

Serum cell-free DNA concentration in BALB/c mice with azoxymethane-dextran sodium sulfate-induced colorectal cancer

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ABSTRAK

Latar belakang: Kanker kolorektal merupakan kanker ketiga tertinggi di Amerika Serikat dengan angka mortalitas menempati peringkat kedua tahun 2012. Mortalitas kanker dapat ditekan melalui deteksi dini saat perkembangan kanker pada tahap polip, salah satunya dengan diagnosis biologi molekuler keberadaan DNA pada tinja maupun serum. Penelitian ini dilakukan untuk menganalisis konsentrasi cell-free DNA (cfDNA) sebagai penanda tumor karsinogenesis kolorektal menggunakan serum darah mencit BALB/c yang sebelumnya diinduksi azoxymethane (AOM) dan dipromosi oleh dextran sodium sulfate (DSS).

Metode: Penelitian eksperimental ini menggunakan enam mencit BALB/c. Mencit ini mendapat 4 perlakuan secara serial dalam rentang waktu tertentu. Perlakuan pertama adalah pengambilan sampel serum sebelum induksi-promosi, pengambilan kedua pada minggu ke-1 (1 minggu setelah induksi AOM), pengambilan ketiga minggu ke-2 (1 minggu setelah induksi-promosi oleh AOM-DSS) dan pengambilan ketiga minggu ke-6 (5 minggu setelah induksi-promosi oleh AOM-DSS). Kuantifikasi cfDNA serum dilakukan dengan metode fluoresensi SYBR Green II menggunakan Rotor Gene 6000 dan pemeriksaan histopatologis karsinogenesis dilakukan pada minggu ke-0, ke-1, ke-2 dan ke-6 dilanjutkan analisis statistik dengan uji T berpasangan.

Hasil: Konsentrasi cfDNA menunjukkan perbedaan yang bermakna antara kelompok sampel sebelum induksi dan promosi ($1238,49 \pm 674,84$ pg/ μ L) dibandingkan serum yang diambil minggu ke-6 dengan gambaran histopatologis sebelum kanker ($2244,04 \pm 726,57$ pg/ μ L). Terdapat kenaikan cfDNA pada minggu ke-1 hanya dengan induksi AOM maupun minggu ke-2 setelah diinduksi dan dipromosi ($1358,57 \pm 803,81$ pg/ μ L dan $1317,23 \pm 735,92$ pg/ μ L), namun perbedaan ini tidak bermakna secara statistik.

Kesimpulan: Konsentrasi cell-free DNA pada serum mencit BALB/c 5 minggu setelah induksi dan promosi karsinogenesis menggunakan AOM dan DSS lebih tinggi dibandingkan konsentrasi cell-free DNA sebelum induksi dan promosi.

ABSTRACT

Background: Colorectal cancer is the third most common cancer in the United States with a mortality rate ranked second in 2012. Early diagnosis such as detection of DNA in serum or faeces at the polyp stage, will reduce colorectal cancer mortality. This study was conducted to analyze the concentration of cell-free DNA (cfDNA) as a tumor marker in colorectal carcinogenesis by using blood serum samples from BALB/c mice previously induced by azoxymethane (AOM) and promoted by dextran sodium sulfate (DSS).

Methods: This experimental animal study used 6 BALB/c mice which had serial intervention in a certain time frame. The first serum samples were taken before induction of carcinogenesis (week-0); then AOM induction of carcinogenesis followed and the second sampling one week after AOM intervention (week-1). Subsequently, promotion of carcinogenesis followed with DSS and the third sampling one week after this intervention (week-2). The fourth sampling was 5 weeks after AOM-DSS intervention (week-6). Quantification of the serum cfDNA was performed with SYBR-Green II fluorescence using Rotor Gene 6000 as a reference. Histopathological examination verified induction of carcinogenesis. For statistical analysis paired T-test was used.

Results: Concentration of serum cfDNA showed significant difference between sampling group at week-0 (1238.49 ± 674.84 pg/ μ L) and sampling group at week-6 (2244.04 ± 726.57 pg/ μ L) the latter group showing pre-cancerous histopathology. Slightly increased cfDNA at week-1 with AOM induction (1358.57 ± 803.81 pg/ μ L) and week-2 after DSS promotion (1317.23 ± 735.92 pg/ μ L) were not significantly different from week-0 samples.

Conclusion: The concentration of cfDNA in the serum of BALB/c mice 5 weeks after AOM induction of carcinogenesis and DSS promotion is significantly higher than before induction.

Keywords: azoxymethane, BALB/c mice, cell-free DNA, colorectal cancer, dextran sodium sulfate

pISSN: 0853-1773 • eISSN: 2252-8083 • <http://dx.doi.org/10.13181/mji.v24i1.730> • Med J Indones. 2015;24:3-7

• Received 26 Feb 2014 • Accepted 19 Nov 2014

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Colorectal cancer is the third most common cancer in the United States with 143,460 new cases in 2012. Overall risk of developing this disease during life is 1 : 20, with a higher proportion in men and dominated by certain people with risk factors. Cancer mortality ranks second highest in the United States with 51,690 deaths during 2012.¹ Early screening for the presence of polyps has been shown to reduce mortality of this disease for last 20 years.²

Molecular biology-based methods have never been used as standard colorectal cancer screening with stool deoxyribose nucleic acid (DNA) test in order to detect the presence of DNA in the feces due to the cancer state in which malignant cells often leave DNA in feces.¹ Nowadays molecular biology techniques are often done either by DNA amplification and quantitative polymerase chain reaction (qPCR) of ribose nucleic acid (RNA) which frequently fails due to an increase in serum ribonuclease (RNase) associated to pathological cancer states.³ Cell-free deoxyribose nucleic acid (cfDNA) is more stable than fragile RNA which is one of the reasons for the development of this method.

The success of treatment of colorectal cancer is determined by the cancer stage at the time of its diagnosis and the start of therapy.^{1,4} The earlier diagnosis of colorectal cancer and start of therapy, the better prognosis will be.⁵ Therefore, analysis of molecular biology can be used as an alternative diagnostic method or serum cfDNA may possibly even become the expected gold standard in the diagnosis of colorectal cancer. The aim of this study was to gain knowledge about differences in cfDNA concentration before and after azoxymethane (AOM)-dextran sodium sulphate (DSS) induction.

METHODS

This experimental animal study used 6 BALB/c mice, 2-3 months old, body weight (BW) of 20-25 g, conditioned in 12 hours light and 12 hours dark. The number of samples was based on the Federer formula with a random sampling technique and intervention allocation of test animals in a certain time frame. The study was approved a part of a larger study by the Ethical Committee of FMUI with the serial clearance number 592/PT02.FK/ETIK/2011.

Each sample was allocated to four sampling and intervention groups in a certain time frame during the study. In each group samples were taken for the measurement of serum concentrations of cfDNA. Cancerogenesis was induced by AOM and promoted by DSS 1% with 10 mg/mL/kg BW as single dose for seven days.

The first serum sample was taken before induction of cancerogenesis (week-0); the second sample 1 week after AOM induction (week-1); the third one, 1 week after AOM induction and DSS promotion (week-2) and the fourth one, 5 weeks after induction and promotion (week-6).

For measurement, cfDNA was isolated from serum samples using a four stages salting out method, namely precipitation of proteins, DNA saturation, washing and rehydration. Precipitation of proteins was performed using protein precipitation solution of Wizard Genomic Purification Kit (Promega™) with continuous saturation by isopropanol. Washing was done with a solution of 70% alcohol and DNA isolates were reconfirmed back to the volume at the beginning of sampling by dissolving cfDNA pellets with tris hydroxymethyl aminomethane ethylenediaminetetraacetic acid (Tris-EDTA) solution from the Wizard Genomic Purification Kit (Promega™).

For the quantification of DNA SYBR-green fluorescence (10.000x, Lonza™) method was used with the same principle in qPCR.^{5,6} Values of cfDNA concentration were analyzed by T-test in three paired groups.

RESULTS

After having salted out the proteins DNA was extracted from the serum samples using lysis buffer for the measurement of cfDNA concentrations (Figure 1 and Table 1).

The results of cfDNA concentration measurements over the experimental time course were shown in table 1.

Before intervention mean value was 1238.49 ± 674.84 pg/ μ L. Further analysis was done by comparing the three cfDNA values after receiving intervention with pretreatment values. All intervention groups had higher mean values

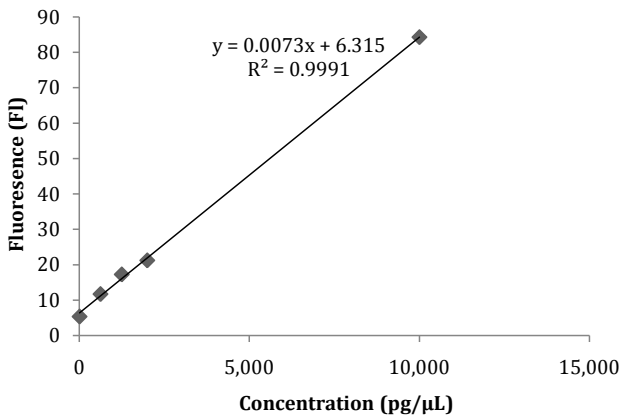


Figure 1. Standard curve of cfDNA

Table 1. Concentration of cell-free DNA in mice

Mice no	Concentration (pg/μL)			
	Week-0	Week-1	Week-2	Week-6
I	364.77	605.15	937.85	1398.10
II	1694.90	577.59	398.74	2345.54
III	377.59	740.41	838.49	2535.28
IV	1566.05	2219.26	2111.56	2518.62
V	1663.49	1785.92	1373.74	1401.31
VI	1764.13	2223.10	2242.97	3265.41
Mean	1238.49	1358.57	1317.23	2244.04

than the pre-intervention group, the maximum mean value of 2244.04 ± 726.57 pg/μL in the week-6 group.

The mean pre-intervention values were always lower than mean values after induction with statistically significant difference between week-0 and week-6 ($p = 0.030$). The means of week-1 and week-2 showed no statistically significant difference compared to week-0 ($p = 0.654$ and $p = 0.809$, respectively).

DISCUSSION

To evaluate methodological sensitivity, quantities of isolated cfDNA must be considered. In this study total amounts of quantitatively extracted DNA were too low to be measured by spectrophotometry. It had been reported that DNA concentration in the sample had to be at least 50 pg/μL in order to obtain linearity in spectrophotometric determinations.⁶ Under these conditions, precision and accuracy

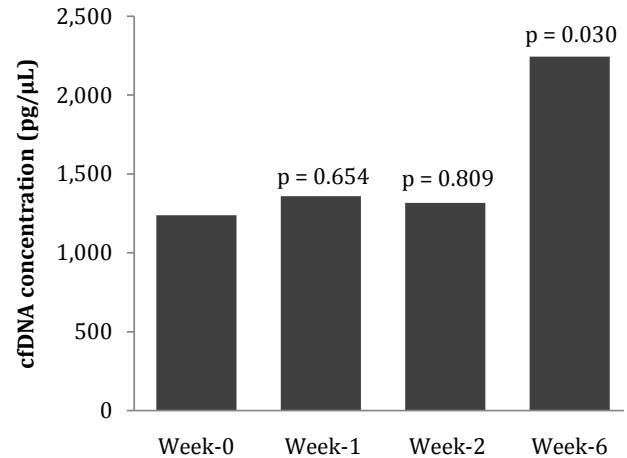


Figure 2. Mean concentration differences of groups (p values indicate the comparison with week-0 period)

of spectrophotometers are insufficient to reach the lowest absorbance value of 0.001 (limit of detection, LoD).⁶

The sensitivity of fluorescence methods especially using SYBR-green is considerably higher than that of spectrophotometry.^{7,8} For double-stranded DNA such as cfDNA we obtained the calibration formula of linearity: $y = 0,0073x + 6.315$. Rotor Gene Q 6000 was used as a reference for fluorescence determination down to 0.01 arbitrary fluorescence units resulting in LoD of 1.37 pg/μL. Hence, SYBR-green fluorescence turned out about 37 times more sensitive than spectrophotometric methods.

We obtained 24 samples with genetic material (DNA) to measure the cfDNA concentration. Fluorescence graph output from Rotor Gene 6000 showed 24 true positive (TP) values and zero (0) false negative (FN) values. From this result, the sensitivity of this method under our experimental conditions can be calculated: $\text{sensitivity} = \text{TP} / (\text{TP} + \text{FN}) = 24 / (24 + 0) = 100\%$. This does not mean “marker sensitivity” in clinical application, which may differ considerably from our experimental conditions.

To obtain specific conditions for measurement, various requirements must be met; it was essential that the isolated DNA was pure without RNA and protein, to rule out falsification of the recorded absorbance due to RNA or protein. On the other hand, SYBR-green is very specific to the nucleic acid double-stranded structure; single-stranded nucleic acids and RNA can not be intercalated. Hence, SYBR-green is capable to detect and

quantify double standard deoxyribonucleic acid (dsDNA) with high specificity.⁶

Specificity of SYBR-green was described by Lu, et al.⁸ They measured 5 groups with known concentrations (mean = 188.5 pg/ μ L) of complementary DNA (cDNA) by SYBR-green fluorescence resulting in 201.19 pg/ μ L. Although SYBR-green is considered very specific to dsDNA, the reason for higher values measured might have been contamination with RNA and/or protein; specificity calculated from these values was 93.7%.⁸

Our mean values before cancer induction were generally lower than after induction, but statistically, only the difference between week-0 and week-6 was significant ($p = 0.030$). The mean values of week-1 and week-2 did not show statistically significant differences compared to week-0 ($p = 0.654$ and $p = 0.809$). Based on the method performed in this study with induction of cancerogenesis by only a single dose of AOM showed a slightly higher mean value in week-1 compared to week-2 group (statistically the same values, $p = 0.5$).

It was proven that cancer occurs due to several simultaneous factors which trigger carcinogenesis through multiple stages.⁹ Colorectal cancer induced by AOM causes mutations in Kras gene that fosters the formation adenoma.^{10,11} The existence of DSS causes inflammation that is promoting delayed colon cancer (DCC) to suppress carcinogenesis through the activation of three pathways, i.e. independent mitogen-activated protein kinase (MAPK) p44/42, c Jun N-Kinases (JNK) and P38 MAPK pathway.^{12,13} The activation of these pathways requires time and eventually leads to malignancy, so in the early post-administration of DSS, increment of cfDNA serum is not meaningful, as shown in the week-2 group.

In healthy individuals, the concentration of circulating DNA is low, since most non-living cells are removed efficiently from circulation by phagocytes. Published studies to date are somewhat unclear in that there is no clear indication whether the serum or the plasma is a better source for circulating DNA to be tested. Studies to date have not yet made it clear whether the method of analysis or the clinical presentation of the patient in terms of cancer

type, tumor location, or tumor stage influence this phenomenon. The methodology of identifying the DNA is primarily the main source of concern. For example, during serum separation, lysis of peripheral blood lymphocytes may cause an artificial increase in DNA integrity.⁷ In addition to viral or virus derived DNA, lymphocytes and cancer cells also proved to be a source of cfDNA in serum (*in vivo*) or in culture medium (*in vitro*). Lymphocytes release their DNA which can be isolated *in vitro* and *in vivo*, although the evidence could not precisely be studied.¹⁴ A basic control level is required in the *in vivo* measurements because blood sampling can trigger cell autolysis affecting the release of DNA. An adequate explanation for these difficulties in the determination can be that the basic autolysis is not enough to release the DNA spontaneously. A report states that freezing and thawing (dilution) repeatedly performed on white blood cells did not cause detectable DNA in the supernatant (non-cellular).¹⁵

The induction of carcinogenesis occurs along with the invisibility of cells undergoing dysplasia and cancer cell infiltration in histopathologic examination (not shown in this article).

At week-0 most normal cells are retained with their intact nucleus structure. These conditions prevent the release of genetic material from the cellular network into circulation. Histopathology of the inflamed colon is different; many cells are not intact, leaky and susceptible to release material including genetic substances. The poly-anion polymeric structure of DSS inhibits RNase activity because it disrupts the interaction between messenger RNA (mRNA) and ribosomes. However, the mechanism of DSS uptake into cells is still not known exactly, e.g. passively or actively through specific receptors. Allegation of complex bonds between DSS and particular polycationic compounds was assumed to facilitate DSS penetration into cells.¹⁶

A week after the AOM-DSS induction of inflammation some cells differ only in the absence of their nuclei (week-2). A review conducted by Raju¹⁷ states that AOM-induced colorectal carcinogenesis in mice occurs in several stages, beginning with the formation of ACF, enhanced ACF and tumor formation. At first, the formation of ACF occurred within 8 weeks after AOM induction,

then entering advanced ACF formation until week-12. Visible tumor formation has eventually completed in 24 weeks after induction with AOM.¹⁷ Results with histopathologic examination of the mice 6 weeks after induction by AOM did not indicate cancer but the stage of pre-cancer. Week-6 with AOM-DSS induction is similar to 12 weeks with AOM induction, only. More rapid progression could be expected because of promotion by DSS the mechanism of which has been described previously.¹⁶ In general, cfDNA concentrations of induced mice were between 1,000 ng/mL and 100,000 ng/mL and exceed the concentrations in control mice without induction. Our cfDNA value of 2,244.04 ng/mL measured at week-6 in induced mice corresponds to this concentration range.

The concentrations of cfDNA in human colorectal cancer patients (quantified by similar fluorescence methods as applied in our study) were higher than in healthy controls with a significance value of $p < 0.001$ (798 ± 409 ng/mL vs 308 ± 256 ng/mL).¹⁸

The application of cfDNA levels in serum as a marker of colon cancer in patients still requires the exact determination of sensitivity and specificity under clinical conditions including diagnostic differentiation versus other cancers.⁷

In conclusion, the concentrations of cfDNA in the serum of BALB/c mice 6 weeks after the induction and promotion of carcinogenesis using AOM and DSS are higher than the concentrations prior to induction.

Acknowledgment

The authors are thankful to Prof. H.-J. Freisleben for his help in writing the manuscript.

Conflict of interest

The authors affirm no conflict of interest in this study.

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