ABBS

Preparation of polyclonal antibody specific for NOR1 and detection of its expression pattern in human tissues and nasopharyngeal carcinoma

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Oxidored-nitro domain containing protein 1 (NOR1) gene is a novel nitroreductase gene first isolated from nasopharyngeal carcinoma (NPC). It plays an important role in the formation of chemical carcinogen and the carcinogenesis of NPC for its nitrosation function. Overexpression of the wild-type *NOR1* gene in nasopharyngeal carcinoma cells is effective to inhibit cell growth and proliferation. In this study, for the first time, we generated a highly specific NOR1 antibody and analyzed NOR1 distribution in the human tissues and NPC biopsies. The results showed that NOR1 protein is predominantly expressed in human nasopharynx and tracheal tissues. Human heart, liver, spleen, stomach, colon, kidney, skeletal muscle, thymus, and pancreas are all deficient of NOR1 protein. More importantly, we performed immunohistochemistry assay of NOR1 protein expression in the NPC tissues, and the result showed that NOR1 protein is frequently down-expressed in NPC. These data shed light on the selectivity of potential physiological functions of NOR1 and provides an indispensable reference to the carcinogenesis process of NPC and to identify or validate tissue-specific drug targets.

Introduction

The nitroreductase family comprises a group of FMN- or FAD- and NAD(P)H-dependent enzymes [1]. It catalyzes the reduction of nitro groups in a wide range of substrates to produce the corresponding hydroxylamine. There is much evidence showing that nitrate reductases and nitroreductases are important enzymes during the formation of nitrosamines due to the nitrosation activity [2–4]. The classical nitroreductase of *Salmonella typhimurium* is a flavoprotein that catalyzes the reduction of nitroaromatics to metabolites that are toxic, mutagenic, or carcinogenic. It has been identified as useful in the metabolism of a number of prodrugs in anti-cancer gene therapy [5–7]. Oxidored-nitro domain-containing protein 1 (NOR1) gene is a novel gene isolated from nasopharyngeal carcinoma (NPC) [8]. Human NOR1 share 40% homology with nitroreductase from *Escherichia coli*, and it may be a novel member of nitroreductases derived from human tissues that exhibit low substrate specificity but have the similar function of reducing nitro [8]. Human NOR1 expression is decreased in NPC cell line HNE1 and tissues when compared with normal nasopharyngeal epithelial cells. Furthermore, mutations were found in the coding region of NOR1 in NPC biopsies [8]. Xiong *et al*. [9] reported that two cSNPs (coding region single nucleotide polymorphism) of NOR1 were found to be associated with NPC. Overexpression of wild-type *NOR1* gene in NPC cells is effective to inhibit cell growth and proliferation [10]. *NOR1* might be a good candidate tumor suppressor gene or related gene in nasopharyngeal tumorigenesis. Since much evidence showed that exposure to nitroso compounds such as nitrosamines is at risk of NPC [11–15], NOR1 may play an important role in the formation of chemical

carcinogen and carcinogenesis of NPC for its nitrosation function. The latest study showed that *NOR1* promoter region was frequently methylated in leukemia cell lines and 63% acute myeloid leukemia (AML) patients, suggesting that downregulation of NOR1 protein resulted from DNA methylation is also involved in the AML progression [16]. However, to date, very few functional studies on NOR1 have been carried out, and the precise tissue and cell localization of NOR1 has not yet been resolved. Understanding the localization of the gene product in humans is important for the characterization of gene function. In this study, we successfully generated a highly specific NOR1 antibody. With this antibody, we analyzed the distribution of the NOR1 protein in the human and NPC tissues. The study showed that NOR1 is predominantly expressed in nasopharynx and trachea, weakly expressed in central nervous system. NOR1 protein is frequently down-expressed in the NPC tissues. These data will help to explain the selectivity of potential physiological functions of NOR1 and provide an indispensable reference to the carcinogenesis process of NPC and to identify or validate tissue-specific drug targets. Furthermore, these data may help to further study the organ targets selectivity of chemical carcinogens such as nitroso compounds. In particular, the prepared NOR1 antibody will be helpful for the study of the bio-functions of endogenously expressed NOR1 protein in progression of NPC.

Materials and Methods

Construction of fusion genes

The sequence encoding the full-length 389 amino acids of the NOR1 protein (GenBank accession No. NM_145047) was amplified by polymerase chain reaction (PCR) using the primers as follows: *NOR1* forward primer, 5′-ATACATATGTCGGTGCGCACGCTACCG-3′; *NOR1* reverse primer, 5′-CTCGAGCTAAAACTCTCCC-ATGATTCTAGC-3′. PCR conditions were 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min; and 72°C for 10 min. The PCR fragment was purified and ligated into *Nde*I/*Xho*I digested pET28b vector (Novagen, Madison, USA), yielding the construct pET28b-NOR1. To identify the positive clones with inserts, plasmid DNA extracted from clones after transformation with recombinant constructs was first subjected to PCR using the same primer pairs mentioned above and confirmed by sequencing.

Expression and purification of the NOR1 protein

A single transformed RosettaBlue(DE3) (Novagen) colony harboring the recombinant construct pET28b-NOR1 was grown in 5 ml Luria-Bertani broth (LB) containing antibiotics (200 μg/ml kanamycin, 34 μg/ml chloramphenicol, and 12.5 μg/ml tetracycline) and grown overnight at 37°C, 220 rpm overnight. Two milliliters of this culture was transferred into 500 ml of fresh LB medium containing 200 μg/ml kanamycin, 34 μg/ml of chloramphenicol and 12.5 of μg/ml tetracycline, and was grown at 37°C to an optical density of 0.6 at 600 nm. Expression of the fusion protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 5 h, the recombinant protein was analyzed by 10% SDS-PAGE. At the same time, the protein extracts of empty vector pET28b-transformed cells induced with the same condition were used as the negative control. After induction, cells were harvested by centrifugation at 12,000 *g* for 2 min at 4°C. The cell pellet was suspended (10 ml/g wet weight) in lysis buffer (PBS, pH 7.4, 1.0 mM phenylmethyl sulfonylfluoride and 1.0 mg/ml lysozyme). This suspension was incubated for 30 min at 4°C with stirring. Following sonication, the suspension was centrifuged at 12,000 *g* for 15 min. The remaining pellet containing inclusion bodies was washed twice with ice cold PBS, then resuspended in 10 ml binding buffer (PBS containing 5 mM imidazole, 6 M guanidine-HCl, pH 7.4) and incubated on ice for 2 h to completely solubilize protein followed by centrifugation at 16,000 *g* for 30 min to remove insoluble materials. The supernatant was filtered through 0.45-μm membrane. Then, the filtrate was loaded on Ni-IDE chromatography resin (Novagen) followed by twice washes with 20 ml binding buffer plus 50 mM imidazole. The target proteins were eluted with 5 ml elute buffer (500 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9). The purified recombinant protein was analyzed by SDS-PAGE and western blot.

Antiserum preparation using purified NOR1

Polyclonal antibody against human NOR1 was prepared according to the protocol described previously [17]. Briefly, two New Zealand white rabbits were immunized intravenously with 400 μg of the His-NOR1 protein plus Freund's complete adjuvant per rabbit, followed by a second immunization of 400 μg of same agent per rabbit 2 days later. After the second injection, additional injections (200 μg recombinant protein with incomplete Freund's adjuvant per injection) were performed at the

28th day. One week later, sera were collected. Serum from a normal rabbit without immunization was also collected. The rabbit IgG fraction was purified from the immune serum or normal serum using the caprylic acid/ammonium sulfate method [18].

Human tissues and NPC tissues

Human tissues (heart, liver, spleen, stomach, kidney, nasopharynx, trachea, lung, brain, spinal, colon, thymus and muscle) were obtained from donation of three victims (20–35 years old, disease free) in traffic accident at Xiangya Hospital, with appropriate written consent and approval from the Central South University Health Authority Joint Ethics Committee and relevant national guidelines. Tissues were collected into cold phosphate buffered saline (PBS containing 150 mM sodium chloride, 150 mM sodium phosphate, pH 7.2). Each type of tissues was obtained from three different sites. A total of 34 nasopharyngeal carcinoma tissue samples and 12 chromatic inflammatory nasopharyngeal epitheliums (NPE) tissue samples were collected from the ENT Department at Xiangya Hospital, Central South University. All nasopharyngeal carcinoma samples were classified as non-keratinizing carcinomas. For the use of these clinical materials for research purposes, prior patient's consent and approval from the Institute Research Ethics Committee were obtained. For immunohistochemistry, the human and NPC tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C overnight.

RNA extraction and RT-PCR analysis

Total RNA from different frozen tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The RNase-free DNase set (Qiagen, Valencia, USA) was used to digest DNA on-column during RNA purification. To generate single-stranded cDNAs, 2 μg total RNA was used with the first-strand cDNA synthesis kit (Promega, Madison, USA) according to the manufacturer's instructions. Human *NOR1* mRNA was amplified with the following specific primers: *NOR1*-forward, 5′-TCAAGGGATTCATCCGAGAC-3′ and *NOR1*-reverse, 5′-CTGGCCAAGAAATTCAGCTC-3′. Human *GAPDH* mRNA was amplified with the following specific primers: *GAPDH*-forward 5′-AACGGATTTGG-TCGTATTGG-3′ and *GAPDH*-reverse 5′-TTGATTTT-GGAGGGATCTCG-3′. PCR was performed using the following protocol: initial denaturation at 94°C for 5 min, 28 cycles of denaturation at 94°C for 30 s,

annealing at 60°C for 30 s, elongation at 72°C for 40 s, and a final 10-min extension at 72°C.

Cell culture and transfection

COS7 cells (African Green Monkey SV40-transf'd kidney fibroblast cell line) were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2. Transfections were performed with either pCMV-Myc-NOR1 plasmid or with pCMV-Myc plasmid using Lipofectamine™ reagent (Invitrogen) according to the suppliers' standard protocols.

SDS-PAGE and Western blot

Proteins were separated by SDS-PAGE, using a 5% stacking gel and 10% separating gel. After electrophoresis, the proteins were electronically transferred onto nitrocellulose membranes. Western blotting was performed as described by Towbin *et al.* [19]. The membranes were incubated with monoclonal anti-His antibody (Invitrogen, 1:10,000), or monoclonal anti c-Myc (Clontech, Palo Alto, USA, 1:1000) and the anti-NOR1 primary antibodies, respectively. After three washings with 0.01 M PBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse antibody or horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Maxin, Fuzhou, China), respectively, and finally visualized using the ECL PLUS Western blotting substrate kit (GE Healthcare, Milwaukee, USA) according to the manufacturer's instructions.

Immunohistochemistry

Tissues sections were deparaffinized in dimethyl benzene and rehydrated through graded alcohols (100%, 90%, 70% and 50% alcohol; 5 min each). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. For antigen retrieval, sections were incubated in sodium citrate buffer (0.01 M, pH 6.0) for 20 min in a household microwave oven. After cooling to room temperature, slides were washed in PBS and immersed in normal goat blocking serum (Maxin, Fuzhou, China) for 30 min. The anti-NOR1 serum (1:5000) was applied at 4°C overnight. Secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) was added according to the manufacturer's instructions. Color reaction was developed using diaminobenzidine chromogen solution (Maxin) and all slides were counterstained with

hematoxylin. Normal rabbit IgG was used as negative control.

Results

Cloning and construction of expression vector of pET28b-NOR1

The sequence encoding the full-length 389 amino acids of the NOR1 protein was amplified by PCR from a cDNA library of human fetal brain and cloned into *Nde*I/*Xho*I-digested pET28b vector. The positive clones with recombinant construct were first identified by PCR with the same primer pairs as amplification of NOR1 from the cDNA library. As shown in **Fig. 1**, a 1170-bp fragment (lane 1) was amplified from a positive clone. DNA sequencing confirmed this fragment to be the coding region of 389 amino acids of the full-length *NOR1* gene (accession No. NM_145047).

Expression and purification of the His-NOR1 fusion protein

The expression plasmid pET28b-NOR1 and empty vector pET28b were transformed into *E. coli* Rosettablue(DE3) strain (Novagen) respectively and induced at various conditions. We found high expression level was achieved under 1 mM IPTG induction and at 37°C for 5 h. To examine the distribution of expressed recombinant protein in soluble and insoluble fractions, both the supernatant and pellet of cell lysate after sonication were analyzed. The most recombinant protein was found in inclusion bodies. The recombinant His-NOR1 protein was purified with Ni-IDE chromatography resin under denature condition. All of the denature substance was removed by dialysis. The purified His-NOR1 was analyzed by SDS-PAGE and Western blot. His-NOR1 protein was highly purified, analyzed by 10%
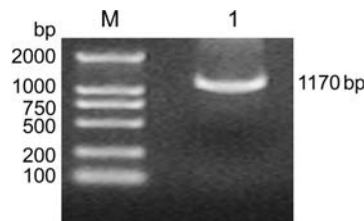
SDS-PAGE gel and stained with Coomassie blue R250 [**Fig. 2(A)**]. The recombinant protein was also analyzed by Western blot using anti-His monoclonal antibody. **Fig. 2(B)** showed the predicted band of 42 kDa recombinant His-NOR1 protein were visualized, no additional bands could be seen. The purified protein was quantified by BCA method.

NOR1 antibody specifically recognizes NOR1 protein

Three immunizations with the His-NOR1 protein were given to two rabbits. The antisera were harvested at the 35th day after primary immunization and subjected to purification. The antibody was purified from the immune serum using the caprylic acid/ammonium sulfate method [18]. The concentration of NOR1 antibody was 130 mg/ml analyzed by BCA method. Western blot results showed that the antibody was able to recognize recombinant His-NOR1 protein with a molecular mass of 43 kDa [**Fig. 3(A)**, lane 2] as that recognized by the anti-His monoclonal antibody [**Fig. 2(B)**]. Normal rabbit IgG was applied as a negative control [**Fig. 3(A)**, lane 1]. More importantly, the purified NOR1 antibody recognized the full-length recombinant fusion protein Myc-NOR1 with a molecular mass of 43 kDa in COS7 cells transfected with pCMV-Myc-NOR1 [**Fig. 3(B)**, lane 3], which corresponds to the molecular mass detected by monoclonal anti-c-Myc in COS7 cells transfected with pCMV-Myc-NOR1 [**Fig. 3(B)**, lane 6]. As shown in lanes 1 and 2, weak endogenous NOR1 protein was detected in COS7 and COS7 transfected with pCMV-Myc vector, respectively.
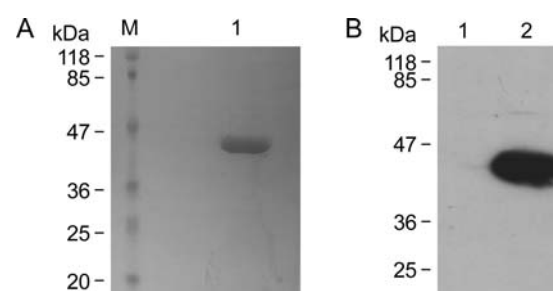

Figure 1 Identification of the recombinant plasmid pET28b-NOR1 Recombinant plasmids were isolated from transformed cells. The insert DNA fragment encoding NOR1 was amplified by PCR using the same primer as amplification of *NOR1* from cDNA library. A band about 1170 bp corresponding *NOR1* open reading frame (ORF) was inserted. M, DNA marker; lane 1, *NOR1*.

Figure 2 Analysis of purification of His-NOR1 fusion protein (A) Purified His-NOR1 fusion protein was separated on a 10% SDS-PAGE gel and stained with Coomassie blue R250. Lane 1, purified His-NOR1 protein, 43 kDa; lane M, protein molecular mass marker. (B) Confirmation of the purified His-NOR1 protein by Western blot analysis with anti-His monoclonal antibody (1:10000). Lane 1, lysate of bacteria with empty vector pET28b; lane 2, the purified His-NOR1 protein.
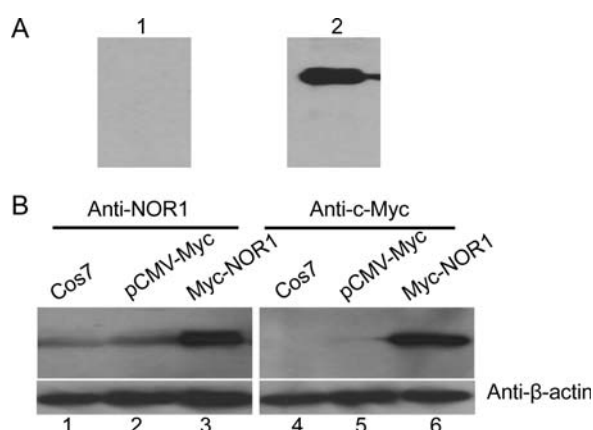
Figure 3 The purified NOR1 antibody specifically recognizes recombinant His-NOR1 and Myc-NOR1 fusion protein (A) Western blot assay of His-NOR1 protein with normal rabbit IgG and NOR1 antibody, respectively. Lane 1, normal rabbit IgG; lane 2, purified NOR1 antibody. (B) Western blot assay of ectopically expressed Myc-NOR1 protein with NOR1 antibody and monoclonal c-Myc antibody (1:1000), respectively. Lanes 1, 2, and 3: COS7, COS7 transfected with pCMV-Myc vector, and COS7 transfected with pCMV-Myc-NOR1, respectively; lanes 4, 5, and 6: COS7, COS7 transfected with pCMV-Myc vector, and COS7 transfected with pCMV-Myc-NOR1, respectively.

Figure 4 Detection of the endogenous NOR1 expression in various human tissues with purified NOR1 antibody by Western blot and RT-PCR (A) Western blot assay of NOR1 protein in various tissues homogenates. NOR1 protein is highly expressed in nasopharynx and trachea, weakly expressed in brain and spinal. (B) The *NOR1* mRNA in various tissues analyzed by RT-PCR. The nasopharynx and trachea have high expression, whereas brain has weak expression of *NOR1* mRNA. Human heart, liver, spleen, stomach, kidney, lung, colon, thymus and muscle showed deficient of *NOR1* mRNA. GAPDH-specific primer was used as controls.

NOR1 protein is highly expressed in epitheliums of upper airway

With the prepared NOR1 antibody, we detected the distribution of NOR1 in human tissues. In the Western blot assay [**Fig. 4(A)**], high expression level of the NOR1 protein was seen in the nasopharynx and trachea. Weak expression level of the NOR1 protein was seen in the homogenates from brain, spinal and lung. Human heart, liver, spleen, stomach, and kidney showed absence of
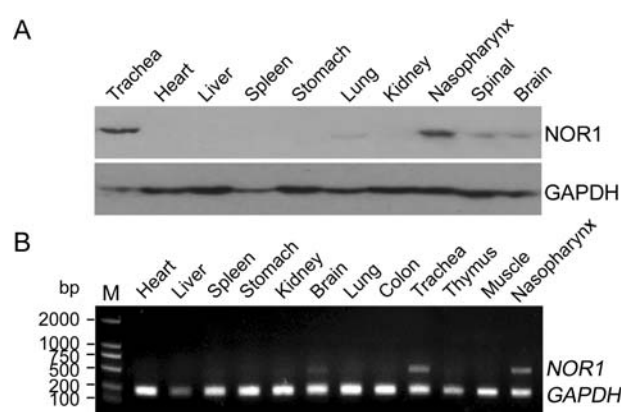
NOR1 protein. We further validated most of the positive expression by RT-PCR [**Fig. 4(B)**], and the results are consistent with the Western blot assay. However, we did not detect *NOR1* mRNA in lung, and this might be due to few small bronchi in lung used in RT-PCR analysis. In immunohistochemistry assays, tissue section from human nasopharynx incubated with normal rabbit IgG, which served as negative control, showed no staining for NOR1 protein [**Fig. 5(A)**]. Intensive cytoplasmic staining for NOR1 protein was seen in the mucosa of human
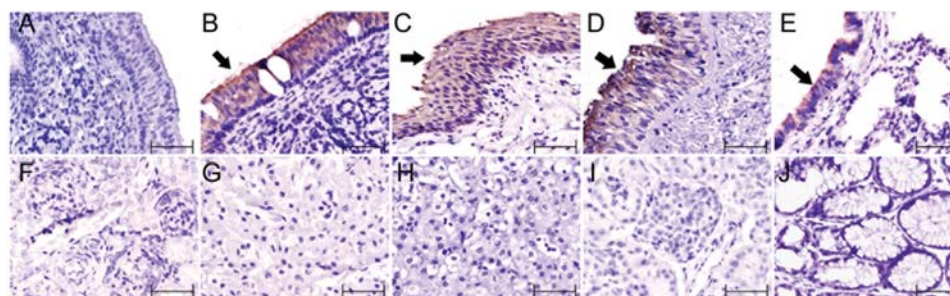

Figure 5 NOR1 protein is predominantly expressed in epitheliums of upper airway Intensive staining for NOR1 protein (brown-stained, arrows) was observed in epitheliums of nasopharynx, trachea, and small bronchi (B,C,D). (A) Normal rabbit IgG was used as primary antibody, which serves as negative control of (B). (B) Intensive immuno-staining for NOR1 protein in columnar epithelium of nasopharynx. (C) Intensive immuno-staining in stratified squamous epithelium of nasopharynx. (D) Intensive immuno-staining in columnar epitheliums of trachea. (E) Intensive immuno-staining in epitheliums of small bronchi, no staining was seen in lung. No staining was seen in kidney (F), heart (G), liver (H), pancreas (I), and stomach (J). Bar = 100 μm.
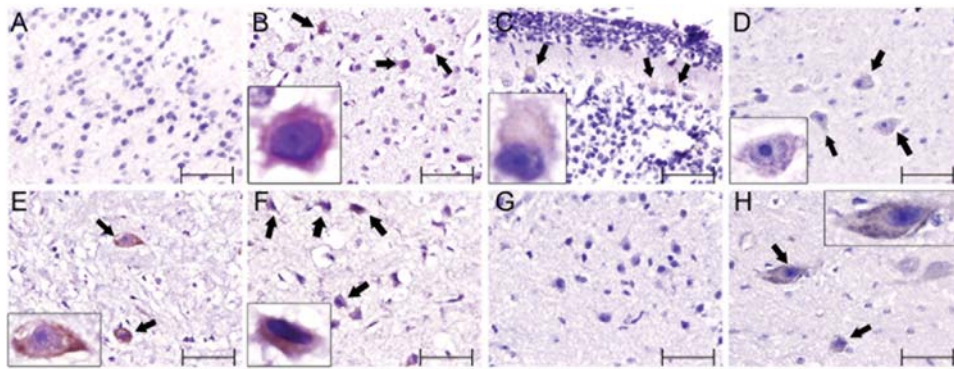
Figure 6 NOR1 protein is predominantly expressed in neurons of central nervous system (brown-stained, arrows) (A) Normal rabbit IgG was used as primary antibody, which serves as negative control of (B). (B) In cerebrum, intensive immuno-staining was seen in neuron cerebrum. (C) In cerebellum, moderate immuno-staining was seen in the Purkinje cells in the molecular layer. (D) In spinal cord, moderate positive staining was present in neurons. In corpus striatum (E) and globus pallidus (F) intensive immuno-staining was seen in neurons. (G) Normal rabbit IgG was used as primary antibody, which serves as negative control of (H). (H) In cingulate gyrus, intensive immuno-staining was seen in neurons. Bar = 100 μm.

nasopharynx, both in the columnar [**Fig. 5(B)**] and stratified squamous epithelia [**Fig. 5(C)**] have strong positive labeling. In trachea and small bronchi [**Fig. 5(D,E)**], intensive staining for NOR1 protein also were seen in epithelial cells, but lung alveoli showed negative staining [**Fig. 5(E)**]. The submucosa matrices of these tissues were negative for staining. Tissue sections from the kidney, heart, liver, pancreas and stomach did not stain positive for NOR1 [**Fig. 5(F–J)**]. Tissue sections from skeletal muscle, smooth muscle, spleen, colon, thymus and adrenal gland also did not stain positive for NOR1 (data not shown).

NOR1 protein is highly expressed in neurons of nervous system

We also observed positive staining for NOR1 protein in neurons of central nervous system. Tissue section from human cerebrum incubated with normal rabbit IgG which served as negative control showed no staining for NOR1 protein [**Fig. 6(A)**]. In tissue sections from human cerebrum, corpus striatum, globus pallidus and cingulate gyrus, intensive staining was seen in neurons, which was characterized by large, central, and achromatic nuclei and prominent nucleolus [**Fig. 6(B,E,F,H)**]. In human cerebellum tissue, moderate immuno-staining of NOR1 protein was seen in the Purkinje cells in the molecular layer [**Fig. 6(C)**]. Moderate positive staining was also present in neurons of spinal cord [**Fig. 6(D)**]. However, glial cells in the central nervous system showed negative staining for NOR1 protein.

NOR1 protein is downregulated in NPC biopsies

By immunohistochemical analysis, 11 of 12 (91.7%) paraffin-embedded chromatic inflammatory NPE biopsies showed moderate-to-strong cytoplasmic staining of NOR1, whereas only 15 of 34 (44.1%) NPC biopsies showed weak-to-moderate staining of NOR1 (**Fig. 7**). The expression of NOR1 was significant in the NPC tissues compared with those in the chromatic inflammatory NPE ($P = 0.004$). Thus, the overall NOR1 expression in the NPC tissues was significantly lower than that in the non-tumor nasopharyngeal tissues.
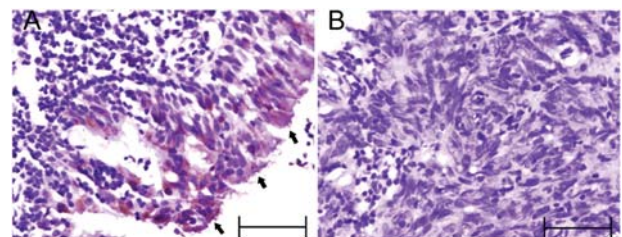

Figure 7 NOR1 protein is downregulated in NPC tissues (A) Intensive staining for NOR1 protein (brown-stained, arrows) was observed in the chromatic inflammatory NPE. (B) Negative staining for NOR1 protein in nasopharyngeal carcinoma tissues. Bar = 100 μm.

Discussion

This work reported the preparation of polyclonal antibodies against human NOR1 and detection of its distribution patterns in the human and NPC tissues. Human NOR1 may be a novel member of nitroreductases

derived from human tissues that exhibit low substrate specificity but have the similar function of reducing nitro [8]. Human *NOR1* gene is mapped to the human chromosome 1p34.2, which is the most frequent loss loci of genetic material in primary NPC biopsies [20–23]. *NOR1* mRNA is decreased in the NPC cell line HNE1 and NPC tissues. Furthermore, mutations were found to occur in the coding region of *NOR1* in the NPC biopsies [8]. Overexpression of wild-type *NOR1* gene in NPC cells is effective to inhibit cell growth and proliferation [10]. *NOR1* might be a good candidate tumor suppressor gene or related gene in nasopharyngeal tumorigenesis. The study of NOR1 may help to understand the relating environmental factors with genetic factors during the carcinogenesis of NPC. However, to date, most of the works are based on the mRNA level and very few functional studies on NOR1 have been carried out, which is mainly because of the unavailability of a specific anti-NOR1 antibody. To obtain sufficient amount of protein to generate polyclonal antibodies against NOR1, a prokaryotic expression system was used. The His-NOR1 fusion protein was highly and rapidly expressed in *E. coli* with 1 mM IPTG induction for 5 h at 37°C. The purified recombinant proteins were found to be immunogenic in rabbits and induced the production of polyclonal antibodies. Western blot results showed that this antibody had the advantages of high specificity and sensitivity. This antibody could serve as a good tool for NOR1 gene function research.

Understanding the localization of gene product in human tissues is important with respect to clarify gene functions [24]. With these antisera, we analyzed NOR1 protein expression in the normal human tissues by Western blot, RT-PCR and immunohistochemistry assays. The study showed that NOR1 protein is predominantly expressed in the human nasopharynx, trachea and bronchi epitheliums. In human nasopharynx section, both the stratified squamous epithelium and the columnar epithelium of these tissues showed intensive cytoplasmic staining. The pseudostratified ciliated columnar epitheliums in trachea and columnar epitheliums in small bronchi also show high expression level of NOR1 protein. Human heart, liver, spleen, lung, kidney, stomach, thymus, muscle, colon, and pancreas showed deficiency for NOR1 protein. However, we detected weak expression level of NOR1 protein in COS7 cells and COS7 cells transfected with pCMV-myc vector [**Fig. 3(B),** lanes 1 and 2], which is an African green monkey SV40-transf'd kidney fibroblast cell line. These differences about the expression of NOR1 protein in

kidney or kidney derived cell line are possibly due to differences in species.

Tissue selectivity considers genes whose expression is enriched to one or a few biologically similar tissue types (such as different portions of the digestive track or various brain sections) [25]. The study showed that NOR1 protein is enriched in upper airway, similar to the expression patterns of NESG1 (nasopharyngeal epithelium-specific gene 1) and PLUNC (Palate lung and nasal epithelium clone protein) family, which was reported as nasopahryngeal specific genes [26,27]. The upper respiratory tract are all exposed to the external environment and, as a result, they can easily be stimulated by physical or chemical irritants and potentially pathogenic organisms [28,29]. There is much evidence that showed that exposure to nitroso compound is risk of nasopharyngeal carcinoma [2–4]. A nitroso derivative of piperazine, 1,4-dinitrosopiperazine (DNP), is particularly carcinogenic to the epithelium of the nasal cavity [12]. Since NOR1 mRNA and protein were predominantly expressed in upper respiratory tract, *NOR1* might be a tissue selective nitroreductase gene that facilitates the clearance or metabolism inactivation of harmful inhaled substances such as DNP. In this study, we also detected NOR1 protein level in the NPC tissues by immunohistochemistry assay and the data showed NOR1 protein is frequently down-expressed in NPC tissues. Decreased expression level of NOR1 protein or genetic alteration of *NOR1* might result accumulation or activation of chemical carcinogens in nasopharyngeal epithelial cells. Although this point should be further elucidated in subsequent studies, the functional study of NOR1 may promote the study of related chemical carcinogens with genetic factors during the carcinogenesis of NPC. Furthermore, tissue specific/selective gene expression is believed to be of physiological importance [25,30]. These data would shed light on the selectivity of potential physiological functions of NOR1 and provide an indispensable reference to carcinogenesis of NPC and to identify or validate tissue-specific drug targets.

We also observed positive staining for NOR1 protein in neurons cytoplasm but negative in glial cells. Neuron is histologically different from respiratory epithelium, so the biological significance of NOR1 protein in neurons remains unknown. In our previous study, yeast two-hybrid assay revealed that NOR1 interacts with cytoplasmic domain of telencephalin (unpublished data), which is a neuronal surface glycoprotein whose expression is restricted to the telencephalon, the most rostral segment of the brain [31]. It has been shown that

telencephalon promotes neurite outgrowth through homophilic interaction [32,33], which suggested that NOR1 might interact with cytoplasmic domain of telencephalin and mediate the signal transduction in neuron. Further study using RNA interference technique to inhibit NOR1 protein expression in isolated neurons or establishing knockout mouse model may help to clarify its significance on neurons function.

In conclusion, we have prepared a high-specificity antibody against human *NOR1* gene with prokaryotic expressed full-length NOR1 protein as immunogen. With this antibody, the expression patterns of NOR1 protein in human tissues were clarified. Our results showed that NOR1 is predominantly expressed in epitheliums of upper airway and neurons. Furthermore, NOR1 protein is frequently downregulated in spontaneous NPC tissues and might serves as a putative NPC biomarker. These data may help to further understand the cellular role of the *NOR1* gene and the mechanism of NPC carcinogenesis. In particular, these data may also help to understand the related environmental carcinogens such as nitroso compounds with genetic factors during the carcinogenesis of NPC.

Acknowledgements

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