Colorectal cancer detection by means of optical fluoroscopy. A study on 494 subjects

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1. ABSTRACT

In the present work we investigated the possible role of the native fluorescence of blood plasma in the management of colorectal cancer (CRC) and its feasibility as a new tumor marker. Sample of blood was collected from 248 asymptomatic blood donors and from 246 CRC patients. The native fluorescence of blood plasma was measured using a conventional spectrofluorimeter. The intensity of fluorescence of blood plasma at 623nm (IF623), reasonably ascribed to endogenous porphyrins, was significantly higher in CRC patients than in healthy subjects. The diagnostic capability of IF623 in the discrimination between healthy subjects and CRC patients was tested by Receiving Operating Characteristic (ROC) curve analysis, which resulted in an Area Under the Curve (AUC) of 0.72±0.01. Fluorescence measurement of blood plasma might be considered diagnostically useful as a candidate for a new tumor marker for CRC management. The procedure is characterised by a great acceptability and by a very low cost, and might be used in a two-step screening wherein an IF623 positive result is followed by colonoscopy.

2. INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer death in the developed world in either sex. In women, it ranks second after breast cancer, and in men, it ranks third after lung and prostate (1). Differently to what happens with these kinds of pathology, death from CRC can be reduced by the detection of early-stage not metastasized disease; the disease itself can be prevented by the detection and subsequent surgical removal of colorectal adenomatous polyps, from which cancer arise in more than 95% of cases (2). The optimal mean to prevent CRC is related to the risk factors that might induce the disease, in order to identify subjects at high risk and the methods for an early detection before the onset of symptoms. The increasing of age is the greatest risk factor for CRC since 85% of cases occur in people aged more than 60 (1). Next to age, family history is the most common risk factor followed by lifestyle and/or environmental factors, such as obesity, excessive intake of processed meat, lack of physical activity and scanty consumption of fruit and vegetables. Among the methods for examining the colorectal tract, the most accurate is the standard (optical)
colonscopy that reaches 97% sensitivity for cancer and large polyps (more than 10mm) (1). The drawbacks of this technique are the low compliance among asymptomatic subjects, the necessity of an adequate preparation and the cost. Also widely diffused techniques are sigmoidoscopy and double contrast barium enema, being however the former limited by the extension of the investigated tract, i.e. left colon only, and the latter by low sensitivity, about 50%, for large polyps.

Alternative techniques for early detection of CRC, typically used in two-step screening, are testing for faecal occult blood and measurements of proper tumor markers, a positive result being followed by imaging of the whole colon. The guaiac based faecal occult blood test (gFOBT) has been proved as a screening investigation for CRC, but the low accuracy make it insensitive to guide the investigation of asymptomatic subjects (3). In fact, gFOBT is frequently negative if bleeding is minimal, such as from adenomas, or positive due to exogenous source of peroxidase activity. Recently, a newer immunochemical faecal occult blood test (iFOBT) has been introduced, which achieves higher specificity than gFOBT being specific for human haemoglobin. At the time, the role of iFOBT in screening for CRC is matter of investigation (4-7).

Table 1. Histological diagnosis and anatomical site of tumoral pathology of the recruited patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Anatomical site</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>Rectum</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Sigma</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Colon</td>
<td>52</td>
</tr>
<tr>
<td>Adenoma</td>
<td>Rectum</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Sigma</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Colon</td>
<td>2</td>
</tr>
<tr>
<td>IAP</td>
<td>~</td>
<td>17</td>
</tr>
<tr>
<td>Local recurrence</td>
<td>Rectum</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>246</td>
</tr>
</tbody>
</table>

To better investigate the role of the native fluorescence of blood plasma in the diagnosis and management of CRC, the purpose of this work was manifold and aimed to evaluate the proposed test in terms of i), sensitivity, specificity and accuracy on an increased pool of CRC patients and healthy subjects; ii), comparison with CEA results obtained on the same population; iii), monitoring after successful therapy; iv), correlation to CRC and to other malignant diseases. Finally, acceptability, simplicity and costs of the test have been examined.

3. PATIENTS AND METHOD

3.1. Patients

Samples of blood were collected from 510 subjects of which 248 were asymptomatic blood donors (156 males, 92 females, mean and median age 53.4 and 59 years, respectively, age range 18-70), reasonably assumed as healthy subjects if both laboratory exams and case-history were judged as regular. In details, subjects were ineligible if they had recent overt bleeding, or a known benign colonic disorder or familial history for colorectal cancer. Preoperative blood sample was available from 262 patients of the Colorectal Surgery Unit of Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy. Thirteen patients were excluded from the study because of history of other malignancy or a chemotherapy treatment not ended since eight weeks, at least. Three patients were excluded because of lack of clinical data. Thus, a total of 246 patients (163 males, 83 females, mean and median age 60.4 and 63 years, respectively, age range 18-70) were included in the study. The distribution of the investigated diseases according to the histological diagnosis and anatomical site is reported in Table 1. The tumors were classified postoperatively according to the TNM classification for UICC stage based on clinical and pathological findings (20). Eleven patients were stage 0, 30 patients stage I, 58 stage II, 88 stage III and 17 patients stage IV. The study was performed in accordance with the ethical standard of our Institute and the informed consent was obtained from all study subjects.
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Figure 1. Normalized fluorescence spectra of blood plasma of 248 blood donors (---) and 246 patients bearing colorectal diseases (--), at 405 nm excitation wavelength.

3.2. Method and study design

For all the recruited subjects, native fluorescence of blood plasma and serum CEA level were measured. The method used to perform the fluorescence analysis has been described in details in a previous work (19). Briefly, the fluorescence measurements were performed by means of a conventional spectrofluorimeter (Model F-3000, Hitachi, Ltd. Tokyo, Japan), selecting an excitation wavelength of 405 nm and recording the fluorescence emission spectra in the range 430-700 nm. Due to the great variability in the fluorescence intensity of plasma from sample to sample, each fluorescence spectrum was normalized by dividing the fluorescence intensity at each wavelength by the maximum value of intensity of the spectrum. CEA level was determined using a commercial kit (Radim CEA IRMA CT, Radim Spa, RM, Italy) available in our laboratory. CEA was quantified with a time-resolved immunofluorimetric assay with a cut-off value of 5 µg/l. The detection limit of the assay is 0.2 µg/l and the inter-assay coefficient of variation was less than 3% in the concentration range 3-100 µg/l. For a subgroup of 10 patients (7 males, 3 females, mean and median age 58.3 and 60 years, respectively, age range 45-69), both markers were re-evaluated after therapeutic treatment. Finally, a randomised subgroup of 35 blood donors (21 males, 14 females, mean and median age 52.7 and 56 years, respectively, age range 41-65) were also submitted to colonoscopy. Data on colonoscopy were recorded on a specific form with information on the quality of the investigation.

3.3. Statistical analysis

The Mann Whitney test for unpaired data was applied to determine the significance of the difference in the native fluorescence of blood plasma and in serum CEA level between CRC patients and healthy subjects. A p value less than 0.05 was considered to be statistically significant. The correlation between the native fluorescence of blood plasma and CEA level as well as the correlation between the two markers and the weight of the surgical specimen were assessed with the Spearman rank correlation test. Additional statistical evaluations were carried out by separately considering patients bearing the following diseases: colon, sigma or rectum-localised adenocarcinoma, local recurrence, single adenoma and familial adenomatous polyposis (FAP).

In order to evaluate the capability of native fluorescence and CEA to discriminate patients with colorectal cancer from healthy subjects, Receiving Operating Characteristic (ROC) curves were determined considering separately native fluorescence and CEA, as well as their combination. The differences in the Area Under the Curve (AUC) values were determined to compare the diagnostic power of the two tests. Statistical calculations were performed by using a commercially available software (Statistica, Stat Soft, Tulsa, OK, USA).

4. RESULTS

The means of the normalized fluorescence spectra of blood plasma collected from 248 blood donors and 246 patients are reported in Figure 1.

According to our previous work, the only significative difference between the fluorescence spectra of blood donors and patients is the emission peak at 615-635 nm reasonably ascribed to endogenous porphyrins (14, 21); the minimum p level (p less than 0.000001) was at 623 nm. The serum level of CEA was significantly higher in CRC patients than in blood donors (p less than 0.000001). Table 2 reports mean value +/- Standard Deviation (SD) and median of the fluorescence intensity at 623 nm-wavelength, from now on named IF623, evaluated from the normalized fluorescence spectra for different groups of subjects, as well as mean value (+/-SD) and median of CEA level.
Table 2. Mean (+/-SD) and median of the fluorescence intensity at 623 nm, evaluated from the normalized fluorescence spectra, and of CEA level for the different groups of subjects

<table>
<thead>
<tr>
<th>Subjects (#)</th>
<th>IF at 623 nm (u.a.)</th>
<th>Median</th>
<th>CEA (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (+/-SD)</td>
<td></td>
<td>Mean (+/-SD)</td>
</tr>
<tr>
<td>Blood donors (248)</td>
<td>13.29 (2.15)</td>
<td>13.09</td>
<td>1.88 (1.11)</td>
</tr>
<tr>
<td>Whole set of patients (246)</td>
<td>15.81 (3.31)</td>
<td>14.90</td>
<td>187.77 (1834.32)</td>
</tr>
<tr>
<td>Patients with adenocarcinoma in</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rectum (96)</td>
<td>15.17 (3.17)</td>
<td>14.67</td>
<td>4.87 (8.40)</td>
</tr>
<tr>
<td>sigma (56)</td>
<td>15.68 (5.72)</td>
<td>14.59</td>
<td>752.21 (3839.47)</td>
</tr>
<tr>
<td>colon (32)</td>
<td>16.36 (3.99)</td>
<td>15.66</td>
<td>82.63 (375.25)</td>
</tr>
<tr>
<td>Patients with local recurrence</td>
<td>15.95 (6.39)</td>
<td>13.96</td>
<td>14.23 (35.14)</td>
</tr>
<tr>
<td>Patients with adenoma (13)</td>
<td>13.97 (3.57)</td>
<td>13.12</td>
<td>2.87 (2.41)</td>
</tr>
<tr>
<td>Patients with FAP (17)</td>
<td>19.29 (12.41)</td>
<td>16.07</td>
<td>1.62 (0.64)</td>
</tr>
</tbody>
</table>

Table 3. Mean (+/-SD) and median of the fluorescence intensity at 623 nm and of CEA level as function of cancer stage.

<table>
<thead>
<tr>
<th>Cancer stage(^1)</th>
<th>Subjects (#)</th>
<th>IF623 (u.a.)</th>
<th>Median</th>
<th>CEA (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (+/-SD)</td>
<td>Mean (+/-SD)</td>
<td></td>
<td>Median</td>
</tr>
<tr>
<td>Stage 0</td>
<td>11</td>
<td>13.17 (2.46)</td>
<td>13.88</td>
<td>1.64 (1.21)</td>
</tr>
<tr>
<td>Stage I</td>
<td>30</td>
<td>14.58 (2.12)</td>
<td>14.76</td>
<td>3.31 (3.46)</td>
</tr>
<tr>
<td>Stage II</td>
<td>58</td>
<td>15.74 (3.26)</td>
<td>14.93</td>
<td>4.44 (4.89)</td>
</tr>
<tr>
<td>Stage III</td>
<td>88</td>
<td>15.92 (5.05)</td>
<td>15.10</td>
<td>628.48 (3422.89)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>17</td>
<td>16.51 (4.84)</td>
<td>15.58</td>
<td>103.20 (336.03)</td>
</tr>
</tbody>
</table>

\(^1\)Respective TNM classification for UICC stage. Stage 0: Tis, N0, M0; I: T1 or T2, N0, M0; II: T3 or T4, N0, M0; III: any T, N1-3, M0; IV: any T, any N, M1.

Figure 2. Comparison of CEA and IF623 with ROC curve analysis in 246 patients bearing colorectal diseases and in 248 healthy subjects.

No correlation was found between IF623 and age, among both patients (r=0.01) and donors (r=-0.22), as well as between CEA level and age (patients, r=0.14; donors, r=-0.01). IF623 mean value evaluated for males did not resulted significantly different from that of females, both in patients (p=0.149) and blood donors (p=0.062), similarly as in case of CEA, p=0.161 and 0.086 for patients and blood donors, respectively. IF623 mean value resulted weakly correlated with the weight of the surgical specimen (r=0.65), whereas no correlation was found between CEA level and weight (r=0.01).

Table 3 reports IF623 and CEA level as function of cancer stage. IF623 shows an increasing trend in passing from stage 0 to stage IV. CEA level shows a similar trend, with the exception of stage IV. However, the great difference shown between values of median and mean in CEA level underlines the not normal distribution of the data.

No correlation was found between IF623 and CEA considering both patients and donors (r=0.07 and 0.10, respectively), thus suggesting the possibility of combining the two test. Figure 2 shows the ROC curves calculated separately for IF623 and CEA as well as combined as a unique test. Area under ROC curves was 0.72+/-0.01, 0.66+/-0.01 and 0.74+/-0.01 for IF623, CEA and their combination, respectively.
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5. DISCUSSION

Colorectal cancer is mainly diagnosed by the presence of macroscopic clinical signs like rectorrhagia, changes in frequency of evacuation, abdominal pain, loss of weight, anaemia and intestinal obstruction. In fact, even if it’s well known that early diagnosis and prompt treatment of CRC before the onset of such symptoms greatly improves survival, at the time screening protocols for CRC diagnosis before development of adenomatous polyps into invasive cancer are not well established (22). This study evaluated a feasible new tumor marker for CRC diagnosis and management and its possible application. The native fluorescence of blood plasma at 623nm, reasonably due to endogenous porphyrins, resulted significantly greater in CRC patients than in healthy subjects. The reasons for the increased amount of endogenous porphyrin in the blood plasma of the patients are not clear. A possible explanation is that a decrease in ferrous form of iron amount, for example due to a cancer related ulcer, may demand to demolish heme, which consists of an iron atom contained in the center of a large heterocyclic organic ring, a porphrin. However, the correlation between IF623 and TNM-UICC stages suggest that the increased amount of endogenous porphyrins might be directly related to the metabolism of cancer cells. In addition, it seems reasonable to take into account even an impairment in the iron / porphyrin balance in the heme synthesis process due to the great request of iron by tumor cells [23].

The ideal tumor marker should be a sensitive indicator of the pathology and, at the same time, greatly specific when tested in normal controls or in different diseases. It should also return to normal levels when successful therapy has been performed and grow again before clinically valuable relapse. It should finally be acceptable and easy to perform with a not prohibitive cost and transportable between laboratories. The aim of this paper was to investigate how IF623 fit the ideal.

5.1. Performance

The performance characteristics of a diagnostic test are typically evaluated by means of ROC curve analysis or by calculating sensitivity and specificity. The first approach has the advantage of giving a global impression of the diagnostic power and especially to allow comparison among different diagnostic tests over the entire spectrum of performances. With regard to IF623, the area under ROC curve resulted 0.72 thus suggesting that the fluorescence test can be considered diagnostically useful (24). Furthermore, on the investigated population the overall diagnostic power of the proposed test resulted greater than that of CEA. Unfortunately, data are not yet available for comparing IF623 and FOBT, but work is in progress. The sensitivity-specificity approach for performance evaluation requests the selection of a threshold or operating point on the outcome, which give rise to a dichotomous response, typically named positive and negative. The best operating point might be chosen so that the classifier gives the best trade off between the costs of failing to detect positive against the cost of raising false alarms. Usually, a threshold is chosen in order to obtain 95% specificity thus limiting the number of false positive and of unnecessary further evaluations. On the contrary, we decided to fix a cut-off on the intensity of fluorescence at 623nm which greatly favour sensitivity with respect to specificity. A value of 13.06 u.a. was imposed as a threshold thus obtaining a sensitivity of 80% and a specificity of 50%. So doing, we accepted a very high number of false positive results which demand further investigations, typically a colonoscopy. In our opinion such a decision is justified by considering the relative low cost of a colonoscopy compared to the cost of the treatment and service of an advanced CRC, and especially considering the importance of submitting subjects at high risk for CRC to a diagnostic investigation. It is worth mentioning that limiting the investigated population to the subjects bearing FAP, the selected cut-off give rise to a sensitivity of 100%. This finding agrees with the correlation found between the weight of the surgical piece and IF623, even though it should be verified on a larger pool of subjects, and suggest a possible application of IF623 in a screening protocol in which people with familiarity for FAP were enrolled. On the same population CEA showed a sensitivity of 0%. On the contrary, when evaluated on patients bearing local relapse, both IF623 and CEA did not result diagnostically useful with a sensitivity of 58% and 25%, respectively. In this phase of our study, the control population was composed of blood donors assumed to be healthy if both laboratory exams and case-history have been judged as regular. Of course this approach might have yield to an underestimation of specificity. Work is in progress to extend our study to subjects submitted to colonoscopy. At the time optical colonoscopy has been performed on a pool of 35 blood donors. All the performed exams resulted negative for CRC and only one positive for adenomatous polyps. IF623 specificity evaluated on these subjects resulted of 57%, in accordance with that obtained on the global population.

5.2. Correlation

Especially if used in screening protocols, the ideal tumor marker should be strongly related to a defined pathology. To evaluate the eventual correlation of IF623 with diseases other than CRC, a preliminary study was performed on a pool of 12 patients bearing breast cancer. IF623 mean value (12.71+/-.1.31) was not significantly different than that obtained on the pool of healthy subjects (13.29+/-.2.15). Work is in progress to investigate the intensity of fluorescence in patients bearing other kind of cancer, as for instance in prostate and in lung.

5.3. Normalization

The ideal tumor marker should return to normal value when successful treatment has been performed. To verify this properties on IF623, a small group of 10 CRC patients, all with an elevate pre-treatment IF623 level and submitted to proper therapy, were re-evaluated two months after any treatment was ended. Colonoscopy, CEA level and IF623 measurements were performed. CEA level, higher than 5µg/l in only four pre-treated subjects, was lower than normality threshold in eight treated subjects. In nine out of 10 patients IF623 value significantly decreased (mean value 12.26+/-.1.42) with respect to pre-treatment
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value (mean value 18.80 ± 7.40). According to colonoscopy executed after proper therapy, all the patients but one were healthy, being that patient characterised by an elevate IF623 value.

5.4. Acceptability

Acceptability of a test is as important to achieving successful screening outcomes as is performance. In fact colonoscopy is of course the best diagnostic approach but only if it is performed, and healthy subjects often reject it. On the contrary, IF623 test is very acceptable since it requires only taking of a blood sample. Combining the acceptability of IF623 test and the performance of colonoscopy, a two step screening programme might be performed where IF623 should result positive.

5.5. Diet

It is well known that both tumor markers measurement and fecal occult blood test may be influenced by the diet and by particular physiological condition, like pregnancy or menstrual cycle. For example, dietary peroxidase found in a range of certain fruit and vegetables, antioxidants such as vitamin C and dietary heme from red meat may cause false positive results when gFOBT is performed. For a preliminary test to evaluate the influence of the diet on IF623 level, the blood of one volunteer (one of the authors) has been submitted to fluorescence measurements the day after the assumption of considerable amount of eggs, chocolate, fresh red meat, fruit or vegetables. No variation greater than 2% was observed on the corresponding fluorescence spectra with respect to the base line. Measurements on the same subject have been made during antibiotic therapy or when different kind of vitaminc integrators were assumed. Only when more than 500mg of vitamin C were assumed, differences in IF623 value greater than 20% with respect to the base line were recorded. Finally, measurements on the same subject during pregnancy and menstrual cycle did not lead to variation greater than 5% with respect to the base line.

5.6. Instrumentation and cost

At present, IF623 measurement requires a spectrofluorimeter, whose cost is about 20.000 US$. However, should the test enter in laboratory practice a close-packed instrument is expected to be realized at a very low cost, being mainly composed by an excitation light, available with led emitting in the blue region, an interference filter and a photodiode to detect the fluorescence emission. Our estimated cost for a single IF623 measurement (blood sample collection, chemical reagents, ancillary materials, labour and overhead) is about 10 US$. Although this cost appears inexpensive, of course hidden costs must be considered, which include the hazards of diagnostic procedures, the emotional cost of worrying of having cancer, as well as the false sense of security engendered in patients with a negative test and expecially the cost of evaluating a false-positive results. As already underline, in our opinion all these costs are compensate by the importance of enrolling healthy subjects into a screening programme.

6. ACKNOWLEDGEMENTS

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7. REFERENCES

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