

# Regulatory role of CDX2 and NOX4 expression associated with recurrent nasopharyngeal carcinoma

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**Abstract.** – **OBJECTIVE:** Nasopharyngeal carcinoma (NPC) is a malignancy caused by Epstein-Barr virus (EBV). NPC is radiosensitive and has a high frequency of treatment failure due to metastasis, which results in recurrent nasopharyngeal carcinoma (rNPC).

**PATIENTS AND METHODS:** In the present study, nasopharyngeal carcinoma biopsies were obtained from NPC and rNPC patients, as well as healthy controls, and reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry, and immunoblotting analyses were performed.

**RESULTS:** The RTPCR data showed expression of CDX2 and NOX4 in rNPC biopsy samples but not in control or NPC samples. Immunohistochemical and immunoblotting analyses confirmed the expression of CDX2 and NOX4 in rNPC samples, but not in NPC biopsy samples.

**CONCLUSIONS:** The finding concludes that an association of CDX2 and NOX4 expression with rNPC was noted; thus, these proteins may have value as prognostic indicators and may facilitate the development of novel therapeutics for rNPC patients.

*Key Words:*

Recurrent nasopharyngeal carcinoma, Metastasis, Epstein-Barr virus, CDX2, NOX4.

## Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor of the head and neck that mainly affects those 40-50 years of age<sup>1,2</sup>. In NPC, the cancer cells line the nasopharynx, which is the upper part of the pharynx that starts behind the nose and ends at the top of the trachea and esophagus<sup>3</sup>. NPC is a malignant tumor and its symptoms include cervical lymphadenopathy, difficulty breathing or speaking, loss of hearing, bleeding from

the nose, and following metastasis, bone pain, and organ dysfunction<sup>3</sup>.

NPC is classified into three types: Type I, squamous cell carcinoma; type II, keratinizing undifferentiated carcinoma; and type III, nonkeratinizing undifferentiated carcinoma<sup>4</sup>. The highest incidence of NPC is in southern China, particularly Guangdong Province. Hence, it is also known as Cantonese cancer<sup>5,6</sup>.

The mechanisms of pathogenesis of NPC are unclear, but include viruses, environmental influences, and heredity<sup>7</sup>. NPC, and particularly type III<sup>8,9</sup>, is linked to Epstein-Barr virus (EBV) infection<sup>10</sup>. The carcinoma cells contain multiple copies of EBV DNA, resulting in the production of EBV-determined nuclear antigen (EBNA)<sup>11,12</sup>. EBV also replicates in the parotid gland, oropharyngeal epithelium, and uterine cervix<sup>13-15</sup>.

EBV produces various proteins, including EBNA1-EBNA6 and LMP1. The latter is associated with the plasma membrane and the EBNA proteins are associated with the nucleus. Only EBNA1 and LMP1 are detected in NPC, and can facilitate its early detection<sup>16,17</sup>.

After treatment, recurrent NPC (rNPC) can develop due to treatment failure caused by metastasis<sup>18,19</sup>. Of 12 patients positive for both LMP1 and EBNA1, 11 developed rNPC<sup>20</sup>. Hence, there is a need to identify prognostic indicators of rNPC to enable timely application of systemic adjuvant therapies.

CDX2 is a transcription factor specific to caudal-related homeobox<sup>21</sup> and NOX4 is an NADPH oxidase homolog expressed by metastatic cancer<sup>22</sup>. CDX2 plays a role in the maintenance and proliferation of intestinal epithelial cells and induces development of intestinal metaplasia<sup>23</sup> whereas NOX4 is abundantly expressed in renal vascular cells and tissues<sup>24</sup>. The levels of these two

proteins increase in the presence of stress and inflammation, which results in metastatic cancer<sup>25</sup>. CDX2 and NOX4 expression is abnormal in rNPC because of inflammation of the nasopharynx and stress, which results in metastasis.

In the current study, we evaluated the regulatory role and expression pattern of CDX2 and NOX4 in rNPC. Our findings will facilitate discovery of prognostic indicators and novel treatment strategies for rNPC patients.

## Patients and Methods

### *Tissue Sample Collection*

Twenty-seven rNPC and ten NPC biopsy samples were obtained from an affiliated hospital with proper consent from the patients, as well as the institutional review board and Ethical Committee. The pathological classification was performed and the project was approved by the Ethics Committee of the hospital. Five nasopharyngeal tissue samples were obtained from healthy controls. The samples were labeled and stored at  $-80^{\circ}\text{C}$  for RNA isolation, reverse-transcription polymerase chain reaction (RT-PCR), immunohistochemistry, and immunoblotting. Histological analysis confirmed that the samples contained  $> 90\%$  tumor cells.

### *RNA Isolation*

RNA was isolated from biopsy samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. After homogenization, the samples were centrifuged for 5 min at 12,000 rpm to remove genomic DNA. RNA was treated with RNase-free DNase (Invitrogen, Carlsbad, CA, USA). RNA quality and quantity were determined by Nanodrop (Nanodrop Technologies, Wilmington, DE, USA) and RNA stability by Agarose gel electrophoresis (Bio-Rad, Hercules, CA, USA).

### *RT-PCR*

Extracted RNA was treated with DNaseI (Invitrogen, Carlsbad, CA, USA) and stored at  $-70^{\circ}\text{C}$  as per the protocol of the RNeasy Mini kit (Qiagen, Valencia, CA, USA). RNA quality and quantity were confirmed by Nanodrop (Nanodrop Technologies, Wilmington, DE, USA). Reverse transcription was carried out using 0.5  $\mu\text{g}$  RNA as a template and PCR was performed with 1/20 dilution of the product using the following primers: CDX2 F, 5'-CAGTCGCTACATCACCATC-3' and

R, 5'-AGAGTCCACGCTCCTCAT-3'; and NOX4 F, 5'-GGTGCTATTTCCTCATGATCAC-3' and R, 5'-AATCTGGGCTCTTCCATACAAA-3'. PCR was performed according to the manufacturer's instructions and the products were resolved in 2% agarose gels and documented using Bio-Rad gel documentation system. RT minus was performed as a control.

### *Immunohistochemistry*

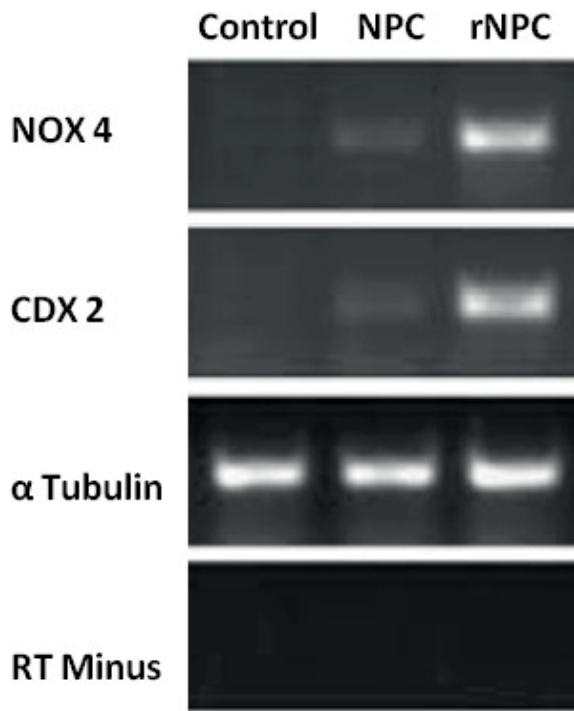
Nasopharyngeal tissue biopsy samples were formalin-fixed and paraffin-embedded. Antigens were retrieved in sodium citrate buffer at  $95^{\circ}\text{C}$  for 30 min (pH 6.0). The biopsy sections were incubated overnight at  $4^{\circ}\text{C}$  with an anti-human CDX2 (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. HPA015475 and Dilution 1:500) or NOX4 (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. ABC459 and Dilution 1:500) antibodies. After washing, secondary antibodies conjugated with horseradish peroxidase (HRP) were added. The slides were developed using appropriate chromogenic substrates and counterstained with Mayer's hematoxylin followed by DPX mounting and observation under a microscope at 40X magnification.

### *Immunoblotting*

Cell lysates were prepared from rNPC, NPC, and control samples and resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes, blocked with 5% skim milk for 1 h, and incubated with anti-CDX2, -NOX, - $\alpha$  tubulin (Abcam, Cambridge, MA, USA, Cat. No. ab18251, Dilution 1:400) and Rad51 (Abcam, Cambridge, MA, USA, Cat. No. ab63801, Dilution 1:500) primary antibodies at the appropriate dilutions according to the manufacturer's instructions. After washing, membranes were incubated with the secondary antibody at an appropriate dilution, and the signals were visualized using DAB (3,3'-Diaminobenzidine) (Sigma-Aldrich, St. Louis, MO, USA).

### *Statistical Analysis*

Analyses were performed using the Statistical Package for Social Sciences (SPSS) for Windows 11.0 (SPSS, Inc., Chicago, IL, USA). All experiments were performed in triplicate and the results are expressed as means and standard errors of the mean. The results were subjected to one-way analysis of variance and the level of significance was set at  $p < 0.05$ .



**Figure 1.** Reverse transcription-polymerase chain reaction analysis of CDX2 and NOX4 expression of control, nasopharyngeal carcinoma (NPC), and recurrent nasopharyngeal carcinoma (rNPC) biopsy samples.

## Results

### Sample Collection and Analysis

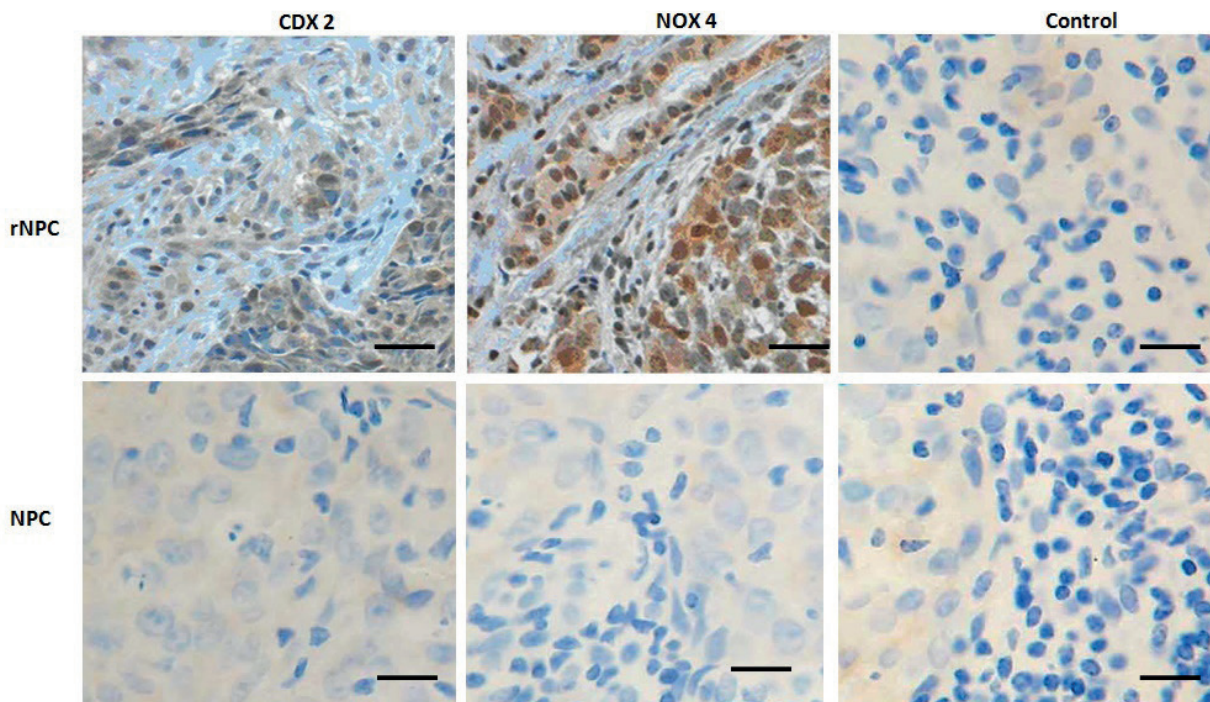
CDX2 and NOX4 expressions were assessed in 27 and 10 biopsy samples from rNPC and NPC patients, respectively, as well as 5 normal nasopharyngeal tissue samples. Of the 27 rNPC samples, 24 and 18 were from males and females, respectively. Similarly in case of NPC 6 samples were from males and 4 were from females. Likewise, 3 and 2 biopsy samples were from males and females of normal nasopharyngeal tissue samples. The age groups of the patients were recorded from 30-73 years. The biopsy samples were analyzed histologically, which confirms to have >90% tumor cells by specialists.

### RT-PCR

CDX2 and NOX4 expressions were detected in all of the rNPC samples, but not in the control or NPC samples (Figure 1). These data suggest that expression of CDX2 and NOX4 is associated with rNPC.

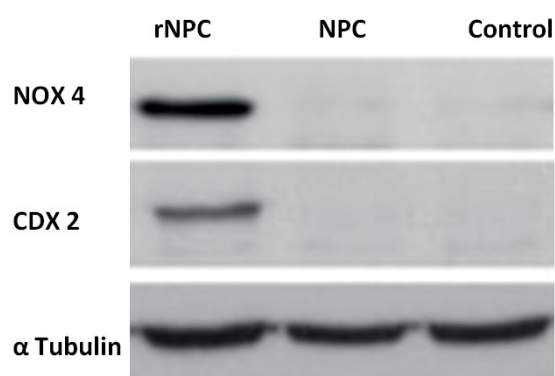
### Immunohistochemistry

CDX2 and NOX4 expressions in rNPC tissues were confirmed by immunohistochemistry (Figure



**Figure 2.** Immunohistochemical analysis using anti-CDX2 and -NOX4 antibodies with hematoxylin counterstaining shows the expression of CDX2 and NOX4 in rNPC, but not NPC or control, tissue samples. The slides were observed under microscope at 40X magnification. Scale bars – 50 μm.





**Figure 3.** Immunoblotting analysis of biopsy samples using anti-CDX2, -NOX4, and - $\alpha$  tubulin antibodies. Lanes 1, 2, and 3 are protein lysates from rNPC, NPC, and control tissue samples, respectively.

re 2). Rad51, which is expressed in NPC patients, was used for comparison. CDX2 and NOX4 expressions were detected in rNPC, but not NPC, samples. These data suggest a link between rNPC and the expression of CDX2 and NOX4.

### Immunoblotting

The immunohistochemistry data were validated by immunoblot analysis. CDX2 and NOX4 expression was detected in rNPC, but not NPC or control, samples (Figure 3). These data suggest that CDX2 and NOX4 are expressed in rNPC tissues. The immunohistochemistry and immunoblotting data confirmed the expression of CDX2 and NOX4 only in rNPC patients. Hence, CDX2 and NOX4 may be useful as prognostic markers in rNPC patients.

### Discussion

NPC is a major health problem in Asian countries, particularly southern China<sup>26,27</sup>. NPC arises from the epithelium, especially from the nasopharyngeal cavity, and is associated with EBV<sup>28-30</sup>. Several EBV proteins have been noted in the malignant phenotype<sup>31,32</sup>. The EBV genes EBNA1 and LMP1 are expressed in NPC<sup>33</sup>. LMP1 is an oncogenic protein that influences various signaling pathways, including the NF- $\kappa$ B, JNK/p38 (SAPK), PI3-kinase, and ERK-MPK9 pathways<sup>19</sup>. Due to its radiosensitivity and propensity to metastasize, 98.1% chances of risk in causing rNPC<sup>20,34</sup>. Hence, we aimed to identify prognostic indicators of rNPC to facilitate timely treatment.

Of note, in the current study, CDX2 and NOX4 expression was detected by RT-PCR in rNPC, but not control or NPC, samples (Figure 1). These findings were confirmed by the results of immunohistochemical (Figure 2) and immunoblotting (Figure 3) analyses. CDX2 is a homeobox gene that plays a vital role in early differentiation, regulation, and proliferation of intestinal epithelial cells<sup>35</sup>, whereas NOX4 is expressed in kidney cells<sup>24</sup>. Both CDX2 and NOX4 play a role in host defense, mitogenic growth, and apoptosis<sup>36</sup>.

Levels of these proteins are high in the presence of stress and inflammation, which is involved in the development of metastatic cancer<sup>25</sup>. Stress impacts downstream events, which may lead to tumor progression<sup>37,38</sup>. Radiotherapy, chemotherapy, other medications, and environmental factors may create stress. Inflammation in the nasopharynx of NPC patients, which results in expression of CDX2 and NOX4, may also lead to the development of rNPC.

### Conclusions

CDX2 and NOX4 expression was detected in rNPC, but not NPC, tissues by RT-PCR, immunohistochemistry, and immunoblotting. Our data may facilitate the development of novel therapies for rNPC.

### Acknowledgments

The authors thank the patients, as well as the Institutional Review Board and Ethics Committee.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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