Low NOX1 expression in the cytoplasm of GISTs correlates with poor prognosis

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Research

Keywords: NADPH oxidase 1, Neoplasm, immunohistochemistry, Gastrointestinal Stromal Tumors

Posted Date: June 18th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-35609/v1

Abstract

Aim To investigate the protein expression and biological significance of NADPH oxidase l (NOX1) on the growth of human gastrointestinal stromal tumor (GIST), and to test the correlation between NOX1 expression level and patient prognosis.

Methods We collected surgical specimens from a series of 146 GIST patients and used immunohistochemistry (IHC) coupled with a GIST tissue microarray to assess the expression pattern of NOXl in GIST. NOX1 mRNA expression in GIST was subsequently confirmed using the online database Oncomine.

Results There was a significance association with mitotic index (χ^2 = 9.476, p = 0.009) and tumor grade (χ^2 = 11.392, p =0.010) in GIST cells with low cytoplasmic NOX1 cytoplasm staining, which comprised 78 of 146 (53.42%) of GIST samples. Univariate analysis showed that overall survival (OS) rate of GIST patients correlated positively with their NOX1 expression level (c^2=2.429, p=0.014), tumor diameter (c^2=2.048, p=0.004), mitotic index (c^2=2.665, p<0.001), and grade (c^2=3.162, p=0.001). Moreover, multivariate analyses demonstrated that low cytoplasm expression of NOX1 (c^2=2.144, p=0.046) and mitotic index (c^2=2.513, p=0.001) had striking effects on OS. Kaplan-Meier survival plots confirmed that patients with low cytoplasmic NOX1 expression and higher mitotic index had an unfavorable prognosis.

Conclusion The present study unveils that cytoplasmic NOX1 cytoplasm expression and mitotic index is associated with the development of GIST, in support of NOX1-based gene therapy in treating GIST.

1. Introduction

Gastrointestinal stromal tumor (GIST) is an extremely common human sarcoma that arises in the gastrointestinal tract, with 10–15 million new cases diagnosed annually around the world [1]. GISTs arise from the stomach followed by the small intestine, and have various clinical factors such as abdominal pain, palpable mass, bowel obstruction, and blood in the stool [2]. Clinicopathological features including tumor size, mitotic rate and location are closely related with overall survival (OS) of GIST patients [3, 4, 5]. To date, complete surgical resection remains the primary treatment for localized GIST [6]. In recent years, an increasing understanding of GIST biology has enabled diagnosis and treatment of GIST patients based on their histologic characteristics and molecular genetics. It's acknowledged CD117 protein is identified as an immunohistochemical (IHC) marker for GIST, whereas c-KIT mutations are detected by genetic analysis. Moreover, the interstitial cells of Cajal have emerged as the origin of GIST precursors [7, 8]. Thus, IHC and molecular analysis now constitute the gold standard of diagnosis [9].

Timely treatment of GIST patients enables a five-year survival of more than 80%. However, there are considerable toxic side effects of conventional treatments. Having a marker for tumor malignancy is indispensable to the validation of new therapeutic approaches. Monoclonal antibodies against KIT/PDGFRA and resistance to many chemotherapeutics present problems for the continued treatment of

GIST [10, 11, 12]. Consequently, effective diagnostic strategies that predict tumor recurrence and metastasis were gaining increasing attention from GIST researchers, and new information about molecular and genetic biomarkers could help to predict the recurrence risk and malignancy potential of GIST.

NADPH oxidase (NOX) is a family of plasma membrane enzymes that can promote the production of reactive oxygen species (ROS) as a significant mechanism for bodily defense, for example in the phagocytic response to pathogenic microbes and tumors. The various NOX family members (NOX1, NOX3, NOX4, NOX5) have tissue specific expression [13, 14, 15]. Furthermore, ROS generated by NOX enzymes act as second messenger molecules participating in the modulation of cell growth, differentiation, apoptosis, and angiogenesis [16, 17, 18]. Since angiogenesis is necessary for tumor development and metastasis, one might suppose that NOX enzymes could contribute to the regulation of tumor growth.

Indeed, high levels of ROS such as superoxide and hydrogen peroxide are found in many types of cancer cells [19]. In the present study, we consider the contribution of the NOXl-based oxidase system to progression of GIST. We measured the expression of NOX1 in surgical specimens from a series of GIST patients and correlated this expression with indices of tumor stage and patient survival.

2. Materials And Methods

2.1 GIST Patient Tissues.

We obtained surgical specimens from a series of 146 GIST patients from the Department of Clinical Pathology of the Affiliated Hospital of Nantong University and Nanjing First Hospital Affiliated to Nanjing Medical University. Samples were collected during the eight-year period (2003–2010). Diagnosis of GIST was based on histopathological assessment validated by positive IHC staining for the proto-oncogene KIT also known as CD177 or mast/stem cell growth factor receptor. Clinical data, including age, sex, tumor diameter, mitotic index, gross classification, tumor location, GIST risk classification, and tumor grade, were acquired from the medical record of GIST patients. In addition, we performed the potential risk classification to examine malignancy based on the Armed Forces Institute of Pathology (AFIP) Miettinen risk classification criterion [20, 21]. Symptomatically treated patients and those received by preoperative radiotherapy or chemotherapy were not included in the research project, which was approved by the human research ethics committees of the two hospitals.

2.2. Tissue Microarray (TMA) Formation and IHC Detection.

The 146 formalin-fixed and paraffin-embedded GIST tissues, were obtained as described above. As described previously, core biopsies (2-mm in size) were obtained from each paraffin-embedded sample and arranged in prepared paraffin blocks [22] for analysis using a Quick-Ray tissue microarray (TMA) system (UT06; UNITMA, Korea) at the Department of Clinical Pathology of the Affiliated Hospital of Nantong University.

We employed TMA-IHC analysis of NOX1 expression in the GIST samples According to standard protocols, paraffin tissue sections of 5 μm thickness were first dewaxed in 100% xylene, and then rehydrated in a graded ethanol series for 5 min each. Next, the GIST TMAs were simultaneously incubated with rabbit polyclonal anti-NOX1 antibody raised against the C-terminus of human NOX1 (1:300 dilution, ab78016; Abcam, Cambridge, MA, USA) overnight at 4° C, and then incubated with horseradish peroxidase-coupled secondary antibody at room temperature. As a negative control, we substituted the primary antibody with phosphate buffered saline (PBS).

We examined the stained sections under a microscope and counted the number of unstained and cytoplasmically NOX1-positive cells in randomly selected fields. These were defined as four grades: 0–29% (0), 30–59% (1), 60–79% (2), and 80–100% (3). Staining intensity of NOX1-positive cells was also graded as absent (0), weak (1), moderate (2) and strong (3). The product of the density and intensity scores served as the final NOX1 staining score. Finally, we established the cutoff point for a significant relationship between NOX1 expression score and patient OS utilizing the X-tile program (the Rimm Lab, Yale University, New Haven, CT) as described previously [23]. The final sum of percentage of NOX1-positive cytoplasm counts and intensity score jointly represented the NOX1 immunostaining score. The NOX1 immunostaining was meticulously score and evaluated independently by two board-certified senior pathologists separately who were blinded to the case clinical data. The mean score from two pathologists was used as the final immunostaining score.

2.3. Statistical Analysis.

The relationship between NOX1 expression scores and clinicopathological parameters was calculated by two different tests. First, the factors associated with OS were assessed by logistic regression, with univariate and multivariate analyses assessed according to Cox proportional hazards regression models. Second, we plotted survival curves utilizing the Kaplan-Meier method and compared differences between curves using the log-rank test. All statistical analyses were calculated with IBM SPSS Statistics 22.0 (SPSS Inc., Chicago, IL).

2.4. Confirmation of NOX1 Expression in GIST.

To validate the association between NOX1 expression in GIST, we explored NOX1 mRNA expression in the online database Oncomine (https://www.oncomine.org). This contained three NOX1 datasets, i.e. the Cho Gastric dataset (90 samples), Detwiller Sarcoma dataset (54 samples), and the Segal sarcoma2 dataset (51 samples).

3. Results

3.1. Clinicopathologic Details.

GIST samples were collected from 73 men and 73 women. 84 GIST patients were aged ≤ 60 years and 62 > 60 years. 35 GIST patients had tumors < 5 cm in diameter, 70 patients had tumors measuring 5–10 cm in size, and 37 patients had tumors ≥ 10 cm in diameter. 68 patients had 0–5 mitotic index, 45 had

6–10 mitotic index, and 29 had > 10 mitotic index. 16 patients had single nodules and 22 patients had multiple nodules. GIST tumors were located in the stomach of 77 patients, the small intestine in 54 cases, and in other organs in 15 cases. What's more, 34 patients were in the very low to low risk group, and 51 patients in the moderate to high risk group, as assessed by AFIP Miettinen risk classification. 46 patients were grade 1, 53 patients were grade 2, 27 patients were grade 3, and 14 patients were grade 4.

3.2. Location and Expression degree of NOX1 Status in GIST by IHC staining.

We confirmed that NOX1 positive staining preferentially localizes to the cytoplasm in human GIST. Representative NOX1 staining patterns by IHC analysis are shown in Fig. 1. Based on the cutoff point for NOX1 expression, tissues samples were categorized and computed using the X-tile software. Low NOX1 cytoplasmic IHC staining of tumor cells was observed in 78 of 146 (53.4%) of the GIST samples and the remaining 68 cases (46.6%) showed high NOX1 cytoplasm staining. Furthermore, there was a significant correlation between NOX1 expression with mitotic index ($p = 0.009$) and tumor grade ($p = 0.010$) in the 78 (53.4%) patients with low expression, but no such correlation in the group with high NOX1 expression (Fig. 1).

3.3 Relationship between cytoplasm Expression of NOX1 and Clinicopathological Details.

The association between NOX1 expression and clinicopathological details of GIST patients is illustrated in Table 1. We found NOX1 Expression was conspicuously related to tumor mitotic index ($\chi^2 = 9.476$, $p = 0.009$) and grade ($\chi^2 = 11.392$, $p = 0.010$). However, there was no evident association between NOX1 protein expression and other clinicopathological characteristics, including gender, age, gross classification, tumor diameter, AFIP Miettinen risk classification, and tumor location (Supplementary Table 1).

3.4. Survival Analysis.

To confirm further the expression profile of NOX1, we undertook univariate Cox's regression analysis to find the factors that significantly influenced survival. Statistically significant differences in OS in relation to low cytoplasmic NOX1 expression ($\chi^2 = 2.429$, $p = 0.014$), tumor diameter ($\chi^2 = 2.048$, $p = 0.004$), mitotic index ($\chi^2 = 2.665$, $p < 0.001$), and GIST grade ($\chi^2 = 3.162$, $p = 0.001$) (Table 2). All the above-mentioned factors correlated with prognosis of GIST patients according to OS rates. In addition, results showed no significant relationship between NOX1 expression and other clinicopathological characteristics, including age, gender, gross classification, tumor location, and AFIP Miettinen risk classification (Supplementary Table 2). Next, multivariate analyses indicated an association between OS with cytoplasm NOX1 expression ($\chi^2 = 2.144$, $p = 0.046$) and mitotic index ($\chi^2 = 2.513$, $p = 0.001$) (Table 2). Meanwhile, Kaplan–Meier survival curves revealed that shorter OS was strongly associated with level of NOX1 expression, and that risk of death increased with each increment of NOX1 expression ($p = 0.011$). Longer OS of the

patients was associated with low mitotic index (< 5 HPFs) compared to those with high mitotic index (> 5 HPFs) ($p < 0.001$). GIST patients with low cytoplasmic NOX1 expression and mitotic index > 5 had significantly ($p < 0.05$) less favorable prognosis (Figs. 2 and 3).

3.5. Association between NOX1 protein expression and prognosis

To assess the NOX1 mRNA expression levels in an independent set of samples from GIST patients, we interrogated Oncomine database, which contains DNA and RNA sequencing information culled from the Gene Expression Library. The three databases including seven analyses were determined indicated that mRNA expression of NOX1 was significantly higher/lower in GIST samples compared to normal tissue. ($p < 0.05$) (Fig. 4).

4. Discussion

Accumulating evidence indicates that ROS generated by members of the NOX family contribute importantly to immunological defense and the regulation of cell differentiation, proliferation, apoptosis, and intercellular signaling pathways. Excessive ROS generation causes oxidative stress, which accelerates the pathogenesis of inflammation, fibrosis, and tumor survival. Thus, NOX can be key factor in cancer initiation and progression, while also serving for diagnosis and prognostic estimation. Previous evidence indicates that loss of NOX1 activity is associated with poor patient survival or drug-resistance in human tumors, and may thus hold some promise as a therapeutic target [24]. NOX1-mediated ROS production regulating exocytosis is a vital factor for development in human cancers, which has an association with unfavorable metastasis risk and poor OS. Thus, NOX1 activity is a biomarker of prognosis for progression in a variety of human cancers[25].

More importantly, recent studies have shown that ROS produced by NOX1 plays a key role in cancer transformation and invasive progression [25, 26], with notable overexpression in cancers of the breast, colon, stomach and prostate, among others [25, 27, 28]. Indeed, NOX1 has been proposed to function as an oncogene and a therapeutic target [29, 30]. In addition, NOX1 may have constitutively high expression in human colon epithelial cells compared with other normal cell types [31].

Given this background, we investigated the association between NOX1 expression and clinicopathological features of GIST. After showing that NOX1 is mainly localized in the cytoplasm, we revealed a low intensity of NOX1 in GIST cells from 78 of our 146 cases, As shown in Table 2, our statistical analyses show that low NOX1 staining and mitotic index were associated with poor survival in GIST patients, suggesting that inadequate NOS generation may favor tumor progression. Indeed, the Kaplan-Meier survival curves showed that longer OS was strongly associated with a low NOX1 expression and a favorable prognosis for GIST patients. Meanwhile, the positive expression rate of NOX1 increased along with the mitotic index (per 50 HPFs).

We also examined the expression of NOX1 mRNA in tissue samples from GIST patients reported in the Oncomine online databases. This revealed that NOX1 protein and mRNA expression levels were both significantly higher in GIST samples compared with normal tissue tissues (Figure. 4), thus concurring with the present findings with IHC.

The main novelty of the present study is our finding that low NOX1 expression is confirmed to be useful prognostic marker for the progression of GIST. This result was obtained in material from patients of all disease stages, none of whom had received preoperative radiotherapy or chemotherapy. GIST patients with high expression of NOX1 did not manifest a significant difference in survival or disease progression. It remains an open matter who NOX1 mediates effects on cancer cell proliferation, migration, and invasion, but we propose that ROS production in macrophages may be decisive in this regard. Pharmacological inhibition of NOX1 or ROS-related redox signaling pathways might be used as tools for preclinical studies of the underlying mechanisms [32]. Another matter for future consideration is the possibility that genetic variants in the NOX-1 or other NOX isoforms may contribute to individual differences in cancer progression.

Taken together, we present an association between low NOX1 expression with high mitotic index in GIST samples with unfavorable prognosis. Therefore, low NOX1 expression emerges as a potentially useful predictive marker for prognosis of GIST. Targeted inhibition of NOX1 could be an attractive strategy for the treatment and management of GIST recurrence and progression.

Abbreviations

NOX1:NADPH oxidase l;

GIST:gastrointestinal stromal tumor;

IHC:immunohistochemistry;

OS:overall survival;

ROS:reactive oxygen species;

AFIP:Armed Forces Institute of Pathology ;

TMA:tissue microarray

Declarations

Acknowledgements

Acknowledgement to the technical staff of Pathology of Nantong University Affiliated Hospital, Jiangsu Province, China.

Authors' Contributions

Wei Wang,Wenjun You and Huijun Zhu were responsible for the study conception and design. Wei Wang and Wenjun You were responsible for the acquisition of data. Xin He and Ao Yuan performed the immunostaining techniques. Wei Wang, Wenjun You and Fang Huang were responsible for the analysis and interpretation of data. Wei Wang and Huijun Zhu were responsible for the drafting of the manuscript. All authors read and approved the final manuscript.

Funding

This research received no external funding.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study was approved by the human research ethics committees of the Department of Clinical Pathology of the Affiliated Hospital of Nantong University and Nanjing First Hospital Affiliated to Nanjing Medical University, Jiangsu Province, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Due to technical limitations, the tables are only available as a download in the supplemental files section.

Figures

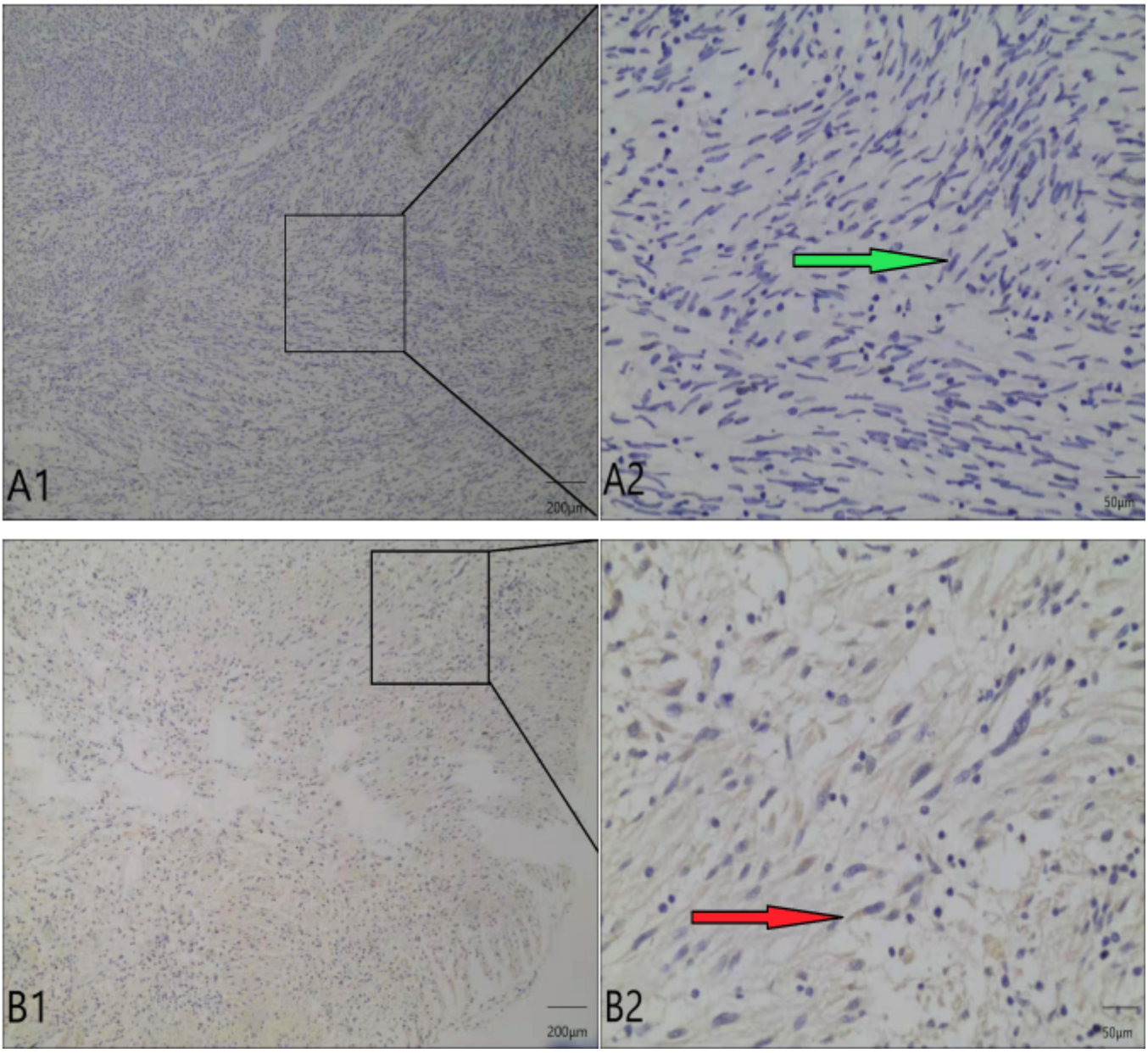

Figure 1

Immunohistochemical staining was determined on paraffin-embedded 5 μm sections. Representative patterns of NOX1 protein expression in GIST are observed. In GIST tissues, green arrow represents negative cytoplasm staining (A1 and A2), red arrow represents positive cytoplasm staining (B1 and B2). A1 and B1: original magnification ×100 (bar = 200 μm) A2 and B2: ×400 (bar = 50 μ m).

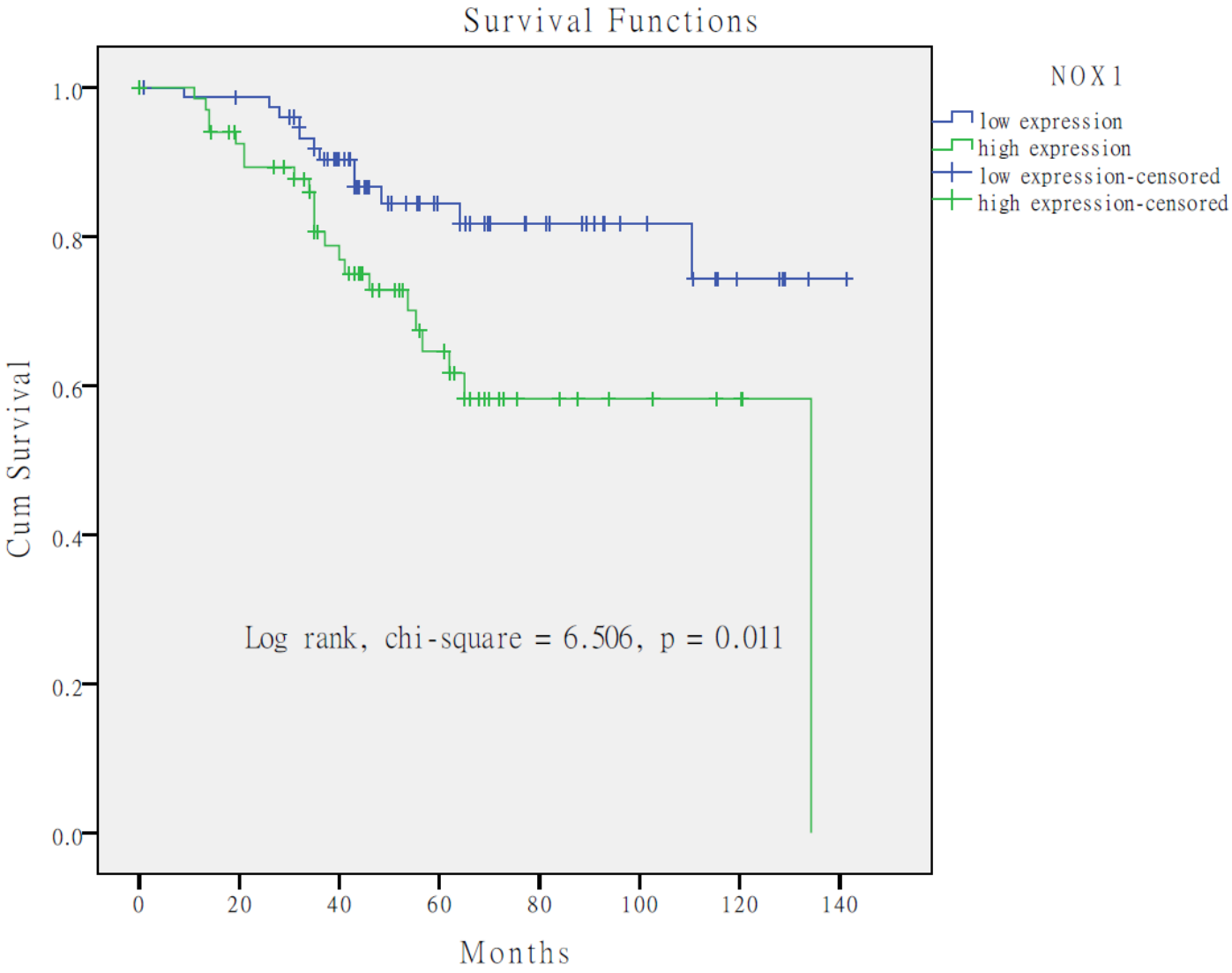

Figure 2

Correlation between overall survival of GIST patients and level of NOX1 expression.

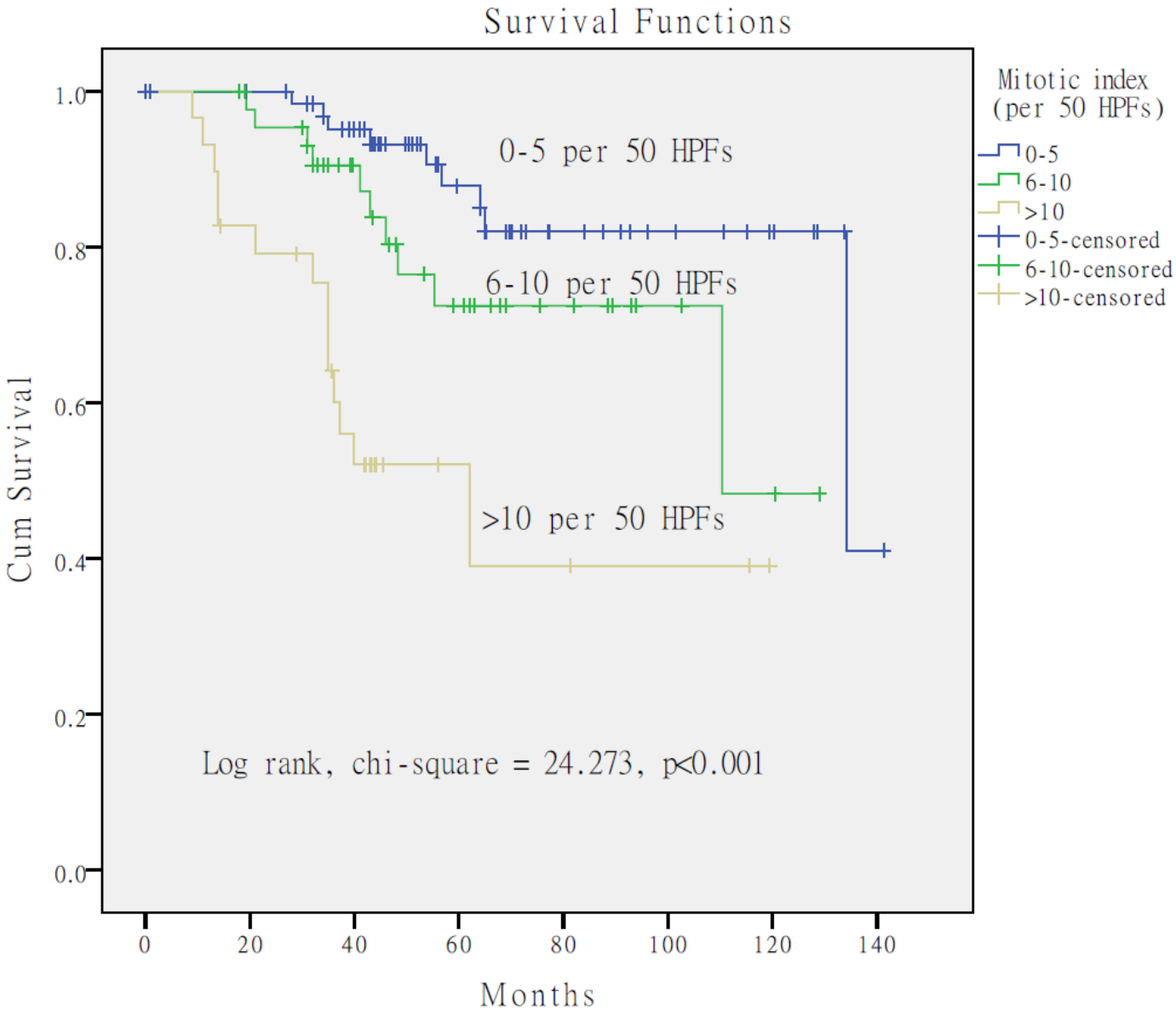

Figure 3

Correlation between overall survival of GIST patients and mitotic index.

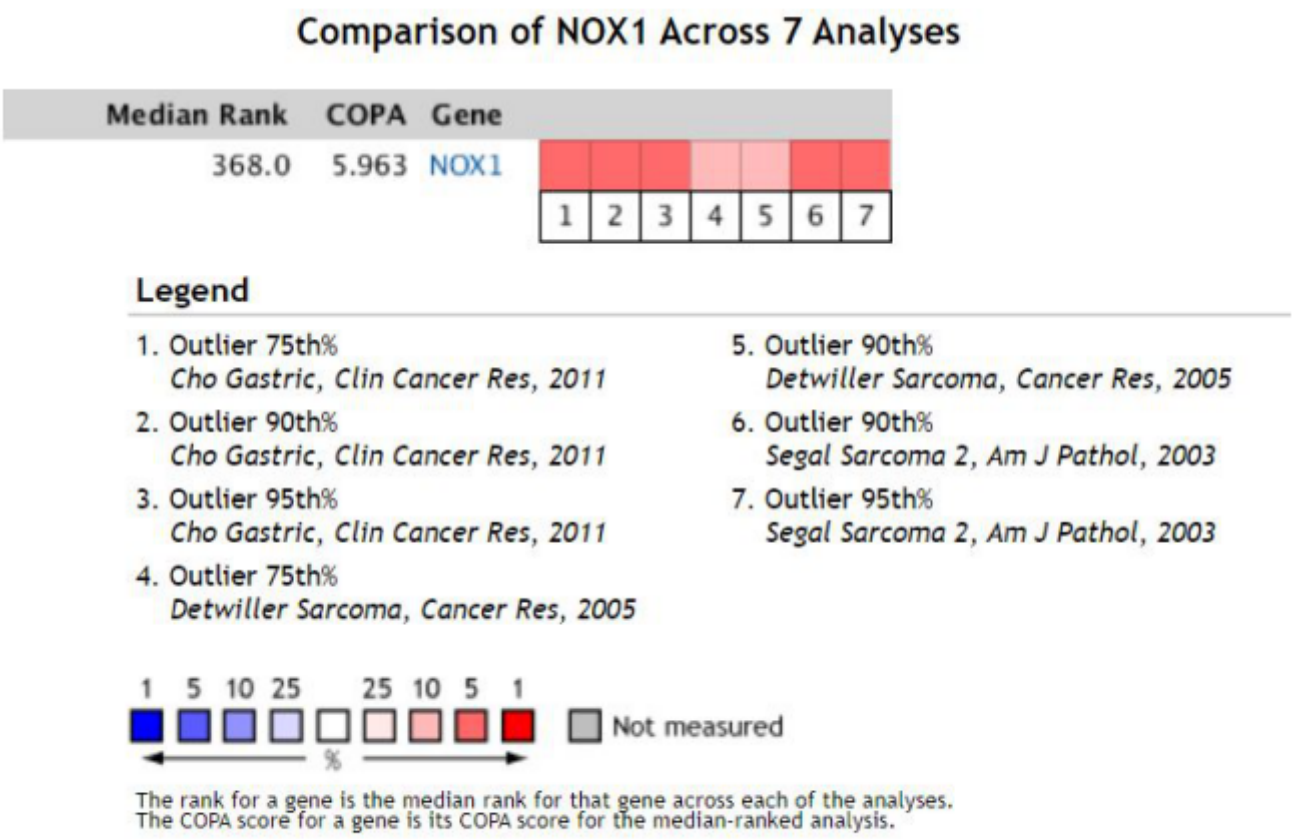

Figure 4

We presented data derived from the Oncomine database. The intensity of color shows the respective mRNA levels of NOX1. The red column means the NOX1 mRNA upregulation.

Supplementary Files

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- 6.12Supplementarytable1and2.docx
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