Expression of tumor pyruvate kinase M2 isoform in plasma and stool of patients with colorectal cancer or adenomatous polyps

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Abstract

Background: Tumor pyruvate kinase M2 isoform (tM2-PK), which is an isoform of PK-glycolytic enzyme and appears on the surface of cancerous proliferating cells, has been used as a diagnostic biomarker for colorectal cancer (CRC). The aim of this study was to evaluate the tM2-PK measurement test for the diagnosis of CRCs and adenomatous polyps in plasma and stool samples in an Iranian population. Methods: In this prospective study, a total of 226 stool and 178 plasma samples were received from patients referred to colonoscopy units. tM2-PK enzyme was measured using two separate ScheBo-Biotech-AG ELISA kits for stool and plasma samples. Results: At the cut-off value of 4 U/ml, in tumor group, the sensitivity of fecal tM2-PK test was 100% and the specificity was 68%, and in polyp group, the sensitivity and specificity were 87% and 68%, respectively. At the cut-off value of 15 U/ml in tumor group, the sensitivity of plasma tM2-PK test was 98% and specificity was 74% and in polyp group the sensitivity and specificity were 98% and 74%, respectively. Based on our results, a cut-off range of 4.8-8 U/ml and >8 U/ml could be used to detect polyp and tumor in stool samples, respectively. Similarly, a cut-off range of 19-25 U/ml and >25 U/ml is recommended in plasma samples for polyp and tumor detection, respectively. Conclusions: This study revealed a high specificity and sensitivity of tM2-PK test for stool and plasma samples in patients with CRC and polyps suggesting it as a non-invasive assay to diagnose CRC and adenomatous polyps.

Background

Colorectal cancer (CRC) is one of the leading causes of cancer morbidity and mortality worldwide [1, 2]. Its incidence rate has increased rapidly since it is associated with several risk factors related to lifestyle such as smoking, sedentary lifestyle, obesity, alcohol abuse and diets containing high red and processed meats [3, 4]. Colonoscopy is
currently claimed as the gold standard screening tool for CRC [5, 6], however, it is expensive and may cause unexpected complications. Moreover, it is uncomfortable and painful for some patients to undergo colonoscopy examination. Thus, the compliance with colonoscopy for CRC screening is quite low [7]. Guaiac fecal occult blood test (gFOBT) is the most widely used noninvasive screening test for stool examination, although it has some limitations [8]. It is inconvenient to perform since patients have to go on a restricted diet for several days prior to the test, which includes avoiding various types of food that may cause false peroxidase reaction and any antioxidants and non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin [9]. Another CRC screening test is an immunological fecal occult blood test (iFOBT) [10]. The low sensitivity of gFOBT and iFOBT may result in missing patients with CRC. Thus, a more effective screening tool is necessary [11, 12]. Based on methylation changes in stool and blood, two approved Food and Drug Administration (FDA) CRC detection kits respectively termed, ColoGaurd™ and Epi proColon® 2.0 CE kits are now available [13]. The relatively low sensitivity of these tests for early CRC and adenomatous polyp detection should be improved.

The majority of human tumors strongly overexpress M2 isoform of the glycolytic enzyme pyruvate kinase (M2-PK). This isoenzyme is released from tumor cells and is quantitatively detectable in body fluids. The measurement of tumor M2-PK has been proposed as a novel approach for early detection of CRC in the stool or blood of CRC patients [10] since adenomas or CRC are usually associated with increased serum and stool levels of tumor M2-PK. Fecal M2-PK detects both bleeding and non-bleeding tumors as well as adenoma. It does not have false positive results originating from various noncancerous sources of bleeding, such as hemorrhoids and fissures. In contrast to FOBT, only one small stool sample (from a single stool passage) is requested without dietary restrictions for the test [14].
Hence, the aim of this study was to evaluate tumor M2-PK measurement test in plasma and stool samples to diagnosis CRC and adenomatous polyps in patients referred to the laboratory. Besides, this study was performed to determine the best cut-off values for tumor M2-PK test in stool and plasma samples.

Methods

In this prospective study, samples were taken in two separate centers including specialty hospital and oncology clinic of Mashhad, Iran, where the study aims and procedures were explained in detail to the participants. The patient information datasheet and written consent were given to all participants. All written consent forms obtained, were kept in the record files specific for each patient at a safe and secure place. The study was carried out by the approval of the Mashhad University of Medical Sciences ethic committee with the ethical code of 1394.512.

Participants from April 2017 to June 2018 admitted prior to colonoscopy, handled their stool samples to the laboratory and at the same day, their blood samples were collected in EDTA tubes. Sampling date were recorded. Minimum sample required for M2-PK test, was 100 mg of feces and 10 µL of plasma. Collected stool and plasma samples were kept frozen at -20°C prior to any experiments. Participants older than 30 years were categorized according to their age, sex, alcohol consumption, diabetes, smoking status and family history of CRC. Patients with inflammatory bowel disease (Crohn and colitis disease) were not included in the study because recent reports indicate that the inflammatory bowel diseases can increase the M2-PK enzyme level [14-16].

Control group was defined as the participants with negative colonoscopy and case group was polyp-positive (adenomatous) or tumor-positive samples in colonoscopy examination. An expert gastrointestinal (GI) pathologist reported all pathology results. Patients suffering from solitary rectal cancer (1 case), hyperplastic (4 cases), retention (3 cases),
inflammatory (2 cases) and mucosal (2 cases) polyps were excluded from the study since we targeted only adenomatous types of polyps. The histopathology report of one polyp resulted in unremarkable lesion which was also excluded.

In the current study, 226 stool and 178 plasma samples were taken from patients prior to colonoscopy. Tumor M2-PK enzyme of samples was measured by two separate ScheBo-Biotech-AG ELISA kits (Giessen, Germany) for stool and plasma. According to colonoscopy and pathology results, participants were categorized as follow: among patients who their stool samples were collected 111 (49.1%) were normal, 76 (33.6%) patients had polyps, and 39 (17.3%) patients were suffering from CRC. In the plasma group, 69 (38.8%) were normal, 53 (29.7%) patients had polyps, and 56 (31.5%) patients were suffering from CRC. Only from 116 participants, both the stool and plasma samples were collected.

**Statistical analysis**

The collected data were analyzed using SPSS version 19 and MedCalc statistical software. In addition to descriptive statistics, student's t-test, Pearson correlation testing, Chi square, and (receiver operating characteristics curve) ROC, ANOVA curve were used where applicable. A p-value <0.05 was statistically significant in this study. Sensitivity and specificity expressed as ROC curve were calculated using colonoscopy results and histology as reference values.

**Results**

In this study, 178 plasma samples were taken from patients including 96 men (53.9%) and 82 women (46.1%). The mean age of the patients whose stool and plasma samples were collected, was 54 and 57.22 years, respectively. Table 1 shows the number of normal, polyps and cancer patients in plasma and stool sample groups based on the pathology results. The lesions were located in rectosigmoid, ascending, descending, and transverse
colon (Table 2). ANOVA test revealed no significant difference (p value < 0.05) in the location of tumor or polyp with a positive M2-PK test in either stool or plasma samples (Table 2).

Neither the stool nor the plasma samples of tumor- and polyp-bearing patients showed significant differences between a positive M2-PK test result and the distribution of age, sex, diabetes, smoking and family history of tumor (p values < 0.05) except for tumor-bearing and normal subjects in terms of smoking with a positive M2-PK test (p value = 0.011).

Although there was no significant difference between a M2-PK positive test and tumor (p value = 0.967) or polyp (p value = 0.074) size in stool samples, it was statistically significant in plasma samples (p values = 0.0001 and = 0.005, respectively). The types of the adenomatous polyps was shown in Table 3 and the pathology results of the stool and plasma samples representing the grading of adenocarcinoma or adenoma was presented in Table 4. As shown, the results of M2-PK stool and plasma samples of cancer- or polyp-bearing patients did not have a significant difference based on the grading of the tumor or adenoma (p value > 0.05).

ANOVA test was used to compare the difference between the results of M2-PK stool and plasma samples in the three groups of normal, patients with polyp, and patients with adenocarcinoma, indicating significant differences between the groups (both tests had p value = 0.0001) (Figure 1). Besides, Chi-square test was used to compare the levels of M2-PK in tumor-/polyp-bearing patients with controls in stool and plasma samples (Table 5).

In the current study, we used ROC curves to determine the best cut-off value for tumor/polyp M2-PK test (Table 6 and Figure 2). In order to have the best sensitivity and specificity for tumor detection in plasma specimens, a cut-off value >25 U/ml is suggested
with a sensitivity and specificity of 90.9% and 91.3%, respectively (Figure 2A). Similarly, for polyp detection, a cut-off value >19 U/ml is recommended in which the test sensitivity is 96.3% and the specificity is 85.5% (Figure 2B). The area under the curve (AUC) of polyp and tumor data was 0.95 and 0.975 respectively, which reveals that the overall discriminatory power of the test is quite high. Also based on our results, to have the best sensitivity and specificity for tumor detection in stool specimens, a cut-off value > 8 U/ml is recommended, in which the test sensitivity is 100% and the specificity is 85.6% (Figure 2C). Besides, for polyp detection, a cutoff value > 4.8 U/ml is recommended in which the test sensitivity and specificity is 81.6% and 74.8%, respectively (Figure 2D). AUC of polyp data was 0.834 and of tumor data was 0.969, which indicates that the overall discriminatory power of the test is high. Based on these results, a cut-off range of 4.8-8 U/ml and > 8 U/ml in stool samples can detect polyp and tumor, respectively. Similarly, a cut-off range of 19-25 U/ml and >25 U/ml in plasma samples can detect polyp and tumor, respectively.

Discussion

In the current study, our stool and plasma study did not show a significant difference between a positive M2-PK test result and the distribution of age, sex, diabetes and family history of tumor in tumor- or polyp-bearing patients. There was only a significant difference between the results of M2-PK test in plasma samples of tumor-bearing subjects and normal subjects in terms of smoking (p = 0.011), although it was not seen in polyp-bearing subjects. These findings were in consistent with the findings of U Haug et al, which reported that the subgroup of the ESTHER study did not differ from the whole ESTHER study population with respect to the distribution of age, sex, body mass index, smoking status and family history of CRC. However, current smokers showed more frequently increased levels of
tumor M2-PK in stool compared to never and former smokers (p value=0.003)[17]. In a similar study, male and female groups showed no significant differences in age or fecal tumor M2-PK levels although a highly significant difference was found between the tumor M2-PK level for participants aged 20-49 years (median M2-PK of 0.66) and 50-79 years (median M2-PK of 0.086)[18]. Besides, in a study with 1082 participants (mean age 63 years, 50% females) the median tumor M2-PK level in the whole study population was 1.3 U/ml (0.3–3.3). Median tumor M2-PK levels did not alter by gender, but tended to be higher in older age groups (p value=0.002). Besides, the sensitivity and specificity did not vary by sex of stool samples. The specificity tended to be lower in older age groups (p value=0.001) but the sensitivity did not vary by age [19]. In another study, average serum M2-PK value among 158 normal individuals was 2.96 U/mL, which was not affected by gender or age [20]. The study of Mohamed El-Tantawy Ibrahim and colleagues revealed that there was no significant difference between patients with colon cancer and control groups considering the age and sex. Moreover, 32% of their patients were smokers compared to only 3.3% of the control group, which was statistically significant (p value less than 0.05) [3].

In our study, although in M2-PK plasma experiment the size of the tumor or polyp was statistically different in the tumor- or polyp-bearing patients in compare to controls, there was no difference between these groups in the M2-PK stool experiment. This was consistent with the study of Yogesh M. et al which reported that in patients undergoing colonoscopy 31 had adenomatous polyps, 21 had small adenomas (<10 mm) and 10 had large adenomas (>=10 mm). Median stool M2-PK in the small and large adenoma groups was 2.9 U/ml and 1.5 U/ml respectively, which was not statistically significant when compared with normal groups. M2-PK was reported positive in 25.8% of adenomas regardless of their sizes; however, FOBT seemed to be more associated with the size of
the lesion [11]. In addition, in a similar study with 50 patients suffering from an
adenomatous disease, 22 were found to have a single polyp greater than 1 cm in size.
There was no significant difference in the M2-PK concentration detectable in the feces of
patients with polyps less or above 1 or even the size of 5 cm [21].
In our study, ANOVA test revealed no significant difference in the location of tumor or
polyp with a positive M2-PK test in either stool or plasma samples. However, Haug et al.
showed that there was a statistically difference (p value <0.001) in tumor M2-PK levels in
stool of ESTHER participants based on the location of the tumor. In their study with the
cut-off value of 4 U/ml, overall sensitivity was 68% with a clear difference between colon
cancer (85%) and rectum cancer (56%) [22].
In the present study, at the cut-off value of 15 U/ml, the test sensitivity for the plasma
samples of tumor- and polyp-bearing groups were both 98%, specificity of 74%, positive
predictive value of 75% and negative predictive value of 98%.
Besides, our results showed that the sensitivity of M2-PK test, at the cut-off value of 15
U/ml, was very high with a NPV of 98%, meaning that if the level of plasma M2-PK in an
individual is determined less than 15 U/ml, the probability for a tumor or polyp is very low.
The specificity of 74% for both groups (tumor and polyp-bearing groups) at the cut-off
value of 15 U/ml indicated that this specificity is acceptable for laboratory testing,
confirmed by the positive predictive value of 74%. At the cut-off value of 4 U/ml, the test
sensitivity, specificity, PPV and NPV for the stool samples of tumor-bearing group was
100%, 68%, 52.7% and 100%, respectively.
Chi-square test was used to compare the normal and abnormal levels of M2-PK in the stool
samples of the normal and polyp-bearing groups, which showed a significant difference
between the two groups (p = 0.0001). At the cut-off value of 4 U/ml, the test sensitivity for
the stool samples of polyp-bearing groups was 87%, specificity was 68%, PPV was 65%
and NPV was 88%.

The sensitivity of fecal M2-PK test was higher in tumor-bearing group (100%) than in polyp-bearing group (87%). Besides, NPV was 100% in tumor-bearing group, meaning that if the level of fecal M2-PK of an individual is determined less than 4 U/ml, the probability for a tumor is almost zero. In contrast, regarding the low PPV of M2-PK test for detecting tumor and polyp in stool, any result higher than 4 U/ml can be false positive indicating a low specificity of the test. Other researchers have shown that the sensitivity of M2-PK measurement in the feces was 77.9% for CRC and specificity ranged from 74.3% to 83.3. Overall sensitivity for adenomas was 45.9%, raising to 61.1% for adenomas larger than 1 cm [16]. In another study, the pooled sensitivity and specificity of fecal M2-PK for the diagnosis of CRC calculated by the bivariate model were 79% and 81%, respectively and the summary PPV and NPV were 74% and 86%, respectively [23]. In a study performed by Kumar et al, at a diagnostic cut-off value of 15 U/ml for plasma tumor M2-pyruvat kinase, sensitivity, specificity, PPV and NPV were 57.3, 89, 85.7 and 64.8%, respectively. In CRC, fecal tumor M2-PK had a sensitivity of 73-92% at a cut-off value of 4 U/ml in compared to 50% sensitivity for Guaiac fecal test [24]. In a multi-center study on 317 subjects with a cut-off value of 4 U/ml, fecal M2-PK assay had a sensitivity, specificity, PPV and NPV of 81.1, 71.1, 61.9, and 86.7% respectively to detect CRC [25].

Besides, with 328 patients according to ROC, the tumor M2-PK cut-off level was 4.00 U/mL (the sensitivity, specificity, PPV, and NPV were 71.4%, 71.0%, 73.5%, and 94.4%, respectively) [20]. In a study by Hisham K. Dabbous et al, M2-PK was the most sensitive and specific test in differentiating CRC from control subjects in fecal samples with sensitivity and specificity of 75%, and 100%, respectively [14].

In the present study, ROC curve was used to determine the best cut-off value for tumor M2-PK test. Based on our results, in order to have the best sensitivity and specificity for
tumor detection in plasma specimens, a cut-off value >25 U/ml was recommended, in which the test sensitivity and specificity was 90.9% and 91.3%, respectively. Besides, for polyp detection, a cut-off value >19 U/ml was suggested, in which the sensitivity of the test was 96.3% and the specificity was 85.5%. AUC of polyp data was 0.95 and for tumor data was 0.975 in plasma, which confirms that the overall discriminatory power of the test is high.

Furthermore, in order to have the best sensitivity and specificity for tumor detection in stool samples, a cutoff value >8 U/ml was recommended, in which the test sensitivity is 100% and the specificity is 85.6%. For polyp detection, a cut-off value >4.8 U/ml was suggested, in which the sensitivity of the test was 81.6% and the specificity was 74.8%. AUC of polyp data was 0.834 and for tumor data was 0.969 in stool that confirmed the overall discriminatory.

Therefore, a cut-off range of 4.8-8 U/ml in stool samples can detect polyp and a cut-off value > 8 U/ml can detect tumor. In addition, a cut-off range of 19-25 in plasma samples can detect polyp and a cut-off value > 25 can detect tumor.

In our study, the specimen size of tumor was not the original size because it was based on the biopsy sample. The same situation applied for polyp samples except for the time when polypectomy was performed, having an original. The specimen size of tumor might affect the analysis related to the M2-PK level and sample size of tumor or polyp in our study.

**Conclusions**

To sum up, high specificity and sensitivity of tumor M2-PK measurement test in stool and plasma samples of patients with CRC and polyp show that this test can be used as a non-invasive method and a diagnostic tool to diagnose CRC and colon adenoma.

**List Of Abbreviations**
**gFOBT:** Guaiac fecal occult blood test; **tM2-PK:** Tumor pyruvate kinase M2 isoform; **CRC:** colorectal cancer; **NSAIDs:** non-steroidal anti-inflammatory drugs; **iFOBT:** Immunological fecal occult blood test; **FDA:** Food and Drug Administration; **ROC:** Receiver operating characteristics curve; **AUC:** Area under the cure; **PPV:** Positive predictive value; **NPV:** Negative predictive value

**Declarations**

**Ethics approval and consent to participate**

This study was approved by Mashhad University of Medical Sciences (MUMS) ethical committee.

**Consent for publication**

This study is approved by all participants for publication.

**Availability of data and material**

All data are included in this published article. Any additional information related to this study is available from the author for correspondence upon reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

HRH and MAK supervised the study. AJ, AI and RR participated in study design and scientific discussion of the data. FR contributed to performing the experiments. All authors read and approved the final manuscript.

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Table 1. The number of normal, polyps and cancer patients in plasma and stool samples based on the pathology results
| Sample test | Lesion type | Frequency | Percent |
|-------------|-------------|-----------|---------|
| Stool       | Normal      | 111       | 49.1    |
|             | Tumor       | 39        | 17.3    |
|             | Polyp       | 76        | 33.6    |
|             | Total       | 226       | 100.0   |
| Plasma      | Normal      | 69        | 38.8    |
|             | Tumor       | 56        | 31.5    |
|             | Polyp       | 53        | 29.7    |
|             | Total       | 178       | 100.0   |

Table 2. The location of tumor and polyps in stool and plasma samples

| Sample test | Lesion type | Ascending colon no. (%) | Transverse colon no. (%) | Descending colon no. (%) |
|-------------|-------------|-------------------------|--------------------------|--------------------------|
| Stool       | Tumor       | 12 (30.8%)              | 6 (15.4%)                | 0 (0%)                   |
|             | Polyp       | 15 (19.7%)              | 7 (9.2%)                 | 11 (14.5%)               |
|             | Tumor       | 14 (25%)                | 6 (10.7%)                | 5 (8.9%)                 |
| Plasma      | Polyp       | 12 (22.6%)              | 6 (11.3%)                | 7 (13.2%)                |

Table 3. The types of the adenomatous polyps.

| Sample | Type of polyp | Tubular adenoma | Tubulovillous adenoma | Villous adenoma | Sessile serrated adenoma | Total |
|--------|---------------|-----------------|-----------------------|-----------------|--------------------------|-------|
| Stool  | Multiple adenomatous | 13 (17.1%) | 8 (10.52%) | 1 (1.31%) | 2 (2.63%) | 24 (31.57%) |
|        | Single adenomatous | 43 (56.57%) | 8 (10.52%) | 0 | 1 (1.31%) | 52 (68.42%) |
| Plasma | Multiple adenomatous | 9 (16.98%) | 4 (7.54%) | 2 (3.77%) | 2 (3.77%) | 17 (32.07%) |
|        | Single adenomatous | 26 (49.05%) | 8 (15.09%) | 0 | 2 (3.77%) | 36 (67.92%) |
Table 4. The pathology results of the stool and plasma samples representing the grading of adenocarcinoma or adenoma

| Sample test | Lesion type          | Low grade no. (%) | High grade no. (%) | Total no. (%) |
|-------------|----------------------|-------------------|-------------------|--------------|
| Stool       | Tumor (adenocarcinoma) | 21 (53.8%)        | 18 (46.2%)        | 39 (100)     |
|             | Polyp (dysplasia)     | 74 (97.4%)        | 2 (2.6%)          | 76 (100)     |
| Plasma      | Tumor (adenocarcinoma) | 45 (80.4%)        | 11 (19.6%)        | 56 (100)     |
|             | Polyp (dysplasia)     | 45 (84.9%)        | 7 (13.2%)         | 53 (100)     |

Table 5. The levels of M2-PK in tumor-/polyp-bearing patients with controls in stool and plasma samples

| Sample type | Lesion type          | Chi-squared p value | Sensitivity | Specificity | Positive Predictive value |
|-------------|----------------------|--------------------|-------------|-------------|--------------------------|
| Stool       | Tumor                | 0.0001             | 100%        | 68%         | 52                       |
|             | Control              | 0.0001             | 87%         | 68%         | 6                        |
| Plasma      | Tumor                | 0.0001             | 98%         | 74%         | 7                        |
|             | Control              | 0.0001             | 98%         | 74%         | 7                        |

Table 6. The cut-off values based on ROC curves for tumor/polyp M2-PK test in stool and plasma samples.

| Sample type | Lesion type | Suggested cut-off value based on ROC (U/ml) | Sensitivity | Specificity |
|-------------|-------------|---------------------------------------------|-------------|-------------|
| Stool       | Tumor       | > 8                                         | 100%        | 85.6%       |
|             | Polyp       | > 4.8                                       | 81.6%       | 74.8%       |
| Plasma      | Tumor       | >25                                         | 90.9%       | 91.3%       |
|             | Polyp       | >19                                         | 96.3%       | 85.5%       |

Figures
Figure 1

Comparison of M2PK plasma and stool test results in 3 groups (normal, polyp, tumor).
Receiver operating characteristic (ROC) curve for tumor/polyp M2PK plasma and stool.

Figure 2