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Original Article

Hypermethylation Status of *NRGN* and *OLFM1* Promoter Regions in Glioblastomas Cell Lines

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Abstract

Glioblastomas are the most malignant astrocytic tumors and the most common brain tumors, representing 50 to 60% of all astrocytic tumors. In a previous study, Marie et al (2005) using SAGE and cDNA microarray analyses, followed by real-time PCR validation in additional tumor samples, identified potential markers for astrocytomas with distinct levels of malignancy. In the present study, we evaluated if the hypoexpression of two of these genes - Neurogranin (*NRGN)* and Olfactomedin (*OLFM1)* – is related to the hypermethylation of their promoter regions. CpGs islands were identified in the promoter regions of both genes and tumour cell lines (A172 and T98G) were submitted to 5 Aza dC treatment. The treatment was able to induct the expression (1.8 to 8.9 folds) of both genes. Nevertheless, the association between *NRGN* and *OLFM1* hypoexpression and CpG island hypermethylation could not be established because the CpGs dinucleotides present in the promoter region of these genes were unmethylated when evaluated by sequencing.

Key words: Glioblastoma. DNA methylation. Neurogranin. Olfactomedin.

Introduction

Astrocytic gliomas are the most frequent primary neoplasms of Central Nervous System (65 to 70% of all gliomas) and according to the World Health Organization they are classified as: pilocytic astrocytomas (grade I), diffuse astrocytomas (grade II), anaplastic astrocytomas (grade III) and glioblastomas (grade IV).[1,2]

Glioblastomas (GBM) are the most malignant astrocytic tumors and the most common brain tumor, representing 50% to 60% of all astrocytic tumors. The annual incidence is 2 to 3/100.000 new cases and the prognosis is extremely unfavourable despite the aggressive treatments with radiotherapy and chemotherapy.[3]

Epigenetic modifications (modifications that do not influence the genetic potential of genes, but lead to inherited alteration of their expression) play an important role in carcinogenesis. Two related components contribute to epigenetic modifications of gene expression: alteration of chromatin conformation (chromatin remodeling) and modification of DNA methylation pattern. DNA methylation is a crucial epigenetic modification of the genome that is involved in regulating many normal cellular processes like embryonic development, transcription,

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chromatin structure, X chromosome inactivation, genomic imprinting and chromosome stability.[4] Alteration in the expression of many genes occurs during tumor formation and progression. It has been demonstrated in many studies that several tumor-suppressor genes that regulate cellular motility, apoptosis, and angiogenesis and genes of the DNA repair system may be inactivated in tumors due to aberrant methylation of their 5´-regulatory regions.[5] Regulatory regions of these genes are located in the specific GC-rich sequences - CpG islands that are not methylated in normal tissues of the adult organism. Aberrant methylation of CpG islands is accompanied by inhibition of gene transcription.[6] Many studies had already been carried out in astrocytomas with the objective of verifing the DNA methylation status of these tumors.[1,7-9] The main purpose of these studies was to better comprehend the molecular pathways involved in astrocytomas tumorigenesis and identify new molecular markers with diagnostic, predictive or prognostic value in astrocytomas.

In a previous study, Marie et al.[10] showed that the combined use of SAGE database and DNA microarray analyses, followed by real-time PCR validation in tumor samples, allowed the identification of potential markers of astrocytomas with distinct levels of malignancy. They found several genes downregulated in astrocytomas and in the present study we evaluated if the hipoexpression of two of these genes - Neurogranin (*NRGN)* and Olfactomedin (*OLFM1)* – is related to the hypermethylation of their promoter regions.

Material and Methods

Cell lines

Two human glioma cells lines derived from glioblastomas (T98G and A172), three cell linesderived from bladder cancer (SCABER, HT-1376 e J82), three cell lines derived from colon cancer (DLD-1, HCT116 e SW480), three cell lines derived from lung cancer (H1299, H1155 e H249) and two cell lines derived from breast cancer (MDA-MB-435 e MDA-MB-231) were used. All of them were obtained from ATCC-American Type Culture Collection. The T98G and A172 cell lines were grown in 10% FBS in DMEM and 20% FBS in RPMI, respectively. The bladder cancer and colon cancer cell lines were grown in 10% FBS in MEM and in 10% FBS in McCoy's, respectively. The lung cancer and breast cancer cell lines were cultivated in 10% FBS in RPMI. The cultures were mantained at 37°C in 5% CO_2 for 5 days, treated with tripsine, harvested and stocked at -70°C.

The cell lines A172 and T98G were cultivated with appropriate medium culture and grown at 90% of confluence. The number of cells was estimated through Newbauer camera counting. $5x10^5$ cells were grown for four consecutives days in appropriated medium supplied with 1mM and 5mM of 5-Aza-dC. The medium culture and drug were changed daily. As a negative control, $5x10^5$ cells of the same cell lines were simultaneously cultivated, under same conditions, but without 5-Aza-dC adition. Twenty-four hours after the last medium change, cells were harvested and stocked at -70°C.

RNA Isolation and cDNA Synthesis

Total RNA was isolated by sedimentation through cesium chloride gradient method as described by Chrigwin et al[11] Briefly, frozen tissue was homogenized in homogenization buffer (4M guanidinium thiocyanate; 25mM sodium citrate; 0.7mM b-mercaptoethanol). The homogenate was loaded onto a cushion of 5.7M cesium chloride and 25m M sodium acetate (treated with diethyl pyrocarbonate), and RNA was pelleted overnight at 20,000 rpm. The CsCl was removed from the centrifuge tube, and the RNA pellet at the bottom of the tube was resuspended in RNase-free water.

Two micrograms of total RNA from different tumor cell lines were reverse-transcribed using oligo(dT) primer and Superscript II (Invitrogen). Each cDNA mixture was diluted 10 folds before use.

Real-time PCR quantification

Quantitative real time polymerase chain reaction assays (RQ-PCR) for the genes *NGRN* and *OLFM1* were performed using the SYBR GREEN I detection system. Primers were positioned in different exons to avoid false-positive results due to possible DNA contamination. All PCR reactions were performed in duplicates using a SDS 7000 machine (Applied Biosystems). Normalization of

quantitative data was based on the housekeeping glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) expression level. The relative mRNA expression of the target genes was calculated using 2^{-DDCt} method.[12] The primers used for amplification of the *NRGN* and *OLFM1* genes were NRGN F (5′-ATGGCGCGGAAGAAGATAAAG-3′) and NRGN R (5′-GCGCACAGAACGAAA GAACTC-3′); OLFM1 F (5′-TGGATGAACTTA GGCCTTTGATACC-3′) and OLFM1 R (5′-TGATGTGGCTCTGGAACTTGTTG-3′), respectively. Primers sequences of GAPDH gene were: GAPDH F (5′-TGCACCACCAAC TGCTTAGC-3′) and GAPDH R (5′-CAGTG TAGCCCAGGATGCCCTTGAG-3′). PCR amplification of cDNA was performed in a final volume of 25 µl containing 2mL of 10X diluted cDNA, 1X PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, DMSO 5%, SYBR Green I 1% (100X diluted) (*Sigma*) and 2 units of Platinum *Taq* DNA polymerase (Invitrogen). The thermal conditions were 95°C for 2 min followed by 45 cycles of 94°C for 15 seconds, annealing temperature (AT) for 30 seconds and 72°C for 30 seconds. The AT for *NRGN* and *OLFM1* genes were 68°C and 65°C, respectively.

Identification of CpG islands in *NRGN* and *OLFM1* promoter regions

The CpG islands present in each gene were determined by the consensus sequences identified using three on line avaliable softwares for CpG island localization: *UCSC Genome Bioinformatics* (http://genome.cse.ucsc.edu), *EMBL-EBI CpGPlot* (http://www.ebi.ac.uk/emboss/cpgplot/) and *MethPrimer* (http://www.urogene.org/ methprimer/ index1.html). The criteria adopted to identify the CpGs islands included (1) CpG island size greater than 200pb, (2) CG percentage more than 50% and (3) CG/GC ratio greater than 60%.[13]

Sodium Bisulfite treatment

Genomic DNA samples cell lines were prepared by use of the Perfect gDNA Blood Mini kit (Eppendorf) according to the manufacturer's protocol. Sodium bisulfite genomic sequencing was performed essentially as described by Jeronimo et al.[14] with a few changes. Basically, two micrograms of genomic DNA from A172 and T98G cell lines without previous 5-Aza-dC treatment was diluted in 18µl of water, and 2µl of 2M NaOH solution was added. The mixture was incubated at 50°C for 20 minutes. After that, 500µl of sodium bisulfite solution (4M sodium bisulfite, 1M hydroquinone, 2M NaOH) was added and the reaction was incubated at 70°C for 3 hours. The treated DNA was purified using the Wizard DNA Clean-up System kit (Promega) according to the manufacturer's protocol, eluted in 45µl of water and disulfide by the addition of 5µl of 3M NaOH and incubation at room temperature for 10 minutes. The DNA was precipitated by the addition of 75µl of 5M ammonium acetate, 350µl of ethanol and 1µl of glycogen (20mg/ml), and incubation at -20°C for 16 hours. After 15 minutes of centrifugation at 13.000 rpm at 4°C, the DNA was washed with 500µl of ethanol 70% and resuspended in 50 µl of water.

Amplification of *NRGN* and *OLFM1* promoter regions

The CpG islands present in *NRGN* and *OLFM1* genes were amplified using a nested PCR strategy. Sodium bisulfite-treated genomic DNAs were amplified using *NRGN* and *OLFM1* gene-specific primers.

The first pair of primers for the *NRGN* gene was designed on -509/-484bp (NRGN GF - 5′-AAGTATGGAGGGATGGGTGAGGGGT-3′) and +301/+335 (NRGN GR - 5′-CTCACTAAAAA AAAAATTCCTCCTATCATCCAAC-3′) positions (the +1 position is the "A" nucleotide of ATG translation start codon). The second primers pair was designed on -453/-428 (NRGN PF - 5′-GTGTTTAGATGTGGGTGTTTGTGTT-3′) and +243/+268 (NRGN PR - 5′-TCCCAATCCA ACCTAAACTATCTAA-3′) positions. The *NRGN* amplified fragment has 724bp.

The first pair of primers for the *OLFM1* gene was designed on -316/-283pb (OLFM1 GF - 5′-GGTTTATTTTTGTTTAGGGGTTTAGGTTTTTA-3′) and +335/+370 (OLFM1 GR - 5′-CCATCAAAATAAAAAAAAAAAACTAAAAAA CTTC-3′) positions. The second primers pair was designed on -221/-195 (OLFM1 PF - 5″-AG ATGGAGTTTTTTATT TTAGTTTA-3′) and +272/+300 (OLFM1 PR - 5′-ACC TAACATAC

AAAACCCTAAATACTAA-3') positions. The *OLFM1* amplified fragment has 524pb.

First-round PCR amplification for both genes was performed in 20µl-reaction mixture containing 2µl of bisulfite-treated genomic DNA, 1mM $MgCl_2$, 0,25mM dNTPs, 0,5mM each of primers, 1X Taq Platinum Buffer (Invitrogen) and 1,5U Taq Platinum DNA Polimerase (Invitrogen). The amplification conditions for the *NRGN* gene were 95ºC for 2 min, 10 cycles of 94ºC for 30 sec, 72°C for 1 min and 72ºC for 1 min again; 10 cycles of 94ºC for 30 sec, 70°C for 1 min and 72ºC for 1 min; 15 cycles of 94ºC for 30 sec, 68°C for 1 min and 72ºC for 1 min. For the amplification of the promoter region of *OLFM1* gene, the PCR conditions were 95ºC for 2 min, 10 cycles of 94ºC for 30 sec, 62°C for 1 min and 72ºC for 1 min; 10 cycles of 94ºC for 30 sec, 60°C for 1 min and 72ºC for 1 min; 15 cycles of 94ºC for 30 sec, 58°C for 1 min and 72ºC for 1 min of final elongation.

To obtain products for sequencing, a second round of PCR (nested PCR) was performed with 1mL of the first PCR product (diluted 5x) for *NRGN* gene and 1mL of the PCR product of the *OLFM1* amplification reaction (diluted 50x). For the second round of PCR the same first PCR mixture was used. Amplification of *NRGN* gene was carried out under the following PCR conditions: 95°C for 2 min, 20 cycles of 95°C for 1 min, 64°C for 1 min, 72°C for 1 min and a final 7 min step at 72°C. The conditions for nested amplification of the *OLFM1* promoter region were: 95ºC for 2 min, 5 cycles of 94ºC for 30 sec, 62°C for 1 min and 72ºC for 1 min; 5 cycles of 94ºC for 30 sec, 60°C for 1 min and 72ºC for 1 min; 10 cycles of 94ºC for 30 sec, 58°C for 1 min and 72ºC for 1 min of final elongation.

Cloning and sequencing

The PCR products were cloned into pGEM-T vector (Promega) using the *pGEM T-Easy vector* kit (Promega) according to the manufacturer's protocol. Sequencing reactions were performed using *Big Dye Terminator Sequencing Kit* (Applied Biosystems) in an ABI 3100 autosequencer.

Results

Relative quantification of NRGN and OLFM1 genes in tumor cell lines

The quantification of the expression level of *NRGN* and *OLFM1* genes in different tumor cell lines showed that *NRGN* gene is highly hypoexpressed in human glioblastomas (A172 and T98G) and lung cell lines (H1299, H1155 and H249) in relation to the expression in normal tissues (figure 1). Some of the bladder (J82) and breast cancer cell lines (MDA-MB-435) showed a moderate expression of this gene. On the other hand, two of the colon cancer cell lines (DLD-1 and HCT116) analyzed showed an expression level of *NRGN* similar to the normal colon tissue. These data seems to indicate that the expression of *NRGN* gene could be highly repressed in brain and lung cancers and have a normal expression in colon cancer. However, experiments with large number of tumor samples are required to confirm these suspects.

The expression of *OLFM1* gene was also evaluated in the same cell lines and it was highly hipoexpressed in all cell lines analyzed (Figure 2).

Cell lines treatment with 5-Aza-dC

5-Aza-2'-Deoxycytidine (5-Aza-dC) is a common methylation inhibitor, which could be used *in vitro* to induce DNA hypomethylation. This drug inactivates the methyltransferase enzyme and causes hypomethylation of the newly synthesized DNA strands. The hypomethylation induced by 5-Aza-dC enables re-expression of silenced genes and cellular differentiation (*15*).

The glioblastoma cell lines (A172 and T98G) where submitted to 5-Aza-dC treatment with increasing concentration of 5-Aza-dC (0, 1, 5mM) for four consecutives days. After that, total RNA was extracted, cDNA molecules were synthesized and the relative expressions of the two genes were accessed by real time PCR. In both cell lines, an increment in the expression level of both genes could be detected after the treatment with the demethylating agent. The expression level of the *NRGN* gene showed an increase of 3 times in A172 and almost 9 times in T98G cell lines (Figure 3). On the other hand, the expression level of the *OLFM1* gene presented an increase of almost 2 times in A172 and more than 3 times in T98G cell lines (Figure 4).

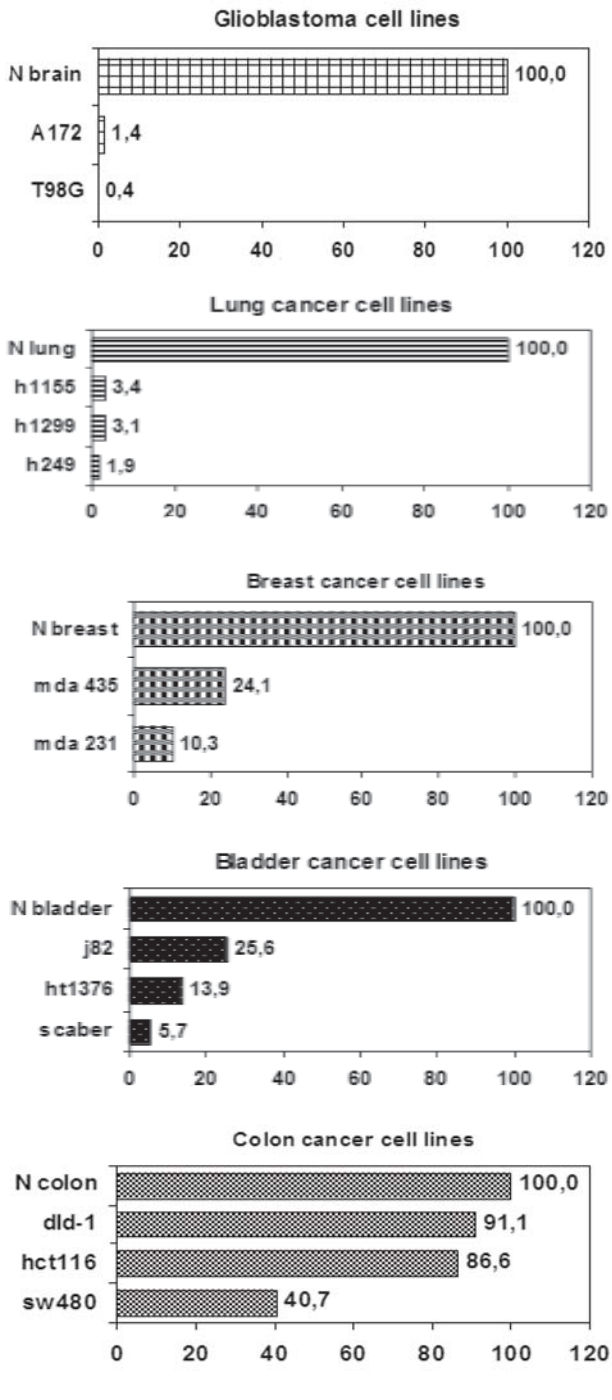

Figure 1 - Relative expression of *NRGN* in different tumors cell lines. The cell lines submitted to this experiment were derived from glioblastoma (T98G and A172), lung (H1299, H1155 and H249), breast (MDA-MB-231 and MDA-MB-435), bladder (J82, H1376 and SCABER) and colon (DLD-1, HCT116 and SW480). The relative expression was calculated using the 2^{-DDCt} method. The values are expressed in arbitrary units (U) in relation to the expression level in the normal tissues.

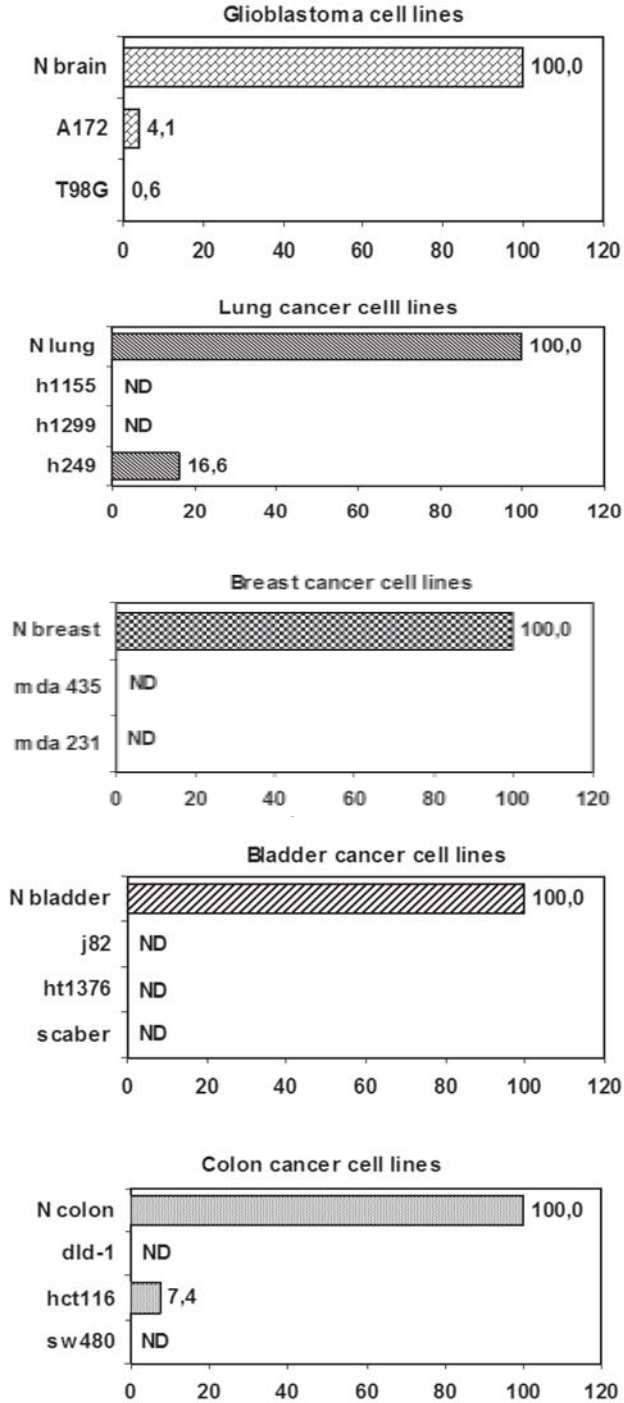

Figure 2 - Relative expression of *OLFM1* in different tumors cell lines. The cell lines submitted to this experiment were derived from glioblastoma (T98G and A172), lung (H1299, H1155 and H249), breast (MDA-MB-231 and MDA-MB-435), bladder (J82, H1376 and SCABER) and colon (DLD-1, HCT116 and SW480). The relative expression was calculated using the 2^{-DDCt} method. The values are expressed in arbitrary units (U) in relation to the expression level in the normal tissues

CpG islands Amplification, Cloning and sequencing

Using on line available tools, a CpG island spanning 698 nucleotides (-427/+268) and containing 57 CpGs were found in the *NRGN* promoter region.

In the *OLFM1* gene, a CpG island with 451 nucleotides (-148/+300) containing 62 CpGs was identified.

The DNA from T98G and A172 cell lines untreated with 5-Aza-dC were extracted and treated with Sodium Bisulfite. The CpG islands present in *NRGN* and *OLFM1* genes were amplified using nested PCRs and cloned in pGEM T vector (Promega). Six clones from each gene from each cell line were sequenced. None of the 6 *NRGN* clones derived from A172 cell line showed methylated CpGs, while the T98G showed 1 clone completely non-methylated, 4 clones with only 1 methylated CpG and 1 clone with 2 methylated CpGs (Figure 5). The CpG on position 2 was found methylated in 3 of the 6 clones analysed.

The analyses of the O*LFM1* clone sequences revealed no methylation in the A172 cell line and the presence of only 1 methylated CpG in two different clones of the T98G cell line (Figure 5).

Those results suggests that although the experiments with the demethylation agent 5-Aza-dC had shown an increase in the expression level of *NRGN* and *OLFM1* genes, the CpGs dinucleotides located in their promoter region did not show hypermethylation in the cell lines analysed.

Discussion

According to Marie et al.[10] the *NRGN* and *OLFM1* genes are hypoexpressed in astrocytic tumors. To verify if the reduction in the gene

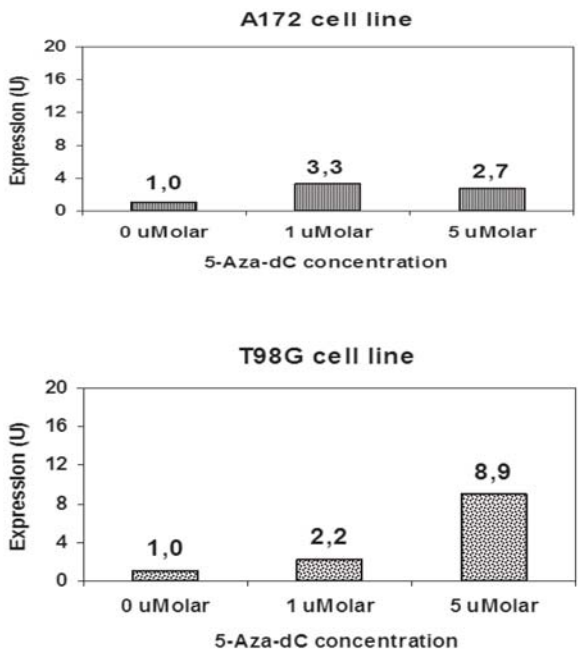

Figure 3 - Quantification of *NRGN* expression in glioblatoma cell lines submitted to 5 Aza dC treatment. The cell lines A172 and T98G were treated for 4 consecutive days with different concentrations of 5 Aza–dC (1 or 5mM). Numbers above each bar represent the expression induction (number of times) after each treatment with 5-Aza–dC in comparison with the corresponding untreated cell line (0mM)

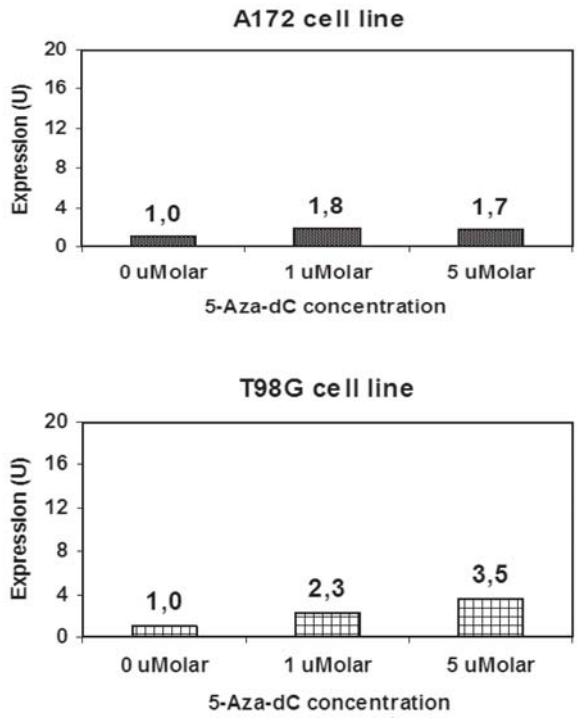

Figure 4 - Quantification of *OLFM1* expression in glioblatoma cell lines submitted to 5-Aza–dC treatment. The cell lines A172 and T98G were treated for 4 consecutive days with different concentrations of 5 Aza–dC (1 or 5mM). Numbers above each bar represent the expression induction (number of times) after each treatment with 5-Aza–dC in comparison with the corresponding untreated cell line (0mM)

expression level could be related with to hypermethylation we analyzed the methylation status of these genes in two glioblastoma cell lines (A172 and T98G). After treatment with 5-Aza-dC, a demethylating agent, it was possible to detect an increase of 1.8 to 8.9 times in expression level of both genes in the analyzed cell lines. This magnitude of increment is similar to those found by others studies. For example, Youssef et al (2004) observed that 5 days of 5-Aza-dC treatment (1 mM) reactivated the expression of the *TIG1* gene (3 times) in colon cancer cell lines when compared to respective non-treated cell lines. In another study, Nishioka et al.[17] had evaluated 14 lung cancer cell lines and had found expression reativaction of *MYO18B* gene in 11 of them (varying between 2 and 15 times) when compared to non-treated cell lines. In a study carried out for Akahira et al.,[18] the *TMS1* gene had an increase of 8 to 12 times in the expression level in ovarian cancer cell line OV90 after 3 days treatment with 5-Aza-dC.

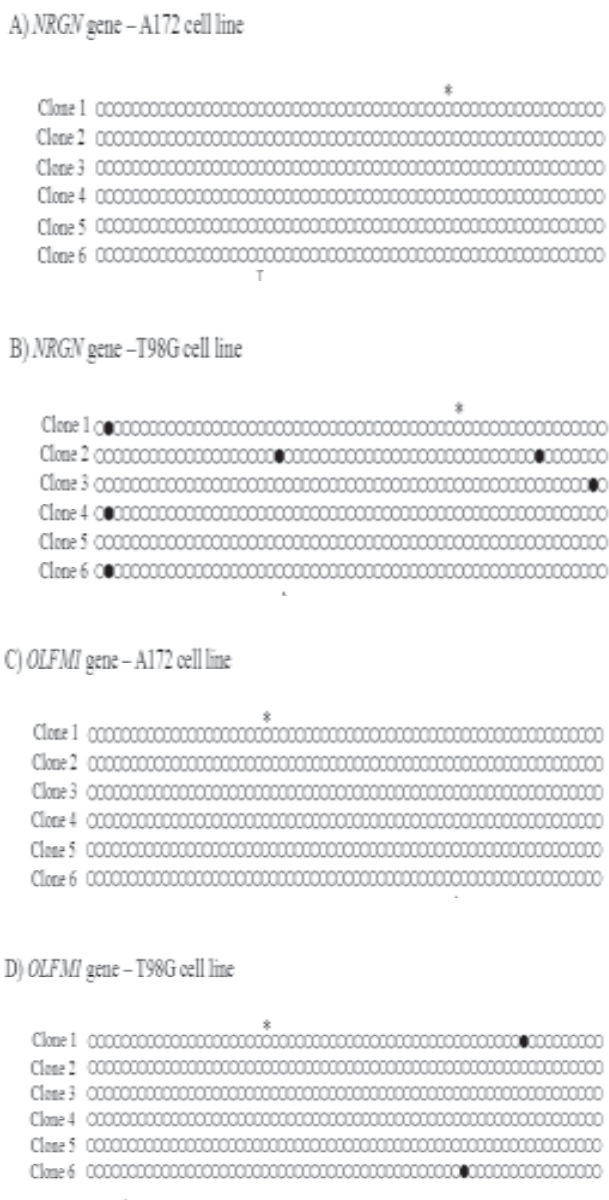

Figure 5 - Methylation status of CpG islands located in *NRGN* (57 CpGs) and *OLFM1* (62 CpGs) promoter regions. The glioblastoma cell lines A172 (A and C) and T98G (B and D) were cultivated and their genomic DNAs were extracted, treated with sodium bisulfite and sequenced. Each line of circles represents the CpGs present in each clone sequenced. The open circles represent the unmethylated CpG dinucleotides and the dark circles represent the methylated CpGs. The ⋆ represents the position of the ATG translation start codon

The DNA of A172 and T98G cell lines untreated with 5-Aza-dC was extracted and treated with Sodium Bisulfite. DNA fragments with the CpG islands situated in *NRGN* and *OLFM1* promoter regions were amplified, cloned and sequenced. The sequencing analysis showed that most of the CpGs dinucleotides were unmethylated in both genes. These results suggest that the reduction in *NRGN* and *OLFM1* expression levels observed in astrocytomas samples should not be directly associated with the hyper-methylation of their promoter regions. Schmelz et al.[19] used the *cDNA microarray* technique to investigate global changes in gene expression in cell lines derived from hematopoetic neoplasias (AML and MDS). They showed that the 5-Aza-dC treatment induced the re-expression of 81 genes. After sequencing the promoter regions of five genes (*RPGR, CD14, PTPN22, calgranulin* and *MPO*) in an AML cell line untreated with 5-Aza-dC, it was not possible to detect the presence of methylated CpG dinucleotides in *RPGR, CD14, PTPN22 and calgranulin* promoter regions. The authors suggest that induction of expression of these genes after treatment with 5-Aza-dC could be caused by demethylation and the consequent activation of transcription activators of these genes. In another study, Zhu et al.[20] observed that *CDKN2A* e *CDKN2D* genes were hypo-expressed in two lung cancer cell lines (H23 and H719). After the analysis of the methylation status, they demonstrated that *CDKN2A* promoter region was methylated and

the CpG island located in *CDKN2D* promoter region was demethylated. They treated cell lines with 5-Aza-dC and obtained an increase of expression level of both of genes. They concluded that the *CDKN2D* induction observed after 5-Aza-dC treatment associated to the ausence of methylated residues in its promoter region indicate that this gene could be under control of transcription factors codified by genes that have their expression controlled by methylation. Furthermore, Nguyen et al.[21] studied the *CDKN2A* promoter region and observed that the 5-Aza-dC treatment induced a substantial demethylation in the promoter region of this gene and reactivation of gene expression. According to authors, this reactivation is preceded by a chromatin remodeling caused by a drastic diminution of H3-K9 (Histone H3-lysine 9) methylation and an increase in the H3-K9 acetylation levels. The same authors analyzed the methylation status of $p14^{ARF}$ gene and verified that the promoter region of this gene is demethylated and the 5-Aza-dC treatment did not cause an increase in its expression. However, using the chromatin immunoprecipitation method (ChIP), they verified an increase in H3-K9 acethylation levels and H3-K4 methylation in $p14^{ARF}$ promoter region. The authors conclude that the 5-Aza-dC treatment induces the regional remodeling of chromatin structure, reduces the H3-K9 methylation, and increases the H3-K9 acethylation and the H3-K4 methylation, independently of its effects on DNA methylation.

Since we did not succeed in finding methylated CpGs in *NRGN* and *OLFM1* promoter regions, we hypothesize three possible theories to explain the expression induction of these genes by the 5-Aza-dC treatment: (1) the 5-Aza-dC treatment could promote the demethylation and expression of *NRGN* and *OLFM1* transcription activators. If this hypothesis is correct, it is the presence of these transcription activators that induces the *NRGN* and *OLFM1* expression and not the direct demethylation of their promoter regions. (2) The 5-Aza-dC treatment could have caused demethylation of enhancers' regulatory elements and this could have induced the *NRGN* and *OLFM1* expression. (3) The 5-Aza-dC treatment could be induced the demethylation and acetylation of the H3-K9 histone. Thus, the chromatin would be remodeled, becoming less condensed and the transcription activators could have access to transcription sites in *NRGN* and *OLFM1* promoters and, in this way, they could increase the gene expression.

The three hypotheses could be better understood if new investigations were carried out to identify the possible transcription factors or regulatory elements of *NRGN* and *OLFM1* genes. Thus, it would be able to evaluate the methylation status of these elements. Moreover, histones H3-K9 and H3-K4 of A172 and T98G DNAs would be studied by verifying their methylation and acethylation status.

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Original Article

Alaryngeal Communication Effectiveness And Long-term Quality Of Life

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Abstract

Background: Primary or salvage total laryngectomy is a surgical procedure used to treat patients with advanced-stage larynx and hypopharynx cancer. The resultant alaryngeal communication is usually considered unsatisfactory and a profound impairment. **Objectives:** The purpose of this study was to evaluate the efficacy of alaryngeal communication after total laryngectomy and its association with long-term quality of life evaluation. **Material and Methods:** 82 patients with squamous cell carcinoma of the larynx and hypopharynx underwent a total laryngectomy associated or not with irradiation therapy. The type of alaryngeal communication was 18 (21.9%) tracheoesophageal voice, 12 (14.6%) esophageal speech, 11 (13.4%) electrolarynx and 41 (50%) non-vocal. Communication effectiveness was judged according perceptual, acoustic and temporal parameters. The European Organization for Research and Treatment of Cancer Quality of Life Core Questionnaire (EORTC QLQ-C30) was used to measure quality of life. **Results:** Tracheoesophageal voice was considered good in 13 cases (72.2%), moderate in 4 (22.2%) and poor in 1 (5.6%); esophageal speech, good in 2 (16.7%), moderate in 8 (66.6%) and poor in 2 (16.7%); electrolarynx, good in 1 (9.1%), moderate in 9 (81.8%) and poor in 1 (9.1%); non-vocal communication, 100% poor. Total range of QLQ score varied from 8.3 to 100 (median, 75). Total QLQ scores were not associated with the effectiveness of communication (p=0.2512). **Conclusion:** Tracheoesophageal voice was more effective than esophageal speech or electrolarynx, but surprisingly alaryngeal communication was not considered by the patients essential to maintain or improve long- term quality of life.

Key words: Laryngeal neoplasm. Laryngectomy. Speech, Alaryngeal. Tracheoesophageal Voice. Speech, Larynx. Voice quality. Quality of life.

Introduction

Primary or salvage total laryngectomy is a surgical procedure traditionally used to treat patients with advanced-stage cancer of the larynx and hypopharynx, when the tumor cannot be resected by a partial surgical procedure. The recent introduction of organ-preservation chemoradiotherapy protocols established an alternative to total ablation of the larynx, but at least one third of the patients will require a salvage laryngectomy. The outcome of patients who underwent organ-preservation protocols, especially quality of life, must be compared with

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