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Inherited cancer predisposition sensitizes colonic mucosa to address Western diet effects and putative cancer-predisposing changes on mouse proteome☆,☆☆

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Abstract

Human epidemiological evidence and previous studies on mice have shown that Western-style diet (WD) may predispose gut mucosa to colorectal cancer (CRC). The mechanisms that mediate the effects of diet on tumorigenesis are largely unknown. To address putative cancer-predisposing events available for early detection, we quantitatively analyzed the proteome of histologically normal colon of a wild-type (*Mlh1*$^{+/+}$) and an *Mlh1*$^{+/-}$ mouse after a long-term feeding experiment with WD and AIN-93G control diet. The *Mlh1*$^{+/-}$ mouse carries susceptibility to colon cancer analogous to a human CRC syndrome (Lynch syndrome). Remarkably, WD induced expression changes reflecting metabolic disturbances especially in the cancer-predisposed colon, while similar changes were not significant in the wild-type proteome. Overall, the detected changes constitute a complex interaction network of proteins involved in ATP synthesis coupled proton transport, oxidoreduction coenzyme and nicotinamide nucleotide metabolic processes, important in cell protection against reactive oxygen species toxicity. Of these proteins, selenium binding protein 1 and galectin-4, which directly interact with MutL homolog 1, are underlined in neoplastic processes, suggesting that sensitivity to WD is increased by an *Mlh1* mutation. The significance of WD on CRC risk is highlighted by the fact that five out of six mice with neoplasias were fed with WD.
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Keywords: 2D DIGE; Colorectal cancer; Lynch syndrome; MLH1; Proteomics; Western diet

1. Introduction

The interactions between genetic and dietary factors are suggested to play a critical role in the etiology of colorectal cancer (CRC) [1–4], the second most common cause of cancer-related deaths in Western countries [5]. Dietary habits of Western populations in particular are recognized as one of its main risk factors. Already in the early 1980s, it was reported that the increased incidence of CRC correlates with the consumption of a typical Western-style diet (WD) that contains high total energy, saturated fats of animal origin and low levels of fiber [6]. Subsequently, low intake of vitamin D, calcium and folic acid was associated with CRC risk [3], and finally, in 2007, the risk of WD was acknowledged by The World Cancer Research Fund and The American Institute for Cancer Research (http://www.dietandcancerreport.org).

Lynch syndrome (LS; previously referred to as hereditary nonpolyposis colorectal cancer) is the most common colon cancer syndrome, accounting for 2%–4% of the whole CRC burden [7]. LS is associated with malfunction of the mismatch repair (MMR) mechanism, and the susceptibility is inherited in an autosomal dominant manner while both alleles are generally inactivated in tumor cells [8]. Although the lifetime risk for CRC in MMR gene mutation carriers can be as high as 80%, disease phenotypes such as age of onset and tumor spectrum vary considerably between mutation carriers [9], suggesting that environmental factors are involved in carcinogenesis also in the CRC syndrome.

Many epidemiological studies on how nutrients affect human diseases such as colon cancer have already been accomplished. However, studies on diet effects are quite infeasible in humans due to

Abbreviations: AIN, AIN-93G purified diet for laboratory rodents; CRC, colorectal cancer; 2D DIGE, two-dimensional differential gel electrophoresis; FAO, fatty acid oxidation; GO, Gene Ontology; LS, Lynch syndrome; MLH1, MutL homolog 1; MMR, mismatch repair; PPP, pentose phosphate pathway; WD, Western-style diet; WD*, WD used in the study.

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a long life span and a high variability in individual dietary habits and genetic background and thus call for use of isogenic model organisms. Indeed, WD has been shown to increase the amount of intestinal neoplasms in Apc^{Min} mouse, the most commonly used rodent model for human intestinal cancer [4]. Moreover, in a long-term feeding experiment, WD induced benign and malignant colonic neoplasms also in wild-type mice without inherited cancer predisposition or carcinogenic treatments [10,11], suggesting that, with increasing age, WD itself is a considerable risk factor.

Since diet does not necessarily generate mutations to DNA sequence as carcinogenic substances but most probably induces expression changes in key regulatory genes affecting normal metabolic processes in a cell, here, by a long-term feeding experiment, we address the expression changes occurring in a proteome of histologically normal colon mucosa. Using a B6.129-$Mlh1^{tm1Rak}$ mouse model, the study allows us to distinguish changes related exclusively to aging from age-related changes induced or accelerated by diet. We further reason that, by using the heterozygote $Mlh1^{+/-}$ mouse, a model for a human LS mutation carrier requiring just a second hit for malignant transformation, it might be possible to discover the most initiative changes occurring prior to the second hit in *Mlh1*and distinguish these from changes occurring later in oncogenesis. Moreover, this is the first study of dietary effects on this mouse strain [12]. During the feeding experiment (from 5weeks until 12months of age), we used our own modification of Western-style diet (WD*) with fat content notably similar to that consumed widely by Western populations and AIN-93G (AIN) as a control diet. In WD*, the amount of fat was increased, while the amounts of folic acid, calcium, vitamin D and fiber were decreased, compared to AIN. Furthermore, fat was mainly milk fat, while in previous Western diet experiments done by Newmark *et al.*, fat came from vegetables [10,11].

In the present study, the proteomic quantitative analysis of proximal colon, the predominant site of cancer formation in LS [13], revealed several significant expression differences between mice fed with WD* and AIN, and the identified proteins mostly point to metabolic disturbances and increased toxicity in the cells. Remarkably, the statistically significant expression changes caused by WD* consumption occurred predominantly in mutation carriers ($Mlh1^{+/-}$) and prior to the second hit in *Mlh1*.

2. Materials and methods

2.1. Mice and diets

Heterozygous B6.129-$Mlh1^{tm1Rak}$ mice ($Mlh1^{+/-}$) (strain 01XA2) [14] were obtained from NCI-MMHCC; National Institutes of Health, Mouse Repository, NCI-Frederick, MD, USA. In B6.129-$Mlh1^{tm1Rak}$ mice, exon 2 is missing in one of the two *Mlh1* alleles, leading to nonfunctional MutL homolog 1 (MLH1) protein. $Mlh1^{+/-}$ mice have been shown to have an increased morbidity compared to their wild-type littermates, and approximately one third develop tumors such as lymphomas as well as tumors of the small and large intestine and a number of other organs during their lifespan [15]. Mice were bred and treated according to the study protocol approved by the National Animal Experiment Board in Finland (ESLH-2008-06502/Ym-23).

At the age of 5weeks (time point 0, tp0), $Mlh1^{+/-}$ and $Mlh1^{+/+}$ mice were randomly divided into two dietary groups fed with AIN control diet [16] or WD* (Harlan Teklad, Madison, WI, USA) (detailed description of diets and their nutritional compositions are in Table 1). AIN-93G control diet is a semisynthetic diet designed to meet the nutritional requirements of growing rodents, while WD* is a modified AIN diet, which contains high dietary fat (39% of total calories) and reduced contents of fiber, calcium, vitamin D and three methyl-transfer donors (i.e., folic acid, methionine, and choline). WD* contains more sucrose and correspondingly less complex carbohydrates than the control diet.

2.2. Sample preparation and proteomics

Mice were sacrificed and sampled at tp0 ($Mlh1^{+/-}$ n=8, $Mlh1^{+/+}$ n=7) and at tp1 [12months of age, which corresponds to the mean age of onset in LS (~45years)] ($Mlh1^{+/+}$ AIN, n=8; $Mlh1^{+/-}$ AIN, n=6; $Mlh1^{+/+W}$D*, n=8; $Mlh1^{+/-}$ WD*, n=7). Histological studies were carried out at The Finnish Centre for Laboratory Animal Pathology, University of Helsinki, Finland.

For protein and mRNA studies, the colonic mucosa (6×4mm) was separated from the underlying submucosa and musculature under a dissecting microscope. Samples for protein extractions were rinsed with solution of 10mM Tris (Sigma-Aldrich, Finland) and 5mM magnesium acetate (Sigma-Aldrich, Germany) (pH8.5), snap frozen and stored at −80°C. Samples for RNA extraction were stored in RNAlater (Qiagen, Valencia, CA, USA) at −80°C.

Table 1
Composition of the diets

Compounds	AIN-93G (g/kg)[a]	WD* (g/kg)[a]
Casein	200	232
L-Cysteine	3	3
Corn starch	397,486	305.63
Maltodextrin	132	95
Sucrose	100	116
Soybean oil	70	–
Anhydrous milk fat	–	**132.8**
Canola oil	–	**55.4**
Sunflower oil	–	**11.8**
Cellulose	50	20
Calcium	5	0.5
Folic acid (mg/kg)	2	0.2
Vitamin D (IU/kg)	1000	100
kcal from proteins (%)	18.8	18.5
kcal from carbohydrates (%)	63.9	42.3
kcal from fat (%)	17.2	39.2

Fat sources that differ in WD* from previously published Western diet in Newmark studies [10,11] are in bold.
[a] If not stated differently.

Proteomic changes were studied using the two-dimensional differential gel electrophoresis (2D DIGE) method for which the reagents (if not stated otherwise) and the equipment were provided by GE Healthcare (Sweden). Colonic mucosa samples were mechanically homogenized in the 2D Protein Extraction Buffer-VI with addition of pH8.5 Tris (30mM) (Sigma-Aldrich) and Protease Inhibitor Mix (1/100) (10–15μl lysis buffer/mg tissue). After homogenization, samples were vigorously shaken for 20min at 4°C followed by centrifugation at 15,000*g* for 20min. Supernatant was collected, snap frozen and stored at −80°C. 2D Quant Kit was used to determine the protein concentrations of total extracts according to the manufacturer's instructions.

For 2D DIGE, half of the samples from each mouse group were labeled with 1-(5-carboxypentyl)-1′-propylindocarbocyanine halide *N*-hydrosuccinimidyl ester (Cy3) and the other four with 1-(5-carboxypentyl)-1′-methylindodicarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy5) (400 pmol of dye for 50μg of sample) according to the manufacturer's instructions. An internal standard was created by pooling 25μg of each protein sample and labeled with 3-(4-carboxymethyl) phenylmethyl-3′-ethyloxacarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy2).

Isoelectric focusing was performed using 24-cm-long immobilized pH3–11 nonlinear gradient (IPG) strips with the Ettan IPGphor II unit. The IPG strips were rehydrated for 8h in IPG box using DeStreak reagent and IPG Buffer, pH3–11 NL. An equal amount (50μg) of internal standard labeled with Cy2 combined with Cy3 and Cy5 labeled samples was introduced actively into the IPG strips using cup-loading method according to the manufacturer's recommendations. Conditions of isoelectric focusing were as follows: 21°C, 75mA/strip, step1: step and hold: 150V, 3h; step and hold: 300V, 3h; gradient: 1000V, 6h; gradient: 8000V, 1h 15min; step and hold: 8000V, 3h 45min. Isoelectric focusing was followed by equilibration of IPG strips in two steps with 1% dithiothreitol and 2.5% iodoacetamide, dissolved in equilibration buffer containing 6M urea (Sigma-Aldrich), 2% sodium dodecyl sulfate (SDS) (Sigma-Aldrich), 50mM Tris pH8.8 (Sigma-Aldrich), 0.02% bromophenol blue (Sigma-Aldrich) and 30% glycerol (Sigma-Aldrich), respectively. Separation of proteins in second dimension was carried out by transferring the equilibrated IPG strips to large 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels and run in homemade Laemmli buffer with constant power of 15W per gel until bromophenol blue front reached the lower edge of the gel.

Gels were scanned immediately after the run with Typhoon 9400 scanner (GE Healthcare). Cy2, Cy3 and Cy5 images were acquired with excitation/emission values of 480/530nm, 520/590nm and 620/680nm, respectively, and maximum values between different channels were optimized to differ by less than 20%–30%. Gel images were analyzed using the software DeCyder 2D 7.0 (GE Healthcare).

2.3. Protein identification

The scanned gels were silver stained using the PlusOne Silver staining kit (GE Healthcare). Selected protein spots were excised and proteins in-gel digested. Gel pieces were treated twice with acetonitrile (Sigma-Aldrich) (200μl) and vacuum centrifuged until dry. Proteins were trypsinized (Trypsin gold; Promega, Sweden), and peptides were desalted using ZipTip (Millipore, Germany), eluted and mixed with α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) on a matrix-assisted laser desorption ionization (MALDI) plate. MALDI-mass spectrometry (MS) and MALDI-MS/MS

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