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ANATOMICAL PATHOLOGY

# High nuclear NADPH oxidase 4 expression levels are correlated with cancer development and poor prognosis in hepatocellular carcinoma

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## Summary

NADPH oxidase (NOX) is a key source of reactive oxygen species (ROS). This study aimed to verify NOX2 and NOX4 expression levels in hepatocellular carcinoma (HCC). A total of 134 matched pairs of HCC cells and non-tumour hepatocytes from 134 patients were examined by immunohistochemical staining, and the association of NOX2 and NOX4 expression with clinicopathological parameters was analysed. Western blotting in four HCC cell lines and reverse transcription digital droplet polymerase chain reaction (RT-ddPCR) in 20 pairs of HCC and non-tumour tissue samples were also performed to detect NOX4. Cytoplasmic NOX2 and nuclear NOX4 expression levels were shown by immunohistochemistry to be higher in HCC cells than in non-tumour hepatocytes ($p$<0.001 each). The western blotting results for NOX4 in four HCC cell lines were consistent with the immunohistochemical results. Increased cytoplasmic expression of NOX2 and NOX4 in HCC cells was significantly correlated with liver cirrhosis ($p$<0.001 and $p$<0.031, respectively). However, decreased cytoplasmic expression of NOX2 and NOX4 was significantly correlated with advanced pathological TNM stage ($p$<0.029 and $p$<0.007, respectively). Multivariate analysis with clinicopathological parameters showed that high nuclear and low cytoplasmic NOX4 expression levels are correlated with short overall survival ($p$=0 .021). Our findings imply that cytoplasmic NOX2 and nuclear NOX4 expression is upregulated during HCC development. In particular, NOX4 translocation into the nucleus may affect the development and progression of HCC. NOX2 and NOX4 could be diagnostic markers and have therapeutic implications in HCC.

*Key words:* NADPH oxidase 2; NADPH oxidase 4; hepatocellular carcinoma; diagnosis; prognosis.

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## INTRODUCTION

Reactive oxygen species (ROS), such as superoxide and $H_2O_2$, have been implicated in inflammatory pathways and cancer initiation and development by molecular damage and disruption of reduction–oxidation (redox) signalling.[1,2] It is generally accepted that ROS are cytotoxic and mutagenic mediators, but recent reports suggest that ROS may be regulators of signal-transduction pathways for cell proliferation and survival.[3] ROS are tumourigenic, as they not only induce DNA damage and, thus, genetic lesions but also participate as second messengers of intracellular signalling cascades that initiate tumourigenicity and sustain tumour progression.[4]

NOX family NADPH oxidases (NOXs) are a major source of ROS in various cell types and mediate critical roles in physiological and pathological processes, including cell signalling, inflammation and proliferation.[3,5,6] The mammalian NOX complex family comprises seven paralogues: five NOXs and two dual oxidases (DUOXs).[4,7] NOX4 expression appears to be involved in anti-apoptosis and cell proliferation and survival in pancreatic cancer cells[8,9] and glioma cells.[3] NOX2 expression was reported to be associated with tumour progression and metastasis by induction of oncogenic KRAS and RAF signalling and upregulation of angiogenesis-related genes.[6]

Hepatocellular carcinoma (HCC) is typically associated with prolonged inflammation in the cirrhotic liver, which results in the transition from chronic inflammation and fibrosis to dysplastic or regenerative nodules or HCC.[10] Altered levels of NOX2 or NOX4 expression may be related to hepatocarcinogenesis. However, our understanding of the association of NOX2 or NOX4 expression with clinicopathological values in HCC is limited. How and where NOX2 and NOX4 are expressed in HCC has not yet been elucidated in detail, and the diagnostic and therapeutic significance of NOX2 and NOX4 expression in HCC has not been studied in detail.

In this study, we investigated the expression of NOX2 and NOX4 and their value as a diagnostic and prognostic factor in HCC by protein analysis according to the location of

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expression through nuclear and cytoplasmic separation of NOX2 and NOX4 in HCC. We performed immunohistochemical staining for NOX2 and NOX4 in 134 HCC cases and analysed various clinicopathological characteristics. Western blotting was performed in four HCC cell lines to support the immunohistochemical staining results. Reverse transcription digital droplet polymerase chain reaction (RT-ddPCR) for NOX4 in 20 pairs of HCC and non-tumour tissue samples was also performed.

## MATERIALS AND METHODS

### Patients and tissue samples

We retrospectively screened 134 cases of HCC between 1999 and 2014 at Chungnam National University Hospital in Daejeon, South Korea. All formalin fixed, paraffin embedded (FFPE) tissue samples were isolated from HCC patients who underwent segmentectomy or lobectomy. The two most representative viable tumour areas and one non-tumour tissue, at least 3.0 cm distant from the tumour, were selected and marked on haematoxylin and eosin (H&E) stained slides. Tissue microarrays (TMAs) were created by punching tissue columns (3.0 mm in diameter) from the original paraffin blocks and inserting the columns into new recipient paraffin blocks (each containing 30 holes to receive the tissue columns).

Twenty matched pairs of fresh-frozen primary HCC and non-tumour liver tissue samples were obtained for RT-ddPCR from the National Biobank of Korea at Chungnam National University Hospital, a member of the Korean Biobank Network.

This study was approved by the Institutional Review Board of Chungnam National University Hospital (CNUH 2017-04-040-001). All frozen tissue samples for RT-ddPCR and clinical data were obtained from the National Biobank of Korea at Chungnam National University Hospital. All patients signed a written informed consent form for biobanking before data were included in the register. The prerequisite for informed consent for this retrospective comparison study involving immunohistochemical analysis of FFPE tissues was waived. In addition, all methods in this study were performed in accordance with the relevant guidelines and regulations and approved by the Institutional Review Board of Chungnam National University Hospital.

### Immunohistochemical study

Immunohistochemical staining of the tissue sections from the TMA paraffin blocks was performed as previously described.[11] A primary mouse monoclonal antibody against human NOX2/gp91phox (ab80897, diluted 1:100; Abcam, UK) and a rabbit polyclonal antibody against human NADPH oxidase 4 (ab79971, diluted 1:100; Abcam) were used; the primary antibody reactions were incubated for 30 min.

The method by Allred *et al.* was modified and applied to evaluate both the intensity of the immunohistochemical staining and the proportion of stained neoplastic or non-tumour hepatocytes on each slide.[12] The proportion scores ranged from 0 to 5 (0, 0; 1, >0 to 1/100; 2, >1/100 to 1/10; 3, >1/10 to 1/3; 4, >1/3 to 2/3; and 5, >2/3 to 1), and the intensity scores ranged from 0 to 3 (0, negative; 1, weak; 2, moderate; and 3, strong). To generate the total immunohistochemical score, the intensity score and proportional score were multiplied for each specimen (range 0–15). For categorical analyses, expression scores greater than the median value of the total score of cytoplasmic NOX2, cytoplasmic NOX4 and nuclear NOX4 expression were regarded as high (total score >5, >3, and >3, respectively). HCC cells demonstrating more nuclear NOX4 expression than the median value, associated with median or lower than median cytoplasmic expression were defined as highN&lowC NOX4 cells. The results were examined separately and scored by KHK and MKY, who were blinded to the patient details. Discrepancies in scores were discussed to obtain a consensus.

### Reverse transcriptase digital droplet PCR (RT-ddPCR)

Based on the review of the H&E stained frozen sections, 20 matched pairs of HCC and non-tumour frozen tissue samples stored in liquid nitrogen were obtained. Total RNA was isolated from frozen samples in liquid nitrogen. The tissues were roughly chopped, dissociated with a gentle MACS dissociator with a human tumour dissociation kit, and filtered through a MACS SmartStrainers (70 μm) (Miltenyi Biotec, Germany). To remove as many nonparenchymal cells as possible, after centrifugation at 1000 rpm for 5 min at 4°C, the supernatant was removed, and a precipitated cell suspension was used for total RNA extraction.[13] Total RNA was reverse-transcribed to complementary DNA (cDNA) using cDNA synthesis master mix (ReverTra Ace qPCR RT Master Mix with gDNA remover; Toyobo, Japan). The following primers were developed: NOX4 forward primer sequence: 5′-TCCACCAGATGTTGGGGGAT-3′ and reverse primer sequence: 5′-GAGTGTTCGGCACATGGGTA-3′ (input PCR template NM_001291926.1). The QX200 Droplet Digital PCR System (Bio-Rad, USA) was used for RT-ddPCR analysis of NOX4. ddPCR was conducted as previously described.[14] A 20 μL PCR mix was prepared with QX200 ddPCR EvaGreen Supermix (Bio-Rad), each primers at 10 pM and approximately 50 ng of cDNA template.

### Western blotting

Nuclear and cytoplasmic proteins were extracted from the HepG2, Hep3B, Huh-7, SK Hep-1 cell lines separately using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific 78833; Pierce, USA). Western blot analyses were performed with 30 μg of protein in each well using anti-NOX4 (Abcam) and anti-β-actin antibodies (Cell Signaling, USA). Immunoreactive bands were visualised on nitrocellulose membranes using alkaline phosphate-linked anti-rabbit antibody and the ECL detection system with a PhosphorImager (GE Healthcare, USA).

### Statistical analyses

Relationships of NOX2 and NOX4 expression with clinicopathological parameters were evaluated using Pearson's chi-square test and the Mann–Whitney U test. Differences in NOX2 or NOX4 expression levels between paired HCC tissue and non-tumour tissue sections were assessed using the Wilcoxon signed-rank test. Postoperative overall and disease-free survival were determined using Kaplan–Meier survival curves and a log-

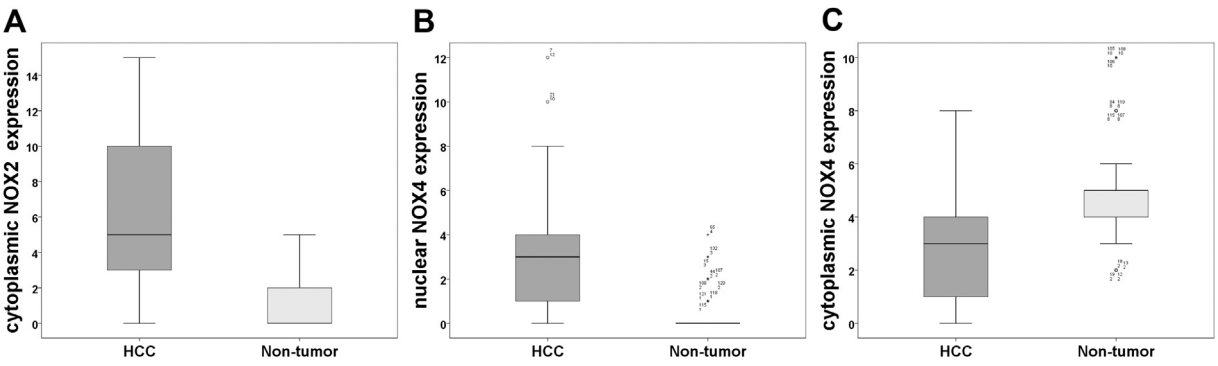


**Fig. 1** In total, 134 matched pairs of HCC and non-tumour tissue samples were assessed for cytoplasmic NOX2 and both nuclear and cytoplasmic NOX4 expression using the Wilcoxon signed-rank test ($p<0.001$, respectively). The line in the middle of the boxes represents the median. The box length indicates the interquartile range. The ends of the whiskers represent the maximum and minimum values.

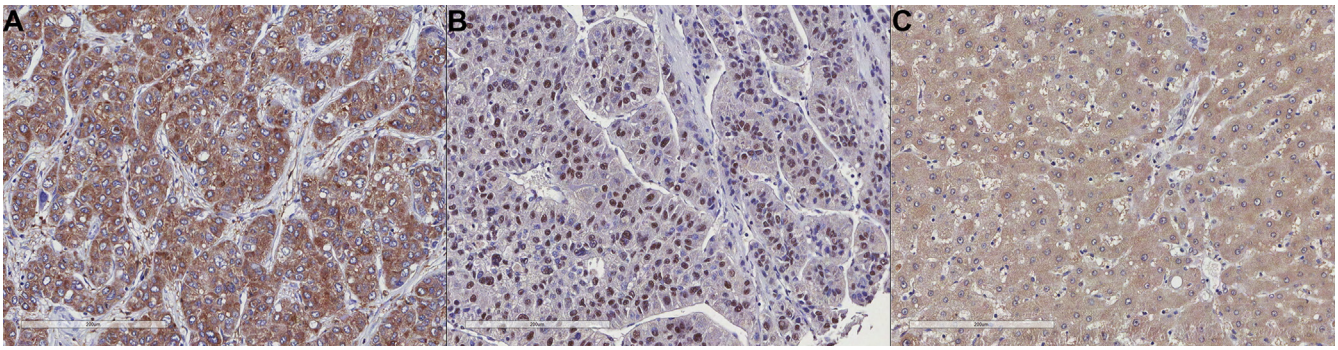


**Fig. 2** Representative NOX2 and NOX4 expression in HCC (A,B). The strong cytoplasmic expression of NOX2 (A) contrasts with the strong nuclear expression of NOX4 (B) in HCC tumour cells. Representative NOX4 expression in non-tumour liver tissue (C). The positive cytoplasmic expression contrasts with the negative nuclear expression of NOX4 in non-tumour hepatocytes (scale bar = 200 μm).

rank test. The Cox proportional hazards model was applied for univariate and multivariate postoperative survival analysis. A $p$ value < 0.05 was considered significant (SPSS v.24; SPSS, USA).

## RESULTS

### Expression patterns of NOX2 and NOX4 in HCC and non-tumour hepatocytes

The 134 matched pairs of HCC and non-tumour tissue samples were immunohistochemically evaluated for NOX2 and NOX4 expression. The cellular localisation of NOX4 was the cytoplasm or nucleus, while NOX2 showed only cytoplasmic expression (Fig. 1 and 2). Cytoplasmic NOX2 expression was generally higher in HCC hepatocytes than in non-tumour hepatocytes ($p$<0.001). Conversely, cytoplasmic NOX4 expression was lower in HCC hepatocytes than in non-tumour hepatocytes ($p$<0.001). In non-tumour hepatocytes, NOX4 was usually expressed in the cytoplasm, although some cells exhibited faint nuclear expression (Fig. 2C). In HCC cells, NOX4 generally showed higher nuclear and/or lower cytoplasmic expression than in non-tumour hepatocytes ($p$<0.001, respectively) (Fig. 1 and 2B). Thirty-five of the 134 HCC cases were identified as highN&lowC NOX4 group. The HCC cells showed higher expression levels of cytoplasmic NOX2 and nuclear NOX4

**Table 1** Summary of the clinicopathological characteristics of patients with hepatocellular carcinoma ($n$=134)

| Characteristics | $n$ | Nuclear NOX4 | | Cytoplasmic NOX4 | | Cytoplasmic NOX2 | |
|---|---|---|---|---|---|---|---|
| | | Median (IQR) | $p$[a] | Median (IQR) | $p$[a] | Median (IQR) | $p$[a] |
| Gender | | | 0.335 | | 0.008 | | 0.124 |
| Male | 103 | 3 (1–5) | | 3 (2–4) | | 5 (3–10) | |
| Female | 31 | 2 (1–3) | | 2 (0–3) | | 4 (2–8) | |
| Age | | | 0.537 | | 0.148 | | 0.953 |
| ≤65 | 110 | 3 (1–4) | | 3 (2–4) | | 5 (3–10) | |
| >65 | 24 | 2 (1–4) | | 2 (0–4) | | 5 (2–10) | |
| HBV or HCV | | | 0.753 | | 0.329 | | 0.087 |
| Negative | 25 | 3 (1–4) | | 3 (1–4.5) | | 5 (3.5–10) | |
| Positive | 109 | 3 (1–5) | | 3 (1–4) | | 5 (2.5–8) | |
| Liver cirrhosis | | | 0.603 | | 0.031 | | <0.001 |
| Negative | 33 | 3 (1–4.5) | | 2 (1–3) | | 2 (1–5) | |
| Positive | 101 | 3 (1–4) | | 3 (1.5–4) | | 5 (4–10) | |
| Tumour size[b] | | | 0.050 | | 0.231 | | 0.305 |
| ≤5.0 cm | 113 | 3 (1–4) | | 3 (1–4) | | 5 (3–10) | |
| >5.0 cm | 21 | 3 (2–4) | | 2 (1–3) | | 5 (1.5–8) | |
| Lymphovascular invasion | | | 0.060 | | 0.810 | | 0.513 |
| Negative | 31 | 2 (1–3) | | 3 (1–4) | | 5 (3–10) | |
| Positive | 103 | 3 (1–5) | | 3 (1–4) | | 5 (3–10) | |
| Histological grade | | | 0.422 | | 0.674 | | 0.968 |
| 1–2 | 113 | 3 (1–4) | | 3 (1–4) | | 5 (3–9) | |
| 3–4 | 21 | 3 (1–5) | | 3 (1–4) | | 5 (2.5–10) | |
| Pathological stage | | | 0.186 | | 0.669 | | 0.416 |
| I | 45 | 3 (1–4) | | 3 (1.5–4) | | 5 (3–10) | |
| II–IV | 89 | 3 (1–5) | | 3 (1–4) | | 5 (2.5–8) | |
| Pathological stage | | | 0.434 | | 0.007 | | 0.029 |
| I–II | 125 | 3 (1–4) | | 3 (1–4) | | 5 (3–10) | |
| III–IV | 9 | 4 (1–4.5) | | 0 (0–2.5) | | 2 (0–5.5) | |

Cytoplasmic NOX2, cytoplasmic NOX2 expression in cancer cells; HBV, hepatitis B viral infection; HCV, hepatitis C viral infection; histological grade, 1–2 well and moderately differentiated, 3–4 poorly differentiated and undifferentiated; IQR, interquartile range; nuclear NOX4, nuclear NOX4 expression in cancer cells.
[a] Mann–Whitney U test.
[b] Greatest diameter.

**Table 2** Univariate analysis of overall survival and disease-free survival in 134 patients with primary HCC

| Prognostic factor | Overall survival | | Disease-free survival | |
|---|---|---|---|---|
| | HR (95% CI) | $p^a$ | HR (95% CI) | $p^a$ |
| Nuclear NOX4 | 1.127 (0.977–1.300) | 0.100 | 1.030 (0.930–1.141) | 0.570 |
| Cytoplasmic NOX4 | 0.823 (0.678–0.998) | 0.048 | 1.013 (0.899–1.141) | 0.831 |
| Cytoplasmic NOX2 | 0.952 (0.874–1.036) | 0.253 | 1.003 (0.952–1.057) | 0.905 |
| Age at operation | 1.000 (0.965–1.035) | 0.982 | 0.978 (0.956–1.001) | 0.063 |
| Sex | | | | |
| Male | 1 (reference) | | 1 (reference) | |
| Female | 0.839 (0.346–2.034) | 0.698 | 1.060 (0.618–1.817) | 0.832 |
| HBV or HCV | | | | |
| No | 1 (reference) | | 1 (reference) | |
| Yes | 1.064 (0.466–2.430) | 0.884 | 1.212 (0.691–2.127) | 0.502 |
| Cirrhosis | | | | |
| No | 1 (reference) | | 1 (reference) | |
| Yes | 0.959 (0.451–2.040) | 0.913 | 1.519 (0.885–2.605) | 0.129 |
| Tumour size[b] | 1.220 (1.084–1.373) | 0.001 | 1.175 (1.079–1.280) | <0.001 |
| Lymphovascular invasion | | | | |
| No | 1 (reference) | | 1 (reference) | |
| Yes | 0.895 (0.403–1.985) | 0.784 | 2.118 (1.116–4.019) | 0.022 |
| Chemotherapy | | | | |
| No | 1 (reference) | | 1 (reference) | |
| Yes | 3.403 (1.488–7.780) | 0.004 | 8.685 (4.665–16.167) | <0.001 |
| Radiation therapy | | | | |
| No | 1 (reference) | | 1 (reference) | |
| Yes | 3.016 (1.243–7.315) | 0.015 | 4.416 (2.323–8.397) | <0.001 |
| Histological grade | | 0.007 | | 0.007 |
| 1 (well) | 1 (reference) | | 1 (reference) | |
| 2 (moderate) | 0.729 (0.306–1.737) | 0.475 | 1.030 (0.560–1.896) | 0.924 |
| 3 (poorly) | 2.497 (0.945–6.597) | 0.065 | 2.438 (1.176–5.053) | 0.017 |
| 4 (undifferentiated) | NA | NA | NA | NA |
| Pathological stage | | <0.001 | | <0.001 |
| I | 1 (reference) | | 1 (reference) | |
| II | 1.142 (0.543–2.404) | 0.726 | 1.711 (1.029–2.845) | 0.039 |
| III | 2.662 (0.736–9.633) | 0.136 | 7.731 (3.029–19.730) | <0.001 |
| IV | 25.405 (6.447–100.110) | <0.001 | 14.668 (3.206–67.116) | 0.001 |

CI, confidence interval; Cytoplasmic NOX2, cytoplasmic NOX2 expression in cancer cells; HR, hazard ratio; HBV, hepatitis B viral infection; HCV, hepatitis C viral infection; Nuclear NOX4, nuclear NOX4 expression in cancer cells; NA, not applicable.
[a] Univariate Cox regression analysis.
[b] Greatest diameter.

than the non-tumour hepatocytes ($p$<0.001, respectively) (Fig. 2).

## Correlation of NOX2 and NOX4 protein expression with the clinicopathological characteristics

We investigated 134 matched pairs of HCC and non-tumour tissue samples from 134 patients with HCC. The clinicopathological characteristics of the 134 HCC patients in association with NOX4 and NOX2 protein expression as determined by immunohistochemical staining are presented in Table 1. The average age of the patients at the time of the operation was 58.10 years, and the male:female ratio was 103:31 (76.9%:23.1%).

Higher cytoplasmic NOX2 and NOX4 expression levels in HCC cells were positively correlated with liver cirrhosis ($p$<0.001 and $p$=0.031). Increased nuclear NOX4 expression showed a trend of positive correlation with tumour size and lymphovascular tumour invasion ($p$=0.050 and $p$=0.060). Meanwhile, decreased cytoplasmic expression of NOX4 and NOX2 was positively correlated with advanced pathological TNM stage ($p$=0.007 and $p$=0.029).[15]

Univariate analyses for overall survival (OS) and disease-free survival (DFS) using Cox's proportional hazard regression model are summarised in Table 2. The multivariate analyses of survival using Cox's proportional hazard regression model were performed for high nuclear and low cytoplasmic expression of NOX4, cytoplasmic NOX2 expression, age, sex, hepatitis B or C viral infection, liver cirrhosis, postoperative chemotherapy, histological grade, and pathological TNM stage in the 134 HCC cases. The multivariate analysis revealed that high nuclear and low cytoplasmic expression of NOX4 was an independent prognostic factor for short OS ($p$=0.021) (Table 3; Supplementary Table 1, Appendix A).

OS was defined as the date of surgery to either death or the last follow-up date. DFS was defined as the date of surgery to the date of recurrence or the date of the last follow-up. The mean follow-up durations for OS and DFS were 49.15±31.828 and 34.92±29.789 months.

## NOX4 mRNA expression levels in 20 matched pairs of HCC and non-tumour liver tissues

RT-ddPCR analysis of NOX4 mRNA in 20 matched pairs of HCC tissue and non-tumour tissue samples from 20 patients showed a trend of higher copy number in non-tumour tissue than in HCC tissue ($p$=0.112, Wilcoxon signed-rank test). The average number of copies of NOX4 in 50 ng of cDNA template derived from HCC/non-tumour tissue was 75.7/81.3 (Fig. 3).

**Table 3** Multivariate analysis of overall survival and disease-free survival in 134 patients with primary HCC

| Prognostic factor | Overall survival | | Disease–free survival | |
|---|---|---|---|---|
| | HR (95% CI) | $p^a$ | HR (95% CI) | $p^a$ |
| HighN&lowC NOX4 | | | | |
| No | 1 (reference) | | 1 (reference) | |
| Yes | 2.787 (1.170–6.635) | 0.021 | 1.227 (0.675–2.232) | 0.502 |
| Cytoplasmic NOX2 | 0.977 (0.888–1.075) | 0.636 | 1.019 (0.957–1.085) | 0.556 |
| Age at operation | 0.993 (0.952–1.036) | 0.750 | 0.988 (0.965–1.011) | 0.298 |
| Sex | | | | |
| Male | 1 (reference) | | 1 (reference) | |
| Female | 1.063 (0.420–2.689) | 0.897 | 1.669 (0.939–1.011) | 0.081 |
| HBV or HCV | | | | |
| No | 1 (reference) | | 1 (reference) | |
| Yes | 1.234 (0.436–3.494) | 0.692 | 1.113 (0.588–2.105) | 0.743 |
| Cirrhosis | | | | |
| No | 1 (reference) | | 1 (reference) | |
| Yes | 1.205 (0.464–3.131) | 0.701 | 1.532 (0.775–3.028) | 0.219 |
| Chemotherapy | | | | |
| No | 1 (reference) | | 1 (reference) | |
| Yes | 2.841 (1.208–6.682) | 0.017 | 8.531 (4.474–16.267) | <0.001 |
| Histological grade | | 0.058 | | 0.084 |
| 1 (well) | 1 (reference) | | 1 (reference) | |
| 2 (moderate) | 0.424 (0.147–1.221) | 0.112 | 0.743 (0.365–1.513) | 0.412 |
| 3 (poorly) | 1.182 (0.357–3.917) | 0.784 | 1.459 (0.635–3.353) | 0.374 |
| 4 (undifferentiated) | NA | NA | NA | NA |
| Pathological stage | | 0.018 | | 0.001 |
| I | 1 (reference) | | 1 (reference) | |
| II | 1.207 (0.515–2.830) | 0.666 | 2.154 (1.189–3.902) | 0.011 |
| III | 2.563 (0.582–11.285) | 0.213 | 7.126 (2.599–19.539) | <0.001 |
| IV | 11.927 (1.999–71.172) | 0.007 | 7.477 (1.379–40.545) | 0.020 |

Chemotherapy, postoperative chemotherapy; CI, confidence interval; Cytoplasmic NOX2, cytoplasmic NOX2 expression in cancer cells; HighN&lowC NOX4, a group of high nuclear and low cytoplasmic NOX4 expression in cancer cells; HBV, hepatitis B viral infection; HCV, hepatitis C viral infection; HR, hazard ratio; NA, not applicable.

[a] Multivariate Cox regression analysis.

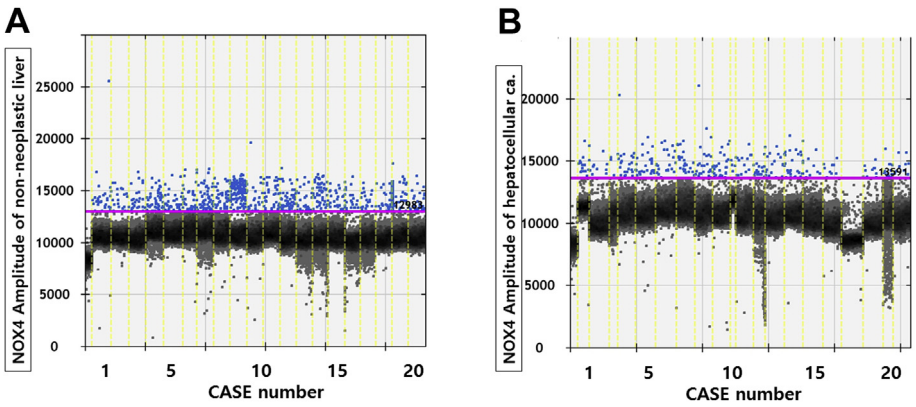


**Fig. 3** Droplet digital PCR amplification plots for individual NOX4 mRNA expression levels in 20 matched pairs of non-neoplastic liver tissues (A) and HCCs (B). Positive droplets are shown in blue and negative droplets are in black/grey. The pink line represents the threshold and dichotomising positive and negative droplets. RT-ddPCR analysis showed a trend of higher copy number in non-neoplastic liver tissue than in HCC tissue ($p$=0.112, Wilcoxon signed-rank test).

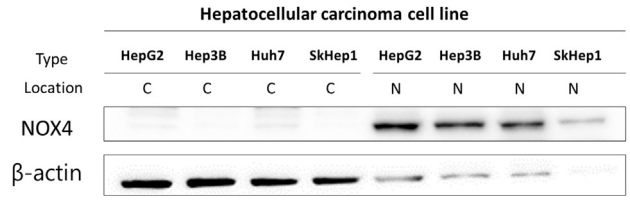


**Fig. 4** NOX4 in four HCC cell lines was detected by western blot in the nuclear fraction (C, cytoplasm; N, nucleus). Four bands indicating NOX4 expression were observed in the nuclear portion of the four HCC cell lines, while no band was observed in the cytoplasm portion. β-actin is generally expressed in the cytoplasmic protein sample but not in the nuclear sample.

## Western blot assay for NOX4 expression in four HCC cell lines

Western blot analysis revealed that the expression of NOX4 was increased in the nuclear portion of the four HCC cell lines, while no band was observed in the cytoplasm portion (Fig. 4). β-actin was used as a loading control. β-actin was clearly expressed in cytoplasmic protein samples but not in nuclear protein samples. This finding suggests that the nucleus and cytoplasm were well separated.

## DISCUSSION

The present study provides evidence that nuclear NOX4 and cytoplasmic NOX2 expression is upregulated in HCC cells. Cytoplasmic NOX2 and NOX4 expression in HCC cells was significantly increased in HCC patients with liver cirrhosis and might be involved in HCC development from liver cirrhosis. NOX4 nuclear expression suggested a potential positive association with HCC proliferation and short OS.

ROS, as a secondary signal transducer, plays an important role in pathways related to transcriptional regulation, differentiation, proliferation, oncogenic transformation and cell death.[16] The liver is an important source of ROS production and NOX enzymes, such as NOX1, NOX2, and NOX4, which mainly produce ROS in the liver.[17,18] According to a previous study, better OS was shown in HCC patients with high NOX4 mRNA expression levels, whereas patients with high NOX2 mRNA expression levels showed poor prognoses.[6] In particular, in our study, we tried to demonstrate experimentally whether NOX2 and NOX4 expression is altered in HCC as well as investigate the association of clinicopathological characteristics with the difference in NOX2 and NOX4 expression in HCC.

Previous studies have shown that NOX2 is involved in the pathogenesis of hepatic fibrosis, which is related to NOX2 activation in hepatic stellate cells as well as macrophages.[19–21] NOX2 has been shown to express profibrogenic genes in hepatocytes and Kupffer cells.[19,20] Moreover, hepatic fibrosis was attenuated in chimaeric mice with NOX2-deficient endogenous hepatocytes.[19] In our study, we found that HCC patients with liver cirrhosis had a significantly higher expression level of NOX2 in HCC cells than those without liver cirrhosis. On the other hand, according to our data, NOX2 expression was not associated with patient survival but rather negatively correlated with advanced tumour stage. This finding suggests that increased expression of NOX2 in HCC does not affect patient survival. NOX2 expression in HCC is not related to the progression of HCC; rather, NOX2 appears to be involved in HCC development from liver cirrhosis. As those previous studies have emphasised the function of NOX2 in contributing to cirrhosis as an important risk factor for HCC,[22] the significant elevation in cytoplasmic NOX2 expression in cirrhotic patients with HCC suggests that NOX2 may also contribute to carcinogenesis of the liver.

Meanwhile, according to our data, cytoplasmic NOX4 expression showed a positive correlation with liver cirrhosis but was decreased in HCC cells compared with non-tumour hepatocytes, while nuclear NOX4 expression was increased in HCC cells. Increased nuclear and decreased cytoplasmic NOX4 expression showed a significant correlation with a short OS, although the tumour survival analysis showed no significant difference in DFS. Generally, NOX4 is detected not only in perinuclear and endoplasmic reticulum areas but also in the plasma membrane and within the nucleus.[7] NOX4 is ubiquitously expressed.[23] Previous studies have shown that the expression of NOX4 is elevated in the nucleus when a hepatitis C viral (HCV) infection is present.[24] There are several hypotheses about transient subcellular translocation of NOX4. It is possible that different stimulation might lead to NOX4 translocation, which is necessary for local ROS production[24] or that NOX4 splicing variants might determine NOX4 subcellular localisation.[25] However, no study has specifically identified the location of NOX4 protein expression in HCC, until now. In our study, nuclear NOX4 expression was significantly elevated in HCC cells compared to paired non-tumour hepatocytes, and high nuclear and low cytoplasmic expression of NOX4 in HCC was significantly correlated with a short OS. This result suggests that NOX4 translocates from the cytoplasm to the nucleus during tumourigenesis and tumour progression.

Previous studies on NOX4 transcript or total NOX4 protein expression do not distinguish between cytoplasmic and nuclear expression but have shown that NOX4 is associated with favourable prognostic factors in HCC.[6] An increase in NOX4 transcript levels results in an increase in TGF-β signalling, which activates TGF-β-induced apoptosis in HCC.[26] In addition, NOX4 expression is involved in the inhibition of proliferation[27] and the positive regulation of E-cadherin level, which maintains parenchymal structures and is therefore inversely correlated with tumour stromal invasion in HCC.[28] As the expression of NOX4 increases, the prognosis of HCC is improved by suppressing EMT-related signalling.[28] In addition, other studies have reported that increased cytoplasmic NOX4 expression in HCC is associated with a good prognosis for DFS and OS.[29] In our study, multivariate analysis with clinicopathological characteristics showed that the increased nuclear and decreased cytoplasmic expression of NOX4 was a significant aggravating factor in OS for HCC patients. This difference was due to the previous studies mainly evaluating cytoplasmic NOX4 expression as a primary endpoint, but our study evaluated NOX4 expression in the cytoplasm as well as the nucleus.

The subcellular localisation of NOX4 to the cytoplasm, cytoplasmic membrane, extracytosolic part and nucleus is cell type specific.[30] For example, in human vascular endothelial cells, NOX4 with a transmembrane domain is located in the nucleoplasm.[31] A major proportion of NOX4 in human umbilical vein endothelial cells and vascular smooth muscle cells is localised to the nucleus.[31,32] In endothelial cells, human pulmonary artery smooth muscle cells, pulmonary artery adventitial fibroblasts and high-grade glioma cells, the role of NOX4 in cell proliferation has been reported.[3,33–35] High-grade gliomas have shown strong NOX4 immunostaining, while few NOX4 positive cells were detected in non-neoplastic tissue, and nuclear staining of NOX4 was predominantly observed in glioma cells, although cytoplasmic expression was also detected.[3]

We suggest that subcellular translocation of NOX4 from the cytoplasm to the nucleus plays an important role in cancer development and progression in HCC.[36] We have demonstrated for the first time that levels of nuclear NOX4 and cytoplasmic NOX2 expression are higher in HCC cells than in non-tumour hepatocytes of the same liver, although the individual molecular mechanism of nuclear NOX4 and cytoplasmic NOX2 expression in hepatocellular carcinogenesis remains unclear. Therefore, further mechanistic studies are needed in this regard.

## CONCLUSIONS

According to this study, the expression of cytoplasmic NOX2 is increased in HCC with liver cirrhosis, but NOX2 expression has not been shown to be associated with prognosis. Therefore, NOX2 expression can be used as a risk factor for HCC with cirrhosis. In the case of NOX4, the short OS of HCC patients was associated with increased nuclear and decreased cytoplasmic expression of NOX4 protein, which may be related to subcellular translocation of NOX4 protein from the cytoplasm to the nucleus. The role of NOX4 in the nucleus may be closely related to that of an oncogene in the development and progression of HCC. Therefore, studies of NOX4 as a diagnostic and therapeutic target are warranted to improve the survival rate of patients with HCC.

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## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2019.05.004.

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