Original Article
Gene expression profile study of the SOX2 siRNA effect on neuroblastoma BE (2)-C cells

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Abstract: Neuroblastoma (NB) is the most common extracranial, solid paediatric tumour, and the molecular mechanisms responsible for the pathogenesis of NB remain elusive. In our previous studies, we found that SOX2 was highly expressed in NB tissues, and its expression correlated with the NB clinical stage. We further found that in I-type neuroblastoma cells (BE (2)-C cells) while SOX2 mRNA expression was knocked down by gene-specific siRNA (small interference RNA), the tumourigenicity of the cells was significantly decreased, but the cells had increased expression levels of marker proteins of N- or S-type cells. The objective of this study was to investigate the effect of SOX2 siRNA on the gene expression profiles of neuroblastoma BE (2)-C cells. According to the results of the gene expression study, 1744 differentially expressed genes (DEGs) were discovered; 596 DEGs related to SOX2 siRNA were determined by cluster analysis. Ten Gene Ontology (GO) functional categories with the smallest *P*-values in the upregulated and downregulated clusters were determined by GO analysis. Some crucial signalling pathways (Wnt, MAPK, ErbB, etc.) are known to be affected by these genes. These results demonstrate that SOX2 regulates several significant genes, which in turn activate important pathways such as the Wnt signalling pathway, ultimately playing a key role in the pathogenesis and progression of NB.

Keywords: Neuroblastoma, Sox2, gene expression profile

Introduction

Neuroblastoma (NB) is the most common extracranial solid paediatric tumour and accounts for 8-10% of childhood cancers [1]. Despite multimodal treatment, the morbidity and mortality still remain high. Therefore, further exploration of the molecular mechanisms responsible for the pathogenesis of NB is essential.

Cancer stem cells (CSCs) have been described as a small subset of cells within the tumour that have higher tumourigenicity, differentiation ability and self-renewal ability [2]. With these characteristics, CSCs have been thought to be related to cancer recurrence and distant metastasis. Additionally, CSCs have been shown to be related to resistance to various treatments. Thus, CSCs are likely to be the most relevant targets for the treatment of NB, and further studies on the characterization of these cells will help in the design of more successful NB therapies. Based on morphological appearances, biochemical properties, and growth pat-

terns, three major cell types have been identified in NB cell lines. These have been designated N-(neuroblastic), S-(substrate-adherent and non-neuronal), and I-type (intermediate) NB cells [3]. I-type NB cells are considered to be the NB cancer stem cells [4, 5].

SOX2 is a member of the SOX (SRY-related high mobility group box) gene family, which contains a high mobility group (HMG) domain that is very similar to what is found in the sex-determining gene SRY [6]. SOX2 plays an important role in the maintenance of self-renewal and the potential for differentiation. Other studies have found that SOX2 is essential for the self-renewing proliferation of many normal and cancer stem cells [7-11]. Although the expression of Sox2 has been reported in several cancers and cancer cell lines, their expression in NB has rarely been reported. Gomez-Mateo *et al* evaluated the expression and clinical significance of Sox2 in NB using immunohistochemical staining and found that high Sox2 protein levels correlate to aggressive disease, indicating that Sox2 pro-

Table 1. Real-time PCR primers

Gene	Primer sequence	Annealing temperature (°C)	Product length (bp)
CARTPT	AGCAACGACGAGTTTCAGAACG GGCATCATCCACGGCAGAGTAG	60	163
HIST1H2BK	GCTGCTCGTCTCAGGCTCGTAG CGCTTCTTGCCGTCCTTCTTCT	63	217
AHNAK2	GAAGGAGGACACGGATGTTGCT CCCGCTTGCTCTTTATGGATTG	60	170
FMN1	ATTTCCACCGATGATGACTGCC ATGCCTTCTGCTGATTTGATGC	60	238
GFRA2	GCTCCTCCTACATCTCCATCTGC TTGGGCTTCTCCTTGTCCTCAT	60	123
PRR11	CACCCTCACCAGAAAGAGTCGG GTGGCAGATACGAGATGGAAAGAT	60	215
EPO	GTCCTGCCTGGCTGTGGCTTCT GCTGCCCGACCTCCATCCTCTT	63	240
TIMP2	GTAGAAATGGGAGCGAGAAACC GCCAGAACAGGCAAGAAGCAAT	60	225
TSPAN8	AGGACAAGCCTGTAACGAATAG ACCACATAGCCAGAACAAGAAG	58	130
PLP2	CCCTGTCGGTGATTGAGATGAT GGTAGAGGATTGCCGCTATGAG	60	135
GAPDH	ACCCAGAAGACTGTGGATGG TTCAGCTCAGGGATGACCTT	59	125

tein levels may be an important prognostic indicator of NB [12]. Bao J *et al* performed microarray data analysis of neuroblastoma and found that Sox2 KCNMB4, FOS, GLI3 and GLI1 may be involved in the pathogenesis of NB, with the expression of Sox2 downregulating the expression of MYCN [13].

In our previous studies, we found that SOX2 was highly expressed in human NB tissues, and its expression correlated with the NB clinical stage [14]. We further found that SOX2 overexpressing I-type NB cells showed higher tumourigenicity than control cells and exhibited decreased expression levels of marker proteins of N or S-type cells after chemically induced differentiation. By contrast, in cells where SOX2 mRNA expression was knocked-down by gene-specific siRNA, their tumourigenicity was significantly decreased, and the expression levels of marker proteins of N- or S-type cells were upregulated [15]. These findings indicate an important function for SOX2 in promoting the tumourigenicity of I-type neuroblastoma cells and inhibiting their differentiation, suggesting

that SOX2 might be a potential therapeutic target in neuroblastoma.

The object of this study was to investigate the effect of SOX2 siRNA on the gene expression profiles of I-type neuroblastoma cells using the human neuroblastoma cell line BE (2)-C as a model. We used microarray analysis to screen genes regulated by SOX2 via comparing expression profiles of BE (2)-C cells and BE (2)-C-SOX2-shRNA cells.

Materials and methods

Cell lines and cell culture

The NB cell line BE (2)-C and the retrovirus packaging cell line 293T were purchased from the American Type Culture Collection (Manassas, VA, USA). BE (2)-C was cultured in DMEM/F12 (Gibco-BRL, New York, NY, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Biochrom, Berlin, Germany) at 37°C in a humidified 5% CO_2 atmosphere; 293T cells were maintained in DMEM (Gibco-BRL) and supplemented with 10% FBS, 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 μg/mL streptomycin (Sigma-Aldrich).

Microarray experiment

BE (2)-C cells were transfected with SOX2 shRNA. Forty-eight hours after transfection, cells were collected. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to microarray analysis. The microarray experiments were performed at the Gminix Company (Shanghai, China) whose GeneChip microarray service was certificated by Affymetrix. Affymetrix Human Gene 1.0ST Arrays (Affymetrix, Santa Clara, CA, USA) were used for microarray hybridization; they carry 760,000 probe sets representing 28,869 well-characterized human genes. Statistically significant, differentially expressed genes (DEGs) were filtered by scatterplot, and genes with a
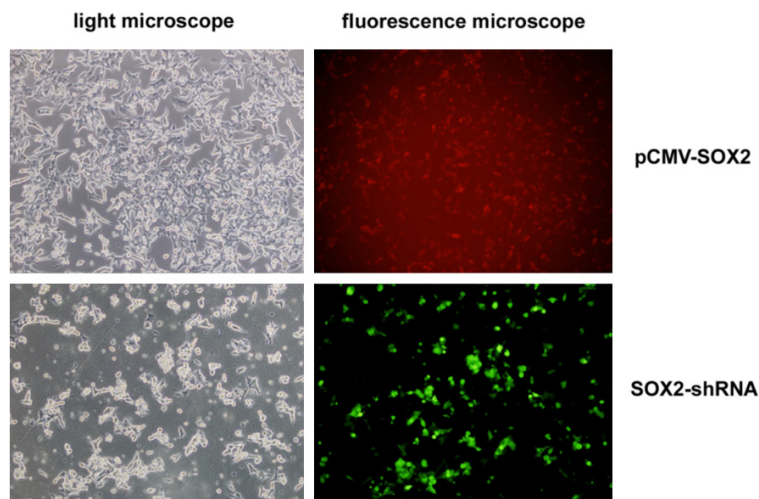
Figure 1. SOX2 shRNA was stably transfected in BE (2)-C cells. Green fluorescence staining demonstrates the efficiency of the transfection (>90%).
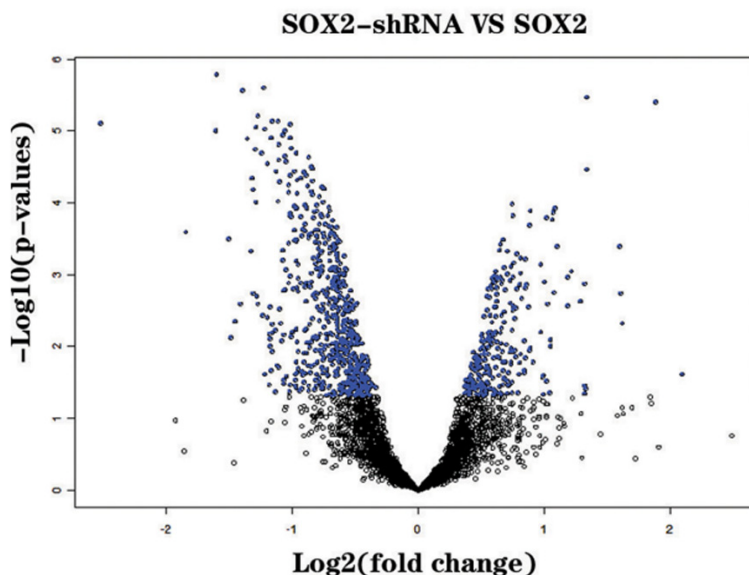

Figure 2. Gene expression scatter plot of SOX2 and SOX2-shRNA groups. Blue and black points represent differentially and non-differentially expressed genes, respectively. X-axis: fold-change; Y-axis: *P*-value; CN101: SOX2-shRNA group; BE (2)-C: SOX2 group.

2-fold change at a *P*-value < 0.05 (as calculated by a T-test) were identified as DEGs (a fold-change ≥ 2 was defined as upregulated expression, and a fold-change ≤ 0.5 was defined as downregulated expression).

Bioinformatics analysis

The genes related to SOX2 downregulation were screened using the Database for Annotation, Visualization and Integrated Discovery (DAVID) and GoSurfer software. Then, the DEGs

were analysed using three methodologies: cluster analysis (Cluster and TreeView software), GO (Gene Ontology) functional classification analysis (DAVID software), and biological pathway analysis (GenMAPP software). The enrichment was quantified using Fisher's exact test. Bonferroni correction was used to adjust for multiple testing. The false discovery rate (FDR) values were determined using the Bonferroni-corrected *P* values for multiple tests.

Real-time PCR verification

To verify the microarray findings, several differentially expressed genes were further analysed in this study using qRT-PCR (quantitative reverse transcription polymerase chain reaction) (**Table 1**). GAPDH was used as a housekeeping gene (Gminix Company, Shanghai, China).

Results

Gene expression study

To investigate the effect of SOX2 siRNA on the gene expression profiles of neuroblastoma BE (2)-C cells, SOX2 shRNA was stably transfected in BE (2)-C cells (**Figure 1**). The Sox2 mRNA level of the Sox2-shRNA cells was less than half of the BE (2)-C group, and the protein expression level of SOX2 was confirmed by western blot analysis. In total, 1744 genes were expressed in the two cell groups. A gene expression scatterplot is shown in **Figure 2**. Through genechip screening, 596 differentially expressed genes were found between the SOX2 and SOX2-shRNA groups (*P*<0.05).

Cluster analysis

Hierarchical clustering results obtained from the TreeView software are shown in **Figure 3**. The SOX2 and SOX2-shRNA groups were clus-
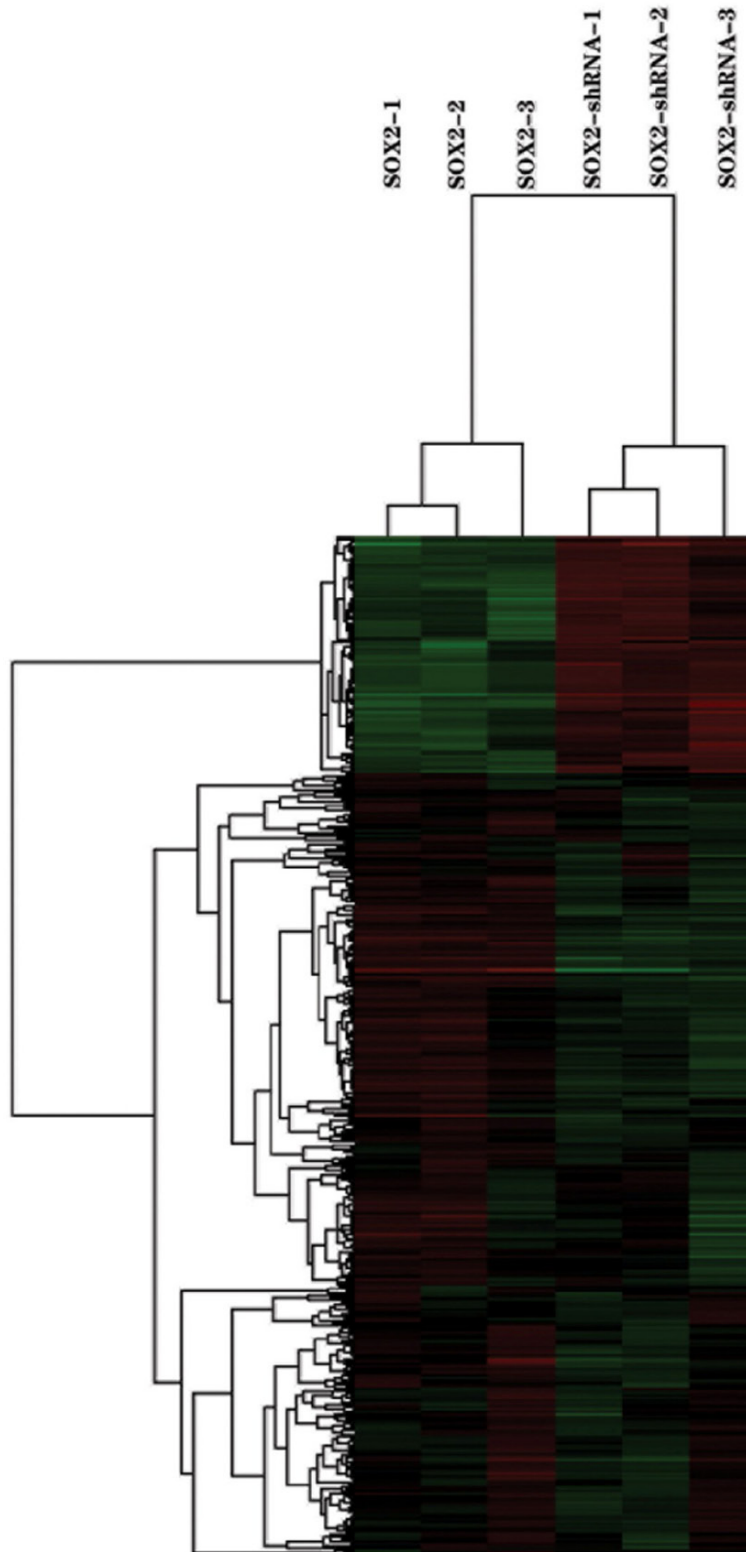
Figure 3. Cluster analysis of genes in the SOX2 and SOX2-shRNA groups. The intensity of the red/green represents the magnitude of change in expression for each gene. Red represents gene upregulation, while green indicates downregulation. Each experiment was done in triplicate.

tered into subgroups. According to the gene expression results, the 596 genes were divided into an upregulated cluster (153 genes) and downregulated cluster (443 genes).

GO analysis

Table 2 shows the GO biological function of the two clusters (the upregulated cluster and the downregulated cluster) as classified using DAVID software. The 10 GO functional categories with the smallest *P*-values in the upregulated and downregulated clusters were divided into two classes: Class I genes were unique functional categories in the upregulated cluster; Class II genes were unique functional categories in the downregulated cluster. The *P*-values in the table indicate the correlation between gene entries and their corresponding functional category. Smaller *P*-values indicate a larger correlation and more credible results.

Biological pathway analysis

Through GenMAPP analysis of the biological pathways related to genes altered by SOX2 silencing, 15 biological pathways were obtained. Among them, the pathways related to NB included the Wnt signalling pathway, the MAPK signalling pathway, the ErbB signalling pathway, and the neurotrophin signalling pathway.

Real-time PCR verification

Real-time PCR results are shown in **Figure 4**. Compared with the control group, all of the 10 genes were differentially expressed, and the expression trends were consis-

Table 2. The distribution of up- and down- regulation gene clusters in GO biological process at third level

NO	GO term	Upregulated cluster			Downregulated cluster		
		P value	Count	Proportion (%)	P value	Count	Proportion (%)
I	1 cell death	1.95e-2	9	9.09			
	2 death	2.03e-2	9	9.09			
	3 positive regulation of protein kinase activity	2.11e-2	5	5.05			
	4 positive regulation of kinase activity	2.37e-2	5	5.05			
	5 positive regulation of transferase activity	2.68e-2	5	5.05			
II	1 homophilic cell adhesion				7.69e-4	10	2.82
	2 biological adhesion				1.38e-3	26	7.34
	3 cell adhesion				1.41e-3	26	7.34
	4 cell-cell adhesion				5.67e-3	13	3.67
	5 female gonad development				6.73e-3	6	1.69

tent with the results of the microarray experiment.

Discussion

Neuroblastoma, the most common extra-cranial malignant tumour in childhood, is the primary cause of death from pediatric cancer for children between the age of 1 and 5 years and accounts for approximately 13% of all pediatric cancer mortality [16]. NB originates from primordial neural crest cells that normally give rise to the adrenal medulla and sympathetic neural ganglia. Its clinical impact and its unique biology have made this aggressive malignancy the focus of numerous translational research effort.

In our previous studies, we found that SOX2 was overexpressed in human NB tissues, and its expression correlated with the clinical stage of NB but not with other clinicopathological parameters, including patient gender and age, tumour size, location and histological classification [14]. These findings suggest that the expression of SOX2 might be related to the pathogenesis and progression of NB. We further established stable cell lines that overexpressed and had downregulated expression of SOX2 by infecting BE (2)-C cells with lentiviral transduction vectors. This was followed by an exploration of the functions of SOX2 in NB. SOX2 promoted BE (2)-C cell proliferation, colony formation and tumourigenesis. Additionally, SOX2 maintained BE (2)-C cells in an undifferentiated state [15]. Our results may provide fur-

ther evidence for the cancer stem cell theory. Additionally, our results may imply the existence of undifferentiated cells in these tumours, in which SOX2 contributes to the characteristics of these cells. These results demonstrate that SOX2 might play an important role in NB tumourigenesis and suggests a possible therapeutic target in NB.

In the current study, we used microarray analysis to screen genes regulated by SOX2 by comparing expression profiles of BE (2)-C cells and BE (2)-C-SOX2-shRNA cells. We found 1744 differentially expressed genes due to SOX2 downregulation. The SOX2 and SOX2-shRNA experimental groups were clustered into subgroups. According to the gene expression results, the 596 DEGs were divided into an upregulated cluster (153 genes) and a downregulated cluster (443 genes). Ten GO functional categories with the smallest P-values in the upregulated and downregulated clusters were divided into two classes: Class I, with unique functional categories in the upregulated cluster; and Class II, with unique functional categories in the downregulated cluster. The influence of the two clusters on the positive and negative regulation of corresponding functions were not the same. The P-values in the downregulated cluster were less than those in the upregulated cluster, and the gene number of the former was more than the latter (**Table 2**). These suggested that SOX2 were dominant in negative regulation.

There were several important biological pathways in the 21 activated biological pathways in
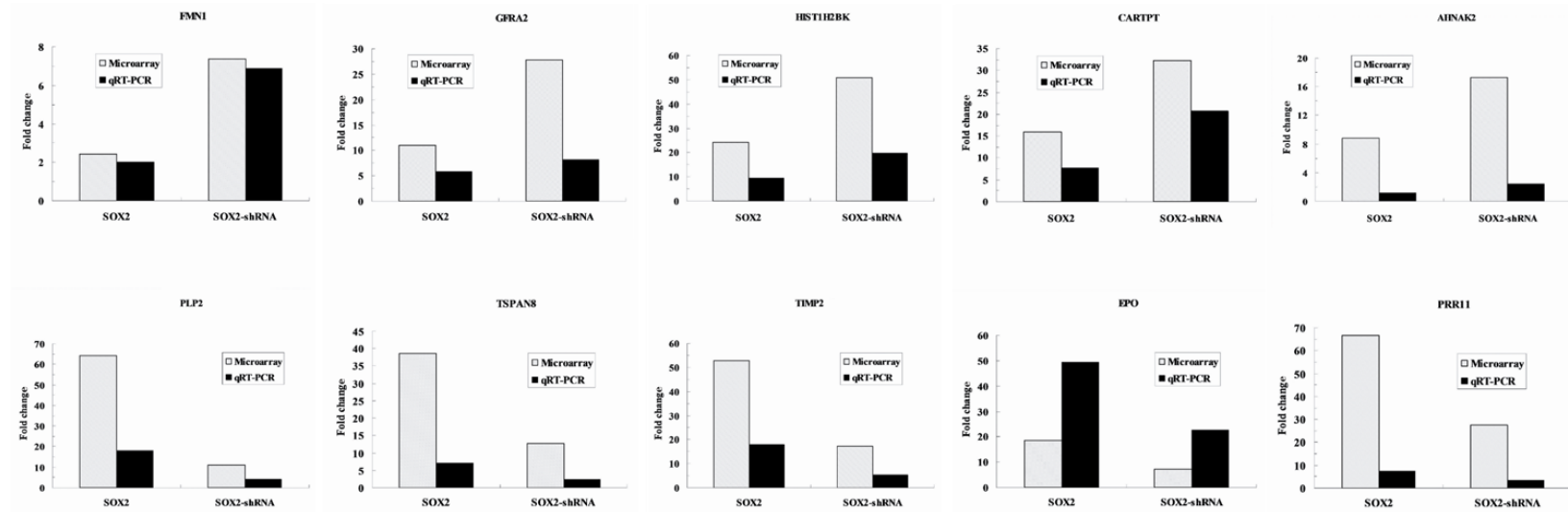
Figure 4. Comparison of the expression of genes affected by SOX2 knockdown in real-time PCR and microarray experiments. The result of Real-time PCR verification is consistent with the result of microarray experiments.

this study (data not shown), such as the ErbB, MAPK, and Wnt signalling pathways. These signalling pathways activated by SOX2-shRNA are closely related to cancer growth, proliferation, and differentiation. These activated pathways work together to regulate NB cell proliferation and differentiation.

Ten differentially expressed genes showed good consistency in both real-time PCR and microarray experiments (**Figure 3**). The PCR results not only confirmed the reliability of the microarray experiments but also demonstrated that SOX2 may promote the biological characteristics of NB by regulating genes related to those characteristics.

In summary, we found that there were 596 differential expressed genes in NB cells after exposure to SOX2 siRNA, and these genes were then subjected to cluster analysis. Ten GO functional categories with the smallest *P*-values in the upregulated and downregulated clusters were determined by GO analysis. Some important signalling pathways (Wnt, MAPK, ErbB, etc.) are known to be affected by these genes. These results demonstrate that SOX2 regulates significant genes and then activates pathways such as the Wnt signalling pathway and ultimately plays an important role in the pathogenesis and progression of NB, and suggests a possible therapeutic target in NB.

Disclosure of conflict of interest

None.

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