# Significance of NQO1 overexpression for prognostic evaluation of gastric adenocarcinoma

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## ARTICLE INFO

## ABSTRACT

NQO1 (NAD(P)H: quinone oxidoreductase, also known as DT-diaphorase) plays a prominent role in maintaining cellular homeostasis. NQO1 is abnormally elevated in many solid cancer types, including those of the adrenal gland, breast, colon, lung, ovary, and thyroid. However, little is known about the status of NQO1 in gastric adenocarcinoma (GAC). To investigate the clinicopathological significance of NQO1 expression in GAC, and thus evaluate its role as a potential prognostic marker, 203 cases of primary GAC, 31 of gastric dysplasia, and 53 of adjacent non-tumor tissues were selected for immunohistochemical staining of NQO1 protein. Correlations between NQO1 overexpression and clinicopathological characteristics were evaluated by $\chi^2$ test and Fisher's exact test, while survival rates were calculated by Kaplan–Meier method. The relationship between prognostic factors and patient survival was analyzed by Cox proportional hazards model.

Through these analyses it was found that the strongly positive rate of NQO1 protein in GAC was significantly higher than that in gastric dysplasia and adjacent non-tumor tissues. Analysis by qRT-PCR also confirmed that NQO1 mRNA levels were increased in GAC compared with those detected in either adjacent non-tumor tissues or normal gastric mucosa. Additionally, the NQO1 expression rate was positively correlated with tumor size, serosal invasion, tumor stage, and both disease-free survival and 5-year survival rates. Further analysis showed that although NQO1 was not an independent predictor of GAC, elevated expression of NQO1 could predict lower disease-free survival and 5-year survival times in late-stage patients. In conclusion, NQO1 plays an important role in the progression of GAC, and might be a potential, but not an independent, poor prognostic biomarker and therapeutic target of GAC.

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## Introduction

Gastric cancer is the fourth most common malignancy and the second leading cause of cancer deaths worldwide (Geng et al., 2013). The development and progression of gastric cancer is a multistage process which involves multiple molecular pathways and abnormal genetic changes. Despite great advances in surgical and medical management of the disease, the prognosis of gastric cancer has not significantly improved. Therefore, identification of reliable criteria for predicting its recurrence and prognosis attracts widespread research interest.

NAD(P)H: quinone oxidoreductase 1 (NQO1, also known as diphtheria toxin diaphorase (DT-diaphorase)), was discovered by Professor Ernster in 1958 (Siegel et al., 2000) and is located on chromosome 16q22 (Zhuet al., 2013). NQO1 is a mainly cytosolic enzyme which uses NADH or NADPH as substrates to directly reduce quinones to hydroquinones (Zhang et al., 2012). It is present in all tissue types with the exception of the liver (Siegel et al., 2000; Strassburget al., 2002) and is induced along with a battery of defensive genes that provide protection against different stresses to prevent organs from carcinogen-induced tumorigenesis. Because there is an increased incidence of disease and xenobiotic-induced toxicity in individuals carrying a polymorphism in NQO1, it has been suggested that it has a role in chemoprotection.

Paradoxically, in spite of this "cell protector" status, NQO1 expression has been found to be increased during malignant transformation in some tumor types including that of the adrenal gland, breast, colon, lung, ovary, and thyroid (Garate et al., 2010; Lewis et al., 2005; Lyn-Cook et al., 2006), and has also been detected following the induction of cell cycle progression and proliferation of melanoma cells (Garate et al., 2010). To date, the role of NQO1 in cancer progression remains
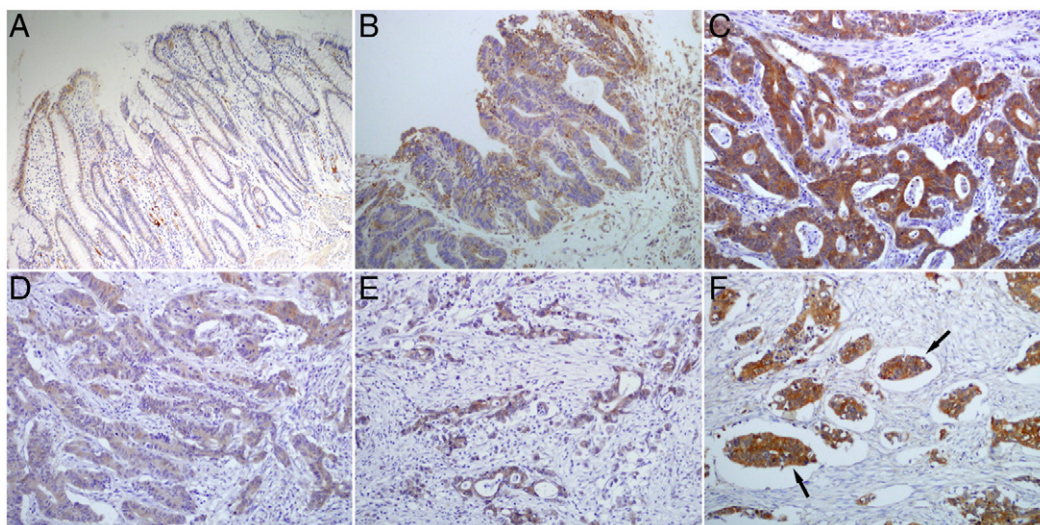
**Fig. 1.** Immunohistochemical staining of NQO1 in gastric lesions. A: Gastric mucosa. B: Atypical cells of gastric dysplasia. C: Gastric adenocarcinoma (GAC) with lymph node metastasis. D: GAC without lymph node metastasis. E: Invasive cancer loci. F: Metastatic cancer cells in blood vessels (*arrows*). Magnification is 100× in A and 200× in B–F.

controversial. Notably, its role in GAC progression has not yet been reported. This study aimed to determine the NQO1 protein expression status in GAC, dysplasia, and adjacent non-tumor tissues, and thereby determine its potential as a prognostic biomarker and therapeutic target in this disease.

## Materials and methods

### Clinical samples

Two hundred and three GAC cases were selected randomly from the Department of Pathology Tumor Tissue Bank, Yanbian University Medical College. These specimens were collected from patients undergoing surgical treatment between 2004 and 2008. The cohort included 135 males and 68 females, ranging from 29 to 72 years old, with a mean age of 49.7. All cases were confirmed as GAC by pathological examination. Tumor stage was determined according to the 7th edition of the American Joint Committee on Cancer (AJCC). Of the 203 samples, 101 cases were stages I–IIa while 102 cases were stages IIb–IIIc. Tumor stage was closely correlated with prognosis. In addition, 80 cases were defined as well-differentiated while 123 cases were poor to mildly differentiated. Fifty-three cases of normal gastric mucosa tissues obtained from the periphery of malignant GAC tissue and 31 cases of gastric dysplasia were also included in the study. None of the patients received radio-chemotherapy before surgery. The 203 patients with GAC had been followed for five years or until death. At the end of the follow-up, 105 patients remained alive.

Fresh samples were also collected and included 12 cases of GAC, 8 cases of adjacent non-tumor tissue and 8 cases of normal gastric mucosa. These were used for RNA extraction and qRT-PCR analysis of NQO1 mRNA.

### Immunohistochemistry

To eliminate endogenous peroxidase activity, 4 μm thick tissue sections were deparaffinized, rehydrated and incubated with 3% $H_2O_2$ in methanol for 15 min at room temperature (RT). The antigen was retrieved by placing the slides in 0.01 M sodium citrate buffer (pH 6.0) at 95 °C for 20 min. The slides were then incubated with NQO1 antibody (1:50, sc-32793, Santa Cruz Biotechnology, Inc. USA) at 4 °C overnight. After incubation with biotinylated secondary antibody at RT for 30 min, the slides were incubated with streptavidin–peroxidase complex at RT for 30 min. Immunostaining was developed using 3,3′-diaminobenzidine, and Mayer's hematoxylin was used for counterstaining. We used tonsil sections as the positive control and mouse IgG as an isotope control. Some positive tissue sections were also processed with omission of the primary antibody (mouse anti-NQO1) as an additional negative control.

All specimens were blind examined by two pathologists. In case of discrepancies, a final score was established by reassessment on a double-headed microscope. The immunostaining for NQO1 was semi-quantitatively scored as '−' (negative) no or less than 5% positive cells; '+' 5–25% positive cells; '++' 26–50% positive cells; and '+++' more than 50% positive cells. Only cytoplasmic staining was considered positive. For statistical analysis, the strongly positive group represents the combined scores of '++' and '+++' positive cells.

### RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of fresh tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using PrimeScript reverse transcriptase (TaKaRa Bio, Dalian, China) and oligo

**Table 1**
NQO1 protein expression in gastric lesions.

| Diagnosis | No. of cases | NQO1 protein expression | | | | Positive rate | Strongly positive rate |
|---|---|---|---|---|---|---|---|
| | | − | + | ++ | +++ | | |
| Gastric adenocarcinoma | 203 | 49 | 29 | 54 | 71 | 75.86% | 61.58% |
| Gastric dysplasia | 31 | 14 | 5 | 6 | 6 | 54.84% | 38.71% |
| Adjacent non-tumor tissues | 53 | 32 | 7 | 9 | 5 | 39.62% | 26.42% |

Statistical analyses were performed using Pearson Chi-square test. Gastric adenocarcinoma compared with Gastric dysplasia, P < 0.05; Gastric adenocarcinoma compared with Adjacent non-tumor tissues, P < 0.01.