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RESEARCH ARTICLE

Increased production of nitric oxide correlates with tumor growth in Algerian patients with nasopharyngeal carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) is thought to arise because of chronic inflammation. The correlation between nitric oxide (NO) production, a biomarker of inflammation and NPC development remains unexplored. To investigate this question, we performed a profile analysis on plasma collected from untreated, treated, remissive, cured and relapsing patients. Nitrites were measured to assess NO activity. We observed that increased nitrites concentrations in untreated and relapsing patients associated with tumor development. Moreover, nitrites levels were similar in remissive, cured and healthy individuals. Altogether, our results suggest that NO might be an interesting blood biomarker to monitor tumor growth in NPC patients.

Keywords: Nasopharyngeal carcinoma, UCNT, nitric oxide, biomarker, inflammation, carcinogenesis

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy found in south east china and in the Mediterranean countries. In North Africa and particularly in Algeria, the disease affects differently young people under 30 years of age and elderly people over 30 years of age, presumably because of differently regulated oncogenic mechanisms (Bourouba et al. 2011b). NPC carcinogenic process is thought to arise as a consequence of a chronic inflammation affecting epithelial cells of the nasopharynx infected with the Epstein–Barr virus conjugated to a tumor immune evasion and/or immunosuppression mechanism (Yip et al. 2009). Ingestion of salted smocked food enriched with nitrosamines, childhood exposure to domestic fumes, expression of some HLA haplotypes (Feng et al. 2007; Feng et al. 2009; Li et al. 2009; Laantri et al. 2011) and genetic polymorphisms of innate immunity genes (Ben Chaaben et al. 2011; Douik et al. 2009) are thought to participate in individual susceptibility towards NPC.

Nitric oxide (NO) is an unstable inorganic free radical gas synthesized from L-Arginine by a family of isoenzymes called NO synthases (NOS). Two of these are constitutively expressed (nNOS and eNOS) and a third called the inducible NO synthase (iNOS) is induced by immunological stimuli (Xie & Nathan 1994). nNOS and eNOS are respectively synthesized by neurons and endothelial cells and act as important signaling molecule in the cardiovascular and nervous systems. These isoforms are regulated primarily by calcium influx and generate low concentrations of NO for brief periods of time (nmol/L range) (Moncada 1994). NO released by iNOS is generated for a sustained period at high concentrations (μmol/L range) by cells of the immune system (monocytes/macrophages) and by epithelial cells

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exposed to pro-inflammatory cytokines (TNFα, IL12, IFNγ.)(Robbins et al. 1994; Chan et al. 1999b).

NO has various effects on tumor biology and may play dual anti-tumoral or pro-tumoral activities depending on its concentration. Indeed, whereas high NO concentrations are generally cytostatic/cytotoxic, low NO concentrations have been reported to protect cells from apoptosis (Mannick et al. 1994). NO may then either act as a suppressor of tumorigenesis (Hussain et al. 2004; Lechner et al. 2005) or contribute to tumor initiation and accelerated tumor development (Hussain et al. 2008; Segawa et al. 2008; Wei et al. 2009). Because NO is a stimulator of angiogenesis and a promoter of formation of stable vessels (Ridnour et al. 2005; Ridnour et al. 2006) it may further contribute to tumor growth and extension (Isenberg et al. 2009; Jenkins et al. 1995). This ambivalence of NO activity was further experimentally confirmed with NO inhibitors and donors, which were found to exhibit anti or pro-tumoral properties *in-vitro* and *in-vivo* (Dhar et al. 2003; Maksimovic-Ivanic et al. 2008; Mijatovic et al. 2008; Flitney et al. 2011), still, the effect of NO concentrations on tumor progression seems to be highly dependent on the type of tissue studied (Aaltomaa et al. 2000; Mazibrada et al. 2008).

In NPC patients with locally advanced cancer, the inflammatory tumors are associated with expression of iNOS and presence of tumor infiltrating lymphocytes (TIL) and macrophages (TIM) (Jayasurya et al. 2003; Soo et al. 2005; Zhang et al. 2010). NPC patients are also characterized with increased concentrations of circulating pro-inflammatory cytokines (ex. TNFα, IFNγ), which may act as positive regulators of iNOS expression (Chan et al. 1999a,b; Li et al. 2007a). Whether, iNOS overexpression is functionally associated with enhanced NO production in patients has not been yet established. To investigate this question, we performed a profiling of plasmatic NO production in untreated and treated NPC Algerian patients and correlated these values with tumor development.

Methods

Biological and clinical samples

Blood samples were collected after informed consent from newly diagnosed NPC patients when they first presented at Algiers Medicinal University Hospital M. Bacha. Samples were also collected from patients following treatments, in remission, at relapse and from cured individuals (Table 1). Taking aseptic precautions, blood samples were collected in sterile vials by venous arm puncture. 5 mL of blood were collected with EDTA for plasma preparation. Plasma was separated by centrifugation at 1.000 g for 15 min. Upon preparation each sample was aliquoted and stored at -20°C until analysis. Venous blood samples were drawn before breakfast. All participants gave their informed consent for the present study as required by the ethic committee of the national agency of research development in health (ANDRS) which supported this study.

Determination of nitrite concentrations

NO production was estimated by measuring the inert end products of its metabolism; namely nitrite concentrations were measured in plasma using the modified Griess reagent as previously described (Belguendouz et al. 2008). Absorbance was read at 543 nm using spectrophotometer.

Tumor staging and histological classification

Patients were classified according to the tumor-node-metastasis staging system promulgated by the American Joint Committee on Cancer AJCC/UICC (1997). The

Table 1. Patients' information.

	Factors	Juveniles	Elderly
Controls	Age	20.7 ± 1.5	40.9 ± 9.25
	Number	10	10
Untreated	Age	20.66 ± 7.37	43.6 ± 8.35
	No. of patients	10	10
	Histology/Stage	UCNT/stage III/IV	UCNT/stage III/IV
Post-therapy	Age	19.3 ± 4.6	47.7 ± 10.18
	No. of patients	11	10
	Histology/Stage	UCNT/stage III/IV	UCNT/stage III/IV
Remission	Age	24.9 ± 3.5	42.3 ± 10.2
	No. of patients	10	11
	Histology/Stage	UCNT/stage III/IV	UCNT/stage III/IV
Cured	Age	26.13 ± 4.2	47.54 ± 9.62
	No. of patients	8	12
	Histology/Stage	UCNT/stage III/IV	UCNT/stage III/IV
Relapse	Age	26 ± 7.07	46 ± 7.89
	No. of patients	6	9
	Histology/Stage	UCNT/stage III/IV	UCNT/stage III/IV

Age values are given as mean years ± standard deviation. Histological type and stages are indicative of the tumor characteristics at the time of first diagnosis.

majority of the 20 untreated NPC patients belonged to stage III and stage IV. Based on morphological examination, all tissues were confirmed to belong to the undifferentiated carcinomas after hematoxylin and Eosin staining (UC, WHO type 3).

Statistical analysis

The data were analyzed with Student's *t* test using the StatSoft. A *p* value ≤0.05 was accepted as statistically significant.

Results

Plasmatic nitrites concentrations are increased in untreated NPC patients compared with controls

Nitric oxide (NO) is a free radical which is associated with inflammation, tumorigenesis and anti-tumoral immunity. To analyze how in Algerian patients with locally advanced NPC NO_2- production is regulated, we measured the concentration of the stable nitrated NO metabolites in the plasma of randomly selected untreated patients. A cohort of age matched healthy individuals with no inflammatory symptoms was used as control (Table 1). We observed with interest that nitrites concentrations in NPC patients were increased compared to controls (40.5 ± 16.4 vs. 56.4 ± 22.6) (Figure 1A). Because NPC development is associated to distinct expression of cellular and viral biomarkers that may influence iNOS activation in patients under and above 30 years of age (Ma et al. 2008; Bourouba et al. 2011a), we next tried to determine whether NO_2- concentrations were significantly different in untreated juvenile and elderly NPC populations. Firstly, we observed that basal NO_2- levels were significantly higher in elderly controls compared to juvenile controls (34.20 ± 6.63 vs. 46.89 ± 9.81). Secondly, we found that all patients displayed significantly higher NO_2^- plasmatic concentrations in comparison to age matched groups (48.05 ± 5 vs. 34.2 ± 6.63) and (64.75 ± 17.6 vs. 46.89 ± 9.81) (Figure 1B). Interestingly, the relative NO increase in absence of any treatment showed to be similar in both groups of age (40% vs. 38% increase). These findings suggest that NPC development in juvenile and elderly patients is accompanied with a positive regulation of nitric oxide synthesis.

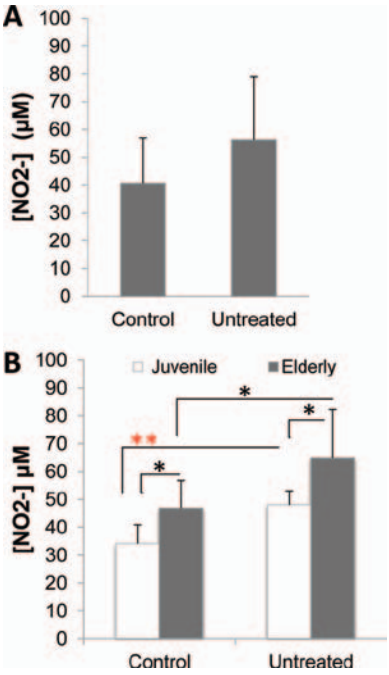

Figure 1. Plasmatic nitrite levels are increased in untreated NPC patients (A) Plasmatic nitrites levels were measured in a cohort of untreated patients (n = 20) and compared to a cohort of healthy individuals (n = 20). (B) Plasmatic nitrites levels were compared between juvenile (n = 10) and elderly individuals (n =10) with NPC. A cohort of age matched healthy individuals was used as control (n = 10, n = 10). Values are given as mean + standard deviation (SD). *p < 0.05. **p < 0.01.

Post-therapy patients display similar nitrite concentrations to healthy individuals

Next, we analyzed the effect of Radiation therapy (RT) and Docetaxel, cisplatin, 5-fluorouracil (TPF)-based induction chemotherapy (CT) on NO production. To this

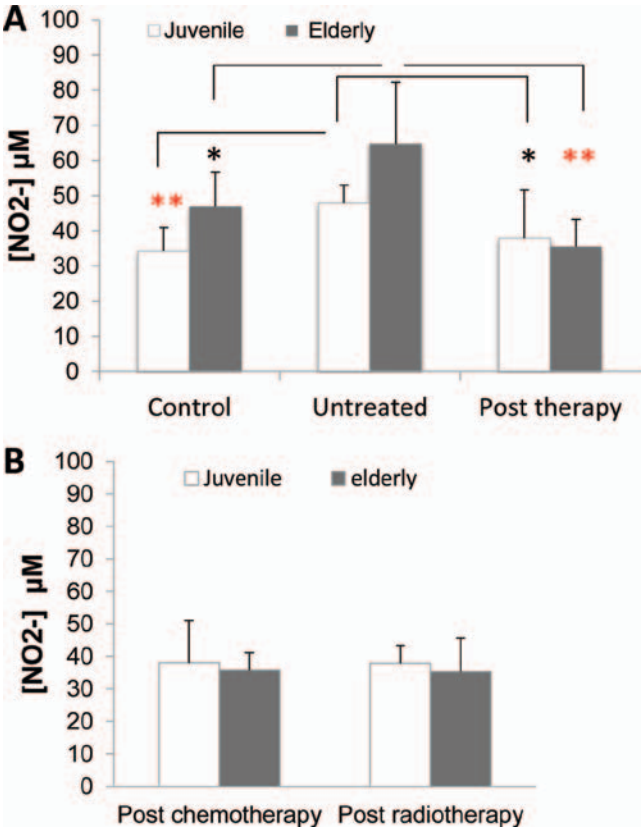

Figure 2. Plasmatic nitrites levels in treated patients and age matched healthy individuals are similar. (A) Plasmatic nitrites levels were measured in patients following therapy (n = 11, n = 10) and compared to those found in age matched patients with NPC (n = 10, n = 10) and matched healthy individuals by the Griess method (n = 10, n = 10). (B) Radiation and chemotherapy (TPF) effect on nitrites production was evaluated in juvenile (n = 4, n = 7) and elderly patients plasma (n = 5, n = 5). Values are given as mean + SD. *p< 0 .05; **p < 0.01.

aim, nitrites levels were assessed 1 month after treatment in NPC patients. Irrespectively of the type of treatment followed, all treated patients experienced a significant reduction in NO_2- levels in response to therapy compared to untreated groups (37.9 ± 15.67 vs. 48.04 ± 5.0) and (35.4 ± 7.84 vs. 64.75 ± 17.57) (Figure 2A). Importantly we found that, regardless of their age, patients after therapy displayed similar nitrites levels compared with healthy individuals (36.7 ± 21.55 vs. 40.55 ± 16,4). We also observed that NO_2- levels in juvenile and elderly patients were reduced to similar levels irrespectively of the type of treatment followed (CT juvenile vs. CT elderly: 38.01 ± 13.06 vs. 35.65 ± 5.46 and RT juvenile vs. RT elderly 37,9 ± 15.04 vs. 35.23 ± 10.42), still, the relative variations in plasmatic nitrite concentrations indicated a more pronounced effect of the treatment on the elderly population (30% vs. 63% reduction juvenile treated/untreated vs. elderly treated/untreated) (Figure 2B).

To determine the specific impact of RT and CT on NO production, we compared plasmatic nitrites concentrations in patients who received each one of the two treatments. No significant differences in nitrites concentrations were found for patients who had CT or RT (juvenile CT vs. RT: 38.01 ± 13.06 vs. 37.9 ± 15.04; elderly CT vs. RT: 35.65 ± 5.46 vs. 35.23 ± 10.42) (Figure 2B). We concluded from these results that radiation and TPF-induced chemotherapy reduces NO synthesis in NPC patients to comparable normal levels upon therapy.

Nitrites concentrations in remissive and cured patients are similar to those found in healthy individuals

Next, we tried to determine whether, in absence of tumor growth, the inflammatory status relative to NO_2- production in remissive and cured patients would be distinct from the one found in patients after therapy. Therefore, nitrites concentrations in plasma of remissive (2 year disease free) and cured (5 year disease free) patients were compared to those of untreated patients and controls. For both remissive and cured groups, nitrites concentrations were independently of patients' age significantly decreased compared to those found in untreated individuals (juvenile remission vs. untreated: 38.64 ± 13.04 vs. 48.04 ± 5; elderly 52.52 ± 21.39 vs. 64.75 ± 17.57) and (juvenile cured vs. untreated 37.23 ± 7.78 vs. 48.04 ± 5; elderly 52.97 ± 19.49 vs. 64.75 ± 17.57) (Figure 3). In contrast, these values were not statistically different from those of age matched healthy controls (juvenile remission vs. control: 38,64 ± 13.04 vs. 34.2 ± 6.62; elderly 52.52 ± 21.39 vs. 46.88 ± 9.81) and (juvenile cured vs. control 37.23 ± 7.78 vs. 34.2 ± 6.62; elderly 52.97 ± 19.49 vs. 46.88 ± 9.81) (Figure 3). We concluded for these results, that absence of tumor growth in remissive and cured patients correlates with normal levels of nitric oxide production.

Relapsing patients and untreated patients display increased plasmatic nitrites concentrations

Because relapse frequently occurs and is one of the main concerns in the treatment of NPC, we next tried to determine how NO_2- concentrations would vary in NPC patients with recurrent growing tumors. We observed that both juvenile and elderly relapsing patients displayed a significant increased NO_2- production compared with controls and cured patients (juvenile relapsing vs. control: 63.92 ± 28.9 vs. 34.2 ± 6.62; elderly 65.3 ± 20.69 vs. 46.88 ± 9.81) and (juvenile cured vs. relapsing 37.23 ± 7.78 vs. 63.92 ± 28.9 ; elderly 52.97 ± 19.49 vs. 65.3 ± 20.69) (Figure 4). We also observed that in relapsing patients NO_2- levels, generated during tumor development, were not different from those observed in untreated patients. Interestingly, the juvenile population developed a more pronounced inflammatory reaction compared with elderly patients during the relapse stage (87% vs.40% increase compared to controls) (Figure 4). Together, these results indicate that tumor growth in untreated or relapsing NPC patients is characterized by an increase in nitric oxide production.

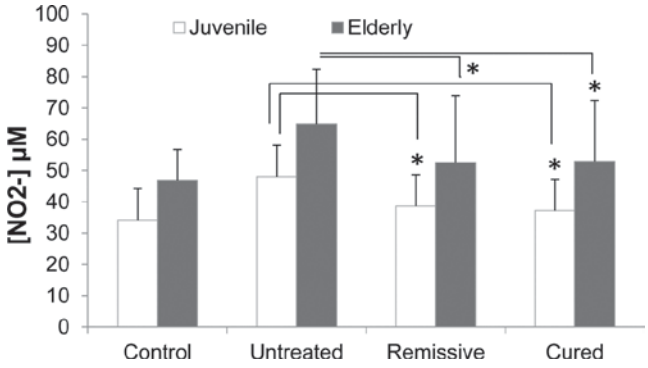

Figure 3. Plasmatic nitrites levels in remissive and cured juvenile and elderly patients are comparable to normal levels. Plasmatic Nitrites levels were measured by the Griess method in 2 year remissive ($n = 10$, $n = 11$) and cured patients plasma ($n = 8$, $n = 12$). Values were compared with those of age matched untreated NPC patients ($n = 10$, $n = 10$) and matched healthy controls ($n = 10$, $n = 10$). Values are given as mean + SD. *$p < 0.05$.

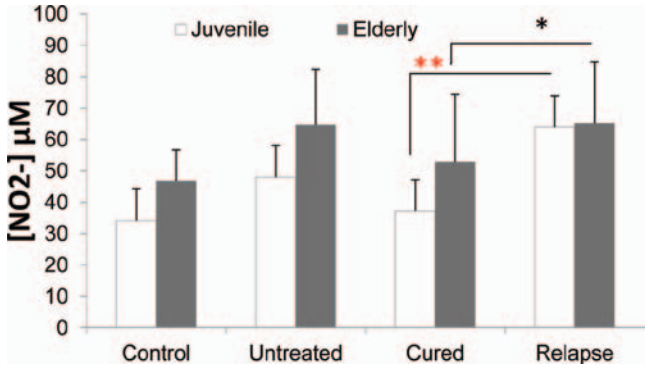

Figure 4. Plasmatic nitrites levels in relapsing patients are increased compared with healthy and cured patients. Plasmatic nitrites levels were measured by the Griess method in relapsing patients ($n = 6$, $n = 9$) and compared with nitrites concentrations in age matched controls, untreated and cured patients. Values are given as mean + SD. *$p < 0.05$; **$p < 0.01$.

Discussion

In this study, we did a profiling analysis of NO production in Algerian patients with developing and relapsing NPC and in treated patients and controls. NO variations in patients with NPC history were correlated with clinical outcome. We observed a significant association between elevated NO concentrations and tumor growth in untreated and relapsing patients. Similarly, we observed a significant association between reduction of NO concentrations, remission and cure. We noticed a tender for NO to be produced at higher levels in elderly patients. Interestingly, the relative variations of NO produced at every stage showed to be independent of patients' age, at the exception of the relapsing stage where a stronger NO response was observed in juvenile individuals.

Our observations show that untreated patients have increased levels of circulating nitric oxide. This finding is in line with previous reports indicating an overexpression of iNOS in tumors of NPC patients(Ma et al. 2008). This is also in agreement with the pro-inflammatory profile of NPC tumors, as indicated by their massive stroma infiltration with T and B lymphocytes and activated macrophages (Li et al. 2011). It is possible that the intense plasmatic nitric oxide levels observed in patients may be due to an enhanced catalytic activity of iNOS generated by the increased levels of pro-inflammatory cytokines produced by the tumoral microenvironnement and by activated circulating peripheral blood mononuclear cells (Li et al. 2011). Recent studies have demonstrated that untreated patients with NPC display increased levels of IL6, IFNγ and TNFα (Li et al. 2007a). These cytokines positively regulate iNOS expression and NO release. It is to mention that epithelial cells exposed to IL6/Stat3 signaling were shown to display increased iNOS products and DNA damage signatures that could be essential to tumor initiation and perpetuation (Ma et al. 2008a). Because expression of the EBV oncogene LMP1 itself induces IL6 expression by epithelial tumor cells (Chen et al. 2003), it is possible that the size of the tumor expressing the viral oncogene may directly influence circulating NO concentrations. To verify this hypothesis, the correlation between the size of the tumor (T), the metastatic extensions (N/M) and NO production should be analyzed in the future on a larger cohort.

It is interesting to note that a more important NO production was found in elderly untreated patients compared with untreated juvenile patients and in relapsing juveniles compared to elderly relapsing patients. The mechanisms by which this phenomenon occurs need to be further investigated, but may possibly be due to distinct expressions levels of EGFR and LMP1 that could in turn differently regulate NO production and tumor development (Bourouba et al. 2011a). Indeed, LMP1 association to EGFR, in presence of IL6/Stat3 signalization, was suggested to potentiate iNOS synthesis and NO dependent DNA damages in NPC (Yu et al. 2002; Soo et al. 2005; Ma et al. 2008). In these conditions, NO synthesis may be hijacked by the transformed cells to positively regulate tumor growth via inhibition of EBV-infected cell apoptosis (Mannick et al. 1994; Gao et al. 1999).

In the NPC context, NO may probably exert its anti-apoptotic activity through S-nitrosylation of mitochondrial caspases and up-regulation of anti-apoptotic proteins expression (Suschek et al. 1999; Mannick et al. 2001). Our recent observation, that NPC tissues are in their majority positive for Bcl2, is in agreement with this hypothesis (Bourouba et al. 2011a). Recent studies showed that transcriptionally active p53 may be stabilized in the nucleus under the impact of NO while serine 15 was phosphorylated (Schneiderhan et al. 2003). NO induced S-nitrosylation and inactivation of HDM2 may also participate in p53 nuclear trapping (Schonhoff et al. 2002). These observations are consistent with our previous study showing the existence of a strong nuclear accumulation of p53 and other studies showing that in NPC: (1) HDM2 half-life was shortened compared to p53's and (2) that LMP1 expression can induce p53 phosphorylation at serine 15 (Li et al. 2007b; Li et al. 2010).

Next, we analyzed the influence of therapy on NO production. Our data show that chemotherapeutic or radiotherapeutic targeting of the tumor reduces the levels of circulating NO. This result may probably result from a reduction of the tumor volume and IL6 expression upon treatment as reported by others (Tan et al. 2006; Orbach et al. 2008).

Finally, we found that patients at relapse displayed increased NO levels. This result suggests that tumor's growth and its relapse is associated to an important inflammatory status in NPC patients. This hypothesis is supported by previous reports describing in relapsing NPC patients a strong up-regulation of protein biomarkers of chronic inflammation like Macrophage inflammatory protein-3α (MIP3α) (Chang et al. 2008) and serum amyloid A (SAA) (Cho et al. 2004). Contrarily to SAA, which was reported to be up-regulated only at relapse, MIP3A was described to be over-expressed in tumors of both untreated and relapsing patients. Moreover MIP3α was described as a biomarker of bad prognosis (Chang et al. 2008). Because MIP3α and iNOS expression are induced by common pro-inflammatory cytokines (IFNγ, TNFα) further investigations should also address the prognostic value of iNOS/NO synthesis in NPC patients. Interestingly, Jayasurya et al. reported that low expression of iNOS in tumors of untreated patients correlates with bad clinical prognosis by promoting tumor recurrence and metastasis after radiotherapy (Jayasurya et al. 2003). Considering our results, this observation would indicate that high iNOS enzymatic activity in untreated NPC patients would be necessary to tumor resolution.

In summary, this study suggests firstly, that in NPC the tumor itself would be a major source of plasmatic nitric oxide and secondly, inflammation, as measured by circulating NO, may be an important factor for NPC development. Our results put forward also that NO might be an interesting non-EBV blood biomarker to monitor patient's inflammatory status during NPC tumorigenesis,

still, further investigation should delineate the role of iNOS signaling in NPC development and should clarify the molecular significance of the strong associations observed between NO production, NPC occurrence and recurrence.

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Declaration of interest

The authors report no declarations of interest.

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