Functional analysis of polymorphisms in the COX-2 gene and risk of lung cancer

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Abstract. The enzyme cyclooxygenase 2 (COX-2) is known to be involved in tumorigenesis and metastasis in certain types of cancer. Nevertheless, the prognostic value of COX-2 overexpression and its polymorphisms in patients with non-small cell lung cancer (NSCLC) have yet to be fully elucidated. The aim of the present study was to investigate the association between the three most commonly studied COX-2 gene polymorphisms (-1195 G/A, -765 G/C and 8473 T/C) with COX-2 expression and lung cancer risk in a Brazilian cohort. In the present hospital based, case-control retrospective study, 104 patients with NSCLC and 202 cancer free control subjects were genotyped for -1195 G/A, -765 G/C and 8473 T/C polymorphisms using allelic discrimination with a reverse transcription quantitative polymerase chain reaction method. COX-2 mRNA expression was analyzed in surgically resected tumors from 34 patients with NSCLC. The results revealed that COX-2 expression levels were higher in tumor tissue compared with normal lung tissue. However, this overexpression of COX-2 was not associated with the patient outcome, and furthermore, none of the analyzed polymorphisms were associated with the risk of developing lung cancer, COX-2 overexpression, or the overall survival of the patients with NSCLC. Taken together, the findings described in the present study do not support a major role for COX-2 polymorphisms and COX-2 overexpression in lung carcinogenesis within the Brazilian population.

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Introduction

Lung cancer is the most common cause of cancer-associated mortality throughout the world (1). The incidence rates and deaths associated with this cancer type have risen markedly over the last century, correlating with an increase in cigarette consumption (1,2). Although accumulative evidence suggests that >80% of lung cancers are attributed to tobacco exposure, <20% of smokers develop lung cancer, suggesting that genetic susceptibility exerts an important role in the etiology of lung cancer (3,4). Several different pathways are involved in the pathogenesis of lung cancer, of which inflammatory processes and genes involved in the associated functional pathways have been of emerging research interest (5).

Cigarette smoke triggers bronchial epithelial cells to produce pro-inflammatory cytokines (e.g., interleukin-1β), and to up-regulate several inflammation-associated genes, including cyclooxygenase-2 (COX-2) (6-8). Cyclooxygenase is an important enzyme required for the conversion of arachidonic acid into prostaglandins (PGs) and thromboxane. Two different COX isoforms have been described, termed COX-1 and COX-2, which possess different properties (9,10). COX-1 is constitutively expressed in the majority of the cells and tissues, whereas COX-2 is inducible, and is expressed in response to cytokines, growth factors and other stimuli (11,12). Different solid tumor types have been demonstrated to overexpress COX-2, including those of the colon (13), prostate (14), breast (15), esophagus (16), lung (17), and pancreas (18). These tumors contained high concentrations of prostaglandin E2 (PGE2), a subproduct of the enzymatic action of COX-2 (19). PGE2 is able to affect cell proliferation, apoptosis and angiogenesis, thus contributing to tumor progression (20).

The mechanisms underpinning the regulation of COX-2 expression have yet to be fully elucidated, and may be influenced by genetic variations. A number of genetic variants that may affect enzyme expression have been described in regions proximal to the regulatory sites of the *COX-2* gene (21,22), and could contribute to an increased risk of

cancer development. It was also suggested that single nucleotide polymorphisms (SNPs) in the *COX-2* gene may alter enzyme function, which could influence an individual's risk of any type of cancer (23).

The polymorphism, -1195 A/G, was revealed to influence transcription levels of *COX-2*, where the A-allele had an increased rate of transcription compared with the G-allele in *in vivo* studies of esophageal tissues and in luciferase reporter assays performed in HeLa cells (22). Another SNP, -765 G/C, resulted in lower promoter activity, which subsequently led to a lower expression of *COX-2* (24). The C-allele of the polymorphism -765 G/C is associated with markedly reduced expression levels of COX-2 compared with the G-allele, and this effect might be mediated by the loss of Sp1 transcription factor binding to its cognate element (21). Recently, the polymorphism -1195 A/G was reported not to be associated with susceptibility to oral cancer, whereas -765 G/C and +837 T/G presented a clear link (25).

Stability of COX-2 mRNA and the synthesis of COX-2 may be increased by genetic variations in the 3'untranslated region (3'UTR) of the prostaglandin endoperoxide synthase 2 (PTGS2) gene (an alternative name for COX-2). The 3'UTR region of *COX-2* has been shown to be an important determinant of the stability of the mRNA, and thus, of the enzyme levels (26). Carriers of the C-allele of *COX-2* 8473 T/C were revealed to exhibit higher basal and induced levels of PGE2 compared with the T-allele (27). In addition, the frequency of SNPs in the *PTGS2* gene may vary between different ethnic groups (28,29). Certain polymorphisms in the *COX-2* promoter region may explain the different levels of COX-2 expression that have been identified in lung tumors. The frequency of these polymorphisms was previously determined in a Brazilian population (30); however, its influence on COX-2 expression has yet to be elucidated. In the present study, the impact of three different polymorphisms in the *COX-2* gene (-1195 G/A, -765 G/C and 8473 T/C) on COX-2 expression was investigated, as well as its influence on the risk of lung cancer in a Brazilian cohort. Furthermore, the expression of COX-2 was analyzed with respect to the overall survival of patients with NSCLC.

Patients and methods

Study population. The present case-control study included 104 patients with NSCLC and 202 cancer-free control subjects. The eligible cases included patients who were diagnosed with primary NSCLC between June 2005 and February 2008 at the Brazilian National Cancer Institute (INCA), Rio de Janeiro, Brazil. There were no age, gender, or stage restrictions; however, patients with prior cancer history were excluded from this study. The control subjects were cancer-free individuals selected from INCA's Tobacco Treatment Program (http://www2.inca.gov.br/wps/wcm/connect/acoes_programas/site/home/nobrasil/programa-nacional-controle-tabagismo).

Volunteers were personally interviewed by trained personnel using a structured questionnaire to determine demographic characteristics and potential risk factors for lung cancer. Information regarding the clinical history was obtained from medical records. All subjects were informed and provided with a written consent form, in order to participate in the study and to allow their biological samples to be genetically analyzed. The study was approved by the Ethics Committee of INCA (Protocol 79/05). The clinicopathological features of the patients with NSCLC are shown in Table I.

COX-2 genotyping. Genomic DNA was extracted from peripheral blood from all volunteers (cases and control subjects) using the QIAamp DNA Mini kit (Qiagen, Inc., Valencia, CA, USA), following the manufacturer's protocol. All subjects were genotyped by allelic discrimination using a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method. The assay reagents for genotyping the SNPs were obtained from the Assays-by-Design service of Applied Biosystems (Applied Biosystems), and consisted of a 40X mix of unlabeled PCR primers and TaqMan minor groove binder probes [fluorescein (FAM) and VIC™ fluorochrome dye-labeled]. These assays were designed for the genotyping of the specific SNPs as follows: -1195 G/A: Forward primer, CCTGAGCACTACCCATGATAGATGT, reverse primer, GGAACATAGTTGGGTGAGGGATTAA; probes: VIC-, AAGATGAAATTCCAACTGTCA, FAM-, ATGAAATTC CAGCTGTCA; -765 G/C: Forward primer, TGCTTAGGA CCAGTATTATGAGGAGAA; reverse primer, CCCCCTCCT TGTTTCTTGGAA; probes: VIC-, CTTTCCCGCCTCTCT, FAM-, CCTTTCCCCCCTCTCT; 8473 T/C: Forward primer, GCATCTTCCATGATGCATTAGAAGTAAC, reverse primer, GCACTGATACCTGTTTTTGTTTGATGA; probes: VIC-, CTTTTGGTCATTTTTC, FAM-, ACTTTTGGTTAT TTTTC. Each assay enabled the scoring of the two genotypes in a single well. The probes were distinguished by labeling them with a different fluorescent reporter dye (i.e., FAM dye or VIC dye). RT-qPCR analysis was performed using an ABI Prism 7500 Fast instrument (Applied Biosystems). A marked increase in either FAM or VIC dye fluorescence indicated homozygosis for the FAM- or the VIC-specific allele, respectively, whereas an increase in the two signals indicated heterozygosis.

RNA isolation and relative quantification via RT-qPCR. A total of 34 tumor tissue samples were obtained from surgically removed specimens of individual patients. Total RNA was isolated from tissues, using TRIzol reagent (Invitrogen Life Technologies). FirstChoice® PCR-Ready Human Lung cDNA (cat. no. AM3327; Ambion®; Applied Biosystems) was used for the analysis. An aliquot of total RNA (2 μg) from each specimen was reverse-transcribed into single-strand complementary DNA (cDNA) using oligo(dT) 15 primer and Superscript II (Invitrogen Life Technologies). Relative gene expression quantification for *COX-2*, with *β-actin* as an internal reference gene, was performed using the ABI Prism 7500 Sequence Detection system (Applied Biosystems) based on the TaqMan method available at the company's website. The primer used for *COX-2* was Hs00153133_m1, and that for β-actin was 4352935E (as featured in the Taqman® gene expression assay).

Relative quantification was performed using the comparative threshold cycle (Cq) method of RT-qPCR, and data were expressed on the logarithmic scale (31). Subsequently, for each tissue sample (n=34), the expression levels of COX-2 and the endogenous control protein, β-actin, were estimated

in duplicate using RT-qPCR for 40 cycles, with the arithmetic average threshold cycle (C_q) used for data analysis. To control variations in the amount of RNA input, reactions were performed with the β-actin probe used as the internal control. Furthermore, for each RNA sample tissue, negative control reactions with: i) Negative controls of cDNA synthesis (i.e., without reverse transcriptase) and ii) no-template controls were performed in duplicate. Subsequently, relative gene expression levels for *COX-2* were calculated according to the $2^{-\Delta Cq}$ method (31), with ΔC_q [COX-2] values determined using the formula: ΔC_q [COX-2]=C_q [COX-2]-C_q [β-actin]. The degree of significance of the mean difference between tumor tissue and the control cDNA (FirstChoice® PCR-Ready Human Lung; Ambion®; Applied Biosystems) was estimated from the log-transformed, normalized expression levels.

Statistical methods. The Hardy-Weinberg equilibrium was examined to compare genotype COX-2 polymorphism frequencies among case subjects and controls. A linkage disequilibrium analysis among the SNPs was performed using the statistical parameters, D' and r^2. Odds ratios and 95% confidence intervals were calculated using logistic regression in order to estimate the risk for lung cancer. SNPs and COX-2 expression association was analyzed using Mann-Whitney's *t*-test. Regarding the overall survival rate, times were obtained from the date of diagnosis to death. Time-to-death parameters were estimated using the Kaplan-Meier method, and data were compared using the log-rank test. Cox proportional hazards models were used to estimate the risk factors in a multivariate model. P<0.05 was considered to indicate a statistically significant value. Statistical analyses were performed using SPSS 20.0 for Windows software (IBM SPSS, Armonk, NY, USA).

Results

Demographic characteristics of patients and control subjects. The general clinical and pathological features of patients with NSCLC are shown in Table I. The demographic variables and risk factors of lung cancer for the cases of the 104 patients with NSCLC and the 200 controls included in the analysis are shown in Table II. The cases and controls appeared to be adequately matched regarding color as suggested by χ^2 test. However, the case group had a higher prevalence of men, people aged >60 years old and non-smokers than did the control group (P<0.05). These differences were controlled in the later multivariate analyses.

COX-2 polymorphisms and occurrence of lung cancer. A total of 340 participants (200 volunteer donors and 104 patients with NSCLC) were genotyped for the *COX-2* polymorphisms, -1195G/A, -765G/C and 8473 T/C, as shown in Table III. For *COX-2* -1195 G/A, 72 (18%) patients and 36 (17%) controls carried the G-allele. For *COX-2* -765 G/C, 299 (75%) patients and 101 (25%) controls carried the G-allele. For *COX-2* 8473 G/C, 244 (61%) patients and 164 (79%) controls carried the T-allele. All genotypic distributions were consistently within the limits defined by the Hardy-Weinberg equilibrium (P>0.05).

Subsequently, the association between the SNPs, -1195G/A, -765G/C and 8473 T/C, and the occurrence of lung cancer was examined. No risk association was identified for the distribution of the genotypes between the overall lung cancer cases and the controls (P>0.05), as shown in Table III. Regarding linkage disequilibrium, the findings of the present study indicated strong pairwise linkage disequilibrium involving the SNPs, -765 and 8473 (D'=0.9807, r^2=0.58). By contrast, no significant association was demonstrated between the SNPs -765 and -1195 (D'=0.6064, r^2=0.027) or -1195 and 8473 (D'=0.7050, r^2=0.069).

Table I. The general clinical and pathological features of our patients with NSCLC.

Patient characteristics	Cases n (%)
Histological cell type	
Adenocarcinoma	54 (51.9)
Squamous cell	41 (39.4)
Other	9 (8.7)
Tumor differentiation	
Poor	12 (11.5)
Moderate	32 (30.8)
Well	4 (3.8)
Undifferentiated	3 (2.9)
Stage	
I/II	38 (38)
III/IV	62 (62)
Performance status	
0-1	14 (13.9)
2-3	87 (86.1)

NSCLC, non-small cell lung cancer.

Overall survival analysis according to COX-2 polymorphisms and COX-2 expression. The overall survival analysis was calculated and correlated with *COX-2* polymorphism and expression. The polymorphisms were grouped according to the variant allele: Homozygous for the variant allele compared with homozygous for the wild-type and heterozygous. The 2-year overall survival rates for patients with the -1195 G/A AA and AG/GG genotypes were 43.1% and 52.8, respectively (P=0.360; Fig. 1A). For the -765 G/C polymorphism, these rates were 39.7% for GG, and 64.1% for GC/CC, patients (P=0.758; Fig. 1B). For the 8473 T/C SNP, the 2-year overall survival rates were 51.8 and 42.6% for the TT and TC/CC alleles, respectively (P=0.684; Fig. 1C). No differences in median overall survival were identified with respect to the three *COX-2* polymorphisms studied. Subsequently, whether the expression of COX-2 may be a potential prognostic biomarker for patients with lung cancer was examined. The 34 patients in which COX-2 expression had been previously determined were stratified into high and low expression groups according to the median. Neither the high nor the low expression of COX-2 was identified to be a prognostic indicator for patients with NSCLC (P=0.235; Fig. 1D).

COX-2 expression in lung tumor vs. normal lung tissue and according to the COX-2 polymorphism. To evaluate COX-2

Table II. Demographic variables and risk factors of lung cancer of cases and controls.

Variables	Cases n (%) 104 (100%)	Controls n (%) 200 (100%)	P-value[a]
Sex			
Male	70 (67.3)	71 (35.5)	<0.001
Female	34 (32.7)	129 (64.5)	
Color			
White	52 (50)	84 (42.0)	0.412
Intermediate	32 (30.8)	71 (65.5)	
Black	20 (19.2)	45 (22.5)	
Age			
>60 years	63 (60.6)	26 (13.0)	<0.001
≤60 years	41 (39.4)	174 (87.0)	
Smoking status			
Non-smokers	7 (6.7)	5 (2.5)	0.036
Light smokers	16 (15.4)	55 (27.5)	
Moderate smokers	41 (39.5)	78 (39.0)	
Heavy smokers	40 (38.4)	62 (31.0)	
Quit smoking			
Non-smokers	7 (6.7)	5 (2.5)	<0.001
≤12 months	42 (40.4)	44 (22.0)	
>12a ≤120 months	14 (13.5)	9 (4.5)	
>120 months	16 (15.4)	0 (0)	
Smokers	25 (24.0)	142 (71.0)	
Drinking			
No	57 (54.8)	62 (31.0)	<0.001
Yes	47 (45.2)	138 (69.0)	

[a]Compared by χ^2 test.

expression in tumor tissue, 34 patients with NSCLC who underwent surgery were included in the present study. The clinical and demographic characteristics of these 34 patients are shown in Table IV. The relative mRNA expression of COX-2 in tumor tissues of cancer patients was determined using RT-qPCR and compared with FirstChoice® PCR-Ready Human Lung cDNA as the reference standard. As shown in Fig. 2A, a difference in COX-2 expression levels between tumor tissue and the commercially available normal lung tissue was observed. The tumor tissue had a higher expression level compared with the normal lung tissue (P=0.001), with an average expression of 2,654 (95% confidence interval =212.08 to -5,096.70).

The expression of COX-2 was also evaluated according to the genotypes for each *COX-2* polymorphism studied. As shown in Fig. 2B-D, no significant differences were identified when compared with COX-2 expression for the -1195 G/A (P=0.446; Fig. 2B), -765 G/C (P=0.843, Fig. 2C) and 8473 T/C (P=0.545; Fig. 2D) COX-2 polymorphisms.

Discussion

COX-2 is overexpressed in premalignant and malignant stages of lung, colon, and breast cancer, suggesting that COX-2 may serve an important functional role from the earliest hyperproliferative stages of the disease to the later stages of invasive carcinoma (32). Therefore, the present study has been, to the best of our knowledge, the first to address the role of COX-2 expression and the -1195G/A, -765G/C and 8473 T/C polymorphisms in lung cancer within the Brazilian context.

In the present retrospective study, the frequency and linkage of COX-2 polymorphisms was analyzed. Unlike previous studies (22,23,33-35), no significant associations were identified between the SNPs, -765 G/C and -1195 G/A. On the other hand, corroborating the results of another Brazilian study (30), a genetic linkage between the -765 G/C and 8473 T/C polymorphisms was demonstrated. This linkage between them reveals a non-random distribution of these proteins. Therefore, the contributions of -765 G/C and 8473 T/C towards *COX-2* expression and activity may be difficult to delineate.

Secondly, the influence of these *COX-2* polymorphisms on the occurrence of lung cancer was analyzed. No association was identified between these polymorphisms and this tumor type in our case-control study. Notably, there are conflicting reports concerning the impact of -1195 G/A, -765 G/C and 8473 T/C polymorphisms on the risk of cancer in the literature (22,35-37). Supporting our findings, a meta-analysis study

Table III. ORs (odds ratios) for lung cancer in relation to the studied polymorphisms.

SNP	Genotype/allele	Cases n (%)	Controls n (%)	OR (95% IC)[a]	P-value
G1195A	A	172 (83)	328 (82)	Ref	
	G	36 (17)	72 (18)	1.049 (0.675-1.629)	0.212
	AA	71 (68.3)	138 (69)	Ref	
	AG	30 (28.8)	52 (26)	1.186 (0.567-2.479)	0.651
	GG	3 (2.9)	10 (5)	3.498 (0.555-22.037)	0.182
	AG+GG	33 (31.7)	62 (31)	1.381 (0.692-2.754)	0.360
G765C	G	164 (79)	299 (75)	Ref	
	C	44 (21)	101 (25)	0.7943 (0.531-1.187)	0.261
	GG	66 (63.5)	112 (56)	Ref	
	GC	32 (30.8)	75 (37.5)	0.967 (0.404-2.314)	0.940
	CC	6 (5.8)	13 (6.5)	1.224 (0.220-6.819)	0.817
	GC+CC	38 (36.5)	88 (44)	1.046 (0.456-2.399)	0.916
T8473C	T	131 (63)	244 (61)	Ref	
	C	77 (37)	156 (39)	0.919 (0.651-1.299)	0.477
	TT	44 (42.3)	69 (34.5)	Ref	
	TC	43 (41.3)	106 (53)	1.803 (0.778-4.182)	0.169
	CC	17 (16.3)	25 (12.5)	1.414 (0.359-5.567)	0.621
	TC+CC	60 (57.7)	131 (65.5)	1.726 (0.751-3.966)	0.198

[a]ORs adjusted by gender, age, smoking status and quit smoking. IC, confidence interval.

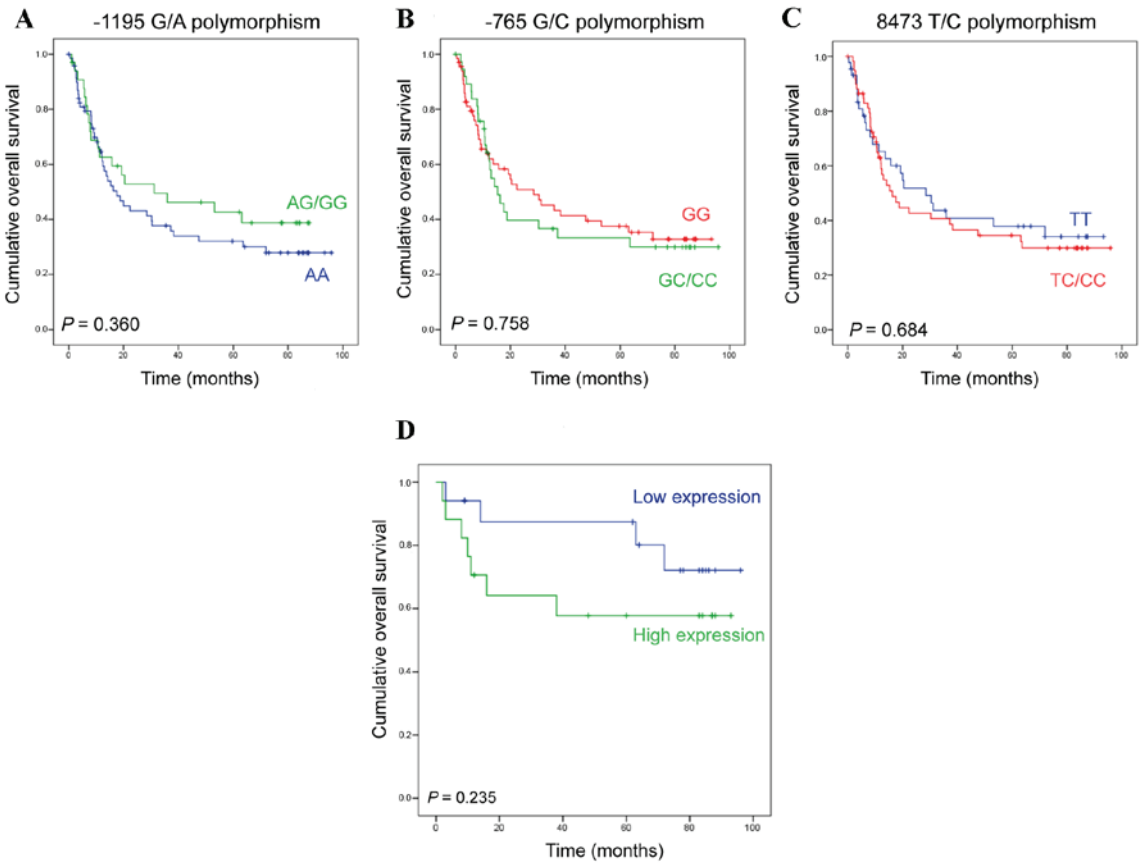

Figure 1. Overall survival curves of patients according to the (A) -1195 G/C, (B) -765 G/C and (C) 8473 T/C COX2 polymorphisms and the mRNA expression of COX2. The polymorphisms were grouped according to the variant allele: Homozygous for the variant allele compared with homozygous for the wild-type and heterozygous, and subjects were genotyped using allelic discrimination by a reverse transcription quantitative polymerase chain reaction method. Survival over time (months) was determined as shown in A, B and C. (D) The 34 patients with non-small cell lung cancer in which COX2 expression had been previously obtained were stratified into high and low-expression groups according to the median. The survival over time (months) was determined.

Table IV. Clinic and demographic variables of lung cancer patients who underwent surgery.

Variable	Cases n (%) 34 (100)
Gender	
Male	19 (55.9)
Female	15 (44.1)
Color	
White	20 (58.8)
Intermediate	9 (26.5)
Black	5 (14.7)
Age	
>60 years	16 (47.1)
≤60 years	18 (52.9)
Smoking status	
Non-smokers	2 (5.9)
Light smokers	7 (20.6)
Moderate smokers	13 (38.2)
Heavy smokers	12 (35.3)
Quit smoking	
Non-smokers	4 (11.8)
≤12 months	15 (44.1)
>12a ≤120 months	4 (11.8)
>120 months	8 (23.5)
Smokers	2 (5.9)
Drinking	
No	16 (47.1)
Yes	18 (52.9)
Histological cell type	
Adenocarcinoma	19 (55.9)
Squamous cell	15 (44.1)
Others	-
Tumor differentiation	
Poor	5 (14.7)
Moderate	20 (58.8)
Well	4 (11.8)
Undifferentiated	0 (0)
Stage	
I/II	22 (64.7)
III/IV	10 (29.4)
Performance status	
0-1	31 (91.2)
2-3	1 (2.9)

performed by Wang *et al* (38) with 29,487 cancer patients and 3,921 controls demonstrated that -765 C carriers are at a significantly increased risk of contracting gastric cancer, leukemia, and pancreatic cancer, but not of other cancer types, including lung cancer. Furthermore, Tang *et al* (39) demonstrated that -1195 G/A is a low penetration risk factor for cancer. In addition, Pan *et al* (40), also in a meta-analysis study with 4,373 lung cancer patients and 5,468 controls, demonstrated that the 8473 T/C polymorphism is not associated with any risk of lung cancer. These negative results could be explained by the fact that the functional mechanisms of such *COX-2* polymorphisms may not be responsible for lung carcinogenesis, or they may be attributable to the source of controls for each study or methodological deficiencies in the analysis.

One possible reason for *COX-2* polymorphisms being only associated with certain cancer types (22,28,36,37,41) might be due to different cancers having distinct molecular signatures. For example, the lung carcinogenesis activation pathway occurs through a variety of mechanisms, including activating mutations in the genes for epidermal growth factor receptor (*EGFR*), *KRAS*, p53, and also echinoderm microtubule-associated protein-like 4 (*EML4*) and anaplastic lymphoma kinase (*ALK*) fusions. The oncogene, KRAS, is mutated in ~30% of cases of lung cancer (42); the mutational status of the *EGFR* gene has been shown to be correlated with responsiveness to small molecule kinase inhibitors (e.g., gefitinib or erlotinib) (43,44); and tumor protein 53 (*TP53*) inactivation is one of the most significant genetic abnormalities described in lung cancer, occurring in ~90% of small cell carcinomas and ~65% of cases of NSCLC (45). Taken together, these differences may also influence the impact of the COX-2 polymorphisms on the lung carcinogenesis process.

The COX-2 8473 T/C polymorphism may contribute to NSCLC cancer susceptibility in the Kashmiri population (46). Bi *et al* (47) demonstrated that the -1195 G/A polymorphism may be able to predict survival in patients with lung cancer. Their functional study revealed that the nucleotide base change of -1195 G to A creates a c-MYB binding site in the COX-2 promoter region and, thus, displays a higher promoter activity. Therefore, the -1195 AA genotype, which results in increased COX-2 expression, was associated with poor overall survival in Chinese patients. To validate this hypothesis, the influence of -1195 G/A was analyzed in the present study in addition to, for the first time to the best of our knowledge, the -765 G/C and 8473 T/C polymorphisms in the outcome of the Brazilian patients with NSCLC. However, differences in overall survival regarding the three *COX-2* polymorphisms studied were not observed. One reason for this discrepancy may be the fact that certain genetic markers are ethnicity-specific. Further prospective clinical trials with a larger sample size comprising different ethnic populations are required to solve these controversial results.

In the present retrospective study, COX-2 mRNA expression in the tumor lung tissue of 34 patients with NSCLC in comparison with a commercially obtained, normal lung tissue mRNA was also analyzed using RT-qPCR. It was observed that the tumor tissue had higher expression levels of COX-2 mRNA compared with normal tissue (Fig. 2A). Bhat *et al* (46) also observed a significant increase in the level of COX-2 in NSCLC tumor tissues when compared with normal lung tissues. Generally speaking, COX-2 is expressed in ~40-80% of neoplastic cells, and the level of expression is higher in cancerous cells compared with non-cancer cells (7,48). Krzystyniak *et al* (49) demonstrated that COX-2 upregulation affects angiogenesis and the production of specific proteases that are critical to lung cancer growth and metastasis. COX-2 also affects tumor progression by stimulating lymphagiogenesis (50).

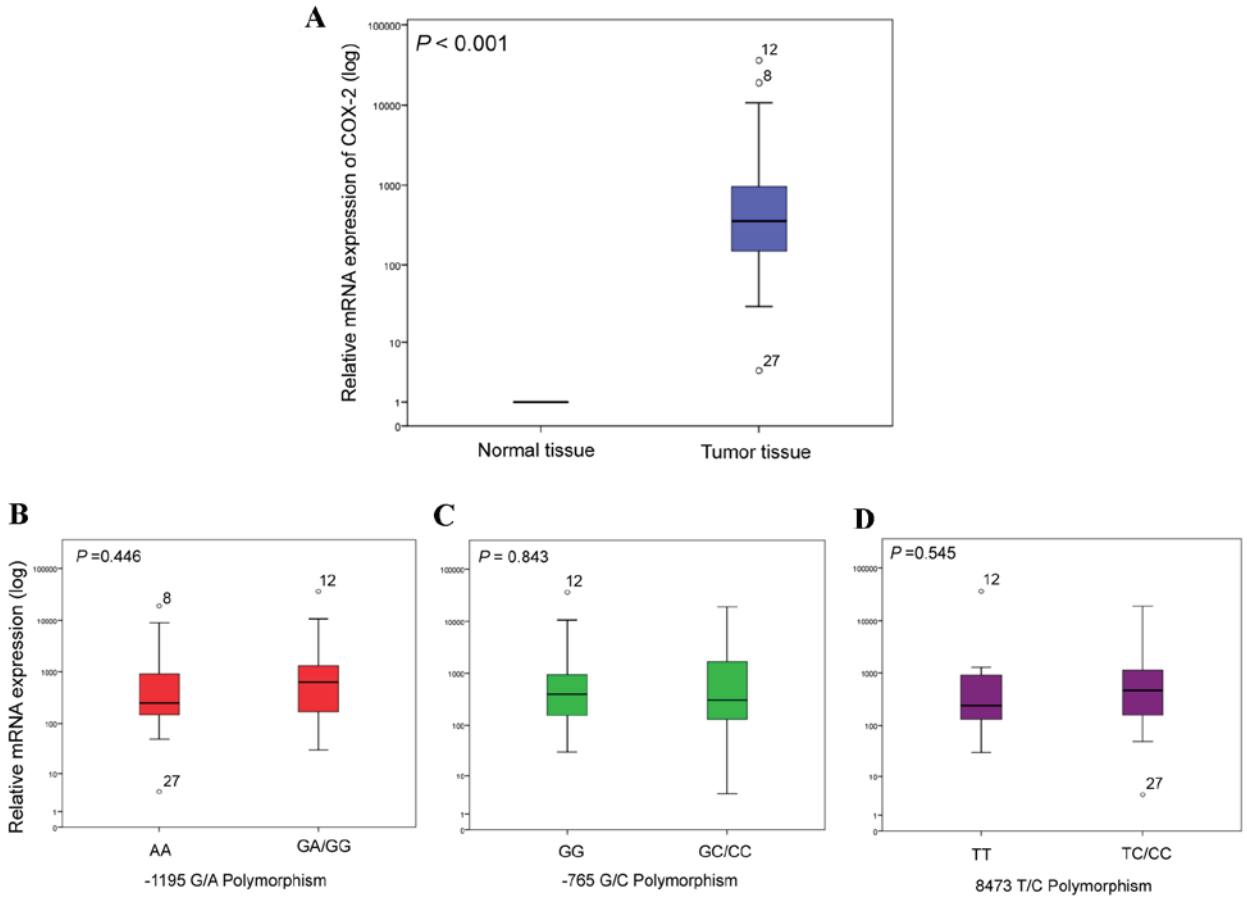

Figure 2. Relative mRNA expression of COX2 by RTPCR according to tumor vs. (A) normal tissue and according to the three COX2 polymorphisms, (B) -1195 G/A, (C) -765 G/C and (D) 8473 T/C. Relative gene expression quantification for COX2, with β-actin as an internal reference gene, was performed. Significance of the mean differences between tumor tissue and the control cDNA from normal lung tissue was estimated on log-transformed normalized expression levels, as shown in (A). (BCD) The relative mRNA expression of COX2 correlated with the COX2 polymorphisms, (B) -1195 G/A, (C) -765 G/C and (D) 8473 T/C.

In spite of these previous results, the prognostic value of COX-2 overexpression in patients with lung cancer remains controversial. Thus, in the present study, the next goal was to analyze whether COX-2 expression was associated with poor prognosis in Brazilian patients with NSCLC. It was revealed that expression of COX-2 was not a prognostic indicator for NSCLC (P=0.235; Fig. 1D) in our Brazilian cohort. Two systematic reviews and meta-analysis studies have corroborated the present study, also demonstrating that COX-2 overexpression does not appear to have any significant impact on the survival of patients with NSCLC (51). By contrast, in a study by Zhan *et al* (51), statistical significance was identified in stage I lung cancer, suggesting that COX-2 expression may be useful as a prognostic indicator during the early stages of cancer to distinguish between those with a worse prognosis.

Evidence derived from a functional analysis study revealed that the -1195 G/A polymorphism creates a cMYB binding site, thus increasing the transcriptional activity of COX-2 through HeLa cell lines (22). The present study has analyzed, to the best of our knowledge for the first time, whether the overexpression of COX-2 was associated with the -1195 G/A, -765 G/C and 8473 T/C polymorphisms in 34 Brazilian patients with NSCLC, and the conclusion drawn from this analysis is that no significant differences were observed (Fig. 2B-D).

In conclusion, the present study has been the first to describe how, in a Brazilian cohort of patients with NSCLC, the -1195 G/A, -765 G/C and 8473 T/C *COX-2* polymorphisms were not associated with any risk of lung cancer, or with the outcome or with COX-2 expression. Nevertheless, it would be interesting to perform a larger prospective study and to compare tumor lung tissue vs. normal adjacent lung tissue in each sample. Even though the present study has disclosed that COX-2 expression was higher in tumor tissue, this parameter was not a prognostic indicator for our cohort of patients with NSCLC. Further functional studies based on a larger sample size are required to determine the effects of COX-2 polymorphisms on the process of lung carcinogenesis.

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