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ORIGINAL ARTICLE

SPLUNC1 and MLL3 regulate cancer stem cells in nasopharyngeal carcinoma

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Summary

Purpose: *Nasopharyngeal carcinoma (NPC) is one of the common types of cancer that originate from the nasopharyngeal region. Recurrence and early metastasis represent major problems associated with NPC mortality. These are mainly caused by various molecular changes that take place during the conversion of normal stem cells into treatment-resistant stem cells. The aim of our study was to investigate the proliferative behavior of cancer stem cells in different stages of NPC and to identify the functional roles of SPLUNC1 and MLL3 associated with cancer stem cells.*

Methods: *We successfully developed a NPC mouse model using C666-1 cells. Immunohistochemistry and Western blotting were used to analyze the expression of SOX2, SPLUNC1 and MLL3.*

Results: *Null BALB/c mice developed initial and aggressive stages of NPC in 3 and 10 weeks, respectively. Histological results showed that the proliferative ability of cells increased as the tumor progressed to the next level. The SOX2 protein showed a peculiar pattern of upregulation in aggressive NPC when compared with control tissues and initial NPC. Remarkably, our study found that SPLUNC1 and MLL3 expression showed upregulation in initial NPC, which indicates their role in the tumor resistance mechanism even if their expression was downregulated in aggressive NPC.*

Conclusion: *Our results conclude that SPLUNC1 and MLL3 expression control the resistance mechanism of cancer stem cells in initial NPC, but their downregulation in aggressive stages contributes to developing resistance in nasopharyngeal cancer stem cells.*

Key words: *nasopharyngeal carcinoma, SLUNGC1, MML3, BALB/C mice, C666-1 cells, SOX2*

Introduction

The nasopharynx is the region situated behind the nose. Nasopharyngeal carcinoma (NPC) is a form of epithelial cancer with complex etiology [1]. The major reasons for developing NPC are infection with the Epstein-Barr virus, host genetic variants, environmental factors and exposure to dietary mutagens [2]. The occurrence of NPC is more common in Southern China and South-East Asia (80% cases) [3], while only 5% of the cases are reported in European countries [4]. The highly invasive nature and aggressive metastatic potential of NPC represent major problems [5]. The nasopharynx is not located in an easily accessible location and is therefore difficult to treat.

At present, radiotherapy is the best choice for treating non-metastatic NPC [4] and technology helps minimize the exposure of normal tissues [6]. NPC in advanced stages shows resistance to both chemotherapy and radiotherapy [7]. Recent research suggests that radioresistance in glioma

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was related with cancer stem cells [8]. Even now, the prognosis of NPC is poor and associated with shorter survival rates [9], possibly due to the resistance of cancer stem cells [10]. Therefore, understanding cancer stem cell behavior in different stages of NPC is important for revealing the resistance mechanism.

The short palate, lung, nasal epithelium clone 1 (SPLUNC1) is a tissue-specific molecule in the upper respiratory tract that plays a role in innate defense mechanisms [11,12]. Interestingly, the expression of SPLUNC1 protein was suppressed in the NPC tissue and its associated cell lines [13]. Similarly, Mixed Lineage Leukemia 3 (MLL3) is a protein able to methylate histone and plays a role in tumor suppression [14,15]. Recent research on MLL3 shows that its expression is downregulated in breast and gastric cancer patients [16,17]. In this current work we investigated the link of SPLUNC1 and MLL3 associated with cancer stem cells in various stages of NPC.

Methods

Experimental animals

To carry out the present investigation, 8-week-old nude female BALB/c mice were chosen. The experimental animals and the protocol followed throughout the experiments were approved by the Institutional animal care unit especially created for this project. Athymic BALB/c mice exhibit immunodeficiency and they were induced with C666-1 cells to develop NPC. Initially, the nude BALB/c mice were subcutaneously injected with 1×10^9 C666-1 cells to initiate NPC [18]. The injected mice were allowed to develop tumors of different stages based on the incubation period. After that, the mice that developed initial and aggressive forms of NPC were euthanized for further experimental analysis.

Immunohistochemical analysis

Initial and aggressive tissues of NPC were cut into small pieces and subjected to fixation using 10% formaldehyde solution. The tissues were then paraffin-embedded to form the final paraffin blocks by following the standard procedure, as previously described [19]. Using a microtome, a thin section of 6 µm size was sliced and placed on a clear glass slide. The tissues were dewaxed and subjected to blocking using 3% endogenous peroxide solution. The non-specific binding of antibodies was blocked using goat serum in 5% tris-buffered saline (TBS) according to a previously described protocol [20]. The tissues were then incubated with different primary antibodies (anti-mouse Sox2 antibody (1:500), anti-mouse SPLUNC1 antibody (1:300) or anti-mouse MLL3 antibody (1:500), separately at 4°C overnight. After washing the slide with phosphate buffer saline (PBS), the sections were overlaid with suitable Horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. After another washing, the sections were developed with a DAB (3,3'-diaminobenzidine) solution to obtain colored signals.

Western blotting

Control, initial and aggressive tissue samples were dissected and crushed with 2X sample buffer. The prepared cell lysate was heated in a boiling water bath for 5 to 7 min. After cooling, the samples were loaded in 12% SDS-PAGE gel and when the sample reached the bottom of the gel they were transferred to a polyvinylidene difluoride (PVDF) membrane. The experiments were carried out according to a previously described protocol [21]. The proteins that were transferred with the membrane were subjected to blocking with 5% bovine serum albumin (BSA) solution for 2h at room temperature. Following blocking, the membrane was incubated with primary antibodies (anti-mouse SOX2 antibody (1:500), anti-mouse SPLUNC1 antibody (1:300) or anti-mouse MLL3 antibody (1:500)), separately at 4 °C overnight. After washing, the membrane was incubated with suitable secondary antibody and further developed to obtain the signal.

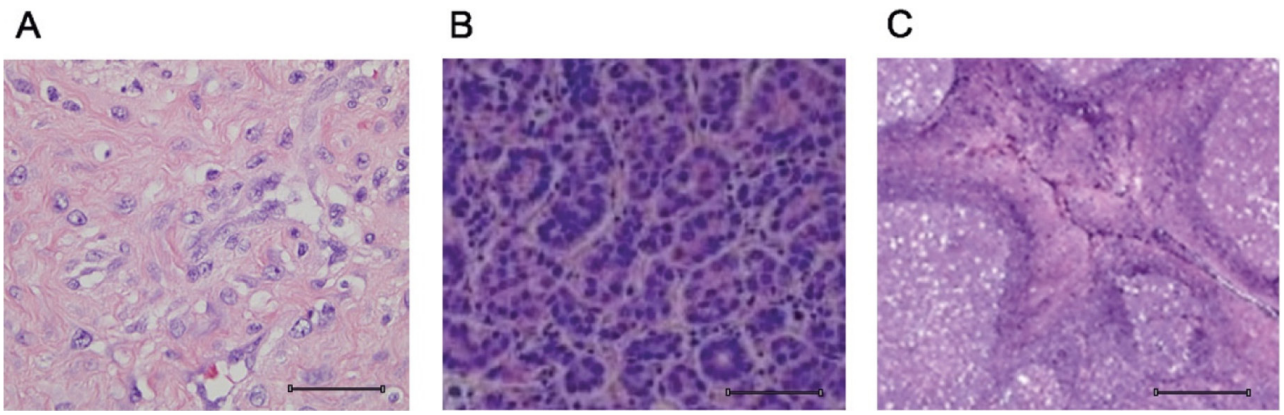

Figure 1. Histological confirmation of NPC. **A:** Histological section of control nasopharyngeal tissue showing evenly arranged cells with prominent nucleus. **B:** Initial NPC tissue section showing higher mass of prolifertative cells that forms clumps. **C:** Aggressive NPC tissue section showing aggressive proliferative cells with hardened tissue. Hematoxylin and Eosin staining, 40X, Scale Bar denotes 100 µm size.

Results

Mouse model developed with NPC

An effective animal model system is essential for understanding the roles of SPLUNC1 and MLL3 associated with NPG cancer stem cells. Tumorigenic cell transformation is a key technique that is successfully utilized to induce NPC [22]. In the present investigation, C666-1 cells were used to develop NPC as described in the method section. Following injection, the mice showed initial stages of NPC in the 3rd week and in the 10th week aggressive NPC developed.

Cellular pattern of different pathological stages of NPC

The mice that developed initial and aggressive stages of NPC were further confirmed through

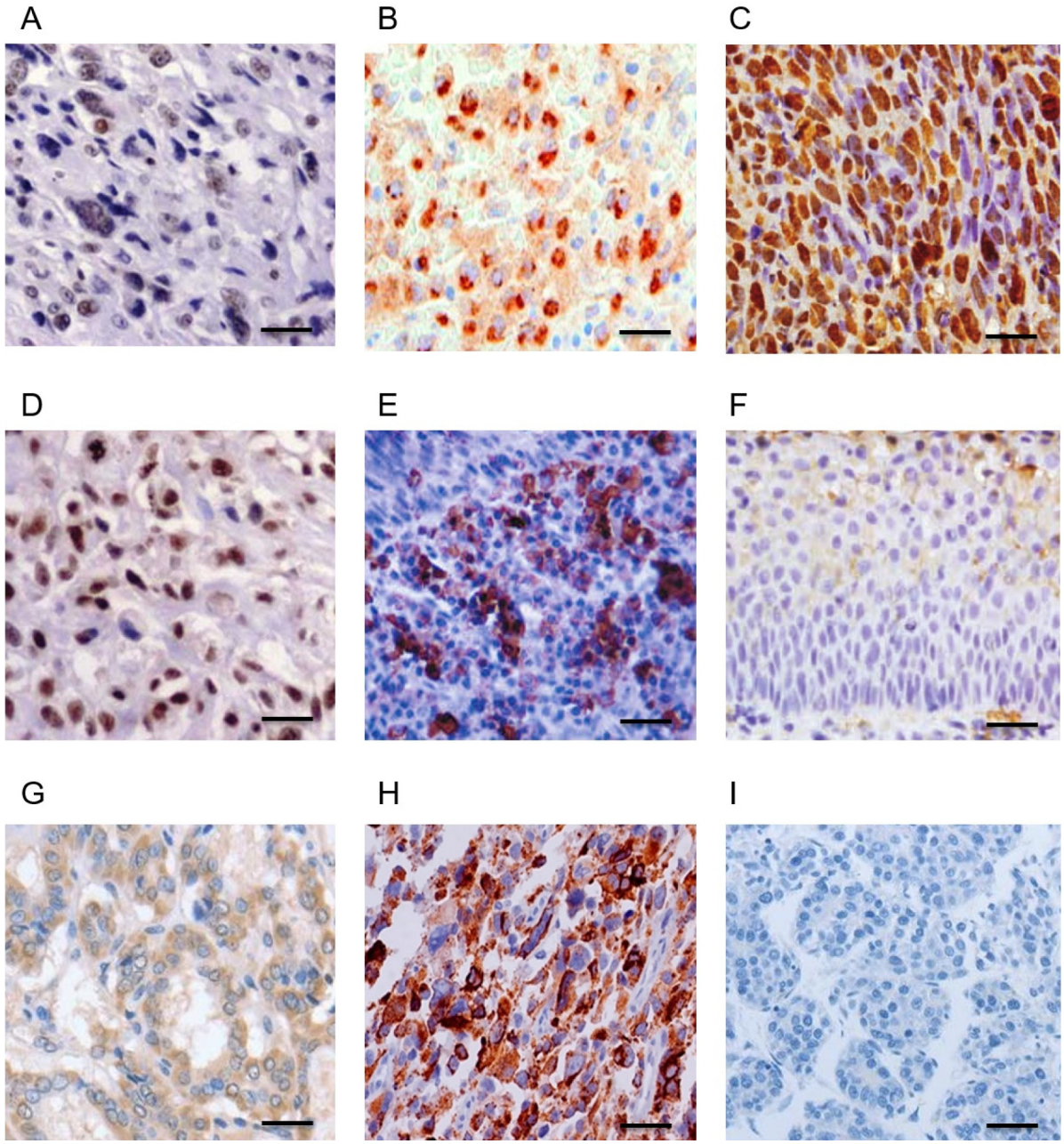

Figure 2. Immunohistochemical expression of SOX2, SPLUNC1 and MLL3 in different stages of NPC. **A:** Slight expression of SOX2 in control nasopharyngeal tissue **B:** Moderate expression of SOX2 in initial NPC tissue section. **C:** Vigorous expression of SOX2 protein in aggressive NPC tissue section. **D:** Moderate expression of SPLUNC1 in control nasopharyngeal tissue. **E:** Upregulated expression of SPLUNC1 in initial NPC tissue section. **F:** Downregulated expression of SPLUNC1 protein in aggressive NPC tissue section. **G:** Moderate expression of MLL3 in control nasopharyngeal tissue. **H:** Upregulated expression of MLL3 in initial NPC tissue section. **I:** Downregulated expression of MLL3 protein in aggressive NPC tissue section. Hematoxylin staining, 40X, Scale Bar denotes 50 μm size.

histological analysis as shown in Figure 1A-C. The control nasopharyngeal tissue showed an uniform arrangement of cellular pattern with a prominent nucleus that was evenly distributed throughout the tissue layer (Figure 1A). However, the mice injected with tumorigenic C666-1 cells developed initial NPC, which was indicated by the abnormal proliferative cells that formed irregular islands of cells (Figure 1B). Similarly, the mice injected with C666-1 cells and maintained for up to the 10th week displayed an aggressive pattern of proliferation with hardened tissue (Figure 1C).

Analysis of nasopharyngeal cancer stem cells in different pathological stages of NPC

The subpopulation of cancer stem cells is responsible for the occurrence, rapid development and metastatic ability of NPC. Understanding the molecular aspects of cancer stem cells in different stages is critical in developing new strategies. SOX2 is one of the key transcriptional factors that help to maintain the pluripotency of stem cells [23]. In this study, the SOX2 expression was studied in initial and aggressive stages of NPC which could help validate the cancer stem cells associated with NPC. The control tissue showed only a few cells positive for SOX2 (Figure 2A) and upon tumor initiation SOX2 showed an increased expression pattern (Figure 2B). In aggressive stages of NPC, SOX2 showed overexpression, which was supported by immunohistological data (Figure 2C).

SPLUNC1 and MLL3 expression in different pathological stages of NPC

The downregulated expression of SPLUNC1 was already reported in NPC [13] but its expression pattern was not extensively studied in different pathological stages of the disease. In this present study, we found that SPLUNC1 was moderately expressed in normal nasopharyngeal tissue (Figure 2D), which may help maintain internal homeostasis. Surprisingly, however, its expression showed an upregulated pattern in initial NPC (Figure 2E). Nevertheless, in an aggressive stage of NPC, SPLUNC1 showed downregulated expression (Figure 2F) when compared with control tissues (Figure 2D).

The role of MLL3 was studied in the context of NPC. The control tissue showed a significantly increased expression of MLL3 (Figure 2G) and on initiation of NPC its expression showed an upregulated pattern (Figure 2H). However, in aggressive stages of NPC, MLL3 showed only a few positive cells that were completely downregulated (Figure 2I).

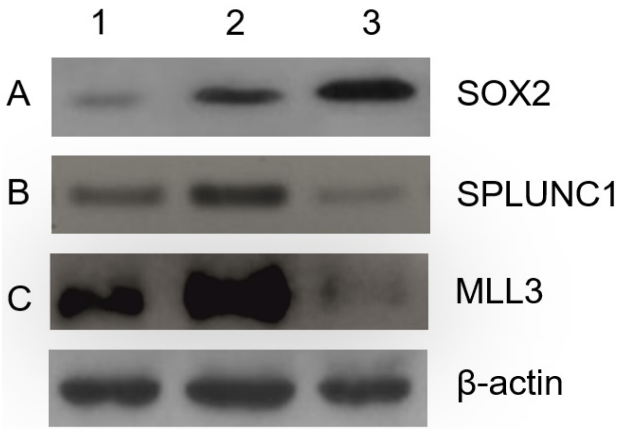

Figure 3. Western blotting. **A:** Expression profile of SOX2 protein is shown in Lanes 1-3 which represent their expression in control, initial and aggressive tissues of NPC. **B:** Expression profile of SPLUNC1 protein is shown in Lanes 1-3 which represent their expression in control, initial and aggressive tissues of NPC. **C:** Expression profile of MLL3 protein is shown in Lanes 1-3 which represent their expression in control, initial and aggressive tissues of NPC. β-Actin was used as a loading control.

Western blotting analysis

Following the immunohistological investigation, we further aimed to confirm the data through Western blotting analysis. To study the role of SPLUNC1 and MLL3 associated with cancer stem cells, the cell lysate was initially prepared from control, initial and aggressive NPC tissues. The prepared samples were subjected to Western blotting analysis using three different primary antibody solutions, namely anti-SOX2 antibody, anti-SPLUNC1 antibody and anti-MLL3 antibody to determine their expression pattern. Our results showed similar patterns of expression as confirmed by the immunohistochemical analysis. SOX2 showed an upregulated expression as the tumor progressed and notably, we observed highly SOX2 positive cells in aggressive NPC (Figure 3). SPLUNC1 and MLL3 showed a downregulated expression only in aggressive NPC, but surprisingly their expression was upregulated in initial NPC (Figure 3).

Discussion

Recent studies show that targeting NPC cancer stem cells is ideal for developing new therapeutic strategies to fight NPC. Cancer stem cells share some similar cell surface markers with normal stem cells, which is the key in identifying the cancer stem cells [24]. Epstein-Barr virus, diet and various genetic factors promote the conversion of normal stem cells into cancer stem cells. Hence, tracking the NPC cancer stem cells associated with different molecular-level changes is important for

understanding the in-depth knowledge of NPC development.

In our experimental work, we used C666-1 cells to successfully induce NPC in BALB/c mice. NPC induction using C666-1 is a preferred method to investigate Epstein-Barr virus infections associated with NPC [25]. The histological data revealed the complex cellular pattern that helps define the initial and aggressive stages of NPC (Figure 1A-C). The proliferative cells and the changes observed in the cellular pattern help to assess the disease status.

The role of CD44 is well established in identifying nasopharyngeal cancer stem cells [26] but less knowledge is achieved using SOX2 [18]. Our immunohistochemical data on SOX2 confirms more variations of SOX2-positive cells in initial and aggressive NPC (Figure 2A-C). The results imply that the population of cancer stem cells or major changes in normal stem cells are well under control in the initial stages of NPC, but later, major changes in stem cells determine the increased proliferation efficiency of cancer stem cells.

Recent studies on MLL3 showed that its mutation plays a direct role in initiating carcinogenesis [27,28]. In the present work, MLL3 and SPLUNC1 clearly showed increased expression in initial NPC (Figure 2E,2H). The correlation of this result with SOX2 expression in the initial stages of NPC reveals the control mechanism behind them. The antibody specificity and sensitivity of the results obtained through immunohistochemistry were cross-checked using Western blotting analysis (Figure 3). The results confirm that the immunohistochemical findings accurately fit with the data obtained from Western blotting.

According to our results, nasopharyngeal cancer stem cells tracked using SOX2 showed vigorous expression in aggressive NPC with a unique pattern when compared with initial NPC. The SOX2 expression correlates with the functional regulation of SPLUNC1 and MLL3, which were initially upregulated in initial NPC, while in aggressive stages of NPC, they showed a downregulated expression.

Conflict of interests

The authors declare no conflict of interests.

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