Clinical interest of the combined use of serum CD26 and alpha-L-fucosidase in the early diagnosis of colorectal cancer

Daniel Ayudea, María Páez de la Cadenaa, Oscar Javier Corderoa, Montserrat Nogueirab, José Ayudec, Almudena Fernández-Brieraa and Francisco Javier Rodríguez-Berrocala,∗

aDepartment of Biochemistry, Genetics, and Immunology, University of Vigo 36200 Vigo, Spain
bDepartment of Biochemistry and Molecular Biology, University of Santiago de Compostela, 15706 Santiago de Compostela, Spain
cDepartment of Computer Sciences, University of Vigo, 36200 Vigo, Spain

Abstract. The purpose of this study was to assess if the combination of CD26 and alpha-L-fucosidase has a role in the diagnosis of colorectal cancer, paying particular attention to the stages in which the tumour is not yet disseminated. CD26 concentration and alpha-L-fucosidase activity were determined in sera from 110 colorectal cancer patients and 46 donors. The combination of CD26 and alpha-L-fucosidase showed a specificity of 100% with a sensitivity of 64% in the diagnosis of colorectal cancer. Interestingly, the combination of both markers had a sensitivity of 75% in the stage I at the highest specificity (100%), providing also high sensitivity levels for the other non-disseminated stages (66% for stages II and III). In conclusion, the combined use of CD26 and alpha-L-fucosidase offers high sensitivity with high specificity in the diagnosis of colorectal cancer, especially at the earliest stage (TNM I).

Keywords: Colorectal cancer, serum, CD26, alpha-L-fucosidase, diagnosis

1. Introduction

Colorectal cancer (CRC) still represents the second most frequent cause of cancer death in Western countries [1], despite that it is a carcinoma with higher expectations of survival when diagnosed at early stages. Thus, it seems reasonable to explore the application of new molecular markers for earlier detection of cancer [2].

Due to the almost complete lack of symptoms at early stages and the absence of highly sensitive, effective and economical methods of screening for CRC, it is difficult to detect the carcinoma before the dissemination of the malignant tumour. Carcinoembryonic antigen (CEA) is the most commonly used serum tumour marker for CRC, but it is not recommended as screening or diagnostic test for this neoplasm, especially in the early stages [3–6]. At this time, the recommended screening methods for CRC are the faecal occult blood test (FOBT) and the sigmoidoscopy/colonoscopy with associated polypectomy [7,8]. Low rates of application of CRC screening methods have been attributed to resistance by physicians, patients, and the health care system [9]. Perhaps the problem is the high cost of applying those methods in screening campaigns for all the population with low or medium risk of CRC (i.e., healthy people over 50 years) [10]. Thus, it would be very useful to have one or several combined tumour markers that could help in the early detection of CRC.
Table 1

Serum levels of CD26, AFU activity and CEA in healthy donors and colorectal cancer patients by TNM stages

| Tumour marker | Healthy donors | Colorectal cancer |
|---------------|----------------|-------------------|
|               | n=46           | All n=110         | Stage I n=12 | Stage II n=55 | Stage III n=29 | Stage IV n=14 |
|               | M ± SD | Me | M ± SD | Me | M ± SD | Me | M ± SD | Me | M ± SD | Me |
| CD26 (ng/ml)  | 557.3±130.14 | 550.0 | 254.3±131.91 | 227.5 | 295.3±152.34 | 292.0 | 226.7±93.06 | 205.0 | 251.5±95.45 | 248.0 | 333.5±243.10 | 312.0 |
| AFU (U/ml)    | 11.1±5.47    | 11.3 | 4.9±2.76 | 4.2 | 4.2±2.68 | 3.3 | 4.9±2.52 | 4.3 | 4.4±2.15 | 3.8 | 6.2±4.31 | 4.9 |
| CEA (ng/ml)   | 2.2±1.68     | 1.7 | 122.5±621.51 | 3.0 | 2.9±3.45 | 1.8 | 7.2±11.23 | 2.9 | 18.7±39.97 | 3.6 | 867.7±1567.60 | 311.0 |

n: number of subjects, M: mean value, SD: standard deviation. \(^1P < 0.001\) when comparing the levels of the serum tumour markers in colorectal cancer patients with regard to healthy donors using the U Mann-Whitney test.
We have previously described the diagnostic value of two novel serum tumour markers: the soluble glycoprotein CD26 (dipeptidyl peptidase IV, EC 3.4.14.5.) and the alpha-L-fucosidase enzyme (EC 3.2.1.51) [11, 12]. A decrease in CD26 concentration and alpha-L-fucosidase activity (AFU) has been detected not only in serum of CRC patients but also in tissue of CRC tumours [13,14] as well as in colorectal transformed cell lines [15,16].

Here we show the results of measuring CD26 and AFU levels in sera of the same patients and donors in order to assess the diagnostic value of the combination of both markers. We have paid especial attention to the stages in which the tumour is not yet disseminated, in order to evaluate their usefulness as diagnostic or screening methods for CRC.

2. Methods

2.1. Patients

Preoperative blood and tumour samples were collected between January 1994 and December 1997 from 110 (54 females, 56 males) consecutive and potentially curable patients, operated on for CRC (74 colon, 36 rectum) at the Xeral-Cíes Hospital, Spain. All primary colorectal adenocarcinoma specimens were processed for regular pathological and histological examination. The grading of tumours was established according TNM classification [17]: 12 samples were classified as stage I, 55 as stage II, 29 as stage III and 14 as stage IV. Blood samples for the control group, consisting of 46 healthy blood donors (26 females, 20 males), were provided by the Galician Transfusion Centre. These controls were healthy and controlled donors. Most of them were habitual and some were new donors obtained in a campaign of the Galician Transfusion Centre at the University of Vigo. Blood from all these donors had been tested by the Galician Transfusion Centre.

2.2. Preparation of samples

The drawn blood was allowed to coagulate at room temperature and centrifuged at $2000 \times g$ for 15 minutes. The sera were stored at $-85^\circ C$ until used, unless otherwise stated.

2.3. CD26 assay

The concentration of serum CD26 was analysed using specific immunoassays (human soluble CD26 ELISA Kit from Bender Medsystems, Vienna, Austria). ELISAs were performed according to the manufacturer’s instructions: mean values of duplicated measurements were calculated and a sigmoid-shaped standard curve was determined by simultaneously analysing a dilution series of standard samples. A CD26 value lower than 410 ng/ml was considered as abnormal [11].

2.4. Alpha-L-fucosidase assay

AFU assays were always performed under linear conditions with respect to the amount of protein and the time of incubation. AFU activity was measured as previously described [12], using 4-methyl-umbelliferyl-α-L-fucopyranoside (4-MU-fucoside) at a final concentration of 0.35 mM as substrate and 100 mM citric acid/K$_2$HPO$_4$ buffer pH 5.0. Fluorescence was read on a Kontron fluorometer (using wavelengths of 365 nm and 460 nm for excitation and emission, respectively), and fluorescence values were corrected by subtracting the serum sample and the substrate blanks. One unit of enzyme (U) is defined as the amount of enzyme necessary to hydrolyse 1 nmol of substrate per minute at 37°C. An AFU activity value lower than 5.6 U/ml was considered as abnormal [12].

2.5. Carcinoembryonic antigen assay

The concentration of CEA was analysed by a commercial immunoassay method using the Enzymun-Test CEA (Boehringer Mannheim). This immunoassay is carried out in one step by means of a sandwich method using the streptavidine technology. A CEA value higher than 5 ng/ml was considered as abnormal [3].

2.6. Calculation of the sensitivity, specificity and efficiency parameters

Normal distribution was assessed using the Kolmogorov-Smirnov test. Variance homogeneity was evaluated by the Levene test. The statistical significance of the results was assessed using the U Mann-Whitney test performed in the SPSS software for Windows (Release 9.0).

The sensitivity was considered as the number of true-positives, and the specificity as the number of true-negatives, expressed as a percentage. The efficiency is
the fraction of patients correctly classified, expressed in percentage.

When the tumour markers were used in combination, the sensitivity value was considered as the number of true-positives, and the specificity value as the number of true-negatives, detected by both tumour markers. Specific software made to calculate directly the sensitivity and the specificity of the tumour markers alone or combined was used.

3. Results

3.1. CD26, alpha-L-fucosidase activity and carcinoembryonic antigen levels in serum from healthy donors and colorectal cancer patients

Soluble CD26 concentration and AFU activity was determined in 46 sera from healthy donors and 110 sera from CRC patients. CEA concentration was also measured as an internal control of the study and to assess its diagnostic value in combination with CD26 and AFU. Means ± standard deviations and medians of the tumour makers for donors and patients by TNM stages are shown in Table 1. CD26 and AFU activity levels were significantly lower \((P < 0.001)\) in CRC patients than in healthy donors, whereas CEA levels were significantly higher \((P < 0.001)\) in patients than in donors. Statistical differences of CD26, AFU and CEA levels between patients with colon versus patients with rectal tumours were not found.

3.2. Diagnostic parameters of the combined use of CD26, alpha-L-fucosidase activity and carcinoembryonic antigen in the detection of colorectal cancer

The diagnostic parameters of the combined use of CD26, AFU and CEA were established using a combination of two of the tumour markers in the detection of CRC. Table 2 shows the sensitivity, specificity and efficiency of the combination of CD26 and AFU, CD26 and CEA, and AFU and CEA in the diagnosis of CRC. The used values of cut-off were 410 ng/ml for CD26, 5.6 U/ml for AFU and 5.0 ng/ml for CEA, determined in previous studies [3,11,12].

The combination of CD26 and AFU in the diagnosis of CRC showed a sensitivity of 64%, with a specificity of 100% and an efficiency of 82%. When CD26 and CEA were used in combination, the diagnostic sensitivity was lower (34%) with a specificity of 100% and an efficiency of 67%. The combination of AFU and CEA showed the lowest sensitivity (25%) with a specificity of 100% and an efficiency of 63%.

### Table 2

| Tumor marker       | Sensitivity | Specificity | Efficiency |
|--------------------|-------------|-------------|------------|
| CD26 and AFU       | 64          | 100         | 82         |
| CD26 and CEA       | 34          | 100         | 67         |
| AFU and CEA        | 25          | 100         | 63         |

CD26 cut-off: 410 ng/ml; AFU cut-off: 5.6 U/ml; CEA cut-off: 5.0 ng/ml. Sensitivity, specificity and efficiency values were calculated as explained in methods, and are expressed in percentage.

3.3. Diagnostic sensitivity of the combined use of CD26, alpha-L-fucosidase activity and carcinoembryonic antigen in the detection of colorectal cancer patients classified by TNM stages

The diagnostic sensitivities of the combinations of the three tumour markers were also evaluated by each TNM stage of the tumours (Table 3).

The results showed that the combined use of CD26 and AFU gave a sensitivity of 75% in stage I, 66% in stages II and III and 43% in stage IV. The combination of CD26 and CEA showed poor sensitivity levels in the stages I (17%), II (27%) and III (35%) and higher sensitivity in the stage IV (71%). The combined use of AFU and CEA also showed poor diagnostic sensitivity levels in the stages I (17%), II (15%) and III (31%), whereas the diagnostic sensitivity in the stage IV was 57%.

Thus, the combination of CD26 and AFU showed the best sensitivity and specificity in the diagnosis of patients at non-disseminated stages.

4. Discussion

We have previously reported significant decreases of more than 50% in the CD26 concentration and AFU
activity of serum from CRC patients, establishing the sensitivity and specificity of each novel marker used alone in the diagnosis of CRC [11,12]. The decrease of serum CD26 and AFU could be related with the diminution of the levels of these proteins in CRC cells [13–16]. However, the impairment of more than 50% in serum CD26 and AFU activity in CRC does not seem to be only originated by alterations of these proteins in tumour cells. The decreased levels of CD26 could be related with an immune defective antitumour response in CRC. For instance, a defect in interleukin-12 production [18] that is a well-known CD26 up-regulator [19] has been described in CRC. The decreased levels of AFU activity could be related with the maintenance of the observed higher levels of fucosylation in tumour cells [20].

In the present paper we have analysed if the diagnostic value of CD26 and AFU is improved when both markers are used together in the detection of CRC. On the other hand, we have also analysed if the results of CEA in the detection of CRC are improved when it is used in combination with CD26 or AFU. Normal levels of each tumour marker were established using as reference the previously described cut-off values: 410 ng/ml for CD26 [11], 5.6 U/ml for AFU [12] and 5.0 ng/ml for CEA [3]. Sensitivity, specificity and efficiency values obtained in the present study for CD26, AFU and CEA, when these markers were used alone in the diagnosis of CRC, were very similar to those previously reported [11,12].

When CD26 is used alone at the 100% of specificity, sensitivity was of 33% in the stage I, 65% in the stage II and 66% in the stage III [11]. These values for AFU were even lower [12]. In this work we have demonstrated that the combined use of CD26 and AFU showed good sensitivity levels (64%) at the highest specificity (100%). Furthermore, the sensitivity at non-disseminated stages was of 75% in stage I and 66% in stages II and III, higher than that observed in the stage IV (43%). However, the combination of CD26 or AFU with CEA offers low sensitivity levels, especially in the non-disseminated stages. These results confirm that CEA is not useful in the diagnosis of CRC.

FOBT screening procedure has been demonstrated to reduce the risk of death of CRC [21–24] and it is the most commonly performed screening test for CRC. It includes an analysis of three serially obtained samples [21–23] and when any of the three tests for each sample is positive, colonoscopy is recommended [25]. We propose similar studies with CD26, AFU, or both, combined with FOBT to assess if the detection rates of CRC could be improved. Other studies have recently been proposed in order to improve the results obtained with FOBT, through the detection of DNA alterations ‘such as APC or K-ras mutation’ in stool samples [26, 27]. The advantages of the methods that we here propose are that they are easy and inexpensive.

In conclusion, our findings provide a new tool especially sensitive in the diagnosis of CRC patients in stage I, but also in stages II and III. The results of the combination of these markers appear promising, and merit further study in a screening setting in order to assess if they could be used alone or combined with other screening tests -such as FOBT- to help clinicians to decide if healthy people with low or medium risk of CRC should undergo other more invasive methods, such as sigmoidoscopy/colonoscopy, which confirm or discard the presence of the tumour.

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