Asian Pac J Cancer Prev, 17 (3), 939-944

Introduction

Colorectal cancer (CRC) is a major health problem, in Europe more than one million individuals develop CRC every year (Ferlay et al., 2013). Colorectal cancer is a major cause of morbidity and mortality, globally ranking as the third most common tumor in men and the second in women and the fourth most common cancer-related cause of death (Labianca et al., 2013).

Small amounts of free DNA circulate in both healthy and diseased human serum. Tumor necrosis causes release of DNA of varying sizes, in contrast to apoptosis in normal cells that releases smaller and more uniform DNA fragments (Fong et al., 2009). DNA integrity index; represented as the ratio of longer to shorter DNA fragments; may be clinically useful as potential serum biomarker for cancer detection (Fong et al., 2009). Recently highly sensitive method was reported to measure the integrity of free circulating DNA in serum of patients with CRC by quantitative polymerase chain reaction (qPCR) for Arthrrobacterluteus (ALU) repeats (Mead et al., 2011). The ALU is the most abundant short interspersed repeated sequence in the human genome, with a copy number of ~1.4 × 106 per genome (Wang et al., 2000).

The current study aimed at investigating the role of DNA integrity index as well as the concentration of circulating cell-free DNA in serum for screening, diagnosis and monitoring the progression of CRC.

Materials and Methods

Eighty participants were enrolled in this study. They were all screened for inflammatory conditions and previous cancer by full history taking before consent. All subjects underwent endoscopic examination and accordingly they were selected. They comprised 20 age and sex matched volunteers without significant clinical findings, as the control group, 10 Patients diagnosed to have benign colonic polyps and 50 Patients with established colorectal cancer (CRC). All subjects were recruited from the National Cancer Institute and were diagnosed by histopathological examination of tumor biopsy taken during colonoscopy/sigmoidscopy and were staged at time of diagnosis. Patients on chemotherapy or Radiotherapy were excluded. The study was approved by Cairo University Hospital research ethics committee and has been performed in accordance with the ethical standards of the Declaration of Helsinki. An informed consent was obtained from all participants. All laboratory
tests were assayed in the Chemical Pathology Unit, National Cancer Institute, Cairo University.

Specimen collection

Six ml of venous blood were collected from all participants in the study and divided as follows: 3 ml dispensed into a sterile plain vacutainer tube for DNA extraction and genetic studies and the remaining 3 ml were dispensed into another sterile plain vacutainer for measurement of tumor markers. Both were centrifuged (1000g for 15 minutes) within 4 hours of collection to separate serum and were stored frozen at -20 °C until the time of analysis.

Laboratory investigations

1-Tumor markers measurement: Carcinoembryonic antigen (CEA) and CA19-9: Both tumor markers were measured by a solid-phase, two-site sequential chemiluminescent immunometric assay performed on Architect i 1000 SR autoanalyzer (Maestranzi et al., 1998). The analyzer and Kits were purchased from Abbott Architect diagnostics-USA.

II- Molecular Studies:

1- Genomic DNA extraction from the serum: was done using QIAGEN DNA extraction mini kit. (Catalog number: 51104/6). Lysis was done with the use of proteinase K enzyme followed by purification on QIAamp Mini spin columns in which the lysate buffering conditions were adjusted to allow optimal binding of the DNA to the QIAamp membrane. DNA was adsorbed onto the QIAamp silica membrane during a brief centrifugation step. Salt and PH conditions in the lysate ensure that proteins and other contaminants which might inhibit PCR were not retained in the QIAamp membrane. Removal of residual contaminants was done by washing DNA bound to the QIAamp membrane in 2 centrifugation steps using 2 different wash buffers AW1 and AW2 which significantly improves the purity of the eluted DNA. Finally the purified DNA was eluted from the QIAamp membrane in a concentrated form in AE buffer.

2- Measurement of the quantity and quality of the DNA

i) Quantification of DNA: The concentration of DNA was determined by measuring the absorbance at 260 nm (A260) using the Nanodrop spectrophotometer using the AE buffer as the blank. The concentration was displayed in ng/ul. ii) Purity of DNA: Samples purity was measured by comparing the CT of the unknown sample against the standard curve with known copy numbers. DNA integrity index was calculated as ratio between Q247/Q 115 (Q247 and Q115 represent the ALU-qPCR results for sample x with ALU247 and ALU115 primers) (Cordaux and Batzer, 2009).

Genomic DNA was obtained from Promega (catalog number: II-5701)

Real-time PCR amplification was performed by the computerized thermocyclers as follows: pre-cycling heat activation of DNA polymerase at 95 °C for 15 min, followed 35 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30s, and extension at 72 °C for 30s in Applied BiosystemReal-Time PCR Detection System (Umetani et al., 2006). Arthrobacterluteus (ALU) repeats; being the most abundant repeated sequence in the human genome; had been used in our study to measure the DNA integrity in the serum by measuring the quantitative PCR for ALU247bp and ALU 115bp using a calibration curve created by performing qPCR on serially diluted genomic DNA. In this method we quantitated concentrations of Alu interspersed segment copy number in DNA of unknown sample by comparing the CT of the unknown sample against the standard curve with known copy numbers. DNA integrity index was calculated as ratio between Q247/Q 115 (Q247 and Q115 represent the ALU-qPCR results for sample x with ALU247 and ALU115 primers) (Cordaux and Batzer, 2009).

Statistical methods

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as median, minimum and maximum as appropriate. Qualitative data were expressed as frequency and percentage. For non normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between 3 groups was done using Kruskal- Wallis test then post-Hoc “Schefe test” was used for pairwise comparison. Spearman-rho method was used to test correlation between numerical variables. The Receiver Operating Characteristic (ROC) curve was plotted for diagnostic test evaluation. All tests were two-tailed. A p-value <0.05 was considered significant.

Results

Fifty patients with histopathologically proven CRC, were 26 males and 24 females with a mean age of 49.4±14years. Patients with benign colonic lesions (50% Tubulovillous adenoma and 50% Colon poly by histopathology) included 7 males and 3 females with a mean age of 33±6.5years, while the control group included 20 healthy individuals, they were 13 males and 7 females with mean age of 49.9 ±11years. Considering
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smoking; 14/50 CRC patients (28%) were smokers and 1/10(10%) patients with benign lesions were smokers, While 5/20(25%) of healthy controls were smokers.

The median levels of DNA integrity index, absolute DNA concentration, CA19-9 and CEA in the 3 studied groups are illustrated in Table 1, showing that the median levels of DNA integrity index and absolute DNA concentration were significantly higher in CRC patients as compared to the benign group and controls (p<0.001 and 0.002 respectively). Meanwhile, the median levels of CA19-9 and CEA were significantly higher in CRC group than that in the benign and the control groups at p<0.001.

A statistically significant positive correlation existed between the DNA integrity index and the absolute DNA concentration (r=0.31) (p=0.006), along with the presence of statistically significant positive correlation between DNA integrity and the studied tumor markers; CA19-9 (p<0.001) (r=0.4) and CEA (P<0.001) (r=0.45).

Table 1. Comparison between the Studied Markers in the 3 Studied Groups

|                      | CRC group (n=50) median (min.-max.) | Benign group (n=10) median (min.-max.) | Control group (n=20) median (min.-max.) | p-value |
|----------------------|------------------------------------|----------------------------------------|-----------------------------------------|---------|
| DNA integrity index  | 1.54 (0.7-3.1)a                     | 0.3 (0.2-1.9)b                         | 0.173 (0.1-1.35)b                       | p<0.001 |
| Absolute DNA concentration ng/µl | 4.6 (1.1-48)a                     | 2.0 (0.6-5.4)b                         | 2.8 (0.8-23.5)b                        | p=0.002 |
| CA19-9 U/ml          | 28.4 (0.8-5835)a                    | 2.8 (0.7-25)b                          | 9.9 (0.8-22)b                          | p<0.001 |
| CEA ng/ml            | 8.7 (0.9-2235)a                     | 1.6 (0.8-2.1)b                         | 1.7 (0.5-3.3)b                         | p<0.001 |

Groups bearing the same lintials were not statistically significantly different; P <0.05 is considered significant

Table 2. The median levels of the Studied Markers in Relation to the Prognostic Factors

|                      | DNA integrity index | P value | Absolute DNA concentration ng/µl | P value | CA19-9U/ml | P value | CEA ng/ml | P value |
|----------------------|--------------------|---------|-------------------------------|---------|------------|---------|-----------|---------|
|                      | Median (min-max)   |         | Median (min-max)              |         | Median (min-max) |         | Median (min-max) |         |
| Grade                |                    |         |                               |         |            |         |           |         |
| Grade II (n=33)      | 1.08 (0.07-3.1)    | p=0.002 | 3.6 (1.1-27.4)                | p=0.003 | 24 (0.8-5835)    | p=0.28 | 4.3 (0.9-2235) | p=0.27 |
| Grade III-IV (n=17)  | 2.03 (0.2-3.1)     |         | 11.6 (1.6-48)                 |         | 38 (2.5-1300)   |         | 12.6 (1.2-246) |         |
| Adenocarcinoma (n=37)| 1.35 (0.07-3.1)    |         | 4.1 (1.1-48)                  |         | 27.4 (0.8-5835)  |         | 8.3 (0.9-2235) |         |
| Mucinous adenocarcinoma | 1.7 (0.09-3.1)  | p=0.3   | 10 (1.5-23.2)                 | p=0.19  | 31.2 (8.1-1300)  | p=0.9  | 12.3 (0.9-130) | p=0.6  |
|                      | (including Signet ring adenocarcinoma) (n=13) | | |
| Site of carcinoma    |                    |         |                               |         |            |         |           |         |
| Proximal (n=25)      | 1.7 (0.07-3.1)     | p=0.6   | 4.8 (1.1-48)                  | p=0.4   | 31.2 (1.8-3099) | p=0.6 | 8.3 (0.9-246) | p=0.9  |
| (Cecum, ascending col, transverse col, descending col) | | | | | | | |
| Distal (n=25)        | 1.3 (0.4-3.1)      |         | 4.2 (1.5-18)                  |         | 25 (0.8-5835)   |         | 9.3 (0.9-2235) |         |
| (Sigmoid colon. and rectum) | | | | | | | |
| Lymph node +ve       | 1.5 (0.09-3.1)     | p=0.06  | 4.6 (1.5-23.2)                | p=0.6   | 33.2 (0.8-5835)  | p=0.18 | 10.8 (0.9-2235) | p=0.24 |
| (n=30)               |                     |         |                               |         |            |         |           |         |
| Lymph node -ve       | 1.0 (0.07-2.1)     |         | 4.6 (1.1-48)                  |         | 16.9 (1.8-1300) |         | 4.2 (0.9-246) |         |
| (n=20)               |                     |         |                               |         |            |         |           |         |
| MO                   | 0.8 (0.07-2.5)     |         | 4.2 (1.1-48)                  |         | 24.9 (0.8-1300) |         | 4.3 (0.9-246) |         |
| (No evidence of metastasis)(n=29) | | | | | | | |
| Status of distant metastasis | 1.7(0.7-3.1) | p=0.004 | 5.3(1.5-23.2) | p=0.86 | 38(1-5835) | p=0.2 | 16.7 (0.9-2235) | p=0.44 |
| (n=21)               |                     |         |                               |         |            |         |           |         |

P<0.05 is considered significant
difference was found among the studied markers in different age or sex (p>0.05).

Studying the markers in relation to prognostic factors of CRC, showed that in more advanced grades (Grades III and IV), the median level of DNA integrity index and absolute DNA concentration demonstrated a statistically significantly higher median levels than that in grade II (p=0.002 and 0.003 respectively), however neither of the median levels of CA19-9 and CEA did. Patients with distant metastasis showed a statistically significantly higher median DNA integrity index than in non-metastatic patients (p= 0.004). No statistically significantly significant difference was demonstrated in the median levels of the other studied parameters (Table2).

ROC curve analysis was plotted for each of the studied markers to evaluate its diagnostic efficacy in differentiation between the CRC and control groups, DNA integrity yielded the highest AUC (0.90) (Table3) (Figure 1). To differentiate between CRC and patients with benign colonic lesions, each of the studied markers showed high area under curve (AUC) > 0.80 (ranging from 0.83-0.89) (Table3) (Figure 2). Only DNA integrity index was found to be able to differentiate between patients with benign

### Table 3. The Diagnostic Significance for Combined Markers to Diagnose the CRC among the Control and Benign Groups

| Markers cutoff | AUC  | Sensitivity | Specificity | Positive predictive value (PPV) | Negative predictive value (NPV) | Diagnostic accuracy |
|----------------|------|-------------|-------------|---------------------------------|---------------------------------|---------------------|
| DNA integrity (0.41) | 0.9  | 90%         | 85%         | 93.90%                          | 81%                            | 92%                 |
| Absolute DNA concentration (3.3ng/µl) | 0.73 | 68%         | 65%         | 82.90%                          | 44.80%                          | 57.2%               |
| CA19-9 (12.3 U/ml) | 0.82 | 78%         | 75%         | 88.6%                           | 57.70%                          | 78.1%               |
| CEA (2.1ng/ml) | 0.86 | 82%         | 80%         | 91.1%                           | 64%                            | 81.5%               |
| DNA integrity (0.41) and CA19-9 (12.3 U/ml) | 96% | 60%         | 85.7%,     | 85.70%                          | 86%                            |
| DNA integrity (0.41) and CEA (2.1ng/ml) | 98% | 65%         | 87.5%,     | 92.90%                          | 88.60%                          |
| Absolute DNA concentration (3.3ng/µl) and CA19-9 (12.3 U/ml) | 96% | 50%         | 90.60%     | 71.40%                          | 88.30%                          |
| Absolute DNA concentration (3.3ng/µl) and CEA (2.1ng/ml) | 100% | 60%         | 92.60%     | 100%                            | 93.30%                          |

CRC among the control group

| DNA integrity (0.55) | 0.83 | 88%         | 90%         | 97.8%,                          | 60%                            | 88.30%               |
| Absolute DNA concentration (2.35ng/µl) | 0.83 | 84%         | 70%         | 93.30%                          | 46.70%                          | 81.70%               |
| CA19-9 (7.35) U/ml | 0.84 | 88%         | 70%         | 93.6%,                          | 53.80%                          | 85%                 |
| CEA (2ng/ml) | 0.89 | 84%         | 90%         | 97.70%                          | 52.90%                          | 85%                 |
| DNA integrity (0.55) and CA19-9 (7.35 U/ml) | 96% | 60%         | 92.3%,     | 75%                            | 90%                            |
| DNA integrity (0.55) and CEA (2.0ng/ml) | 98% | 80%         | 96%         | 88.90%                          | 92%                            |
| Absolute DNA concentration (2.35ng/µl) and CA19-9 (7.35 U/ml) | 96% | 50%         | 90.60%     | 71.40%                          | 88.30%                          |
| Absolute DNA concentration(2.35ng/µl and CEA (2.0ng/ml) | 100% | 60%         | 92.60%     | 100%                            | 93.30%                          |
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The median level of DNA integrity index was significantly higher in CRC group when compared to the control group (p<0.001). These results were supported by other studies (Umetani et al., 2006; da Silva et al., 2013; Leszinski et al., 2013, Hao et al., 2014). A statistically significant increase in median level of DNA integrity index between CRC group and the benign group (p<0.001) was found, which was also consistent with several studies (da Silva et al., 2013; Hao et al., 2014; Zaher et al., 2014), but was contrasted by one study (Leszinski et al., 2013), which reported a statistically non-significant difference between CRC and benign colonic patients (p=0.14). The current study failed to demonstrate a statistically significant difference in the median level of DNA integrity index between benign and control groups (p=0.2), this finding was opposed earlier by a study demonstrating a statistically significant difference between the benign group and healthy controls at p=0.001 (Mead et al., 2011), while comes in agreement with a more recent one (Zaher et al., 2014). As for absolute DNA concentration, it’s median value was statistically significantly higher in CRC as compared to the control (p=0.004) and the benign groups (p=0.002), close results were previously reported (Mead et al., 2011; Zaher et al., 2014). Nevertheless, this study reported a non-significant difference between the benign and control groups (p=0.6) which was contrasted by an earlier study (Mead et al., 2011), while comes in accordance with another (Zaher et al., 2014). The discrepancy between the results reported by different studies might be contributed to the difference in ethnic and racial groups studied, the differences in methodology and lack of standardization in these methodologies. Several studies use plasma to quantify the circulating cell-free DNA, while other studies use serum as a template. Moreover, some studies performed DNA extraction (Agostini et al., 2011) and measured the levels of circulating cell-free DNA by qPCR, while other studies use serum (Umetani et al., 2006) or plasma (Mead et al., 2011) as a direct template to quantify cell-free DNA.

In the current study, a significant positive correlation existed between DNA integrity index and absolute DNA concentration (P=0.006), which was previously suggested (Umetani et al., 2006). On comparing the DNA integrity index and absolute DNA concentration, with tumor grade, advanced grades (Grades III and IV) showed a statistically significantly higher median values than patients with grade II at p=0.002 and 0.003 respectively. This was opposed by other studies that failed to demonstrate a significant difference in the DNA integrity index (Umetani et al., 2006) and absolute DNA concentration (Zaher et al., 2014) in different tumor grades.

Moreover, patients in the current study with distant metastasis at the time of presentation showed statistically significantly higher median level of DNA integrity index than that in non -metastatic patients at p=0.004, which was opposed earlier (Zaher et al., 2014). No statistically significantly significant difference exists in the median levels of neither DNA integrity index nor absolute DNA concentration in different states of lymph node, sites or types of the tumor (p>0.05) as was previously reported.
ROC curve for DNA integrity index, showed an AUC of 0.90 and at cutoff value of 0.41; it can differentiate CRC from healthy control with a sensitivity of 90% , a specificity of 85% and overall accuracy of 92%. Regarding absolute DNA concentration, ROC curve showed AUC = 0.73 and at cutoff value of 3.3 ng/µl had a sensitivity of 68%, specificity of 65% and diagnostic accuracy of 57.2%. These results were slightly different from a previous study in which ROC curve for DNA integrity index yielded an AUC of 0.78, at a cutoff of 0.22 showed a sensitivity of 56% and specificity of 90%, while for absolute DNA concentration, it showed AUC =0.75, at a cutoff value of 1.73ng/µl, showed a sensitivity of 40% and a specificity of 90% (Umetani et al., 2006).

Regarding CRC and the benign group; ROC curve for DNA integrity index showed an AUC of 0.86 , at cutoff 0.55, sensitivity was 88% , specificity was 90% and diagnostic accuracy was 88.3%. ROC curve for absolute DNA concentration yield an AUC of 0.83, at cutoff 2.35 ng/µl, it had a sensitivity of 84%, specificity of 70% and diagnostic accuracy of 81.7%, which suggested that DNA integrity index was superior to absolute DNA concentration considering the diagnosis of CRC among healthy control or benign subjects that comes in agreement with one study (Mead et al., 2011) and is contrasted by an earlier one concluding that DNA integrity index and absolute DNA concentration were equivalent to each other considering the diagnosis of CRC among healthy subjects (Umetani et al., 2006).

In this study, the currently used markers CA19-9 and CEA have a lower diagnostic value for CRC than both DNA integrity index and absolute DNA concentration, as was previously suggested (Mead et al., 2011).

We concluded that DNA integrity index could be clinically used as a serum biomarker in discriminating colorectal cancer patients from healthy subjects or patients with benign colonic polyp as well as serving as a potential indicator for the assessment of disease progression in CRC patients. Furthermore, Genetic markers (DNA integrity index or absolute DNA concentration) could be a clinically useful surrogate markers in CRC patients in combination with the conventional tumor markers (CEA and CA19-9), which was proved to be better than the use of either of them alone for the diagnosis of CRC. A simple blood draw test could even replace or minimize the use of the invasive colonoscopy, as the preferred mode for screening and diagnosis of CRC.

Acknowledgements

To National Cancer Institute, Cairo University for providing the facilities during the research, including the laboratories, equipment, analyzers and consumables.

References

Agostini M, Pucciarelli M, Vittoria M (2011). Circulating cell-free DNA: a promising marker of pathologic tumor response in rectal cancer patients receiving preoperative chemoradiotherapy. Ann Surg Oncol, 18, 2461-8.
Casadio V, Foca F, Zingaretti C, et al (2013). Gene methylation in rectal cancer: predictive marker of response to chemoradiotherapy. J Cell Physiol, 228, 2343-9.
Chen H, Sun LY, Zheng HQ, et al (2012). Total serum DNA and DNA integrity; diagnostic value in patients with hepatitis B virus-related hepatocellular carcinoma. Pathol, 44, 318-24.
Cordaux R, Batzer MA (2009). The impact of retrotransposons on human genome evolution. Nature Reviews Genetics, 10, 691-703.
da Silva Filho BF, Gurgel AP, Neto MA, et al (2013). Circulating cell-free DNA in serum as a biomarker of colorectal cancer. J Clin Pathol, 66, 775-8.
David A, Lieberman D, Douglas K, et al (2012). Guidelines for colonoscopy surveillance after screening and polypectomy: a consensus update by the US multi-society task force on colorectal cancer. Gastroenterol, 143, 844-85.
Ferlay J, Steliarova-Foucher E, Lorant-Tieulent J, et al (2013). Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer, 49, 1374-403.
Fong SL, Zhang JT, Lim CK, et al (2009). Comparison of 7 methods for extracting cell-free DNA from serum samples of colorectal cancer patients. Clin Chem, 55, 587-9.
Gao YJ, He-YJ, Yang ZL (2010). Increased integrity of circulating cell free DNA in plasma of patients with acute leukemia. Clin Chem Lab Med, 48, 1651-6.
Hanley R, Christ KM, Canes D (2006). DNA integrity assay: a plasma-based screening tool for the detection of prostate cancer. Clin Cancer Res, 12, 4569-74.
Hao TB, Shi W, Shen XJ2, et al (2014). Circulating cell-free DNA in serum as a biomarker for diagnosis and prognostic prediction of colorectal cancer. Br J Cancer, 111, 1482-9.
Labianca R, Nordlinger B, Beretta G, et al (2013). Early colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol, 24, 64-72.
Leszinski G, Lehner J, Geyer U, et al (2013). Increased DNA integrity in colorectal cancer. In Vivo, 3, 299-303.
Luo L, Ting W, Qian H, et al (2014). Changes of TCR repertoire diversity in colorectal cancer after erbitux (cetuximab) in combination with chemotherapy. Am J Cancer Res, 4, 924-33.
Maestranzi S, Przemioslo R, Mitchell H, et al (1998). The effect of benign and malignant liver disease on the tumor markers CA 19-9 and CEA. Ann Clin Biochem, 35, 99-103.
Maio G, Rengucci C, Zoli W, et al (2014). Circulating and stool nucleic acid analysis for colorectal cancer diagnosis. World J Gastroenterol, 20, 957-67.
Mead R, Duku M, Bhandari P, et al (2011). Circulating tumour markers can define patients with normal colons, benign polyps, and cancers. Br J Cancer, 105, 239-45.
Otero E, Chiara L, Rodriguez L, et al (2015). Serum sCD26 for colorectal cancer screening in family-risk individuals: comparison with fecal immunochemical test. British. J Cancer, 112, 375-81.
Pinzani P, Salvianti F, Zaccara S (2011). Circulating cell-free DNA in plasma of melanoma patients: qualitative and quantitative considerations. Clin Chim Acta, 412, 2141-5.
Umetani N, Kim J, Hiramatsu S, et al (2006). Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: direct quantitative PCR for ALU repeats. Clinical Chemistry, 52, 61062-9.
Wang H, Nekrutenko A, Li WH (2000). Densities, length repeats. Nature Reviews Genetics, 35, 81-8.
Wang M, Li Y, Wang Z, et al (2015). The PKA RIα/A-kinase anchoring proteins 10 signaling pathway and the prognosis of colorectal cancer. J Gastroenterol Hepatol, 30, 496-503.
Zaher MN, Mahpar E, Rafiq S, et al (2014). Clinical features and outcome of sporadic colorectal carcinoma in young patients: a cross-sectional analysis from a Developing Country. ISRN Oncol, 2014, 461570.