

Assessment of *MLL* methyltransferase gene expression in larynx carcinoma

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Abstract. Larynx cancer is the second most common type of cancer among all head and neck cancers. Dereglulation of epigenetic effectors, including altered expression of histone methyltransferases from the *MLL* (mixed lineage leukemia) family, have been reported in many cancer types, yet little is known concerning their involvement in larynx cancer. Our objective was to determine the expression profile of *MLL* genes in larynx carcinoma and normal adjacent tissues and correlate this profile to tumor characteristics. We analyzed the expression profile of 5 *MLL* genes in 13 cases of larynx carcinoma and their adjacent non-tumor tissues using quantitative real-time PCR. *MLL3* was significantly downregulated in tumor samples compared to their normal counterparts, and all *MLL* genes showed decreased expression in advanced tumors compared to tumors in the initial stage. Altered expression in a single *MLL* gene was associated with a similar alteration in the other *MLL* genes, revealing a strong correlation of expression in each individual patient. In conclusion, *MLL* genes may have similar transcriptional control, and decreased expression of these genes may contribute to larynx cancer progression.

Introduction

Larynx cancer is the second most common among all head and neck cancers, representing 2% of all malignant diseases worldwide, with 140,000 cases occurring every year (1). In

Brazil alone, there is an estimated risk of 7 cases for every 100,000 men. Usually, affected men are over the age of 40 years. The registered incidence of this cancer in women is very low, accounting for less than 1 case for every 100,000 women. The disease can cause hoarseness, dysphagia, odynophagia, pain and other symptoms, according to the affected site (supraglottis, glottis, subglottis). Early detection leads to a better prognosis, enabling a cure in the majority of the cases, while patients with advanced tumors usually succumb to the disease (2). Tobacco is the main associated risk factor, with a positive correlation of duration and intensity of smoking and increased risk for larynx cancer. Other risk factors include alcohol consumption, occupational exposures and various dietary habits. Moreover, the risk for larynx cancer increases with the combined effect of smoking and alcohol consumption (2). Studies concerning the association of human papillomavirus (HPV) infection with larynx cancer are heterogeneous and inconclusive, yet HPV infection appears to be associated with the risk of larynx cancer, particularly with the high-risk type HPV16 (3).

Almost all cancers occurring in the head and neck are squamous cell carcinomas. Although they have the same histological characteristics, they appear to have genetic and epigenetic differences, depending on the site. Allelic loss leading to the loss of function of tumor-suppressor genes are an important alteration in larynx tumors, such as the loss of heterozygosity (LOH) at 9p21, a region harboring the cell cycle regulator p16 gene (4). LOH at 8p was also found to be common in larynx and oral squamous cell carcinoma and appears to be involved in early carcinogenesis (5). Some studies have shown that promoter hypermethylation of cancer-related genes are common events in head and neck cancers, which include the larynx. Hypermethylation of *p16*, *DAP-kinase*, *E-cadherin* and *MGMT* genes have been reported in larynx cancer (6). Another study showed that promoter methylation of at least one of these genes (*p16*, *DAP-kinase*, *E-cadherin* and *RASSF1A*) was present in 60% of head and neck cancers,

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including larynx cancer (7). However, to date, little information is available concerning the epigenetic alterations occurring in larynx cancer.

Although DNA methylation is one of the most studied epigenetic changes in cancer, it is believed that other regulators of chromatin methylation are involved in the process of carcinogenesis. Recently, post-translational modifications of histone tails through methylation of specific lysine residues, catalyzed by histone methyltransferases (HMTase), have been associated with many types of cancer (8). Deregulation of histone methylation affects important biological processes, such as transcription regulation, DNA repair and chromosome stability, but differently from genetic mutations, epigenetic alterations are potentially reversible (9).

The *MLL* (mixed lineage leukemia) family of genes [*MLL* (*ALL-1*), *MLL2/KMT2D*, *MLL3*, *MLL4/KMT2B* and *MLL5*] encodes histone-modifying enzymes characterized by a conserved catalytic SET domain, responsible for the methyltransferase activity in histone 3 - lysine 4. Many of the *MLL* genes have been described to be involved in different types of cancer (8), but to date there are no data concerning the relationship of these genes with larynx cancer.

While prevention can be the most important strategy for the control of larynx cancer, the knowledge of molecular alterations can provide a better understanding of the disease, providing more predictive information and contributing to early diagnosis, risk stratification of patients and new perspectives of treatment. Despite the development of new technologies and the advances achieved in cancer research, little is known concerning the molecular changes occurring in larynx cancer as well as the epigenetic alterations involved in the initiation and progression of this disease. In addition, the survival of patients with larynx cancer has not improved over the last 30 years, with data showing a downward trend (10). In the present study, we analyzed the expression profile of the 5 *MLL* family genes in 13 cases of larynx carcinoma and their adjacent non-tumor tissues.

Materials and methods

Patients and controls. Thirteen patients diagnosed with larynx squamous cell carcinoma were included in the present study. The adjacent non-tumor tissue from each of the patients was used as a control. Written informed consent was obtained from all participants and, based on resolution 196/96 of the National Health Council/Brazilian Ministry of Health, approval for this study was obtained from the ethics committee at the University Hospital of the Medical School of Ribeirão Preto, University of São Paulo, Brazil. Identification of tumor type and content (minimally 80% tumor) was determined by microscopic pathological analysis.

Tissue microdissection. Fragments of 0.5-1 cm of the tumor and of the adjacent non-tumor tissues were removed during tumor resection surgery. The tissues were immediately immersed into liquid nitrogen and stored at -180°C. Before RNA extraction, a cryostat section of the tumor sample was examined under a microscope, and the frozen blocks were dissected by apposition, trimming off non-neoplastic or necrotic areas.

RNA extraction and cDNA synthesis. Total RNA was isolated from the surgically removed specimens using TRIzol LS reagent, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized from total RNA obtained from the clinical samples with reverse transcriptase and random primers, using the Applied Biosystems High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's protocol.

Quantitative real-time PCR (qPCR). qPCR was performed on a StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) using TaqMan Universal PCR Master mix and TaqMan Gene Expression Assays for *MLL* gene family (Hs01061596_m1 for *MLL*, Hs00231606_m1 for *MLL2/KMT2D*, Hs00419011_m1 for *MLL3*, Hs00207065_m1 for *MLL4/KMT2B* and Hs00218773_m1 for *MLL5*; Applied Biosystems). Amplification conditions were as follow: 2 min at 50°C and 10 min at 95°C on holding stage, and then 40 cycles of 15 sec at 95°C and 1 min at 60°C. Gene expression in tumor samples was represented as the ratios between the amplification levels of the *MLL* genes and the levels of the endogenous control gene *GAPDH*, used as a normalization factor (Hs02758991_g1 for *GAPDH*, Applied Biosystems). This ratio was then normalized to the ratio obtained in the control samples (relative expression level), as explained below in 'qPCR data and statistical analysis'.

qPCR data and statistical analysis. Relative expression was calculated using the formula $2^{-\Delta\Delta Ct}$ (11), using *GAPDH* as an endogenous control to normalize sample loading. Each normal sample was used as reference for its tumor counterpart. Briefly, the mean of Ct (cycle threshold) values of the replicates were calculated and normalized by subtracting the Ct value of the co-amplified endogenous control gene to yield a ΔCt value. The ΔCt of control samples (1X sample) was subtracted from the ΔCt of the tumor samples to yield a $\Delta\Delta Ct$ value. The amount of target gene, normalized to an endogenous control and relative to a reference was converted into relative quantification by the formula: $2^{-\Delta\Delta Ct}$.

Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform statistical analyses. The level of significance was set to 5% (CI 95%). The Wilcoxon signed rank test was performed to examine differences in the expression of the *MLL* genes between pairs of normal and tumor samples of the same patient. The Mann Whitney test was used to check differences in *MLL* gene expression between initial and advanced stage tumors. The non-parametric Spearman test was performed to check the correlation of relative quantification between *MLL* gene pairs.

Results

Profile of patients with larynx squamous cell carcinoma. The clinical and histopathological characteristics of the 13 patients are summarized in Table I. The age of the patients ranged from 57 to 80 years, with an average age of 65 years. The majority of the samples were collected from male patients, reflecting the higher incidence of larynx carcinoma in men. The tumor size according to the TNM classification was T1, T2, T3 and T4 in

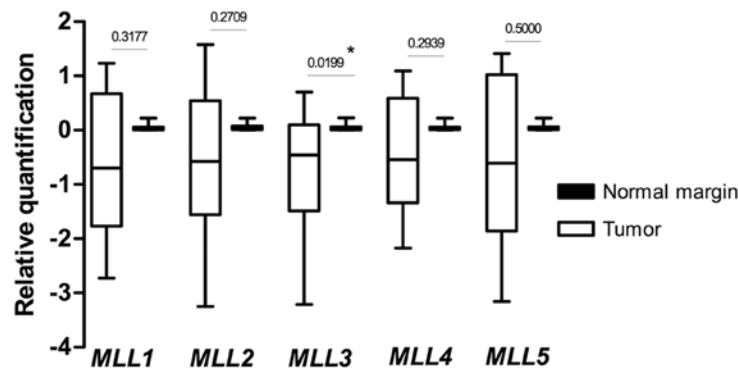


Figure 1. Expression analysis of *MLL* genes in 13 pairs of normal and tumor clinical samples. Each normal sample was used as a reference for its tumor counterpart. Wilcoxon signed rank test (CI 95%).

Table I. Clinical characteristics of the 13 larynx cancer patients.

Characteristics	Data
Total, n (%)	13 (100)
Gender, n (%)	
Male	12 (92.3)
Female	1 (7.7)
Age, years	
Average	65
Range	57-80
Age at diagnosis, years	
Average	59
Range	51-75
Use of tobacco, n (%)	
Yes	11 (84.6)
No	0 (0.0)
NA	2 (15.4)
Use of alcohol, n (%)	
Yes	11 (84.6)
No	0 (0.0)
NA	2 (15.4)
Treatment, n (%)	
Surgery only	6 (46.15)
Surgery and radiotherapy	6 (46.15)
Surgery, radio and chemotherapy	1 (7.70)
Stage, n (%)	
T1	3 (23.0)
T2	2 (15.4)
T3	1 (7.7)
T4	7 (53.9)

NA, not available.

MLL3 is downregulated in the tumor samples. The paired expression analysis between normal and tumor samples from the same patient is shown in Fig. 1. The quantification analysis for the tumor and their matching non-tumor samples (normal margins) (n=13) revealed a decreased expression level tendency for all genes in the tumors when compared to the normal samples. Importantly, *MLL3* was significantly downregulated in the tumor samples when compared to the level in their normal counterparts (p=0.0199).

Correlation of the expression of MLL genes. Non-parametric Spearman test was performed to check the correlation of relative quantification among the *MLL* gene pairs. The mRNA expression level of all *MLL* genes was significantly correlated in each individual patient. The altered expression in one gene resulted in a similar alteration in the rest of the family (Fig. 2). The correlation of each pair of *MLL* genes was clearly significant in our cohort of larynx cancer as shown in Fig. 3. These results suggest that the *MLL* family could have synchronized regulatory mechanisms.

MLL genes show decreased expression levels in advanced tumors. We examined the expression levels of the *MLL* genes in subgroups of advanced stage tumors versus initial stage tumors. Although the statistical difference was not significant, this analysis indicated that all of the *MLL* genes had a decreased expression level in the advanced tumors when compared to the level in the initial stage of the disease (Fig. 4).

Discussion

Epigenetic alterations, such as deregulated histone methylation appear to be involved in many types of human cancers. Mutations in histone methyltransferases or alterations in their expression may strongly contribute to a pattern of deregulated methylation in histones as well as other non-histone proteins (12,13). Although increasing new data are revealing the involvement of methyltransferases in cancers, their exact role in carcinogenesis remains to be elucidated. In this scenario, our knowledge concerning the epigenetic modifications in larynx cancer is even poorer. While prevention and early diagnosis are essential for the management of larynx cancer, which improves the quality of life and survival of patients, the understanding of epigenetic alterations involved

3, 2, 1 and 7 of the determined cases, respectively. Based on this classification, the patients were stratified in initial (6 cases) or advanced stage (7 cases) groups for statistical analysis.

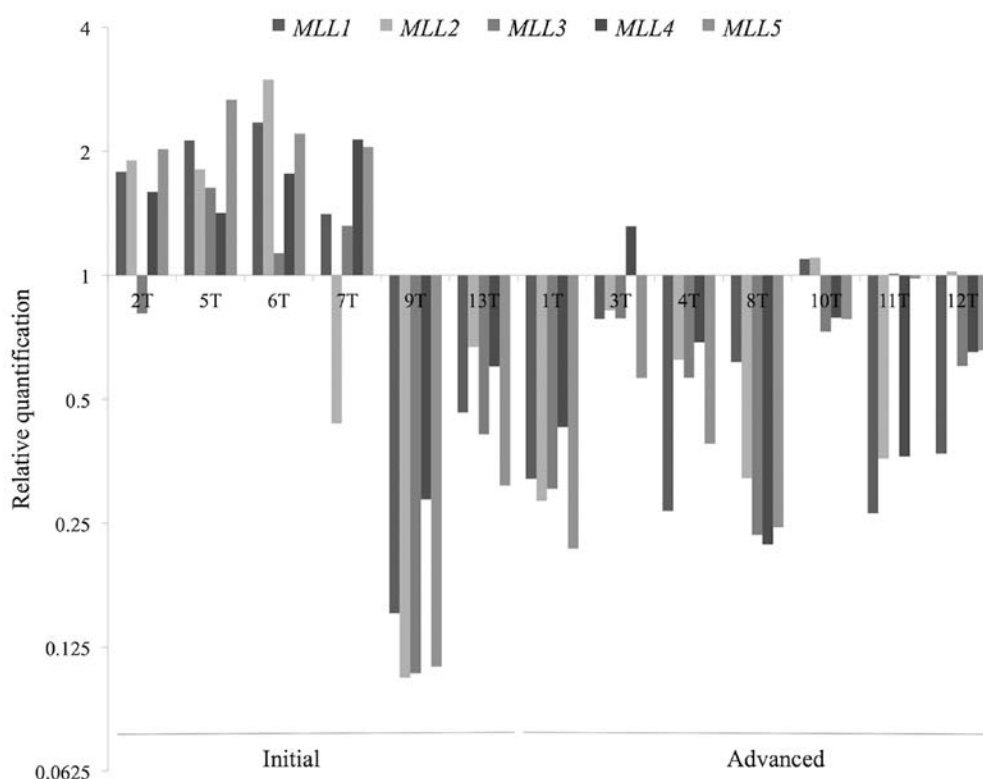


Figure 2. Relative quantification of the *MLL* genes for each patient, including initial and advanced cases of larynx cancer.

in this disease could provide valuable insight into the development of new diagnostic or prognostic markers and new perspectives of therapy.

Sparse information is available concerning the epigenetic alterations occurring in larynx squamous cell carcinoma. A recent study showed that DNA methyltransferase 1 (*DNMT1*) was overexpressed in tobacco-induced larynx cancer, which was associated with a worse prognosis (14). Another study revealed an increased expression of *H3* (Histone 3) in larynx cancer when compared to normal tissue and suggested that since H3 synthesis is coupled with DNA replication, it can be used as a marker of cell proliferation (15). Corroborating this observation, *HIST1H3F*, a member of the histone 3 family, showed elevated expression levels in high-risk larynx cancer (16). However, to date, there is no information concerning the role of the *MLL* gene family in larynx cancer. Nonetheless, recent studies have linked alterations of these genes with different types of cancer. *MLL* genes appear to maintain the expression of *Hox* genes during development (17) and are frequently associated with chromosomal translocations in leukemia (18). A comprehensive study of the *MLL* family in breast cancer revealed a reduced expression of *MLL2/KMT2D* in that cancer (19). In addition, *MLL2/KMT2D* was shown to be mutated in lymphoma patients (20,21). *MLL3* is located in a region (7q36.1) usually deleted in leukemia (22). *MLL4/KMT2B* maps to 19q13.1, a region frequently rearranged in human cancer (23). *MLL5* has a different SET domain sequence and may function in a different manner from the rest of the *MLL* family (24). Acute myeloid leukemia patients with low *MLL5* expression were found to have a worse outcome, when compared to patients with high transcript levels (25).

Herein, we analyzed the expression profile of the *MLL* genes in 13 cases of larynx carcinomas and their adjacent non-tumor tissues. The paired analysis, using adjacent non-tumor tissue of each patient as its normal control, provided a reliable control group for the expression profile analysis. In addition, tissue microdissection allowed an accurate selection of the tumor area, trimming off all non-neoplastic or necrotic areas that might affect the gene expression results.

In our cohort, the *MLL3* gene showed a decreased expression in tumor samples compared to that in the normal counterparts ($p=0.0199$). *MLL3* is located on chromosome 7q36.1 and has been associated with different types of cancers. *MLL3* presents inactivating mutations in medulloblastoma (26) and colorectal cancer (27). Moreover, it shows reduced expression in primary breast tumor samples (28) and radioresistant esophageal cancer cell lines (29). A recent study showed that exome sequencing identified a germ line mutation in *MLL3*, producing a truncated protein, in a pedigree of colorectal cancer and acute myeloid leukemia (30). These results suggest that *MLL3* acts as a tumor suppressor in cancer development. *MLL3* forms a steady-state complex with ASC-2, a multifunctional coactivator of nuclear receptors and other transcription factors. This complex called ASCOM interacts with p53 and is required for expression of p53-target genes in response to DNA damage. These results indicate a role of *MLL3* in the DNA damage response pathway through p53 activation (31). The downregulation of *MLL3* in larynx cancer may therefore impair the DNA damage response contributing to the proliferation of cancer cells. However, this hypothesis must be further investigated.

Although *MLL3* was the only member of the *MLL* family genes to be significantly downregulated in all tumor samples,

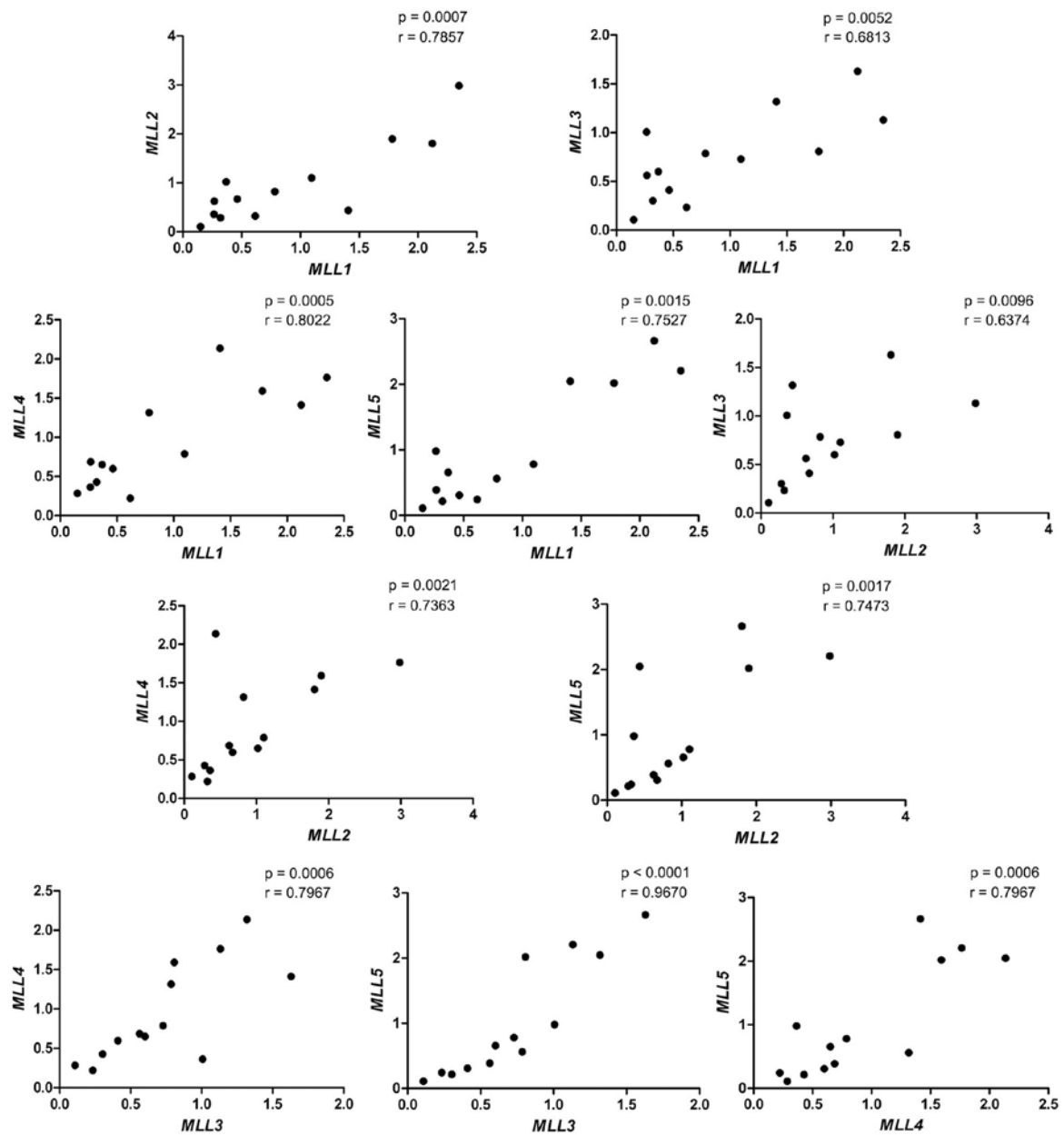


Figure 3. Correlation analysis of the relative quantification of the *MLL* genes in 13 tumor clinical samples. Spearman correlation test (CI 95%).

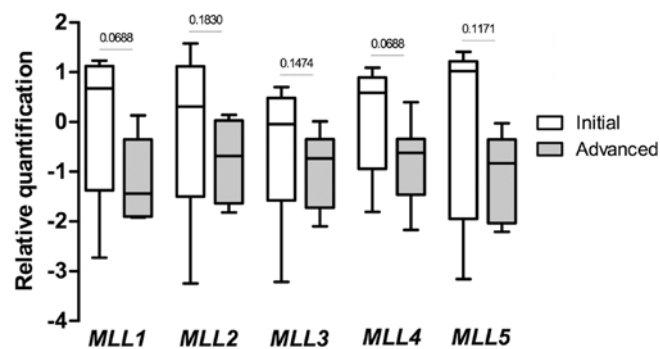


Figure 4. Expression analysis of *MLL* genes in advanced (n=7) and initial stage (n=6) tumor samples. Mann Whitney U test (CI 95%).

we observed a strong correlation in the expression of all 5 genes in each individual patient. Using the non-parametric Spearman

correlation analysis, we found that an altered expression in one *MLL* gene was correlated to a similar alteration in the other

genes of the family, for every combination of 2 genes. These results suggest that all *MLL* family genes may have a common regulatory mechanism. To date, there is no clear information on a possible complementary function of all 5 *MLL* genes, and further studies are necessary to unveil whether they have the same transcription factors, microRNA regulators or other types of synchronized regulation.

Based on clinical and histopathological classification, we stratified the larynx cancer patients in groups according to advanced and initial stage disease. Of note, we found that all of the *MLL* genes showed decreased expression in the advanced stage tumors when compared to the initial stage tumors. Although the statistical difference was not significant, which was probably due to the small number of cases, we observed a clear tendency of decreased expression levels for all *MLL* genes in patients with advanced disease.

The correlation among the *MLL* genes in larynx cancer highlights the importance of this family in the progression of this disease. Further studies will reinforce the significance of the downregulation of *MLL3* for larynx carcinogenesis and will help to elucidate the mechanisms by which *MLL* genes act on larynx cancer progression.

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