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Diet, cancer and aging in DNA mismatch repair deficient mice

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Diet is an important risk factor for many cancers. High fat/low calcium (HFLC) diets are associated with increased tumorigenesis, whereas caloric restriction (CR) reproducibly increases lifespan and decreases tumors. Mutations are involved in aging and cancer, and different diets may alter mutagenesis. However, a number of repair pathways normally counteract mutations by correcting errors before they can be fixed in the genome. To further understand interactions between diet, aging and cancer, mice deficient in a major repair pathway called DNA mismatch repair (MMR) were fed HFLC, CR or control diets. Mlh1 deficient mice are prone to lymphomas and intestinal adenomas and carcinomas. No significant changes in adenocarcinoma or lymphoma incidence were observed with HFLC or CR diets. Significantly more (2.2-fold) adenomas occurred with HFLC diets although adenoma numbers were unchanged with CR. Only a small increase in lifespan (116% of control) was achieved with CR. In addition, levels of microsatellite mutations in the small and large intestines were unchanged with the different diets. Our studies indicate that MMR deficiency may be epistatic to certain otherwise strong environmental influences on carcinogenesis or aging.

Introduction

A number of studies have demonstrated associations between diet and human cancer (1–3). Colorectal cancer prevalence varies among populations and immigrants tend to adopt the incidence of their new countries (2). These associations have been explored experimentally with animal models that facilitate manipulations of genetic and environmental factors. Diets high in fat and low in calcium (HFLC) are associated with increased rates of human colorectal cancer (1–3) and similarly increase intestinal tumors in mouse models (4–9). In contrast, caloric restriction (CR) reproducibly increases rodent lifespan and decreases tumor incidence (10).

The mechanisms underlying tumor frequency variations with different diets are uncertain. Colorectal cancer progression is associated with the accumulation of somatic mutations in oncogenes and tumor suppressor genes (11), and diet may influence mutagenesis. In general, more mutations may accumulate either by increasing mutation and/or mitotic rates (12). Mutagens may be classified as either genotoxic or mitogenic (12–14). It is unclear to what extent various diets are genotoxic or mitogenic.

One approach to better understand carcinogenesis is to modulate mutation rates with both genetic and environmental interventions. Mice deficient in DNA mismatch repair (MMR) due to homozygosity for a null allele of Mlh1 develop normally but are prone to lymphomas, intestinal adenomas and carcinomas, and skin gland tumors (15–19). Heterozygous germline MLH1 mutations are also seen in hereditary non-polyposis colorectal cancer (HNPCC), a familial cancer syndrome characterized by early adult-onset colorectal and other cancers (11). Mlh1 deficient mice facilitate experimental dietary manipulations because tumors appear relatively late (>6 months) after birth and about half the mice develop intestinal tumors (15). MMR is the primary mechanism responsible for the correction of polymerase errors after DNA replication but also has roles in recombination, transcription-coupled repair, DNA damage surveillance and apoptosis (18). Dietary effects on lifespan and tumorigenesis may be enhanced in Mlh1 deficient mice because many types of mutations normally corrected by MMR can accumulate. Conversely, dietary effects may be minimal because unlike other mouse models of cancer (4–9), MMR deficient mice have extremely elevated (~100-fold greater) background mutation rates due to uncorrected DNA polymerase errors (15–19). To investigate interactions between diets and MMR deficiency, Mlh1 deficient mice were fed diets that, a priori, are expected to increase (HFLC) or decrease (CR) tumor formation.

Materials and methods

Mice analyzed in this study were generated from Mlh1+/– mice (16) and were >90% C57Bl/6. The mice were fed three different diets (Table I). Fat in the HFLC diet (TD 97170, Harlan Teklad, Madison, WI) was corn oil (160 g/kg) and hydrogenated coconut oil (48 g/kg), and was fed *ad libitum*. The CR (TD 91351) and control or normal (NL) diets (TD 91349) were identical except the CR diet contained 70% of the calories of the NL diet. These mice were fed defined amounts of food every 2–3 days and the CR mice were caged individually. CR mice weights were ~70% of the NL diet mice with average maximum weights of 26 versus 17 g with CR. One other NL diet (TD 97247) was fed *ad libitum*. The two NL diets were combined because their compositions were similar and outcomes of the mice were not significantly different. The mice were monitored every 2–3 days and killed at signs of morbidity (weight loss, decreased activity). Autopsies were performed and all tumors were verified by microscopic examination. Intestinal tumors were considered cancers if invasion was noted. Mice found dead were not included in the tumor analysis because necrosis precluded microscopic verification.

DNA was extracted from microdissected small or large intestinal epithelium (estimated to be at least 70% epithelial cells) and diluted to essentially single molecules prior to PCR at two non-coding CA-repeat Dmit38 (CA_{20}) and Dmit129 (CA_{21}) microsatellites (MS) loci (20). The variance of an MS size frequency distribution is proportional to the average number of mutations per locus (21).

Survival comparisons used the log-rank analysis. The *t*-test or Fisher's

Abbreviations: CR, caloric restriction; HFLC, high fat/low calcium; HNPCC, hereditary non-polyposis colorectal cancer; MMR, mismatch repair; MS, microsatellite; NL, normal diet.

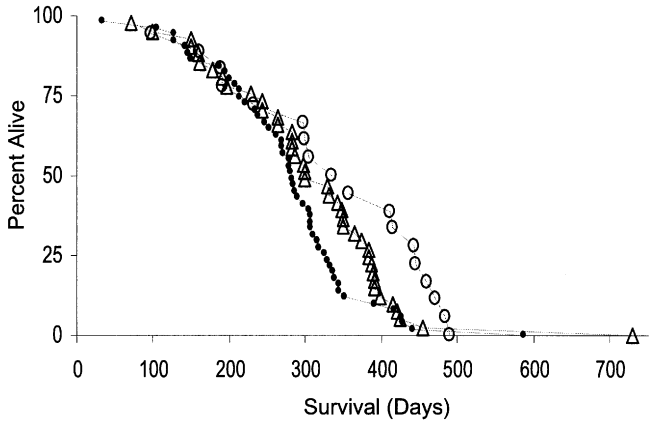

Fig. 1. Mlh1–/– survival curves on HFLC (filled circles), NL (triangles) and CR (open circles) diets. Only lifespan differences between HFLC and CR diets were significant ($P = 0.016$, log-rank).

Table I. Diet compositions

	HFLC	NL	CR[a]
Fat (%)	21	8.8–13.8	13.8
Fat (% of kcal)	41.3	20.8–30.2	30.8
kcal/g	4.6	3.8–4.1	4.0
Calcium (%)	0.1	0.6	0.6
Vitamin D (IU)	500	1500–2200	2200
Phosphorus (%)	0.54	0.5–0.56	0.56

[a]Values for calcium, phosphorus and vitamin D reflect final intake adjusted for the reduced amount of food provided for CR.

exact test were used to compare the average numbers of tumors with tumor incidences. Cox regression was used to compare times of tumor appearance between diets.

Results

The Mlh1 deficient mice were fed three diets primarily differing in fat, calcium and caloric content (Table I). The HFLC diet was similar in composition to the 'Western style' diet previously used in dietary mouse studies (6). Survival (Figure 1) was longest with a CR diet and shortest with a HFLC diet. Median lifespan was 299 days on a NL diet. Compared with the NL diet, median lifespan was 95% less (283 days) with a HFLC diet, and 16% longer (346 days) with a CR diet. Only the survival differences between HFLC and CR diets were significant ($P = 0.016$).

Tumors were found in the majority of mice at death. The most common tumors were intestinal adenomas or carcinomas, and lymphomas. Proportions of mice with tumors were not significantly different between diets (Table II). Lymphomas were found in 31–47% of mice and intestinal tumors were present in 65–69% of mice on the different diets. Numbers of adenocarcinomas per mouse were similar between diets.

Adenoma numbers were unchanged with CR but there were significantly more adenomas per mouse with the HFLC diet compared with the NL diet (2.2-fold increase, $P = 0.0029$). In addition, gastric and colonic adenomas were only found with the HFLC diet. Lifespan for mice with adenomas or cancers was decreased with a HFLC diet and increased with CR (Figure 2). These lifespan differences were significant for adenomas ($P = 0.003$) but not for cancers.

Mutations in short simple repeats or MS accumulate in phenotypically normal appearing tissue in MMR deficient mice (20). We determined levels of mutation in normal small or large intestines by PCR following dilution of the DNA to essentially single molecules (Figure 3). Numbers of MS mutations [proportional to the variance of their distributions (21)] were variable between mice, probably due to the stochastic nature of MS mutation (20). Importantly there were no consistent differences in the accumulation of MS mutations between the diets. However, there was a trend for increased numbers of MS mutations with age, and mutations were 1.2-fold higher in the small compared with the large intestines ($P = 0.03$).

Discussion

Human colorectal cancers can be divided into two general types based on genetic instability. One type is characterized by chromosomal instability (CIN) or aneuploidy, and the other type (~15%) is characterized by loss of MMR and MS instability (22). Compared with MMR proficient cancers, MMR deficient cancers have distinctive pathologies and clinical responses (11,23,24). Human MMR deficient colorectal cancers are also associated with different environmental exposures with higher risks observed with cigarette smoking and consumption of heterocyclic amines (25,26).

Similar to the dichotomy between MMR proficient and deficient human cancers, mice deficient in MMR respond differently to diet compared with MMR proficient mice. In general, HFLC diets and CR tend to, respectively, increase or decrease tumorigenesis in MMR proficient mice, including tumor prone mice with specific tumor suppressor gene deficiencies in Trp53 or Apc. However, in the setting of MMR deficiency, the effects of diet on carcinogenesis and lifespan are reduced as CR led to a small increase in lifespan (16% longer) but had no significant influence on the numbers or timing of lymphomas or adenocarcinomas. Similarly, a HFLC diet had no significant influence on the timing or numbers of adenocarcinomas or lymphomas compared with a NL diet. In contrast, CR of MMR proficient (normal or p53 deficient) mice increased lifespan 50–60% (10,27). Numbers of adenocarcinomas were observed to increase 3–6-fold with a HFLC diet in MMR proficient but Apc deficient (Apc1638) mice (6).

Unlike with malignant tumors, numbers of adenomas per mouse were increased ~2-fold with a HFLC diet, and mice with adenomas succumbed sooner. This increase in adenoma number is similar to the magnitude observed with high fat diets in Apc heterozygous deficient mice (6–9). Numbers of adenomas per mouse were unchanged with CR although the lifespan of mice with adenomas was significantly increased with CR.

CR or HFLC diets may influence tumorigenesis by modulating mitotic rates because intestinal proliferation has been reported to increase with high fat or low calcium diets (28–30), or decrease with CR (29,30). However, these studies primarily measured the mitotic activity of differentiated cells and not of the crypt stem cells that are probable targets for transforming mutations (31). In contrast, the MS mutations measured in this study of Mlh1 deficient mice probably reflect the number of stem cell divisions because mutations in non-stem cells will not accumulate (31). Mutations in CA-repeat MS sequences occur due to unrepaired mispaired loops due to polymerase slippage after DNA replication (32). Therefore, total numbers of MS mutations are proportional to the numbers of stem cell divisions (20).

Table II. Lifespan and tumor frequencies

	HFLC	NL	CR	Significant differences
n	51	41	18	
Median lifespan (days)	283	299	346	HFLC versus CR ($P = 0.016$)
Tumor analysis, *n*	46	34	16	
Any intestinal tumor (%)	65	68	69	
(Average number/mouse)	(2.59)	(1.50)	(1.62)	HFLC versus NL ($P = 0.017$)
Median lifespan (days)[a]	307	350	444	$P = 0.02$
Adenoma (%)	65	53	50	
(Average number/mouse)	(2.24)	(1.00)	(1.06)	HFLC versus NL ($P = 0.0029$)
				HFLC versus CR ($P = 0.013$)
Median lifespan (days)[a]	307	350	438	$P = 0.003$
Cancer (%)	26	38	50	
(Average number/mouse)	(0.35)	(0.50)	(0.56)	NS
Median lifespan (days)[a]	328	348	444	NS
Lymphoma (%)	41	47	31	NS

[a]Calculated for the subset of mice with the tumor.
n, Number of mice in each group; NS, not significant ($P > 0.05$).

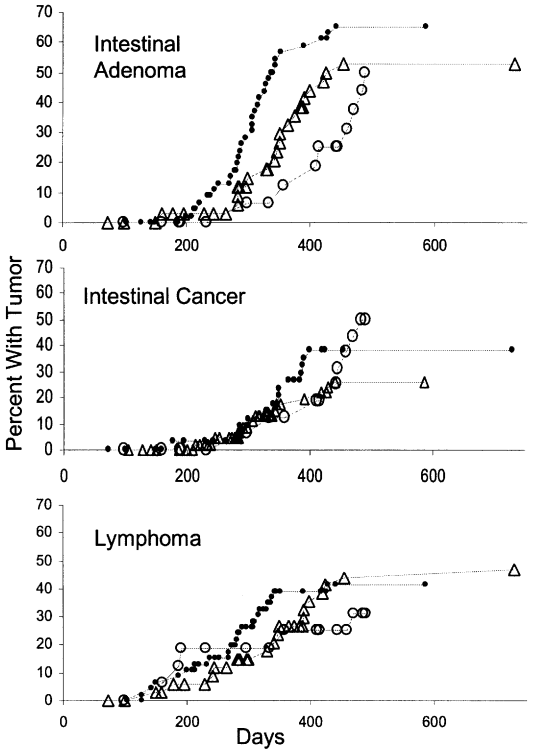

Fig. 2. Incidence of mice dying with intestinal adenomas, cancers or lymphomas on the three different diets. Differences between times of death were only significant for adenomas. Symbols are the same as Figure 1.

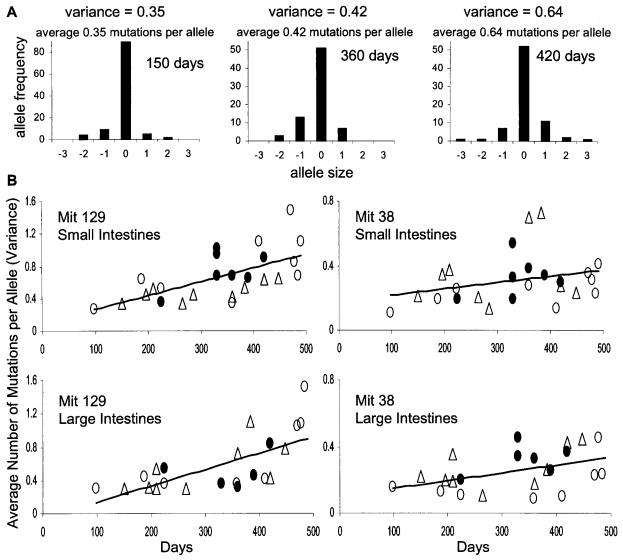

Fig. 3. (**A**) Frequency distribution of MS alleles (Mit129) in the small intestines of NL diet mice of different ages. Alleles become polymorphic with age. The variances of the frequency distributions are equal to the average number of mutations per allele (21) because stepwise additions (+1) and deletions (–1) are possible. (**B**) Accumulation of MS mutations in small or large intestines with different diets. Differences in mutation numbers were not significant between the different diets but were 1.2-fold higher in the small intestines ($P = 0.03$). Symbols are the same as Figure 1.

Significantly fewer MS mutations were found in the colon compared with the small intestines, consistent with crypt models that postulate longer stem cell cycles in the murine colon compared with the small intestines (33). However, no consistent mutation differences were found between the different diets. Therefore, diets may influence tumorigenesis or aging not by markedly changing stem cell division rates but rather through alternative mechanisms. Evidence of genotoxic dietary effects has been ambiguous. Studies using lacI transgenic mice have not found significant changes in mutation frequencies with CR or high fat diets (34,35). However, Hprt mutation frequencies in lymphocytes are decreased with CR (36,37). Cellular responses to a CR diet may be complex as demonstrated in recent studies using microarray gene expression analysis (38).

The relative insensitivity of the current MMR deficient mice (Mlh1–/–) to dietary changes may not reflect risks for most human MMR deficient cancers because congenital human MMR deficiencies are rare (21,39). Most human MMR deficient cancers arise in MMR proficient individuals that somatically lose MMR in individual tumor cell progenitors. Indeed, it appears that other than heterocyclic amine consumption, dietary risk factors are not very different between MMR deficient and proficient human colorectal cancers (25,26). Interestingly, molecular tumor clock studies estimate that human MMR loss usually occurs ~6 years before tumor

removal (40), allowing for a significant exposure to diet before MMR loss because MMR deficient tumors rarely arise before the age of 30, even in HNPCC individuals (41). Dietary studies of mice with heterozygous germline mutations (Mlh1+/–) would better mimic HNPCC but are infeasible because they rarely develop tumors (17). Nevertheless, the current studies illustrate that the relative influence of diet on tumorigenesis may change during progression. Conceptually, diet may have a greater effect on tumorigenesis before the somatic onset of genomic instability caused by loss of MMR or some other mechanism. The relatively modest effects of diet on survival and cancer incidence in MMR deficient mice are consistent with the strong inherent carcinogenic potential of a mutator phenotype (42). An elevated mutation rate may partially obscure otherwise strong environmental influences on carcinogenesis or aging.

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