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Glycosilated Nucleolin as Marker for Human Gliomas

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ABSTRACT

Nucleolin is a multifunctional DNA and RNA binding protein involved in regulation of gene transcription, chromatin remodeling, RNA metabolism, and ribosomal RNA synthesis. Nucleolin seems to be over-expressed in highly proliferative cells and is involved in many aspect of gene expression: DNA recombination and replication, RNA transcription by RNA polymerase I and II, rRNA processing, mRNA stabilization, cytokinesis, and apoptosis. Although nucleolin is localized predominantly in the nucleolus, it has also been shown to be localized in a phosphorylated/glycolsilated form on the cell surface of different cells. Numerous articles dealing with surface nucleolin targeting for tumor therapy have been recently published. However, at present, no extensive informations are so far available for the presence of nucleolin in human gliomas. In the present work we investigated on the presence and localization of nucleolin in glioma on glioma specimens at different grade of malignancy and on primary glioma cell cultures derived by surgical resection, trying to correlate the presence of glycosilated membrane nucleolin with the malignancy grade. To this purpose an antibody produced by us against gp273 protein, demonstrated to recognized the glycosilated surface nucleolin, has been used. The results obtained demonstrate that surface nucleolin increase with the malignancy grade thus suggesting that it may constitute a histopathological marker for glioma grading and a possible tool for targeted therapy. J. Cell. Biochem. 113: 571–579, 2012.

KEY WORDS: GLYCOSILATED NUCLEOLIN; HUMAN GLIOMAS; TARGETED THERAPY

Nucleolin is a multifunctional DNA and RNA binding protein involved in regulation of gene transcription, chromatin remodeling, RNA metabolism, and ribosomal RNA synthesis [Lapeyre et al., 1987; Borer et al., 1989; Yang et al., 1994; Hanakahi et al., 1997; Srivastava and Pollard, 1999; Grinstein et al., 2002; Sengupta et al., 2004; Angelov et al., 2006; Mongelard and Bouvet, 2007]. Nucleolin is found in the nucleolus, in the nucleus, in the cytoplasm, and at the cell surface [De Verdugo et al., 1995; Derenzini et al., 1995].

Nucleolin seems to be over-expressed in highly proliferative cells and is involved in many aspect of gene expression: chromatin remodeling, DNA recombination and replication, RNA transcription by RNA polymerase I and II, rRNA processing, mRNA stabilization, cytokinesis, and apoptosis. Interestingly, nucleolin is also found on the cell surface in a wide range of cancer cells, a property which is being used as a marker for the diagnosis of cancer and for the development of anti-cancer drugs to inhibit proliferation of cancer cells [Hovanessian et al., 2010; Destouches et al., 2011; Guo et al., 2011; Krust et al., 2011; Meng et al., 2011]. In addition to its implication in cancer, nucleolin has been described not only as a marker or as a protein being involved in many diseases like viral infections, autoimmune diseases, Alzheimer's disease pathology but also in drug resistance. Nucleolin is present at low levels in non-dividing cells and is preferentially associated with chromatin [Sirri et al., 1995]. The amount of nucleolin is low in serum-deprived cells; nucleolin expression is induced in mid and late G1, and thus is likely

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to be necessary for cell-cycle progression [Gillet et al., 1993]. In ABAE cells undergoing the G0–G1 transition, b-FGF enters the nucleolus and stimulates nucleolin, which in turn activates the transcription of ribosomal genes [Bouche et al., 1987]. Even in plants, Nucms1 (a plant homologue of nucleolin) expression is induced in the G1 phase on mitogenic stimulation of G0-arrested leaf cells. No expression has been reported in cells that have exited the cell cycle and are undergoing differentiation or polar growth [Bogre et al., 1996]. In addition, retinoic acid and dibutyryl cyclic AMP increase neurite outgrowth during differentiation of human neuroblastoma cells and have an inhibitory effect on cell proliferation. Simultaneously, the expression of N-myc, nucleolin, and hsp70 are down-modulated, indicating a possible association of expression of these three genes [Murakami et al., 1991]. In cancer, the relationship of nucleolin (a major nucleolar Ag-NOR protein) and cell proliferation represents a reliable parameter predicting the tumor growth rate [Roussel and Hernandez-Verdun, 1994; Roussel et al., 1994]. Although nucleolin is localized predominantly in the nucleolus, it has also been shown to be localized in a phosphorylated form on the cell surface of different cells [Bogre et al., 1996]. The significant levels of nucleolin in mature brain and in differentiating neural cells suggest that nucleolin may not only function in signaling by extracellular matrix molecules, but may also be important in differentiation and maintenance of neural tissue [Kibbey et al., 1995]. However, no extensive informations are so far available for the presence of nucleolin in human gliomas. Gliomas are the most common primary brain tumors. In terms of symptoms and signs, seizures are a common presenting manifestation of the tumors, although subtle abnormalities, such as speech difficulties, changes in sensation, vision, or some motor change, might have been present earlier but gone unnoticed [Kleihues, 2000]. The clinical history of GBM is usually short (<3 months in more than 50% of cases), unless the neoplasm has developed from diffuse astrocytoma or anaplastic astrocytoma [Kleihues, 2000]. Gliomas are divided into four grades, each with different biological behavior. Furthermore, because different gliomas share a predominant histological appearance, the final classification includes both histological features and degree of malignancy. Obviously, the prognosis and biological behavior of malignant gliomas are closely related to the different molecular backgrounds of each type of glioma. Furthermore, the ability of several low-grade gliomas to progress into more aggressive tumors has allowed cancer researchers to elucidate several pathways implicated in the molecular biology of these devastating tumors. In the present work we investigated on the presence and localization of nucleolin in glioma patients at different degree of malignancy trying to correlate the presence of glycosilated membrane nucleolin with the malignancy grade. To this purpose an antibody produced by us against gp273 protein, demonstrated to recognized the glycosilated surface nucleolin [Aldi et al., 2009] has been used both on glioma specimens at different grade of malignancy and on primary glioma cell cultures derived by surgical resection. The results obtained demonstrate that surface nucleolin increase with the malignancy grade thus suggesting that it may constitute a histopathological marker for glioma grading and a possible tool for targeted therapy.

MATERIALS AND METHODS

MATERIALS

Triton X-100, dimethylsulfoxide (DMSO), sodium dodecylsulfate (SDS), Tween-20, bovine serum albumine (BSA), HOECHST, Nonidet P40, sodium deoxycolate, ethylen diamine tetraacetate (EDTA), phenylmethanesulphonylfluoride (PMSF), sodium fluoride, sodium pyrophosphate, ortovanadate, leupeptin, aprotinin, pepstatin, NaCl, polyvinylidene difluoride (PVDF) sheets, Harris' H&E, fluorescein-labeled anti-rabbit and anti-mouse IgG antibodies, were all purchased from Sigma Chemical Co (St. Louis, CO). Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS) were all purchased from Sigma Chemical Co. The penicillin/streptomycin solution, trypsin/EDTA solution, and L-glutammine were purchased Euroclone. ProteoExtract Subcellular Proteome Extraction Kit and ProteoExtract Tissue Dissociation Buffer Kit were purchased from Calbiochem. Mouse monoclonal anti-glial fibrillar acidic protein (GFAP) antibody was purchased from Chemicon (Temecula, CA) Alexafluor 488-conjugated goat anti-rabbit IgG and Alexafluor 546-conjugated goat anti-mouse IgG antibodies were purchased from Molecular Probes, Invitrogen (CA), Vectashield mounting medium was purchased from Vector Laboratories Inc. (Burlingame, CA) Micro-BCA kit was purchased Pierce Biotechnology (Rockford, IL). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody were from S. Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) Bio-Rad Laboratories (Hercules, CA). All other chemicals were of the highest analytical grade.

PATIENT POPULATION

The study was approved by the Hospital Ethics Committee, and all patients signed an informed consent before participating in the study. Between April 2006 and December 2010 98 patients underwent surgical resection for a newly diagnosed primary supratentorial brain glioma at the Department of Neurosurgery, S. Salvatore Hospital, L'Aquila, Italy.

Of these patients, 65 were diagnosed according to the World Health Organization (WHO) Grade IV astrocytoma, 23 with WHO Grade III astrocytoma, 10 with Grade II astrocytoma. Age ranged from 45 to 75 years, with a mean age of 57 years. All patients underwent a complete clinical and neurological evaluation at the admission in order to evaluate clinical conditions and Karnofsky Performance Status. Before the surgical procedure a complete neuro-radiological study, including CT scan without contrast enhancement (c.e.), MRI with and without gadolinium, Technetium 99 MIBI brain SPECT, was performed in all patients; in cases of tumors seated near motor and/or speech areas functional MRI (f-MRI) was performed. For this study we selected patients whose lesions were suitable for gross total removal. The surgical technique used foresees tumor aggression from the borders avoiding initial lesion debulking. Surgical removal starts from the edematous brain surrounding the tumor. When possible the resection includes apparently normal brain tissue and in some selected cases as in case of tumors involving brain lobes, especially in non-dominant sites, lobotomy was performed. In all case surgical intervention with the aid of Image Guided Surgery was performed and in selected cases of

suspected grade IV astrocytoma 5-aminolevulinic acid, before surgical procedures, was orally administered in order to control the extent of tumor resection by photo dynamic diagnosis (PDD), as described by Stummer et al. [1998]. Complete post-operative neuro-radiological investigation was performed in all patients including CT scan with and without c.e. within 48 h from surgery and MRI with and without gadolinium performed immediately before and at the end of radiotherapy and following each 3 months, in association with Technetium 99 MIBI brain SPECT.

TISSUE SPECIMENS

For this study we used tissue samples from surgical resection corresponding to a well defined pathological portion, preferably a portion corresponding to the main contrast enhanced part on preoperative MRI, assessed intra-operatively with the use of Image Guided Surgery, and confirmed by intra-operative histological examination.

CELL CULTURES

Human astrocytes were seeded at density of 5,000 cells/cm^2 in poly-L-lysine coated flask with astrocyte complete medium (AM). The culture medium was changed every 3 days until 90% of confluence. The culture medium was changed every 2 days. Cells were detached by using trypsin/EDTA solution, enzymatic digestion was neutralized added complete medium.

Glioma patients who underwent a resection of their tumor in the neurosurgery department were included in the study. Individual tumor biopsies excluding necrotic fragments were maintained in culture medium and addressed to our laboratory. The fragment was rinsed with Hank's balanced salt solution (HBSS), the necrotic areas and red endothelial parts were moved aside. The remaining fragments were finely minced into 0.5 mm^3 section pieces approximately. The suspension was centrifuged twice at 1,000*g* for 5 min in HBSS and the supernatant discarded. The enzymatic digestion was carried out by incubating the pellet with 4 ml 0.125% trypsin and 0.125% EDTA at 37°C for 10 min. Growth culture medium containing 20% FBS was added to stop the enzymatic digestion. After centrifugation (5 min, 1,000*g*), the fragments were in culture growth medium, and then were transferred to 75 cm^2 flask precoated with 2 $\mu g/cm^2$ poly-L-lysine and maintained at 37°C in 5% CO_2, 95% air atmosphere. The cells were cultured in DMEM supplemented with 20% FBS, 2 mM L-glutammine, 50 UI/ml penicillin–streptomycin until confluence. The culture medium was changed every 2 days. The subculture was assessed by using trypsin (0.05%)/EDTA (0.02%), for 10 min at 37°C. Enzymatic digestion was neutralized by adding an equal volume of culture medium and cells were resuspended and transferred to new flask. The subculture cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutammine, 50 UI/ml penicillin–streptomycin until confluence. The culture medium was changed every 2 days.

GBM stem cells were prepared starting from U87 Glioblastoma cell line. Human GBM cell line U87 (ATCC, Manassas, VA) was cultured, according to Yu et al. [2008], with minor modification. Briefly, U87 cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 0.1 mg/ml penicillin, and 0.1 mg/ml streptomicyn (Sigma) and incubated at 37°C in humidified 95% air–5% CO_2 atmosphere. Thereafter, U87 the culture medium was replaced with serum-free neural stem cell medium, DMEM-F12 (Sigma), containing 20 ng/ml of both recombinant human epidermal growth factor (EGF) and basic fibroblast growth factor (b-FGF) (PeproTech, Hamburg, Germany) until several primary tumorspheres were visible under microscopy.

After several passages tumorspheres were dissociated and single cells were seeded at 1×10^4/well. The secondary spheres derived from single cells of primary tumor spheres were analyzed under phase contrast microscopy Leica DMIL.

PROTEIN EXTRACTION

Protein extraction was conducted by ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem). The Calbiochem Kit is used for the differential extraction of protein from mammalian cells according to their subcellular localization. It takes advantage of differential solubility of certain subcellular compartments in specific reagent mixtures (buffer). The extraction procedure provide four fraction: cytosolic proteins (fraction 1), membranes and membrane organelles proteins (fraction 2), nuclear proteins (fraction 3), and cytoskeletal proteins. The Calbiochem ProteoExtract Tissue Dissociation Buffer Kit was used to isolate viable cells from tissue. Tissue was first minced into small pieces, washed with PBS, and incubated with tissue dissociation buffer and collagenase. Following incubation the tissue was strained using a tissue sieve. Isolated cells proteins were extracted by ProteExtract Subcellular Proteome Estraction Kit.

PROTEIN ASSAY

Proteins were assayed by the micro-BCA kit. Briefly, this assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. The method combines the reduction of Cu^{+2} to Cu^{+1} by protein in alkaline medium (the biuret reaction) with the high sensitive and selective colorimetric detection of the cuprous cation using a reagent containing BCA. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This complex exhibits a strong absorbance at 562 nm.

WESTERN BLOTTING

For Western blotting, 20–50 μg of proteins were electrophoresed through a 7.5–15% SDS–polyacrilamide gel under reducing conditions [Laemmli, 1970]. Proteins were transferred onto PVDF membrane sheets and non-specific binding sites were blocked for 1 h at room temperature (RT) in 20 mM TRIS–HCl buffer, 55 mM NaCl, and 0.1% Tween-20, pH 7.4 (TST), containing 5% non-fat dry milk (blocking solution). Membranes were then incubated overnight at 4°C with anti-gp273 antibody (1:1,000) dissolved in blocking solution). After extensive washings with TST, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG secondary antibody (1:2,000). Immunoreactive bands were visualized by ECL, according to the manufacturer's instructions. Band relative densities were determined using TotalLab software (ABEL Science-Ware srl, Italy) and values were given as relative units (RU). Immunoblot data were normalized to

total protein load by quantification of all samples in a single assay before loading and confirmation of equal loading by image analysis and scanned Coomassie blue-stained gels after blotting.

IMMUNOFLUORESCENCE

Surgical specimens were fixed in 10% buffered formalin, routinely processed and paraffin-embedded to obtain 4-μm thick sections. The pathological diagnosis of tumor was based on criteria from the histological classification of the WHO. Sections from healthy and glioma brains were deparaffinized by using xylene and graded ethanol, and rehydrated. Slides were then immersed in 10 mM sodium citrate buffer, pH 6.1, and processed for the antigen-retrieval procedure, using a microwave oven operated at 720 W for 10 min. After cooling, slides were transferred to phosphate-buffered saline (PBS) containing 4% (w/v) BSA and 0.05% Tween-20, for 2 h at RT, then incubated overnight at 4°C with 1:200 rabbit polyclonal anti-gp273 antibody, diluted in PBS containing 4% (w/v) BSA. Sections were thoroughly rinsed with PBS and incubated for 2 h at RT with Alexafluor 488-conjugated goat anti-rabbit IgG, diluted 1:2,000 with PBS containing 4% BSA. For double immunofluorescence experiments sections were incubated, overnight at 4°, with a mixture of 1:200 rabbit polyclonal anti-gp273 with 1:200 mouse monoclonal GFAP antibodies. Sections were thoroughly rinsed with PBS and incubated for 2 h at RT with a mixture of Alexafluor 488-conjugated goat anti-rabbit IgG and Alexafluor 546-conjugated goat anti-mouse IgG antibodies (1:2,000). Both primary and secondary antibodies were diluted with PBS containing 4% BSA. Controls were performed in parallel omitting the primary antibody. Slides were finally mounted in Vectashield mounting medium and observed and photographed under an AXIOPHOT, Zeiss microscope.

Normal human astrocytes and glioma primary cells at different grades of malignancy as well as GBM stem cells, grown on coverslips were fixed with 4% paraformaldehyde in PBS for 10 min RT and permeabilized with 0.1%Triton X-100 in PBS for 5 min at RT. Non-specific binding sites were blocked with 3% BSA in PBS, for 10 min at RT. For single immunofluorescence staining, cells were treated with 1:200 rabbit polyclonal anti-gp273 primary antibody and then incubated for 30 min at RT with 1:2,000 Alexafluor 488-conjugated goat anti-rabbit IgG secondary antibody. Both primary and secondary antibodies were diluted with PBS containing 3% BSA. For double immunofluorescence, GBM stem cells were incubated, overnight at 4°C, with a mixture of 1: (rabbit anti-SOX2) to 1 (mouse anti-CD133) (1:500, Abcam, Cambridge Science Park, Cambridge, UK). Slides were then incubated for 2 h at RT with a mixture of Alexafluor 488-conjugated goat anti-rabbit IgG and Alexafluor 546-conjugated goat anti-mouse IgG antibodies (1:2,000). Controls

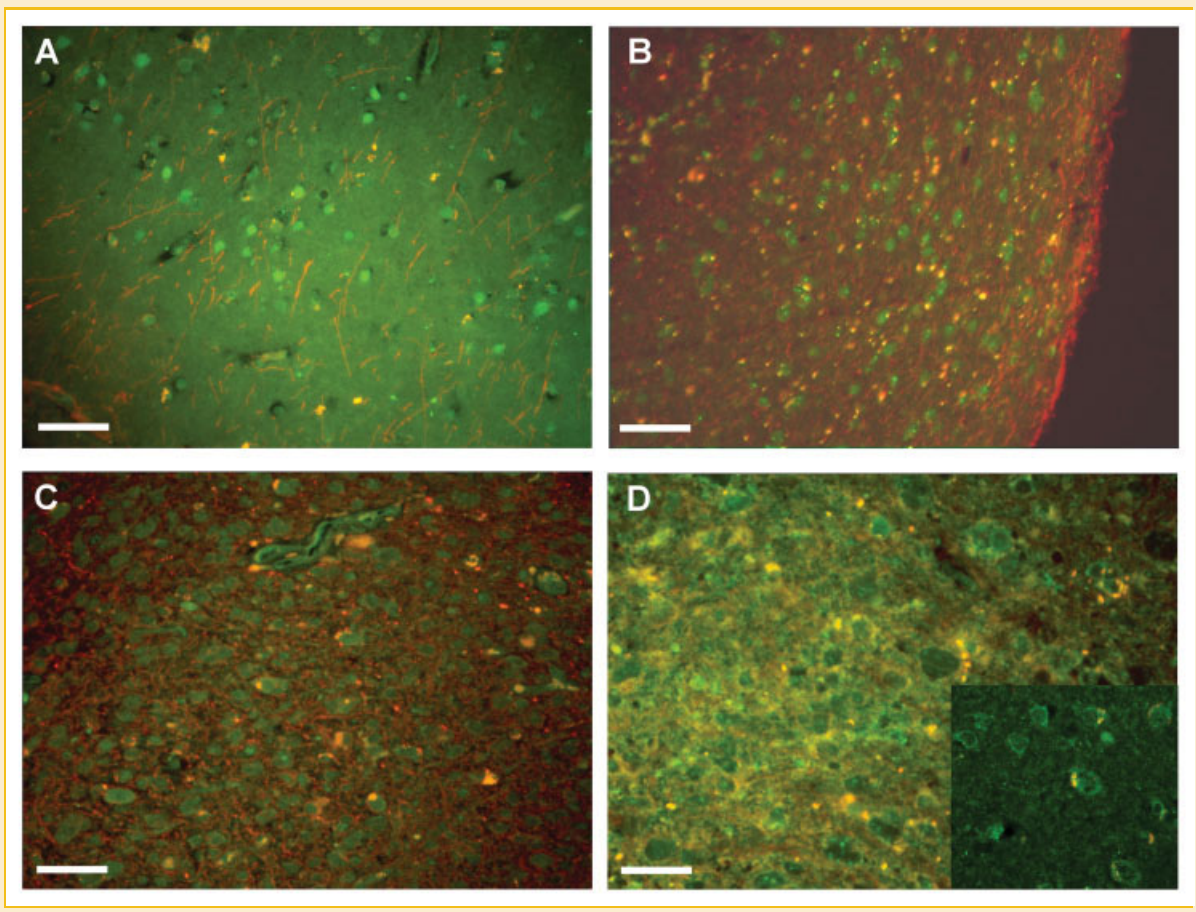

Fig. 1. Double immunostaining GFAP/nucleolin in healthy (A), II grade glioma (B), III grade glioma (C), and IV grade glioma (D) sections. Bar = 60 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

were performed in parallel omitting the primary antibody. Coverslips were finally mounted in Vectashield mounting medium and observed and photographed under confocal Laser scanning microscope (Sarastro 2000).

STATISTICAL ANALYSIS

Statistical significance of paired samples was analyzed by Student's *t*-test. $^{*}P < 0.01$; $^{**}P < 0.005$. Samples were processed by SPSS software and analyzed by ANOVA test, followed by Scheffe's post hoc test analysis. $^{*}P < 0.01$; $^{**}P < 0.005$. All data are mean ± SD of three separate experiments.

RESULTS

Figure 1 shows the double immunostaining GFAP/nucleolin in healthy (A), II grade glioma (B), III grade glioma (C), and IV grade glioma (D) sections. It is possible to observe a clear nuclear localization of the protein in healthy brain section; in II grade glioma section an increase of the fluorescence intensity is observed both for GFAP and nucleolin, this latter still localized to the nuclei. In III grade glioma section an increase of the fluorescence intensity is still apparent, but also a nuclear/cytoplasmic/membrane localization of nucleolin is observed. Finally in IV grade glioma a marked increase of the fluorescence intensity is evident as well as a pronounced membrane localization in several cells within the section. The endothelium is always strongly positive.

In Figure 2 the Western blotting analysis for gp273 in the different cellular compartments, nuclear, cytoplasm, and membrane, is reported. As shown by the densitometric analysis, membrane and cytoplasmic nucleolins increase with the malignancy grade with IV grade glioma expressing the higher membrane and cytoplasmic content. Moreover, in this analysis also a IV grade recurrency has been analyzed, showing that also in recurrences membrane and cytoplasmic nucleolins are significantly higher with respect to normal brain tissue.

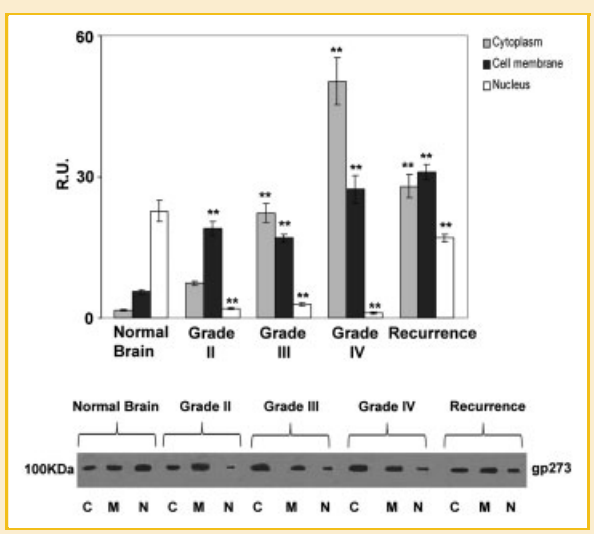

Fig. 2. Western blotting analysis for gp273 in the different cellular compartments, nuclear, cytoplasm, and membrane, in glioma specimens at different grade of malignancy. Also an example of GBM recurrency it is also shown. Data are mean of five experiments ± SD. $^{**}P < 0.005$.

Figure 3 shows gp273 immunolocalization in normal human astrocytes and in glioma primary cells at different grades of malignancy. In normal astrocytes (A) nucleolin is mainly present in the nuclear/cytoplasmic compartments. In glioma cells, an evident membrane localization of gp273 is observed with the increase of the malignancy grade (B–D), with glioblastoma cells (D) showing a strong membrane/cytoplasmic localization, thus indicating that with the increase of malignancy grade nucleolin seems increased and gradually localized to the membranes.

Figure 4 shows the Western blotting analysis of gp273 for cell cultures, in the different cellular compartments, nucleus, cytoplasm, and membrane. The densitometric analysis shows that in normal AS nucleolin is mainly present in the nuclear compartment and to a lesser extent in the others, with the increase of malignancy nucleolin starts to be much concentrated in the cytoplasmic/membrane compartments than in the nucleus. In GBM cells, nucleolin is overall increased and mainly localized to the membrane.

Finally, since it is known that in glioblastoma tumor stem cells are also present and that they may be responsible for recurrences appearance, in preliminary experiments, GBM stem cells were isolated, characterized by contrast phase microscopy (A) stem cell markers presence, such as SOX2 and CD133 (B), and probed against gp273 (C,D). Also in this cellular model, we observe a clear cytoplasmatic/membrane localization of the protein, thus confirming the proliferative potential of GBM stem cells and suggesting gp273 as a possible marker for GBM stem cells (Fig. 5).

DISCUSSION

Glioblastomas are the commonest and the most malignant of all adult brain tumors, characterized by genetic instability, intratumoral histopathological variability, and unpredictable clinical behavior. The utility of tumor markers that reflect their underlying biology is becoming increasingly important with respect to patient prognosis and their potential role as molecular targets for therapy is being recognized. In this study, we aimed to identify the presence and localization of glycosilated nucleolin and to propose it as immunohistochemical malignancy marker.

Nucleolin is abundant in proliferating cancerous cells, and high levels of nucleolin expression are related to poor clinical prognosis for certain types of cancer [Jordon et al., 1994; Zhou et al., 1997]. In B-cell lymphomas, nucleolin contributes to activation of the c-myc gene and the Epstein–Barr virus nuclear antigen 1 gene [Semenkovich et al., 1990; Tuteja et al., 1995; Hanakahi et al., 1997], which are known to promote induction of these malignancies [Arends et al., 1990]. Nucleolin is also directly involved in post-transcriptional inhibition of the p53 gene. The synthesis of nucleolin is positively correlated with increased rates of cell division. It is not surprising, therefore, that nucleolin levels are highest in tumors or other rapidly dividing cells [Derenzini et al., 1995]. Indeed, nucleolin is used in studies of different cancer cell lines as a useful marker for cell proliferation [Roussel and Hernandez-Verdun, 1994; Roussel et al.,

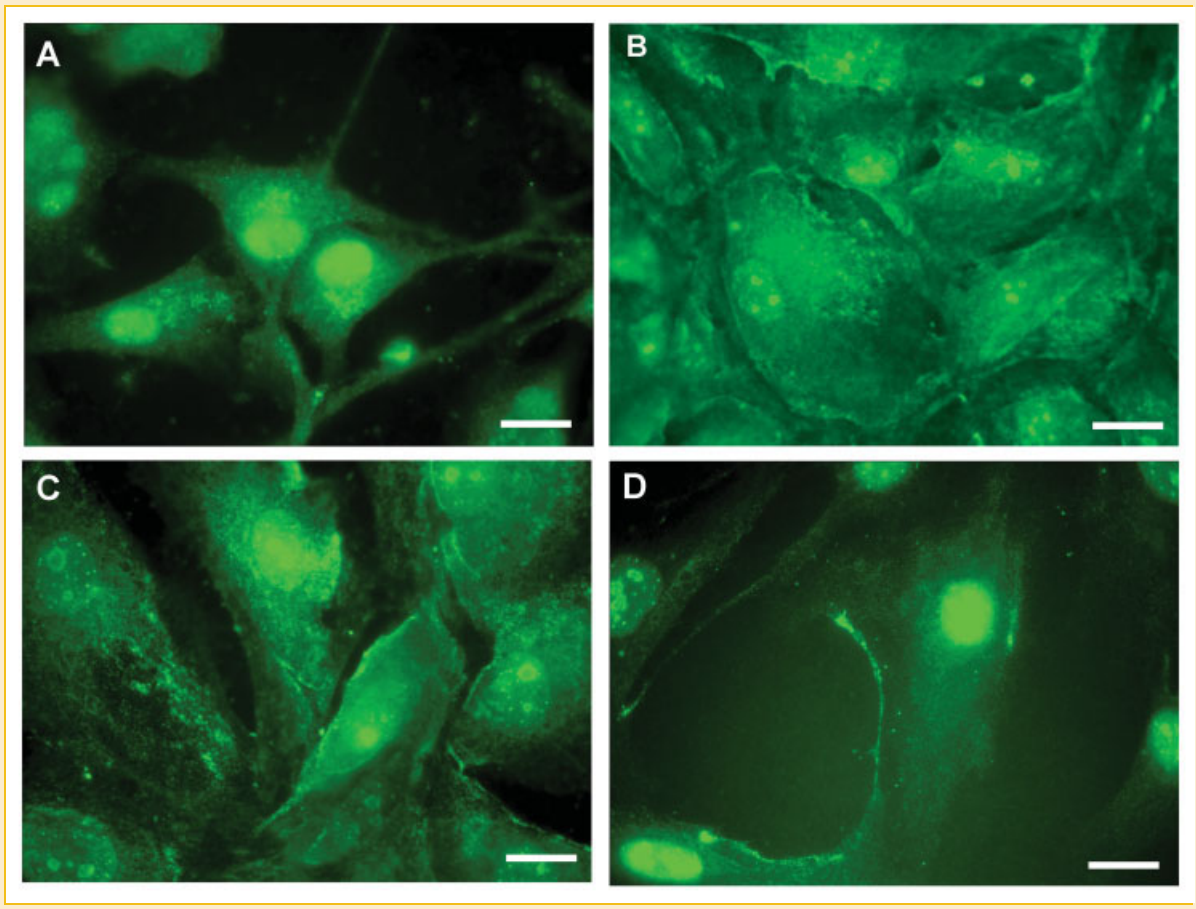

Fig. 3. gp273 immunolocalization in normal human astrocytes and in glioma primary cells at different grades of malignancy. Bar = 18 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

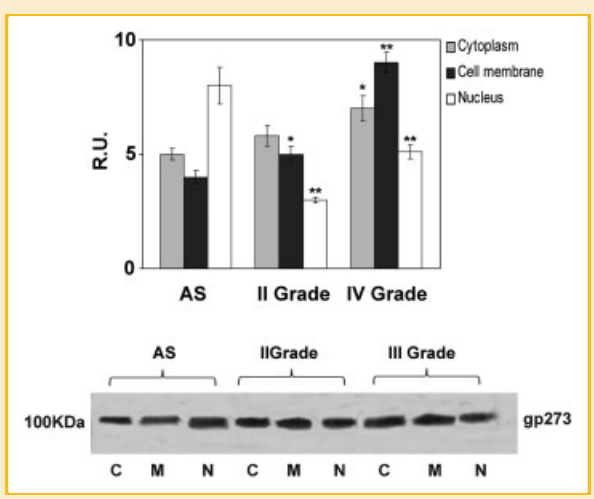

Fig. 4. Western blotting analysis of gp273 in the different cellular compartments, nucleus, cytoplasm, and membrane in normal human astrocytes, II grade and IV glioma primary cells. Data are mean of five experiments ± SD. $^{**}P < 0.005$.

1994; De Verdugo et al., 1995; Sirri et al., 1995]. In cancer, the relationship of nucleolin (a major nucleolar Ag-NOR protein) and cell proliferation represents a reliable parameter predicting the tumor growth rate. Although nucleolin is localized predominantly in the nucleolus, it has also been shown to be localized in a phosphorylated or glycosylated form on the cell surface of different cells [Pfeiffe and Anderer, 1983]. Nucleolin is highly expressed by exponentially growing eukaryotic cells and was primarily found on the nucleus [Lapeyre et al., 1987]. Recent studies have indicated that cell surface forms also exist [Said et al., 2002; Sinclair and O'Brien, 2002]. In contrast to nuclear nucleolin, surface nucleolin is glycosylated and is constantly induced in proliferating tumor and endothelial cells [Krust et al., 2011]. Plasma membrane nucleolin has been reported to act as a binding molecule for agents involved in proliferation such as the tumor-homing peptide F3 in endothelial cells of angiogenic blood vessels [Christian et al., 2003]. The protein has therefore been indicated as a shuttle molecule between the cell surface and nucleus and indeed as a mediator for the extracellular regulation of nuclear activity [Srivastava and Pollard, 1999]. Cell surface expression of nucleolin has been further substantiated by other investigators. Nucleolin is the protein that specifically binds apoB and apoE-containing lipoprotein to the surface of the HepG2 cells [Semenkovich et al., 1990]. The neurite-promoting IKVAV site

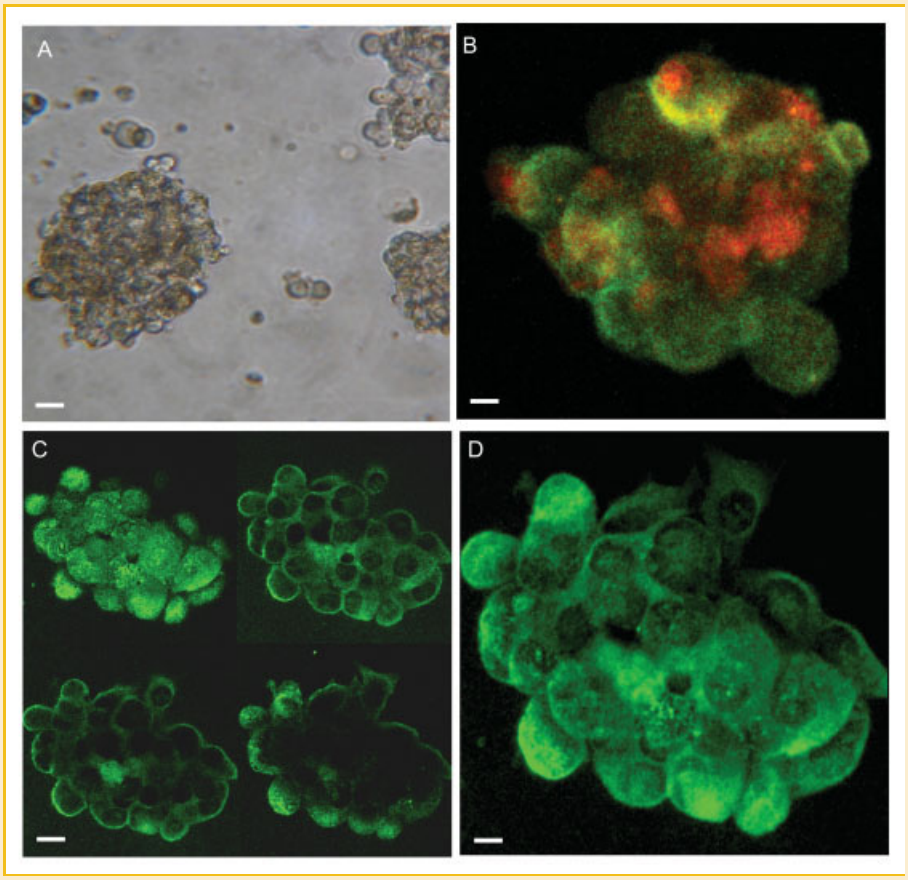

Fig. 5. Confocal laser scanning microscopic analysis of gp273 immunolocalization in GBM stem cells: (A) contrast phase microscopy of GBM neurospheres, bar = 20 μm; (B) SOX2/CD133 double immunofluorescence in GBM cells is shown, bar = 10 μm; (C) laser sections of a neurosphere probed with anti-gp273, bar = 20 μm; (D) sections reconstruction, bar = 10 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

of laminin-1 (basement membrane protein) binds to nucleolin on the cell surface and has been found to promote the differentiation of primary neurons and a variety of neural cell lines [Kleinman et al., 1991]. The significant levels of nucleolin in mature brain and in differentiating neural cells suggest that nucleolin may not only function in signaling by extracellular matrix molecules, but may also be important in differentiation and maintenance of neural tissue [Kibbey et al., 1995].

We have previously demonstrated the existence and localization of fucosyl-containing *O*-glycoforms of nucleolin in cultured bovine endothelial cells (CVEC) and malignant cultured human A431 cells [Aldi et al., 2009]. The tool for this discovery was an antibody raised against gp273, a glycoprotein ligand for the sperm–egg interaction in the mollusc bivalve *Unio elongatulus*. The function and immunological properties of gp273 mainly depend on clustered Lewis-like, fucose-containing *O*-glycans. We found that anti-gp273 strongly and exclusively interacted with a 110-kDa protein in CVEC and A431 tumor cells. After partial purification, mass spectrometry identified the protein as nucleolin. This was confirmed by comparing anti-gp273 and anti-nucleolin antibody immunoblotting after nucleolin depletion. We further confirmed that anti-gp273 binding to nuclear and extranuclear nucleolin was against a fucose-containing *O*-glycoepitope by immunoblot analysis of the protein after chemically removing *O*-glycans and by lectin-blot analysis of control and nucleolin-depleted samples. Using anti-gp273 IgG, we detected nucleolin on the plasma membrane and cytoplasm [Palumberi et al., 2010].

Numerous articles dealing with surface nucleolin targeting for tumor therapy have been recently published [Hovanessian et al., 2010; Destouches et al., 2011; Guo et al., 2011; Krust et al., 2011; Meng et al., 2011], in this work the results here obtained by utilizing the same antibody against gp273 to characterize human gliomas, both on ex vivo specimens and on primary cell cultures, clearly demonstrated that membrane nucleolin is increased with the malignancy grade and proliferation rate also in human gliomas. In fact, with the increase of malignancy the higher cellularity observed in patients sections is accompanied by the increase of nucleolin present at cytoplasmatic and membrane levels, accordingly also in primary cell cultures, the presence of cytoplasmatic/nuclear nucleolin is increased as function of the malignancy grade. It is worthnoting that nucleolin is mainly present at cytoplasmatic/membrane level also in GBM stem cells, thus suggesting its possible use as glioma stem cells surface marker.

On the overall our data strongly suggest the use of an antibody against the glycolsylated form of nucleolin as specific tool for glioma histopathological grading. Moreover, once produced as

monoclonal antibody, anti-gp273, since its low expression in healthy cells, it may be used for the formulation of targeted therapies.

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