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Original Article

Reactive oxygen species, NF-κB, and p53 levels in tissue of undifferentiated nasopharyngeal carcinoma

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Key Words
Lipid peroxidation; NF-kB; p53; Reactive oxygen species

Abstract
This study aimed to evaluate the level of reactive oxygen species, oxidative stress, and NF-κB and p53 in undifferentiated nasopharyngeal carcinoma. Twenty-four nasopharyngeal carcinoma patients and ten normal subjects were involved in order to compare the level of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and peroxidative index (PI). The level of reactive oxygen species (ROS), expression of NF-κB and p53 from a biopsy specimen of nasopharyngeal carcinoma tissue (histologically confirmed to be undifferentiated WHO III) were also compared with normal nasopharyngeal tissue. The Student t-test was used to analyze the different level of MDA, H_2O_2 and PI. Analysis of MDA level and H_2O_2 was done by colorimetric method. Levels of ROS, NF-κB, and p53 were analyzed using laser scanning confocal microscopy. MDA and H_2O_2 levels as well as PI of nasopharyngeal carcinoma patients were significantly higher compared to control. The levels of ROS and expression of NF-κB and p53 were higher in nasopharyngeal carcinoma tissue than those in normal nasopharyngeal tissue. We conclude that the tissue of nasopharyngeal carcinoma is one source of ROS and oxidative stress in nasopharyngeal carcinoma. NF-κB and p53 levels in nasopharyngeal carcinoma tissue may contribute to oxidative stress in undifferentiated nasopharyngeal carcinoma.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignant tumor with a high incidence of metastasis. Previous studies have reported a disturbance of the oxidative profile in NPC patients. Oxidative stress contributes to tumor initiation and progression due to genomic instability. Beside that, oxidative stress directly contributes to tumor metastasis [1, 2]. Undifferentiated NPC is 100% associated with Epstein-Barr virus (EBV) [3]. When EBV enters the lytic cycle, the virus first expresses transcription factors to activate the transcription of EBV lytic gene [4]. A previous *in vitro* study in the Raji (human Burkitt's lymphoma) cell line study showed that transition of latent phase into lytic phase was mediated by hydrogen peroxide (H_2O_2) [5].

Hydrogen peroxide is a weak oxidant with dual faced function. Ambient production H_2O_2 at low-level is necessary for cell growth and proliferation [6]. Large amounts of H_2O_2 can be generated by activating inflammatory cells through oxidative burst mechanisms. H_2O_2 can be converted to another reactive oxygen species (ROS) or highly reactive hydroxyl radicals in the presence of reduced transition metals, such as ferrous and cupreous ions [7]. Malondialdehyde (MDA) is a decomposition product of peroxidized polyunsaturated fatty acids that is widely preferred for detection of ROS reactivity toward lipid cells [8, 9]. Modification of proteins with MDA changes antigenicity, function, and turnover kinetics of various proteins [10]. MDA is also mutagenic in bacterial and mammalian cells and carcinogenic in rats [11]. A

previous study showed that MDA was significantly higher in the blood of NPC patients compared to control [12], but the source of the ROS is unclear. Besides, the involvement of the redox sensitive transcription factors, such as NF-κB and p53 in oxidative stress is also warranted to be clarified.

To date, no studies have assessed the source of ROS in oxidative stress of NPC patients. In addition, the involvement of the redox sensitive transcription factor, such as NF-κB and p53 in its mechanisms is unknown. Accordingly, this study aimed to to evaluate the level of ROS, oxidative stress, and NF-κB and p53 in NPC patients.

MATERIALS AND METHODS

Subjects

In order to compare H_2O_2 and oxidative stress in serum, we compared NPC patients with normal healthy volunteers. Twenty-four untreated NPC patients were recruited from Otorhinolaryngology Department, Ulin General Hospital, Faculty of Medicine, University of Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia. The mean age of the patients was 45.8 ± 14.9 years. Ten normal healthy volunteers comprised the control group. These patients were not taking any drugs or antioxidants before blood samples were taken. The mean age of the healthy subjects was 38.1 ± 5.2 years. Informed consent was obtained from all patients and controls. In addition, we also compared the level of reactive oxygen species, the expression of NF-κB, the expression of p53 from a biopsy specimen of NPC patients (histologically confirmed to be undifferentiated WHO III) compared with normal nasopharyngeal tissue.

Samples

Five milliliters of blood were taken from the cubital median vein of the patients and normal healthy volunteers. The blood samples were centrifuged at 1400 rpm for 10 min at 4°C. The upper serum layers were carefully pipetted out into polypropylene tubes and were stored at –80°C until biochemical analysis. The biopsy specimen was obtained from NPC patients which evaluated by experts of histopathology as NPC or normal nasopharyngeal tissue.

Malondialdehyde analysis

In the samples, which are serum, MDA levels were determined using the method of Ohkawa [13], based on the reaction of MDA with thiobarbituric acid (TBA) at 95°C. In the TBA test reaction, MDA and TBA react to form a pink pigment with maximum absorption at 532 nm. The reaction was performed at pH 2-3 at 95°C for 15 min. The sample was mixed with 2.5 volumes of 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation and an aliquot of supernatant was reacted with 0.67% TBA in a boiling water-bath for 15 min. After cooling, the absorbance was read at 532 nm. Arbitrary values obtained were compared with a series of standard solutions (acid hydrolyzed 1,1,3,3-tetramethoxypropane into MDA). Results were expressed as mol/liter.

Hydrogen peroxide analysis

A commercial hydrogen peroxide detection kit (Oxis Biotech, Portland, OR, USA) was used to measure hydrogen peroxide in serum. Results were expressed as mol/liter.

Peroxidative index analysis

Peroxidative index (PI) was analyzed by the equation according to a previous study with modifications [14]:

$$\textbf{PI = serum MDA/serum } H_2O_2 \textbf{ level}$$

Reactive oxygen species analysis

ROS analysis was done using laser scanning confocal microscopy [15]. A 25 μM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was added to nasopharyngeal tissue. After that, the tissue was washed with phosphate buffer saline (PBS) and fixated by 10% buffer formalin for 10 min. Mounting was done on a slide glass by Prolong Antifade (Invitrogen). The green fluorescence in nasopharyngeal tissue expressed as arbitrary units was estimated as ROS level.

Double-labeling immunofluorescent staining of p53 and NF-κB

Slides were incubated at 37°C (overnight), then deparaffinized with xylol, absolute ethanol, ethanol 90% and 70% (5 min). Washing solution was PBS (pH 7.4). The washed slides were heated in buffer citrate (pH 6) at high temperature microwave (10 min). Non-specific protein-binding sites were blocked by incubation of skim milk 2% in PBS buffer. Slides were then washed and incubated with primary anti-NF-κB p50 Rabbit polyclonal (dilution range 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-p53 mouse monoclonal (dilution range 1:1000; Dako, Glostrup, Denmark) antibodies for 1 h in dark conditions. Slides were then washed and incubated with secondary antibodies: goat anti-rabbit IgG Rhodamine (dilution range 1:1500; Santa Cruz) and goat anti-mouse IgG-FITC (dilution range 1:1500; Santa Cruz) for 1 h in dark conditions. Slides were then washed and the expression of NF-κB and p53 were analyzed using laser scanning confocal microscopy (Olympus).

Statistical analysis

Statistical analysis was carried out using the Student t-test to assess the statistical significance of the differences between patients and controls. Data are presented as mean ± SD and differences between groups were analyzed using SPSS 16.0 software; $P < 0.05$ was considered statistically significant.

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RESULTS

The MDA levels of NPC patients were found to be significantly higher compared to control ($P < 0.001$). In NPC patients, we found 3.18 fold increase of the MDA level compared with control group as to see in Fig.1.

H_2O_2 levels in serum of NPC patients are significantly higher than those in control ($P < 0.001$). In NPC patients, we found 1.61 fold increase of the H_2O_2 levels compared to control group as shown in Fig.2.

To measure the proportion of the involvement of H_2O_2 on reaction series of MDA production and degradation we used PI (see Fig.3). The PI of NPC patients was significantly higher compared to control ($P < 0.05$). In NPC patients, we found 1.78 fold increase of the PI level than that of the control group.

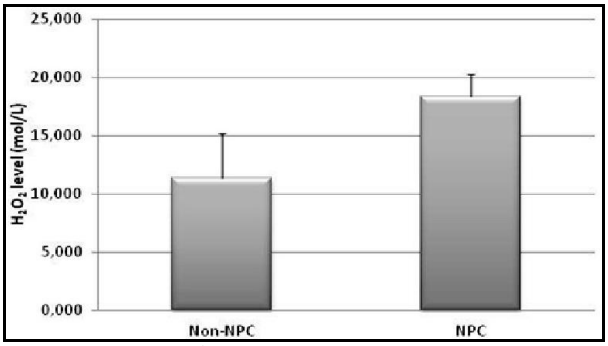

Figure 2. The level of H_2O_2 in NPC patients and non-NPC control group. In NPC patients, H_2O_2 levels were found 1.61 fold higher than control values.

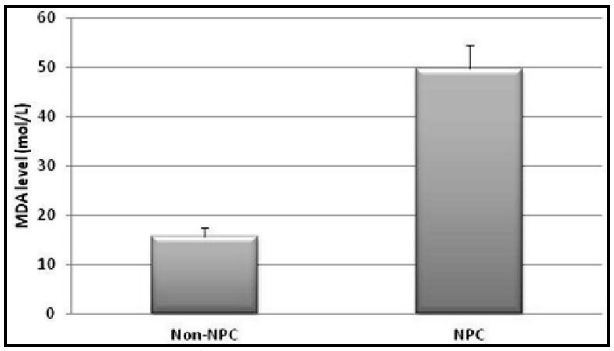

Figure 1. The level of MDA in NPC patients and non-NPC control group. In NPC patients, MDA levels were found 3.18 fold higher than control group.

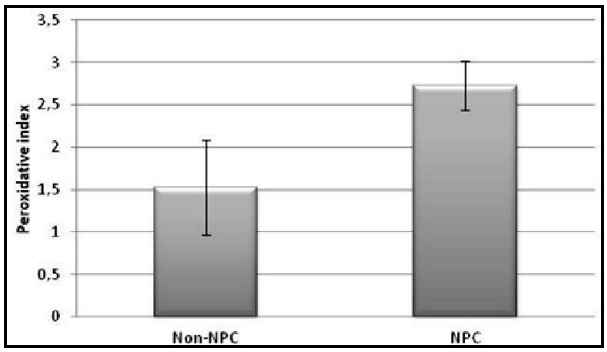

Figure 3. The level of PI in NPC patients and non-NPC control group. In NPC patients, PI was 1.78 fold higher than control.

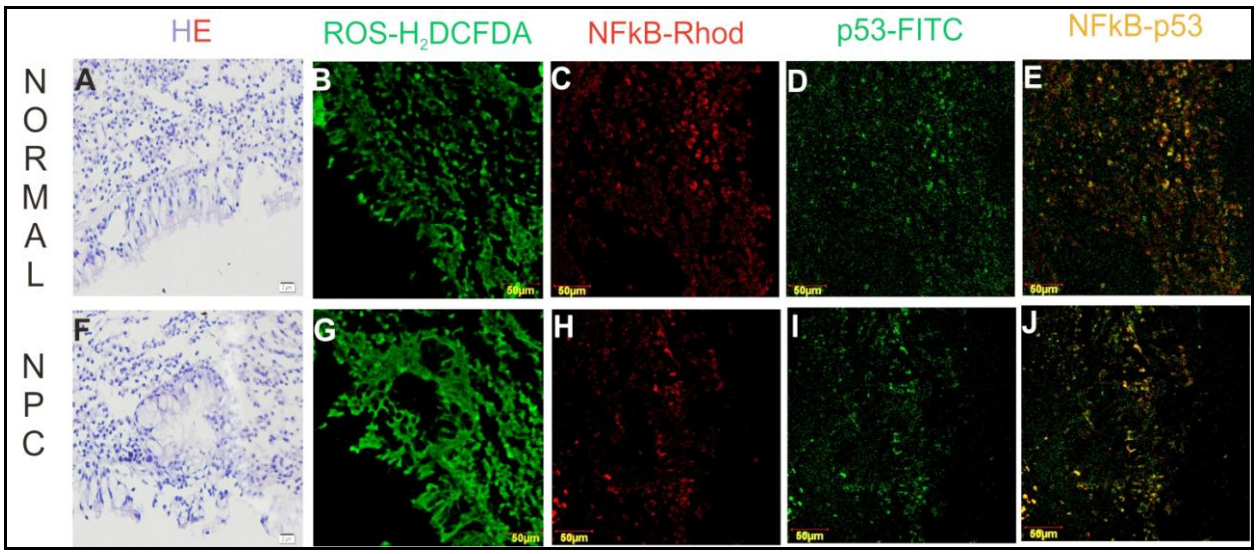

Figure 4. Confocal micrograph of ROS, NF-κB and p53 in NPC tissue and normal nasopharyngeal tissue. Immunofluorescence of ROS **(B-G)** was labeled with H_2DCFDA (green color); NFκB expression **(C-H)** was labeled with Rhodamin (red color); p53 expression **(D-I)** was labeled with FITC (green color); merge expression of NFκB and p53 showed (yellow color) **(E-J)**. Higher expression of ROS was detected in NPC tissue **(G)** compared with normal nasopharyngeal tissue **(B)**. We also found higher expression of NF-κB **(H)** and p53 levels **(I)** in NPC tissue compared to normal nasopharyngeal tissue **(C, D)**. [Magnification x400; scale bar 50 µm]

To obtain the source of ROS and underlying pathway we used laser scanning confocal microscopy to measure ROS, NF-κB and p53 levels in NPC tissue and normal nasopharyngeal tissue as shown in Fig.4. We found higher expression of ROS in NPC tissue than in normal nasopharyngeal tissue. Besides, we also found higher expression of NF-κB and p53 levels in NPC tissue compared to normal nasopharyngeal tissue.

DISCUSSION

The main finding of this study showed that serum MDA and H_2O_2 level significantly increase in NPC patients compared to healthy subjects. This finding indicates the involvement of MDA and H_2O_2 level in carcinogenesis of nasopharyngeal tissue. Lipid peroxidation in human diseases can be better explained by following the reaction sequence; disease induced cell damage, then increase in lipid peroxidation, since the disrupted tissues undergo peroxidation more quickly than healthy ones [16]. The severity of lipid damage is related to the concentration of the oxidants in the tissue, and hence, to the efficiency of lipid repair mechanisms. This study is confirmed by a previous study showing that MDA was significantly higher in the blood of NPC patients compared to control [12].

Hydrogen peroxide is poorly reactive; it does not oxidize most biological molecules. The danger of H_2O_2 largely relates to its ease of conversion to the indiscriminately reactive hydroxyl radicals by interaction with transition metal ions. In millimolar levels, H_2O_2 is involved in the oxidative degradation and oxidation of lipid peroxidation [17, 18]. In blood, H_2O_2 can contribute to Fenton chemistry by not only providing one of the substrates but also liberating hemoglobin to releasing iron from hem proteins [17]. Hydrogen peroxide can act in all stages of carcinogenesis [8]. A previous *in vitro* study performed in Raji cell line showed that transition of latent phase into lytic phase was mediated by H_2O_2 [5]. Therefore, the involvement of H_2O_2 in NPC acts not only as a substrate for lipid peroxidation process, but also as an oxidant signaling or inducer for transition of latent into lytic phase. The rates of peroxide generation and use for signaling and control appear to be considerably greater than the rates of free radical reactions contributing to macromolecular damage [19]. In addition, increase of H_2O_2 reflects the elevation of superoxide dismutase (SOD) activity. Previous study showed that SOD level was increased in the nuclear fraction of NPC cells which indicated protection to the genomics integrity [20].

Previous study stated that the level of MDA is the collective result of MDA production and degradation at low pH conditions. The ratio of MDA:H_2O_2 serves to know the involvement of H_2O_2 on reaction series of MDA production degradation, not acting as an oxidant signaling agent [14]. In NPC patients, we found 1.78-fold increase of the PI level than that of the control group. This finding indicates that involvement of H_20_2 in MDA formation is higher in NPC than that in non-NPC individuals' serum.

The level of oxidants and oxidative stress in blood may be supported by levels of ROS in nasopharyngeal tissue. Using laser scanning confocal microscopy, we found higher expression of ROS in NPC tissue than those in normal nasopharyngeal tissue. Besides, we also found higher expression of NF-κB and p53 levels in NPC tissue compared to normal nasopharyngeal tissue. Higher levels of ROS and NF-κB in NPC tissue represent a conductive environment for cell growth and survival as oncogenesis pathway. NF-κB more often promotes progression and survival in cancer [21]. A NF-κB-dependent control pathway of energy metabolism to metabolic adaptation in cancer was previously reported *in vivo* [22]. The ROS also contributed to cystein oxidation of p53 then inhibit p53 activation. In addition, p53 protein is also able to transactivate genes involved in the production of ROS [23]. The lower level expression of p53 in normal nasopharyngeal tissue indicated the action of p53 in maintenance of genomic integrity. In resting conditions, p53 protein is maintained at low levels by MDM2 (murine double minute 2) gene-mediated proteasomal degradation and at this low level of expression reduces ROS levels by inducing the expression of anti-oxidative stress proteins [24].

In conclusion, the tissue of NPC seems to be a source of ROS and oxidative stress in NPC. NF-κB and p53 levels within the NPC tissue may contribute to oxidative stress in the pathomechanisms of undifferentiated NPC.

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