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Quantitative detection of cyclooxygenase- 2 gene expression in carcinoma of larynx by real- time polymerase chain reaction*

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[ABSTRACT] **AIM:** To quantify cyclooxygenase- 2 (COX- 2) mRNA in carcinoma of larynx and evaluate the correlation between the quantity of COX- 2 mRNA and clinical staging or histological grade. **METHODS:** The expression of COX- 2 mRNA in 30 cases of carcinoma of larynx tissue and adjacent non- cancerous tissues were evaluated by PCR, which includes a fluorescence dye, SYBR green Ⅰ, and the sequence specific primer. The GAPDH was used as control. **RESULTS:** The specificity of products was proved to be COX- 2 and GAPDH by the analysis of the melting curve of the amplified products and agarose gel electrophoresis. The expression of COX- 2 mRNA was detected in all cancerous tissues of 30 patients (100%), but only in 12 adjacent non- cancerous tissues of 30 patients (40%). The N_{COX} value of carcinoma of larynx tissue and adjacent non- cancerous tissues was 16.54 ±13.27 and 9.24 ±6.91, respectively, and the expression levels of COX- 2 mRNA elevated significantly in laryngeal squamous cell carcinoma tissue and there were significant correlation between the expression levels of COX- 2 mRNA and clinical stage or histological grade. **CONCLUSION:** The expression of COX- 2 mRNA in carcinoma of larynx can be determined by real- time PCR technique. An increase in COX- 2 mRNA may be associated with carcinogenesis of carcinoma of larynx, and it may be useful as a biomarker in laryngeal cancer.

[KEY WORDS] Cyclooxygenase- 2; Laryngeal neoplasms; Fluorescent dyes; Gene expression

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Cyclooxygenase (COX) is the rate- limiting enzyme involved in the biosynthesis of prostaglandins (PG) that takes part in keeping the body homeostasis from arachidonic acid (AA). Recent studies have indicated that two isoforms of COX exist, referred as COX- 1 and COX- 2. COX- 1 is constitutively expressed in most tissues and thought to be responsible for maintaining gastric mucosal integrity, regulating the blood of kidney, controlling platelet aggregation etc. The expression of COX- 2 is very low or not detectable in most tissues, but can be highly induced in response to cell activation by a variety of stimuli[1]. COX- 2 has been shown to overexpress in a wide variety of epithelial tissue tumors (such as gastrointestinal cancer, cancer of colon, carcinoma of prostate, pulmonary carcinoma, cancer of breast, tumor of head and neck, squamous epithelial cell carcinoma of skin etc.)[2]. The expression of COX- 2 in carcinoma *in situ* and precancerous lesion were significantly higher than normal tissues, the value of expression is 20- 80 times more than that in normal[3]. So the purpose of the present study was to determine the expression of COX- 2 mRNA in laryngeal carcinoma and its adjacent non- cancerous tissues at different stages by real- time PCR method. We want to elucidate the role of COX- 2 in occurrence and development of laryngeal carcinomas, which would provide preliminary experimental evidence for therapy of laryngeal carcinoma and help estimate prognosis.

MATERIALS AND METHODS

1 Tissue samples

Tumor tissue and adjacent non- cancerous tissues of laryngeal carcinomas were obtained from 30 cases admitted to our department from 2002- 2003. No radiotherapy or chemotherapy had been taken before surgery. 24 patients were male, and 6 were female. The mean age of the patients was 61 (range, 41- 79 years). The specimens were classified into 3 stages according to the Tumor- Node- Metastasis (TNM) stage determined by UICC in 1997,

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these 30 cases of laryngeal carcinomas were classified as stage Ⅰ (10), stage Ⅱ (8), and stage Ⅲ/Ⅳ (12). All 30 specimens were diagnosed as the squamous cell carcinoma according to the pathologic examination and classified into 3 histological degree of differentiation: well differentiated (WD) (10), moderately differentiated (MD) (9), and poorly differentiated (PD) (11). The adjacent non-cancerous normal tissues came from the same specimen but 2 cm outside to tumor tissue. All samples were kept in liquid nitrogen.

2 Main reagents and instrument

Trizol (Invitrogen), Oligo (dT), M-MLV, RNasin inhibitor (Promega), dNTP, Taq enzyme (Jingmei company in shenzhen), SYBR Green Ⅰ (OPE Technology Development Company). Lightcycler Ⅱ (Fluorescence PCR instrument, Roche, Switzerland), microcapillary tube (Roche, Switzerland), COX-2 and GAPDH primers were synthesized by Saibaisheng company in Beijing. The sequences were as follow: GAPDH sense 5'-TGGGTGTGAACCATGAGAAG-3'; antisense 5'-GCTAAGCAGTTGGTGGTGC-3'; 80 bp in length COX-2 sense 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'; antisense 5'-AGATCATCTCTGCCTGAGTATCTT-3'[4]; 305 bp in length.

3 RNA extraction and synthesis of cDNA

50-100 mg frozen tissue was pulverized in a mortar containing 1 mL trizol, and then chloroform was added to precipitate protein. RNA was isolated by precipitation with isopropanol. RNA pellets were washed in 75% ethanol, air-dried and dissolved in water treated with DEPC. RNA stored at -80 ℃, A_{260}/A_{280} ratio of the extracted RNA samples were measured by RNA/DNA calculator. cDNA was synthesized from 1-25 μg of RNA in a total volume of 25 μL including of 100 mg/L Oligo(dT) 5 μL, dNTPs (10 mmol/L) 2.5 μL, 5× buffer 5 μL, M-MLV 200 U, RNasin treated H_2O to 25 μL. The cDNA synthesis was done in 37 ℃ 60 min, then 95 ℃ 5 min.

4 Real-time PCR

Real-time PCR was carried out using a LightCycler (Roche). Reactions were set up in microcapillary tubes. To optimize the concentration of $MgCl_2$ and annealing temperature by laryngeal carcinoma cell line (Hep-2), the concentrations of $MgCl_2$ used is 2.5, 3.0, 3.5 mmol/L, respectively, choose the best one. Taq enzyme was 2.0 U as usual. The reaction mixture consisted of 10× buffer 2.0 μL, cDNA 1 μL, 10mmol/L dNTP 0.5 μL, Taq enzyme 2.0 U, 100 mmol/L of each primer 0.5 μL and 20× SYBR green 1 (1 μL), a total volume was 20 μL. Amplified protocol of COX-2 is as follow: 95 ℃ 120 s, 95 ℃ 10 s, 53 ℃ 10 s, 72 ℃ 30 s, 83 ℃ 1 s (fluorescent data were acquired), 40 cycles, 95 ℃ 0 s, 72 ℃ 0 s, 95 ℃ 0 s, 40 ℃ 0 s (a melting curve program).

Amplified protocol of GAPDH was as follow: 95 ℃ 120 s, 95 ℃ 10 s, 55 ℃ 10 s, 72 ℃ 30 s, 83 ℃ 1 s (fluorescent data were acquired), 40 cycles, 95 ℃ 0 s, 72 ℃ 0 s, 95 ℃ 0 s, 40 ℃ 0 s (a melting curve program).

5 Measure the samples

With the best condition, assay the 30 cases samples; H_2O was used as a negative control every time. Dissociation curve were analyzed using dissociation program.

6 Calculation

To calculate the expression level of COX-2 mRNA by $N_{COX}= CT_{COX-2}/CT_{GAPDH}$, CT (threshold cycles).

7 Agrose gel electrophoresis

Product in microcapillary tube was confirmed by electrophoresis on a 1.5% agrose gel stained with ethidium bromide.

8 Statistical analysis

Data were analyzed using SPSS 10.0 software. All the data were shown as $\bar{x} \pm s$. Comparisons between groups were made by the Student's t test.

RESULTS

1 Quality identification of the extracted RNA

The A_{260}/A_{280} ratio of the extracted RNA samples determined with RNA/DNA calculator was 1.7-1.9, which suggested that the purity of the extracted RNA samples was good.

2 Optimal reaction condition

The efficiency of amplification was measured with real-time PCR by comparing the threshold cycles (CT), the less CT, the higher efficiency. We found that the CT was 16, 12, 11.5 with 2.5, 3.0, 3.5 mmol/L of $MgCl_2$, respectively, so the specificity and efficiency of amplification were good with 3.5 mmol/L of $MgCl_2$ (Fig 1). The suitable annealing temperature of COX-2 was 53 ℃, GAPDH was 55 ℃.

3 Dissociation curve

The specificity of the real time PCR product was examined by both running the product in agarose gel and dissociation curve analysis. Dissociation curve plots (melting temperature analysis) showed only one peak for each product of COX-2 and GAPDH (Fig 2a, b), thus indicating

that specific amplification formation had occurred during the PCR. The peak of primer- dimmers in dissociation curve was eliminated because of increasing the temperature - detected fluorescence. Agarose gel electrophoresis showed single GAPDH and COX- 2 fragment with the predicted size of 80 and 305 bp, respectively (Fig 3a, b).

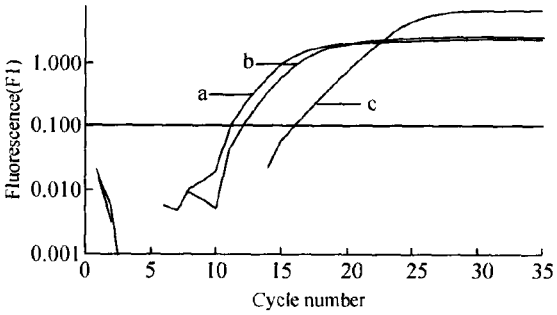

Fig 1 Influence of $MgCl_2$ on GAPDH PCR amplification. Curve, a is the amplification at the concentration of 3.5 mmol/L $MgCl_2$, b is 3.0 mmol/L and c is 2.5 mmol/L. The CT values were measured as 11.5, 12 and 16 respectively when using three kinds of concentrations of $MgCl_2$ (3.5, 3.0, 2.5 mmol/L). The concentration of 3.5 mmol/L was optimal by comparing the threshold cycles (CT) after real- time PCR using SYBR green Ⅰ.

4 Expression of COX- 2 mRNA in the carcinomas and adjacent non- cancerous tissues

When the CT value of COX- 2 is above 40, we think that the sample hasn't the expression of COX- 2. The expression of COX- 2 mRNA was detected in all 30 cases in laryngeal carcinomas; however, the positive rate of COX- 2 mRNA in adjacent non- cancerous tissues was 40% (12 out of 30). The N_{COX-2} in carcinoma and adjacent non- cancerous tissues was 16.54 ± 13.27 and 9.24 ± 6.91 (average $\pm$variance), respectively. A significant difference was detected in the expression of COX- 2 mRNA between in carcinoma and in adjacent non- cancerous tissues ($t = 2.438$, $P < 0.05$).

5 The relation of pathological factors and the expression of COX- 2

According to the N_{COX} value calculated from the formula ($N_{COX} = CT_{COX-2}/CT_{GAPDH}$), we found that the expression of COX- 2 mRNA had a significant difference in clinical stage among Ⅰ, Ⅱ and Ⅲ/Ⅳ and histological grade among WD, MD and PD (pathological grade and stage of 30 cases are shown in Tab 1).

DISCUSSION

Gene expression analysis plays an increasingly important role in many fields of biological research. The recently developed real- time PCR quantification method has many

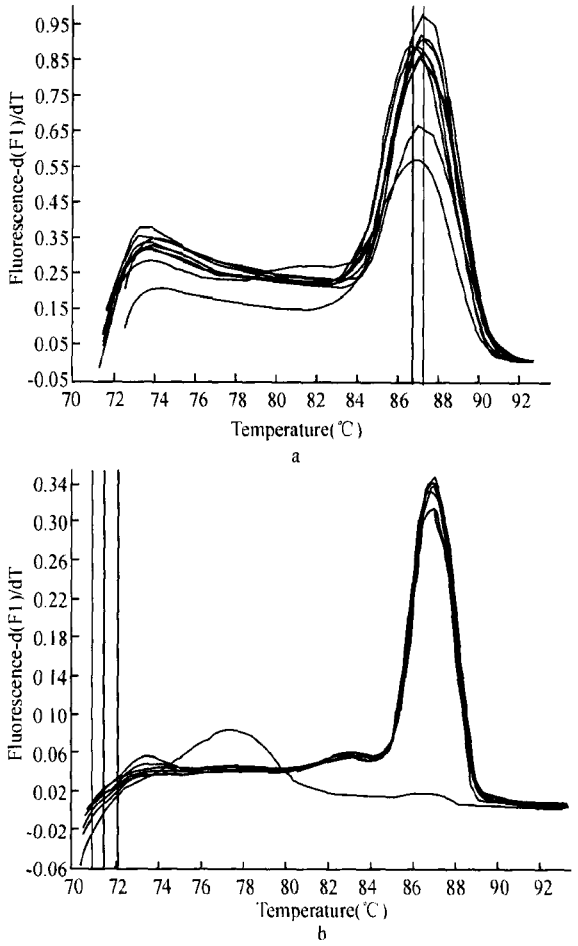

Fig 2 The GAPDH (a) and COX- 2 (b) melting curve. Both curves showed a single melting peak, indicating the specialty were good. Because of increasing the temperature - detected fluorescence, the peak of primer- dimmers in dissociation curve was eliminated.

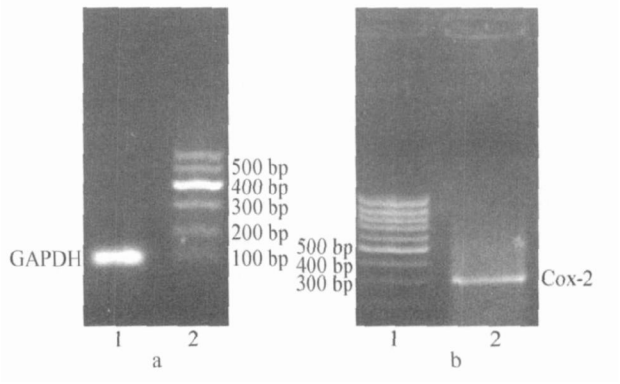

Fig 3 Agarose gel electrophoresis showed the GAPDH (a, with the lane 1 was GAPDH and lane 2 was marker) and COX- 2 (b, with the lane 1 was marker and lane 2 was COX- 2). The expected amplicon size for the GAPDH and COX- 2 gene is 80 bp and 305 bp, respectively. Product lengths on 1.5% agarose gel match the expected size.

advantages over the conventional quantifications in terms of accuracy, sensitivity, dynamic range, high-throughput capacity, and absence of post-PCR manipulations. Sequence-specific fluorescence-labeled probes (eg., TaqMan) have been considered as a standard detection format in many diagnostic and research applications[5]. However, it is not very well suited for quantification of a large number of different sequences, because a new and relatively expensive probe is generally required for each amplicon under investigation. We have therefore optimized and validated a reverse transcriptase PCR (RT-PCR) assay for accurate expression profiling using the double stranded DNA-binding dye SYBR green Ⅰ, which is a much more economical alternative to quantify any given transcript in a reaction.

Tab 1 The quantity of COX-2 mRNA according to clinicopathologic characteristics in 30 squamous laryngeal cancer patients

	Number of patients	N_{COX-2}
Histological grade		
WD	10	$8.68 \pm 1.32^{*}$
MD	9	$11.86 \pm 1.91^{\#}$
LD	11	15.71 ± 3.42
Tumor stage		
Ⅰ	10	$8.54 \pm 1.33^{\triangle}$
Ⅱ	8	$11.66 \pm 2.19^{\blacktriangle}$
Ⅲ/Ⅳ	12	14.89 ± 3.49

WD: well differentiation; MD: moderate differentiation; PD: poor differentiation. * $P<0.01$ *vs* MD; $^{\#}$ $P<0.05$ *vs* LD; $^{\triangle}$ $P<0.05$ *vs* Ⅱ; $^{\blacktriangle}$P< 0.05 vs Ⅲ/Ⅳ.

SYBR green Ⅰ, a dye that emits light only when bound to double-stranded DNA, have high affinity with dsDNA. The intensity of fluorescence gradually increases according to the amplified products. The intensity is dependent on the quantity of the original template. SYBR green Ⅰ allow detection of DNA in a sequence-independent way. This means that both specific and nonspecific PCR fragments can be binded by it. To visualize nonspecific PCR a melting curve analysis can be performed. Ball et al[6] used a modification of the LightCycler PCR strategy at a temperature greater than the melting point of primer-dimmers (PDs) for eliminating the contaminating fluorescence induced by the formation of primer-dimmers (PDs).

Many studies showed that COX-2 is highly expressed in human colon carcinoma, squamous cell carcinoma of the esophagus, and skin cancer. The function of COX-2 in tumor development and progression remains to be fully elucidated. Several different mechanisms could provide an important link between COX-2 and tumor. Enhanced synthesis of PGs, a consequence of up-regulation of COX-2, can increase cell proliferation, promote angiogenesis, and inhibit immune surveillance. All of these effects favored the growth of malignant cells. Additionally, over expression of COX-2 inhibited apoptosis and enhanced invasiveness[7]. COX-2 has oxidase activity and activates protooncogene to oncogene. For example, COX-2 catalyzes the oxidation of the tobacco procarcinogen benzo[a]pyrene-7, 8-dihyrodiol to benzo[a]pyrene-diol epoxide, which is a highly reactive and strongly mutagenic carcinogen[8]. It was recently reported that COX-2 specific inhibitors, nimesulide and celecoxib, had chemopreventive effects in many tumors[9].

In this experiment, the CT value is the minimum when the concentration of Mg^{2+} is 3.5 mmol/L, suggesting the amplification efficiency is the best and the specificity the highest. So the concentration of Mg^{2+} in our reaction mixture is 3.5 mmol/L. The quantification of COX-2 mRNA in carcinoma of larynx and adjacent non-cancerous tissues was detected with SYBR green Ⅰ. Because GAPDH's quantity is permanent in all tissues, the quantification of the sample and the total RNA, as well as the efficacy of RT-PCR can be estimated by GAPDH as the control.

The COX-2 mRNA was detected in all of the 30 samples, indicating the expression of COX-2 mRNA in laryngeal carcinomas was 100%, which was not the same with the result of Lu by immunohistochemistry (the positive rate is 58.7%, unpublished data). We supposed there were two reasons. One was that mRNA was the more sensitive marker than the protein; the other was that the real-time PCR quantification method was more sensitive than the immunohistochemistry technique. The N_{COX} values of COX-2 mRNA were significantly higher in laryngeal carcinomas than that in adjacent noncancerous tissues ($P<0.05$), showing that the expression COX-2 gene could be up-regulated in laryngeal carcinomas, which was consistent with the conclusions of Chan et al[10]. The relation between the COX-2 mRNA in tumor and the histopathological factors was observed by comparing the value of N_{COX}. Significant differences were found in clinical stage between Ⅰ and Ⅱ or Ⅱ and Ⅲ/Ⅳ. We supposed that as lesions of laryngeal mucose progress from no

malignant alterations to laryngeal carcinomas, the expression of COX- 2 mRNA increase gradually, which was indicated by our results. Furthermore, there was significant difference in histological grade between WD and MD or MD and PD with the progression of histological differentiation. It demonstrated the expression of COX- 2 mRNA in tumor increased gradually from well differentiation to poor differentiation. So COX- 2 may be used as a biomarker in laryngeal cancer, we could categorize the laryngeal cancers on the basis of the quantity of COX- 2 mRNA and choose the best therapy for them. In addition, our studies could raise the possibility that selective inhibitors of COX - 2 may be useful in the prevention or treatment of laryngeal cancer.

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喉癌组织中环氧化酶- 2(COX- 2)基因表达的实时定量 PCR 研究

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[摘 要] **目的**: 测定环氧化酶- 2(COX- 2)mRNA 在喉鳞癌组织中的含量, 并分析环氧化酶- 2(COX- 2)mRNA 与喉鳞癌临床分期及组织分化程度的关系。**方法**: 用序列特异性引物和荧光染料 SYBR green Ⅰ 聚合酶链式反应(PCR)方法, 检测 30 例原发性喉鳞癌和癌旁组织中 COX- 2 mRNA 表达, 采用 GAPDH 作对照。**结果**: 30 份标本的扩增产物经熔解曲线分析及 DNA 琼脂糖凝胶电泳, 证实均为 COX- 2 和 GAPDH。COX- 2 mRNA 在喉鳞癌中的阳性率是 100%, 而在癌旁级织为 40%(30 例中 12 例阳性), N_{COX}分别为 16.54±13.27 ,9.24±6.91, 两者之间差异显著($P<0.05$), 且喉癌组织中的 COX- 2 mRNA 表达水平与喉癌组织分化程度及临床分期密切相关。**结论**: 应用荧光染料 SYBR green Ⅰ 的实时定量 PCR 技术能够对 COX- 2 基因表达水平进行定量分析, COX- 2 基因表达的上调与喉癌的发生有关, 且可以作为一种有效的喉癌分子标志。

[关键词] 环氧化酶- 2; 喉肿瘤; 荧光染料; 基因表达

[中图分类号] R734.2 [文献标识码] A