Nucleostemin Expression in Squamous Cell Carcinoma of the Head and Neck

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Abstract. *Background: This study presents initial data on presence of nucleostemin – a nucleolar protein typical of stem cells in the normal squamous epithelium of the oropharynx and larynx – in squamous cell carcinoma originating from these epithelia. Materials and Methods: Differentiation and proliferation markers such as keratins, β-catenin, galectin-1, and Ki-67 were studied in parallel with nucleostemin for defining cell characteristics. Results: Nucleostemin was detected in nucleoli of both proliferating basal cells and terminally differentiated suprabasal cells of normal epithelium and in tumor cells. Importantly, malignant transformation was connected with a significant enlargement of nucleostemin-positive nucleoli in these cell types. Conclusion: Detection of nucleostemin in head and neck cancer cells, together with the size of nucleoli, may be important in the evaluation of tumor differentiation and biology.*

Head and neck cancers represent about 6% of malignant tumor cases worldwide; at least 90% of these tumors are squamous cell carcinomas. Despite rapid progress in diagnosis and therapy, the overall 5-year survival rate for this malignancy is among the lowest of the major cancer types (1). This unfavorable situation calls for research activities to aim at finding new markers to better characterize the biological behavior of tumors in order to serve as a rational guideline to improve therapeutic modalities (2-5). Respective candidates may originate from applying the stem cell concept to this tumor class.

Adult tissue stem cells have several similarities with cancer cells, and the idea of stem cells as a source of solid cancer was put forward recently (6, 7). As a consequence, potential roles of epidermal stem cells in cancer, especially in squamous cell carcinoma, have been proposed (8). Fitting this concept, characteristics of the epidermal stem cell phenotype could be detected in *in vitro* propagated cells from cancer lines of squamous cell epithelial origin (9, 10). Moreover, cells of a very low differentiation level, akin to epidermal stem cells, have been observed on the periphery of tumor lesions in the so-called "aggressive front" of carcinomas. Tumors abundantly populated by these cells exhibit a highly anaplastic aggressive phenotype (11). At present, no single specific marker of adult tissue stem cells (including stem cells of squamous epithelia) has yet been discovered. These cells are currently identified by the detection of a combination of markers. In this situation, the systematic study of individual proteins will help characterize the phenotype of these cells thoroughly. This rationale prompts the study of nucleostemin, a nuclear/nucleolar protein present in neural and bone marrow stem cells and their related malignancies (12, 13). Nucleostemin, of note, participates in the control of proliferation in these cells and also in early embryonic development (14) and tissue regeneration (15), explaining why monitoring of its presence in cancer is warranted. In the human epidermis, this protein is not exclusively expressed by cells of the stem cell pool, and even nucleoli of terminally differentiated suprabasal cells reveal the presence of nucleostemin (16). However, nucleostemin expression is up-regulated in follicular bulge epidermal stem cells when measured by microarray

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technology at the mRNA level (17); *in vitro* only those cells co-cultured with non-tumor feeder cells contain nucleoli positive for nucleostemin expression (16).

This study demonstrates the expression of nucleostemin in nucleoli of cells of normal squamous cell epithelium (namely of the larynx and oropharynx) and in squamous cell carcinomas originating from these epithelia. The scope of these results was extended to nucleostemin presence in FaDu cells, a model line of human squamous cell carcinoma from the hypopharynx, *in vitro* and *in vivo* after tumor development in *nu/nu* mice. To relate nucleostemin presence to other cellular characteristics, the presence of the following well-established markers was determined: Ki-67, β-catenin and keratin 10. Proliferating cells were detected by the nuclear expression of Ki-67 (18). β-Catenin is usually a membrane-associated protein in the majority of cells of the squamous cell epithelium; its shift to the cytoplasm and nucleus is related to tumor progression (19). Keratin 10 expression is associated with terminal differentiation in cells of squamous epithelia under physiological conditions and in cancer (11, 20). In addition, the presence of a key member of the adhesion/growth-regulatory galectins, galectin-1 was determined. These endogenous lectins can interact with distinct glycan epitopes and proteins at different sites of the cell to trigger efficient signaling leading to diverse cell responses (21-23). In this context it is noteworthy that nuclear presence of galectin-1 has been observed in cells of the bulge region of the hair follicle which are phenotypically similar to epidermal stem cells (24).

Materials and Methods

Clinical material. Five specimens of laryngeal squamous cell carcinoma, three specimens of squamous cell carcinoma of the tongue and four specimens of oropharyngeal squamous cell carcinoma at stage T3 and without previous therapy as well as five control samples of normal laryngeal mucosa and three control samples of oropharyngeal mucosa (control samples were obtained from tumor-free organs as verified by histology) were taken. All samples were donated with the informed consent of the patients. The tissue donors had not undergone previous cytostatic (chemo)therapy. The samples were frozen in liquid nitrogen using Tissue-Tek (Christine Gröpl, Tulln, Austria) as a cryoprotective medium and stored at –85˚C until further processing.

Tissue culture and animal experiments. The human hypopharyngeal squamous cell carcinoma line FaDu (HTB-43) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, antibiotics (100 units/mL of penicillin, 100 μg/mL of streptomycin; Sigma, St. Louis, MO, USA), 1.5 g/L $NaHCO_3$, 0.11 g/L sodium pyruvate, 0.292 g/L *L*-glutamine, and 10 mM *N*-(2-hydroxyethyl) piperazine-*N*'-(2-ethanesulfonic acid) (HEPES). FaDu cells were also cultured on coverslips as described previously (10, 16). The cells were cultured under standard conditions, *i.e.* under 5% CO_2 tension at 37˚C. Three independent experimental series were immunohistochemically evaluated. For xenotransplantation, two female *nu/nu* CD-1 mice, aged 8-12 weeks, were purchased from the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic. The mice were housed in accordance with approved guidelines and provided food and water *ad libitum*. A total of $1x10^6$ FaDu cells from tissue culture were resuspended in 100 μl of phosphate buffered saline (PBS) and mixed with 50 μl of BD Matrigel™ (BD Biosciences, Erembodegen, Belgium) according to supplier instructions. The resulted suspension was then subcutaneously injected into each *nu/nu* CD-1 female mice.

The animals were sacrificed after 49 days and the specimens were frozen as described above.

Immunohistochemistry. Frozen sections, 7 μm each, were prepared using Cryocut E (Reichert-Jung, Vienna, Austria). The tumor sections and the FaDu cells grown on coverslips were washed with PBS, briefly fixed with 4% paraformaldehyde in PBS (pH 7.3) at room temperature, and then washed once with PBS. Diluted porcine serum (1%) (DAKO, Brno, Czech Republic) was used as a blocking solution to prevent the nonspecific binding of first and second step antibodies. Nucleostemin was detected by goat polyclonal antibody (Neuromics, Bloomington, MN, USA). Ki-67, a panel of keratins, and keratin 10 were visualized by commercial mouse monoclonal antibodies (DAKO) and β-catenin by a rabbit polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Rabbit polyclonal antibody against galectin-1 (non-crossreactive with other galectins) was employed to visualize this antigen (25, 26). TRITC-labeled donkey anti-goat (Jackson Laboratories, West Grove, PA, USA) along with FITC-labeled swine anti-mouse (SwAM-FITC; AlSeVa, Prague, Czech Republic) and FITC-labeled swine anti-rabbit (SwAR-FITC, AlSeVa) were used as second-step reagents. All commercial antibodies were diluted according to supplier recommendations. Five sections from the each tumor samples were employed for the each antibody combinations. Sections and cultured cells were stained at room temperature for 60 minutes. Specificity controls were performed by omitting the first-step antibody or by replacing it with monoclonal/polyclonal antibodies against thyroglobulin (not expressed in the studied tissues; DAKO) to exclude any interaction of an antibody with sections of the studied tissues *via* Fc receptor. The nuclei were then counterstained with DAPI (4',6'-diamidino-2-phenylindole dilactate) (Sigma-Aldrich, Prague, Czech Republic). The specimens were mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA) to prevent the UV bleaching of fluorochromes. A Nikon Eclipse-90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with filter blocks specific for DAPI, FITC and TRITC, a cooled CCD Vosskühler Cool-1300Q camera (Vosskühler, Osnabrick, Germany) and a computer-assisted image analyzer LUCIA 5.1 (Laboratory Imaging, Prague, Czech Republic) were used for imaging. The image analyzer was also used for measuring the size of nucleolar area positive for nucleostemin. A total of 300-500 cells were analyzed in each specimen. The results were statistically processed using Student's unpaired *t*-test.

Results

Normal oropharyngeal and laryngeal epithelium exhibited nucleostemin-positive nucleoli in both basal and suprabasal cells (Figure 1A). This observation is in accordance with a

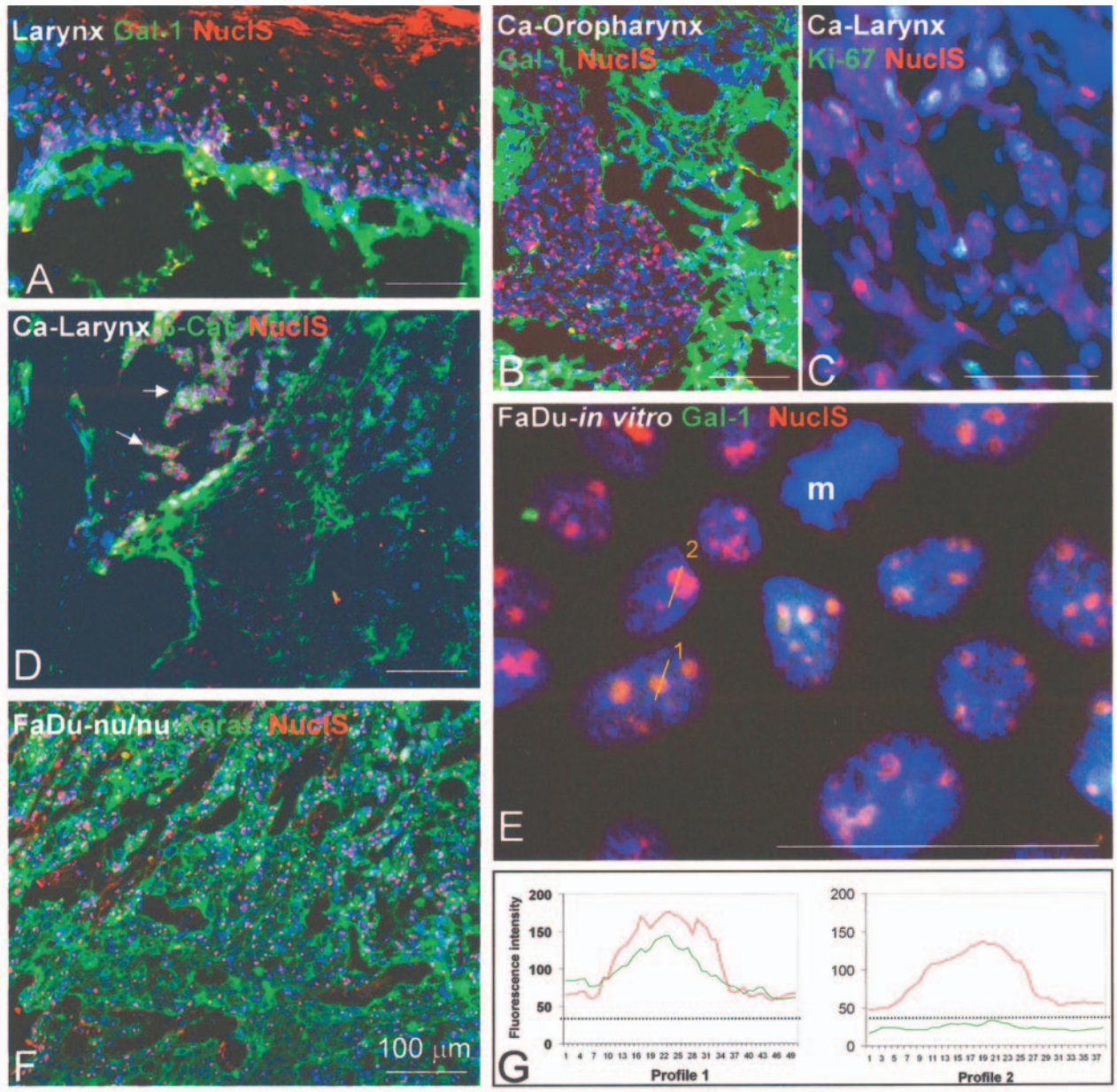

Figure 1. *Detection of nucleostemin (NuclS, red) (A-E), galectin-1 (Gal-1, green) (A, B, E), Ki-67 (green) (C), β-catenin (β-cat, green) (D) and of a panel of keratins (Kerat, green) (F) in normal laryngeal epithelium (A), squamous cell carcinoma of the oropharynx (B), squamous cell carcinoma of the larynx (C, D),cultured FaDu cells (E) and in FaDu cells grown in nu/nu mice (F). All nuclei are counterstained with DAPI. Arrows indicate cells with cytoplasmic/nuclear expression of β-catenin. Mitotic cells are marked by "m". Fluorescence intensity profiles were measured for FaDu cell nucleoli marked 1 and 2 (G).*

previous study of normal epidermis, hereby serving as internal quality control (16). The size of nucleostemin-positive nucleoli was identical in both compartments (Figure 2A). In order to support this notion the presence of Ki-67 was measured and found to be restricted to cells of the basal layer in samples of normal epithelium (not shown). The nuclei of cells from squamous cell carcinomas contained nucleoli which gave a strong nucleostemin signal (Figure 1B-D). Similar findings were also obtained in cultured FaDu cells (Figure 1E) and in tumors from FaDu cells grafted into mice (Figure 1F). In addition to the signal intensity, the size of nucleostemin-positive nucleoli was significantly larger in cells of squamous cell carcinomas than in cells of normal epithelia (Figure 2A). This property was also detectable in FaDu cells grown both *in vivo* and *in vitro*, although it was not statistically verified (Figure 2A). Analyzing the distribution

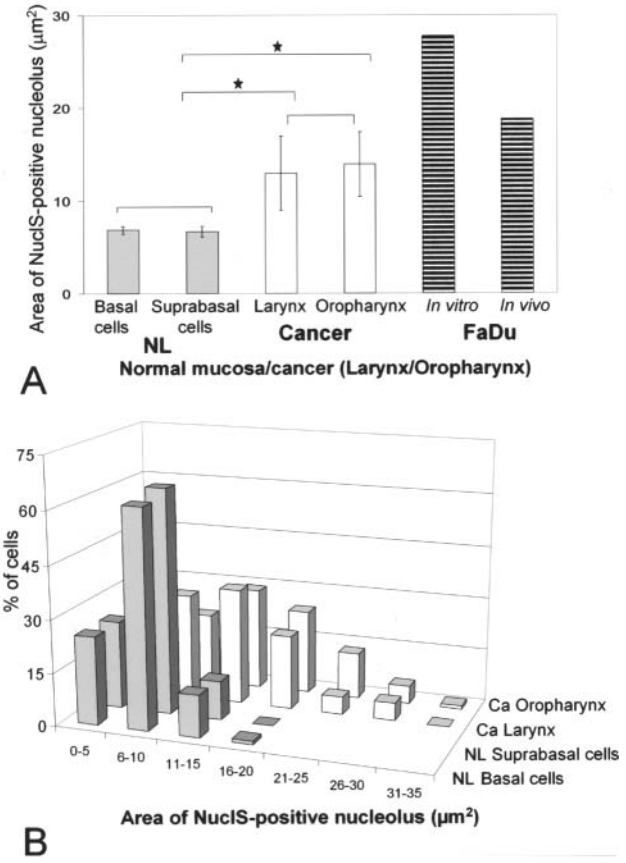

Figure 2. *A) Size of nucleostemin-positive nucleoli in basal layer cells (NL Basal cells) of normal laryngeal epithelium, in suprabasal layer cells (NL Suprabasal cells) of normal laryngeal epithelium, in laryngeal (Ca Larynx) and in oropharyngeal (Ca Oropharynx) cancer cells, in cultured FaDu cells (FaDu in vitro), and in cells of tumors formed by grafting FaDu cells in vivo. Statistically significant differences are marked by asterisks; p=0.05. B) Size-dependent distribution of nucleostemin-positive nucleoli in basal and suprabasal layer cells, as well as in carcinoma cells of the oropharynx and larynx.*

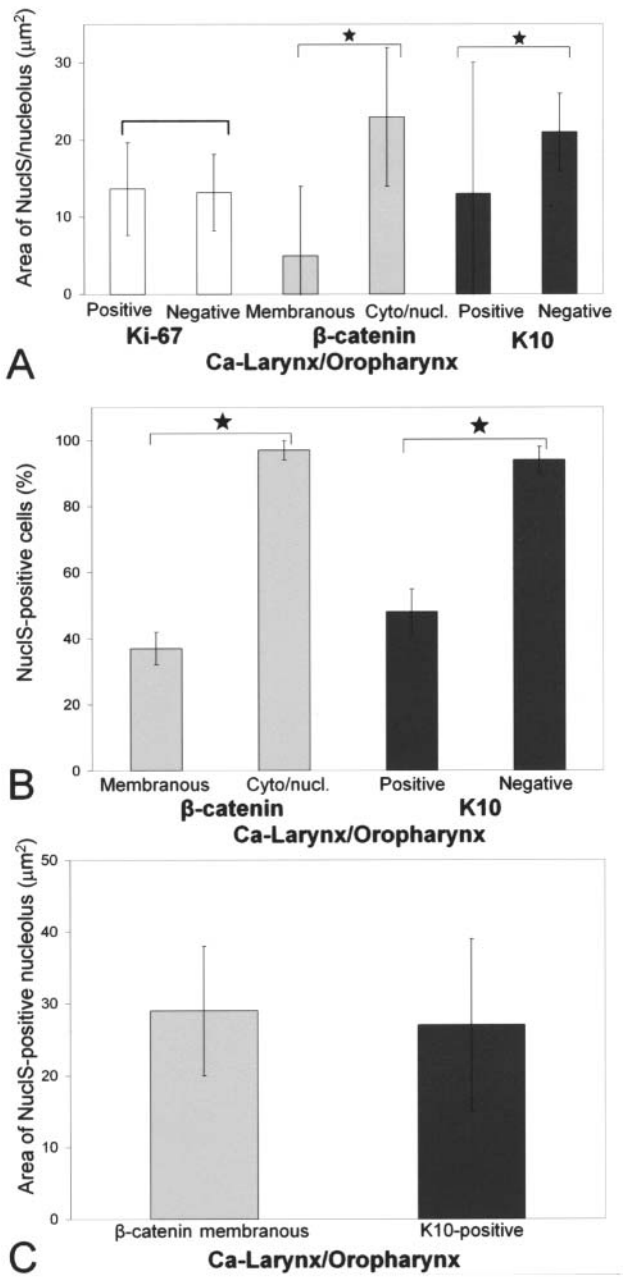

Figure 3. *A) Nucleostemin positivity per nucleolus in cancer cells, in relation to the expression of the proliferation marker Ki-67, the expression pattern of β-catenin (membranous, cytoplasmic/nuclear) and the expression of keratin 10. B) Incidence of cancer cells according to their phenotype. C) Comparison of the size of the area expressing nucleostemin in cells positive for this marker and presenting a membrane-associated signal for β-catenin, and for keratin 10. The difference is statistically insignificant (*p=0.05).*

of nucleoli according to their size, the nucleolar area in normal epithelium was rather uniform with a high incidence of nucleoli in the range of 6 to 10 µm^2 (Figure 2B). In contrast, the size distribution of nucleoli in both laryngeal and oropharyngeal squamous cell carcinomas was broad, with occurrence of very large nucleoli up to 35 µm^2 (Figure 2B).

Having first focused on features of nucleostemin presence, we next set the immunohistochemical data in relation to proliferation and other cellular markers.The studied tumors contained groups of cells with membrane-associated signals for β-catenin with the cytoplasmic and nuclear presence of this protein (Figure 1D) that is associated with tumor progression. The mean size of the nucleostemin-positive area per nucleolus was smaller in cells with membrane-associated positivity for β-catenin than in cells with positivity in the cytoplasm/nucleus (Figure 3A). However, this result should be be considered cautiously due to the rather low degree of nucleostemin positivity in cells with membrane-associated β-catenin (Figure 1D), where approximately one half of the cells contained

nucleostemin-positive nucleoli (Figure 3B). When the signal for nucleostemin was evaluated based on positive cells only, these were found to exhibit large nucleostemin-positive nucleoli (Figure 3C). Interestingly, the same phenomenon was observed for keratin 10-positive cells (Figure 3A-C). Nuclear/nucleolar expression of galectin-1, known to be expressed in cells sharing features with epidermal stem cells was detected in cultured FaDu cells (Figure 1E). No signal for the expression of this endogenous lectin was found in the nuclei of cells from normal epithelia or carcinomas (Figure 1A, B), or in tumors from FaDu cells grown in *nu*/*nu* mice (not shown). Of note when examining the tumor sections was the abundant presence of galectin-1 in the tumor stroma (Figure 1B); its level was significantly higher than in the connective tissue of the normal mucosa (Figure 1A).

Discussion

Evidently, expression of nucleostemin is not dependent on the proliferation status of cells in squamous epithelia of either ectodermal (epidermis) or endodermal (larynx) origin, knowing that only basal cells are able to proliferate (4, 27). Similarly, the proliferation status of tumor cells has no influence on the expression of nucleostemin in their nucleoli. However, the nucleostemin-positive nucleoli are larger than these in the normal epithelia. Surprisingly, nucleostemin-positive nucleoli of a very large area were found in cancer cells exhibiting membrane attached β-catenin and keratin 10, markers indicating differentiated phenotype in the normal cells (11, 19). This finding is similar to our observation in a previous study comparing the expression of keratins, ligands for galectin and Ki-67 where difference between expression of markers of the terminal differentiation and Ki-67 can be explained by the disparity between cell maturation and differentiation in cells of squamous cell carcinomas of the head and neck (11). Galectin-1 expression in the cell nucleus and/or nucleolus was observed in cells sharing features of epidermal stem cells (24) and it was also observed in FaDu cells (10). While nucleostemin was expressed in all cultured FaDu cells nucleoli, galectin-1 was detected in one half of studied cells where the good agreement of both proteins localization was present.

Extensive expression of galectin-1 in the tumor stroma represents one of dominant features of all the studied carcinomas. Increased presence of galectin-1 in the stroma has been observed, for example, in basal cell carcinomas (28) and the dermis of psoriatic skin (29).

The presented results document the presence of nucleostemin in squamous cell carcinoma of the head and neck. A high level of expression of this nuclear protein has also been observed in brain tumors (12), basal cell carcinomas (16), stomach and liver cancers (30) and cancer of the kidney (31). By immunohistochemical means it is not possible to determine whether this high level is an inherent property of tumor cells or is induced by a crosstalk between the cancer epithelium and tumor stromal cells (28). Looking at functional aspects, nucleostemin is likely not involved in the production of rRNA (32), but it may exert other regulatory functions during malignant transformation (33). One proposed function of nucleostemin is the control of proliferation and the inhibition of senescence, a potential means by which tumor cells avoid restrictions to their growth potential also related to galectins (34-36).

Conclusion

The presence of nucleostemin was documented in head and neck cancer here, and its detection, together with the size properties of positive nucleoli, may relate to tumor cell features (37).

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