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Identification of potential therapeutic targets for melanoma using gene expression analysis

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Metastatic melanoma represents a significant cause of death in patients with melanoma and the frequency is increasing. The aim of this study was to identify potential therapeutic targets for metastatic melanoma.

Gene expression profile GSE44660 was downloaded from Gene Expression Omnibus database. A total of 22 samples were analyzed in our study, including 3 specimens of normal melanocytes, 12 specimens of melanoma LNM (lymph node metastasis) and 7 specimens of MBM (melanoma brain metastasis). DEGs (differentially expressed genes) in LNM and MBM were identified respectively using Limma package. GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways analyses of common DEGs between two comparison groups were performed using DAVID, followed by cancer-related genes and transcription factor analysis. PPI (protein-protein interaction) network was constructed by STRING, and significant key genes were selected.

Totally, 401 common DEGs were identified. Disease analysis showed that *ICAM1* (intercellular adhesion molecule 1) and *NBN* (nibrin) were related to melanoma. In the PPI network, *BIRC5* (baculoviral IAP repeat containing 5), *BUB1* (BUB1 mitotic checkpoint serine/threonine kinase), *GMNN* (geminin, DNA replication inhibitor), *AURKA* (aurora kinase A), *TOP2A* (topoisomerase (DNA) II alpha) and *BUB1B* (BUB1 mitotic checkpoint serine/threonine kinase B) were with higher degree more than 50.

ICAM1, *NBN*, *BIRC5*, *BUB1*, *BUB1B*, *GMNN*, *AURKA* and *TOP2A* may play key roles in the progression and development of melanoma. They may be used as specific therapeutic targets in the treatment of metastatic melanoma. However, further experiments are still needed to confirm our results.

Key words: melanoma, metastasis, differentially expressed gene, protein-protein interaction network

Melanoma is considered as a type of skin cancer that originated from melanocytes [1]. High incidence is particularly in Caucasians, especially northwestern and northern Europeans, who live in sunny climates [2]. In 2012, the estimated rates of new melanoma cases in Europe are 11.0 per 100,000 for females and 11.4 per 100,000 men [3]. Metastatic melanoma represents a significant cause of death in patients with melanoma and the frequency is increasing. Primary melanoma can commonly metastasize to regional and distant lymph nodes and then by hematogenic dissemination into distant organs including liver, brain and lung [4]. Despite the advances in surgery, it causes the majority of deaths (75%) related to skin cancer [5]. Therefore, it is essential to develop more effective methods for its treatment.

Recently, an increasing number of studies have focused on the prevention and treatment of melanoma, and then found that targeted therapy trials are promising. Carvajal *et al*. have shown that *KIT* (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) is a therapeutic target in metastatic melanoma [6]. Notably, the Ras/Raf/MEK/ERK pathway has been considered as a major, druggable

Abbreviations: BP – biological process; CC – cell component; DAVID – Database for Annotation, Visualization and Integrated Discovery; DEGs – differentially expressed genes; FC – fold change; FDR – false discovery rate; GO – Gene Ontology; ICAM1 – intercellular adhesion molecule 1; KEGG – Kyoto Encyclopedia of Genes and Genomes; LNM – lymph node metastasis; MAPK/ERK – mitogen-activated protein kinase/extracellular regulated kinase; MBM – melanoma brain metastasis; MF – molecular function; PPI – protein-protein interaction; STRING – Search Tool for the Retrieval of Interacting Genes

regulator of melanoma [7]. *BRAF* (v-raf murine sarcoma viral oncogene homolog B) mutation is commonly observed in human melanoma, which resulting in melanocyte hyperproliferation and overactive of MAPK/ERK (Mitogen-activated protein kinase/Extracellular Regulated Kinase) signaling [8]. Nevertheless, resistance to BRAF inhibitors is a major clinical challenge. Moreover, Ericsson *et al*. have found that *IGF1R* (insulin-like growth factor-1 receptor) is a predictor for metastatic disease and a potential therapeutic target [9]. Cotargeting MEK and IGF1R/PI3K can overcome resistance to BRAF inhibitors [10]. Rappa *et al*. have indicated that the stem cell-associated antigen CD133 is a molecular therapeutic target for metastatic melanoma [11]. Although tremendous efforts have been made to discover novel targets for melanoma treatment and novel targeted therapies are showing promising, resistance to these agents and patient relapse rapidly ensue. Therefore, more novel therapeutic targets for metastatic melanoma into brain and lymph node are needed to improve clinical treatment.

DNA methylation profiling associated with MBM (melanoma brain metastasis) and LNM (lymph node metastasis) have been performed by Marzese and colleagues [12]. In this study, DEGs (differentially expressed genes) in MBM and LNM compared with normal controls were identified respectively. Then function annotation of common genes between two comparison groups was conducted, followed by PPI (protein-protein interaction) network construction and key genes screening. Through the identification of key genes, the possible molecular mechanism and the potential therapeutic targets for melanoma were explored. The aim of this study was to identify novel gene targets to improve metastatic melanoma therapy.

Materials and methods

Affymetrix microarray data. We obtained the gene expression profile data GSE44660 from Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) which was deposited by Marzese *et al.* [12]. The gene expression profiling was based on the platform of GPL5175 ([HuEx-1_0-st] Affymetrix Human Exon 1.0 ST Array). Totally, 22 samples were analyzed in our study, including 3 specimens of normal melanocytes, 12 specimens of melanoma LNM and 7 specimens of MBM.

Identification of the common DEGs. The raw data were preprocessed using the Affy package (http://www.bioconductor.org/packages/release/bioc/html/affy.html) [13] in R language. DEGs in melanoma LNM and DEGs in MBM compared with normal melanocytes were screened respectively by Limma package (http://www.bioconductor.org/packages/release/bioc/html/limma.html) in R [14]. The multiple testing correction was performed to control the FDR (false discovery rate) [15]. FC (fold change) of individual gene was also calculated. The genes with FDR < 0.01 and $\log_2|FC| \geq 1$ were considered to be significant. Finally, the common DEGs between two comparison groups were obtained.

Functional and pathway enrichment analysis of DEGs. The GO (Gene ontology, http://geneontology.org/) functional analysis has become a commonly used approach for functional studies of large-scale genomic or transcriptomic data [16]. KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/pathway.html) pathway database [17] contains information of graphical diagrams of biological pathways including some of the known regulatory pathways and most of known metabolic pathways. DAVID (Database for annotation visualization and integrated discovery, http://david.abcc.ncifcrf.gov/) online tool [18] was applied to systematically extract biological meanings from large gene or protein lists. GO function in BP (biological process), CC (cell component), MF (molecular function) and KEGG pathway enrichment analysis of common DEGs were performed using DAVID (version 6.7) with $P < 0.05$.

Analysis of cancer-related genes. Genetic-association-DB-Disease analysis was performed by uploading the common DEGs to DAVID system. Then, cancer-related genes were screened out, and heatmap of cancer-related genes was generated using the heatmap.2 function of the "gplots" package in R (http://www.inside-r.org/packages/cran/gplots/docs/heatmap.2) [19].

PPI network construction. The STRING (search tool for the retrieval of interacting genes, http://string-db.org/) [20] database was used to retrieve the predicted interactions for the common DEGs. All interactions obtained from STRING are provided with a confidence score, and each score represents a rough estimate of how likely a given association describes a functional linkage between two proteins [21]. The common DEGs with a confidence score more than 0.4 including cancer-related genes were selected to construct the PPI network, visualized using the Cytoscape software (http://cytoscape.org/) [22].

Transcription factor analysis. The online tool Whole Genome RVista [23] {Zambon, 2005 #1006}(http://genome.lbl.gov/cgi-bin/WGRVistaInput5.pl?cfg_dir=gp_r4099_169), a publicly available bioinformatics program, was used to analyze the significantly enriched transcription factor binding sites in the regulatory promoter region of cancer related genes (5000 bp upstream of the transcriptional start sites). The transcription factor with $P \leq 0.1$ was screened out. Then, the network including transcription factor and cancer-related genes was visualized using Cytoscape software.

Results

Functional and pathway enrichment analysis of common DEGs. A total of 401 common DEGs between two comparison groups including 352 up-regulated genes and 49 down-regulated genes were identified (Figure 1). Functional enrichment analysis showed that the up-regulated DEGs were significantly enriched in GO terms of RNA processing, nuclear division, mitosis and M phase of mitotic cell cycle (Table 1); the down-regulated DEGs were mainly involved in regulation

of cell development, negative regulation of cell differentiation and embryonic appendage morphogenesis (Table 2). Pathway enrichment analysis showed that the up-regulated DEGs were significantly enriched in spliceosome and pyrimidine metabolism (Table 1). However, no significant pathway of down-regulated DEGs was identified.

Analysis of cancer-related genes. The functions of these common DEGs were preliminarily investigated by DAVID online servers under the Genetic Association DB Disease. The results revealed that 18 out of 401 common genes were significantly associated with cancers (Figure 2). Moreover, most of DEGs were up-regulated in both two comparison groups excluding down-regulated *EPHX1* (epoxide hydrolase 1, microsomal). Especially, only two genes of *ICAM1* (intercellular adhesion molecule 1) and *NBN* (nibrin) were related to melanoma.

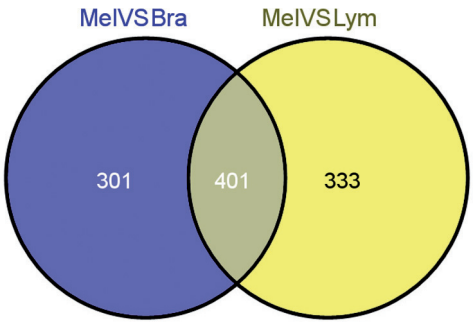

Figure 1. The venn diagram of selected differentially expressed genes (DEGs) in melanoma brain metastasis (MBM) and lymph node metastasis (LNM). MelVSBra indicates DEGs between normal melanocytes and MBM; MelVSLym indicates DEGs between normal melanocytes and melanoma LNM. The overlapped genes were common DEGs between two comparison groups.

Table 1. Gene ontology (GO) functional and pathway enrichment analysis of the up-regulated differentially expressed genes.

Category	Term	Count	P Value
GOTERM_BP_FAT	GO:0006396~RNA processing	49	4.94E-19
GOTERM_BP_FAT	GO:0000280~nuclear division	30	2.14E-16
GOTERM_BP_FAT	GO:0007067~mitosis	30	2.14E-16
GOTERM_BP_FAT	GO:0000087~M phase of mitotic cell cycle	30	3.48E-16
GOTERM_BP_FAT	GO:0048285~organelle fission	30	7.42E-16
GOTERM_CC_FAT	GO:0043232~intracellular non-membrane-bounded organelle	127	6.86E-28
GOTERM_CC_FAT	GO:0043228~non-membrane-bounded organelle	127	6.86E-28
GOTERM_CC_FAT	GO:0031981~nuclear lumen	93	2.96E-27
GOTERM_CC_FAT	GO:0070013~intracellular organelle lumen	103	4.40E-27
GOTERM_CC_FAT	GO:0043233~organelle lumen	103	2.81E-26
GOTERM_MF_FAT	GO:0003723~RNA binding	42	7.20E-11
GOTERM_MF_FAT	GO:0008135~translation factor activity, nucleic acid binding	11	1.26E-05
GOTERM_MF_FAT	GO:0003743~translation initiation factor activity	9	1.49E-05
GOTERM_MF_FAT	GO:0000166~nucleotide binding	66	7.70E-05
GOTERM_MF_FAT	GO:0032555~purine ribonucleotide binding	53	8.38E-04
KEGG_PATHWAY	hsa03040:Spliceosome	12	4.39E-06
KEGG_PATHWAY	hsa00240:Pyrimidine metabolism	7	0.004055211

BP: biological process; CC: biological process; MF: molecular function; Count: numbers of DEGs in each GO term; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Table 2. Gene ontology (GO) functional enrichment analysis of the down-regulated differentially expressed genes.

Category	Term	Count	P Value
GOTERM_BP_FAT	GO:0060284~regulation of cell development	4	0.01339419
GOTERM_BP_FAT	GO:0045596~negative regulation of cell differentiation	4	0.015398758
GOTERM_BP_FAT	GO:0035113~embryonic appendage morphogenesis	3	0.018971394
GOTERM_BP_FAT	GO:0030326~embryonic limb morphogenesis	3	0.018971394
GOTERM_BP_FAT	GO:0035108~limb morphogenesis	3	0.024158796
GOTERM_CC_FAT	GO:0016023~cytoplasmic membrane-bounded vesicle	6	0.007299266
GOTERM_CC_FAT	GO:0031988~membrane-bounded vesicle	6	0.008338855
GOTERM_CC_FAT	GO:0031410~cytoplasmic vesicle	6	0.013712789
GOTERM_CC_FAT	GO:0031982~vesicle	6	0.016251043
GOTERM_CC_FAT	GO:0016021~integral to membrane	18	0.04640683
GOTERM_MF_FAT	GO:0008134~transcription factor binding	5	0.03610483

BP: biological process; CC: biological process; MF: molecular function; Count: numbers of DEGs in each GO term.

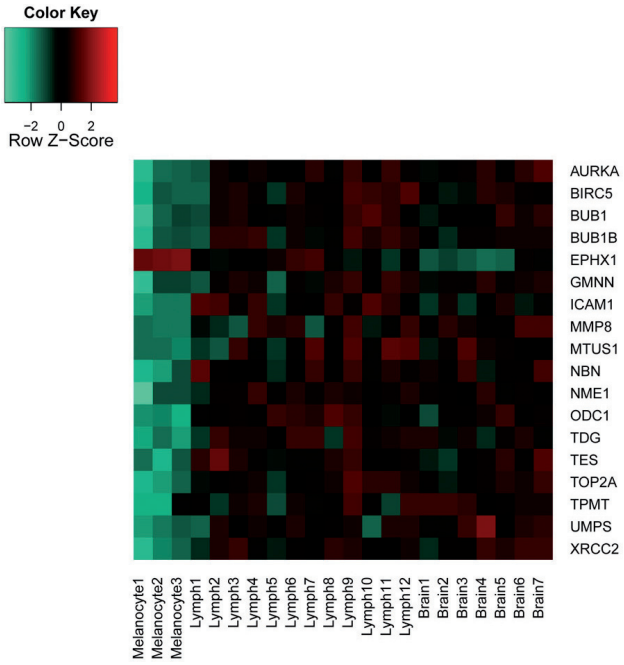

Figure 2. Heatmap of cancer-related genes.
Average gene expression value was log 2 converted. Each row indicates the common differentially expressed gene (DEG); each column indicates the sample. > 2 Red for high expression in metastatic melanoma compared with normal control and < 2 green for low expression in metastatic melanoma compared with normal control.

PPI network and transcription factor analysis. In the PPI network (Figure 3), most of genes interacted with cancer-associated genes were up-regulated. Six cancer-associated genes including *BUB1* (BUB1 mitotic checkpoint serine/threonine kinase), *GMNN* (geminin, DNA replication inhibitor), *BIRC5* (baculoviral IAP repeat containing 5), *AURKA* (aurora kinase A), *TOP2A* (topoisomerase (DNA) II alpha) and *BUB1B* (BUB1 mitotic checkpoint serine/threonine kinase B) with higher degree more than 50 were identified as key genes in metastatic melanoma. Transcription factor analysis showed the lrx2 (iroquois homeobox 2) was the key transcription factor regulating cancer-related genes *AURKA* and *BUB1B* (Figure 4).

Discussion

Melanoma causes the majority of deaths related to skin cancer [5]. Now, the large health burden of melanoma in population is enormous [24]. Thus, the potential use of new therapeutic targets appears to be the most promising area of research. In this work, we used bioinformatics approaches to predict the potential therapeutic targets for melanoma, and 401 common DEGs between two comparison groups were screened out. In addition to the known melanoma-related genes *ICAM1* and *NBN*, six abnormally expressed genes including *BIRC5*, *BUB1*, *BUB1B*, *GMNN*, *AURKA* and *TOP2A* were identified to be key genes that may play an important role in melanoma metastasis.

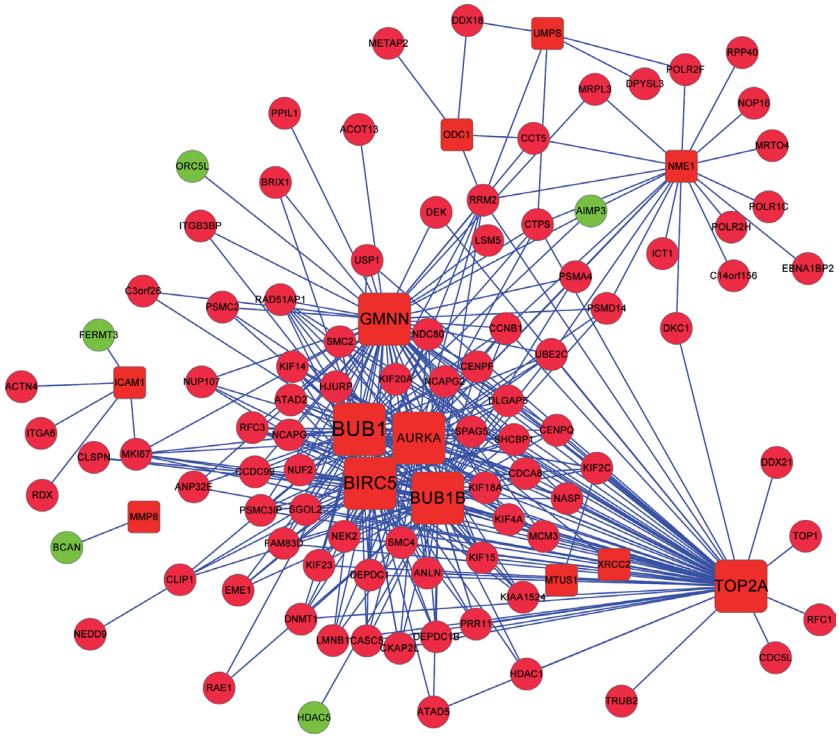

Figure 3. The protein-protein interaction network of common genes in metastatic menaloma.
Red nodes represent up-regulated genes and green nodes represent down-regulated genes. Square nodes represent cancer-related genes and large square nodes represent key genes in metastatic melanoma.

ICAM1 encodes a cell surface glycoprotein which is typically expressed in endothelial cells [25]. Johnson *et al*. have found that the increased expression of *ICAM-1* on melanoma cells may positively correlated with a greater risk of metastasis [26]. Moreover, Banks and colleagues have found that levels of *ICAM1* were significantly increased in melanoma, and *ICAM-1* has been implicated in tumor progression and metastasis [27]. Kageshita *et al*. have suggested that monitoring of serum *ICAM-1* level may contribute to monitor the clinical course of the malignant melanoma [28]. Additionally, another known melanoma-related gene *NBN* has been proved to be overexpressed in aggressive uveal melanoma, and thus considered as a prognostic marker [29]. In our studies, *ICAM-1* and *NBN* were up-regulated and related to metastatic melanoma in Genetic-association-DB-Disease analysis which was consistent with the previous study, therefore, *ICAM-1* and *NBN* may be a therapeutic target in metastatic melanoma.

In PPI network, six genes with higher degree more than 50, including *BUB1*, *GMNN*, *BIRC5*, *AURKA*, *TOP2A* and *BUB1B* were identified to be key novel genes in metastatic melanoma. *BUB1* and *BUB1B* encode a serine/threonine-protein kinase that plays a central role in mitosis [30]. In a previous study, Grabsch *et al*. have found that overexpression of *BUB1* and *BUB1B* in gastric cancer may contribute to tumor cell proliferation [30]. Pinto *et al*. have shown that overexpression of *BUB1* is associated with genomic complexity in clear cell kidney carcinomas [30]. Ricke *et al*. have proved that *BUB1* overexpression induces tumor formation through Aurora B kinase hyperactivation [31]. Notably, higher levels of *BUB1* has been shown in metastatic melanomas than primary melanoma indicating the key role of *BUB1* in metastasis of melanoma [32]. Disappointed, few studies have reported the association between *BUBIB* and metastatic melanoma. In our study, *BUB1* and *BUB1B* were up-regulated, and mainly involved in mitosis biological process which was consistent with previous study. As a result, *BUB1* and *BUB1B* might play vital role in melanoma metastasis and be predicted therapeutic targets.

BIRC5 is a member of inhibitor of apoptosis gene family and product preventing apoptotic cell death [33]. Abnormal expression of BIRC5 has been proved to be associated with prognosis and metastasis of melanoma [34]. Importantly, survivin, an anti-apoptotic protein encoded by *BIRC5*, has been shown to be correlated with progression in human melanoma and LNM [35]. *GMNN* encodes a protein that plays a critical role in cell cycle regulation [36]. Increased expression of *GMNN* may play a role in several malignancies including colon, rectal and breast cancer [37]. More recently, *GMNN* has been observed to be overexpressed in distant metastatic patients and is correlated with prognosis of melanoma [34]. *AURKA* overexpression may play a role in tumor development and progression via promoting cancer cell survival, stimulating the PI3K pathway and activating Akt [38]. Additionally, *AURKA* has been down-regulated in differentiation of metastatic HO-1 human melanoma cells, and therefore contributes to HO-1 cell proliferation [39]. Notably, *TOP2A* is considered as an oncogene in tumor and significantly involved in cell cycle, thus associated with tumor cell growth, proliferation and migration [40]. Particularly, *TOP2A* has been shown to be overexpressed in patients with metastatic melanoma [41]. In our studies, up-regulated *BIRC5*, *GMNN*, *AURKA* and *TOP2A* may be obviously involved in the melanoma development through regulating cell growth, and thus, *GMNN*, *AURKA* and *TOP2A* may be considered to be therapeutic targets in melanoma. Furthermore, transcription factor Irx2 regulated expression of *AURKA* and *BUB1B*. As we all know, vertebrate *Irx* genes are involved in neural development [42]. Specifically, Irx2 is shown to be involved in cerebellum formation through regulating FGF8/MAP kinase signaling pathway [43]. As a result, transcription factor Irx2 may play a key role in MBM by targeting *AURKA* and *BUB1B*.

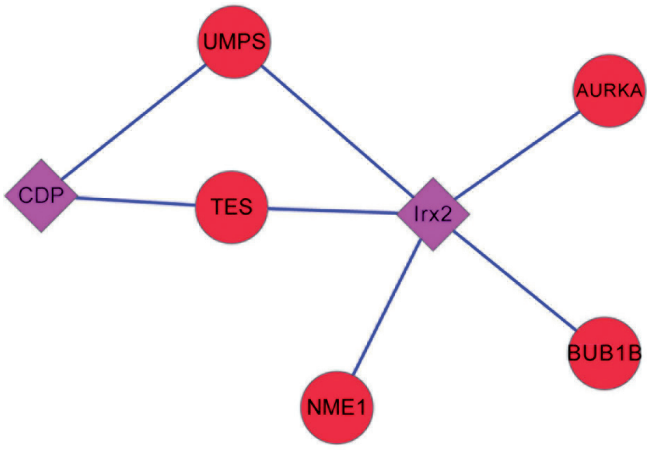

Figure 4. The transcription factor regulatory network.
The diamond nodes represent the transcription factors and the red circle nodes represent up-regulated genes.

There are some limitations in this study. First, the sample size for microarray analysis is relatively small. Other datasets will be collected in further study. Second, the genes identified in our preliminary study are required to be further confirmed by RT-PCR or western blot. As a result, further experimental studies based on a larger sample size are needed to validate our results.

Conclusions

In conclusion, the *ICAM1*, *NBN*, *BIRC5*, *BUB1*, *BUB1B*, *GMNN*, *AURKA* and *TOP2A* may play key roles in the progression and development of melanoma. They might be specific therapeutic targets in the treatment of melanoma. This report represents a comprehensive gene expression analysis of melanoma metastasis and opens up new avenues to identify novel molecular markers and therapeutic targets for metastatic melanoma therapy. However, further experiments are still needed to confirm our results.

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