Tumor type M$_2$ pyruvate kinase expression in gastric cancer, colorectal cancer and controls

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AIM: Tumor formation is generally linked to an expansion of glycolytic phosphometabolite pools and aerobic glycolytic flux rates. To achieve this, tumor cells generally overexpress a special glycolytic isoenzyme, termed pyruvate kinase type M$_2$. The present study was designed to evaluate the use of a new tumor marker, tumor M$_2$-PK, in discriminating gastrointestinal cancer patients from healthy controls, and to compare with the reference tumor markers CEA and CA72-4.

METHODS: The concentration of tumor M$_2$-PK in body fluids could be quantitatively determined by a commercially available enzyme-linked immunosorbent assay (ELISA)-kit (ScheBo® Tech, Giessen, Germany). By using this kit, the tumor M$_2$-PK concentration was measured in EDTA-plasma of 108 patients. For the healthy blood donors a cut-off value of 15 U/mL was evaluated, which corresponded to 90% specificity. Overall 108 patients were included in this study, 54 patients had a histological confirmed gastric cancer, 54 patients colorectal cancer, and 20 healthy volunteers served as controls.

RESULTS: The cut-off value to discriminate patients from controls was established at 15 U/mL for tumor M$_2$-PK. The mean tumor M$_2$-PK concentration of gastric cancer was 26.937 U/mL. According to the TNM stage system, the mean tumor M$_2$-PK concentration of stage I was 16.324 U/mL, of stage II 15.290 U/mL, of stage III 30.289 U/mL, of stage IV 127.31 U/mL, of non-metastasis 12.854 U/mL and of metastasis 35.711 U/mL. The mean Tumor M$_2$-PK concentration of colorectal cancer was 30.588 U/mL. According to the Dukes stage system, the mean tumor M$_2$-PK concentration of Dukes A was 16.638 U/mL, of Dukes B 22.070 U/mL, and of Dukes C 48.024 U/mL, of non-metastasis 19.501 U/mL, of metastasis 35.711 U/mL. The mean Tumor M$_2$-PK concentration of stage I was 16.638 U/mL, of stage II 15.290 U/mL, of stage III 30.289 U/mL, of stage IV 127.31 U/mL, of non-metastasis 12.854 U/mL and of metastasis 35.711 U/mL. The mean Tumor M$_2$-PK concentration of colorectal cancer was 30.588 U/mL. According to the Dukes stage system, the mean tumor M$_2$-PK concentration of Dukes A was 16.638 U/mL, of Dukes B 22.070 U/mL, and of Dukes C 48.024 U/mL, of non-metastasis 19.501 U/mL, of metastasis 35.711 U/mL. The mean tumor M$_2$-PK concentration allowed a significant discrimination of colorectal cancers (30.588 U/mL) from controls (10.965 U/mL) (P<0.01), and gastric cancer (26.937 U/mL) from controls (10.965 U/mL) (P<0.05). The overall sensitivity of tumor M$_2$-PK for colorectal cancer was 68.52%, while that of CEA was 43.12%. In gastric cancer, tumor M$_2$-PK showed a high sensitivity of 50.47%, while CA72-4 showed a sensitivity of 35.37%.

CONCLUSION: Tumor M$_2$-PK has a higher sensitivity than markers CEA and CA72-4, and is a valuable tumor marker for the detection of gastrointestinal cancer.

INTRODUCTION

Pyruvate kinase plays a key role in the glycolytic pathway. One of its functions is to control nucleotide triphosphate generation[4-5]. Different isoforms of this enzyme exist (pyruvate kinases L, R, M$_1$, M$_2$, Tumor M$_2$), which are tissue-specifically expressed in various organisms. All isoforms are known to be homotetramers in their active state. In tumor cells, however, tetrameric pyruvate kinase M$_1$ isoenzyme is disrupted and predominant in a dimeric form. It has been suggested that at least a part of the mechanisms is to disrupt the tetrameric form of pyruvate kinase M$_2$ phosphorylated by receptor tyrosine kinases. The concentration of dimeric pyruvate kinase M$_2$ isoenzyme is dramatically increased in a metabolic state characteristic for tumor cells. It is thus called tumor M$_2$-PK[5, 13]. Tumor M$_2$-PK is also present in body fluids, most likely released from tumor cells by tumor necrosis and cell turnover. It can be detected by a sandwich-ELISA based on two monoclonal antibodies. Furthermore, it has been demonstrated that tumor M$_2$-PK determination should be carried out in EDTA-plasma for its stability[6-9].

Circulating tumor markers are an established index of monitoring systemic therapies in a number of solid tumors. In the diagnosis of gastrointestinal cancer, CA19-9, CA72-4 and CEA are the major tumor markers. Since the diagnosis of gastrointestinal cancer was dependent on endoscopies and cytology more than these tumor markers, the present study was designed to evaluate the use of a new tumor marker tumor M$_2$-PK in discriminating gastrointestinal cancer patients from healthy donors in order to increase the sensitivity of the diagnosis for gastrointestinal cancer[10-13].

It was previously shown for renal and pancreatic carcinoma that tumor M$_2$-PK determination in circulation could provide a good discrimination of benign disease from malignant one and might correlate with stage of disease[14-18]. Only limited data are available on tumor M$_2$-PK in gastrointestinal cancer. Thus, we investigated this new marker in patients with gastrointestinal cancer focusing on whether tumor M$_2$-PK plasma levels increased in gastrointestinal cancer patients in comparison to healthy controls, whether tumor M$_2$-PK was correlated with the classical tumor marker CEA, whether tumor M$_2$-PK gave any predictive information on response to therapy.

MATERIALS AND METHODS

Patients

A total of 108 consecutive patients with histological confirmed primary gastrointestinal cancer were included in the study. The
mean age was 47.9 years (ranging from 32 to 66 years). There were 76 men and 32 women. Among them, 54 were gastric cancer patients (25 non metastasized, 29 metastasized), 54 were colorectal cancer patients (20 non-metastasized, 34 metastasized). According to TNM stage system, 5 gastric cancer patients were classified as stage I, 24 as stage II, 23 as stage III, 2 as stage IV. According to Dukes stage system, 14 colorectal cancer patients were classified as Dukes A, 19 as Dukes B, 21 as Dukes C. Twenty healthy donors served as a control group.

Methods
The test kit (ScheBo- Tu M₂-PK, ScheBo®Tech GmbH, Giessen, Germany) required 10 µL EDTA-plasma per sample and was performed according to the manufacturer’s instructions. Samples were collected as EDTA-blood, followed by centrifugation (2 000 r/min, 10 min) and removal of the supernatant plasma. Tumor M₂-PK concentration in EDTA-plasma was determined immunologically using a sandwich enzyme-linked immunosorbent assay (ELISA) based on two monoclonal antibodies (clones I and II) specific for tumor M₂-PK. The antibodies did not cross-react with other isoforms of pyruvate kinase.

The ELISA plate was coated with a monoclonal antibody that only recognized tumor M₂-PK. Tumor M₂-PK from EDTA-plasma samples and standards bound to the antibody and thus were immobilized on the plate. EDTA-plasma samples were diluted (1:100) with sample/washing buffer, 50 µL of diluted sample and ready-to-use standard were transferred into wells, incubated for 60 min at room temperature. Then the wells were emptied of the sample and each well was washed 3 times with sample/washing buffer (250 µL/well). The plate was inverted and tapped on a clean paper towel to remove any remaining liquid. Fifty µL/well of the 1:100 biotin-conjugated second monoclonal antibody was added and incubated for 30 min at room temperature. After washing, 50 µL/well of ready-to-use POD-streptavidin was added and incubated for 30 min in dark at room temperature. After washing, 100 µL of ready-to-use substrate solution was added to each well, and incubated for 30 min in dark at room temperature. The substrate reaction was stopped by adding 100 µL of stop solution per well. The contents were mixed well by agitating the plate. The optical density was read at 405 nm wavelengths with a micro titer plate reader between 5 and 30 min after addition of the stop solution. The contents were mixed well before measuring. The 492 nm was used as a reference wavelength.

For determination of CEA and CA72-4, serum samples (25 µL, undiluted) were measured using the fully automatic, competitive chemiluminescent immunoassay with a diagnosis kit.

Statistical analysis
Data were statistically analyzed with origin 6.1 for windows. All P values were resulted from a two-sided test. P value less than 0.05 was considered statistically significant.

RESULTS
The mean tumor M₂-PK concentration of gastric cancer was 26.937 U/mL. According to the TNM stage system, the mean tumor M₂-PK concentration of stage I was 16.324 U/mL, of stage II 15.290 U/mL, of stage III 30.289 U/mL, of stage IV 127.31 U/mL, of non-metastasis 12.854 U/mL and of metastasis 35.711 U/mL (Table 1).

The mean tumor M₂-PK concentration of colorectal cancer was 30.588 U/mL. According to the Dukes stage system, the mean tumor M₂-PK concentration of Dukes A was 16.638 U/mL, of Dukes B 22.070 U/mL, of Dukes C 48.024 U/mL, of non-metastasis 19.501 U/mL, and of metastasis 49.437 U/mL (Table 2).

The mean tumor M₂-PK concentration allowed a significant discrimination of colorectal cancers (30.588 U/mL) from controls (10.965 U/mL) (t=3.173, P=0.0022, P<0.01), gastric cancer (26.937 U/mL) from controls (10.965 U/mL) (t=2.314, P=0.024, P<0.05) (Table 3 and Figure 1).

Table 1 Concentration of tumor M₂-PK in patients with gastrointestinal tumors and controls.

| No. | Tumor M₂-PK (U/mL) | TNM stage | Metastasis | No. | Tumor M₂-PK (U/mL) | TNM stage | Metastasis |
|-----|--------------------|-----------|------------|-----|--------------------|-----------|------------|
| 1   | 9.26               | II        | N          | 24  | 24.52              | IIIB      | M          |
| 2   | 36.5               | IIIA      | M          | 28  | 16.6               | II        | M          |
| 3   | 23.08              | II        | N          | 30  | 9.26               | II        | N          |
| 4   | 23.08              | IIIB      | M          | 31  | 37.85              | IA N      |            |
| 5   | 165.3              | IIIA      | M          | 32  | 17.06              | II        | N          |
| 6   | 18.06              | II        | N          | 33  | 34.81              | IIIA M    |            |
| 7   | 8.81               | II        | N          | 34  | 14.81              | IIIA M    |            |
| 8   | 17.34              | II        | N          | 35  | 23.49              | II        | M          |
| 9   | 6.87               | IB        | M          | 36  | 28.25              | IIIA M    |            |
| 10  | 17.91              | IIIB      | M          | 37  | 12.87              | II        | N          |
| 11  | 24.07              | IIIB      | M          | 38  | 10.62              | II        | M          |
| 12  | 12.67              | II        | N          | 39  | 118.02             | IV M      |            |
| 13  | 28.4               | IIIB      | M          | 40  | 28.75              | IIIA N    |            |
| 14  | 56.76              | IIIA      | N          | 41  | 14.31              | II        | M          |
| 15  | 6.42               | IIIA      | M          | 42  | 32.4               | II        | M          |
| 16  | 28.7               | IIIB      | M          | 43  | 7.61               | II        | N          |
| 17  | 7.47               | IA        | N          | 44  | 12.23              | IA N      |            |
| 18  | 13.9               | IIIA      | M          | 45  | 18.1               | II        | M          |
| 19  | 14.5               | II        | M          | 46  | 25.17              | IIIA M    |            |
| 20  | 69.24              | IIIB      | M          | 47  | 10.63              | II        | N          |
| 21  | 12.33              | IIIA      | M          | 48  | 8.84               | II        | N          |
| 22  | 28.66              | IIIB      | M          | 49  | 19.35              | IIIA N    |            |
| 23  | 136.6              | IV        | M          | 50  | 41.11              | II        | M          |
| 24  | 11.36              | II        | N          | 51  | 11.38              | IIIA N    |            |
| 25  | 7.62               | II        | N          | 52  | 21.75              | IIIA M    |            |
| 26  | 17.2               | IB        | N          | 53  | 30.03              | IIIB M    |            |
| 27  | 11.36              | IIIA      | M          | 54  | 11.3               | II        | N          |

Figure 1 Concentrations of tumor M₂-PK in patients with gastrointestinal tumors and controls.

N=non metastasis, M=metastasis.

The sensitivity of tumor M₂-PK for a cutoff point of 15 U/mL was compared to the established tumor markers CEA (cutoff point of 3.0 µg/mL) and CA72-4 (cut-off point of 4 KU/L). The overall sensitivity of tumor M₂-PK to colorectal cancer was 68.52%, while that of CEA was 43.12%. In gastric cancer, tumor M₂-PK showed a higher sensitivity of 50.47%, while CA72-4
showed a sensitivity of 35.37% (Figure 2).

**DISCUSSION**

The metabolic state of tumor cells is different from that of normally proliferating cells. Tumor cells exhibit an increased glycolysis to lactate initiated by multiple steps, including a switch of isoenzyme pattern and activity. Pyruvate kinase is a key enzyme of glycolysis. Different isoforms of this enzyme exist (pyruvate kinases L, R, M, G, tumor M2) and are tissue-specifically expressed in various organisms. The L-type is found in the liver and proximal tubules of normal kidneys, erythrocytes express the R-type, the M1-type predominates in skeletal muscle, heart and brain, and the M2-type is expressed in the lung, distal tubules of normal kidney, fetal and undifferentiated or proliferating tissues. All isoforms are known to be homotetramers in their active state.

In tumor cells, pyruvate kinase isoenzyme M2 is strongly overexpressed and shifted into the dimeric state. The tetrameric form has a high affinity to phosphoenolpyruvate (PEP), whereas the dimeric form has a considerably lower PEP affinity, which consequently leads to an increase of phosphometabolite pool. The concentration of the dimeric pyruvate kinase M2 isoenzyme is dramatically increased in a metabolic state characteristic of tumor cells. It is thus called tumor type M2 pyruvate kinase (tumor M2-PK) by means of specific monoclonal antibodies against tumor M2-PK, which does not cross-react with the pyruvate kinase M1 tetramer and other pyruvate kinase isoforms. A sensitive immunoassay (ELISA) was employed to measure tumor M2-PK in body fluids.

It was previously shown that tumor M2-PK determination in the circulation provided a good discrimination of benign disease from a malignant one and might correlate with stage of disease[19-22]. Only limited data are available on tumor M2-PK in gastrointestinal cancer. In the present study, tumor M2-PK in the diagnosis of gastrointestinal cancer was evaluated.

Since proliferating cells had an altered metabolism with an over-expression of the dimeric form of the tumor-specific pyruvate kinase isoenzyme tumor M2-PK, the present study was initiated to evaluate tumor M2-PK in the diagnosis of gastrointestinal cancer, in comparison with the established tumor markers CEA and CA72-4. Significant discrimination of tumor patients from healthy controls was observed[19-22]. In the diagnosis of colorectal cancer, the sensitivity of tumor M2-PK was higher than that of CEA. But the sensitivity of tumor M2-PK in the diagnosis of gastrointestinal cancer was lower than that of CA72-4, whereas it was higher than that of CA72-4.

From the presented data, it is concluded that tumor M2-PK can be used as a valuable diagnostic marker for gastrointestinal cancer. Further studies should focus on disease monitoring, therapy evaluation and the combination of tumor M2-PK with other tumor markers[29-32].

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