Microarray data analysis of neuroblastoma: Expression of *SOX2* downregulates the expression of *MYCN*

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Abstract. The present study aimed to identify the genes directly or indirectly correlated with the amplification of *MYCN* in neuroblastoma (NB). Microarray data (GSE53371) were downloaded from Gene Expression Omnibus, and included 10 NB cell lines with *MYCN* amplification and 10 NB cell lines with normal *MYCN* copy numbers. Differentially expressed genes (DEGs) were identified using the Linear Models for Microarray Data package, and a false discovery rate of <0.05 and $|\log_2$FC (fold change)$|>1$ were selected as cut-off criteria. Hierarchical clustering analysis and Gene Ontology analysis were respectively performed for the DEGs using the Pheatmap package in R language and The Database for Annotation, Visualization and Integrated Discovery. A protein-protein interaction network (PPI) was constructed for the DEGs using the Search Tool for the Retrieval of Interacting Genes database. Pathway analysis was performed for the DEGs in the PPI network using the WEB-based GEne SeT AnaLysis Toolkit. The correlation between *MYCN* and the key gene associated with *MYCN* was determined using Pearson's correlation coefficient. In total, 137 downregulated and 35 upregulated DEGs were identified. Functional enrichment analysis indicated that *KCNMB4* was involved in the regulation of action potential in neuron term, and the *FOS*, *GLI3* and *GLI1* genes were involved in the extracellular matrix-receptor interaction pathway. The PPI network and correlation analysis revealed that the expression of *SOX2* was directly correlated with the expression of *MYCN*, and the correlation coefficient of *SOX2* and *MYCN* was -0.83. Therefore, *SOX2*, *KCNMB4*, *FOS*, *GLI3* and *GLI1* may be involved in the pathogenesis of NB, with the expression of *SOX2* downregulating the expression of *MYCN*.

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Introduction

Neuroblastoma (NB) is a cancer of the peripheral sympathetic neurons, which occurs during childhood, and the percentage of NB of all pediatric oncology-associated mortality is ~10% worldwide (1). NB predominately occurs in young children, of which the average age is 17 months (2) NB derives from undifferentiated neural crest cells and metastasizes to other organs (3). Patients with NB have poor prognosis with a 5-year survival rate of no more than 30% (4).

With the development of the field of biology, increasing numbers of markers correlated with NB have been identified. *MYCN* is a well-known prognostic marker of NB (5), and *MYCN* amplification is the most significant molecular marker of risk in NB (6). Tang *et al* demonstrated that patients with NB exhibiting low expression levels of *MYCN* and *TrkA* have a 5-year survival rate of 63.7%, whereas the 5-year survival rate of patients with high expression levels of the two is 88.1% (7). In addition, the alternative *TrkAIII* splice variant is involved in the pathogenesis of advanced stage human NB through microtubules, which are involved in the promotion and maintenance of NB cells (8). In addition to these reports, *NCYM* has been observed to act as a *cis*-antisense gene of *MYCN*, and contributes to the stabilization of *MYCN* in human NB by inhibiting *GSK3β* (9). *NCYM*, co-amplified with *MYCN*, is involved in the pathogenesis of NB (10). Although several studies have reported that *MYCN* is key in the pathogenesis of NB, the genes correlated with *MYCN* amplification remain to be fully elucidated. Therefore, the present study aimed to examine the molecular mechanism of *MYCN* amplification in NB.

Wu *et al* analyzed expression profiles using Ingenuity Pathway Analysis, and found that the aryl hydrocarbon receptor downregulates the expression of *MYCN* through the mediation of *E2F1* in the protein-protein interaction (PPI) network, and further confirmed that the aryl hydrocarbon receptor regulates the activity of the *MYCN* promoter and results in the downregulated expression of *MYCN* (11). To identify more genes correlated with *MYCN* in the present

study, the determination of differentially expressed genes (DEGs) between NB cell lines exhibiting *MYCN* amplification (*MYCN* amplification group) and NB cell lines with a normal *MYCN* copy number (control group) were identified. Hierarchical clustering and Gene Ontology (GO) analysis were performed for these DEGs, and a protein-protein interaction (PPI) network was constructed for the DEGs to identify the key genes associated with *MYCN*. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was also performed for the DEGs in PPI network. The correlation of *MYCN* and the key gene associated with to *MYCN* was determined using Pearson's correlation coefficient, in order to investigate whether their association was synergistic or antagonistic.

Materials and methods

Microarray data. Microarray data (accession. no. GSE53371) were downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) (11). The microarray platform of GSE53371 was GPL887 Agilent-012097 Human 1A Microarray (V2) G4110B (Feature Number version; Agilent Technologies, Inc., Santa Clara, CA, USA). A total of 20 samples were available, which included 10 NB cell lines with *MYCN* amplification and 10 NB cell lines with a normal *MYCN* copy number.

Data preprocessing and identification of DEGs. Normalization of the microarray data were performed using a median normalization method (12). The DEGs were identified using the Linear Models for Microarray Data (Limma) package (13). The raw P-value was adjusted into a false discovery rate (FDR) using the Benjamini & Hochberg method (14,15). The genes with an FDR <0.05 and $|\log_2 FC$ (fold change)$|>1$ were considered significantly different between the *MYCN* amplification group and the control group.

Hierarchical clustering analysis of DEGs. Based on the Euclidean distance, which is the actual distance between two points that may be calculated using the Pythagorean formula, hierarchical clustering analysis (16) was performed to evaluate the sample specificity. In the hierarchical clustering analysis, the expression value of the DEGs were extracted, and subsequent analysis was performed of the DEGs using the Pheatmap package in R language (http://cran.r-project.org/web/packages/pheatmap/index.html) (16,17).

Functional enrichment analysis of DEGs. The Database for Annotation, Visualization and Integrated Discovery (DAVID) is a comprehensive functional annotation tool (18). Gene Ontology (GO) analysis was performed for the DEGs using the DAVID database (http://david.abcc.ncifcrf.gov/). The raw P-value was adjusted into the FDR using Benjamini & Hochberg's method and an FDR <0.05 was selected as the cut-off criterion.

Construction of the PPI network. The Search Tool for the Retrieval of Interacting Genes (STRING) database is used to collect and predict PPI associations (19). In the present study, a PPI network was constructed for the DEGs using STRING, and a confidence score >0.4 was selected as the cut-off criterion. Subsequent visualization of the PPI network was performed using Cytoscape (http://cytoscape.org/) (20).

Pathway analysis of DEGs in the PPI network. The WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) aims to examine large sets of genes, and is used for functional enrichment analysis, including GO enrichment, pathway enrichment and transcription factor analysis, by integrating functional categories (21,22). KEGG (http://www.genome.ad.jp/kegg) pathway enrichment analysis was performed for the DEGs in the PPI network using WebGestalt, and P<0.05 was selected as the cut-off criterion.

Correlation analysis. Based on the PPI network, the expression values of the genes correlated directly with *MYCN* were extracted, and correlation analysis was performed using the expression values of the genes. For this correlation analysis, the correlation of *MYCN* and the key gene associated with *MYCN* was determined using Pearson's correlation coefficient (23) with SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Identification of DEGs. In total, 172 DEGs between the *MYCN* amplification group and the control group were identified, which included 137 downregulated DEGs and 35 upregulated DEGs.

Hierarchical clustering analysis of the DEGs. Following hierarchical clustering analysis, there were two distinct gene clusters identified, including the *MYCN* amplification cluster and control cluster (Fig. 1). These results suggested that there were two different gene expression patterns with marked color differences, which were available for use to distinguish between the *MYCN* amplification samples and the control samples.

GO enrichment analysis of the DEGs. GO enrichment analysis revealed that the five enriched terms were as follows: Regulation of action potential in neuron (P=1.24E-05), myelination (P=2.95E-05), regulation of action potential (P=4.71E-05), ensheathment of neurons (P=4.85E-05) and axon ensheathment (P=4.85E-05), as listed in Table I. The *KCNMB4*, *PLP1*, *CLDN1*, *LGI4*, *MAL*, *PMP22* and *GAL3ST1* genes were involved in the regulation of action potential in neuron biological process, and the percentage of the genes enriched in this term was 21.88% (Fig. 2).

PPI network analysis of DEGs and pathway analysis. Analysis of the PPI network revealed that there were 85 nodes and 142 gene links, and *SOX2* was identified as the key gene correlated directly with *MYCN* (Fig. 3).

KEGG pathway enrichment analysis of the DEGs in the PPI network indicated that the *ASPA*, *MAOA* and *ALDH1A3* genes were involved in the histidine metabolism pathway (P=0.012376), and the *LAMA2*, *FOS*, *ITGA6*, *GLI3*, *GLI1* and *RAD51* genes were involved in the extracellular matrix (ECM)-receptor interaction pathway (P=0.040716; Table II).

Table I. Top five most enriched GO terms for the differentially expressed genes.

GO ID	GO term	Gene count	P-value	FDR	Gene
GO:0019228	Regulation of action potential in neuron	7	1.24E-05	0.015898	*KCNMB4, PLP1, CLDN1, LGI4, MAL, PMP22, GAL3ST1*
GO:0042552	Myelination	6	2.95E-05	0.018878	*PLP1, CLDN1, LGI4, MAL, PMP22, GAL3ST1*
GO:0001508	Regulation of action potential	7	4.71E-05	0.020080	*KCNMB4, PLP1, CLDN1, LGI4, MAL, PMP22, GAL3ST1*
GO:0007272	Ensheathment of neurons	6	4.85E-05	0.015539	*PLP1, CLDN1, LGI4, MAL, PMP22, GAL3ST1*
GO:0008366	Axon ensheathment	6	4.85E-05	0.015539	*PLP1, CLDN1, LGI4, MAL, PMP22, GAL3ST1*

GO, Gene Ontology; FDR, false discovery rate.

Table II. Top two most enriched KEGG pathways of the differentially expressed genes in the protein-protein interaction network.

ID	KEGG pathway	Gene count	P-value	Gene
hsa00340	Histidine metabolism	3	0.012376	*ASPA, MAOA, ALDH1A3*
hsa04512	ECM-receptor interaction	6	0.040716	*LAMA2, FOS, ITGA6, GLI3, GLI1, RAD51*

KEGG, Kyoto Encyclopedia of Genes and Genomes; ECM, extracellular matrix.

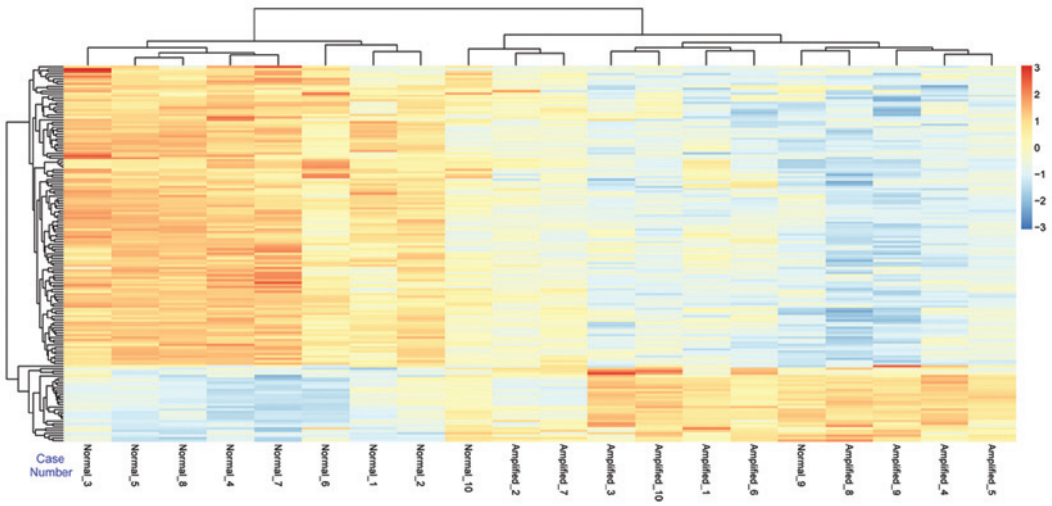


Figure 1. Hierarchical clustering analysis of differentially expressed genes. Red represents the higher levels of gene expression; blue represents lower levels of gene expression.

Correlation analysis of genes. Correlation analysis indicated that the expression of *SOX2* was inversely correlated with the expression of *MYCN* in the NB cell lines, and the correlation coefficient of *SOX2* and *MYCN* was -0.83 (Fig. 4).

Discussion

In the present study, 172 DEGs between the *MYCN* amplification group and control group were identified, which included 137 downregulated and 35 upregulated DEGs. Functional enrichment analysis indicated that the *KCNMB4* gene was involved in the regulation of action potential in neuron pethway, and the *FOS*, *GLI3* and *GLI1* genes were involved in the ECM-receptor interaction pathway. Correlation analysis demonstrated that the expression of *SOX2* was inversely correlated with the expression of *MYCN* in the NB cell lines.

Neurons encode and convey information by generating action potentials (24). According to a previous report by Hall *et al*, the expression of specific Kv3.1 glycoprotein, which is a voltage-gated potassium (Kv) channel, has an

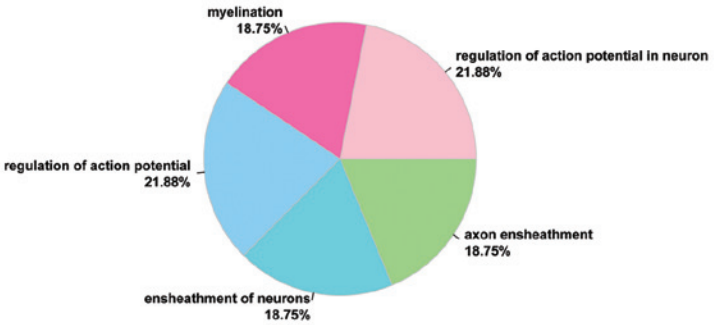


Figure 2. Pie chart of Gene Ontology enrichment analysis for the differentially expressed genes. The percentage was proportional to the differentially expressed genes enriched in each function.

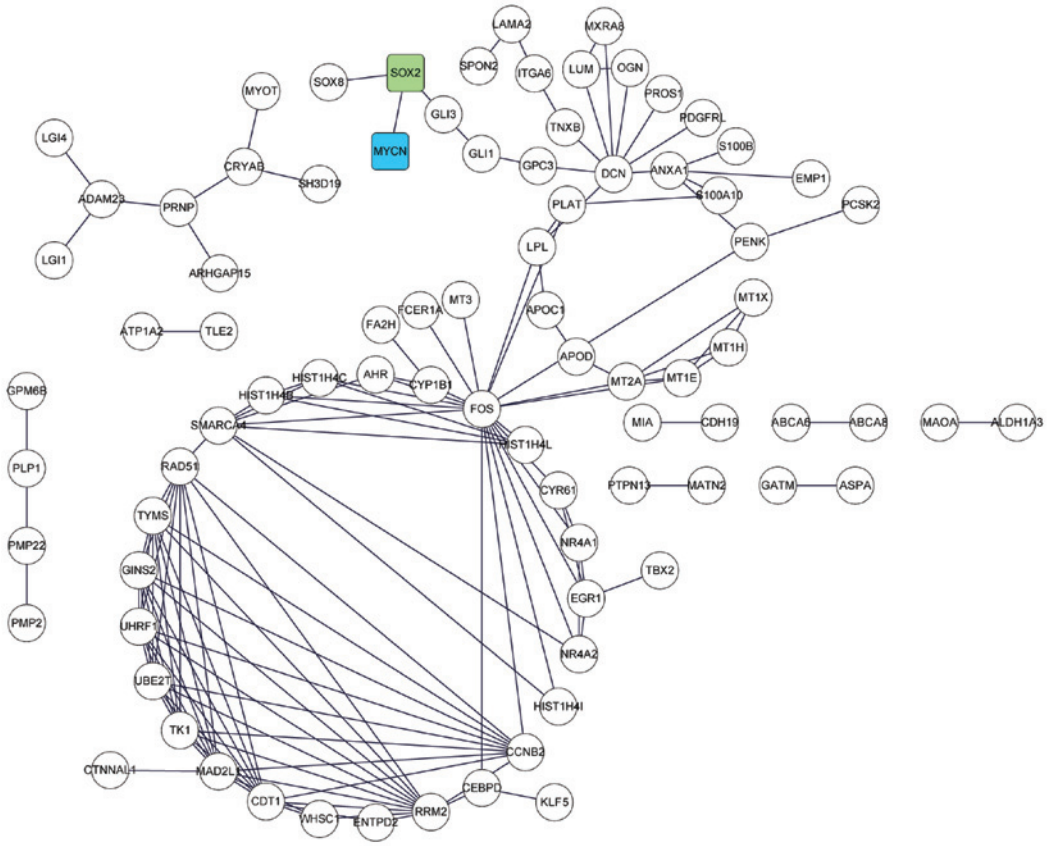
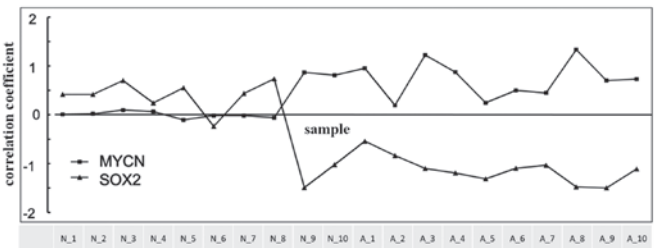


Figure 3. Protein-protein interaction network of differentially expressed genes. The blue square node represents *MYCN*; the green square node represents *SOX2*. The line between two genes represents their interaction.

impact on the wave form of action potentials and *N*-glycans associated with the Kv3.1 protein affect the migratory rate of The NB cell (25). In addition, Leung *et al* demonstrated that the voltage-gated Kv channel has an effect on cAMP-stimulated neuritogenesis in mouse NB N2A cells (26). These findings suggest that the voltage-gated Kv channels are important in the development of NB through the modulation of action potentials. Large-conductance Ca^{2+}-activated K^+ (BK) channels contribute to the excitability of neurons (27). The two subunits of BKβ are BKβ3 (KCNMB3) and BKβ4 (KCNMB4) (28). Therefore, KCNMB4 may be involved in the pathogenesis of NB through the voltage-gated Kv channel. In the present study, *KCNMB4* was involved in the regulation of action potential in neuron biological process.



	N_1	N_2	N_3	N_4	N_5	N_6	N_7	N_8	N_9	N_10	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_10
MYCN	0.008	0.02	0.097	0.065	-0.11	-0.02	-0.02	-0.06	0.867	0.808	0.952	0.194	1.224	0.873	0.244	0.499	0.448	1.334	0.701	0.73
SOX2	0.416	0.416	0.699	0.242	0.552	-0.24	0.437	0.736	-1.49	-1.02	-0.54	-0.84	-1.1	-1.19	-1.31	-1.1	-1.03	-1.48	-1.5	-1.11

	MYCN
SOX2	-0.83

Figure 4. Correlation analysis of the expression levels of *SOX2* and *MYCN*. Correlation coefficient= -0.83. Values were obtained relative to the control.

Based on these findings, it was hypothesized that *KCNMB4* is involved in the development of NB by the regulation of action potentials in neurons.

SOX2, located in chromosome 3q26.33, is a transcription factor which is involved in the regulation of stem cell properties (29,30). Previously, it has been reported that neural stem cell-like cells are isolated from certain types of neural cancer, including glioblastoma, medulloblastoma and NB (31,32). In addition, Das *et al* demonstrated that *SOX2* is involved in the maintenance of undifferentiated stem cells by the regulation of all-trans retinoic acid, which induces the differentiation of NB cells. Furthermore, *miR-340* has been found to be involved in the pathogenesis of NB by mediating the expression of *SOX2* (33). Wang *et al* found that *FoxM1* is involved in the tumorigenicity of aggressive NB cells by activating the expression of *SOX2* (34). According to a report by Yang *et al*, the expression of *OCT4* and *SOX2* are involved in the progression of NB (35). These results indicate that *SOX2* is important in the pathogenesis of NB. The results of the present study, which revealed that the expression of *SOX2* was directly correlated with the expression of *MYCN*, were consistent with previous findings. Therefore, *SOX2* may be involved in the pathology of NB through the regulation of *MYCN*.

In the present study, PPI network analysis indicated that *GLI1* regulated *SOX2* by the mediation of *GLI3*, and *FOX* also regulated the expression of *SOX2* by the mediation of *DCN*. *GLI1* and *GLI3* are mediators of the *SHH* pathway, which has an effect on the early development of the central nervous system (36). In addition, *SHH-GLI1* is involved in the self-renewal of cancer stem cells (37). Furthermore, Shahi *et al* reported that the expression of *SMO* and *GLI3* are partially correlated in NB (38). These results suggest that *GLI1* and *GLI3* may be involved in the development of NB by the mediation of *SOX2*. In addition, *FOX* proteins are a conserved transcriptional regulator superfamily, and *FOX* family transcription factors are key in the progression of cancer (39). In NB, *FOXR1*, fused to *MLL* or *PAFAH1B* due to interstitial deletions, function as oncogenes (40). Santo *et al* demonstrated that events of intra-chromosomal deletion/fusion at 11q23 activate the expression of *FOXR1* in NB (41). This suggested that *FOX* may also be involved in the pathogenesis of NB by the regulation of *SOX2*. According to these findings, the present study hypothesized that *FOS*, *GLI3* and *GLI1* may regulate the expression of *MYCN* by the mediation of *SOX2*. Although the findings of the present study were consistent with previous observations that the *FOS*, *GLI3* and *GLI1* genes are involved in the pathogenesis of NB, the present study revealed that the *FOS*, *GLI3* and *GLI1* genes were involved in the ECM-receptor interaction pathway. Therefore, *FOS*, *GLI3* and *GLI1* may be involved in the ECM-receptor interaction pathway to contribute to the development of NB.

In conclusion, the present study identified 172 DEGs between the *MYCN* amplification group and the control group, including 137 downregulated and 35 upregulated DEGs. *KCNMB4* may contribute to the development of NB by the regulation of action potentials in neurons. *FOS*, *GLI3* and *GLI1* may be involved in the pathogenesis of NB by the regulation of *SOX2*, the expression of which was inversely correlated to the expression of *MYCN* in the NB cell lines. These findings have provided a novel opportunity to investigate the pathogenesis of NB, however, these results require confirmation by further investigations.

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