Research Article

Survivin as a therapeutic target for neuroendocrine neoplasms

Yuma Hane [a], Takahiro Tsuchikawa [a*], Toru Nakamura [a], Kanako C. Hatanaka [b, c], Katsunori Sasaki [a], Noriko Kawai [a], Shintaro Takeuchi [a], Kimitaka Tanaka [a], Yoshitsugu Nakanishi [a], Toshimichi Asano [a], Takehiro Noji [a], Toshiaki Shichinohe [a], Yutaka Hatanaka [b], Yoshihiko Hirohshi [d], Toshihiko Torigoe [d], and Satoshi Hirano [a]

[a] Department of Gastroenterological Surgery II, Hokkaido University Faculty of Medicine, West-7, North 15, Kita-ku, Sapporo 060-8638, Japan

[b] Research Division of Genome Companion Diagnostics, Hokkaido University Hospital

[c] Clinical Research and Medical Innovation Center, Hokkaido University Hospital

[d] Department of Pathology, Sapporo Medical University School of Medicine

Short Title: Targeting survivin for neuroendocrine neoplasms

Corresponding Author:

Takahiro Tsuchikawa

E-mail address: tsuchi-t@med.hokudai.ac.jp; Tel: +81-11-706-7714; Fax: +81-11-706-7158

Keywords: neuroendocrine neoplasms; survivin; prognosis; metastasis; immunotherapy.

Abstract

Introduction: Although neuroendocrine neoplasms (NENs) have a good prognosis, distant metastasis remains a crucial prognostic factor. Survivin, a tumor-associated antigen, is overexpressed in several solid tumors, indicating poor prognosis. We aimed to evaluate the clinical significance and role of survivin as a therapeutic target for NEN. Methods: We assessed the cytotoxicity of Survivin-2B (a splicing variant of Survivin) 80-88 specific CTL clone with HLA-A24 restriction against NEN cell lines using intracellular staining for interferon γ and assessed the frequency of Survivin-2B 80-88 CTL precursors in nine HLA-A24 positive patients with NEN using tetramer staining and compared it before and after resection. Finally, we evaluated the association between survivin expression and prognosis in 74 patients with pancreatic NEN using immunohistochemistry. Results: Survivin-2B 80-88 CTL clone showed high cytotoxicity against QGP-1 (HLA-A24 positive) cocultured with the Survivin-2B 80-88 peptide. Only three patients were positive for tetramer staining; two showed decreased Survivin-2B 80-88 CTL precursor following resection. The nuclear survivin-low group had a significantly better prognosis than the nuclear survivin-high group. Conclusion: Survivin in NEN is antigenic and may induce cellular immunity via the Survivin-2B CTL precursor. Survivin-targeting immunotherapy can be used to treat NEN with highly expressed Survivin-2B.

Introduction

Although gastroenteropancreatic (GEP) neuroendocrine neoplasm (NEN) is a rare tumor derived from the enterochromaffin cells, its reported incidence is 3.56 per 100,000 and has been increasing [1]. NENs usually grow gradually, and the recently reported 5-year survival rate of all originating NENs is 39.4% [2]. Distant metastasis is an important prognostic factor. In a previous report, the 5-year survival of patients with liver metastases was 13–54% compared with the 75–99% for patients without liver metastases [3]. Several guidelines recommend multidisciplinary treatment, such as surgical treatment, and if feasible, molecular targeted drugs, transcatheter arterial chemoembolization, and radiotherapy for the management of metastases. However, existing treatments have demonstrated limited efficacy, necessitating novel treatments.

Immunotherapy is one of the most potent anticancer treatments that has demonstrated remarkable progress in the treatment of NEN. Somatostatin receptors and CDH17 can be exploited by chimeric antigen receptor T cells (CART) in NEN; moreover, a phase I study on anti-CDH17 CART therapy is underway [4,5]. In active immunotherapy, several tumor-associated antigens, which can be recognized by the presence of cytotoxic T lymphocytes and induction of cancer-specific immune reaction, have been identified in melanoma and other cancers [6–8]. Survivin, a tumor-associated

antigen, is an inhibitor of the apoptosis protein (IAP) family with a single baculovirus IAP repeated domain [9]. Along with its role as an IAP, survivin is a subunit of the chromosomal passenger complex and a regulator of microtubule dynamics [10–12], and its overexpression in several solid tumors has been linked to poor prognosis.

Survivin-2B 80-88 (AYACNTSTL), a 9-mer in Survivin-2B, one of the splicing variants of survivin, is presented by HLA-A24. Survivin-2B 80-88 specific CTL, induced with this peptide, could recognize survivin-positive tumor cells. Survivin-2B 80-88 specific CTL clones derived from various types of cancers demonstrated cytotoxicity with restriction of HLA-A24 *in vitro*. Moreover, tetramer analysis has revealed an increased number of CTL precursors in the peripheral blood mononuclear cells (PBMC) of HLA-A24-positive patients with cancer [13,14]. However, data on the immunological effects of the survivin antigen in patients with NEN remain limited. Therefore, in this study, we evaluated the clinical significance of the survivin antigen and determined its potential use as a therapeutic target for NEN.

Methods

Cell lines

QGP-1 (pancreatic neuroendocrine tumor), H727 (lung neuroendocrine tumor), SW480 (colorectal cancer), and K562 (chronic myeloid leukemia) cells were purchased from the American Type Culture Collection (Manassas, VA). Normal human dermal fibroblasts (NHDFs), a negative survivin control, were purchased from Lonza. T2-A24 as *HLA-A*2402* gene-transfected T2 cell line was a kind gift from Dr. Kiyotaka Kuzushima (Aichi Cancer Center, Nagoya, Japan). Cell lines were cultured in the appropriate medium (SW480 in Dulbecco's Modified Eagle's Medium, QGP-1, H727, T2-A24, K562 in RPMI 1640, all media from WAKO, Tokyo, Japan) containing 10% fetal bovine serum (Cell Culture Bioscience, Tokyo, Japan) and 1% penicillin-streptomycin (Life Technologies, Tokyo, Japan) at 37 °C with 5% CO_2. NHDFs were cultured in Clonetics™FGM™-2 BulletKit™ (CC-3132, Lonza) at 37 °C with 5% CO_2.

Survivin-2B 80-88 CTL clone

Survivin-2B 80-88 CTL clone derived from the PBMC of patients with pancreatic cancer was a kind gift from Dr. Yoshihiko Hirohashi (Sapporo Medical University, Sapporo, Japan). The clone was

cultured in AIM-V containing 10% human serum, 10 mM HEPES, 50 μM 2-ME, and 1% penicillin-streptomycin (Life Technologies, Tokyo, Japan) at 37 °C with 5% CO_2.

Measurement of survivin expression

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, and stained with phycoerythrin (PE)-conjugated mouse anti-survivin monoclonal IgG1 (R&D Systems, Minneapolis, MN, USA) and PE-conjugated mouse IgG1 (R&D Systems) as isotype controls. After washing twice with flow cytometry staining buffer, flow cytometry was performed using MACSQuant 10 (Miltenyi Biotec, Bergisch Gladbach, Germany). The data were analyzed using FlowJo software (Tree Star Inc., Oregon, USA).

Real-time polymerase chain reaction analysis of Survivin-2B expression

Total RNA was isolated from the cultured cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The cDNA mixture was synthesized from 1 μg of total RNA via reverse transcription according to the manufacturer's protocols. Polymerase chain reaction (PCR) amplification was performed in 25 μl of PCR mixture containing 2 μl cDNA mixture, KOD Plus DNA polymerase (Toyobo, Osaka, Japan), and 0.4 μM of primers. 5'-TCAAGGACCACCGCATCTCTAC-3' was used as a forward primer for real-time reverse transcription (RT)-PCR analysis. The following reverse primers were used: 5'-GTGCTGGTATTACAGGCGTAAG-3' for survivin-2B. As an internal control, β-Actin was detected using a forward primer 5'-AGAAAATCTGGCACCACACC-3' and a reverse primer 5'-AGAGGCGTACAGGGATAGCA-3'. Real-time PCR was performed using a Light Cycler 96 (Roche, Basel, Switzerland), and amplifications were performed using SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). The thermal cycling conditions were composed of an initial denaturation step at 95 ℃ for 20 s, followed by 45 cycles of denaturation at 95 ℃ for 5 s, and annealing and extension at 60 ℃ for 30 s. The experiments were performed in triplicate for each data point. The relative quantification in gene expression was determined using the 2-ΔΔCt method [15]. Using this method, we obtained the fold changes in gene expression normalized to β-Actin as an internal control gene.

Semiquantitative PCR for HLA-A24 expression

Genomic DNA was isolated from the NEN cell lines and PBMCs that were confirmed HLA-A24 positive and negative, using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). 5'-ACTGACCGAGAGAACCTGCGGAT-3' was used as a forward primer, and 5'-TCTCCAGGTATCTGCGGAGCCCG-3' as a reverse primer for semiquantitative PCR. The thermal cycling conditions were composed of initial denaturation at 94 ℃ for 2 min, followed by 35 cycles of denaturing at 94 ℃ for 15 s, annealing at 58 ℃ for 30 s, and extension at 72 ℃ for 30 s. PCR products

were visualized by ethidium bromide staining under ultraviolet light after electrophoresis on a 1.5% agarose gel.

Intracellular staining for interferon-γ

Each cell line and the Survivin-2B 80-88 CTL clone were cocultured for 5 h. QGP-1 was stimulated with Survivin 80-88 (HLA-A*24:02) peptide (NovoPro Bioscience, Shanghai, China) for 2 h before coculturing with Survivin-2B 80-88 CTL clone. As a positive control, Survivin-2B 80-88 CTL clone was stimulated for 5 h with phorbol 12-myristate 13-acetate (PMA, 20 ng/ml, Sigma-Aldrich), ionomycin (IO, 500 ng/ml, Sigma-Aldrich), and GolgiStop (1:500, BD Biosciences). Cells were stained with Peridinin Chlorophyll Protein Complex (PerCP)-conjugated anti-CD8 mAb (Bio Legend, California, USA) for 30 min at 4 °C followed with fixation/permeabilization solution (BD Cytofix/Cytoperm™ Kit, BD Biosciences, New Jersey, USA) according to the manufacturer's instructions. After washing three times with Perm/Wash buffer, fluorescein isothiocyanate-conjugated anti-IFN-γmAb (Thermo Fisher Scientific, Massachusetts, USA) was added for 1 h at 4 °C and the cells were analyzed using MACSQuant 10.

Tetramer staining

PBMCs were isolated from the blood samples via Ficoll–Conray density gradient centrifugation and stained with PE-labeled HLA-A*24:02 Survivin-2B tetramer (MBL, Tokyo, Japan), with HLA-A*24:02 HIV env tetramer as negative controls, at room temperature for 20 min, followed by staining with PerCP-conjugated anti-CD8 mAb (BioLegend, California, USA) at room temperature for 20 min. The cells were then washed twice with phosphate buffered saline and fixed in 1% formaldehyde. The frequency of the CTL precursors was calculated as the number of tetramer-positive cells relative to the number of CD8-positive cells. We defined tetramer staining as HLA-A*24:02 Survivin-2B positive in cases where the frequency of CTL precursors was > 0.1%, as previously described [14].

Measurement of the Serum Survivin concentration

Serum survivin concentrations were determined using enzyme-linked immunosorbent assay (ELISA) with Quantikine Human Survivin Immunoassay (R&D System, Minnesota, USA) according to the manufacturer's instructions. The experiments were performed in duplicate for each data point.

Patients and samples for immunohistochemistry

This study included 74 patients diagnosed with pancreatic NENs between 2004 and 2019 who underwent surgical resection at the Department of Gastroenterological Surgery II at the Hokkaido

University Hospital. The Clinical and pathological data were obtained through a detailed retrospective review of the patients' medical records.

Immunohistochemical analysis of Survivin

The tissue sections were deparaffinized in xylene and rehydrated using a graded ethanol series. Heat-induced antigen retrieval was performed in a low-pH antigen retrieval buffer (Dako, Glostrup, Denmark). Endogenous peroxidases were blocked by incubating the tissue sections in 3% H_2O_2 for 5 min. The sections were incubated for 30 min with a primary antibody against survivin (1:250, NB500-201; Novus Biological, Colorado, USA) and subsequently visualized using the horseradish peroxide (HRP)-labeled polymer method (EnVision FLEX System, Dako, Glostrup, Denmark). The immunostained sections were counterstained with hematoxylin, dehydrated in ethanol, and cleared in xylene. All the tissue sections obtained from the formalin-fixed paraffin blocks were analyzed for semi-quantitative evaluation of nuclear and cytoplasmic survivin using HALO (v3.4, Indica Labs, New Mexico, USA).

Briefly, the immunohistochemical score was calculated as the product of the percentage of tumor cell positivity and staining intensity (0, none; 1+, weak; 2+, intense). The lower-limit intensities of 1+ and 2+ are shown in Figure 1. The percentage of tumor cells showing different staining intensities was evaluated by two pathologists (K.H. and Y.H.).

Statistical analysis

A receiver operating characteristic (ROC) curve was used to determine the cut-off values of the H-score for survivin. The H-score, as a continuous variable, and 5-year recurrence (recurrence or no recurrence), as a binary variable, underwent a modified ROC analysis as previously described [16]. Survival was estimated using the Kaplan–Meier method, and survival estimates were compared using the log-rank test. Overall survival (OS) was calculated from the date of surgery to the date of death due to any cause or the last contact/follow-up. Recurrence-free survival (RFS) was calculated from the date of surgery to the date of recurrence. Student *t*-tests were used to compare the differences in the serum survivin concentrations between the patient and healthy groups. The threshold for significance was $P < 0.05$. All statistical analyses were conducted using the JMP software package (version 17.0; SAS Institute, Cary, NC, USA).

Results

HLA-A24 expression for NEN cell lines using Semiquantitative PCR

Semi-quantitative PCR for HLA-A24 indicated QGP-1 and H727 to be HLA-A24 positive and negative, respectively (Fig. 2).

Survivin-2B expression using real-time RT-PCR

We assessed the expression profile of Survivin-2B in NEN cell lines using real-time RT-PCR. SW480 cells were used as positive controls for survivin expression. Our results indicated that Survivin-2B mRNA in SW480 and H727 cells was higher than that in NHDF. In contrast, Survivin-2B mRNA in QGP-1 cells was as low as that in NHDF (Fig. 3).

Expression of Survivin for NEN cell lines using flowcytometry

We assessed survivin expression on the cell surface using flow cytometry and demonstrated that the expression of survivin on the NEN cell surface was sufficiently higher than that in NHDF (Fig. 4).

Cytotoxicity of Survivin-2B 80-88 CTL clone for NEN cell lines

To assess the cytotoxicity of Survivin-2B 80-88 CTL clone for NEN cell lines, we performed intracellular staining for interferon (IFN)-γ. Our results indicated that the Survivin-2B 80-88 CTL clone showed high cytotoxicity for QGP-1 (HLA-A24 positive) cocultured with the Survivin-2B 80-88 peptide and little cytotoxicity for QGP-1 alone and H727 (HLA-A24 negative) (Fig. 5).

The frequency of Survivin-2B 80-88 CTL precursor in patients with NEN

We first assessed the frequency of Survivin-2B 80-88 CTL precursors in seven patients with NEN and three healthy donors with HLA-A24 positive using tetramer staining. The primary sites of NEN were the duodenum (1), stomach (1), rectum (1), and pancreas (4). Of all the patients with NEN and healthy donors, only three patients (Cases 1, 2, and 4) were positive for tetramer staining. Representative images of positive tetramer staining (Case 1) are shown in Figure 6.

Moreover, we compared the frequency of Survivin-2B 80-88 CTL precursor in three HLA-A24 positive patients with NEN after resection with that before resection. The Survivin-2B 80-88 CTL precursor after resection decreased compared with that before resection in two (Cases 1 and 2) of the three patients (Fig. 7). Two patients in whom Survivin-2B 80-88 CTL precursor levels decreased after surgery exhibited an uneventful course without recurrence for 3 years. One patient in whom the Survivin-2B 80-88 CTL precursor level did not decrease after surgery (Case 4) had a liver recurrence. Patient characteristics and corresponding data on the frequency of the Survivin-2B 80-88 CTL precursor are summarized in Table 1.

Serum survivin levels in patients with NEN

We assessed the serum survivin levels in 36 patients with NEN and 4 healthy donors using ELISA. The mean survivin level in the sera of patients was 10 pg/ml (range, 8–57 pg/ml), significantly lower than that in healthy donors (mean, 13; range, 12–16 pg/ml) (P = 0.0045) (Fig. 8).

Association between the survivin expression using immunohistochemistry and the prognosis and clinicopathological features

ROC analysis revealed the cut-off value of the H-score for nuclear and cytoplasmic survivin was 8.9 and 84.9, respectively. We classified patients with an H-score greater than the cut-off value into the survivin-high group and those with an H-score less than the cut-off value into the survivin-low group. No significant difference was observed in the OS between the nuclear survivin-high and survivin-low groups (Fig. 9A). Similarly, no significant difference was observed in the OS between the cytoplasmic survivin-high and survivin-low groups (Fig. 9B).

In contrast, in terms of the RFS, the high nuclear survivin expression group had a significantly poorer prognosis than the low nuclear survivin expression group (P = 0.0371) (Fig. 9C). No significant difference was observed in the RFS between the cytoplasmic survivin-high and survivin-low group (Fig. 9D).

Table 2 shows the association between nuclear survivin expression and clinicopathological features. Higher nuclear survivin expression showed a significant correlation with tumor size >2 cm, lymph node metastasis, liver metastasis, and function.

Discussion

Recently, an increasing number of clinical trials utilizing peptide vaccination have been conducted [17–19]. In the case of GEP-NEN, nuclear survivin expression was upregulated during progression, associated with poor prognosis, and correlated with a high ki-67 index [20,21]. Therefore, we focused on survivin among many tumor-associated antigens. Previous studies on clinical trials utilizing a survivin vaccine demonstrated its safety and limited effectiveness in inducing cellular and humoral immune responses [22–24]. Indeed, a recent phase IIa study of survivin peptide vaccine plus adjuvant chemotherapy for glioblastoma revealed a significantly longer progression-free survival than radiotherapy and adjuvant chemotherapy (progression-free survival rate for 6 months: 95.2% vs. 54%, respectively; P < 0.0001) [25]. In this study, Survivin peptide vaccine induced survivin specific CD8+ T cells and aitibody/IgG titers. Moreover, a phase I study of the survivin peptide vaccine (SurVaxM, 15 amino acid synthetic peptide vaccine targeting survivin, developed initially to treat glioblastoma by Roswell Park's spin-off company Mimivax) on metastatic NENs is underway [26]. HLA-A24 is found in approximately 60% of the Japanese population; therefore, HLA-A24-restricted

immunotherapy is suitable for Japanese patients. To our knowledge, this study is the first to determine whether the Survivin-2B 80-88 peptide, which may induce HLA-A24 restricted immune reaction, could be useful for treating NEN in Japanese patients.

Although survivin has been considered an intracellular protein, it is present on the outer cell membrane of many cancer cells [27]. Therefore, antibody-mediated immunotherapy is a potential targetable strategy. Patients with cancer reportedly possess anti-survivin antibodies and survivin-specific CTLs. Moreover, the presence of Survivin-2B 80-88 CTL precursor in PBMC from patients with breast cancer, colon cancer, pancreatic cancer, and melanoma has been reported [14,22,24]. In our study, the tetramer assay demonstrated that the Survivin-2B 80-88 CTL precursor was present in three of the seven patients with NEN. In addition, the Survivin-2B 80-88 CTL precursor decreased to < 0.1% after tumor resection in two of the three patients.

These observations indicate that survivin expressed in NEN tissues is antigenic, and the Survivin-2B 80-88 peptide could be recognized by T cells in HLA-A24-positive patients with NEN. However, the reason for the Survivin-2B 80-88 CTL precursor levels not reducing in the remaining patient remains to be determined. Circulating tumor cells (CTCs), cells shed from the primary tumor, migrate and circulate in the bloodstream [28]. CTCs escape immune surveillance by modulating MHC class I and CD47, Programmed cell death ligand 1, and FASL expression. CTCs interact with immune cells during extravasation from vessels forming metastases [29]. In this study, liver resection was performed for treating liver metastasis; therefore, CTCs were presumed to exist in the blood of the Case 4 patient, and the Survivin-2B 80-88 CTL precursor did not decrease after liver resection due to induction of Survivin-2B 80-88 CTL precursor by CTCs.

Hirohashi et al. reported that HLA-A24- restricted CTLs from PBMCs of HLA-A24-positive patients with cancer demonstrated cytotoxicity against HLA-A24 and Survivin-2B positive cell lines, such as 888-mel cells (melanoma) and LHK-2 cells (lung adenocarcinoma) [29–31]. Similarly, we determined whether HLA-A24- restricted CTLs show cytotoxicity against HLA-A24 positive neuroendocrine cell lines. Survivin-2B 80-88 CTL clone did not show cytotoxicity against the HLA-A24 positive neuroendocrine cell line alone, although it did against the HLA-A24 positive neuroendocrine cell line pulsed by Survivin-2B 80-88 peptide. Our data indicated that Survivin-2B mRNA in QGP-1 was low; therefore, QGP-1 was not considered sufficient for inducing cytotoxicity in the Survivin-2B 80-88 CTL clone.

An important prerequisite for targeted therapy is the expression of the target structure within the tumor. Therefore, we performed immunohistochemistry to detect survivin in 74 patients with pancreatic NENs and demonstrated that survivin expression significantly correlated with poor

prognosis in various types of tumors [30–32]. A meta-analysis by Krieg et al. revealed that high expression levels of nuclear survivin in GEP-NEN correlated with shorter OS, and highly proliferative G3 NEC showed increased expression levels of nuclear survivin compared with G1/G2 tumors [32]. In our study, the high nuclear survivin group experienced recurrence significantly earlier than the low nuclear survivin group. Moreover, higher nuclear survivin expression significantly correlated with lymph node and liver metastases. Survivin has been shown to promote melanoma cell metastasis by upregulating integrins [33]. Chu et al. reported that high survivin expression is an independent predictor of lymph node metastasis in colorectal cancer [34]. Several patient studies have suggested a correlation between survivin overexpression and increased tumor invasion and metastasis. Ki-67 is expressed in all phases of the cell cycle and used as a marker of the proliferative potential of tumors. Similarly, survivin regulates cell division [35].

Survivin is an integral component of the chromosome passenger complex (CPC), ensuring proper association of chromosomes and cytoplasmic division during cell division [36]. Yosikawa et al. reported a significant direct correlation between Ki-67 and survivin expression in patients with rectal cancer treated with preoperative chemotherapy [37]. In our study, although Ki-67 expression was higher in the survivin-high group, the value was not significant (P = 0.079), owing to the small number of cases.

Recently, liquid biopsy, a concept different from that of conventional tissue biopsy, has attracted attention. The potential of liquid biopsy is increasing not only for diagnosis and pretreatment prognosis but also for monitoring during treatment owing to its simplicity in terms of repeated sampling. Tumor-associated proteins in the peripheral blood are widespread as tumor markers and may be considered pioneers of liquid biopsy. Few tumor markers for NENs without neuron-specific enolase have been identified. Khan et al. reported that survivin was released from tumor cells via exosomes in the sera of patients with breast cancer and that survivin levels were significantly higher in all breast cancer samples than in controls [38]. Moreover, serum survivin concentrations were significantly elevated and considered an independent prognostic factor in patients with pancreatic ductal carcinoma [39,40]. In contrast, serum survivin levels were significantly lower in patients with colorectal cancer than in healthy controls, and no correlations were observed between the serum survivin levels and the selected clinicopathological parameters [41]. In the present study, the serum survivin levels were significantly lower in patients with NENs than in healthy controls. It is possible that survivin is not released from neuroendocrine tumor cells via exosomes, in contrast to that in breast cancer and pancreatic ductal carcinoma. Serum survivin levels in NENs may be diagnostically ineffective for clinical use.

This study has some limitations. First, NENs are rare tumors; therefore, the sample size was limited. Moreover, the Survivin-2B 80-88 CTL clone was not derived from the PBMC of patients with NENs, and the Survivin-2B 80-88 CTL clone did not show cytotoxicity against the HLA-A24 positive neuroendocrine cell line without a survivin peptide pulse owing to low Survivin-2B expression. Thirdly, only the cases of highly expressed survivin-2B are suitable for this immunotherapy. Initial screening to detect survivin-2B by RT-PCR or immunohistochemistry obtained from biopsy or surgical resection is useful to select candidate for this immunotherapy.

In conclusion, survivin in NEN is antigenic, and NENs may induce cellular immunity via the Survivin-2B CTL precursor. Survivin-targeted immunotherapy may be suitable for the treatment of NEN, especially when survivin is highly expressed.

Statements

Acknowledgment

The authors would like to thank Ayae Nange for technical assistance and Sapporo Medical University (Sapporo, Japan) for allowing the use of its facilities and for technical support.

Statement of Ethics

Study approval statement: Opt-out informed consent protocol was used for use of participant data for research purposes. This consent procedure was reviewed and approved by the Ethics Committee of Hokkaido University Hospital, approval number 018-0163, date of decision January 9th, 2020.

This study protocol was reviewed and approved by the Ethics Committee of Hokkaido University Hospital, approval number 018-0163.

Consent to participate statement: An opt-out approach was used to obtain informed consent from the patients, and personal information was protected during data collection.

Consent for publication: An opt-out approach was used to obtain informed consent from the patients. Written consent was obtained for age, sex and medical history.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was not supported by any sponsor or funder.

Author Contributions

Conceptualization, T.T. and Y.H.; methodology, T.T. and K.S.; software, K.S.; validation, Y.H. and K.S.; formal analysis, Y.H.; investigation, Y.H., Y.H. (Yutaka Hatanaka) and K.C.H.; resources, T.T and S.H.; data curation, H.Y.; writing—original draft preparation, Y.H.; writing—review and editing, Y.H., T.T. , T.N., N.K., S.T., K.T., Y.N., T.A., T.N.(Takehiro Noji), T.S., Y.H.(Yoshihiko Hirohashi) , T.T. (Toshihiko Torigoe) and S.H.; visualization, Y.H.; supervision, S.H.; project administration, T.T.; funding acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

All data that support the findings of this study are contained within the article.

Further enquiries can be directed to the corresponding author.

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Figure Legends

Fig. 1. Criteria of staining intensity for immunohistochemistry. (A) Lower limit of 1+ for cytoplasm (arrow). (B) Lower limit of 2+ for cytoplasm (arrow). (C) Lower limit of 1+ (red circle) and 2+ (red square) for nucleus.

Fig. 2. HLA-A24 expression for NEN cell lines using semiquantitative PCR.

Fig. 3. Expression of Survivin-2B in NEN cell lines using real-time RT-PCR.

Fig. 4. Expression of Survivin on NEN cell lines using flow cytometry.

Fig. 5. Cytotoxicity of Survivin-2B 80-88 CTL clone against NEN cell lines by intracellular staining for IFN-γ.

Fig. 6. Tetramer staining of HLA-A*24:02 survivin-2B (Case 1).

Fig. 7. Comparison of the frequency of Survivin-2B 80-88 CTL precursor for 2 HLA-A24 positive patients with NEN after resection with that before resection (Cases 1 and 2).

Fig. 8. Serum Survivin level in 36 patients with NEN and 4 healthy donors using ELISA.

Fig. 9. Kaplan-Meier survival analysis to compare survivin-high with survivin-low groups. (A) Comparison of overall survival between nuclear survivin-high with nuclear survivin-low groups. (B) Comparison of overall survival between cytoplasmic survivin-high with cytoplasmic survivin-low groups. (C) Comparison of recurrence-free survival between nuclear survivin-high with nuclear survivin-low groups. (D) Recurrence-free survival compared cytoplasmic survivin-high group with cytoplasmic survivin-low group.

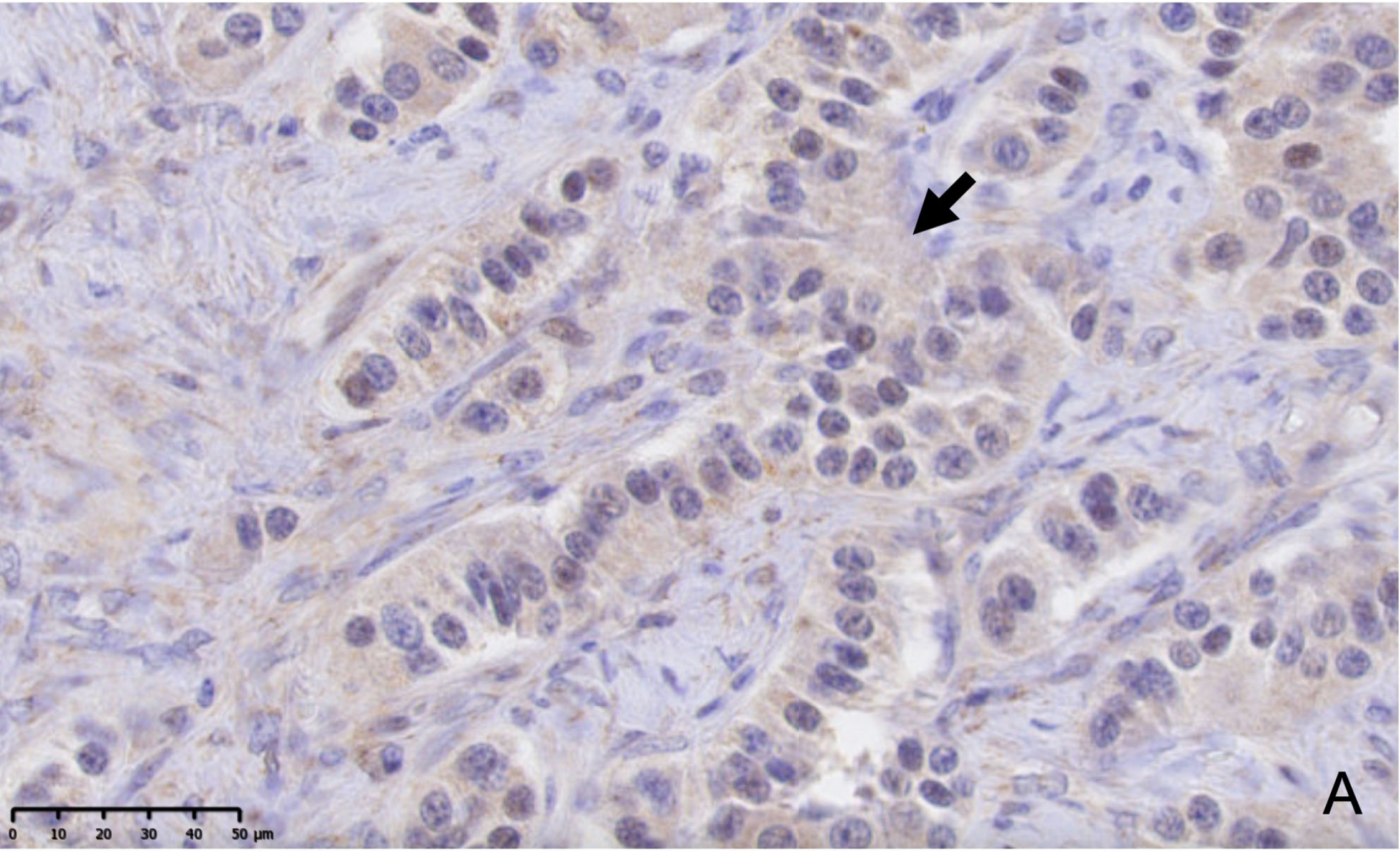


Figure1A

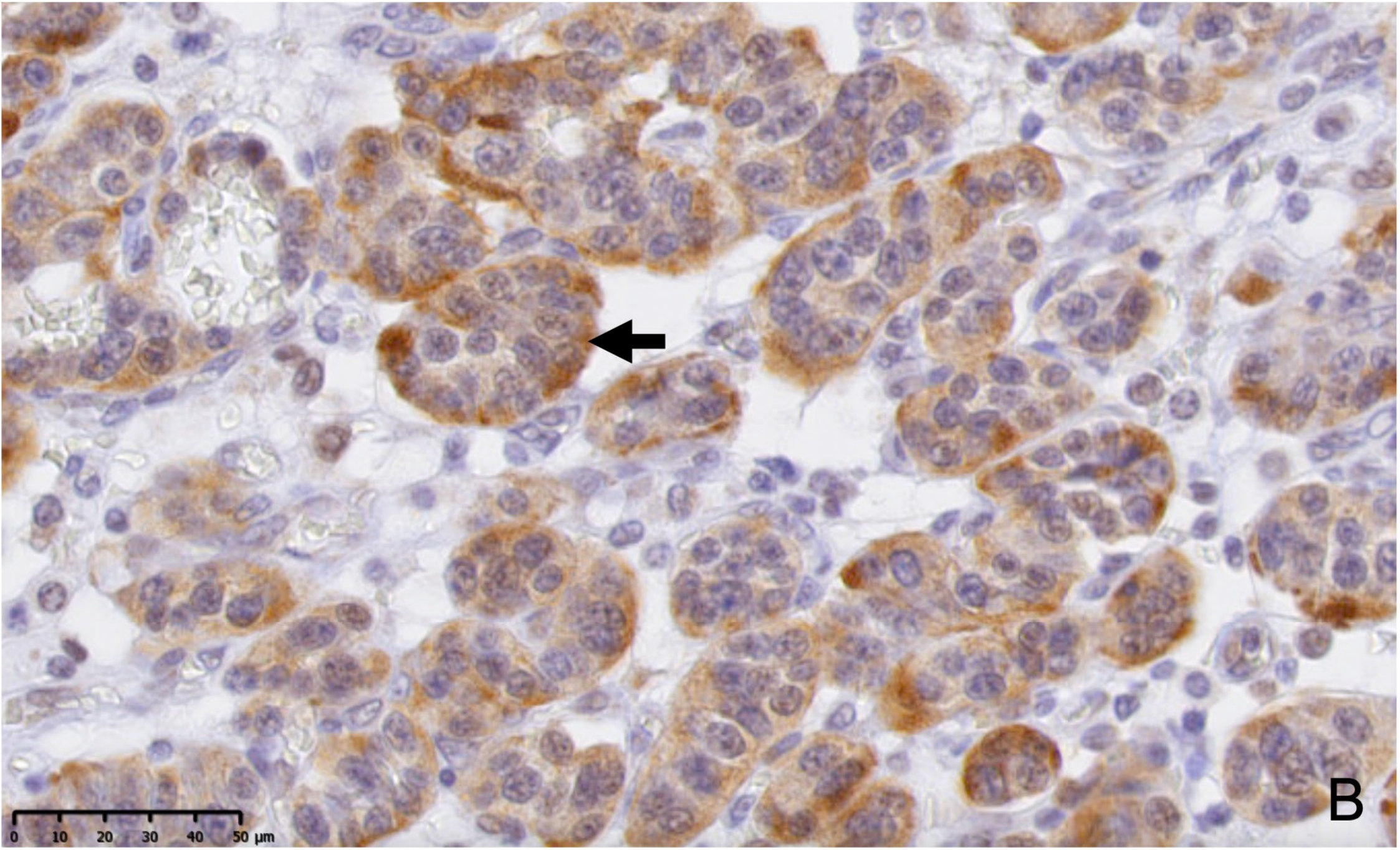


Figure1B

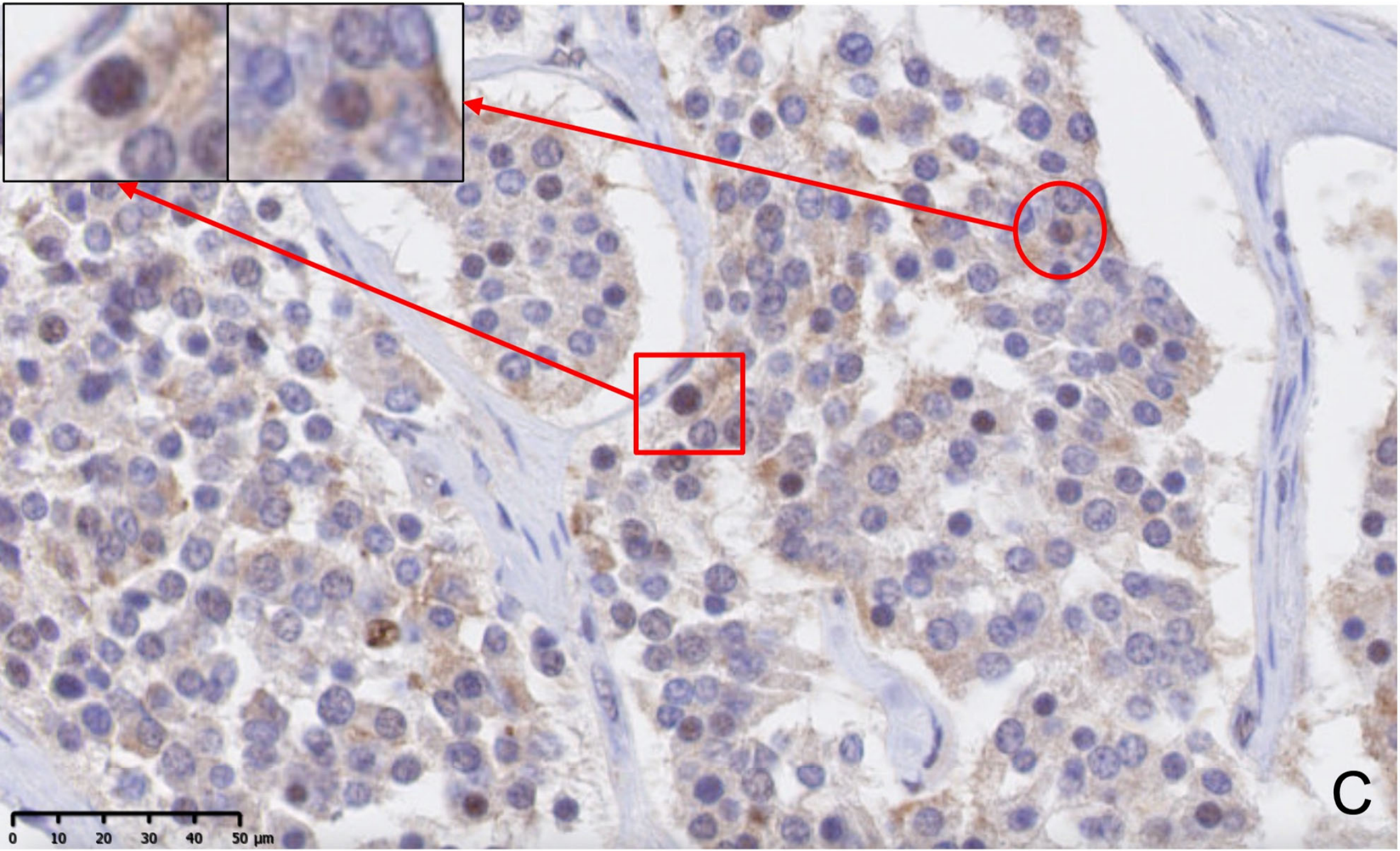


Figure1C

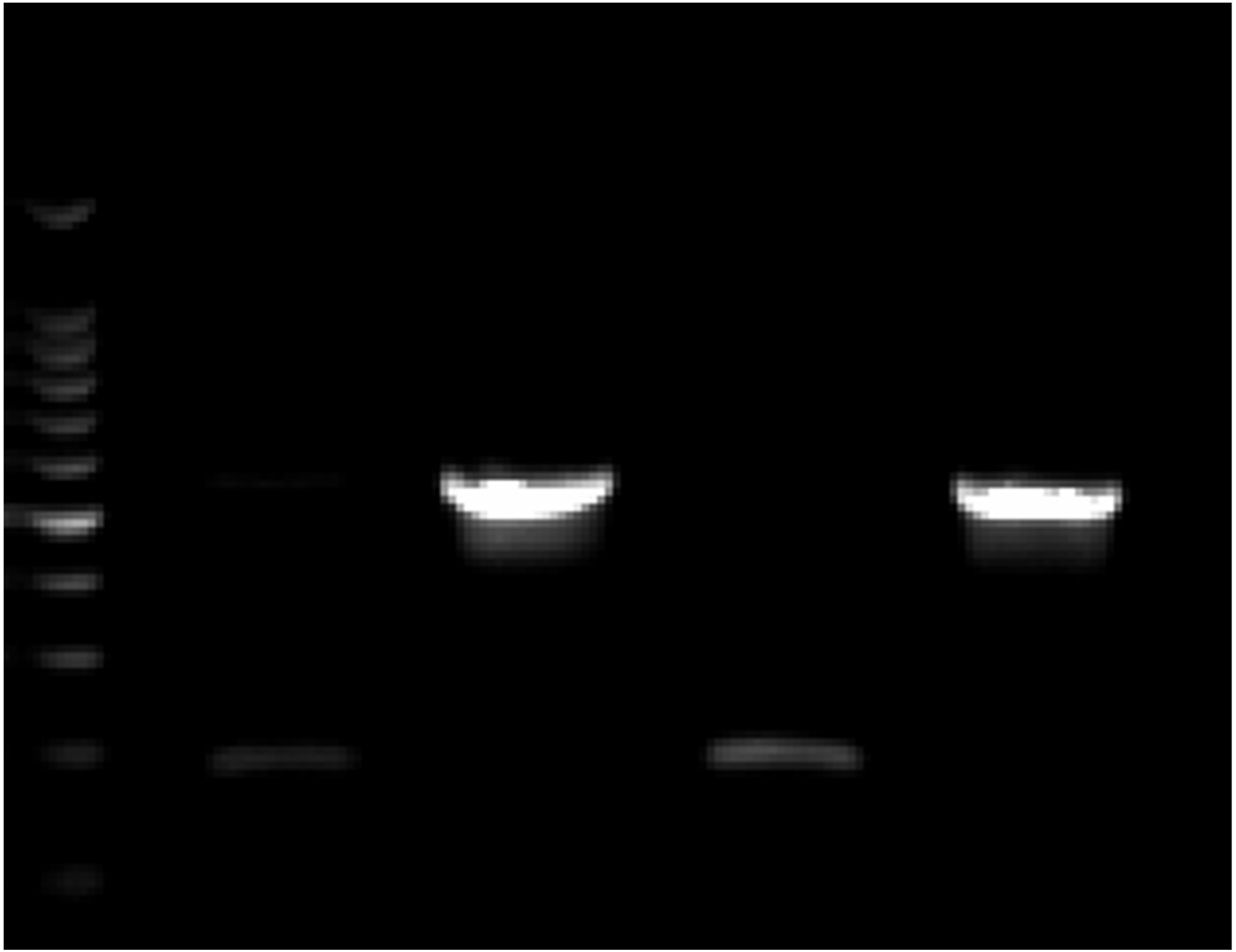


Figure2

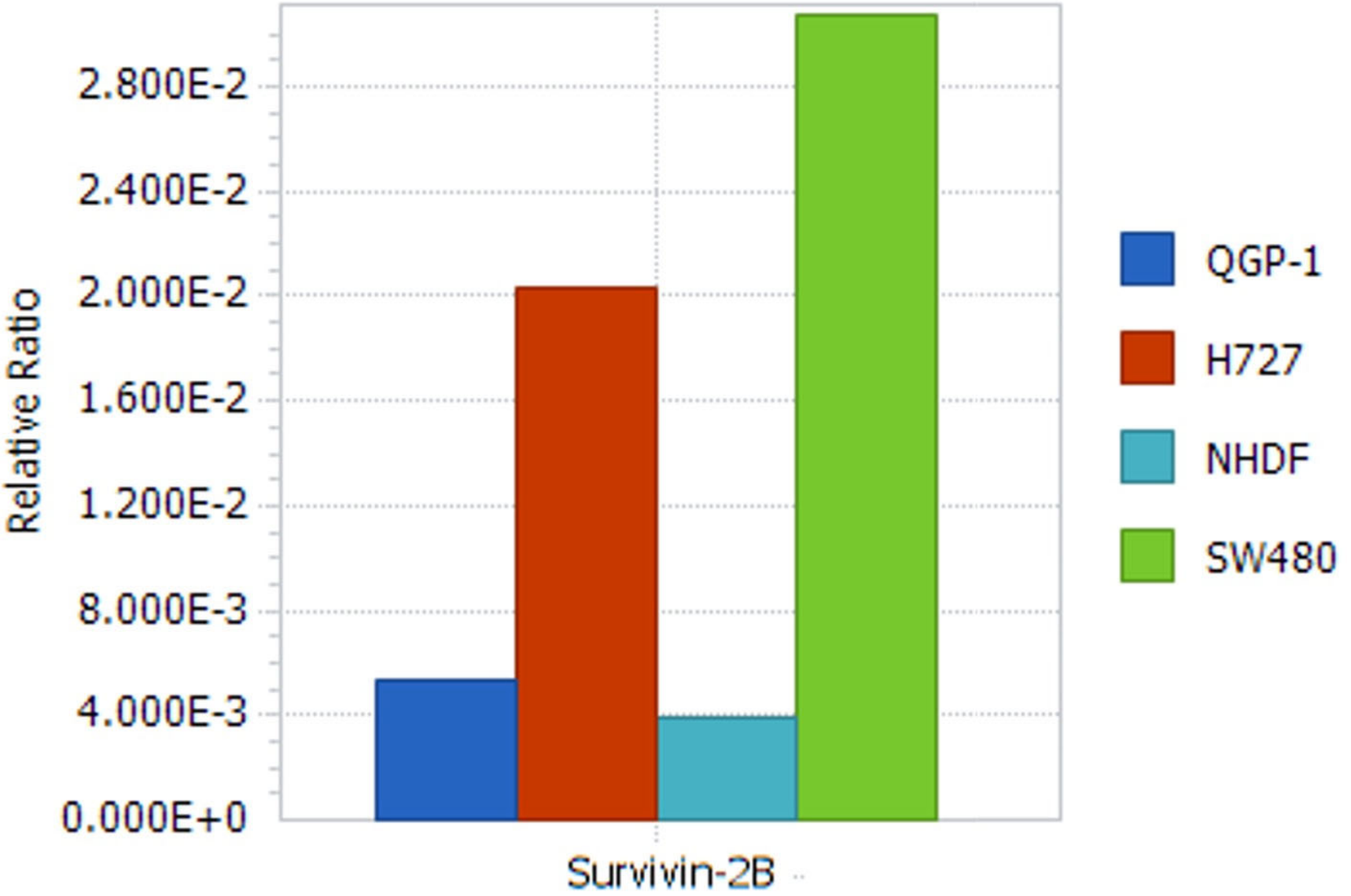

2.800E-2
2.400E-2
2.000E-2
1.600E-2
1.200E-2
8.000E-3
4.000E-3
0.000E+0
Relative Ratio
Survivin-2B
QGP-1
H727
NHDF
SW480

Figure3

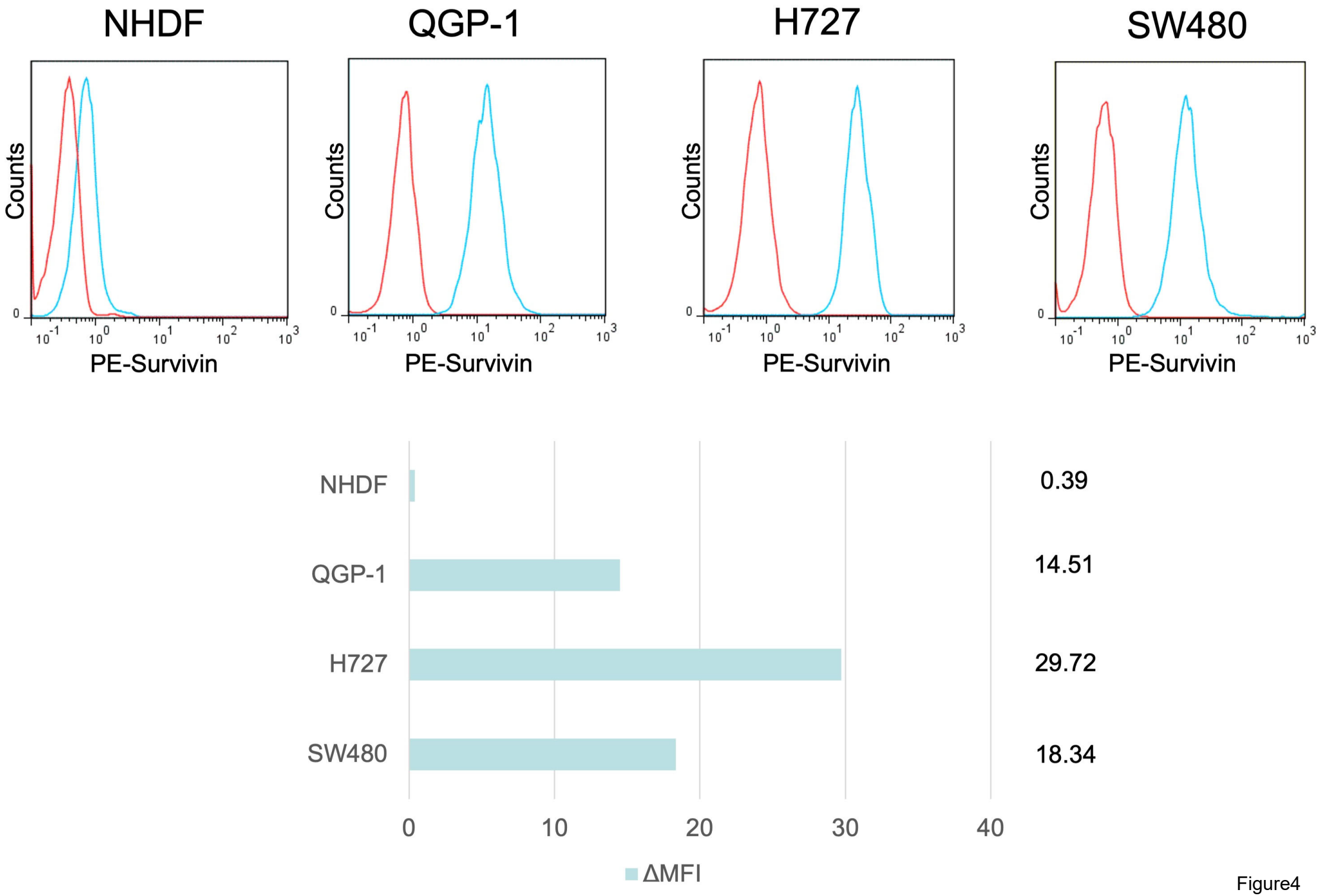

NHDF
QGP-1
H727
SW480
Counts
PE-Survivin
0
NHDF
QGP-1
H727
SW480
0.39
14.51
29.72
18.34
0
10
20
30
40
ΔMFI

Figure4

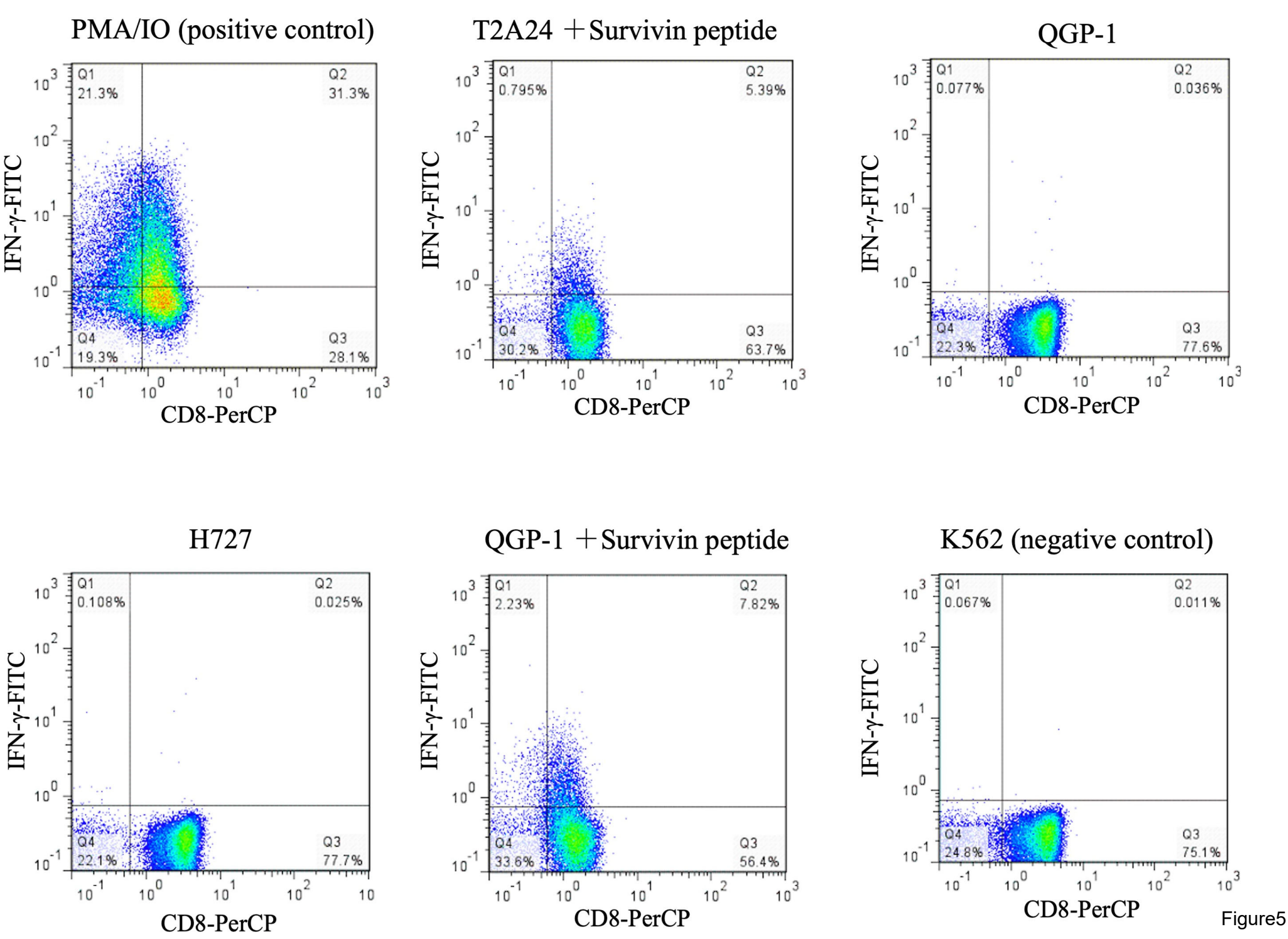
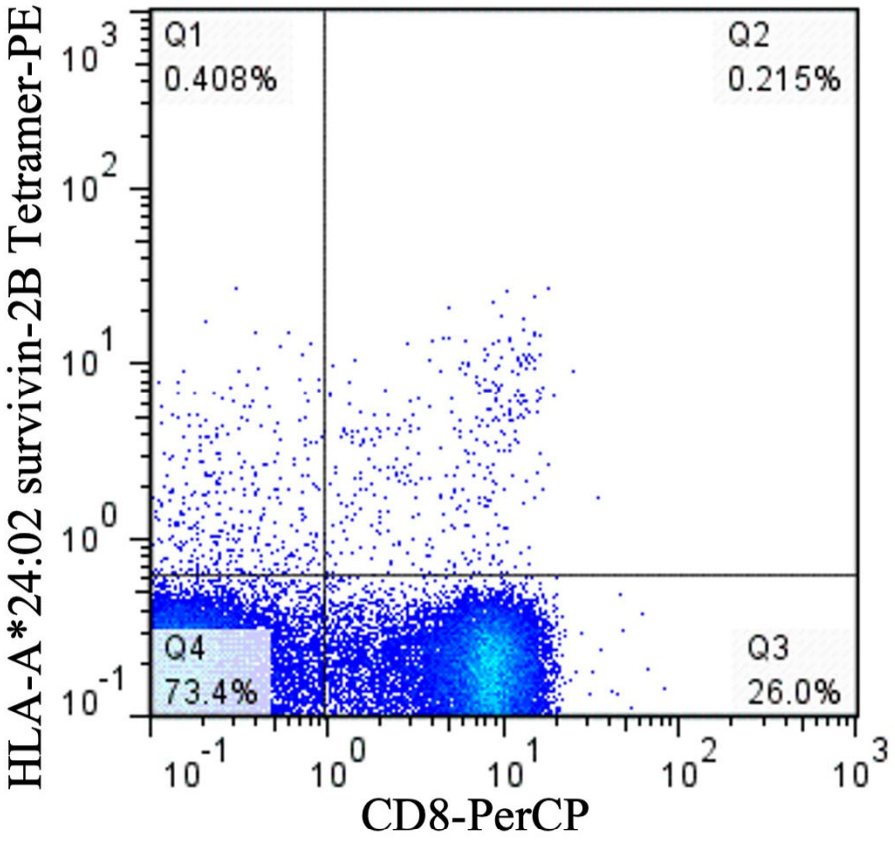

PMA/IO (positive control)
Q1 21.3%
Q2 31.3%
Q4 19.3%
Q3 28.1%
T2A24 ＋Survivin peptide
Q1 0.795%
Q2 5.39%
Q4 30.2%
Q3 63.7%
QGP-1
Q1 0.077%
Q2 0.036%
Q4 22.3%
Q3 77.6%
H727
Q1 0.108%
Q2 0.025%
Q4 22.1%
Q3 77.7%
QGP-1 ＋Survivin peptide
Q1 2.23%
Q2 7.82%
Q4 33.6%
Q3 56.4%
K562 (negative control)
Q1 0.067%
Q2 0.011%
Q4 24.8%
Q3 75.1%
IFN-γ-FITC
CD8-PerCP

Figure5


HLA-A*24:02 survivin-2B Tetramer-PE
Q1
0.408%
Q2
0.215%
Q4
73.4%
Q3
26.0%
CD8-PerCP

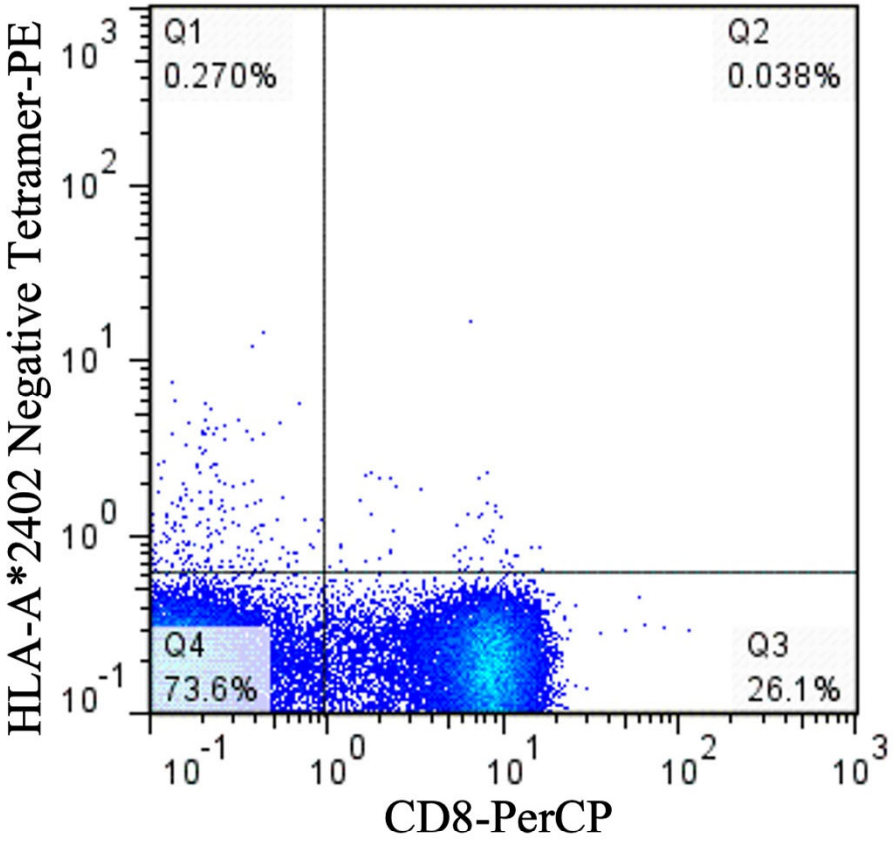

HLA-A*2402 Negative Tetramer-PE
Q1
0.270%
Q2
0.038%
Q4
73.6%
Q3
26.1%
CD8-PerCP

Figure6

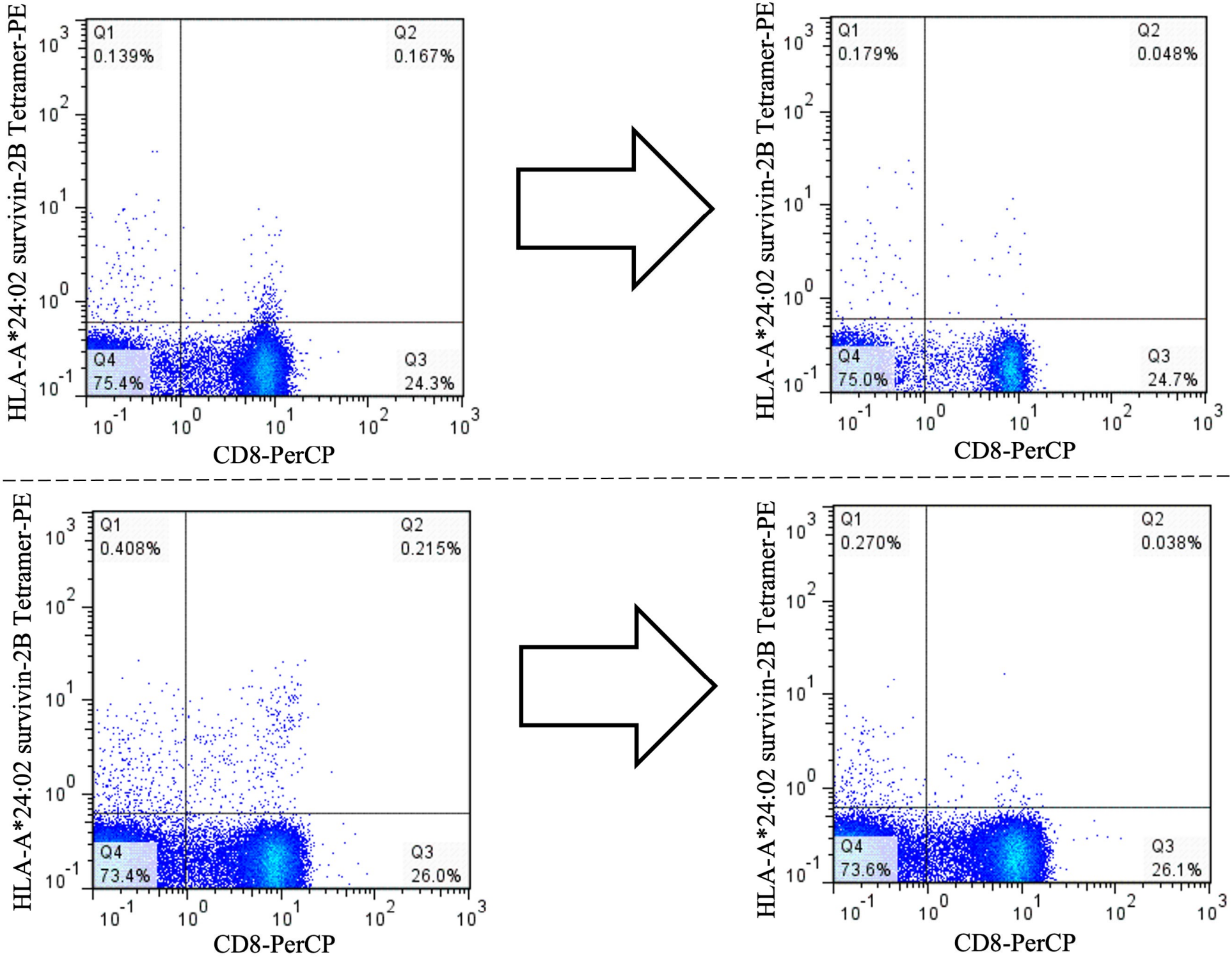

HLA-A*24:02 survivin-2B Tetramer-PE
CD8-PerCP
Q1
0.139%
Q2
0.167%
Q4
75.4%
Q3
24.3%
Q1
0.179%
Q2
0.048%
Q4
75.0%
Q3
24.7%
Q1
0.408%
Q2
0.215%
Q4
73.4%
Q3
26.0%
Q1
0.270%
Q2
0.038%
Q4
73.6%
Q3
26.1%

Figure7

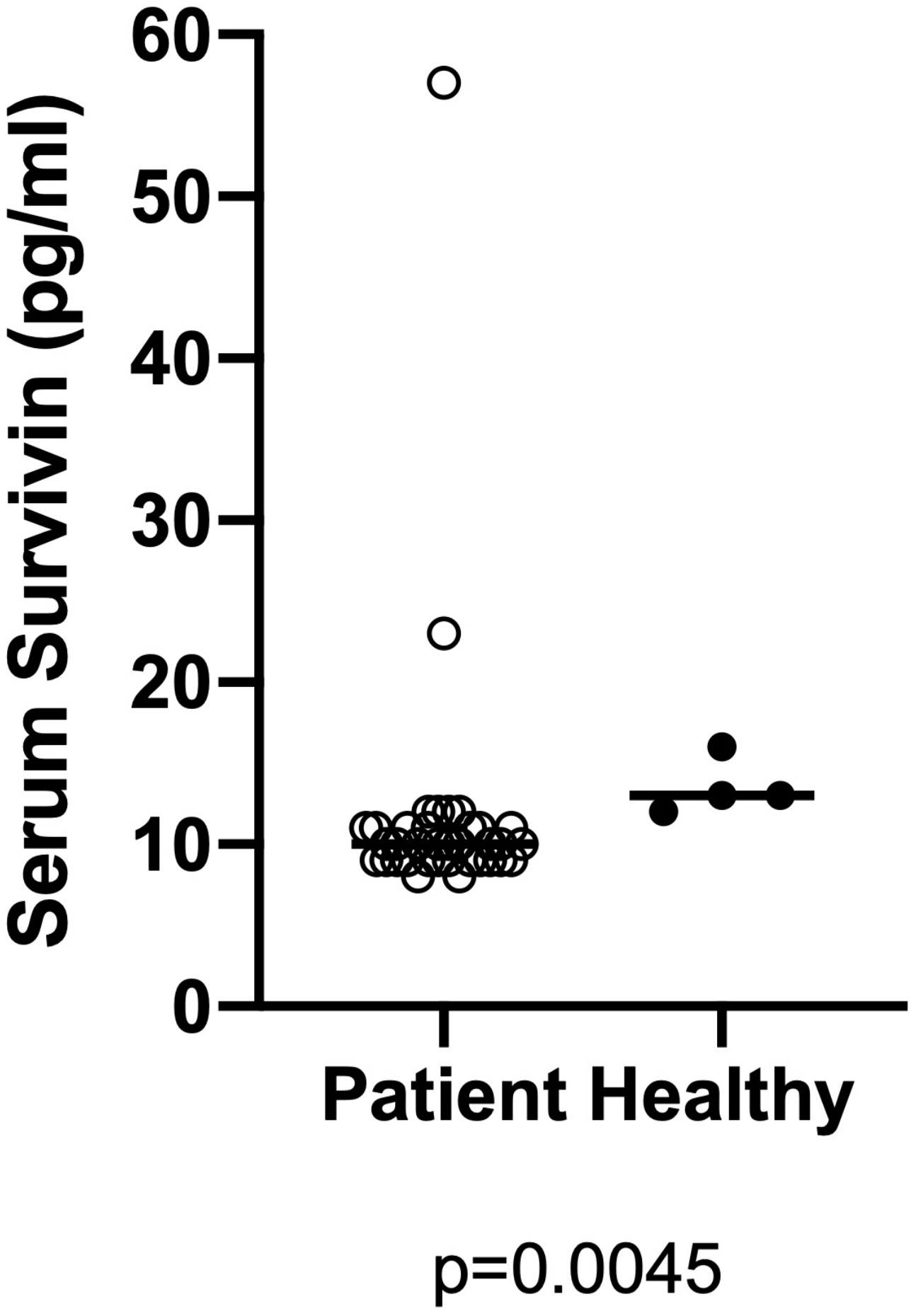

60
50
40
30
20
10
0
Serum Survivin (pg/ml)
Patient
Healthy
p=0.0045

Figure8

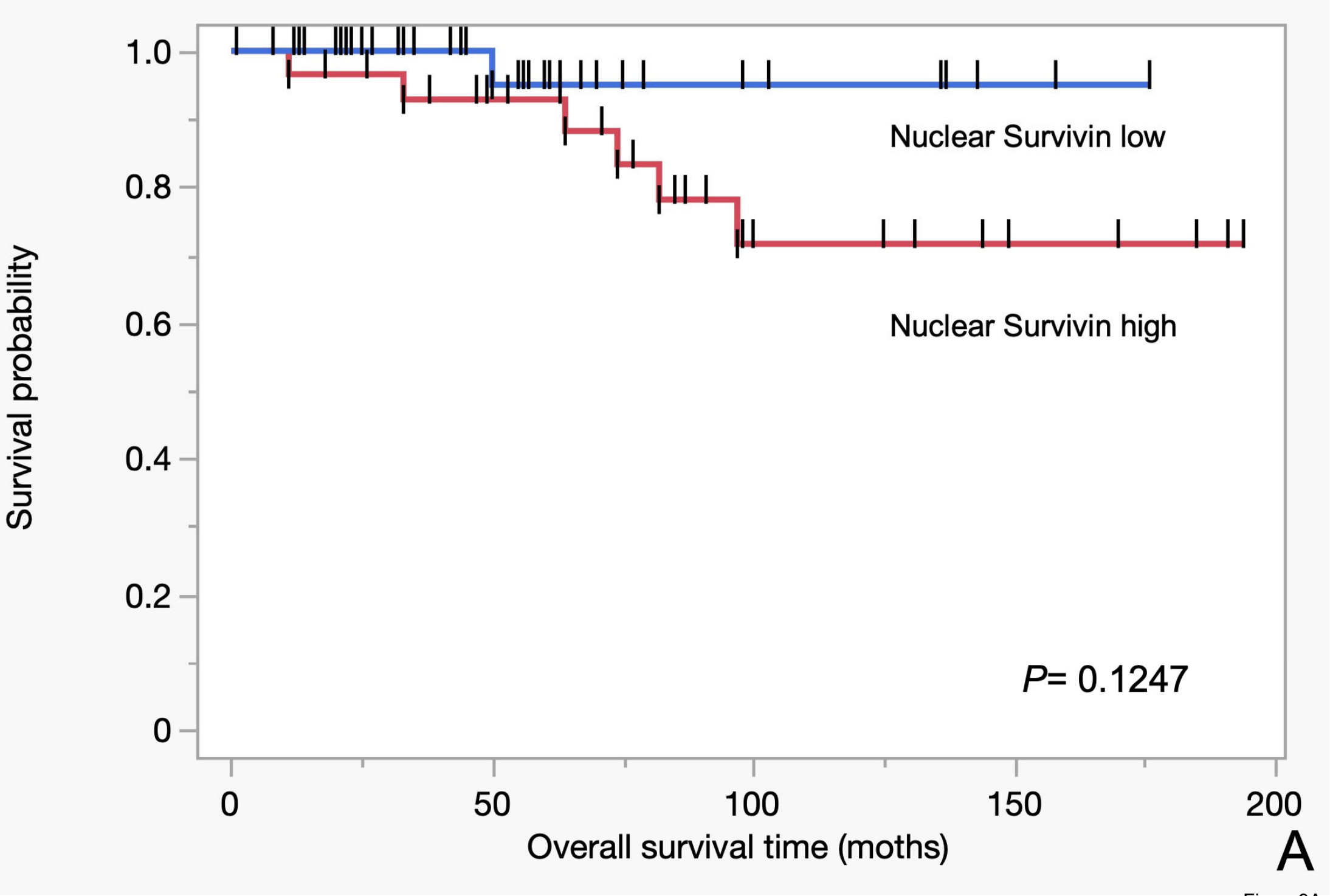

1.0
0.8
0.6
0.4
0.2
0
Survival probability
Nuclear Survivin low
Nuclear Survivin high
P= 0.1247
0
50
100
150
200
Overall survival time (moths)
A

Figure9A

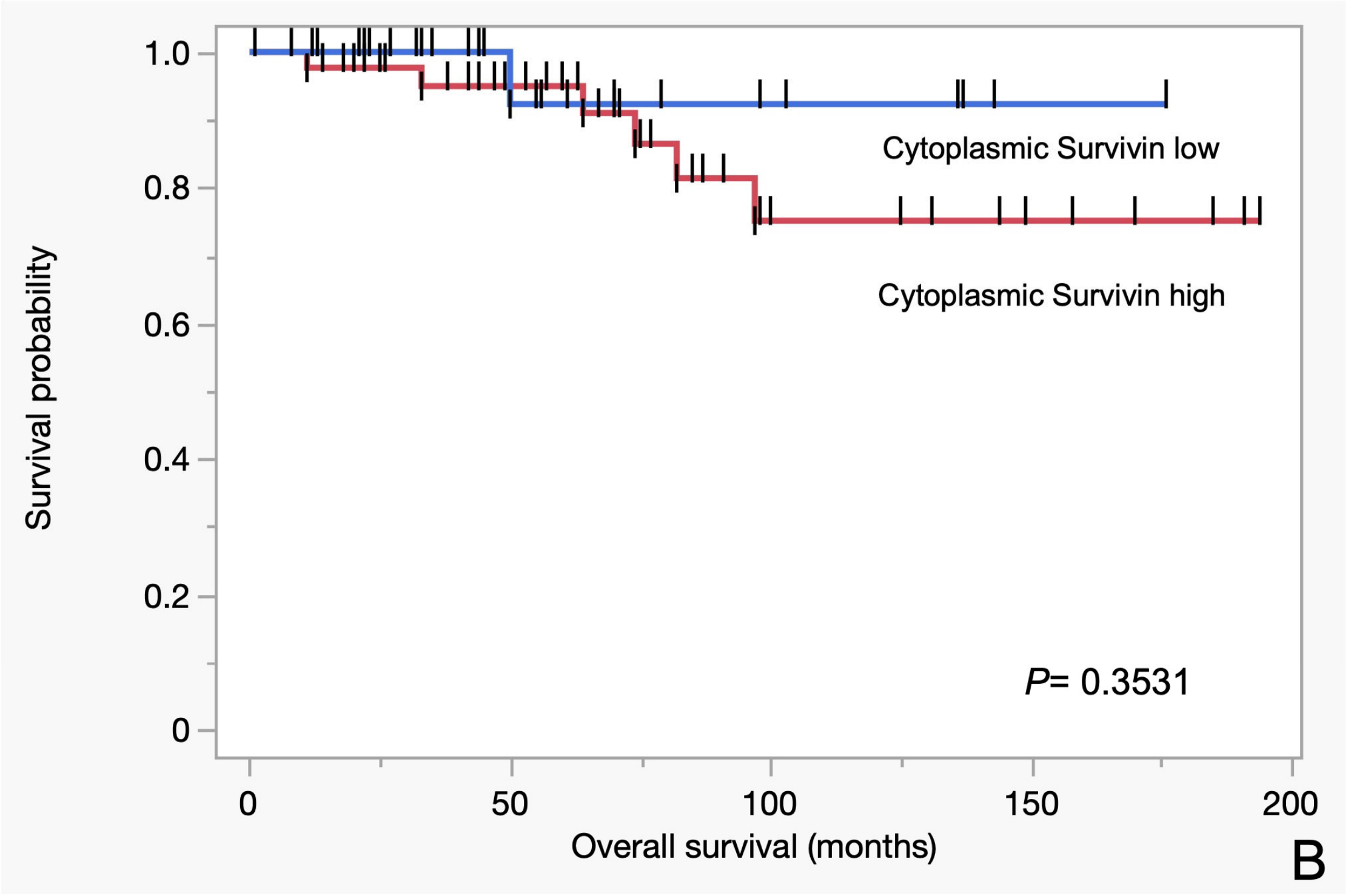

1.0
0.8
0.6
0.4
0.2
0
Survival probability
Cytoplasmic Survivin low
Cytoplasmic Survivin high
P= 0.3531
0
50
100
150
200
Overall survival (months)
B

Figure9B

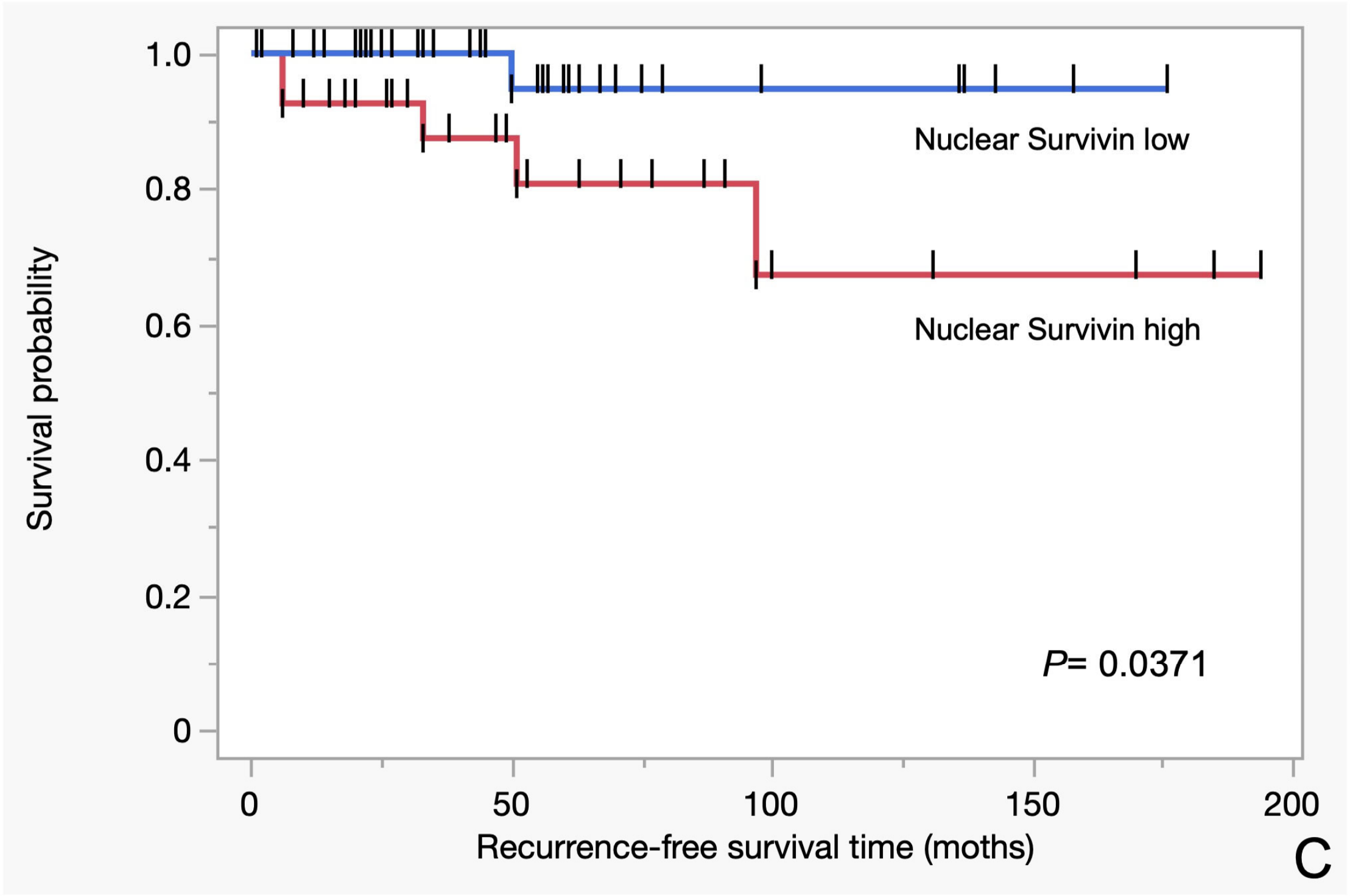

Survival probability
1.0
0.8
0.6
0.4
0.2
0
Nuclear Survivin low
Nuclear Survivin high
P= 0.0371
0
50
100
150
200
Recurrence-free survival time (moths)
C

Figure9C

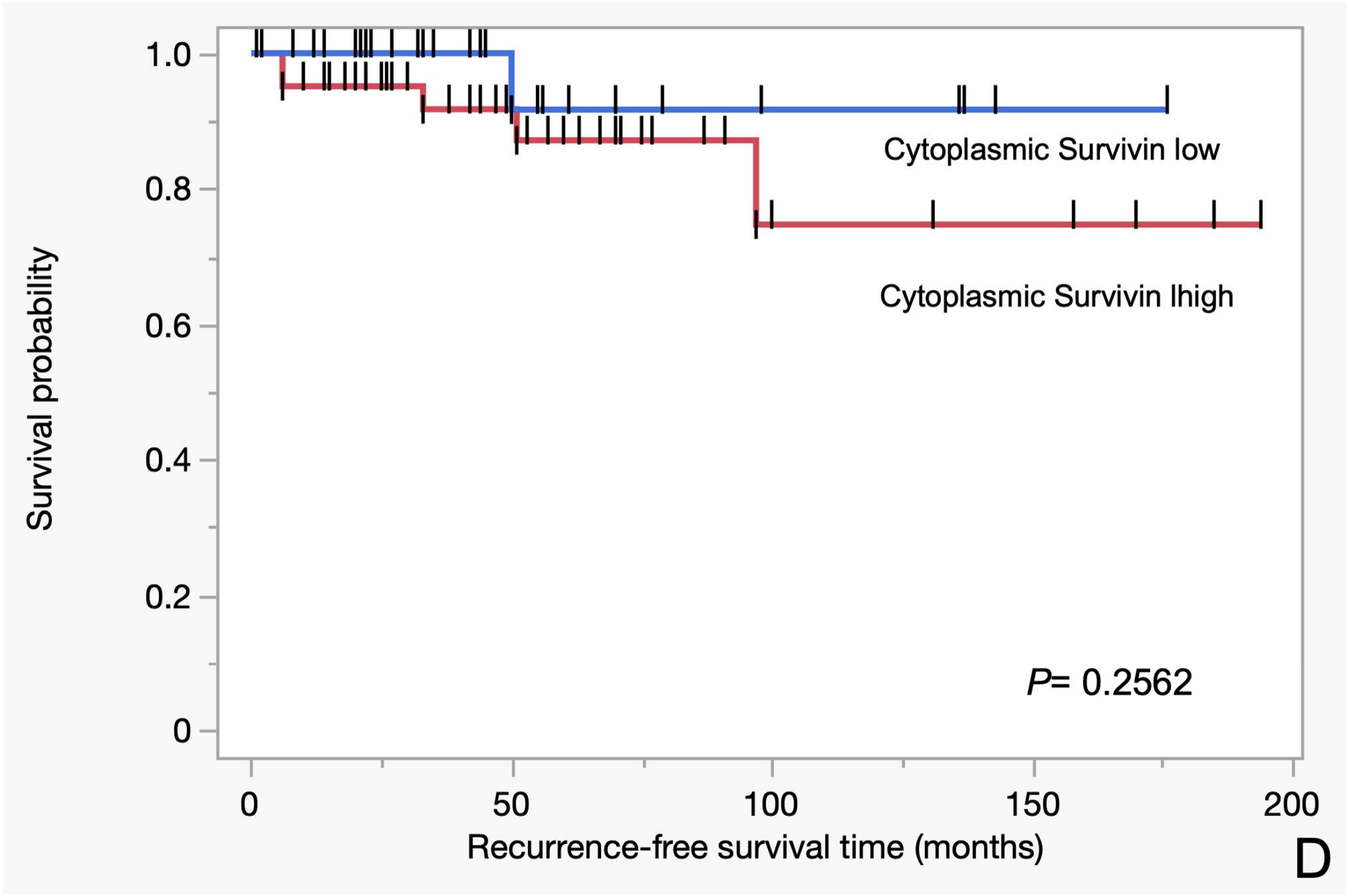

1.0
0.8
0.6
0.4
0.2
0
Survival probability
Cytoplasmic Survivin low
Cytoplasmic Survivin lhigh
P= 0.2562
0
50
100
150
200
Recurrence-free survival time (months)
D

Figure9D

Table 1. Characteristics of HLA-A24 positive patients with NEN who underwent assessment for the frequency of Survivin-2B 80-88 CTL precursors.

Case no.	Age range	Sex	Primary site	Tumor size (mm)	TNM classification	Stage	Histology (Ki-67)	Functional	CTLp frequency (%)	Recurrence
Patient. 1	35-45	M	Duodenum	11	T3N1M0	Ⅲ	NET G2 (4.4%)	Nonfunctional	0.21	(-)
Patient. 2	35-45	F	Pancreas	20	T1N0M0	Ⅰ	NET G1 (2.1%)	Insulinoma	0.16	(-)
Patient. 3	35-45	F	Stomach	30	T2N1M1	Ⅳ	NET G1/2 (N/A)	Gastrinoma	0.01	SD
Patient. 4	55-65	F	Rectum	23	T2N0M1	Ⅳ	NEC G3 (>50%)	Nonfunctional	0.18	Liver metastasis
Patient. 5	45-55	M	Pancreas	8	T1N0M0	Ⅰ	NET G1 (1.9%)	Nonfunctional	0.09	(-)
Patient. 6	45-55	M	Pancreas	22	T3N1M0	Ⅲ	NET G2 (4.4%)	Nonfunctional	0.014	Liver metastasis
Patient. 7	45-55	F	Pancreas	14	T1N0M0	Ⅰ	NET G2 (9.8%)	Nonfunctional	0.005	(-)
Healthy. 1	25-35	M	N/A	N/A	N/A	N/A	N/A	N/A	0.041	N/A
Healthy. 2	25-35	M	N/A	N/A	N/A	N/A	N/A	N/A	0.033	N/A
Healthy. 3	25-35	M	N/A	N/A	N/A	N/A	N/A	N/A	0.036	N/A

Table 2. Association between nuclear survivin expression and clinicopathological features.

Characteristics	Number of patients	nuclear Survivin-High	nuclear Survivin-Low	P-value
All cases	74	45	29	
Age				
<60	40	18	22	0.0025
≧60	34	27	7	
Sex				
Male	30	18	12	0.9061
Female	44	27	17	
Tumor location				
Ph	29	14	15	0.0762
Pbt	45	31	14	
Tumor size				
<2cm	42	33	9	0.0003
≧2cm	32	12	20	
Node status				
N0	57	40	17	0.0025
N1	17	5	12	
Liver Metastasis				
M0	66	44	21	0.0018
M1	9	1	8	
Functionality				
Nonfunctional	53	27	26	0.0037
Functionali	21	18	3	
Ki-67				
<3%	40	28	12	0.079
≧3%	34	17	17	
mitotic index				
<2%/10HPF	59	37	22	0.5064
≧2%/10HPF	15	8	7	