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NKCC1 a Regulator of Glioblastoma Progression

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Running title: Targeting NKCC1 in Glioblastoma

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Abstract

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults, with poor prognosis despite multimodal therapy. Chloride cotransporters NKCC1 and KCC2 are key regulators of intracellular chloride levels and thereby determine whether GABA acts inhibitory or excitatory. In GBM, disrupted chloride homeostasis promotes proliferation, migration, and stem-like properties, but its clinical relevance is not fully understood.

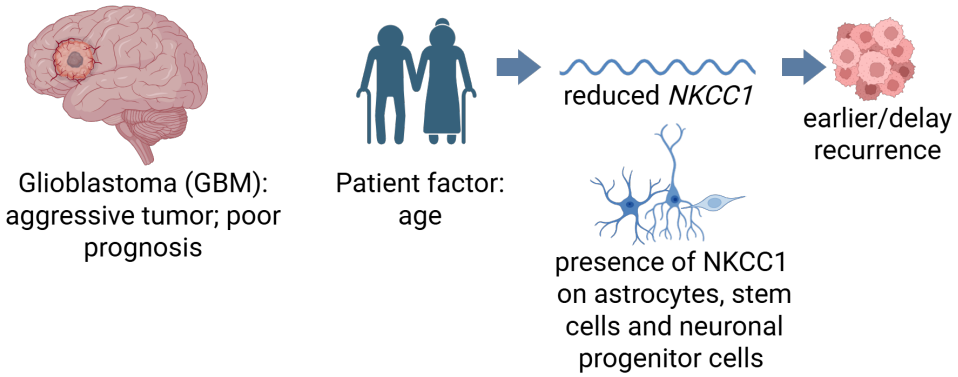
We analysed *NKCC1* and *KCC2* expression in glioblastoma samples, considering clinical parameters such as age, gender, and MGMT promoter methylation. Statistical analyses included ROC-based cutoff determination, Kaplan–Meier survival analysis and subgroup. Immunohistochemistry was performed to identify cell types expressing NKCC1.

NKCC1 expression was significantly higher in older patients and emerged as a prognostic marker for recurrence-free survival, with lower levels correlating with delayed recurrence, although overall survival was unaffected. *NKCC1* was expressed in stem-like, astrocytic, and progenitor cells, but not in mature neurons.

These findings identify *NKCC1* as a regulator of GBM progression and recurrence, linking chloride transporter imbalance to GABAergic signalling. Targeting *NKCC1* and restoring chloride homeostasis may provide promising new treatment strategies.

Graphical abstract

NKCC1 as a regulator of glioblastoma progression and recurrance



Introduction

Glioblastoma (GBM) is the most common and most aggressive primary brain tumor in adults [1]. Despite intensive multimodal treatment approaches, including surgical resection, radiotherapy, and chemotherapy with temozolomide, the prognosis for patients remains extremely poor. The median survival time is approximately 15 months after diagnosis [2] and the five-year survival rate is less than 5% [3]. This unfavorable outcome is largely driven by the high invasiveness of the tumor, its genetic and epigenetic heterogeneity, and the ability of tumor cells to evade therapeutic strategies and develop resistance. Consequently, a central challenge in glioblastoma research is to identify molecular mechanisms that regulate tumor growth, invasiveness, and therapy resistance in order to develop novel therapeutic strategies. In this context, ion channels and transporters have received increasing attention, as they are indispensable for maintaining cellular homeostasis, regulating cell volume, and mediating signal transduction.

Among these, the cation-chloride cotransporters NKCC1 and KCC2 are of particular interest. They are essential regulators of intracellular chloride concentration and play a pivotal role in the functional development of the central nervous system. NKCC1 is mainly active in immature neurons, where it increases intracellular chloride levels, whereas KCC2 is expressed in mature neurons and lowers chloride concentration by extruding ions [4, 1, 5]. Through their opposing functions, these transporters shape the action of the neurotransmitter GABA, which depolarizes immature neurons but hyperpolarizes mature ones [6-9].

In GBMs, dysregulation of chloride transport appears to contribute directly to tumor progression. NKCC1, in particular, has emerged as a critical factor in glioma biology [10, 11, 5, 12-14]. By mediating chloride influx, NKCC1 facilitates changes in cell

volume and cytoskeletal organization that are essential for GBM cells to migrate through the dense extracellular matrix of the brain. Upregulated NKCC1 activity has been associated with enhanced proliferation, invasiveness, and survival of GBM cells [4]. Pharmacological inhibition of NKCC1 has been shown in experimental models to reduce glioma cell migration and invasiveness, highlighting its potential clinical relevance [15-17]. Given its dual role in regulating both chloride homeostasis and glioblastoma cell behavior, NKCC1 represents a promising candidate for therapeutic intervention aimed at limiting tumor progression and improving patient outcomes.

The role of NKCC1 in GBM and its potential therapeutic implications highlight the relevance of these transporters for neuronal function and the pathogenesis of neurological and oncological diseases.

102 **Materials and Methods**

103 ***Human samples***

104 The transcription analyses were performed with 61 glioblastoma samples surgically
105 obtained from the Department of Neurosurgery at the University Hospital Jena. 10
106 FFPE samples were used for localization and co-localization of specific cells after
107 immunofluorescence staining and confocal microscopy, which were provided by the
108 Institute of Neuropathology, Charité-University Berlin. All tissue samples were
109 histopathologically classified according to the current 2016 WHO classification for
110 tumors of the central nervous system by the Institute of Neuropathology at Charitè
111 Berlin, University Berlin [18]. All patients provided informed consent for the
112 pseudonymized use of the extracted tumor material. The study was approved by the
113 local ethics committee for human research (*Reg.-Nr. 2019-1400*).

114

115 ***Total RNA extraction, reverse transcription and quantitative polymerase chain***
116 ***reaction***

117 Surgically removed tissue was immediately stored at -80°C until processing. Total
118 RNA from tissue sample was isolated, lysed in 1 ml Qiazol (Qiagen GmbH, Hilden,
119 Germany) and homogenized with TissueLyser LT (Qiagen GmbH, Hilden, Germany)
120 at 50 Hz for 5 min. The lysed tissue sample was mixed with 0.2 volume units (PU) of
121 chloroform, centrifuged at 12,000 x g at 4 °C for 15 minutes. The upper aqueous
122 RNA phase was removed and mixed with 1 PU isopropanol and centrifuged at
123 12,000 x g at 4 °C for 10 minutes. The resulting pellet was dissolved twice in 75 %
124 ethanol and precipitated at -20 °C for one hour. The resulting pellet was air-dried for
125 10 minutes and resuspended in 40 µl RNASE-free water.

126 The RNA quality and concentration was determined spectrophotometrically
127 (NanoDrop2000; Thermo Scientific, Wilmington, DE, USA). Complementary DNA
128 synthesis was performed using the GoScriptTM Reverse Transcription System
129 (Promega Corp., Mannheim, Germany) in a volume of 20 µl per reaction according to
130 the manufacturer's instructions.
131 The prepared cDNA was used as the basis for qPCR with the DyNAmo Flash SYBR
132 Green qPCR Kit (Thermo Scientific Inc., Wilmington, DE, USA) to determine the
133 mRNA levels of the transporters *NKCC1* and *KCC2*. The human-specific primers
134 used for qPCR were designed based on mRNA coding sequences (NCBI Nucleotide)
135 with NetPrimer (PREMIER Biosoft, Palo Alto, CA, USA) and NCBI Primer-BLAST and
136 checked for hairpins and other secondary structures. The specificity of the
137 amplification reaction was analyzed by gel electrophoresis and melting curve
138 analysis.
139 The primer sets are listed in Table 1. The specific transcripts were amplified in a
140 RotorGeneG (Qiagen GmbH, Hilden, Germany) with the following program: 7 min
141 polymerase activation, 40 amplification cycles (95°C for 10s, 55°C for 20s, 72°C for
142 30s). To quantify the expression of target genes, we normalized the Ct value using
143 *RPL13A* and *CYC1* as reference genes and normalized expression using the
144 efficiency-based ∆Ct method (Pfaffl, 2001).
145

146 ***Immunofluorescence staining***

147 The FFPE sections were deparaffinized by a descending ethanol series. Antigen
148 retrieval was performed by incubating citrate buffer (pH 6.0) at 60 °C for 30 minutes.
149 After washing with phosphate buffered saline (PBS), the non-specific binding sites
150 were blocked with 20 % normal donkey serum (NDS) for 120 min. Hybridization of

151 the primary antibodies: rabbit anti-NKCC1 (Proteintech™; 1:250), chicken anti-DCX
152 (Abcam; 1:500), mouse anti-nestin (Abcam; 1:500), mouse anti-NeuN (Merck
153 Millipore KGaA; 1:500) and/or mouse anti-S100b (Sigma Aldrich Chemie GmbH;
154 1:500) was performed overnight at 4 °C. The next day the slides were incubated at
155 4 °C. After two washes, the secondary antibodies were hybridized: Alexa488 donkey
156 anti-mouse (Jackson Immunoresearch; 1:200), Alexa488 donkey anti-chicken
157 (Jackson Immunoresearch; 1:200), Cy5 donkey anti-mouse (Jackson
158 Immunoresearch; 1:200) and Rhodamine donkey anti-rabbit (Jackson
159 Immunoresearch; 1:200) for 2 hours. Nuclear staining was performed with DAPI.
160

161 ***Colocalization of NKCC1 with stem cell, glial, progenitor and neuronal marker***
162 ***using immunofluorescence staining***

163 Ten GBM samples were used for fluorescent immunohistochemistry. Tissue sections
164 were pre-embedded in paraffin and provided by Institute of Neuropathology, Charité-
165 University Berlin. Colocalization analysis was performed for NKCC1 with markers of
166 stem cells (Nestin), mature astrocytes (S100b), neuronal progenitor cells
167 (Doublecortin, DCX), and mature neurons (NeuN).
168 Fluorescence imaging was performed using the Keyence microscope at 4×
169 magnification for overview images. High-magnification images (40×) were acquired
170 using the LSM980 confocal microscope (Zeiss, Jena, Germany). Individual cells were
171 also imaged to confirm the specificity of the staining.
172

173 ***Statistics***

174 Statistical analyses were carried out using SPSS software (version 23). Gene
175 expression (qPCR) data were first tested for normal distribution. Depending on the

distribution, differences between two groups were analyzed using the Mann–Whitney U test.

For survival analysis, receiver operating characteristic (ROC) curves were generated in SPSS, and an optimal cutoff value was determined using the Youden index (J = sensitivity + specificity – 1). This cutoff was applied to stratify normalized expression values for subsequent analyses. Progression-free survival (PFS) and overall survival (OS) were evaluated using Kaplan–Meier survival analysis. A p-value of ≤ 0.05 was considered statistically significant.

Results

Age-dependent increase of NKCC1 in Glioblastoma

The patients' age, sex, and MGMT methylation status were analyzed (Fig. 1). Significant differences were observed in *NKCC1* expression based on age: younger patients (≤ 60 years) exhibited approximately threefold lower NKCC1 expression (mean = 0.02374) compared to older patients (> 60 years) (mean = 0.07220), which was statistically significant (p = 0.027) (Fig. 1A/B).

Regarding sex, *NKCC1* and *KCC2* expression levels were similar between males and females (*NKCC1*: males mean = 0.04089, females mean = 0.06978; *KCC2*: males mean = 0.00213, females mean = 0.00129) (Fig. 1C/D). Likewise, no significant differences were found based on MGMT methylation status (*NKCC1*: unmethylated promoter mean = 0.06367, methylated promoter mean = 0.04881; *KCC2*: unmethylated promoter mean = 0.00164, methylated promoter mean = 0.00175) (Fig. 1E/F).

Decreased NKCC1 levels correlate with progression-free survival

201 To assess the impact of *NKCC1* expression on recurrence-free survival, a cutoff
202 value of NE = 0.01401 was determined for individual patients (n = 48) using ROC
203 analysis (Fig. 2A). Kaplan–Meier analysis demonstrated that patients with *NKCC1*
204 expression below this cutoff (NE < 0.01401) had significantly longer recurrence-free
205 survival during the first nine months after surgery compared to patients with higher
206 *NKCC1* expression (NE > 0.01401; p = 0.019) (Fig. 2A).
207 For overall survival analysis, 43 patients were included (16 patients excluded due to
208 missing death data), and a cutoff value of NE = 0.04028 was established (Fig. 2B).
209 Kaplan–Meier analysis revealed no significant association between *NKCC1*
210 expression and overall survival (log-rank test; p = 0.078). Kaplan-Meier analysis
211 based on gender revealed no differences in both overall survival and progression-
212 free survival. In contrast, when stratified by age, the analysis showed differences in
213 overall survival but not in progression-free survival.
214

215 ***NKCC1 Colocalization with Specific Glial and Progenitor Cell Types in***
216 ***Glioblastoma***

217 Ten GBM samples were subjected to fluorescence staining and subsequently imaged
218 at both 4x and 40x magnifications for visual evaluation. NKCC1 was stained
219 alongside various glial and neuronal markers, including Nestin, S100b, DCX, and
220 NeuN. Nestin served as a marker for neural stem cells, S100b for astrocytes, DCX
221 for neuronal progenitor cells, and NeuN for mature neurons.
222 Overview images at 4x magnification revealed a heterogeneous distribution of the
223 labeled cell types throughout the tissue (Fig. 3). Rather than a uniform pattern, all
224 samples displayed distinct regional clusters of specific cell populations. The
225 fluorescence intensity of each marker protein (Nestin, S100b, DCX, or NeuN) was

notably increased in defined areas, indicating local accumulations of the respective cell types.

High-resolution imaging at 40x magnification demonstrated colocalization of the chloride cotransporter NKCC1 with multiple cell types within the GBM tissue (Fig. 3). A pronounced overlap was observed between NKCC1 signals and Nestin-positive cells, suggesting expression in neural stem cells. Additionally, colocalization occurred with S100b-positive astrocytes and DCX-positive neuronal progenitor cells. Conversely, no significant colocalization was detected between NKCC1 and NeuN-positive mature neurons.

Discussion

The present study examined the expression and potential role of the chloride cation cotransporters NKCC1 and KCC2 in human glioblastomas, taking into account clinical parameters such as age, sex, and MGMT promoter methylation status. The results contribute to understanding the molecular heterogeneity of GBMs [19] and suggest possible implications for prognosis and therapy.

An age-dependent increase in *NKCC1* expression was observed, indicating a potential association between patient age and transporter activity. Elevated *NKCC1* may be linked to processes such as cell volume regulation and migratory capacity, which are relevant for tumor invasion [12]. This observation may partly explain the generally more aggressive tumor behavior observed in older patients [15, 5].

NKCC1 also emerged as a potential marker for progression-free survival. Patients with lower *NKCC1* expression exhibited longer progression-free intervals, while overall survival was not significantly associated with *NKCC1* levels. These findings suggest that *NKCC1* gene expression may be particularly relevant for early tumor

251 progression and recurrence rather than overall survival. From a translational
252 perspective, *NKCC1* represents a potential therapeutic target. Current inhibitors,
253 such as bumetanide, are limited by side effects and poor brain penetration,
254 highlighting the need for the development of more selective and brain-penetrant
255 compounds [15, 17].
256 Immunohistochemical analyses demonstrated cell type-specific NKCC1 expression in
257 GBM tissue. NKCC1 colocalized with stem cell (Nestin), astrocyte (S100b), and
258 neuronal progenitor cell (DCX) markers, but not with mature neurons (NeuN),
259 consistent with its role in developmental and progenitor cell populations [20].
260 Overall, these results indicate a multifaceted role of NKCC1 in glioblastoma biology,
261 particularly in tumor progression and recurrence. They support further investigation
262 into targeting chloride homeostasis as a therapeutic strategy in GBM and provide a
263 rationale for developing selective *NKCC1* inhibitors and evaluating the effects of
264 modulating the NKCC1/KCC2 balance in vivo.
265

266 **Conclusions**

267 This study offers new insights into the molecular heterogeneity of glioblastomas and
268 identifies NKCC1 as a key regulator of tumor progression. The observed age-related
269 increase of *NKCC1* underscores the importance of personalized treatment strategies
270 that consider patient-specific molecular profiles. These findings suggest several
271 promising avenues for future research, including the development of selective
272 NKCC1 inhibitors with enhanced clinical applicability. In summary, this work
273 advances our understanding of GBM biology and provides a foundation for
274 developing more effective therapeutic approaches against this highly aggressive
275 brain tumor.

Conflict of interest Statement

The authors declare that there is no conflict of interest.

Author Contributions

All authors approved the final submitted version of the manuscript. A.T., D.F., M.H., S.K.: Data acquisition, data curation, and writing. C.S., F.S.: review & editing, final approval. S.K, D.F.: Conceptualization, writing, review, and editing.

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Disclosures

None

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366

367

368

369 **Table 1** qPCR primers (fw: forward, rv: reverse)

370

Gene	Sequence 5' → 3'	Accession number	Product (bp)
CYC1	fw: GAG GTG GAG GTT CAA GAC GG rv: TAG CTC GCA CGA TGT AGC TG	NM_001916.5	160
KCC2	fw: CGA AAT CCT GCT GGC TTA CC rv: AGA GGA TGA CAC AAC CCA GG	NM_020708.5	197
NKCC1	fw: AAG ACC AAG ACA TAC CGG CA rv: GGA GGA TTG GTG GTA GGT CC	NM_001256461.2	169
RPL13A	fw: CCT GGA GGA GAA GAG GAA AGA GA rv: TTG AGG ACC TCT GTG TAT TTG TCA A	NM_001270491.2	126

371

372

Figures

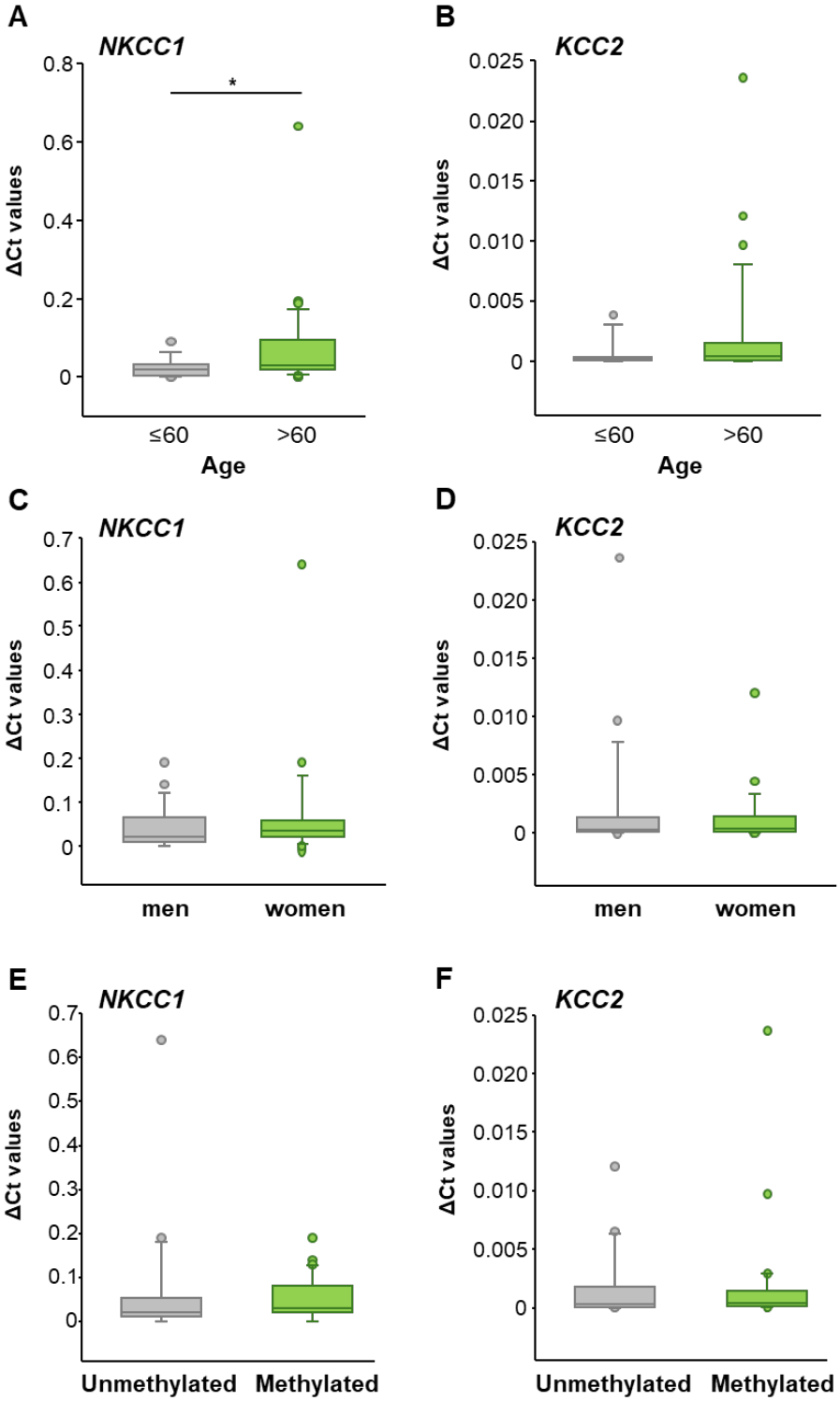

Fig. 1. A *NKCC1* was expressed significantly more strongly in individuals over 60 years of age compared to those under 60. **B** There were no age-related differences in *KCC2* expression in glioblastoma The expression of *NKCC1* and *KCC2* was not affected by **C/D** sex or **E/F** methylation status. Mann–Whitney U test, *$p \leq 0.05$.

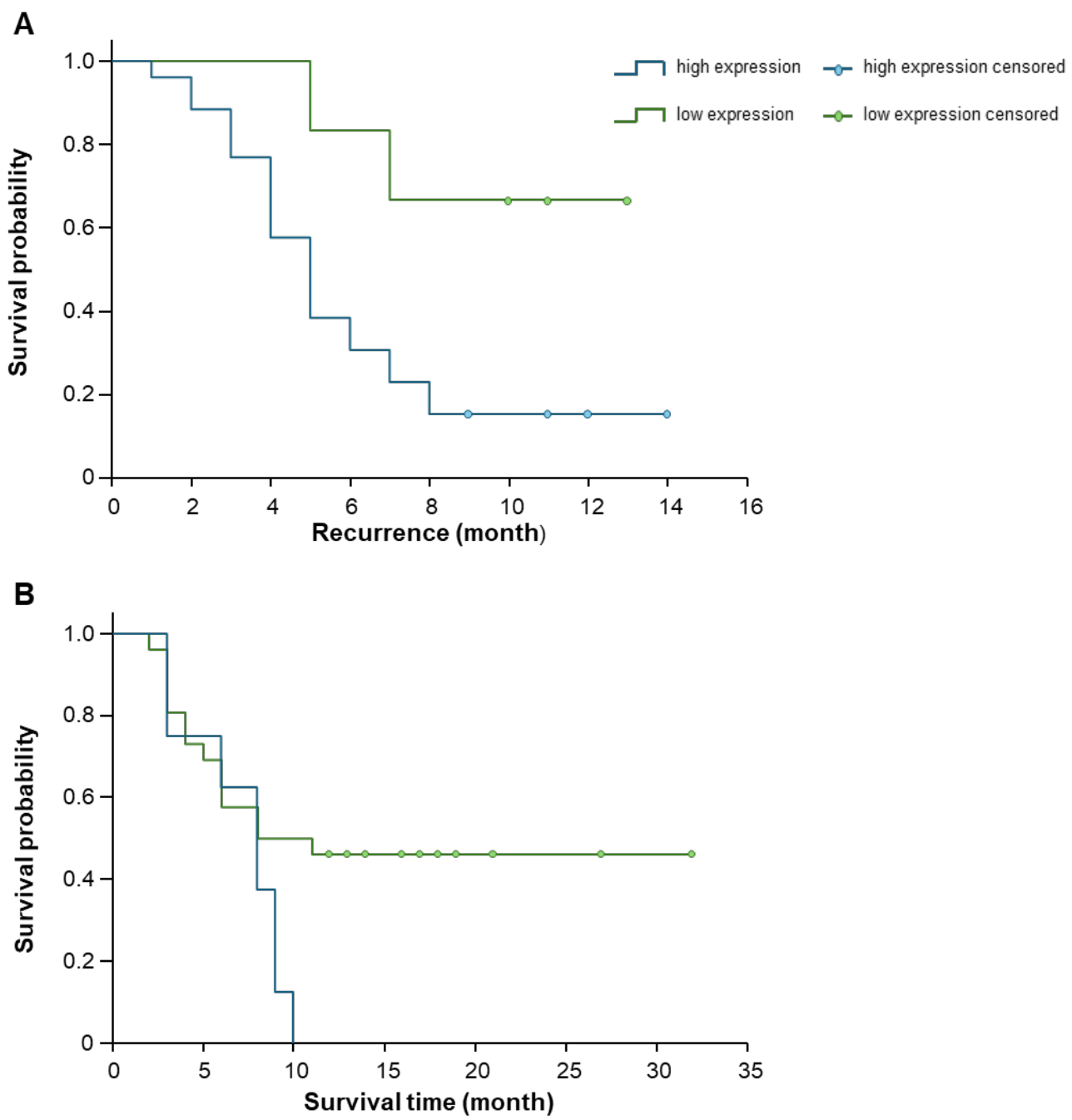

Fig. 2. Progression-free and overall survival in relation to *NKCC1* expression. **A** Kaplan–Meier analysis showing that patients with low *NKCC1* expression had significantly longer progression-free survival after 9 months ($p \leq 0.05$). **B** Overall survival tended to be longer in patients with low *NKCC1* expression after 12 months, but this difference was not statistically significant.

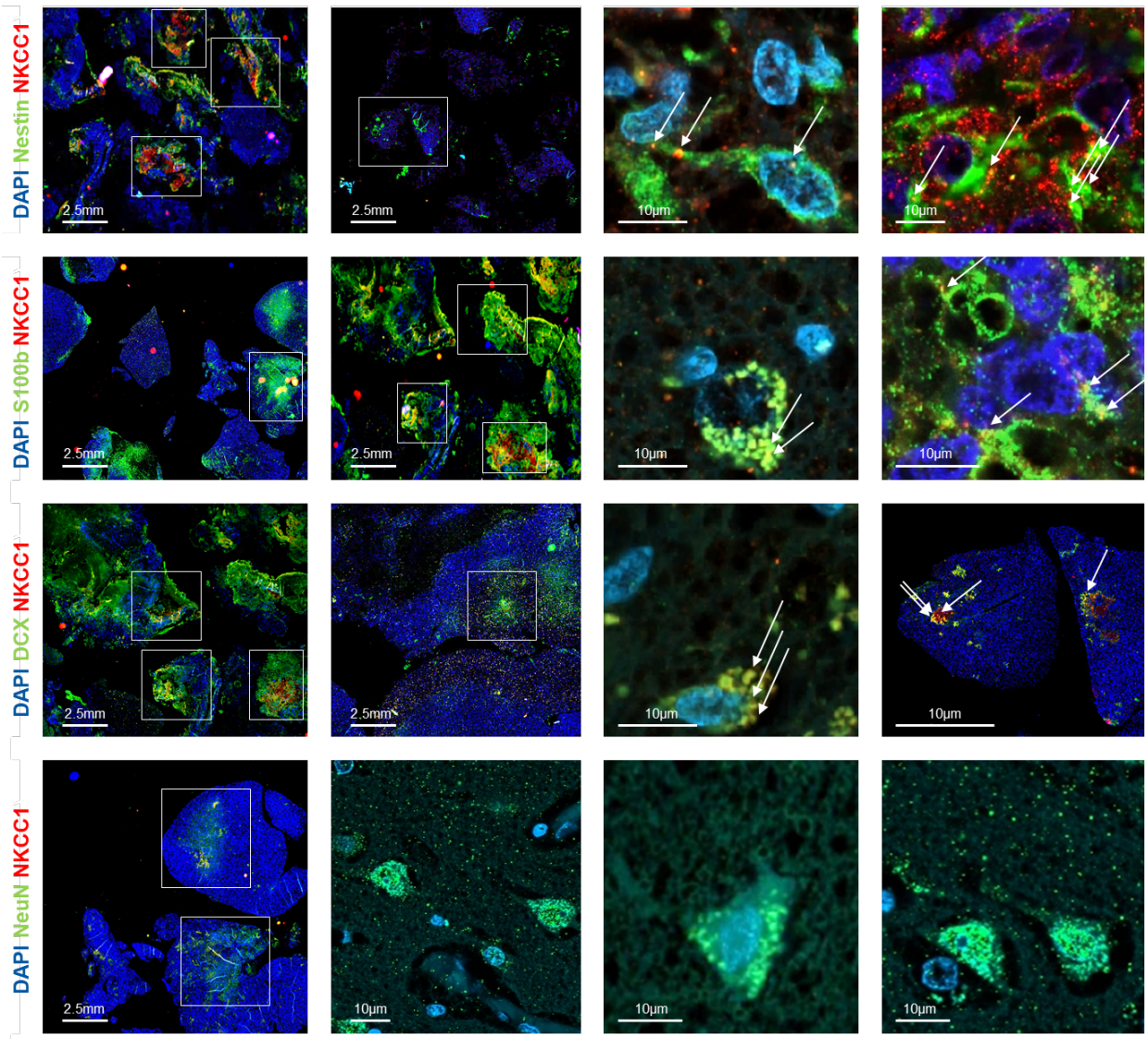

Fig. 3. Immunohistochemical analysis of glioblastoma samples showing NKCC1 expression in different cell types. The first two columns show stained GBM samples at 4x magnification. These overview images illustrate the clustering behavior of different cell types: stem cells, astrocytes, neuronal progenitor cells, and mature neurons each co-stained with NKCC1. Clear colocalization of NKCC1 (red) was observed with Nestin-positive stem cells, S100b-positive astrocytes, and DCX-positive neuronal progenitor cells (green) and DAPI (blue). In contrast, no colocalization was detected with NeuN-positive mature neurons (green). Images in the third and fourth columns, acquired at 40x magnification, provide higher-resolution views of NKCC1 expression (red) in various cell types (green) within GBM tissue. White arrows mark the presence of NKCC1 in the different cell types.