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IDENTIFICATION OF CD133$^+$/NESTIN$^+$ PUTATIVE CANCER STEM CELLS IN NON-SMALL CELL LUNG CANCER

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Aims. No effective treatment for lung cancer exists currently. One reason for this, is the development of drug resistance, assumed to be associated with cancer stem cell (CSCs) emergence within the tumour. This pilot study aimed to identify CSCs in 121 non-small cell lung cancer (NSCLC) patient samples via detection of the expression of stem cell markers – CD133 and nestin.

Material and methods. Archived paraffin blocks of 121 patient samples were prepared as Tissue Microarrays (TMA). Indirect immunohistochemical staining was used to determine the level of expression of CD133 and nestin. Double immunofluorescence staining was used to investigate the co-expression of these two markers. To determine the correlation between expression of nestin and CD133 with the length of asymptomatic period and overall patient survival we used the Kaplan-Meyer analysis.

Results. CD133 expression was detected in 22 (19%), nestin in the epithelium in 74 (66%) and vasculature in 78 (70%) of patients. Co-expression of these two markers was found in 21 (17%) patients in less than 1% of positive cells without impact on disease free or overall survival.

Conclusions. We identified CD133$^+$/nestin$^+$ cells as novel potential markers of lung cancer CSCs.

INTRODUCTION

The cancer with the highest mortality rate worldwide is now lung cancer[1] and a major problem in finding treatments is the frequent resistance to drugs which emerges. This is linked to the development and maintenance of cancer stem cells (CSCs) considered to underlie the ability for self renewal, tumourigenicity, plasticity, resistance to chemotherapy and radiation that these cancers display[2,3]. Effort is thus being made to discover and characterize CSCs in lung cancer patients.

CD133 appears to be a good marker of CSCs as its presence has been demonstrated in the membrane of various stem cells, both normal and cancer. This surface antigen is a member of the pentaspan membrane protein family. It was first observed in haematopoetic stem cells[4] but later detected in a variety of solid tumour CSCs: brain[5], melanomas[6], lung[7], pancreas[8], liver[9], kidney[10], colon[11] and prostate[12]. Since CD133$^+$ cells support neovascularisation they are frequently considered to be endothelial cells[7,10]. In CD133$^+$ cells signalling pathways controlling self-renewal and differentiation are activated[13]. After radiation, glioma CD133$^+$ cells activate the DNA damage checkpoint and thus trigger repair mechanisms conferring them radioresistance[14]. Recent studies have shown that CD133 is also ubiquitously expressed in apical or apicolateral membranes of differentiated epithelial cells where it probably regulates secretion, cell polarity and migration[15–17].

As the population of CD133$^+$ cells in NSCLC may represent a relatively large portion of cells (up to 22%) and only a fraction of CD133$^+$ cells possesses the abilities of stem cells[18], we need to identify other molecular characteristics of stem cells. Due to its very frequent occurrence in NSCLC and also brain metastasis, we selected nestin, since CD133$^+$/nestin$^+$ cells in brain tumours are considered to be CSCs[17].

Nestin is a 220 kDa[19] intermediate filament (IF) protein that takes part in cell division, proliferation and transfer of material in cytoplasm[20]. It is expressed in stem and precursor cells in developmental and regenerating tissues as neuroepithelial stem cells and newly vascular endothelial cells[21], liver, pancreas, gastrointestinal tract (GIT), testes[22], and in a spectrum of tumors such as gliomas, melanomas, gastrointestinal stromal tumors (GIST) and adrenocortical tumors. Nestin expression correlates with tumour malignancy[6,20,23,24]. In differentiated cells, nestin is replaced by vimentin, eventually with glial fibrillary acidic protein (GFAP) in glial cells[25]. However, sometimes the presence of nestin is observed during the differentiation to neuronal or glial cells. For this reason, we cannot consider nestin to be an explicit marker of "stemness" (ref.[26]).

In this pilot study, we aimed to detect $CD133^+/nestin^+$ cells using immunohistochemistry and immunofluorescence.

MATERIALS AND METHODS

Patients and samples

Formalin-fixed paraffin-embedded tissues were obtained from the archives of non-small cell lung carcinomas derived from patients operated in the period between 1996–2000 at the 1st Department of Surgery Faculty of Medicine and Dentistry Palacky University Olomouc and Faculty hospital Olomouc. Each sample was diagnosed by two independent pathologists according to the World Health Organization classification[27]. Of the 121 patient samples (95 male and 26 female), 82 were squamous cell carcinomas and 39 were adenocarcinomas.

Tissue microarray construction

Tumour tissue microarrays (TMA) were constructed with 121 formalin-fixed primary lung cancers, as stated above. The tissue area for sampling was selected on the basis of visual alignment with the corresponding hematoxylin-eosin (HE)-stained section on the slide. Two tissue cores (diameter and height of 2 mm and 3–4 mm, respectively) taken from a donor tumour block were placed onto a recipient paraffin block with an ultimate computer assisted Tissue Microarrayer Galileo TMA CK 3500 (Integrated Systems Engineering S.r.l., Italy). Cores of non-tumour lung tissue and non-tumour tissue from other organs (heart, liver, spleen, kidney and lymph node) for better orientation were punched onto each recipient paraffin block. For immunohistochemical and immunofluorescence analysis, 4-µm sections of the resulting microarray block were used.

Immunohistochemical staining

An indirect immunohistochemical technique was used. The sections were deparaffinised and antigens were unmasked in citrate buffer (pH 6) for nestin or in Target Retrieval Solution, High pH (10x) (Dako, Denmark) for CD133. Primary antibodies used were rabbit polyclonal to CD133 – Stem Cell Marker (Ab16518, Abcam, UK) or mouse anti-nestin human specific monoclonal antibody (MAB5326, clone 10C2, Chemicon International, USA) at a dilution of 1:100. Visualisation was made by EnVisionTM+ Dual Link (Dako, Denmark). Nuclei were counterstained with hematoxylin. Stained tissue sections were assessed semiquantitatively by estimation of the "histoscore" (percentage of positive cells x intensity of staining) as low (< 10%), moderate (< 30%), medium (< 60%) or high (> 60%) expression.

Immunofluorescence technique

For detection of nestin and CD 133, indirect double-staining was used. The tissue sections on Poly-L-lysine solution (Sigma-Aldrich, USA) charged slides were deparaffinised and antigens unmasked in Target Retrieval Solution, High pH (10x) (Dako) for 20 min at 98°C. Autofluorescence was reduced with filtered solution 0.5% Sudan Black in 70% ethanol for 5 min and 3 min flushed in running tap water[28]. Each of the below indicated immunostaining steps was followed with a brief washing using the PBS solution (1x) with 0.1% Tween-20. Nonspecific antigens were then blocked with an Image-iT™ FX Signal Enhancer (Invitrogen, USA) for 30 min. After washing,

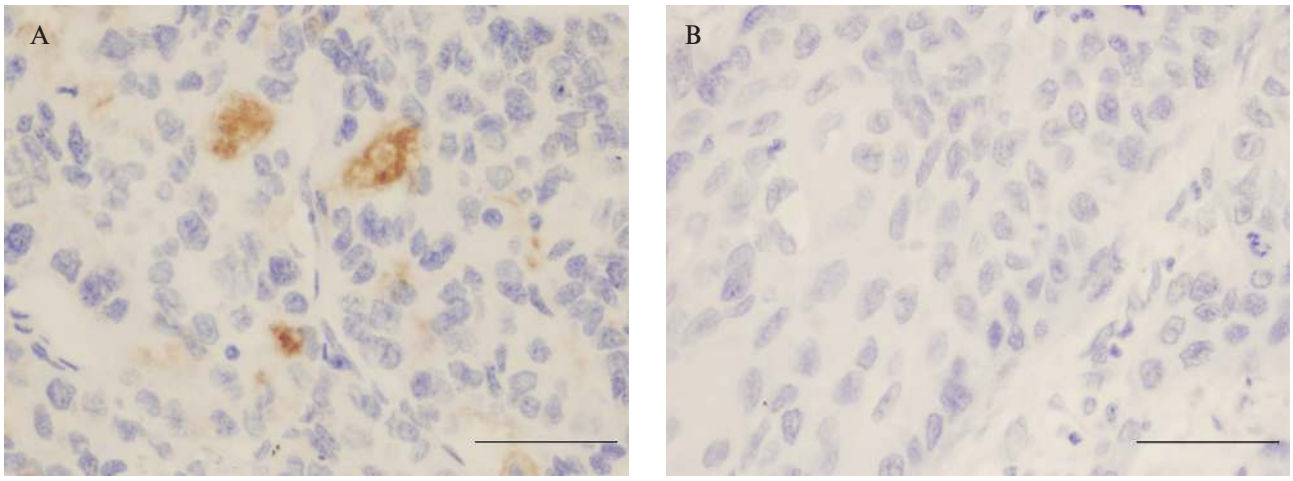

Fig. 1. Immunohistochemical detection of CD133. A – positivity (adenocarcinoma), B – negativity (squamous cell carcinoma). Magnification 400x. Scale bar is 50 µm.

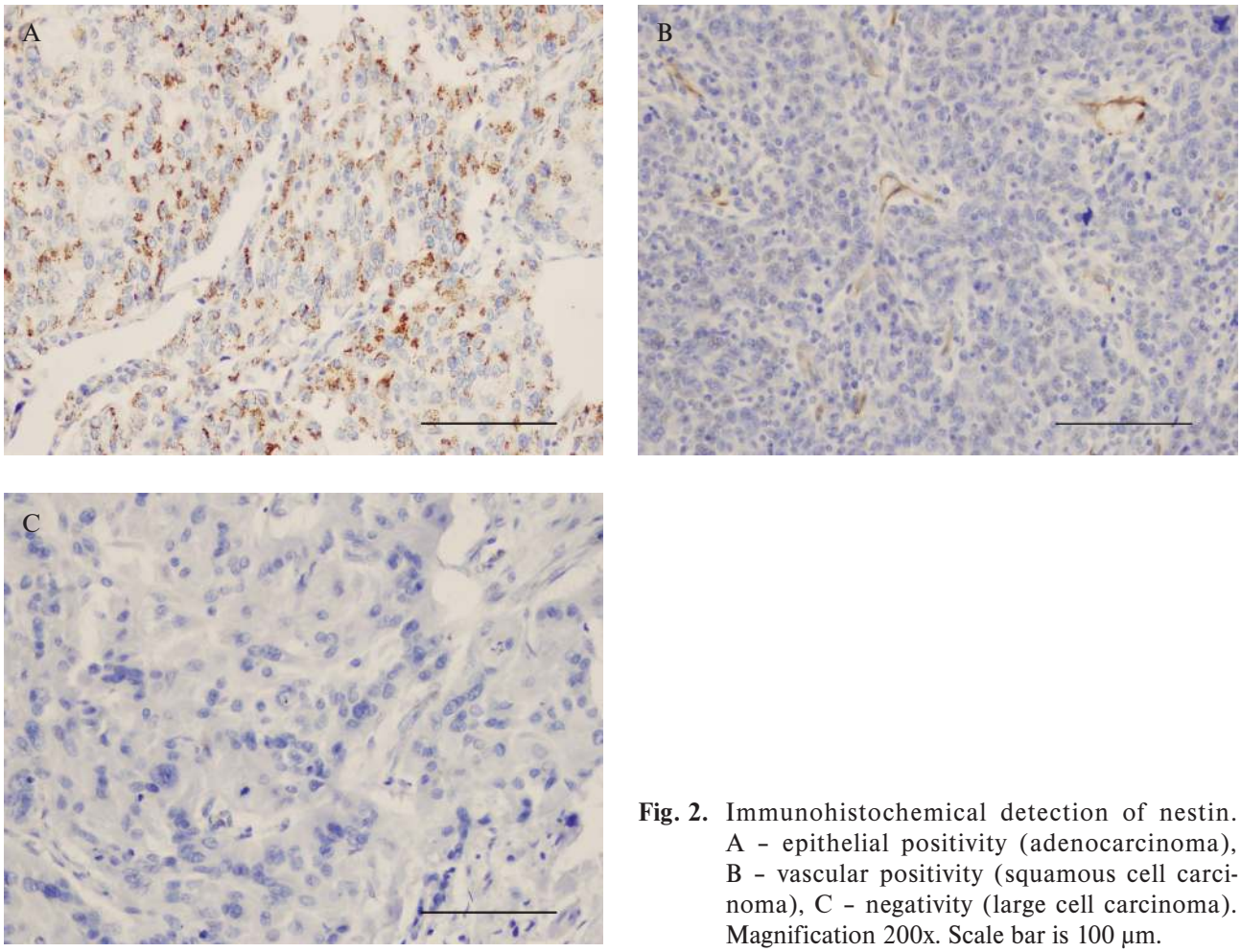

Fig. 2. Immunohistochemical detection of nestin. A – epithelial positivity (adenocarcinoma), B – vascular positivity (squamous cell carcinoma), C – negativity (large cell carcinoma). Magnification 200x. Scale bar is 100 μm.

both primary antibodies rabbit polyclonal to CD133 – Stem Cell Marker (Ab16518, Abcam) and mouse anti-nestin human specific monoclonal antibody (MAB5326, Chemicon International) diluted in Dako REAL™ Antibody diluent (Dako) in the ratio 1:100 were applied for 1 hour at room temperature. Both secondary antibodies Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (A11034, Invitrogen) and Alexa Fluor® 594 goat anti-mouse IgG (H+L) (A11032, Invitrogen) were applied in a dilution 1:200 in Dako REAL™ Antibody diluent (Dako) for 30 min at room temperature. Sections were mounted with Fluorecence Mounting Medium (Dako) containing DAPI (4',6-diamidino-2-phenylindole). The slides were observed on a fluorescence microscope and images were captured with a DP71 camera (Olympus, Japan). Sections without primary antibodies were used as negative controls.

RESULTS

19 TMA blocks from 121 patients were immunohistochemically analysed for CD133 and nestin expression. The TMAs were also used for the detection of the two proteins by immunofluorescence. Due to the loss of material during TMA processing, expression of CD133 was examined in only 116 tumour samples and that of nestin in 112 tumour samples.

In 22 (19%) patients we detected CD133⁺ cells. In 17 of these patients only a few CD133⁺ cells were detected but in the remaining 5 (4%) patients a higher expression of CD133 was observed (up to 20% of cells) (Fig. 1). Nestin expression was significantly enhanced in the epithelium and especially in the vasculature. Positivity in the epithelium was detected in 74 (66%) patients, in 33 (29%) it was high. In the vasculature, we detected nestin expression in 78 (70%) patients. This expression was high in 53 (47%) patients (Fig. 2). Using the Kaplan-Meyer analysis we found no relationship between the expression of CD133 or epithelium nestin with disease free (DFS) and/or overall survival (OS) (fixed probability level $p \leq 0.1$) (Fig. 3).

Using double immunofluorescence staining, we identified double positive CD133⁺/nestin⁺ cells in 21 (17%) patients, in less than 1% cells (Fig. 4).

DISCUSSION

Our goal was to detect the presence of nestin⁺ and CD133⁺ cells by double immunofluorescence in formalin-fixed and paraffin-embedded tumour samples of patients with NSCLC. Cells express other molecules, characteristic of stem cells, such as certain transcription factors responsible for the maintenance of stemness (Okt4/3 and

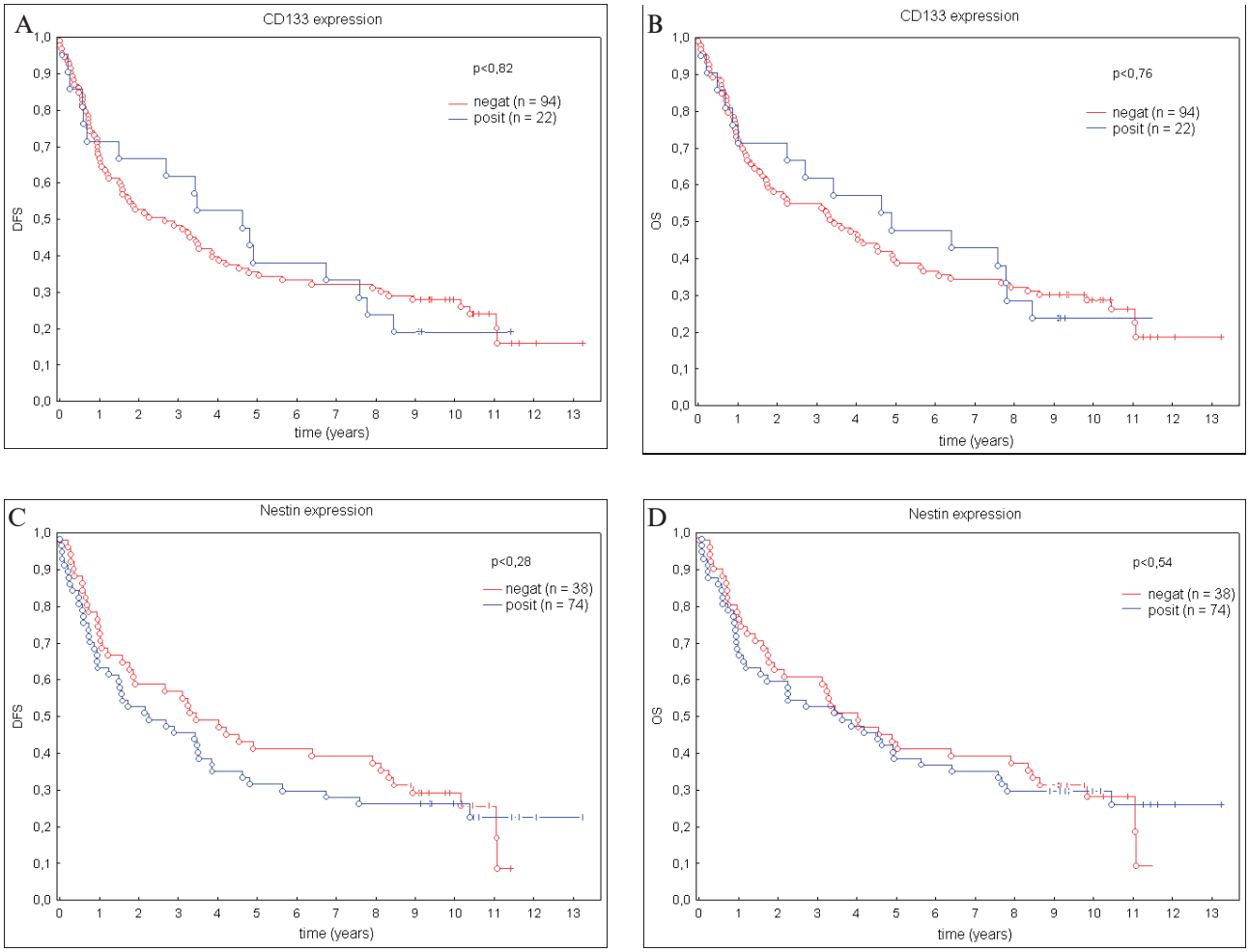

Fig. 3. Kaplan-Meyer analysis of CD133 expression (in A and B) in 116 patients and epithelial nestin expression (in C and D) in 112 patients. Probability level $p \leq 0.1$.
(OS – overall survival, DFS – disease free survival)

Homeobox protein NANOG), membrane transporters (ABCB1 and ABCG2), detoxifying enzymes (glutathione S-transferase) and motility proteins (CXCR4). Studies show that these underlie the failure of treatment and the emergence of drug resistance and metastasis[29–31]. Using immunohistochemistry, we detected the expression of CD133 in 22 (19%) patients. In the most patients, the expression of CD133 was sporadic (less than 5 cells per dot) while $CD133^+$ cells were detected in 5 patients were presented in 20%.

A similar frequency (0.3 to 22%) was reported by Eramo et al. 2008, but the tumourigenic potential was only 5 to 30% $CD133^+$ cells. Thus, the $CD133^+$ cell population can be divided into two groups: a population of tumourigenic stem-like cells and a non-tumourigenic population of precursor/progenitor cells[18]. The incidence of $CD133^+$ cells was low and it is uncertain that they are CSCs.

To date, for the isolation of $CD133^+$ cells from NSCLC, epithelial markers (EpCAM or ESA) have been used to prevent contamination by hematopoietic and endothelial precursors. Thus the obtained cells were considered to be CSCs despite the presence of the above mentioned non-tumourigenic population[7,18,29]. Earlier studies using CD133 and nestin as markers of CSCs were designed for other types of tumours (glioblastoma, osteocarcoma, ...)[17,32]. The NSCLC epithelial precursors described so far have been only $CD133^+$ cells. For this study we examined the co-expression of nestin and CD133 in pulmonary tumours.

Nestin expression was significantly stronger than the expression of CD133, and thus we could follow the positivity, particularly in the epithelium and especially in the vessels. Nestin expression was not observed in 38 (34%) patients in tumour epithelium and in 34 (30%) patients in tumour vascular bed. High nestin expression was observed in 53 patients (47%) in the vasculature and in 33 patients (29%) in the epithelium. The higher nestin expression in the vasculature is consistent with the knowledge that nestin is a marker of endothelial precursors[33].

Our results suggest that expression of neither CD133 nor nestin had an impact on patient survival or length of asymptomatic periods. Other research groups have found a correlation between CD133 expression and the emergence of resistance phenotype[7].

Co-expresion of CD133 and nestin was intended originally for identification of CSCs in brain tumours[34] but has recently been reported for osteosarcomas as well[32]. Joint

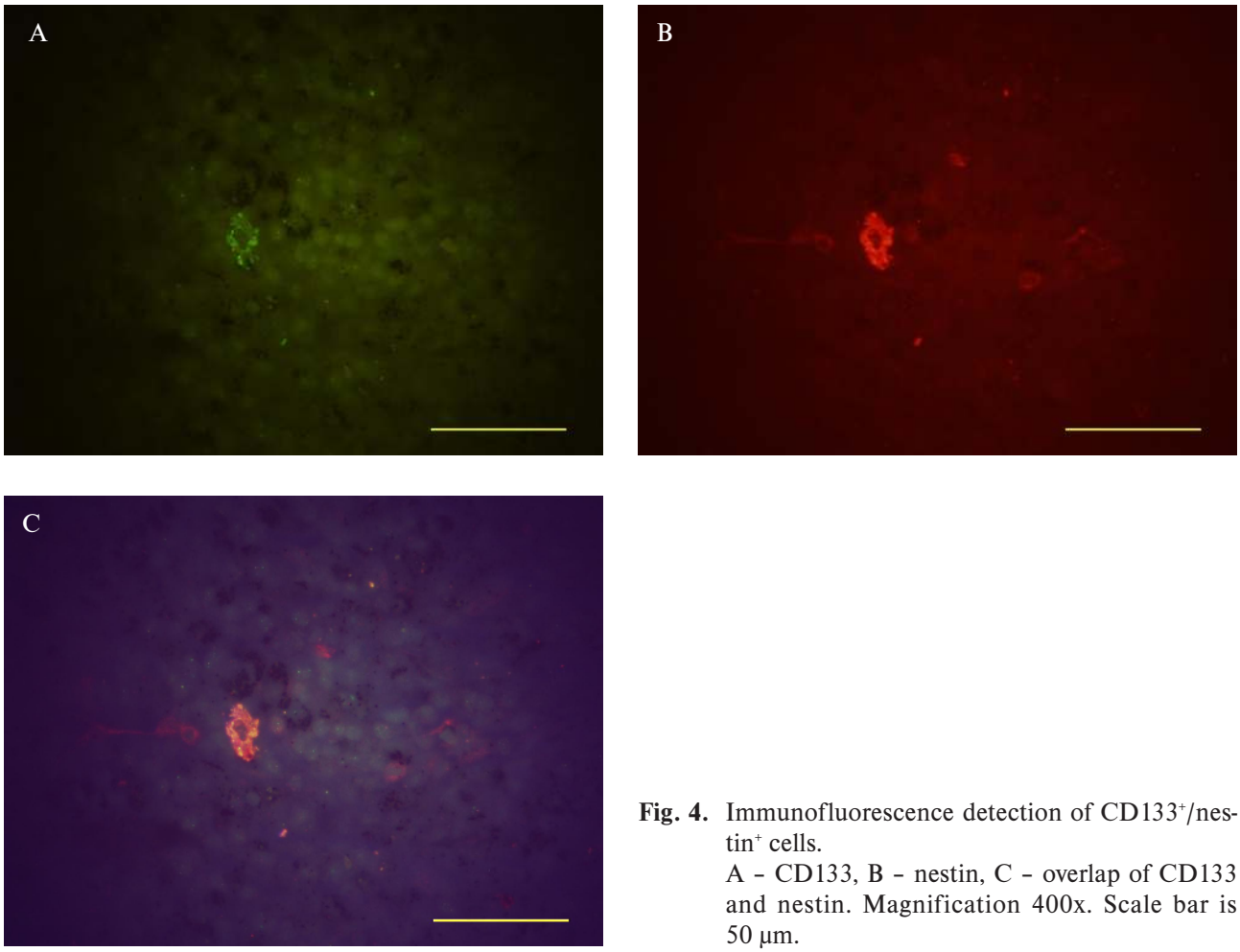

Fig. 4. Immunofluorescence detection of CD133⁺/nestin⁺ cells.
A – CD133, B – nestin, C – overlap of CD133 and nestin. Magnification 400x. Scale bar is 50 μm.

CD133 and nestin expression was found in biologically aggressive gliomas in patients with very poor survival[17]. In our case, we detected CD133⁺/nestin⁺ cells in 21 (17%) of 121 patients with NSCLC but quantitatively this represented <1 % positive cells. The prognostic impact of CD133⁺/nestin⁺ cells in NSCLC needs to be followed up in a larger sample of patients.

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