Folate levels in hepatocellular carcinoma patients with portal vein thrombosis

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Abstract

Background: Portal vein thrombosis (PVT) occurs frequently in hepatocellular carcinoma (HCC) and is often diagnosed in the course of a routine patient evaluation and surveillance for liver cancer. The purpose of this study is to investigate the relationship between folate status and portal vein thrombosis.

Methods: HCC with PVT patients were 78, HCC without PVT were 60 and control subjects were 70 randomly selected. We evaluate serum and red blood cellular folate, homocysteine, alpha fetal protein cholesterol, triglycerides, prothrombin time.

Results: HCC patients with PVT showed lower levels of serum folate, respect HCC patients without PVT, with an average difference of 1.6 nmol/l \( p < 0.01 \) (95% CI −2.54 to −0.66), red cell folate 33.6 nmol/l \( p < 0.001 \) (95% CI −43.64 to −23.55) and albumin 0.29 g/dl \( p < 0.001 \) (95% CI −0.42 to −0.15); PVT patients displayed higher levels of bilirubin 0.53 mg/dl \( p < 0.001 \) (95% CI 0.23 to 0.78), INR 0.91 \( p < 0.001 \) (95% CI 0.72 to 1.09), γGT 7.9 IU/l (95% CI 4.14 to 11.65) and homocysteine 4.6 μmol/l \( p < 0.05 \) (95% CI 0.32 to 8.87)

Conclusion: The low folate concentration and higher levels of homocysteine are associated with the loss of antithrombotic function, and with a more aggressive course of HCC and with a higher change of complications related to portal vein thrombosis

Keywords: Folate, Hepatocellular carcinoma, Thrombosis, Portal vein thrombosis, Homocysteine, Red cell folate

Background

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer mortality [1].

There are striking variations in its incidence in various parts of the world, although the 83% of the total deaths occur in developing countries [2, 3].

Many studies have focused on the investigation of markers to predict the outcomes of HCC and to exploring accurate prognostic factors for the clinical examination [4, 5]. Many HCC patients display portal hypertension, which cause the blood flow velocity of the portal vein to slow down. Changes in haemodynamic of the portal vein are the anatomical base of Portal Vein Thrombosis (PVT) formation. This complication is a multifactorial process, characterized also by the increase of systemic prothrombotic factors and the presence of local inflammatory foci. As a result, there is hypercoagulation, accompanied by the slowing of blood flow and damage to the vessel walls [6–8].

Patients with HCC with PVT have poor prognoses than those without PVT [9–11].

Evidence show a relationship between nutrition and environmental factors in relation to the progression of HCC. For instance, folate (known also as vitamin B9) and its derivatives are found abundantly in leafy green
vegetable and fresh fruit. These compounds play an essential role in the production and maintenance of new cells and is involved in DNA methylation, DNA synthesis and DNA repair.

Folate is a water-soluble beta-vitamin critical for health. It participates in numerous single-carbon exchange reactions that are essential for the synthesis of nucleotides purine/pyrimidine. Folates have been studied as a potential chemo-preventive agent.

Dietary folate is digested in the jejunal brush border surface by intestinal glutamate carboxy peptidase, followed by the transfer, through the portal vein to the liver where it is transported across membranes by the reduced folate carrier, possibly together with folate binding protein or the proton coupled folate transporter. These molecules are mainly stored in the liver, where are used in several reaction. Then, dietary folate is transported to the systemic blood circulation and in part, eliminated by the biliary excretion [12].

Both dietary and endogenous folate plays an important role in epigenetic methylation reactions, in lipid export, antioxidant defence and the hepatic methionine metabolism, which in turn regulates homocysteine levels [13, 14].

The objective of our study is to investigate, the relationship among blood folate and PVT in HCC patients, comparing these variables in controls subjects and in patients with and without PVT.

**Methods**

**Patients recruitment**

The subject with HCC included in this study were 138 patients with HCC: 78 HCC with PVT (31 females and 47 males) aged 65 or more years, and 60 HCC without PVT (24 female and 36 males), aged 60 or more years, living in Sicily (Italy).

The control subjects were 70 (35 female/35 males), aged 60 years or more. They were randomly selected and healthy without any history of cancer, major organ failure or active intravenous drug abuse.

The inclusion criteria were patients older than 18, with histologically proven HCC and with written informed consent.

This study used similar recruitment strategies, inclusion and exclusion criteria and measurement protocol adopted in previous studies on HCC patients with PVT and in controls subjects.

The questionnaires were administered by trained interview and focused on dietary, sociodemographic, environmental and lifestyle information.

**Serum and whole blood folate assay**

Serum and whole blood samples were collected into citrate tubes during the screening visit. They were immediately put on ice and centrifuged (2000 × g, 4 °C, 10 min) within 60 min. Both serum and whole blood samples were stored at −70 °C until analysis and were measured using QuantaPhase radioassay II Kit (Bio-Rad Laboratories, Hercules, California).

**Statistical analysis**

Data were expressed as mean ± standard deviation. To assess relationship and differences between folate, Hcy, alfa-fetoprotein of different patent groups, parametric, non-parametric Fisher’s exact test and 95% CI Odds Ratio (O.R.) Baptista–Pike method or ordinary one-way ANOVA with Tukey’s multiple comparisons test. A p value < 0.05 was considered statistically significant. Data were analysed using the GraphPad Prism 8 statistical software package (8.4.2 Macintosh Version; GraphPad Software San Diego, CA).

Univariate and multivariate linear regression were built to assess the relationship between folate concentration and sociodemographic data, area of residence (urban, metropolitan, semi urban, rural), social class (managers, skilled, semiskilled, unskilled) and clinical features of HCC patients.

**Results**

PVT patients’ ages ranged between 60 years and over; of these 35.90% were smokers, 12.82% were diabetics, 12.82% complained of renal failure; 15.38% of Hypertension; 23.08% of cardiovascular disease; 19.23% of dyspepsia, 12.82% experienced alcohol consumption. Etiologic factors in PVT patients were 44.87% for HCV, 32.5% for HBV and 10.26% unknown (Tables 1 and 2).  

HCC without PVT patient’s ages ranged 60 years and over; of these 45% were smokers or previous smokers, 15% were diabetics, 23.33% complained of hypertension, 26.67% of cardiovascular disease, 20% of renal failure, 35% of dyspepsia, 26.67% of alcohol consumption. Etiologic factors were 41.67% HCV, 15% HBV, 16.67% unknown.

In the control group ages ranged between 60 years and over; of these 28.57% were smokers, 11.43% were diabetics, 14.29% complained of hypertension, 14.29% of cardiovascular disease, 17.14% of renal failure, 17.14% of dyspepsia (Tables 1 and 2).

**Comparison from HCC patients with PVT to HCC patients without PVT**

We observed that were lower serum folate 1.6 nmol/l p<0.01 (95% CI −2.54 to −0.66), red cell folate
Table 1 Demographic and baseline characteristics of the study population

|                  | With PVT | NPVT | Controls | PVT versus NPVT | PVT versus Control | NPVT versus Control |
|------------------|----------|------|----------|-----------------|--------------------|---------------------|
| Age (range)      | 60–75    | 60–75| 70–75    | -               | -                 | -                   |
| Female/male      | 31/47    | 24/36| 35/35    | 0.9999          | 0.5042 to 1.275    | 0.2913             |
| BMI (Kg/m²)      | 23.8     | 25.0±2| 24.7±4.2| 0.2336          | -2.934 to 0.5336   | 0.409              |
| Heart rate (b.p.m)| 83.7     | 82.2±8.4| 81.4±9.7| 0.9644          | -3.283 to 8.497    | 0.9988             |
| Systolic blood pressure (mmHg) | 140.2±11.4 | 137.0±12.8 | 140.1±14.9 | 0.3293 | -2.097 to 8.497 | 0.0988 |
| Diastolic blood pressure (mmHg) | 82.2±8.4 | 81.8±9.2 | 81.4±9.7 | 0.9644 | -3.283 to 4.083 | 0.8543 |
| Heart rate (b.p.m) | 83.7±9.68 | 83.8±9.7 | 81.4±10.2 | 0.9981 | -4.099 to 3.899 | 0.3344 |
|                  |          |      |          |                 |                    |                     |

Cl, confidence interval; pt, patients; bpm, beats per minute; BMI, Body Mass Index

33.6 nmol/l $p<0.001$ (95% CI $-43.64$ to $-23.55$), albumin 0.29 g/dl $p<0.001$ (95% CI $-0.42$ to $-0.15$); were higher bilirubin 0.53 mg/dl $p<0.001$ (95% CI 0.23 to 0.78), INR 0.91 $p<0.001$ (95% CI 0.72 to 1.09), γGT 7.9 IU/l (95% CI 4.14 to 11.65) and homocysteine 4.6 μmol/l $p<0.05$ (95% CI 0.32 to 8.87) (Table 3).

Comparison between HCC patients with PVT and controls

We observed that were lower serum folate 3.28 nmol/l $p<0.001$ (95% CI $-42.20$ to $-2.31$), red cell folate 150 nmol/l $p<0.001$ (95% CI $-159.53$ to $-140.46$), albumin 0.83 g/dl $p<0.001$ (95% CI $-0.96$ to $-0.65$); were higher bilirubin 1.63 mg/dl $p<0.001$ (95% CI 1.46 to 1.79), INR 1.69 $p<0.001$ (95% CI 1.54 to 1.84) ALT 16.70 IU/l $p<0.001$ (95% CI 13.27 to 20.11) AST 16 IU/l $p<0.001$ (95% CI 12.62 to 19.58) γGT 55.4 IU/l $p<0.001$ (95% CI 51.28 to 58.81), αFP 232.9 mg/l $p<0.001$ (95% CI 229.16 to 236.63) and homocysteine 22.10 μmol/l $p<0.001$ (95% CI 51.28 to 58.81). The results are showed in Table 3.

Comparison between HCC patients without PVT and controls (Table 3)

We observed that serum folate was lower 1.66 nmol/l $p<0.001$ (95% CI $-2.66$ to $-0.66$), red cell folate 116.4 nmol/l $p<0.001$ (95% CI $-126.14$ to $-106.66$), Albumin 0.52 g/dl $p<0.001$ (95% CI $-0.68$ to $-0.35$); Bilirubin was higher 1.12 mg/dl $p<0.001$ (95% CI 0.86 to 1.38), INR 0.78 $p<0.001$ (95% CI 0.61 to 0.94) ALT 13.70 IU/l $p<0.001$ (95% CI 9.86 to 17.53) AST 15.60 IU/l $p<0.001$ (95% CI 11.84 to 19.36) γGT 47.50 IU/l $p<0.001$ (95% CI 43.52 to 51.47), αFP 237.00 mg/l $p<0.001$ (95% CI 233.64 to 240.36), total homocysteine 17.50 μmol/l $p<0.001$ (95% CI 13.93 to 21.06).

The median value of serum folate levels was 5.9 nmol/l. Patient were divided into two groups each of 69 patients (Table 4): ≤ 5.9 nmol/l and > 5.9 nmol/l.

We observed deficient serum folate levels in PVT patients in high grade (IV stage) of HCC ($p=0.028$) and in presence of extrahepatic metastases ($p=0.016$) (Table 4).

To identify the prognostic factors of PVT both univariate and multivariate analyses were used to evaluate red cellular folate and other clinical pathological variables.

The results suggested that red cellular folate ($p<0.01$), higher homocysteine level ($p<0.01$) and higher αFP ($p<0.05$) were related to PVT. The other variables included in multivariate models were not statistically significant.

Discussion

Serum folate, RBC folate and plasma total Hcy are the three most used biochemical indicators to assess folate status [15].

In our study we observed that serum folate and red cells folate in HCC patients with PVT were lower than in no PVT and controls subjects, whereas homocysteine was higher than in no PVT and in control subjects. These conditions induce atherosclerotic and thrombotic vascular disorders and promote oxidative stress, inflammation and endothelial injury, as well prothrombotic effect. Portal vein thrombosis is a relatively frequent event in cirrhosis and HCC, where its incidence varies from 7.4 to 17.8% in different studies [16–18].

In PVT, the concentration of folate in red cells result as a strong indicator of folate status, because the transitory changes of the diet do not control it. The concentration of folate in red cells is much higher than in plasma. Moreover, its concentration is established during erythropoiesis, for this reason its levels last for approximately 4 months [19].

The patients with PVT and HCC display an aggressive disease course, worsening of liver conditions, complications related to portal hypertension, low tolerance to treatment and lower serum folate and HHcy if compared with HCC subject without PVT [20–23]. Numerous variables such as agents, drugs, diseases and...
### Table 2 Characteristics of the patients and risk factors

| Risk Factor                | With PVT = 78 pt | NPVT = 60 pt | Controls = 70 pt | PVT versus NPVT | PVT versus control | NPVT versus control |
|----------------------------|-----------------|--------------|------------------|-----------------|--------------------|---------------------|
| **N** | **%** | **N** | **%** | **N** | **%** | **p** | **95% CI** | **O.R.** | **Sig** | **p** | **95% CI** | **O.R.** | **Sig** | **p** | **95% CI** | **O.R.** | **Sig** |
| Smokers/no smokers       | 28              | 35.90        | 27               | 45.00           | 20                 | 28.57              | 0.2975       | 0.3451 to 1.347 | ns      | 0.3821   | 0.7087 to 2.877 | ns      | 0.0672   | 0.9742 to 4.156 | ns      |
| Diabetes mell             | 10              | 12.82        | 9                | 15.00           | 8                  | 11.43              | 0.8049       | 0.3208 to 2.078 | ns      | 0.09999  | 0.4387 to 3.093 | ns      | 0.6078   | 0.5114 to 3.884 | ns      |
| Hypertension              | 12              | 15.38        | 14               | 23.33           | 10                 | 14.29              | 0.2757       | 0.2682 to 1.431 | ns      | 0.09999  | 0.4575 to 2.783 | ns      | 0.2569   | 0.7175 to 4.602 | ns      |
| Renal failure             | 10              | 12.82        | 12               | 20.00           | 12                 | 17.14              | 0.3484       | 0.2274 to 1.425 | ns      | 0.4952   | 0.2786 to 1.697 | ns      | 0.8211   | 0.5195 to 2.809 | ns      |
| Cardiovascular dis        | 18              | 23.08        | 16               | 26.67           | 10                 | 14.29              | 0.6921       | 0.3726 to 1.791 | ns      | 0.2096   | 0.8020 to 4.219 | ns      | 0.1226   | 0.9064 to 5.360 | ns      |
| Dyspepsia                 | 15              | 19.23        | 21               | 35.00           | 12                 | 17.14              | 0.0501       | 0.2122 to 0.9495 | ns      | 0.8325   | 0.5049 to 2.610 | ns      | 0.026    | 1.132 to 5.798  | *       |
| Alcohol                   | 10              | 12.82        | 16               | 26.67           | 0                  | 0                  | 0.0489       | 0.1659 to 0.9651 | *       | 0.0016   | –                  | **       | <0.0001 | –                  | ****     |
| HCV                       | 35              | 44.87        | 25               | 41.67           | 0                  | 0                  | 0.732        | 0.5902 to 2.235 | ns      | <0.0001  | –                  | ****     | <0.0001 | –                  | ****     |
| HBV                       | 25              | 32.05        | 9                | 15.00           | 0                  | 0                  | 0.028        | 1.135 to 6.349  | *       | <0.0001  | –                  | ****     | 0.0007  | –                  | ***      |
| HCC unknown aetiology     | 8               | 10.26        | 10               | 16.67           | 0                  | 0                  | 0.3132       | 0.2084 to 1.480 | ns      | 0.0007   | –                  | **       | 0.0003  | –                  | ***      |

*Fisher's exact test, 95% CI Odds Ratio (O.R.) Baptista-Pike method

pt, patients; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus

Summary: *<0.05; **<0.005; ***<0.001; ****<0.0001
lifestyle factors have a relevant impact on folate status. Especially those substances that act directly or indirectly on enzymes where folates operate as cofactors, but also as a consequence of the increase of disulfide exchange reactions, or the impairment of folate absorption. In fact, folate deficiency may be caused by the inadequate intake, the reduced absorption from the gastrointestinal tract, or by increased drug consumption and interactions. Subjects without a balanced diet, patients with renal disease, inflammatory bowel disease or malignant disease are more at risk for folate deficiency and, as a consequence, they have elevated levels homocysteine [24–29].

The role of folate deficiency and the hyperhomocysteinemia in PVT and in venous thromboembolism deserve also a relevant attention to evaluate the role of

### Table 3 Laboratory parameters and classifications of subjects included in the study

| Parameter                     | PVT (n = 78) | NPVT (n = 60) | Controls (n = 70) | PVT versus NPVT (p value) | PVT versus Controls (p value) | NPVT versus Controls (p value) |
|-------------------------------|-------------|---------------|------------------|--------------------------|------------------------------|-------------------------------|
| Bilirubin (mg/dl)             | 2.87 ± 0.6  | 2.36 ± 1.02   | 1.24 ± 0.38      | < 0.001                  | < 0.001                      | < 0.001                        |
| Albumin (g/dl)                | 3.15 ± 0.42 | 3.44 ± 0.38   | 3.96 ± 0.55      | < 0.001                  | < 0.001                      | < 0.001                        |
| INR                           | 2.91 ± 0.54 | 2.00 ± 0.57   | 1.22 ± 0.36      | < 0.001                  | < 0.001                      | < 0.001                        |
| Tot cholesterol (mmol/l)      | 5.77 ± 1.08 | 5.69 ± 1.01   | 5.50 ± 1.04      | 0.659                    | 0.124                        | 0.297                          |
| HDL (mmol/l)                  | 1.40 ± 0.36 | 1.41 ± 0.37   | 1.46 ± 0.40      | 0.873                    | 0.338                        | 0.463                          |
| LDL (mmol/l)                  | 3.44 ± 0.38 | 3.48 ± 0.39   | 3.46 ± 0.37      | 0.545                    | 0.747                        | 0.765                          |
| Triglycerides (mmol/l)        | 1.57 ± 0.70 | 1.50 ± 0.68   | 1.48 ± 0.65      | 0.556                    | 0.421                        | 0.864                          |
| ALT (IU/l)                    | 51.20 ± 10.80 | 48.20 ± 11.90 | 34.50 ± 10.20    | 0.124                    | < 0.001                      | < 0.001                        |
| AST (IU/l)                    | 49.20 ± 10.60 | 48.70 ± 10.80 | 33.10 ± 10.80    | 0.786                    | < 0.001                      | < 0.001                        |
| γGT (IU/l)                    | 82.80 ± 10.20 | 74.90 ± 12.10 | 27.40 ± 10.80    | < 0.001                  | < 0.001                      | < 0.001                        |
| AFP (mg/l)                    | 236.10 ± 15.80 | 240.20 ± 14.20 | 3.20 ± 0.60     | 0.117                    | < 0.001                      | < 0.001                        |
| Serum folate (nmol/l)         | 5.24 ± 2.81  | 6.84 ± 2.71   | 8.50 ± 3.01      | < 0.001                  | < 0.001                      | 0.001                          |
| Red cell folate (nmol/l)      | 144.20 ± 30.60 | 177.80 ± 28.20 | 294.20 ± 27.80   | < 0.001                  | < 0.001                      | < 0.001                        |
| Total homocysteine (μmol/l)   | 32.80 ± 12.20 | 28.20 ± 13.10 | 10.70 ± 6.90    | 0.035                    | < 0.001                      | < 0.001                        |

PVT, portal vein thrombosis; INR, International normalized ratio; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALT, alanine transferase; AST, aspartate transferase; γGT, gamma-glutamil-transferase

### Table 4 Clinical features of the HCC patients

| Serum folate | ≤ 5.9 mm/l (69 pt) | > 5.9 mm/l (69 pt) | p value | Signif | Odds-ratio | 95% CI O.R. |
|--------------|--------------------|-------------------|---------|--------|------------|-------------|
| N            | %                  | N                 | %       |        |            |             |
| PVT          |                    |                   |         |        |            |             |
| HCC stage I  | 16                 | 23.19             | 20      | 28.99  | 0.5612     | ns          | 0.7396      | 0.3337 to 1.573 |
| HCC stage II | 18                 | 26.09             | 23      | 33.33  | 0.4564     | ns          | 0.7059      | 0.3412 to 1.519 |
| HCC stage III| 20                 | 28.99             | 21      | 30.43  | > 0.9999   | ns          | 0.9329      | 0.4610 to 1.876 |
| HCC stage IV | 15                 | 21.74             | 5       | 7.25   | 0.0276     | *           | 3.556       | 1.265 to 9.289 |
| Clip Score 0–2| 31               | 44.93             | 28      | 40.58  | 0.7309     | ns          | 1.195       | 0.6229 to 2.310 |
| Clip Score 3–6| 38               | 55.07             | 41      | 59.42  | 0.7309     | ns          | 0.8371      | 0.4329 to 1.605 |
| Cirrhosis    | 25                 | 36.23             | 33      | 47.83  | 0.2272     | ns          | 0.6198      | 0.3139 to 1.194 |
| < 50 mm      | 25                 | 36.23             | 33      | 47.83  | 0.2272     | ns          | 0.6198      | 0.3139 to 1.194 |
| > 50 mm      | 44                 | 63.77             | 36      | 52.17  | 0.2272     | ns          | 1.613       | 0.8373 to 3.185 |
| Tumour Invasion | 26             | 37.68             | 32      | 46.38  | 0.3886     | ns          | 0.6991      | 0.3570 to 1.345 |
| Vascular Invasion | 29             | 42.03             | 31      | 44.93  | 0.8637     | ns          | 0.8887      | 0.4619 to 1.701 |
| Extra-hepatic MTS | 44            | 63.77             | 29      | 42.03  | 0.0166     | *           | 2.428       | 1.190 to 4.875 |

PVT, portal vein thrombosis; HCC, hepatocellular carcinoma; Mtd, max tumour diameter

Summary:*< 0.05; **< 0.005; ***< 0.001; ****< 0.0001
these findings in the pre-operative, operative and post-operative thrombotic complication of HCC.

PVT prophylaxis with anticoagulants is still controversial and relatively less investigated; for this reason, it can be recommended on an individual basis. Despite these results, the reduction of thrombosis with folate supplementation in patients with PVT and HCC is scarce [30–32].

The identification of the high-risk patients with hypofolatemia and/or hyper homocysteinemia, through the evaluation of genetic mutation and nutritional deficiency is essential to plan clinical management in subjects who are candidate for major surgery or liver transplantation [33].

Folate carry out their metabolic functions when it is converted to 5-methyl tetrahydrofolate-. 5-methyltetrahydrofolate and it is associated with improvements in NOS coupling and nitoxide (NO) synthesis in vivo [34–38].

NO is a potent vasodilator that improves vascular function through its anti-inflammatory, antithrombotic and anti-angiogenic properties.

The study has established an inverse association between the folate level and tumour size, multiplicity and metastases; disease progression was categorized into stages 1 to 4, and serum folate decreased as disease stage progressed [39–43] (Table 4).

Therefore, folate deficiency aids the incorporation of uracil into DNA, which can lead to DNA breaks and chromosome instability; such breaks could contribute to the increased risk of cancer [44–49].

Conclusion

Our study supports the hypothesis that the folate status shows a protective role in HCC prevention, development and progression.

Further longitudinally designed and interventional studies with a large sample size may help to determine the optimal strategies to improve folate status of HCC patients [50] and to evaluate the effects in coagulation disorders in HCC patients with PVT [51].

Abbreviations

PVT: Portal vein thrombosis; HCC: Hepatocellular carcinoma; γGT: γ-Glutamyltransferase; DNA: Deoxyribonucleic acid; αFP: Alpha-fetoprotein; CT: Computed tomography; MR: Magnetic resonance; CLIP: Cancer Liver Italian Program score; TNM: (Tumour Nodes Metastases) Classification of Malignant Tumours; US: Ultrasonography; °C: Degree Celsius; G: Gravitational accelera-
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