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Main Article

Chemiluminescence assay of reactive oxygen species in laryngeal cancer

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Abstract

Objective: This study aimed to evaluate the presence of reactive oxygen species in laryngeal cancer tissue, using a luminol-amplified chemiluminescence method.

Materials and methods: Fourteen patients with histopathologically diagnosed laryngeal squamous cell carcinoma were enrolled. Patients with recurrent tumours or a history of prior chemotherapy or radiotherapy were excluded. Tissue specimens were harvested both from the tumour itself and from the neighbouring, apparently normal mucosa (immediately after tumour removal). Tissue specimens were washed with ice-cold saline solution and processed immediately, without storage. The level of reactive oxygen species was measured quantitatively by a luminol-amplified chemiluminescence method.

Results: The mean luminol-amplified chemiluminescence values for tumour and control tissue were 140.52 (standard error of the mean 40.21) and 121.36 (standard error of the mean 35.33) relative light units/mg tissue, respectively. Furthermore, mean tumour and control luminol chemiluminescence values were compared for stage one and two tumours versus stage three and four tumours. Both the tumour and the control luminol chemiluminescence values for the latter tumour group were significantly higher than those for the former tumour group.

Conclusion: This study measured directly the levels of reactive oxygen species in samples of laryngeal cancer tissue and normal mucosa. Higher levels of reactive oxygen species were found in laryngeal cancer tissue, suggesting a relationship between reactive oxygen species and laryngeal cancer.

Key words: Larynx Cancer; Reactive Oxygen Species; Chemiluminescence; Luminol

Introduction

Laryngeal carcinoma is a multifactorial disease which has been linked to several aetiological factors, including tobacco use, alcohol consumption, infectious agents (e.g. Epstein–Barr virus), ionising radiation and genetic factors.[1]

Reactive oxygen species are highly reactive substances that can cause a wide variety of cellular damage, including lipid peroxidation, enzyme inactivation and DNA damage. Exposure to reactive oxygen species leads to several chemical reactions within DNA, which can result in mutagenesis.[2] Recent studies have focused on the role of reactive oxygen species in laryngeal cancer.

The effect of reactive oxygen species in laryngeal cancer has been demonstrated indirectly using blood and tissue indicators.[3–7] However, none of these studies has directly evaluated the presence of reactive oxygen species in laryngeal cancer tissue.

Chemiluminescence involves the generation of electromagnetic radiation as light via the release of energy from a chemical reaction. Chemiluminescence assessment is a sensitive technique for estimating reactive oxygen species generation, which measures light production as a byproduct of oxidative metabolism. It has been used to demonstrate the involvement of reactive oxygen species in various diseases.[8]

The present study aimed to compare the direct, quantitative levels of reactive oxygen species, as measured by a luminol-amplified chemiluminescence method, within laryngeal cancer tissue and neighbouring, apparently normal tissue from the same cases. The study also compared reactive oxygen species levels in tumour stage (T) T_1 and T_2 tumours versus T_3 and T_4 tumours, in order to assess the effect of tumour stage on reactive oxygen species level.

Materials and methods

The study was conducted in the otolaryngology and head and neck surgery department of the Marmara

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University School of Medicine, and was approved by the university ethical committee.

Fourteen patients with histopathologically diagnosed laryngeal squamous cell carcinoma were enrolled. Patients with recurrent tumour or a history of prior chemotherapy or radiotherapy were excluded. Patients comprised 13 men and one woman. The mean age for the male patients was 59.6 years (range 38–74 years); the female patient was aged 63 years.

All patients underwent a routine head and neck examination. Tumour localisation and spread and vocal fold mobility were noted.

Computed tomography was used to radiologically evaluate tumour localisation, extension, cartilage invasion, extralaryngeal spread and cervical metastasis.

Tissue specimens were harvested both from the tumour itself and also from neighbouring, apparently normal mucosa (immediately after surgical specimen removal). Tissue specimens were washed with ice-cold saline solution and processed immediately, without storage.

Chemiluminescence measurements were made at room temperature using a Mini Lumat LB 9506 luminometer (EG&G Berthold, Bad Wildbad, Germany), in the presence of 0.2 mmol/l luminol in buffer (the latter containing 0.5 mol/l phosphate-buffered saline plus 20 mmol/l HEPES (N-2-hydroxyethylpiperazine-N-2 ethanerulphonic acid)). Light counts were obtained at 5-second intervals. The results were collated to show the area under the curve for a luminol chemiluminescence counting period of 5 minutes. At the end of each experiment, the solution was drained and the tissue weighed; specimens weighed approximately 30–40 mg each. Results were presented as the area under the curve for chemiluminescence, and expressed as relative light units per milligram of tissue.

Study findings were statistically analysed using the Number Cruncher Statistical System 2007 and the Power Analysis and Sample Size 2008 software program (Kaysville, Utah, USA). Data were described in terms of means, standard errors of the mean (SEM). Non-normal distribution parameters were analysed using the Mann–Whitney U test, while the Wilcoxon signed rank test was used for intra-group comparisons. A p value of less than 0.05 was used to denote statistical significance.

Results

The mean luminol-amplified chemiluminescence values for tumour and control tissue specimens were 140.52 (SEM 40.21) and 121.36 (SEM 35.33) relative light units/mg tissue, respectively. Chemiluminescence values for laryngeal cancer tissue were found to be statistically significantly greater than those for control tissue ($p < 0.05$).

The luminol-amplified chemiluminescence values for T_{1-2} ($n = 7$) and T_{3-4} ($n = 7$) tumour specimens were compared, in order to investigate the relationship between tumour stage and reactive oxygen species level. In the T_{1-2} tumour specimens, the tumour and control mean chemiluminescence values were 47.40 (SEM 9.99) and 37.48 (SEM 3.70) relative light units/mg tissue, respectively. In the T_{3-4} tumour specimens, the tumour and control mean chemiluminescence values were 233.64 (SEM 63.39) and 205.24 (SEM 55.22) relative light units/mg tissue, respectively (Table I). The luminol-amplified chemiluminescence values for both tumour and control tissue from T_{3-4} tumours were statistically significantly greater than those for tumour and control tissue from T_{1-2} tumours (Figure 1).

Discussion

Recent studies have focused on the role of reactive oxygen species in carcinogenesis. Reactive oxygen species have been shown to cause more than 100 kinds of chemical reactions (by interacting with membrane lipids, DNA and amino acids) which may result in DNA mutation.[2] Reactive oxygen species lead to DNA chain breaks, chromosomal anomalies, base modifications and cell transformation, and therefore facilitate malignant transformation.[9]

Various studies have examined the role of reactive oxygen species in laryngeal cancer by using either blood or tissue indicators of oxidative damage. Seven *et al.* evaluated oxidative stress parameters in the blood of laryngeal carcinoma patients.[3] Taysı *et al.* demonstrated that oxidative stress is increased in patients with advanced laryngeal cancer, by measuring plasma levels of malondialdehyde and nitric oxide.[4] Kalaycı *et al.* investigated superoxide dismutase and glutathione peroxidase enzyme activity in laryngeal cancer tissue.[5] Munnia *et al.* reported significantly greater levels of malondialdehyde DNA and aromatic DNA products in larynx cancer tissue.[6]

However, no previous study has undertaken direct, quantitative measurement of reactive oxygen species in laryngeal cancer tissue.

TABLE I

CHEMILUMINESCENCE MEASUREMENTS FOR TUMOUR AND CONTROL TISSUE SPECIMENS

Parameter	Tumour stage			p^*
	$T_{1-2}^{\dagger}$	$T_{3-4}^{\ddagger}$	T_{1-4}^{**}	
Cancer CL (rlu/mg)	47.67 ± 9.99 (46.5)	233.64 ± 63.99 (244.3)	140.52 ± 40.21 (78.6)	0.006[§]
Control CL (rlu/mg)	37.48 ± 3.70 (35.4)	205.24 ± 55.22 (223.3)	121.36 ± 35.33 (54.7)	0.002[§]
$p^{\#}$	0.237	0.018[a]	0.013[a]	

Data represent area under the curve means ± standard errors of the mean (medians), unless otherwise specified. $^{\dagger}n=7$; $^{\ddagger}n = 7$; $^{**}n = 14$. *Mann–Whitney U test; #Wilcoxon signed rank test. $^{\S}p < 0.01$; $^{a}p < 0.05$. T = tumour stage; CL = chemiluminescence; rlu = relative light units

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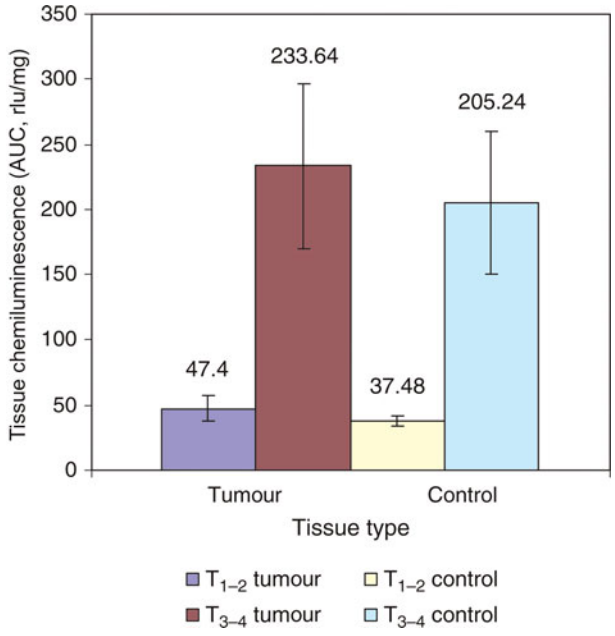

FIG. 1

Luminol-amplified chemiluminescence values for tumour stage (T) T_{1-2} and T_{3-4} tumour tissue and control tissue. AUC = area under the curve

The half-life of reactive oxygen species is very short, and they are thus difficult to investigate under normal circumstances. Chemiluminescence measurement is a noninvasive, direct assessment method which uses the light formed during chemical reactions.[10] Chemiluminescence is a universal property of organic substances that are able to undergo an oxidation reaction sufficiently exothermic to produce a light-emitting state.[11] Because of limitations such as potential variability and the low intensity of native chemiluminescence, enhancer compounds such as luminol have recently been used. In the current study, luminol was selected primarily because of its high quantum efficiency after oxidation. It also functions as a bystander substrate for oxygenation and forms high levels of excited state products and chemiluminescence when added to an *in vitro* biological system.[11]

In the present study, luminol-amplified chemiluminescence values were found to be significantly greater in laryngeal cancer tissue compared with control tissue ($p < 0.05$). In addition, T_{3-4} tumours were found to have increased reactive oxygen species levels, both in cancerous and normal tissue specimens, compared with these specimen types from T_{1-2} tumours. Although the number of cases was limited, the difference was statistically significant ($p < 0.05$). Nevertheless, the correlation between reactive oxygen species level and T stage should be evaluated in a larger group.

Several studies have evaluated the free radical damage associated with well known laryngeal cancer aetiological factors such as tobacco and alcohol. Panda *et al.* have demonstrated oxidative damage in the proteins and lipids of liver, lung and heart tissue, in guinea pigs exposed to tobacco smoke.[12] The same study confirmed that ascorbic acid treatment significantly decreased oxidative damage and lipid peroxidation. In a previous study, we found that vitamin E decreased cigarette smoke induced reactive oxygen species levels in the laryngeal and lung tissue of a rat model.[13]

In the current study, control tissue consisted of apparently normal, neighbouring mucosal biopsies harvested from patients undergoing surgical tumour removal. Since all our patients were heavy smokers, such adjacent, apparently normal tissue had been exposed to similar smoking effects as the tumour tissue. However, it is not possible to create a true control group for this type of study, because it is not ethical to take biopsies from completely normal, healthy subjects. This lack of a true control group could be seen as a limitation of the current study.

- **Oxidative stress can be defined as the state in which an organism's production of oxidants exceeds its capacity to neutralise them; oxidative stress is now thought to contribute significantly to carcinogenesis**
- **This study demonstrated increased levels of reactive oxygen species in laryngeal cancer tissue, compared with apparently normal, adjacent mucosa**
- **Further investigations are required to establish the exact role of reactive oxygen species in laryngeal cancer development, and the potential for new therapeutic strategies for laryngeal cancer treatment and prevention**

In the present study, the reactive oxygen species levels of cancerous and control tissues were measured quantitatively using a chemiluminescence method; levels in tumour tissue were found to be significantly higher than those in control tissue. The presence of reactive oxygen species within laryngeal cancer tissue has been indicated but not directly measured by previous studies; however, in the present study it was quantitatively measured. To the best of our knowledge, this is the first study in which reactive oxygen species levels have been measured directly, using a chemiluminescence method, in laryngeal cancer tissue.

Conclusion

Oxidative stress can be defined as the state in which an organism's production of oxidants exceeds its capacity to neutralise them. Oxidative stress is now thought to make a significant contribution to carcinogenesis. In the current study, we demonstrated increased reactive oxygen species production in laryngeal cancer tissue, compared with apparently normal control mucosa.

Although several studies have indicated a relationship between reactive oxygen species and laryngeal cancer, the exact role of reactive oxygen species in laryngeal cancer development has not yet been fully elucidated. Further investigations will be required

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to establish both the exact role of reactive oxygen species in the development of laryngeal cancer, and the potential for new therapeutic strategies for laryngeal cancer treatment and prevention.

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