

# Genomic instability in blood cells is able to predict the oral cancer risk: an experimental study in rats

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Received: 12 June 2008 / Accepted: 18 July 2008 / Published online: 1 August 2008  
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**Abstract** This study was undertaken to investigate the genomic instability on blood cells during 4-nitroquinoline 1-oxide (4NQO)-induced rat tongue carcinogenesis by means of single cell gel (comet) and micronucleus assays. Male Wistar rats were distributed into three groups of 10 animals each and treated with 50 ppm 4NQO solution through their drinking water for 4, 12, and 20 weeks. Ten animals were used as negative control. Although no histopathological abnormalities were induced in the epithelium after 4 weeks of carcinogen exposure, genetic damage was found in blood cells as depicted by the mean tail moment and an increase of micronucleated polychromatic erythrocytes. After 12 and 20 weeks treatment, the same picture occurred, being the strong effect observed in the micronucleus induction. These periods correspond to pre-neoplastic lesions and well-differentiated squamous cell carcinomas, respectively. Taken together, our results support the idea that genomic instability on blood cells appears to be associated with the risk and progression of oral cancer, being a reliable tool for detecting early systemic conditions of malignancy.

**Keywords** Genomic instability · Rat tongue mucosa · Oral squamous cell carcinoma · 4-Nitroquinoline 1-oxide

## Introduction

Squamous cell carcinoma is the most common malignancy that affects the human oral cavity (Nagpal et al. 2002). Despite recent advances in therapy, the prognosis of patients with oral squamous cell carcinoma has not been improved significantly in recent decades (Charabi et al. 1997; Xi and Grandis 2003). It is desirable to examine the precise pathobiological mechanisms involved in oral tumorigenesis in order to identify reliable biomarkers for prevention of oral squamous cell carcinomas, especially during neoplastic conversion. The most often used animal models in this line of research are the hamster buccal pouch by fat-soluble 7,12-dimethylbenzanthracene (DMBA), and the rat tongue by water-soluble 4-nitroquinoline 1-oxide (4NQO). Considering one of the most important routes of oral carcinogenesis is through liquid containing water-soluble carcinogens, 4NQO is well suited to examine the role of xenobiotics in experimental oral carcinogenesis (Tanaka et al. 2002). Based on the multi-step process of carcinogenesis characterized by initiation, promotion, and tumor progression, chronic administration of 4NQO in drinking water simulates rat tongue carcinogenesis similar to its human counterpart (Ohne et al. 1985; Nishimura 1999; Okazaki et al. 2002; Vered et al. 2003; Ribeiro et al. 2004; 2005).

It has been established that DNA damage and subsequent cell proliferation can establish genomic instability through multiple pathways (Limoli et al. 1997). Accumulation of such abnormalities in the genome is associated with cell transformation from benign to malignant phenotype (Califano et al. 1996). However, little is known about the genetic basis in the multistage process of oral carcinogenesis, particularly the relationship between oral tumorigenesis and the systemic host response. To date, a variety of assays has been proposed as potential biomarkers

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for detecting genomic instability, including those that assess metaphase chromosomal aberrations, sister chromatid exchanges and host cell reactivation. However, these methods are typically laborious and time-consuming or require highly trained technicians to accurately read and interpret slides.

In this regard, a great deal of enthusiasm was raised by the application of the micronucleus test to mammalian cells (Belien et al. 1995). Micronucleus arises from acentric fragments or whole chromosomes which are not included into the main nuclei of the daughter cells. The formation of micronuclei can be induced by substances that cause chromosome breakage (clastogens) as well as by agents that affect the spindle apparatus (aneugens) (Stich et al. 1992). The single cell gel (comet) assay, in the alkaline version, is a rapid, simple, and reliable biochemical method for evaluating DNA damage in mammalian cells (Tice et al. 2000). This technique includes embedding cells in agarose gel on microscope slides and lysing with detergent and high salts. During electrophoresis under alkaline conditions, cells with damaged DNA display increased DNA migration resulting of DNA strand breaks, alkalilabile lesions including a basic sites, and incomplete repair sites toward anode. Broken DNA migrates farther in the electric field and the cell resembles a 'comet' with brightly fluorescent head and a tail region (Olive et al. 1990). The extent of the comet is related to increased DNA damage. These images can be analyzed and compared in a cell-to-cell basis. In the present study, we used the single cell gel (comet) and micronucleus assays as a putative biomarker to predict genomic instability in blood cells during the 4NQO-induced rat tongue multistep carcinogenesis.

## Material and methods

### Animals and experimental design

All experimental protocols involving animals conformed to procedures described in the Guiding Principles for the Use of Laboratory Animals and the study approved by the Animal Committee of Federal University of Sao Paulo, UNIFESP.

Forty male outbred Wistar rats (8 weeks old) weighing approximately 250 g, were obtained from Centro de Bioterrorismo (CEMIB), Universidade Estadual de Campinas, SP, Brazil. They were maintained under controlled conditions of temperature ( $24 \pm 2^\circ\text{C}$ ), light-dark periods of 12 h, and with free access to water and commercial diet (Nuvital PR, Brazil). The animals were divided into 3 groups of 10 and were treated with 50 ppm 4NQO (Sigma Aldrich, St. Louis, USA) solution by drinking water for 4, 12 or 20 weeks. Ten animals were used as negative control, in which were sacrificed at the beginning the experiment (zero week). At the end of

the experimental period, the rats were sacrificed by 0.4% sodium pentobarbital (1 ml/kg, i.p.). The tongues were longitudinally bisected for histopathological examinations. The tissues were fixed in 10% buffered formalin (Merck, Darmstadt, Germany), embedded in paraffin blocks, and stained with hematoxylin and eosin (H.E., Merck).

### Histopathological analysis

Histopathological evaluation was performed by light microscopy. Analyzes of the tongue sections were graded as normal, hyperplasia, dysplasia, and carcinoma per animal according to Ribeiro et al. (2005).

### Single cell gel (comet) assay

The single cell (comet) assay with blood cells was carried out as previously described by Tice et al. (2000) with some modifications. Peripheral blood cells were collected from cardiac puncture after anesthesia and the cell suspensions ( $\sim 10 \mu\text{l}$ ) were used for single cell gel (comet) assay. Thus, a volume of  $10 \mu\text{l}$  was added to  $120 \mu\text{l}$  of 0.5% low-melting point agarose at  $37^\circ\text{C}$ , layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and the slides immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for about 1 h. Afterwards, the slides were washed in ice-cold PBS for 5 min, left in electrophoresis buffer (0.3 mM NaOH and 1 mM EDTA, pH > 13) for DNA unwinding during 20 min, and electrophoresed in the same buffer for 20 min at 25 V (0.86 V/cm) and 300 mA. Following electrophoresis, slides were neutralized in 0.4 M Tris-HCl (pH 7.5), fixed in absolute ethanol and stored at room temperature until analysis in a fluorescence microscope at  $400\times$  magnification. All steps were performed under reduced light.

An automatized analysis system (Comet Assay 2.2: Perceptive Instruments, UK) was used to measure the level of DNA damage induced by 4NQO. Two parameters were estimated to determine the level of DNA damage: tail moment (product of tail DNA/total DNA by the center of gravity) and tail intensity (percentage of DNA in the tail) from 50 cells per animal. In none of the experiments there was a significant difference between these parameters. Therefore, we chose tail moment for the presentation of the results.

### Micronucleus test

The bone marrow micronucleus test was performed according to Sugui et al. (2003). One thousand polychromatic erythrocytes were analyzed per animal. Slides were

scored blindly using a light microscope with a 100× immersion objective.

Statistical methods

Statistical analyses for single cell gel (comet) and micronucleus data were assessed by Kruskal–Wallis non-parametric test followed by post-hoc analysis (Dunn’s test) if a significant effect was detected using SPSS software pack (version 1.0). A *P* value < 0.05 was considered statistically significant.

Results

Histopathological evaluation following 4NQO treatment

No histopathological changes in tongue epithelia were observed in the control group (Fig. 1a) nor after treatment for 4-weeks with 4NQO. The primary histopathological change, i.e., hyperplasia and hyperkeratosis with the spinous cell layer gradually thickened was evidenced after 12-weeks-treatment (Fig. 1b). In this period, epithelial dysplasia was also found in mild and moderate forms (Fig. 1c). At 20 weeks, moderate and/or severe oral dysplasia (Fig. 1a, b, respectively) and squamous cell carcinoma in the tongue (Fig. 1c) were found, being that in the majority of animals consisted of squamous cell carcinoma. The histopathological grade was usually squamous cell carcinoma of a well-differentiated type. The tumors spread into the submucosa and underlying muscle layer, forming small nests with typical keratin pearl formation. In

**Table 1** Incidence of histopathological lesions in tongue of rats in the 4-nitroquinoline 1-oxide (4NQO)<sup>a</sup> model for oral carcinogenesis

Groups (week)	No. of animals	Lesions			
		Normal	Hyperplasia	Dysplasia	Carcinoma
0 (Control)	10	10	0	0	0
4	10	10	0	0	0
12	10	0	7	3	0
20	10	0	0	3	7

<sup>a</sup> 4NQO—50 ppm by drinking water

advanced cases, severe atypia was frequently found. The histopathological findings are summarized in the Table 1.

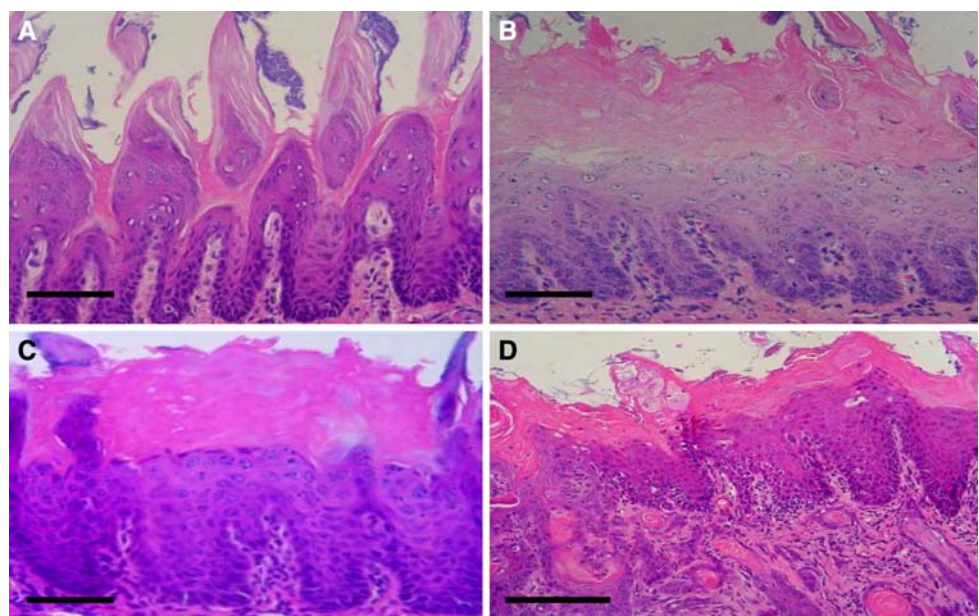
Genotoxicity data

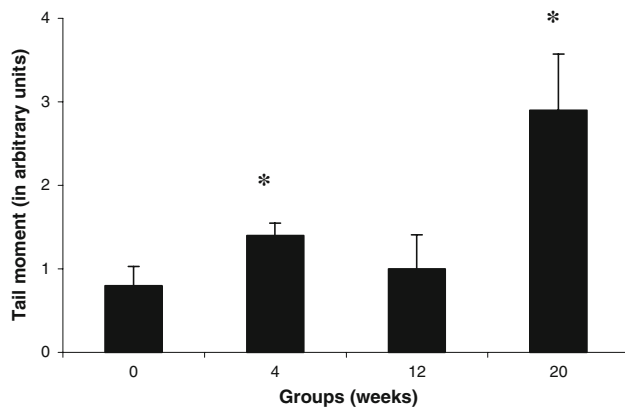
The results of the single cell gel (comet) assay are shown in Fig. 2. Statistically significant (*P* < 0.05) increase of DNA damage was observed at 4, and 20 weeks administration of 4NQO in blood cells as depicted by the mean tail moment (Fig. 3). Regarding micronucleus data, a gradual increase of micronucleus frequency was detected at all periods evaluated. In addition, the effect was in a time dependent manner. Such findings are summarized in Table 2. Figure 4 illustrates a micronucleated polychromatic erythrocyte.

Discussion

Carcinogenesis is a multi-step process, which is characterized by genetic, epigenetic, and phenotypic changes (Sugimura et al. 1992). Such changes involve genetic

**Fig. 1** Photomicrographies showing the multi-step process of rat tongue carcinogenesis. (a) no histopathological change (control); (b) hyperplasia and hyperkeratosis; (c) epithelial dysplasia; (d) squamous cell carcinoma of well-differentiated type. (Hematoxylin and Eosin stain; Bar = 56 μm)





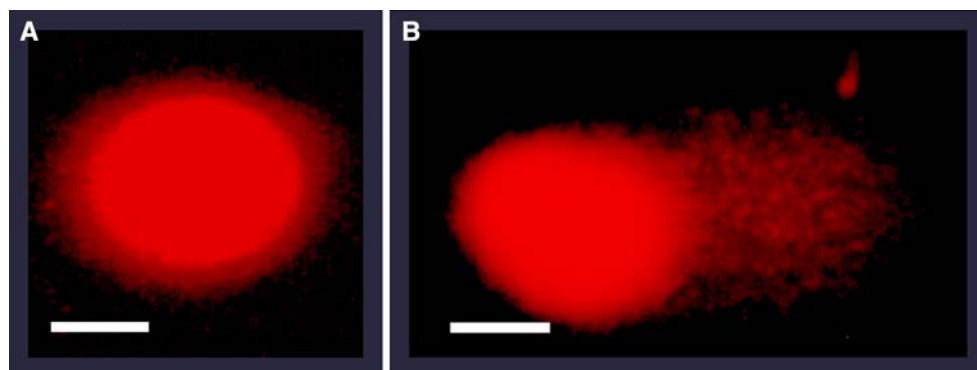
**Fig. 2** DNA damage (tail moment) in the rat blood cells following 4NQO administration. \*  $P < 0.05$  when compared to control (zero)

damage, mutation in critical genes related to the control of cell division, cell death, and metastatic potential, and activation of signaling or metabolic pathways that give the cells favorable growth and survival characteristics (Sarasin 2003). In patients, the molecular analysis of these multiple steps is hampered, due to the unavailability of biopsies at all the stages of carcinogenesis. Animals models of carcinogenesis allow the isolation of all stages under controlled conditions, including normal tissues, which are then amenable to pathological, genetic, and biochemical analysis, and at lower costs (Herzig and Christofori 2002). Moreover, the chemical carcinogenesis models help to investigate hazard risk caused by environmental agents as well as to determine which putative precancerous lesions will progress. Several medium term duration assay systems for oral carcinogenesis offer particular promise. Our results, using 4NQO as a carcinogen inducer, demonstrated histopathological changes in rat tongue mucosa along a time-course from hyperplasia, pre-malignant dysplasia, and carcinoma in situ, to invasive squamous cell carcinoma. Therefore, it should be assumed that tongue carcinogenesis was 4NQO dependent, because these lesions did not occur in control rats and the rats that developed tumors were

younger than 28 weeks of age, when spontaneous tumors are not common in this species (Hayashi et al. 1989).

Genomic instability, either spontaneous or mutagen-induced, has been considered a pre-disposing factor for neoplastic transformation (Wu et al. 2002). The accumulation of these genetic alterations is the basis for the progression from normal to a cancer cell, referred as multistep carcinogenesis (Califano et al. 1996). The nature and genetic role of the alterations occurring at each step of oral malignant transformation is still unclear, particularly the relationship between oral tumorigenesis and the systemic host response. A good candidate to detect the genetic abnormalities would be a reliable predictor of a wide spectrum of significant genetic changes at an early stage of carcinogenesis. In this study, we used the single cell (gel) comet assay to assess a wide variety of 4NQO-induced DNA damage on blood cells to determine whether genomic instability as a result of systemic host-response is associated with the risk of oral cancer. 4NQO is an alkylating compound and potent mutagen that requires metabolic activation to be converted to 4-acetoxyaminoquinoline 1-oxide which reacts with DNA causing damage as single strand breaks, incomplete repair sites and alkali-labile sites. Our results showed increased DNA damage in blood cells four weeks following 4NQO administration as depicted by increase of tail moment and micronucleated polychromatic erythrocytes. This is important to stress that no histopathological changes in oral mucosa cells were noticed in this experimental period. Micronucleated cell index may reflect genomic instability (Maluf and Erdtmann 2001; Neri et al. 2003). The detection of an elevated frequency of micronuclei in a given population indicates increased risk of cancer (Hagmar et al. 1998). Taking into account available data, some studies have reported increased DNA damage in blood cells exposed to 4NQO in vitro (Frenzilli et al. 1997; Andersson et al. 2003). In a study conducted by Nakajima et al. (1999), 4NQO was able to induce genetic mutation, and also chromosomal aberration in mice after oral administration on days 7, 14 and 28 after treatment. Therefore, our results support the hypothesis that genomic

**Fig. 3** Representative comet images from control rat blood cell (a), and 4NQO exposed cell (b) (DNA was stained with ethidium bromide; Bar = 4  $\mu\text{m}$ )

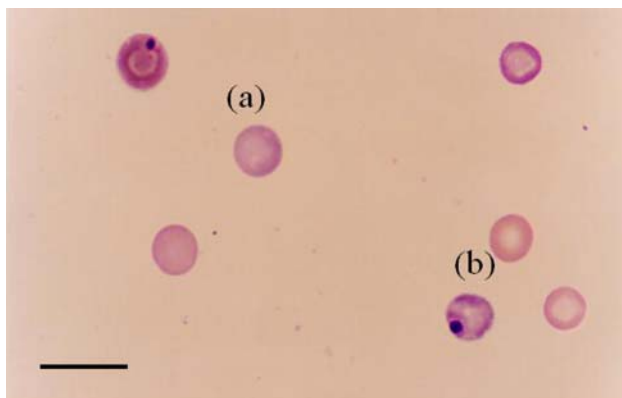




**Table 2** Frequency of micronucleated polychromatic erythrocytes (PCE) following rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide (4NQO)<sup>a</sup>

Groups (weeks)	Total number of PCE analyzed	Micronucleus	
		Total number	%
0 (Control)	10,000	3	0.03
4	10,000	21	0.21*
12	10,000	48	0.48*
20	10,000	124	1.24*

\*  $P < 0.05$ ; <sup>a</sup> 4NQO—50 ppm by drinking water

**Fig. 4** Normal polychromatic erythrocytes (a) and micronucleated cell (b). (Giemsa stain, Bar = 12  $\mu$ m)

instability in blood cells may be present in early phases of oral tumorigenesis. Therefore, this condition may trigger an increased risk of oral cancer.

In humans, only a small portion of initial lesions develop oral carcinomas (Ribeiro et al. 2005). Therefore, the challenge is to identify which lesions have real malignant potential. In this study, high levels of micronucleated cells were found in blood cells in the period corresponds to oral pre-malignant lesions and oral squamous cell carcinomas following 12 and 20 weeks exposure to 4NQO, respectively. Herein, it seems that the levels of 4NQO-induced DNA damage also progressed during tumorigenesis. We assumed that these multiple genetic alterations are not generated suddenly by a single event, but instead were generated early and continuously by 4NQO chronic administration during tumor progression. Nevertheless, endogenous cellular processes may induce DNA damage and serve as a source of genomic instability. Previous studies have showed DNA damage in blood cells of oral cancer patients assessed by the single cell gel (comet) assay (Rao et al. 1997). The results support the concept of a systemic host response in malignancy. Moreover, others have suggested that the level of background DNA damage before irradiation measured by comet

assay as well as the level of micronuclei were significantly higher in the head and neck cancer patient group than in the healthy donors in isolated lymphocytes (Palyvoda et al. 2003).

In conclusion, our results suggest that genomic instability appears to be associated with the risk and progression of oral cancer, being a reliable tool for detecting early systemic conditions of malignancy. This kind of approach should be considered to persons with high risk of oral cancer, such as in smokers or alcohol consumers as well as human patients diagnosed with oral dysplasia or carcinoma.

**Acknowledgments** The authors are grateful to Paulo Roberto Cardoso and Maria Luiza Falaguera Ardanaz for their technical assistance. This work was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo (Grant number: 07/01228-4). D.G. Grilli is a recipient CNPq student's fellowship (PIBIC).

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