Original Article

Hypermethylation Pattern of COX-2 and Rb1 in Head and Neck Squamous Cell Carcinomas

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Abstract

Head and neck cancer is among the ten most common human cancers worldwide, being 90% of all cases squamous cell carcinoma histological subtype. These tumors are always associated with high rates of mortality and patients with disease presenting in the same site with the same stage that under go the similar treatment, may have different oncologic outcomes. These aspects show the necessity of developing effective molecular markers that may be able to increase survival rates. Epigenetic mechanisms contribute to the carcinogenesis process, especially by the methylation of cytosines in CpG islands. The identification of aberrantly methylated DNA may help carcinogenesis understanding as well as potential clinical targets for studies in cancer. We analyzed genes *RB1* and *COX-2* methylation status by the quantitative, high-throughput Q-MSP assay in cell lines, 30 tumor samples and 10 normal oral cavity mucosa samples. These two genes evaluation was not informative because the incidence of hypermethylation was completely absent (*RB1*) or ubiquitous (*COX-2*), RB1, regardless of tissue type. These results suggest that the hypermethylation pattern of both *RB1* and *COX-2* might not be a reasonable biomarker for head and neck squamous cell carcinomas.

Keywords: Head and Neck Neoplasms. Carcinoma, Squamous Cell. Methylation. Cicloxygenase-2.

Introduction

Head and neck carcinomas are among the ten most common human cancers worldwide.[1] Around 90% of all cases are classified as squamous cell carcinoma (HNSCC) histological subtype[2] The upper aero-digestive tract, specially the oral cavity, pharynx and larynx, are the most affected areas.

This type of cancer is always associated with high rates of mortality, especially due to the advanced stage of the disease at diagnosis. Another important aspect that contributes to these rates is the high incidence of local-regional recurrences. However, it is important to take into account that patients with disease in similar sites and stages that undergo the same treatments may have different oncologic outcomes.These characteristics make necessary the development of effective molecular markers that may be able to detect the disease in early stages, determine prognostic, predict a better treatment and follow-up according to the specific features of each lesion and maybe lead to both an increase of overall survival and a reduction of treatment costs.

Squamous cell carcinoma is a result of different genetic changes that lead to alterations in the crucial metabolic pathways that maintain cellular homeostasis.

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These changes will contribute to an increase in growth factors production, membrane receptors, intracellular signaling and transcriptional factors. When this combines with the loss of activity of tumor suppressor genes, cells increase their proliferative potential, lose cell adhesion and acquires the ability of infiltrating tissues and spreading to distant areas. These changes are cumulative and can lead to the formation of atypical cells and eventually cancer.[3-4]

Besides genetic changes, epigenetic mechanisms can also contribute to silencing tumor suppressor genes, especially by the methylation of cytosines in CpG islands.[5] These alterations are inherited during cell-division and are defined as mechanisms that can change the phenotype of a particular gene, without affecting its nucleotide sequence or genotype.[6] CpG islands are clusters of CG nucleotides that usually occur in the promoter region of a gene. When a CpG island is methylated, the expression of this gene is usually repressed.[7] This repression is caused by changes in DNA structure, with the binding of specific proteins in the methylated region of the DNA and affinity reduction between transcriptional factors and the promoter region. This process has been described as the major cause of tumor suppressor genes inactivation.[8-9] Genes involved in the cell cycle control, DNA repair, apoptosis and angiogenesis have already been described as inactivated by methylation in many human cancers.[8-9]

The identification of aberrantly methylated DNA sequences in different tumor types represents a clinical target for studies in cancer.[7,10] Such process has been studied as potential molecular markers for many tumors, including HNSCC, and demethylating agents have already been studied as potential chemotherapic agents, confirming the role of methylation in the carcinogenesis process.[11-16]

Considering the important roles that the genes *COX-2* and *RB1* play in the carcinogenesis process and the existence of other studies showing that expression silencing of these genes, caused by promoter hypermethylation, can be correlated with tumor progression (Table 1), the aim of this study was to analyze the hypermethylation pattern of *COX-2* and *RB1* in HNSCC tumor samples and oral cavity normal mucosa.

Methods

Cell Culture

The HNSCC cell lines O12, O19 and FaDu, and the immortalized normal epithelium cell line HaCaT were maintained in a medium supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C at atmosphere with 5% of oxygen. After growth, cells were trypsinized and centrifugated. Cells were stocked at -70°C after removal of feeders.

Tissue Samples

Twenty-six primary paraffin-embedded HNSCC samples were selected from previously untreated patients submitted to surgery at the Department of Head and Neck Surgery and Otorhinolaryngology of A.C. Camargo Cancer Hospital, São Paulo, between 1989 and 2000. Ten normal mucosa tissue samples from healthy patients were obtained from odontological and pre-prosthetic surgeries at Faculdade Bahiana de Odontologia, Bahia, and frozen at -70°C. Written informed consent was obtained from all patients, and the study was approved by the institutional Ethics Committee.

DNA Extraction

The paraffin embedded samples were microdissecated in 10 sections of 10μm, which were scraped and de-paraffinated with xylol. The Genomic DNA was isolated from tumor and normal samples and cell lines by the standard proteinase K digestion and phenol-chloroform extraction method.[17] The DNA obtained was quantified with spectrophotometer and stored at -20°C. In order to check the presence and integrity of the DNA, a fragment of the *Albumin* gene (F: 5'-GCTGTCATCTCTTGTGGGCTGT-3'; R: AAACTCATGGGAGCTGCTGGTT-3') was amplified in cell lines and paraffin embedded samples and in normal samples, a fragment of the *BRCA1* gene (F: 5'-GAGAGGCATCCAGAAAAGTATCAG-3'; R: 5'-

Table 1 - List of gene names analyzed by QMSP in HNSCC

Gene Symbol	Name	Gene ID	Proposed funcion	Refs
RB1	Retinoblastoma 1	NM_000321	Cell cycle regulator, tumor supressor	4, 15, 17
COX-2	Cyclooxygenase 2	NM_000963	Prostaglandin synthesis from arachidonic acid	8, 26, 30

GGCTTAAGTTGGGGAGGT-3').The PCR conditions were 94˚C for 5 min; 35 cycles of 94˚C for 30 s, 66˚C and 61˚C respectively for 45 s, 72˚C for 45 s and a final extension at 72˚C for 7 min. The amplification was performed using 2 μL of MgCl2 (25 mM), 0,5 μL of dNTPs (10mM) (Invitrogen), 1,6μL of each primer (5 μM), 2,5 μL of reaction buffer 10X, 0,25 μL of TaKaRa Ex Taq (5 U/μl -TaKaRa Biomedicals) and 2μL of the DNA template in a total volume of 25μL. The PCR products were then analyzed in an 8% polyacrylamide gel and 1% agarose gel, respectively.

Sodium Bisulfite Conversion

DNA from either tumor or normal samples was treated with sodium bisulfite. This treatment results in the conversion of cytosine residues to uracil, while methylated cytosine remains unaffected. During PCR amplification, uracil residues are replaced by thymine, and methylcytosine residues are replaced by cytosine. These differences in sequence can be used to determine the methylation status of the original DNA. Sodium bisulfite treatment was conducted as described previously.[18] Briefly, 2μg of genomic DNA from each sample were firstly denaturated by incubating with 1μL of Hering Sperm DNA (10mg/mL) (INVITROGEN) and 2μL of 0,3M NaOH at 50ºC for 20 minutes. 500μL of a freshly made sodium bisulfite solution (sodium bisulfite 2.5M, hydroquinone 125mM and NaOH 350mM) were added to each sample which were incubated in the dark at 70ºC for 3 hours.The bisulfite-converted samples were purified using Wizard DNA Clean-up system kit (PROMEGA) and eluted in 45μL of water preheated to 80ºC.The eluted DNA was denatured in 0.3M NaOH for 10 minutes at 37oC and. Ammonium acetate 5M was added at the mixture and incubated for 2 minutes at room temperature. The DNA was then precipitated with 100% ethanol and glycogen (20μg/mL). Pellets were washed with 70% ethanol, left to dry at room-temperature, re-suspended in 110μL of water and stored at -80oC.

QMSP (Quantitative Methylation-Specific PCR or Methylight)

Sodium bisulfite converted DNA was used as a template for a fluorescence-based, real-time quantitative PCR, specific to detect the methylation pattern of DNA samples. In QMSP, primers and probes for the genes of interest (in this case *RB1* and *COX-2*) were designed considering only the methylated situation of the genes and were located in a high CpG content region around the transcriptional start codon of the genes. For the reference gene *ACTB*, the primers and the probe were designed in a region without CpG and it is supposed to amplify either methylated or unmethylated DNA, as long as it has been correctly converted by bisulfite treatment.The sequences were created using the softwares Gene Runner and MethPrimer (www.urogene.org/methprimer/index.html) (Table 2).

Table 2 - List of primer sequences of genes analyzed by QMSP assay in HNSCC

Gene symbol	Oligonucleotide sequences (5´-3´)
ACTB	Forward Primer TGGTGATGGAGGAGGCTCAGCAAGT Reverse Primer ACCAATAAAACCTACTCCTCCCTTAA Probe ACCACCACCCAACACACAATAACAAACACA
COX-2	Forward Primer CGGAAGCGTTCGGGTAAAG Reverse Primer AATTCCACCGCCCCAAAC Probe TTTCCGCCAAATATCTTTTCTTCTTCGCA
RB1	Forward Primer TTAGTTCGCGTATCGATTAGC Reverse Primer ACTAAACGCCGCGTCCAA Probe TCACGTCCGCGAAACTCCCGA

Serial dilutions of a control sample (*in vitro* methylated lymphocyte by the action of SssI-methyltransferase enzyme (New England Biolabs) were used in each reaction plate to generate a standard curve which allows the quantification of the methylated DNA amount in each sample. The proportion of methylation was calculated by the ratio *GENE of interest / ACTB* and multiplying by 100. We use the abbreviation PMR (percent of methylated reference) to indicate measurement.[19] Each set of primers was first optimized using cell line DNA and confirmed using patients' DNA.

The efficiency of all reactions was controlled by including a methylated controlled DNA.

Results

Total DNA was isolated from the cell lines (~155 µg), tumor (~36 µg) and control specimens (~37 µg). The presence and integrity of tumor samples and cell lines DNA were confirmed by amplification of 141bp fragment of *Albumin* gene (Figure 1A). A 664bp fragment of the *BRCA1* gene was amplified from the normal samples (Figure 1B). The amplification of all samples confirmed the quality of all DNA.

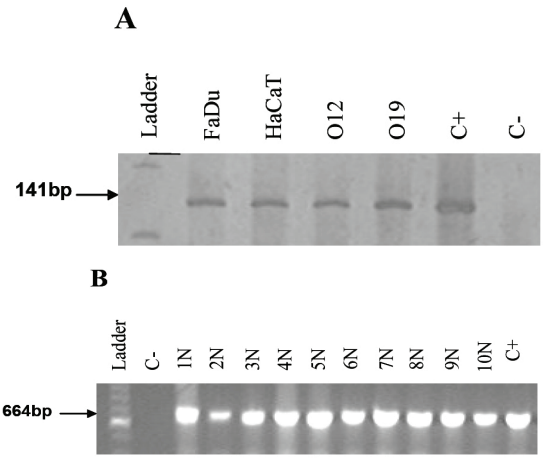

Figure 1 - PCR analysis for the presence and integrity of DNA extracted and efficiency of sodium bisulfite conversion. A. Amplification of DNA extracted from cell lines for albumin gene (141bp). Ladder: 100bp DNA Ladder (Invitrogen); C+: cell line A549; C-: reaction without any input of DNA. B. PCR reaction for gene *BRCA1* (664bp) using DNA extracted from normal oral cavity tissues as template. Ladder: 100bp DNA Ladder (Invitrogen); C+: cell line HeLa; C-: reaction without any input of DNA.

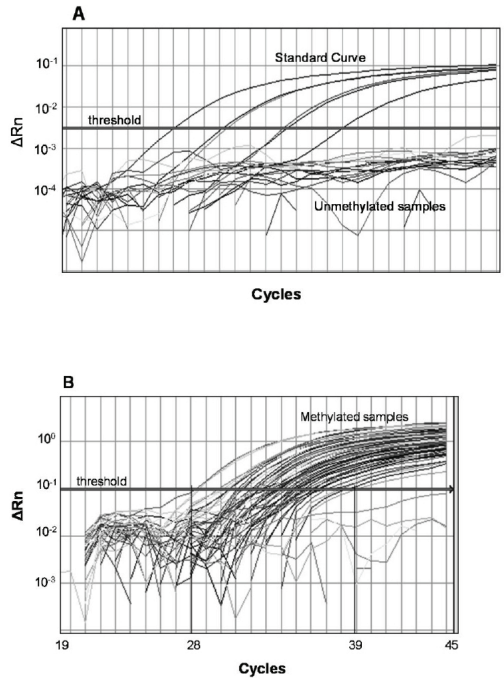

Figure 2 - Amplification plots showing the different patterns of methylation for genes *RB1* and *COX-2*. A. Plot showing the lack of amplification in the methylation pattern analysis of *RB1* in tumor samples and the standard curve obtained by reactions using human lymphocyte DNA methylated by treatment in vitro with SssI methyltransferase, diluted in 10-fold increments up to 1:100 000 with the purpose of creating a standard curve. B. Amplification plot obtained from the analysis of *COX-2* in tumor samples, showing hypermethylation in most cases. The threshold used for the calculation of initial template amounts is indicated by the dark horizontal line.

QMSP reactions for the gene *RB1* did not detect any amplification in cell lines (0/4) or in tumor samples (0/30), showing that this gene is not aberrantly methylated in analyzed cases (Figure 2A). For this reason, we did not perform the analysis in the normal samples. All samples scored positive for DNA input as measured by the amplification of control gene *ACTB*. Therefore, the lack of amplification cannot be attributed to a lack of input DNA. The integrity and specificity of all QMSP reactions were also confirmed by using in vitro methylated human lymphocytes as positive controls and to create a standard curve to normalize DNA input (Figure 2A).

On the other hand, gene *COX-2* is methylated in the cell lines FaDu, O12, O19 and HaCaT, in the normal oral cavity tissues and in most tumor samples, regardless of tissue type (Figure 2B). However, the relative quantification obtained based on PMR values showed a great range of values. The oropharyngeal squamous cell carcinoma cell line FaDu and the normal epithelium cell line HaCaT showed a low proportion of methylated molecules (0.46 and 0.42%, respectively), while the oral SCC cell lines O12 and O19 exhibit 92 and 52% of methylated molecules, respectively). The evaluation of this gene in the HNSCC cases found a frequency of 94% (28/30) of methylated samples. However, we also found methylation of *COX-2* in all normal oral cavity samples evaluated (10/10). For both the tumor and the normal samples, the PMR values also varied greatly among cases (Figure 3). No correlation could be established between these results and the clinic-pathological information from these patients.

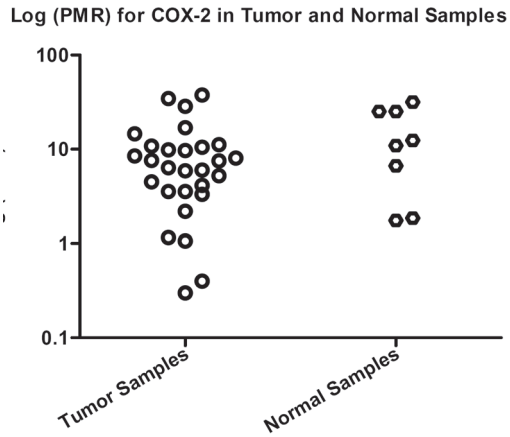

Figure 3 - Graphic showing the distribution of cases using Log (PMR) for gene *COX-2* in HNSCC and normal tissues. The percentage of fully methylated molecules at a specific locus (PMR) was calculated by dividing the *GENE/ACTB* ratio of a sample by the *GENE/ACTB* ratio of a control sample and multiplying by 100.

Discussion

We have used a high-throughput, fluorescence-based methylation assay (QMSP) to examine the hypermethylation patterns of CpG islands from 2 different genes, *RB1* and *COX-2*, with the purpose of identifying pattern differences between head and neck tumor and normal samples. The extensively methylated molecules assayed by this technique are likely to represent alleles that have been completely silenced by CpG island hypermethylation, although this was not investigated in this study. The evaluation of these two genes was not informative because the incidence of hypermethylation was either completely absent or ubiquitous, regardless of tissue type (Figure 3).

RB1 is a gene participating in DNA transcriptional regulation, and it controls the passage from G1 stage to S stage of the cell cycle. Retinoblastoma protein (pRB) is controlled by CDKs (cyclin-dependent kinase) and CDK inhibitors and acts in the negative regulation of cell progression.[20] The inactivation of pRB regulation is an early event necessary to the development of retinoblastoma and is also involved in the tumorigenesis of other cancers, like osteosarcomas and melanomas. The promoter CpG island methylation of this gene is observed in around 10% of cases of retinoblastomas, being this mechanism involved in significant reductions of gene expression.[21-22] Our results in QMSP analysis of *RB1* suggest that the hypermethylation pattern of this gene might not be a reasonable biomarker for head and neck squamous cell carcinomas, although it is frequently hypermethylated in retinoblastoma tumors. This suggests that there may be different tissue-specific interaction factors that modulate methylation changes in CpG islands of this gene during tumorigenesis according to cancer type, HNSCC or retinoblastomas. Also, there may be a lack of selective silencing by methylation of this gene in HNSCC cancer type. Although this gene could possibly be inactivated by a different mechanism, this may not be the case as we have evaluated the expression pattern in the HNSCC cell lines O12, O19 and FaDu by reverse-transcriptase reaction and found this gene expressing in all of them (data not shown). Maybe this gene do not have a role in tumor suppression in HNSCC.

Cyclooxygenase enzyme expressed by the gene *COX-2* is responsible for the conversion of arachidonic acid in prostaglandins, especially PGE-2, potential inflammatory mediators. This enzyme is expressed in a reduced number of tissues and can be associated with carcinogenesis by contributing to the proliferation, invasion and metastasis of various solid tumors as a consequence of an increase in cell proliferation, differentiation, immunosuppression, angiogenesis, apoptosis inhibition, cell motility and adhesion to epitheliums.[23-24] When overexpressed, the protein gives tumors an invasive phenotype in many epithelial cancers and can be used as a predictor factor for a low response to radiotherapy.[23] This gene is hypermethylated and downregulated in colorectal tumors and gastric cancers.[25] Studies have shown that therapies like 5-aza-2'-deoxycytidine were able to induce the re-expression of *COX-2* in several cell lines. Therefore, demethylating agents might be an appropriate treatment for lesions in which this gene is silenced.[26-28]

Many previous studies that analyzed the expression pattern of *COX-2* in HNSCC show a close relation between the aberrant expression of this gene and a worse prognosis.[29] Such influence seem to vary according to the tumor nature, suggesting that this gene might be involved in many pathways such as cell proliferation, angiogenesis and immunosuppression.[29] On the other hand, the presence of this gene expression in head and neck tumors allows the use of *COX-2* inhibitor drugs (coxibs) that might act as chemotherapic agents. Moreover, this drugs seem to be safer than nonsteroidal anti-inflammatory drugs (NSAIDs).[30-32] This discussion suggests that the high percentage of hypermethylation in some tumors might confer a better prognostic to these patients, but the presence of methylation prevents the use of coxibs as chemotherapic agents. Furthermore, we found a similar pattern of methylation both in the tumors and in normal samples. This fact suggests that

the hypermethylation pattern of *COX-2* might not be a useful molecular marker for HNSCC. Also, no correlation could be found between the methylation patterns and the clinic-pathological characteristics of tumor samples.

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References

1. Parkin DM, Pisani P, Ferlay J. Estimates of the world wide frequency of eighteen major cancers in 1990. Int J Cancer 1999; 80:827-41.
2. Collins SL. Squamous cell carcinoma of the oral cavity, oropharynx, pharyngeal wall, and nasopharynx. In: Ballenger JJ, snow JB, editors. Otorhinolaryngology – Head and Neck Surgery. Media-PA: Williams & Wilkins; 1996. p.249-368.
3. Califano J, van der Riet P, Westra W, Nawroz H, Clayman G, Piantadosi S, Corio R, et al. Genetic progression model for head and neck cancer: implication for field cancerization. Cancer Res 1996; 56:2488-92
4. Williams HK. Molecular pathogenesis of oral squamous carcinoma. J Clin Pathol Mol Pathol 2000; 53:165-72.
5. Ha P, Califano JA. Promoter methylation and inactivation of tumour-supressor genes in oral squamous cell carcinoma. Lancet Oncol 2006; 7:77-82.
6. Feinberg AP, Tycko B. The history of cancer epigenetics. Nat Rev Cancer 2004; 4:143-53.
7. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002; 3:415-28.
8. Ushijima T, Watanabe N, Okochi E, Kaneda A, Sugimura T, Miyamoto K. Fidelity of the methylation pattern and its variation in the genome. Genome Res 2003; 13:868–74.
9. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003; 33 Suppl:245-54.
10. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003; 349:2042-54.
11. Belinsky SA. Gene-promoter hypermethylation as a biomarker in lung cancer. Nat Rev Cancer 2004; 4:707–17.
12. Rosas SL, Koch W, Costa Carvalho MG, Wu L, Califano J, Westra W, et al. Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer Res 2001; 61:939-42.
13. Viswanathan M, Tsuchida N, Shanmugam G. Promoter hypermethylation profile of tumor-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. Int J Cancer 2003; 105:41-6.
14. Ishida E, Nakamura M, Ikuta M, Shimada K, Matsuyoshi S, Kirita T, et al. Promotor hypermethylation of p14ARF is a key alteration for progression of oral squamous cell carcinoma. Oral Oncol 2005; 41:614-22.
15. Ogi K, Toyota M, Ohe-Toyota M, Tanaka N, Noguchi M, Sonoda T, et al. Kohama G, Tokino T: Aberrant methylation of multiple genes and clinicopathological features in oral squamous cell carcinoma. Clin Cancer Res 2002; 8:3164-71.
16. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004; 429:457–63.
17. Wolff RK, Frazer KA, Jackler RK, Lanser MJ, Pitts LH, Cox DR. Analysis of chromosome 22 deletions in neurofibromatosis type 2-related tumors. Am J Hum Genet 1992; 51:478-85.
18. Jeronimo C, Henrique R, Oliveira J, Lobo F, Pais I, Teixeira MR, et al. Aberrant cellular retinol binding protein 1 (CRBP1) gene expression and promoter methylation in prostate cancer. J Clin Pathol 2004; 57:872-6.
19. Trinh BN, Long TI, Laird PW. DNA Methylation Analysis by MethyLight Technology. Methods 2001; 25:456-62.
20. Ducket AS, Dagnino L, Gallie BL. Retinoblastoma: patients, tumors, gene and protein contribute to the understanding of cancer. Mol Gen Cancer 2001; 297-315.
21. Ohtani-Fujita N, Dryja TP, Rapaport JM, Fujita T, Matsumura S, Ozasa K, et al. Hypermethylation in the retinoblastoma gene is associated with unilateral, sporadic retinoblastoma. Cancer Genet Cytogenet 1997; 98:43-9.
22. Klutz M, Horsthemke B, Lohmann DR. RB1 gene mutations in peripheral blood DNA of patients with isolated unilateral retinoblastoma. Am J Hum Genet 1999; 64:666-7.
23. Ferrandina G, Lauriola L, Distefano MC, Zannoni GF, Gessi M, Legge F, et al. Increased cyclooxygenase-2 expression is associated with chemotherapy resistance and poor survival in cervical cancer patients. J Clin Oncol 2002; 20:973-81.
24. Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE, Schror K. Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res 1999; 59:198-204.
25. Wang B-C, Guo C-Q, Sun C, Sun Q-L, Liu G-Y, Li D-G. Mechanism and clinical significance of cyclooxygenase-2 expression in gastric câncer World J Gastroenterol 2005; 21:3240-4.
26. Van Rees BP, Saukkonen K, Ristimaki A, Polkowski W, Tytgat GN, Drillenburg P, et al. Cyclooxygenase-2 expression during carcinogenesis in the human stomach. J Pathol 2002; 196:171-9.
27. Yu J, Leung WK, Lee T-L, Tse PC, To KF, Sung JT. Promoter hypermethylation of cyclooxygenase-2 in gastric carcinoma. International Journal of Oncology 2003; 22:1025-31.
28. Toyota M, Shen L, Ohe-Toyota M, Hamilton SR, Sinicrope FA, Issa J-PJ. Aberrant methylation of the Cyclooxygenase 2 CpG Island in Colorectal Tumors. Cancer Research 2000; 60:4044-8.
29. Jaeckel EC, Raja S, Tan J, Das SK, Sudhansu KD, Girod DA, et al. Correlation of Expression of cyclooxygenase-2, vascular endothelial growth factor, and peroxissome proliferators-activated receptor δ with head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2001; 127:1253-9.
30. Oshima M, Taketto MM. Cox selectivety and animals models for colon cancer. Curr Pharm Des 2002; 8:1021-34.
31. Kawzmori T, Rao CV, Seibert K, Reddy BS. chemopreventive activity of Celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res 1998; 58:409-12
32. Eads CA, Lord RV, Wickramasinghe K, Long TI, Kurumboor SK, Bernstein L, et al. Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res 2001; 61:3410-8.