Oct4 and Sox2 are overexpressed in human neuroblastoma and inhibited by chemotherapy

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Abstract. Neuroblastoma (NB) is the most common extracranial solid tumor in childhood and the most frequently diagnosed neoplasm during infancy. Oct4 and Sox2 are essential transcription factors for embryonic development and play key roles in determining the fate of stem cells. In this study, we aimed to investigate the expression of Oct4 and Sox2 in NB tissues, and evaluated their relationship with various clinicopathological parameters. Oct4 and Sox2 expression in 65 samples of NB and paracancerous tissues was examined by real-time PCR. The relationship between Oct4 and Sox2 expression and clinical data was assessed. To detect Oct4 and Sox2 expression at the protein level, western blot analyses and immunohistochemical staining were employed. We found that the expression levels of Oct4 and Sox2 in NB tissues were significantly higher than those in paracancerous tissues ($P<0.05$). Oct4 and Sox2 expression was significantly correlated to the clinical stage of NB, but not other clinicopathological parameters including patient gender and age, tumor size, location and histological classification. In stage III and IV tumors, Oct4 and Sox2 expression was significantly decreased in the chemotherapy subgroup as compared with that of the non-chemotherapy subgroup ($P<0.05$). These findings suggest that the expression of Oct4 and Sox2 may correlate with the genesis and progression of NB. In addition, Oct4 and Sox2 expression can be inhibited by chemotherapy.

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Abbreviations: NB, neuroblastoma; CSCs, cancer stem cells; NBSC, neuroblastoma stem cells; ESCs, embryonic stem cells; SOX, SRY-related high mobility group box; HMG, high mobility group; PanINs, pancreatic intraepithelial neoplasias; VCR, vincristine; CTX, cytoxan; VM-26, teniposide; INSS, international neuroblastoma staging system; qRT-PCR, quantitative reverse transcription polymerase chain reaction; HRP, horseradish peroxidase

Key words: neuroblastoma, Oct4, Sox2, chemotherapy

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in childhood and the most frequently diagnosed neoplasm during infancy (1). NB is characterized as biologically and clinically heterogeneous tumors that range from spontaneously regressing growth to aggressively malignant and incurable. The tumor originates from embryonal neural crest cells that are committed to development of the sympathetic nervous system. The clinical outcome is associated with patient age, tumor stage, MYCN amplification and histological classification. Although major advances have been made in surgery and chemotherapy of NB, the morbidity and mortality remain high. Thus far, the molecular mechanisms responsible for the pathogenesis of NB remain elusive. Because the self-renewal capacity of cancer cells is similar to that of stem cells, the theory that cancer is a stem cell disease has recently received considerable attention. Various cancers, such as brain, breast, bladder, colon, pancreas and prostate cancers, have been shown to contain tumor-initiating stem cells, also known as cancer stem cells (CSCs) (2-6). Moreover, NB stem cells (NBSCs) have been isolated from NB cell lines (7,8).

Stem cell transcription factors Oct4 and Sox2 are expressed in both embryonic and adult stem cells and are necessary to maintain self-renewal and pluripotency in embryonic stem cells (ESCs) and germ cells (9,10). Cancer cells, particularly in poorly differentiated and undifferentiated tumors, have been characterized by numerous phenotypic traits that are similar to undifferentiated embryonic cells. These similarities suggest the expression of genes that participate in the regulation of stem cell properties. In recent years, Oct4 and Sox2 expression has been detected in various tumors and are proposed as useful markers of these tumors.

Although the expression of Oct4 and Sox2 has been studied in multiple tumor types, little is known about their expression in NB. In this study, we investigated the expression of Oct4 and Sox2 in human NB, the relationship between the expression levels of Oct4 and Sox2 and various clinicopathological parameters and the effect of chemotherapy on Oct4 and Sox2 expression.

Materials and methods

Human tissue samples. NB samples were obtained from 65 children with primary NB during surgical operation at the Department of Surgery, Children's Hospital of Fudan University, China. The matched adjacent non-cancerous tissue samples were obtained from a segment of the resected specimens that were located at more than 5 cm away from the primary site. All specimens were collected between July 2007 and August 2010 from 28 males and 37 females ranging from 1 month to 13 years of age (median age 5.2 years). Clinical staging was carried out according to the International Neuroblastoma Staging System (INSS) (11). No preoperative treatment was conducted in stage I and II patients (26 cases) and 15 stage III and IV patients, while 24 stage III and IV patients received chemotherapy prior to surgery. The samples were snap-frozen in liquid nitrogen and stored at -80˚C until analysis. One section of each sample was sectioned and fixed in 10% formalin for immunohistochemical staining. All the samples were confirmed by pathologic examination. The study was conducted in accordance with the regulations of the Institutional Review Board. Informed consent was obtained from the guardian of each patient. The clinicopathological characteristics of the 65 patients are sumarized in Table I.

RNA extraction and reverse transcription. Tissue samples were homogenized with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then 200 μl chloroform was added to the homogenate. The samples were centrifuged at 12000 g for 15 min at 4˚C, and then the upper aqueous layer was transferred to a clean Eppendorf tube containing 500 μl isopropanol. The samples were then incubated at room temperature for 10 min. RNA was precipitated at the bottom of the tube after the samples were centrifuged at 12000 g for 10 min at 4˚C. Total RNA was washed with 1 ml 75% alcohol and then air-dried. Finally, the RNA was dissolved in RNase-free water and the quality was evaluated by gel electrophoresis. RNA concentrations were measured by a spectrophotometer (260 nm). For cDNA synthesis, 2 μg total RNA was reverse transcribed at 37˚C for 1 h using MMLV reverse transcriptase (Promega, Madison, WI, USA) and an oligo(dT) primer cDNA was stored at -20˚C until analysis.

Real-time PCR. Real-time PCR was performed in a reaction mixture containing 10 μl SYBR Premix (Takara Bio, Shiga, Japan), 0.8 μl each primer (10 μM), 0.4 μl ROX reference dye II, 2 μl cDNA and 6 μl dH_2O. PCR primers are listed in Table II. β-actin was used as an internal control. All real-time PCR reactions were performed using an ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: initiation at 95˚C for 30 sec; amplification for 40 cycles at 95˚C for 5 sec and 60˚C for 34 sec; dissociation stage: 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec. Reactions were performed in duplicate. Fold differences were calculated using the ΔΔCT method with normalization to β-actin and expressed as a fold difference compared with non-cancerous tissues.

Western blot analysis. Tissues were lysed in ice-cold RIPA lysis buffer containing proteinase inhibitors. Cell lysates were incubated on ice for 30 min and then centrifuged at 12000 g for 10 min at 4˚C. Supernatants were collected, and protein concentrations were measured by an enhanced BCA protein assay kit (Beyotime Biotech, Haimen, Jiangsu, China). Protein samples (20 μg) were separated in a 10% SDS-PAGE gel and then transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). Membranes were blocked in 5% non-fat milk for 1 h and then probed with primary antibodies followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies. Primary antibodies used were as follows: rabbit polyclonal Oct4 antibody (1:1000, ab19857; Abcam Biotechnology, Cambridge, UK) and rabbit polyclonal Sox2 antibody (1:1000, cst2748, Cell Signaling Technology, Beverly, MA, USA). A mouse monoclonal anti-β-actin antibody (1:1000, sc-69879; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the internal

Table I. Clinicopathological characteristics of neuroblastoma patients.

Characteristics	N (%)
Gender	
Male	28 (43.1)
Female	37 (56.9)
Age (years)	
≥5	21 (32.3)
<5	44 (67.7)
Clinical stage	
I	14 (21.5)
II	12 (18.5)
III	16 (24.6)
IV	23 (35.4)
MYCN amplication	
Yes	30 (46.2)
No	35 (53.8)
Tumor size (cm)	
≥5	36 (55.4)
<5	29 (44.6)
Tumor location	
Adrenal gland	37 (56.9)
Aboral peritoneum	22 (33.8)
Neck	2 (3.1)
Mediastinum	4 (6.2)
Histology	
Favourable	35 (53.8)
Unfavourable	30 (46.2)
Preoperative chmotherapy	
Yes	24 (36.9)
No	41 (60.1)

Table II. Real-time PCR primers.

Gene	Primer sequence	Annealing temperature (˚C)	Product length (bp)
Oct4	For: GGTATTCAGCCAAACGACCATCT Rev: TCATTGTTGTCAGCTTCCTCCAC	55	114
Sox2	For: ATCCCATCACCCACAGCAA Rev: TCGGCATCGCGGTTTTT	60	80
β-actin	For: TAG TTG CGT TAC ACC CTT TCT TG Rev: TGC TGT CAC CTT CAC CGT TC	59	156

control. Membranes were developed using an ECL detection system (Thermo Fisher, Waltham, MA, USA). Band intensities were determined using Image-lab 3.0 software.

Immunohistochemical staining. Paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated via a graded ethanol series, and endogenous peroxidase was inhibited with 3% H_2O_2. Sections were then boiled in 0.01 M sodium citrate buffer (pH 6.0) for 10 min for antigen retrieval. After blocking with 5% bovine serum albumin, sections were incubated with primary antibodies (Oct4, 1:250, ab19857; Abcam Biotechnology, and Sox2, 1:100, cst2748; Cell Signaling Technology) at 4˚C overnight. Following incubation with a biotinylated secondary antibody, a streptavidin-biotin/HRP complex was applied. The visualization signal was developed with DAB reagent (Genetech, Shanghai, China), and the sections were counterstained with hematoxylin. Negative controls were prepared by omitting the primary antibody.

Statistical analysis. Data were expressed as mean ± SD from at least three separate experiments. Differences in mean values were analyzed by one-way analysis of variance and Student's t-test with SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). P<0.05 was considered statistically significant.

Results

Overexpression of Oct4 and Sox2 in NB tissues. Using a qRT-PCR method, Oct4 and Sox2 were detected in all 65 pairs of NB tissues and their matched adjacent non-cancerous tissues. In the 41 patients that did not receive preoperative treatment, Oct4 and Sox2 mRNA relative expression levels were significantly higher in tumor tissues than in the adjacent non-cancerous tissues (P=0.006, P=0.011) (Fig. 1A). Western blot analysis confirmed that the protein levels of Oct4 and Sox2 were higher in tumor tissues than in the adjacent non-cancerous tissues (Fig. 1B). Oct4 and Sox2 protein bands were detected in all the 41 non-chemotherapy tumor tissues, while weaker bands were detected in non-cancerous tissues, and in some cases no band was detected.

Distribution and localization of Oct4 and Sox2. The immunohistochemical staining showed that Oct4 was localized in the cytoplasm of NB cells (Fig. 2A), while Sox2 was primarily localized in the nuclei (Fig. 2C). Oct4 and Sox2 immunostaining

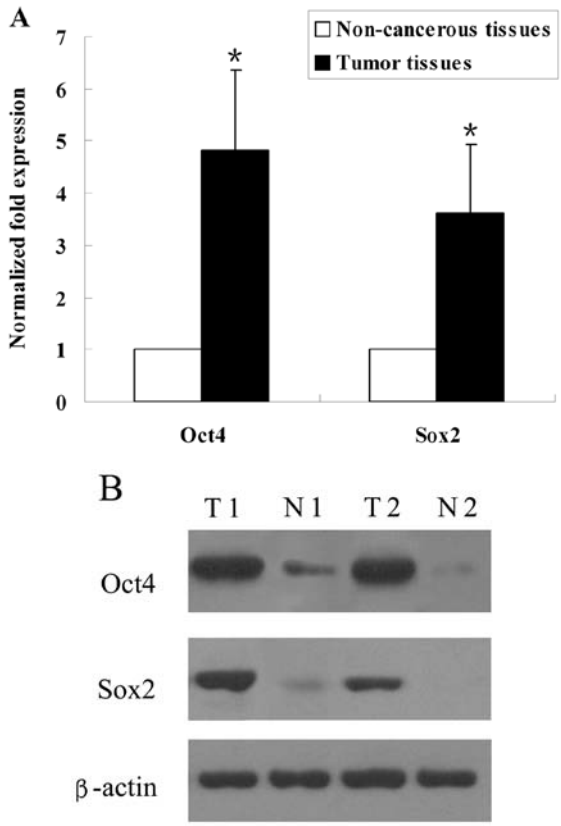

Figure 1. The expression of Oct4 and Sox2 in tumor and neighboring non-cancerous tissues. (A) Real-time PCR analysis of mRNA levels of Oct4 and Sox2 in the two groups. Compared with non-cancerous tissues, Oct4 and Sox2 were overexpressed at different expression levels in tumor tissues (Oct4, 4.83±1.54-fold; Sox2, 3.61±1.32-fold). *P<0.05. (B) Oct4 and Sox2 protein expression in the paired tumor (T) and non-cancerous (N) tissues as determined by Western blot analysis.

was not observed in paracancerous cells (Fig. 2B and D). The intensity of immunostaining was variable among Oct4 and Sox2 positive cells. Immunoreactivity was not observed in negative controls.

Correlation between Oct4 and Sox2 expression and clinicopathological characteristics. The expression levels of Oct4 and Sox2 were significantly correlated with the clinical stage in NB patients. Their relative expression levels in stage III and IV NB were higher compared with those in stage I and II NB (P=0.025,

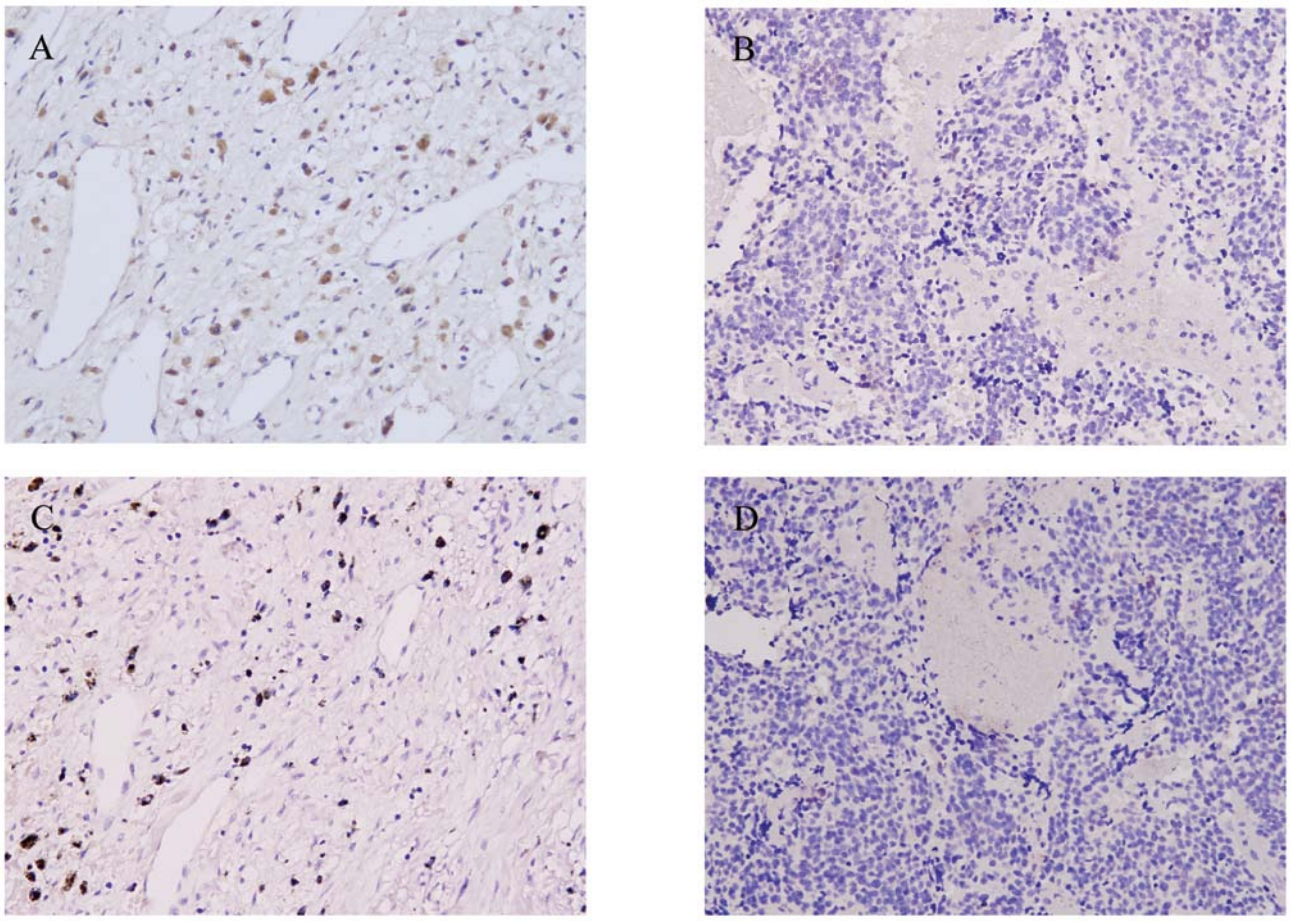

Figure 2. Immunohistochemical staining of Oct4 and Sox2 in NB and matching paracancerous tissues. (A) Oct4 is primarily located in the cytoplasm of NB cells (x200). (B) The matched paracancerous tissue is negative for Oct4 (x200). (C) Sox2 is mainly localized in the nuclei of NB cells (x200). (D) Sox2 staining is negative in matched paracancerous tissue (x200).

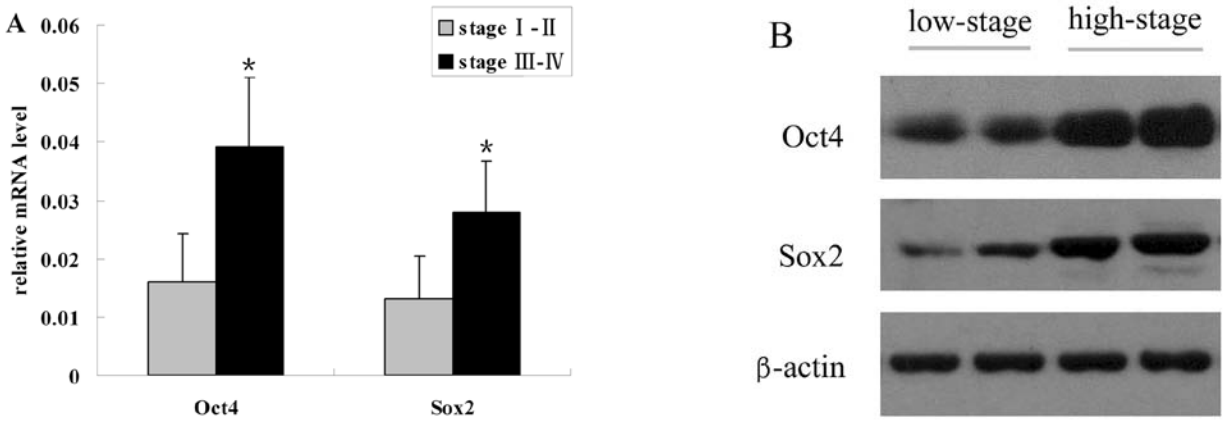

Figure 3. Analysis of Oct4 and Sox2 expression in stage I-IV NB. (A) Relative mRNA levels of Oct4 and Sox2 in stage I-II and III-IV NB. ΔCT values of the two genes were normalized to β-actin. *P<0.05. (B) Western blot analysis of Oct4 and Sox2 in stage I-II (low-stage) and III-IV (high-stage) NB.

P=0.017) (Fig. 3). Oct4 and Sox2 expression was not associated with any other clinicopathological characteristics including patient gender and age, tumor size, location, MYCN amplication and histological classification (Table III).

Chemotherapeutic effect on Oct4 and Sox2 expression. To investigate the effect of chemotherapy on Oct4 and Sox2 expression, we analyzed the data according to the chemotherapy subgroup. In stage III and IV tumors, the preoperative non-chemotherapy subgroup expressed higher levels of Oct4 and Sox2 compared with those of the chemotherapy subgroup (P=0.009 and P=0.013) (Fig. 4A). Oct4 and Sox2 protein bands were detected in all 15 cases of non-chemotherapy tumor tissues, while weaker bands or even no band were detected in 24 cases of chemotherapy tumor tissues. Moreover, the expression levels of Oct4 and Sox2 in one particular sample were higher compared with those of any

Table III. Correlation between the expression levels of Oct4 and Sox 2 with clinicopathological features in neuroblastoma patients.

Parameters	Oct4 (mean ± SD)	P-value	Sox2 (mean ± SD)	P-value
Gender				
Male	0.0271±0.0104	0.1655	0.0246±0.0085	0.1814
Female	0.0353±0.0167		0.0294±0.0132	
Age (years)				
≥5	0.0375±0.0128	0.3258	0.0283±0.0150	0.5360
<5	0.0287±0.0174		0.0196±0.0108	
Clinical stage				
I+II	0.0169±0.0084	**0.0254**	0.0135±0.0075	**0.0173**
III+IV	0.0392±0.0126		0.0284±0.0087	
MYCN amplication				
Yes	0.0326±0.0183	0.4267	0.0287±0.0160	0.5032
No	0.0261±0.0144		0.0255±0.0093	
Tumor size (cm)				
≥5	0.0304±0.0171	0.3783	0.0253±0.0139	0.6069
<5	0.0246±0.0136		0.0241±0.0148	
Tumor location				
Adrenal gland	0.0326±0.0152	0.7235	0.0305±0.0143	0.4750
Aboral peritoneum	0.0275±0.0124		0.0242±0.0096	
Neck	0.0183±0.0142		0.0164±0.0117	
Mediastinum	0.0228±0.0157		0.0186±0.0145	
Histology				
Favourable	0.0237±0.0166	0.4582	0.0224±0.0135	0.6256
Unfavourable	0.0316±0.0158		0.0297±0.0140	

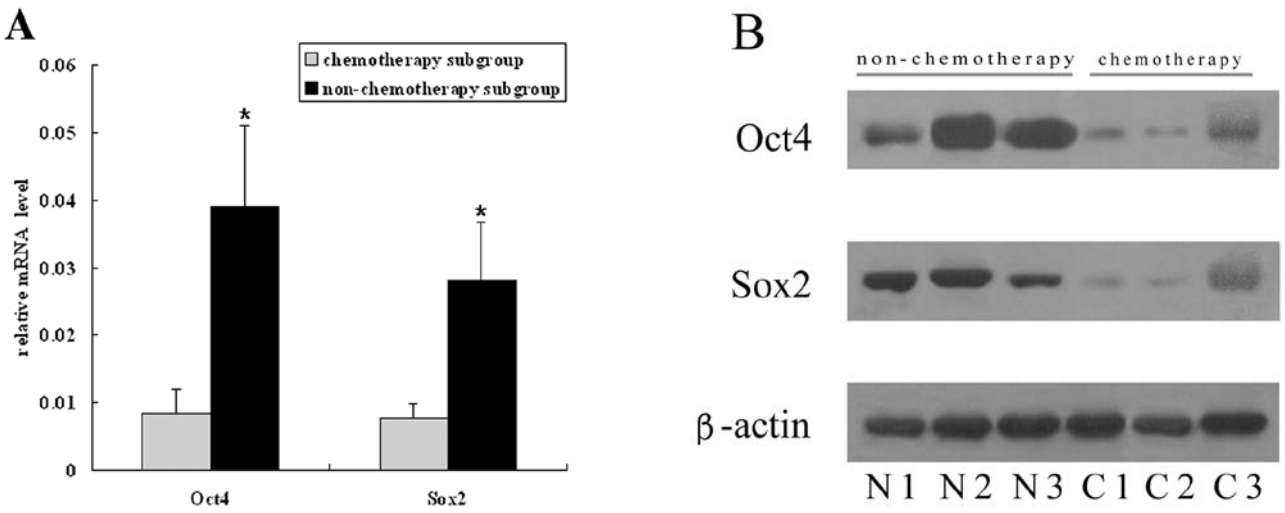

Figure 4. Analysis of Oct4 and Sox2 expression in subgroups with and without preoperative chemotherapy. (A) Relative mRNA levels of Oct4 and Sox2 in the two groups. The relative mRNA levels of Oct4 in non-chemotherapy and chemotherapy subgroups were 0.039±0.012 and 0.0083±0.0036, respectively, while the Sox2 mRNA relative levels were 0.028±0.0087 and 0.0076±0.0022, respectively. *P<0.05. (B) Western blot analysis of Oct4 and Sox2 in non-chemotherapy (N) and chemotherapy (C) subgroups. The expression level in one particular sample (C3) was higher compared with other samples in the preoperative chemotherapy subgroup.

other sample in the preoperative chemotherapy group (Fig. 4B). The chemotherapy scheme of this sample was OPEC according to the clinical data.

Discussion

Oct4 (also known as Oct-3 and POU5F1), is a transcription factor expressed in both embryonic and adult stem cells, and has been associated with pluripotency, proliferative potential and self-renewal of ESCs and germ cells (12). Oct4 belongs to the family of POU-domain transcription factors that are involved in regulation of cell growth and differentiation (13,14). Oct4, as well as Sox2 and Nanog, plays a pivotal role in regulation and maintenance of pluripotency. Expression of Oct4 is restricted to pluripotent cells and its level decreases with the onset of differentiation and loss of pluripotency (9,15). In recent studies, Oct4 expression has been detected in various carcinomas including breast, prostate, bladder, head and neck squamous cell carcinomas and lung adenocarcinoma, which correlates with an unfavorable prognosis (16-20). Oct4 expression was only found in the immature neuroepithelium of high-grade immature teratomas, which indicates that Oct4 may be a promising biomarker for the diagnosis of highly malignant immature teratoma (21). These studies suggest that Oct4 not only plays a crucial role in maintaining stem cell characteristics, but also correlates with the genesis and progression of the tumor involving cancer stem cells.

The Sox2 gene is located in chromosome 3q26.3-q27 and is a member of the SOX (SRY-related high mobility group box) gene family, which all contain a high mobility group (HMG) domain that is very similar to that in the sex-determining gene SRY (22). The SOX family of transcription factors is expressed during various phases of embryonic development, which affects cell fate and differentiation (23). Sox2 is initially expressed in all blastomeres of the 4-cell embryo and later becomes restricted to the inner cell mass and epiblast, and then to the germ cells. Sox2 plays an important role in the maintenance of self-renewal and differentiation potential. Recently, Sox2 has been studied in several types of malignant human tumors. Sanada *et al* reported that Sox2 is overexpressed in human pancreatic carcinoma, and the protein level correlates to the pathological grade (24). Similar results have been observed in breast cancer (25). In stage I lung adenocarcinomas, Sox2 appears to be an independent predictor of a poor clinical outcome and may help to identify patients with an increased risk of recurrence (26). Sox2 was also found to be involved in the later events of carcinogenesis, such as invasion and metastasis, rather than the early progression of pancreatic intraepithelial neoplasias (24). However, decreased expression of Sox2 is also associated with a few types of carcinoma such as gastric cancer and choriocarcinoma (27,28).

Although the expression of Oct4 and Sox2 has been reported in several cancers and cancer cell lines, their expression in NB has rarely been reported. Melone *et al* detected the expression of Oct4 in NB cells and tissue samples, but Sox2 expression was not found (29). 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 protein levels may be an important prognostic indicator of NB (30). However, in the present study for the first time, we investigated the expression of Oct4 and Sox2 in paired NB tissues and matched adjacent non-cancerous tissues and investigated their relationship with clinicopathological parameters. First, we observed the expression of Oct4 and Sox2 in NB tissues was significantly higher compared with those in paracancerous tissues. Furthermore, Oct4 and Sox2 expression in stage III and IV NB was higher compared with those in stage I and II. These data suggest that the expression of Oct4 and Sox2 may correlate with the genesis and progression of NB. This result is consistent with most previous studies (17,18,21,24,25). However, further research is needed to validate the correlation, and we will carry out further experiments to demonstrate the effects of Oct4 and Sox2 in NB carcinogenesis. We found that Oct4 and Sox2 expression was not associated with any other clinicopathological parameters including patient gender and age, tumor size, location and histological classification. Additionally, we found that the Oct4 expression level was higher compared with that of Sox2 in NB tissues.

To determine the tissue distribution and subcellular localization of Oct4 and Sox2 in paired NB and paracancerous tissue sections, we performed immunohistochemical staining. We found that Oct4 was primarily localized in the cytoplasm of NB cells, while Sox2 was localized in the nuclei, without positive staining in paracancerous cells. The intensity of immunostaining was variable among Oct4- and Sox2-positive cells, indicating that the tumor cells were heterogeneous in terms of Oct4 and Sox2 expression.

To explore the effect of chemotherapy on Oct4 and Sox2 expression, we analyzed the data according to the chemotherapy subgroup. In stage III and IV tumors, Oct4 and Sox2 expression in the chemotherapy subgroup was significantly lower compared with those of the non-chemotherapy subgroup. This observation indicates that Oct4 and Sox2 expression may be inhibited by chemotherapy. To our knowledge, similar findings have not been found in other tumors, and our study is the first to report the effect of chemotherapy on Oct4 and Sox2 expression in NB. More research is needed to investigate the potential mechanisms. Interestingly, we also found that the expression levels of Oct4 and Sox2 in one particular sample were higher compared with those of any other sample in the preoperative chemotherapy subgroup. After analyzing the clinical data, we found that the chemotherapy scheme of this patient was OPEC, and the only participant to receive this chemotherapy scheme. OPEC is a chemotherapy scheme with joint use of VCR (vincristine), CTX (cytoxan), Cisplatin and VM-26 (teniposide), which is often used to treat stage II tumors after postoperative chemotherapy, and is the least vigorous of several commonly used chemotherapy schemes. This incidental finding indicates that the chemotherapeutic inhibition of Oct4 and Sox2 expression may be associated with the dynamics of chemotherapy schemes. However, this presumption requires further validation. For example, experiments can be conducted to detect the expression of Oct4 and Sox2 after the same course of treatment with various chemotherapy schemes *in vitro*.

In conclusion, we have found that Oct4 and Sox2 are overexpressed in human NB tissues compared with those of paracancerous tissues. Oct4 and Sox2 expression correlates to the clinical stage in NB. Moreover, Oct4 and Sox2 expression can be inhibited by chemotherapy, and this inhibition may be associated with the dynamics of chemotherapy schemes.

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