Detection of Malondialdehyde DNA Adducts in Human Colorectal Mucosa: Relationship with Diet and the Presence of Adenomas


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Abstract

Colorectal biopsies from normal mucosa of participants in the United Kingdom Flexible Sigmoidoscopy Trial and European Prospective Investigation on Cancer (EPIC; \( n = 162 \)) were analyzed for the presence of malondialdehyde-deoxyguanosine (M1-dG), a DNA adduct derived from lipid peroxidation. The aim was to investigate whether dietary factors can modulate M1-dG levels and whether M1-dG in normal mucosa is a risk factor for colorectal adenomas. Samples were analyzed using a sensitive immunoslot blot assay. This study has shown for the first time that M1-dG is present in human colorectal tissue. M1-dG levels ranged from undetectable (\( n = 13 \)) to 12.23 per \( 10^7 \) total bases. Mean levels were 4.3 ± 3 and 4.6 ± 2.9 per \( 10^7 \) total bases in men and women, respectively. In men, there were positive associations of adduct levels with height and age, and inverse associations with body mass index. Legumes, fruit, salad, and whole meal bread were inversely associated with M1-dG adducts, whereas consumption of offal, white meat, beer, and alcohol were positively associated with elevated levels. In women, there was an inverse association of the adduct with the ratio of polyunsaturated:saturated fatty acids (\( P = 0.019 \)) and a weak positive correlation with saturated fat (\( P < 0.061 \)). When levels of adducts were compared in individuals with and without adenomas, there was a trend for higher levels in individuals presenting with adenomas especially in the highest category of M1-dG adducts (\( P < 0.005 \)).

Introduction

Cancer (1) is the second most common cause of death from malignant diseases in Western Europe and the United States, both in men and in women. Approximately 94% of CRC is sporadic in nature (1), and it is estimated that 75–80% might be attributable to environmental causes (2, 3). CRC develops through a multistep sequence of dysplastic morphological change, most commonly including an adenoma, characterized by an accumulation of genetic defects (4, 5). The risk of developing cancer rises with the number and size of adenomas and villous histology.

Diet is regarded as the most important environmental influence on CRC (6, 7). Increased risk of CRC has been associated with high intake of red meat and total fat (6–9). High consumption of vegetables and fruit has been shown to reduce the risk (reviewed in Refs. 6 and 7). In addition, Lee et al. (10) and Slattery et al. (11) reported that individuals who maintained high levels of physical activity throughout their lives were at a lower risk for developing colon cancer.

Dietary fat, lipid peroxidation, and arachidonic acid metabolism have all been implicated in colorectal carcinogenesis (9, 12, 13). Lipid peroxidation is initiated by free-radical attack of membrane lipids, generating large amounts of reactive products, which have been implicated in tumor initiation and promotion. Because modification of DNA is believed to be an important early step in carcinogenesis, endogenous DNA adducts derived from oxidative stress, lipid peroxidation, or other endogenous processes have been proposed as contributors to the etiology of human cancer (14).

MDA is a major genotoxic carbonyl compound generated by lipid peroxidation (14, 15). It is also a by-product of the arachidonic acid metabolism in the synthesis of prostaglandins (16). Both of these endogenous processes are modulated by dietary factors. For example, lipid peroxidation is stimulated by the presence of high levels of n-6 PUFAs and is inhibited by dietary antioxidants. Increased levels of MDA, together with increased levels of PUFAs and prostaglandins, e.g., PGE\(_2\), have been reported in tumor tissues of CRC patients as compared with their normal mucosa (13).

MDA is mutagenic in bacterial and mammalian systems (17). It reacts with DNA to form adducts with deoxyguanosine, deoxyadenosine and deoxyctydine (18). The adduct formed on reaction with deoxyguanosine, the highly fluorescent cyclic pyrimidopurinone, M1-dG, was originally detected in liver.

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1 CRC, colorectal cancer; MDA, malondialdehyde; PUFAs, polyunsaturated fatty acids; M1-dG, 1,3-di(malondialdehyde-deoxyguanosine; EPIC, European Prospective Investigation on Cancer; CT-DNA, calf thymus DNA; HPLC, high-pressure liquid chromatography; BMI, body mass index; ISB, immunoslot blot; PTM, PBS-Tween 20 + fat-free milk powder.

4 The abbreviations used are: CRC, colorectal cancer; MDA, malondialdehyde; PUFAs, polyunsaturated fatty acids; M1-dG, 1,3-di(malondialdehyde-deoxyguanosine; EPIC, European Prospective Investigation on Cancer; CT-DNA, calf thymus DNA; HPLC, high-pressure liquid chromatography; BMI, body mass index; ISB, immunoslot blot; PTM, PBS-Tween 20 + fat-free milk powder.
DNA from healthy individuals, at levels of 5–11 adducts per $10^7$ total bases (19). Since then, M$_1$-dG has been detected in several human tissues at levels ranging from 0.02 to 21 per $10^7$ normal bases (20–24). Very interestingly, it has been shown that M$_1$-dG can also be formed after direct oxidative attack on DNA (25). M$_1$-dG is a premutagenic lesion and can induce guanine-to-thymine transversions and guanine-to-adenine transitions in DNA (26, 27). The adduct is repaired by bacterial and mammalian nucleotide excision repair and by bacterial mismatch repair pathways (15).

Fang et al. (28) reported that volunteers on a diet containing high amounts of PUFAs had higher levels of M$_1$-dG in leukocyte DNA than individuals on a diet rich in monounsaturated fatty acids. The difference between adduct levels after the two diets was greater in women than in men. These results suggested a role of diet in modulating adduct levels. Results from our group have also suggested a dietary influence on M$_1$-dG levels in human leukocyte DNA (29).

The United Kingdom Flexible Sigmoidoscopy Screening Trial is a multicenter randomized controlled trial designed to investigate the efficacy and effectiveness of a single flexible sigmoidoscopy, with removal of all polyps observed during screening, in decreasing morbidity and mortality from CRC (30). The Norfolk branch of the study is unique because one-third of the 3000 participants also independently participated in the EPIC study. As part of EPIC, dietary, health, and lifestyle (31) information on diet, smoking status, weight, height, and BMI —[weight (kg)/height$^2$ (m$^2$)]— was obtained at the initial EPIC health check. Patients. Ethical approval for this study was obtained from the Norwich District Ethics Committee in 1997. Patients (2999) from general practices were screened in Norfolk as part of the EPIC study. Among the 3000 participants also independently participated in the EPIC study. As part of EPIC, dietary, health, and lifestyle (31) information on diet, smoking status, weight, height, and BMI —[weight (kg)/height$^2$ (m$^2$)]— was obtained at the initial EPIC health check.

Materials and Methods

Materials. CT-DNA, proteinase K, RNase A (from bovine pancreas), RNase T1, and propidium iodide were purchased from Sigma Chemical Co. Ltd. (Dorset, United Kingdom). PBS tablets (Dulbecco A) were purchased from Oxoid Ltd. (Hampshire, England). All other reagents and solvents of analytical or HPLC grade were obtained from either BDH or Fisher Scientific Ltd. (Loughborough, Leicestershire, United Kingdom).

Human Study

Patients. Ethical approval for this study was obtained from the Norwich District Ethics Committee in 1997. Patients (2999) from general practices were screened in Norfolk as part of the United Kingdom Flexible Sigmoidoscopy Study. Among the EPIC patients, at the first health check from 1992–1997, 144 were found to have one or more polyps (adenomatous or metaplastic polyps) and to have completed a detailed diary for 7 days of all food and drink consumed (32). Food diaries from 144 controls, age-, sex-, and general practitioner-matched, were also available. The food diaries were coded and analyzed for nutrients and foods using methods described elsewhere (33). Only patients with histologically proven adenomas (83 cases) and polyp-free controls (79 controls) were included in the study. Information on diet, smoking status, weight, height, and BMI —[weight (kg)/height$^2$ (m$^2$)]— was obtained at the initial EPIC health check.

Biopsies and Polyp Identification. During flexible sigmoidoscopy screening, colorectal biopsies were taken from the normal mucosa of the posterior wall of the rectum, 3 cm above the dentate line. When polyps were present, they were removed at the same time. Biopsies were transferred to cryovials, immersed in liquid nitrogen immediately and stored at −80°C. Patients with multiple or large (>1-cm) adenomas or with >20% villous histology were considered high risk.

DNA Extraction. Biopsies were defrosted and washed in PBS twice to minimize bacterial contamination.

For DNA extraction, biopsies were homogenized using glass mortars and PTFE pestles (Polytron or Fisher). DNA was extracted using Qiagen genomic DNA extraction kit (Qiagen Ltd., Crawley, Sussex, United Kingdom) and was digested using proteinase K (160 units), RNase A (400 units), and RNase T1 (400 units). DNA was dissolved in ultrapure water. DNA purity was assessed by 260/280 nm ratio using a GeneQuant II RNA/DNA calculator (Pharmacia Biotech) and by reverse-phase-HPLC after digestion to deoxynucleotides (34).

ISB

Standard MDA-modified CT-DNA was prepared as described previously (34). The amount of M$_1$-dG was measured by HPLC-fluorescence using a calibration curve obtained with synthetic M$_1$-dG (35).

The ISB assay was performed as described previously (34), with several modifications. MDA-modified CT-DNA was diluted with control CT-DNA (both at a concentration of 0.1 μg/μl) to obtain decreasing amounts (5 fmol to 0.2 fmol) of M$_1$-dG adduct and to generate standard curves for the ISB. Standard CT-DNA and colorectal DNA (3.5 μg) were dissolved in 10 mM potassium hydrogen P$_i$, (pH 7; 100 mM) and PBS (150 μl). DNA samples were sonicated for 20 min in a water bath sonicator, heat-denatured for 5 min in a boiling water bath, cooled on ice for 10 min and mixed with an equal volume of 2 mM ammonium acetate. Resulting single-stranded DNA (143 μl containing 1 μg DNA/sample, in triplicate) was loaded onto nitrocellulose (NC) filters (0.1 μm, BA79; Schleicher & Schuell, Dassel, Germany) using a Minifold II, 72-well slot blot microfiltration apparatus (Schleicher & Schuell). The slots were rinsed with 200 μl of 1 mM ammonium acetate. The filters were subsequently removed from the support and baked at 80°C for 90 min to immobilize the DNA. Filters were then bathed in 100 ml of PTM [PBS-Tween 20 (0.1% Tween) + 0.5% fat-free milk powder] for 1 h at room temperature to inhibit unspecific antibody binding. After two 5-min washes with PBS-Tween 20 (0.1% Tween), the filters were bathed in 40 ml of PTM containing the anti-M$_1$-dG monoclonal antibody D10A1 (36), diluted 1:48,000. Filters were incubated for 2 h at room temperature, followed by overnight incubation at 4°C. After one 1-min and two 5-min washes with PBS-Tween, the filters were incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibody (goat antimouse IgG: Dako A/S, Glostrup, Denmark), diluted 1:4,000 in 32 ml of PTM. Additional washes in PBS-Tween, the filters were incubated for 5 min with the chemiluminescent reagent consisting of 4 ml of luminol/enhancer solution plus 4 ml of stable peroxide buffer (SuperSignal; West Dura, Pierce, Rockford, IL). An image of the filters was acquired using a Fluor-S Multimager (Bio-Rad, Hercules, CA) and the following setting: filter: chemiluminescence; integration: manual (time ranging from 5 to 20 min depending on the intensity of the signal); light source: Chemi; no light; scan width: 80 mm; high sensitivity. The intensity of chemiluminescent signal for each band was determined using the image analysis software. Adduct level in each sample was
determined from the calibration line generated by MDA-modified DNA (diluted with control DNA) containing known amounts of M₁-dG.

**Propidium Iodide Staining for Quantitation of DNA Bound to the Nitrocellulose Filter**

The nitrocellulose filters were washed overnight in PBS and incubated with propidium iodide (250 μg) in 50 ml of PBS for 3 h at room temperature and in the dark. The filters were then washed with PBS for 90 min. An image of the filter was captured using the Bio-Rad Fluor-S Multimager with the following settings: filter: 520 nm; integration: automatic; light source: Epi UV light; high resolution. The intensity of fluorescent signal for each band, which was proportional to the amount of DNA bound to the filter (data not shown), was determined using the image analysis software. The level of adducts in each sample was corrected for the amount of DNA bound to the filter (37). M₁-dG levels are given as adducts per 10⁷ total normal bases.

**Statistical Methods**

Results for DNA adducts were subjected to Kruskal-Wallis ANOVA followed by Mann-Whitney test. The difference between distributions of cases and controls was evaluated using the χ² test and Fisher’s exact test. Minitab (for Windows) was used in the analysis. Associations of food and nutrients with modified DNA (diluted with control DNA) containing known amounts of M₁-dG.

**DNA Adducts.** DNA samples were analyzed using a previously developed ISB assay, which has a limit of detection of ~0.2 adducts per 10⁷ total bases (34). This assay, which uses a specific monoclonal antibody against M₁-dG (36), requires only 1 μg of DNA/sample (in triplicate). DNA extraction from colorectal biopsies using a Qiagen kit, as described in “Materials and Methods,” gave a yield that varied between 5 to 200 μg of DNA. Enough DNA for ISB analysis was therefore obtained from all biopsy samples. In the present work, adduct levels measured by ISB in each sample, were corrected for the amount of DNA bound to the filter, as determined by propidium iodide staining.

M₁-dG adducts were detected in 92% of the samples analyzed. Thirteen samples had adduct levels below the limit of detection of the assay.

Substantial interindividual variation was observed, with adduct levels ranging from undetectable to 12.23 M₁-dG per 10⁷ total bases (Fig. 1). The mean adduct levels were 4.30 ± 3.0 and 4.60 ± 2.9 per 10⁷ bases in men and women, respectively.

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>57.7 ± 3.4</th>
<th>56.7 ± 2.9</th>
<th>57.4 ± 3.1</th>
<th>58.4 ± 3.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td>169.8 ± 5.2</td>
<td>175.3 ± 6.4</td>
<td>171.7 ± 8.8</td>
<td>175.0 ± 6.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>73.2 ± 10.7</td>
<td>78.4 ± 14.0</td>
<td>76.5 ± 9.2</td>
<td>78.4 ± 10.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.3 ± 3.9</td>
<td>29.8 ± 4.1</td>
<td>26.3 ± 3.8</td>
<td>29.9 ± 4.1</td>
</tr>
</tbody>
</table>

| M₁-dG per 10⁷ bases (range) | 4.11 ± 1.64 | 4.13 ± 2.8 | 3.41 ± 1.64 | 4.52 ± 3.2 |

*Values are means ± SD. Pearson’s correlation test was used in the analyses.*
Diet and Polyps Modulate M1-dG Levels in Human Colorectum

failed to reach significance, although there was a weak positive correlation with antioxidant vitamins. All of the associations with adduct levels, except tomatoes, were not significant (data not shown). There were significant associations between adduct levels and beer, offal, white meat, and whole meal bread. Cereals, fruit, vegetables, salads, and raw nuts showed inverse association with adduct levels, but these were not significant (data not shown). There were significant associations between adduct levels and age, but these were not significant (Fig. 1). In men, M1-dG levels were also significantly correlated with BMI (P = 0.061). No correlation with smoking status was observed either in men or in women.

DNA Adducts and Diet. Information from the 7-day dietary diaries was computerized and converted into estimates of intake for a series of more than 30 food items and nutrients. Significant associations between various food components and adduct levels were observed, with marked differences between men and women (Table 2).

In men, M1-dG levels were inversely associated (P ≤ 0.05) with the reported consumption of legumes, nuts, and white meat. Cereals, fruit, vegetables, salads, and raw tomatoes showed inverse association with adduct levels, but these were not significant (data not shown). There were significant correlations with adduct levels and beer, offal, white meat, and alcoholic drinks.

In women, there were no significant associations between adduct levels and food consumption. Intakes of nutrients were also related to adduct levels. Table 3 shows associations for those nutrients that would be expected to relate to MDA adducts, e.g., fat, fatty acids, and antioxidant vitamins. All of the associations with adduct levels failed to reach significance, although there was a weak positive association with saturated fatty acids in women (P = 0.061). The ratio of monounsaturated:saturated fatty acids was inversely associated with M1-dG levels in women, although the correlation was only slightly significant (P = 0.09). In addition, the ratio of polyunsaturated:saturated fatty acids was inversely associated with adduct levels in women (P = 0.019). No association between fatty acids and M1-dG was observed in men.

DNA Adducts and Adenomatous Polyps. Table 1 also shows mean adduct levels in participants found to have adenomas, compared with adenoma-free individuals. Mean differences were not significant, although Fig. 1 showed that cases tended to have higher levels. Twelve individuals with polyps had adducts levels above 9 per 10^7 bases, compared with 1 polyp-free individual. The difference in adduct levels between the distributions (Fig. 1) in cases and controls failed to reach statistical significance (P < 0.065). However, Fisher’s exact test, not taking into account that the analysis was data driven, showed that there was a large excess of cases (12) compared with controls (1) in this category (P < 0.005). Five of the 12 samples with M1-dG levels above 9 per 10^7 bases were from patients presenting with high-risk adenomas.

In females, a trend in relation to the severity of the adenomas was observed (Fig. 2). M1-dG levels (per 10^7 total bases) increased from 4.06 ± 2.0 in controls to 4.76 ± 3.6 in low-risk and to 5.40 ± 2.6 in high-risk women. The increase from controls to high-risk women was marginally significant (P = 0.09) by Kruskal-Wallis ANOVA followed by Mann-Whitney U test. There were no differences in men.

Discussion

Colorectal biopsies from normal mucosa of 162 participants in both the United Kingdom Flexiscope Sigmoidoscopy Screening Trial and the EPIC study were analyzed for the presence of M1-dG, the major DNA adduct formed by reaction of MDA with DNA. The objectives of the study were to investigate whether this adduct can be regarded as a biomarker of specific dietary intake and whether it can be considered a risk factor for the presence of premalignant lesions in humans.

Samples were analyzed using a quick and sensitive ISB assay, which allows a high throughput of samples in large population-based studies, using only small amounts of DNA.

The present study has shown for the first time that M1-dG is present in the normal colorectal mucosa in humans. The levels measured had an average value, considering all participants, of 4.42 ± 2.95 per 10^7 total bases. These levels are lower than those detected in our laboratory in human gastric biopsy DNA (23). It is likely that this difference in M1-dG levels is attributable to tissue-specific differences in adduct formation and removal.

In the present study, there was substantial interindividual variation in adduct levels. Levels in women were slightly, but not significantly, higher than in men. Fang et al. (28) reported higher levels of adducts in WBCs of women as compared with men. Similar findings were reported in the same leukocyte samples for etheno adducts, another product of lipid peroxidation (38). Similar to our findings for colorectal biopsies, no sex differences in M1-dG levels were reported for gastric tissue (23).

In general, associations between adduct levels and anthro-
M₁-dG levels in normal colorectal mucosa were modulated by age in men but not in women. No relationship with smoking mass is positively correlated with CRC risk. No correlation with protection against colon and rectal cancer. Antioxidants present in fruits and vegetables could decrease formation of M₁-dG by inhibiting either lipid peroxidation or direct DNA oxidation, which are believed to give rise to M₁-dG through the formation of base propenal intermediates. Alternatively, micronutrients and bioactive compounds, such as phenols and flavonoids, could stimulate detoxification pathways and DNA repair mechanisms.

Table 3  Associations of selected nutrient intake with M₁-dG levels

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>All (Males)</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fat</td>
<td>0.1224</td>
<td>0.1131</td>
</tr>
<tr>
<td>Monounsaturated fat</td>
<td>0.0473</td>
<td>0.0096</td>
</tr>
<tr>
<td>Polyunsaturated fat</td>
<td>-0.0869</td>
<td>-0.0828</td>
</tr>
<tr>
<td>M:P ratio</td>
<td>-0.1173</td>
<td>-0.0711</td>
</tr>
<tr>
<td>P:S ratio</td>
<td>0.1436 (P &lt; 0.07)</td>
<td>-0.0438</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>-0.0364</td>
<td>-0.1542</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>-0.0954</td>
<td>-0.1387</td>
</tr>
<tr>
<td>Alcohol</td>
<td>0.1117</td>
<td>0.1629</td>
</tr>
<tr>
<td>Fiber</td>
<td>-0.1126</td>
<td>-0.1703</td>
</tr>
<tr>
<td>Fat</td>
<td>-0.0048</td>
<td>-0.0527</td>
</tr>
<tr>
<td>Iron</td>
<td>0.0405</td>
<td>0.0308</td>
</tr>
<tr>
<td>Carotene equivalents</td>
<td>-0.0755</td>
<td>-0.0672</td>
</tr>
</tbody>
</table>

* a: Pearson’s correlation test was used in the analyses.

Fig. 2  M₁-dG levels in low-risk (LR) adenomas, high-risk (HR) adenomas, and controls (C) for all of the participants, men and women. Values are means ± SD.

The present results could be explained by the fact that different tissues have been analyzed, compared with a Fang et al. study (colorectal mucosa versus leukocytes), and that there was no differentiation between ω-6 and ω-3 fatty acid intake. However, because of the complexity of the dietary data collected in EPIC and only limited food composition data on specific fatty acids in the United Kingdom food tables, we are not yet able to accurately differentiate between ω-6 PUFAs and ω-3 PUFAs. Whereas ω-6 PUFAs have been shown to enhance colon tumorigenesis in rodents, ω-3 PUFAs can act as growth inhibitors of initiated cancer cells.
PUFAs could, therefore, have had a confounding effect in the present analysis. However, no associations with fatty fish consumption (the major source of ω-3 fatty acids) were shown.

The other objective of our study was to analyze M1-dG levels in relation to the presence or absence of adenomatous polyposis, to examine whether adducts could be related to disease outcome. A trend toward higher levels of adducts in cases than in controls was observed for both men and women, although the difference failed to reach significance. The difference in adduct levels between women presenting with high risk adenomas and polyp-free controls was statistically significant, although only marginally (Fig. 2). This is probably attributable to the wide interindividual variation in adduct levels and consequent high SDs from the adduct means. In addition, the number of women in the high-risk group was quite small (n = 11).

At present, the relationship between MDA-DNA damage and the risk for adenomatous polypos remains unclear. What is probably very important is the location of M1-dG in DNA, i.e., which genes are modified, and the rate to which the damage is converted into mutations. DNA repair rate and fidelity are also extremely important. Moreover, other endogenous as well as exogenous DNA damage has been reported in human colorectal tissue (44–47). A previous study (48) has demonstrated increased levels of bulky and/or aromatic DNA adducts associated with increased CRC risk. It is likely that the contribution of DNA damage derived by both exogenous and endogenous sources, together with host susceptibility factors such as polymorphisms in relevant genes (49), could be of importance in colorectal carcinogenesis.

In conclusion, M1-dG was detected in the normal colorectal mucosa of participants in the United Kingdom Flexiscope Sigmoidoscopy Screening Trial and the EPIC study. Food items that, in epidemiological studies, were associated with modulation of CRC risk, modulated M1-dG levels in men. Saturated fat was correlated to increased adduct levels in women. Cases showed higher adduct levels than did controls, although the difference was not statistically significant. Results from the present study raise the possibility that M1-dG plays a role in human colorectal carcinogenesis, certainly in combination with other genetic and environmental factors.

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References


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