Peripheral Blood Mononuclear Cell (PBMC) Inducible Nitric Oxide Synthase (iNOS) Expression and Activity as a Potential Tumor Marker in Hepatocellular Carcinoma (HCC)

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ABSTRACT

Background: PBMC iNOS expression was evaluated as a potential tumor marker in HCC.

Methods: Forty five HCC patients, 40 cirrhotic (30 hepatitis and 10 Bilharzial) and 20 healthy subjects were studied. PBMC iNOS expression was evaluated using real time PCR and normalized for GAPDH. PBMC and plasma nitrite/nitrate was measured by Griess reaction. AFP was measured by ELIZA.

Results: iNOS was undetectable in normal individuals; minimal expression was encountered in chronic hepatitis and Bilharzial group with a ratio of 0.45±0.12 and 1± 0.48 respectively. A high level was encountered in HCC patients with an iNOS/GAPDH ratio of 5.7±1.2 (p <0.0001). The ratio was higher in patients with tumor size ≥3cm in diameter (6.2± 0.9 versus 5.2±1.2, p <0.05). PBMC nitrite/nitrate was detectable at comparable low levels in normal and hepatitis groups with a mean of 16.4±7.8 and 16.9±7. It was insignificantly elevated in Bilharzial group (22.1±7.5) and markedly elevated in HCC (72.9±14.9 umol/ml, p< 0.001). Strong positive correlation was encountered between PBMC iNOS expression, activity and plasma nitrite/nitrate (r=0.86, 0.89 and 0.83). Plasma nitrite/nitrate was elevated in HCC patients in a fashion comparable to its PBMC level. AFP measurement showed a level >50 ng/ml in 84.5% of the HCC patients.

Conclusion: PBMC iNOS expressions, activity in PBMC and plasma nitrite/nitrate are increased in all HCC patients compared to 84.5% with elevated AFP. These findings would strongly recommend iNOS expression and activity as a potential tumor marker in HCC.

Key Words: iNOS - HCC - Tumor markers.

INTRODUCTION

Nitric Oxide (NO) is a molecule of high versatility where in small amounts, it is essential for many physiologic functions while in high concentrations it might be noxious [10]. Expression of inducible nitric oxide synthase (iNOS) and its product NO are known to play an important role in carcinogenesis as well as in promoting tumor growth and angiogenesis [21].

Chronic viral hepatitis (CVH), one of the risk factors for the development of HCC, is known to cause elevation of iNOS transcription [14] which might be a factor contributing to carcinogenesis [9].

NO in the form of nitrite/nitrate, as a tumor marker in HCC, was claimed to be of sensitivity comparable to AFP; however its production is compromised by increased hepatic dysfunction [16-18]. The source of nitrite/nitrate in HCC is both Kupffer cells and tumor cells. Increased iNOS expression might be induced by some factors released by the tumor itself as well as by the endotoxemia and/or increased circulating cytokines [21]. Moreover INFγ, a major player in the immune response to cancer, is known to increase the expression of iNOS [17].

Previous studies on iNOS in HCC were all performed on hepatic tissue investigating the enzyme in tumor cells, kupper cells and surrounding tissues [11,21]. However tissue biopsy, taken for diagnosis, may not be adequate for such studies. Also iNOS production by the liver may be compromised by increased hepatic dysfunction [16].
Accordingly we have put forward a hypothesis that the factors causing increased iNOS in the liver tissue whether endotoxins, cytokines or factors released by the tumor might as well reach the circulation and increase iNOS expression in peripheral blood mononuclear cells (PBMC).

If this is the case PBMC would work as a simple accessible surrogate system, not affected by hepatic dysfunction that might serve as a tumor marker in HCC.

Thus we estimated PBMC iNOS mRNA and protein expression in 45 HCC cases and compared the results to 30 patients with cirrhosis on top of CVH, 10 with Bilharzial liver cirrhosis and 20 healthy controls. We also evaluated plasma nitrite/nitrate and AFP in the HCC group to verify how the standard markers would correlate to the suggested ones.

**PATIENTS AND METHODS**

*Patients:*

The study comprised 45 HCC patients including 28 male and 17 female with an age range of 42-73 with a median of 56 years.

Thirty viral hepatitis cases (20 HCV and 10 HBV), 10 with Bilharzial liver cirrhosis as well as 20 healthy subjects of comparable age and sex were included as controls.

Diagnosis of HCC was confirmed by liver biopsy, diagnosis of HBV by HbsAg and detection of viral DNA by PCR while diagnosis of HCV by positive antibody and detection of viral RNA by RT PCR. HCC cases presented to the NCI, Cairo University and Ain-Shams University Hospital in the period from June 2001 to February 2002. Exclusion criteria included co-existing chronic inflammatory conditions, active allergy or infection, patients receiving ribavirin, nitroglycerin or other nitrate containing medications as well as pregnant women. None of the subjects had renal dysfunction or clinical or laboratory evidence of bacterial infection; all patients fasted for at least 12 hours.

Hepatitis, Bilharzial and normal controls presented to Ain-Shams University during the same time period.

The 45 HCC patients were divided according to underlying pathology into 3 groups:

- Twenty patients with no underlying pathology; they will be referred to thereafter as HCC De Novo group.
- Fifteen patients of HCC on top of viral hepatitis: HCC hepatitis group.
- Ten patients of HCC on top of Bilharzial cirrhosis: HCC Bilharzial group.

The study was approved by the Institutional Research Board of the NCI, Cairo University and the Medical Research Center, Ain-Shams University.

All patients and controls gave consent to participate in the study after detailed explanation of its nature.

*Methods:*

All study groups were subjected to the following investigations:

- Liver function tests including: bilirubin, total protein, albumin AST, ALT, GGT & ALP.
- PBMC iNOS mRNA expression presented as a ratio to glyceraldehyde phosphate dehydrogenase (GAPDH) expression.
- PBMC iNOS protein expression presented as nitrite/nitrate (umol/ml) measured in 48 hours’ culture supernatant by the Griess reaction [5] using the Total NO assay kit (R&D, 1600, USA).
- Plasma nitrite/nitrate (umol/ml) and serum AFP in the HCC group to verify how the standard markers would correlate to the suggested ones.

**PBMC iNOS determination:**

Mononuclear cells were separated on Ficoll Hypaque gradient [2]. RNA was extracted using total RNA extraction kit (Boehringer Manheim, Germany) according to manufacturer’s instructions; concentration and purity were determined using UV spectrophotometer at 260 and 280 nm wavelengths. One µg was reverse transcribed in a 25 µl reaction containing 25 pmole oligo DT and 5 µ AMV-RT enzyme for one hour at 37°C. Reactions were stopped by heating to 99°C for 5 minutes and kept on ice for 5 minutes. The resulting cDNA was used for conventional PCR amplification and analyzed by Gel Documentation system (Gelpro-analyser, version 30, MEDIA. CYBERNETICS. USA) as well as by Real Time PCR 5700 (Perkin-Elymer) using...
SYBR green 1 assay. Two sets of primers were used for iNOS and GAPDH after Sharara et al. [23], the sequence of the primers is presented in table (1). Results were expressed as iNOS/GAPDH ratio.

Conventional PCR amplification was performed in a 50 µl reaction containing 10 mM Tris HCl (PH 8.3), 1.5 mM MgCl, 25mM KCl, 200uM each dNTPs, the 4 primers (sense and antisense) each 50 pmole, 2.5 units Taq polymerase and 200 ng cDNA. Amplification was performed using thermal cycler (Biometra, Uno II Thermo block) starting with denaturation at 94 ºC for 5 minutes followed by 40 cycles of 94 ºC for 30 seconds, 55 ºC for 30 seconds and 72 ºC for 30 seconds. A final extension step at 72 ºC for 7 minutes was performed and samples kept at 4 ºC. The amplified products were separated on 1.5% agarose gel for 30 minutes at 100 volt; the run included a DNA molecular marker 80-587 bp (PBR 322, Sigma). Gels were stained in ethidium bromide for 30 minutes and destained in 1mM MgSO4 for 15 minutes. Fig. (1) illustrates the amplified GAPDH and iNOS.

Amplification for real time PCR was performed in a 50 µl reaction containing 1 X SYBR Green reaction buffer (SYBR Green dye, 250 uM of each dGTP, dCTP, and dATP, 500 uM dUTP, 0.5 units UNG, 3mM MgCl, 1.5 units AmpliTaq Gold), 50 Pmole of each of the sense and antisense primers of either iNOS or GAPDH and 200 ng cDNA.

**Statistical analysis:**

Results were expressed as mean ± SD. Statistical analysis was performed using the unpaired Student’s T test to compare means. Ranked Spearman correlation test was used to calculate correlation coefficient between various parameters within individual cases. Analysis was performed using SPSS Software Package, Echo Soft Corporation USA, 1998 version 9.05.

**RESULTS**

In this work 45 HCC patients were tested for PBMC iNOS mRNA and protein expression as well as for plasma nitrite/nitrate and serum AFP.

The PBMC iNOS mRNA expression was increased in all the patients with marked statistically significant difference from control, hepatitis and Bilharzial groups (p < 0.001, Table 2).

According to the underlying pathology (Table 3) the HCC De Novo showed the highest expression and activity; the difference from the other 2 groups (cirrhotic) was found to be statistically significant (p < 0.05).

PBMC iNOS mRNA and protein expression were also found to be higher in patients with tumor size ≥ 3 cm compared to those with a size < 3 cm (Table 3); the difference was found to be statistically significant (p<0.05). Strong positive correlation was encountered between PBMC iNOS expression and activity (r = 0.86, Fig 2).

In this work plasma nitrite/nitrate was also evaluated in the HCC group. Elevated level was found in all patients, maximum in HCC De Novo and least in HCC Bilharzial; the difference was found to be statistically significant (p < 0.05). It was also significantly higher in patients with tumor size ≥ 3 cm compared to those with < 3 cm (p < 0.05). Strong positive correlation was encountered between PBMC iNOS and plasma nitrite/nitrate (r = 0.89, Fig. 3) as well as between PBMC iNOS and plasma nitrite/nitrate (r = 0.83, Fig. 4).

In this work, AFP was evaluated at a cutoff value of 50 ng/ ml; 7 / 45 cases had lower values, all with tumor size < 3 cm. Three of the 7 had HCC De Novo, 2 had HCC on top of hepatitis and 2 on top of Bilharziasis. The other 38 cases showed values varying between 199 and 1855 with a mean of 1057±476 ng/ml. The level of AFP in patients with values > 50 ng/ml was found to be comparable in the 3 groups of HCC with different underlying pathology. However it was significantly higher in cases with tumor size ≥ 3 cm (p < 0.05, Table 4).

The iNOS expression and activity as well as plasma nitrite/nitrate were highest in the group with > 50 ng/ml AFP and ≥ 3 cm tumor size followed by the group with > 50 ng/ml AFP and < 3 cm tumor size and least in the group with < 50 ng/ml AFP and < 3 cm tumor size (Table 4), the difference was found to be statistically significant (p < 0.05). However no or poor correlation was encountered between AFP on one side and each of plasma nitrite/nitrate, PBMC iNOS expression and activity on the other side (r = 0.22, 0.3 and 0.13 respectively).
Fig. (1): Agarose gel electrophoresis of amplified iNOS (290 bp) and GAPDH (390 bp).
Lane 1: DNA Molecular Weight Marker, 80-587 bp, Lanes 2, 3: Control Amplified GAPDH, Lanes 4, 5: GAPDH and iNOS in Hepatitis C, Lanes 6–10: GAPDH and iNOS in HCC.

Fig. (2): Correlation between PBMC iNOS Expression and Activity.

Fig. (3): Correlation between PBMC and Plasma Nitrite/Nitrate.

Fig. (4): Correlation between PBMC iNOS and Plasma Nitrite/Nitrate.

Table (1): Primer sequences for iNOS and GAPDH.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS Sense</td>
<td>5’ CCT GAG CTC TTC TTC GAA ATC C3’</td>
</tr>
<tr>
<td>iNOS Antisense</td>
<td>5’ AGG ATG TTG TAG CGC TGG A C3’</td>
</tr>
<tr>
<td>GAPDH Sense</td>
<td>5’ CTA CTG GCG CTG CCA AGG CTG T3’</td>
</tr>
<tr>
<td>GAPDH Antisense</td>
<td>5’ GCC ATG AGG TCC ACC ACC CTG T3’</td>
</tr>
</tbody>
</table>

Sharara et al. (1997)

Table (2): PBMC iNOS mRNA and protein expression in HCC, CVH and Bilharziasis.

<table>
<thead>
<tr>
<th>Test group</th>
<th>Number</th>
<th>iNOS/GAPDH Ratio</th>
<th>Nitrite/Nitrate (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>0.0</td>
<td>16.4±7.8* (7.4-36)</td>
</tr>
<tr>
<td>CVH</td>
<td>30</td>
<td>0.4±0.12 (0.27-0.65)</td>
<td>16.9±7.0 (7.0-33)</td>
</tr>
<tr>
<td>Bilharzial</td>
<td>10</td>
<td>1.0±0.48 (0.33-2.0)</td>
<td>22.1±7.5 (11.0-36)</td>
</tr>
<tr>
<td>HCC</td>
<td>45</td>
<td>5.7±1.2 (4.8-8.0)</td>
<td>72.9±14.9 (45.0-99.0)</td>
</tr>
</tbody>
</table>

* mean±SD  
\( p \text{ value} < 0.001 \) (range)
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HCC, probably for the first time. We also investigated, in parallel, the classical tumor marker, AFP as well as plasma nitrite/nitrate, which was recently claimed to be a valuable complementary tumor marker [16-18].

Previous work on iNOS expression was all performed on liver tissue. Using immunohistochemistry iNOS expression was found to be significantly higher in the hepatocytes of both tumor and surrounding tissue in HCV positive HCC [14]. It was reported to be significantly higher in the surrounding liver in cirrhotic patients [21]. Several factors are responsible for induction of iNOS in these conditions including endotoxemia, cytokines as well as factors released by the tumor; both hepatocytes and kupffer cells are involved in NO production [17].

Liver infiltrating T lymphocytes were also reported to express iNOS [22].

Thus a majority of PBMC would be capable of iNOS expression including T cells and monocytes which are the precursors of all fixed tissue mononuclear phagocytes including kupffer cells [20]. The presence of markedly increased iNOS mRNA and protein expression in cases of HCC encountered in the current study validates our proposed hypothesis that iNOS could be induced in the PBMC by the same factors causing its induction in the liver tissue and that these factors readily gain access to the circulation. As PBMC are not directly affected by the tumor or hepatic dysfunction, they might be better responders.

In this work, strong positive correlation between PBMC iNOS mRNA and protein expression was encountered. Accordingly, if both prove to be relevant as tumor markers, the latter

Table (3): iNOS mRNA and protein expression in HCC in relation to underlying pathology and tumor size.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HCC De Novo 20 cases</th>
<th>HCC CVH 15 cases</th>
<th>HCC Bilharzial 10 cases</th>
<th>HCC Cirrhosis* 25 cases</th>
<th>HCC ≥ 3 cm 23 cases</th>
<th>HCC ≤ 3 cm 22 cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC iNOS/GAPDH Ratio</td>
<td>6.6±1.0 (4.8-8.0)</td>
<td>5.4±0.62 (4.6-6.7)</td>
<td>4.6±1.0 (2.9-5.6)</td>
<td>5.0±1.1 (2.9-6.7)</td>
<td>6.2±0.9 (4.8-8.0)</td>
<td>5.2±1.2 (2.9-7.9)</td>
</tr>
<tr>
<td>Plasma Nitrite/Nitrate umol/L</td>
<td>83±12.6 (58-99)</td>
<td>70±10.2 (58-89)</td>
<td>57±8.4 (45-69)</td>
<td>64±11.3 (45-89)</td>
<td>77.2±13.6 (58-98)</td>
<td>68±15.2 (45-99)</td>
</tr>
<tr>
<td>PBMC Nitrite/Nitrate umol/L</td>
<td>87±10.3 (67-110)</td>
<td>76±12.2 (60-99)</td>
<td>66±8.3 (52-75)</td>
<td>71.4±11 (52-99)</td>
<td>82±14.11 (56-110)</td>
<td>74.8±12.2 (52-99)</td>
</tr>
</tbody>
</table>

*p value < 0.05

Table (4): Serum AFP in relation to plasma nitrite/nitrate and tumor size in HCC.

<table>
<thead>
<tr>
<th>Variable</th>
<th>&lt; 3 cm</th>
<th>&gt; 3 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP &lt; 50 (ng/ml) Number=7 cases</td>
<td>4.6±1.2</td>
<td>60±7±13.7</td>
</tr>
<tr>
<td>Plasma Nitrite/Nitrate umol/L</td>
<td>72.0±13.6</td>
<td>67.0±13.1</td>
</tr>
<tr>
<td>iNOS / GAPDH Ratio</td>
<td>5.4±1.2</td>
<td>6.2±0.9</td>
</tr>
<tr>
<td>Plasma Nitrite/Nitrate umol/L</td>
<td>80.0±12.4</td>
<td>54.0±14</td>
</tr>
<tr>
<td>PBMC Nitrite/Nitrate umol/L</td>
<td>74.0±14</td>
<td>70.0±13.1</td>
</tr>
</tbody>
</table>

p value : < 0.05

DISCUSSION

The dual role of NO in cancer has raised a lot of interest [8]. NO can induce DNA damage via multiple pathways [3&19]. Endogenously formed NO could modify the structure of p53, thus affecting its tumor suppressor function [1& 6]. NO also plays a role in promoting tumor growth and angiogenesis [21]. On the other hand, high NO levels inhibit mitochondrial respiration, citric acid cycle glycolysis and DNA replication. The action of NO is exacerbated by other factors including locally high levels of reactive oxygen species with generation of even more reactive compounds such as peroxynitrite [7,12&24].

Accordingly, a lot of research was directed to study NO, iNOS (the gene responsible for its production) as well as nitrite/nitrate (its end products) in various types of cancer.

In this work, PBMC iNOS mRNA and protein expression were evaluated in patients with HCC, probably for the first time. We also investigated, in parallel, the classical tumor marker, AFP as well as plasma nitrite/nitrate, which was recently claimed to be a valuable complementary tumor marker [16-18].

Previous work on iNOS expression was all performed on liver tissue. Using immunohistochemistry iNOS expression was found to be significantly higher in the hepatocytes of both tumor and surrounding tissue in HCV positive HCC [14]. It was reported to be significantly higher in the surrounding liver in cirrhotic patients [21]. Several factors are responsible for induction of iNOS in these conditions including endotoxemia, cytokines as well as factors released by the tumor; both hepatocytes and kupffer cells are involved in NO production [17]. Liver infiltrating T lymphocytes were also reported to express iNOS [22].
would be preferred on account of its simplicity.

In this study plasma nitrite/nitrate was found to be elevated in the 45 HCC cases tested with strong positive correlation to PBMC nitrite/nitrate. Basically, this sounds as simple logic; yet plasma nitrite/nitrate was previously reported to be elevated in only a fraction of cases [17-18]. However the number of cases in our study, 45 and in theirs, 39 and 22 respectively is too small to make solid conclusions. The value of plasma nitrite/nitrate is compromised by the multiplicity of factors affecting its level including diet, renal function, inflammation and others [17]. However if all these factors were controlled, it would be the simplest of the 3 parameters. But even though, it would not be reliable in patients with renal insufficiency, chronic inflammatory diseases or acute inflammation; we cannot rely upon a tumor marker that would be suitable for only a fraction of patients.

In the present study, the 3 parameters were found to be higher in De Novo HCC compared to those associated with cirrhosis. It has been suggested that NO is reactively produced by the hepatic tissue components surrounding HCC including macrophages, kupffer cells and hepatocytes; by the progress of cirrhosis the number of macrophages and kupffer cells decreases with deterioration of the hepatic reserve [4&16]. However this would not explain the difference in PBMC iNOS mRNA and protein expression. It must be that the inducing factors whether from the tumor or surrounding hepatic tissue are compromised in the presence of cirrhosis. The tumor itself is apparently a contributor as patients with ≥ 3 cm tumors exhibited significantly higher values of the 3 parameters making them a possible tool to monitor tumor burden and response to therapy.

It was suggested that there is a possibility that an immune response against HCC occurs and correlates to nitrite/nitrate concentration [17]. This explanation might be appealing in view of our results. First, the immune response will be systemically evident in PBMC. Second, immune response is known to be impaired in both CVH and Bilharziasis [13&15], augmenting the compromise caused by cancer alone.

In this work, we have evaluated the classical HCC tumor marker namely AFP. The results, as expected, showed a level > 50 ng/ml in about 84% of cases with marked variation among individual patients. As previously reported by Moriyama et al. [6], it showed no correlation to plasma nitrite/nitrate; it also showed no correlation to PBMC iNOS mRNA or protein expression.

In conclusion, we have reported, probably for the first time, increased expression of PBMC iNOS mRNA and its protein product (nitrite/nitrate) in HCC. Both parameters correlated to each other and to plasma nitrite/nitrate level. The 3 parameters were higher in patients with ≥ 3 cm tumors. These findings, collectively, qualify the 3 parameters as potential tumor markers for the support of diagnosis and for follow up and monitoring therapy in HCC patients. The superiority of one or the other of the 3 parameters needs to be put to the test on a large number of cases taking in consideration the simplicity, the cost and the liability to be affected by other factors besides the standard evaluation of sensitivity and specificity as potential tumor markers.

REFERENCES

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