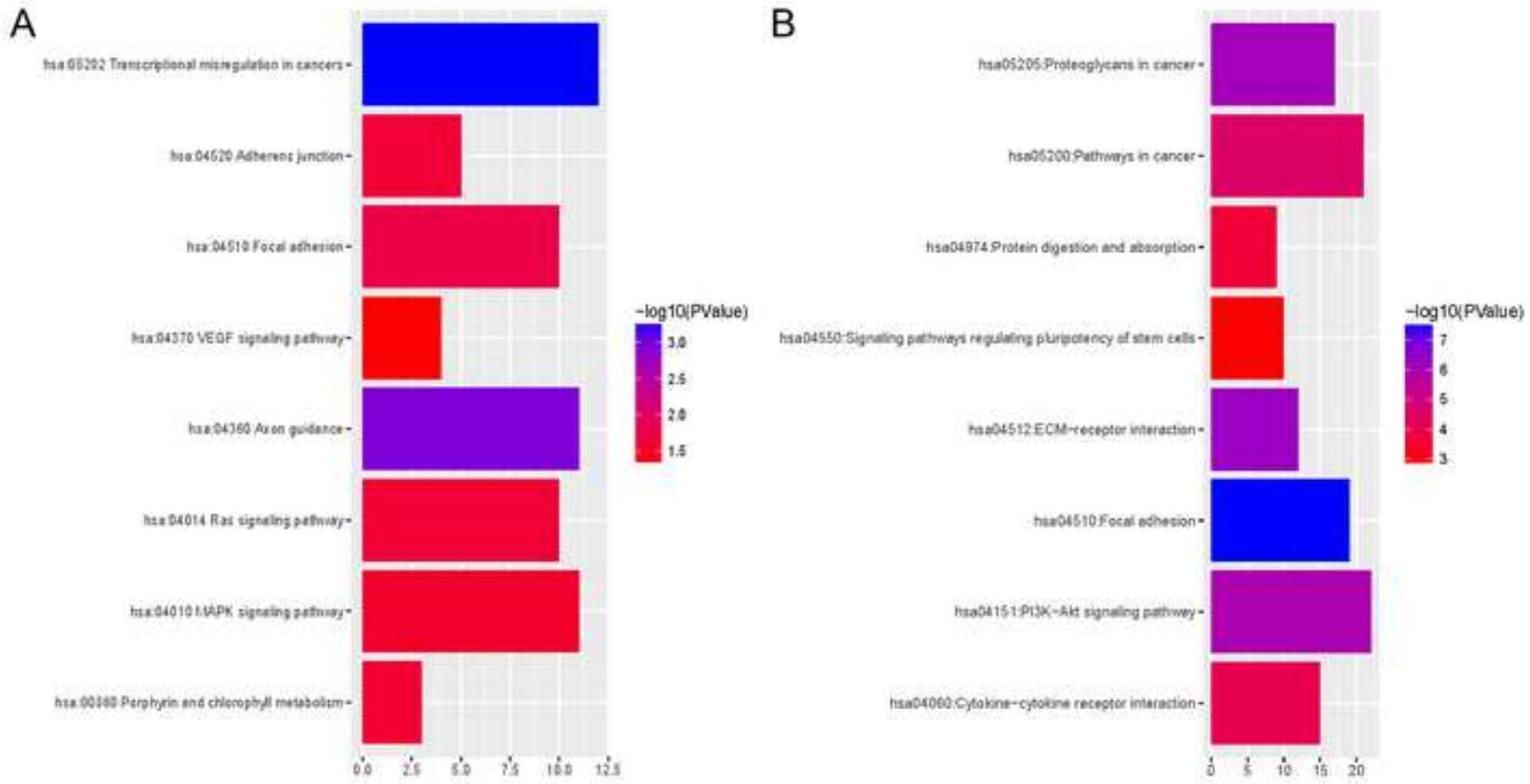

Figure(s)

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