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Laboratory Investigation

Effect of dietary vitamin A or N-acetylcysteine on ethylnitrosourea-induced rat gliomas

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Summary

It is our hypothesis that low grade gliomas are the glial counterparts of other precancerous lesions such as colon polyps and, therefore, suitable targets for chemoprevention. Steps in the molecular progression of gliomas have been described, indicating that an accumulation of abnormalities is required for progression to a high grade and interruption of this progression might be possible. An animal model of chemical glial carcinogenesis was used to test this hypothesis. Pregnant rats were injected intravenously with ENU (ethylnitrosourea) on the 18th day of gestation to induce gliomas in the offspring, which were randomized to receive control diet, diet supplemented with vitamin A palmitate, or diet supplemented with N-acetylcysteine. Animals exposed to ENU and receiving a control diet developed brain tumors and had a shortened life expectancy compared with rats unexposed to ENU. The animals treated with NAC showed no statistically significant delay in the time to tumor and no change in the histologic grade of the tumors when compared with animals receiving control diet, but the time to death from any cause of NAC treated animals differed significantly from untreated animals. Animals receiving high dose VA had statistically significantly prolonged time to tumor, survived significantly longer than untreated animals, but had no reduction in the total number of tumors or change in the histologic grade of their tumors. The theoretical basis of these results is likely due to the putative mechanism of action of these agents. These data indicate that glioma chemoprevention is possible and deserves further exploration.

Introduction

The annual incidence of primary malignant brain tumors in the United States is estimated to be about 17,500 cases per year [1], of which 50 to 60% are malignant astrocytomas [2]. Despite many experimental protocols utilizing surgery, radiation, chemotherapy, immunotherapy, and other modalities [3], the treatment of these tumors has improved little in the last 30 years. Most of these tumors are inexorably fatal, with 90% of Grade III and IV astrocytoma patients dead within two years of diagnosis [4].

Low grade gliomas are relatively common, with low grade astrocytic tumors alone comprising as much as 15% to 32% of surgical brain tumors [5, 6]. While a benign histologic picture, indolent growth, and relatively prolonged survival are characteristic of these lesions, their biologic behavior is unpredictable [7–10]. Some lesions appear curable [9, 11, 12], but up to 85% of histologically identical low grade tumors eventually develop anaplastic features [9–11, 13] and progress to take the life of the patient [14], sometimes decades after diagnosis [8].

In recent years, some of the molecular events in glial carcinogenesis have been elucidated and indicate that an accumulation of genetic abnormalities,

including both gene loss and gain of function, is required for progression to a high grade malignancy [see reviews in 15–19]. Because current treatment for low grade gliomas is unsatisfactory and because the progression from a low grade to a high grade glioma appears to require the accumulation of additional genetic events, a potential treatment for low grade gliomas might be designed to prevent the occurrence of the additional mutational events which contribute to anaplastic transformation and thereby maintain the indolent state of low grade glial tumors. Such a treatment strategy would be called chemoprevention, which has been defined as 'the administration of chemical agents to prevent the initiational (mutational) and promotional events that occur during the process of neoplastic development' [20] or the use of 'chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer' [21]. The chemoprevention strategy presupposes a multistep process of carcinogenesis (initiation, promotion, progression, clonal evolution) which can be interfered with at any of these points [21].

While an enormous amount of animal chemoprevention research has already been done [20, 21], and a number of human chemoprevention trials are already underway [20, 22, 23], little of this work has been directed at central nervous system carcinogenesis. A January, 1998 Medline literature search discloses no references matching both 'glioma' and 'chemoprevention'. This may be because the most likely targets for chemoprevention trials are diseases with easily detected precancerous lesions. A recent review [23] lists 7 tumors and their precancerous counterparts (in parentheses) as the subjects of ongoing chemoprevention trials: colorectal (adenomas), prostate (early stage or intraepithelial neoplasms), lung (one primary at risk for a second), breast (carcinoma *in situ*), bladder (carcinoma *in situ*), oral (leukoplakia), and cervical (intraepithelial neoplasia). It is our hypothesis that Grade II astrocytomas, oligodendrogliomas, and mixed low grade gliomas are the glial counterparts of these precancerous lesions and, therefore, suitable targets for chemoprevention trials as well.

Because human low grade gliomas do not become established in culture nor is there an *in vitro* model of progression from low grade glioma to high grade glioma, screening for potential chemopreventative agents requires an animal model. An animal model of central nervous system carcinogenesis which may be suitable for screening chemopreventative agents has been described. The offspring of pregnant rats given a single intravenous dose of ethylnitrosourea (ENU) after the 12th day of gestation develop malignant neuroectodermal tumors of the central and peripheral nervous systems [24–26]. We have established this model in our laboratory and utilized it to screen a number of agents with putative chemopreventative activity against other tumor types for the ability to prevent or delay the development of gliomas. We report the results of this experiment.

Materials and methods

All experiments were conducted with the approval of the University of Michigan Committee on the Use and Care of Animals in accordance with NIH regulations in the care and handling of vertebrate animals in experimental protocols. Twelve pregnant female Sprague-Dawley rats (average wt 325 ± 20 g), free of specific pathogens (Charles River, Wilmington, Massachusetts) were injected with 75 mg/kg of a 0.1 M solution of ENU (Sigma, St. Louis) dissolved in citric acid: disodium phosphate buffer, pH 6.0 immediately prior to injection via the lateral tail vein on the 18th day of gestation as described previously [27, 28]. Four additional pregnant animals received the buffer solution alone. The animals were allowed to deliver naturally, were whelped by their natural mothers, and weaned at 21 days of age. Animals from ENU treated dams were randomized to 3 groups by using a random number table after stratifying by dam, and all animals housed in groups based upon sex and weight. The three groups were vitamin A palmitate (VA), N-acetylcysteine (NAC), observation alone.

Animals were weighed weekly in all groups to document growth rates. Chow and water consumption were measured to document intake of chemopreventative agents. All animals had access to food and water ad libitum. Control offspring were fed

standard lab chow containing approximately 15,000 IU vitamin A and 6.4 mg beta-carotene per kg. This amount of total vitamin exceeds the recommended minimum daily allowance for rodents and allows for normal growth and longevity.

Administration of chemopreventative agents began after weaning at 21 days of age. VA treated animals receive standard chow supplemented with 150,000 IU vitamin A palmitate per kg as previously described [29]. This was accomplished by stirring standard chow in an ethanol solution of Vitamin A palmitate for 1.5 hours in the dark, during which most of the ethanol evaporates. Control chow was treated with ethanol alone. Treated offspring receiving NAC received dietary NAC (1% w/v in drinking water) as previously described [30]. Water was changed every 3 days to assure stability of the compound. Fresh batches of food and NAC solution were prepared at approximately one week intervals.

Control and treated offspring were examined for the type and distribution of tumors and for longevity. At monthly intervals approximately 6 animals (3 male, 3 female) were randomly selected, euthanized, and brain and spinal cord removed. Samples were fixed in 10% buffered formalin and processed for paraffin sectioning (3–4 μm sections) and hematoxylin and eosin staining. Additionally, animals displaying progressive neurologic signs, weight loss, and other decrements in normal health were euthanized. Brains and spinal cords from these animals were processed for histology as described above. All surviving animals were sacrificed after 280 days of life.

All paraffin section were examined by a neuropathologist (MB) blinded as to the treatment group from which the specimens were derived. Each neoplasm was graded as low, intermediate, or high grade based on the following criteria:

Low	*Intermediate*	*High*
low cellularity	more cellular	hypercellular, multinucleated cells
< 2 mitoses	> 1 mitoses	conspicuous mitoses
no karyorrhexis	karyorrhexis present	conspicuous karyorrhexis
no endothelial proliferation	endothelial proliferation	conspicuous endothelial proliferation
no necrosis	no necrosis	necrosis

Statistical analysis was performed with the collaboration of a biostatistician (MS). Survival was analyzed by Kaplan-Meier statistics. Survival was measured from birth to death with or without tumor. Sacrificed animals were censored. The tumor incidence rates were compared between groups using two methods: one proposed by Malani and Van Ryzin [31] and the poly-3 test proposed by Bailer and Portier [32]. Both tests are based upon the chi-squared distribution.

Results

Two hundred and four animals entered the trial distributed between groups as 51 control, 46 ENU, 56 ENU and VA, and 51 ENU and NAC. Weight gain was similar in all 4 groups (data not shown). No control animals died spontaneously nor did any have tumors of the brain or spinal cord visible at autopsy. Eleven ENU animals, 8 NAC animals, and 4 VA animals were euthenized for deteriorating health or died spontaneously. Kaplan-Meier survival curves for the three treatment groups are shown in Figure 1. The three curves are statistically significantly different from ech other by the logrank test with a $p = 0.0005$. Similarly, the ENU survival differed from the NAC survival ($p = 0.0312$) and also from the VA survival ($p = 0.0002$).

Central nervous system tumors were first apparent in sacrificed animals in the ENU treated group at 79 days of life (Figure 2). Both NAC and VA treated animals' tumors were first apparent at 165 days of life, with the proportion of tumors observed at sacrifice still rising at 280 days. The time to death or sacrifice with tumor present was compared between groups using the logrank test. We noted no significant difference in time to 'observed' tumor between NAC and ENU ($p = 0.1547$). However, the VA group appeared to have a longer time to 'observed' tumor compared to the ENU group ($p = 0.0001$) and the NAC group ($p = 0.0048$). We recognize that this analysis is flawed due to the occult nature of the tumor onset, the competing risk of non-tumor mortality, and the differential effect of agents on tumor lethality. These issues have been dis-

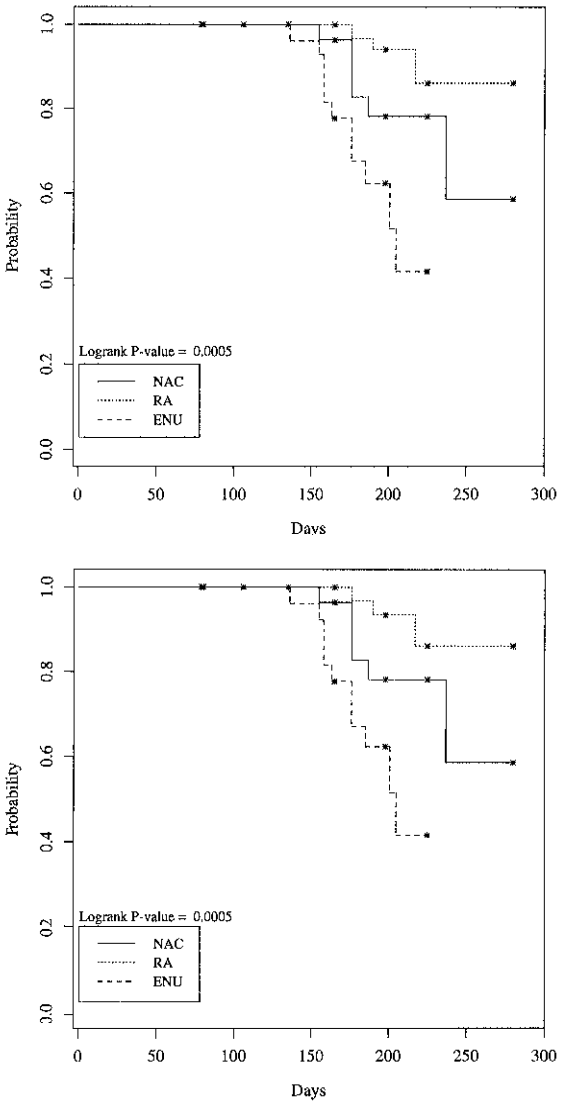

Figure 1. A) Kaplan-Meier curves comparing probability of survival in all animals entering the trial receiving dietary vitamin A palmitate (VA) or N-acetylcysteine (NAC) following an *in utero* exposure to ethylnitrosourea (ENU). Logrank P value for NAC verus ENU = 0.0312, for VA verus ENU = 0.0002, and for NAC versus VA = 0.1. * indicates censored animals due to interim sacrifice or trauma. B) Number of remaining living animals versus time reflecting sacrifice or death secondary to illness or other complications.

cussed in the literature by McKnight and Crowley [33] and others [31, 32, 34].

The nonparametric methods of Malani and van Ryzin [31], which test incidence rates in the presence of sacrificed animals, only assume the same sacrifice scheme was used in all groups and that there is no difference between groups in mortality for animals without tumors. No significant difference in tumor incidence was detected between VA and ENU ($p = 0.6029$) or NAC and ENU ($p = 0.9592$). However, it is known that this test performs poorly when there are few observed natural deaths in the intervals between sacrifices. Also, the high proportion of sacrificed animals relative to the entire cohort size probably adversely affected the power to detect differences in incidence rates. We also examined incidence rates using the Poly-3 test, which is not affected by tumor lethality and adjusts for differential survival. Again, no difference in incidence of tumors over the study period was detected between VA and ENU groups ($p = 0.5824$) or between NAC and ENU ($p = 0.8051$). Although no difference in tumor incidence was seen over the entire study period, we wondered if the (imperfect) analysis of time to 'observable' tumor (tumor detected by random sacrifice or when clinically symptomatic) might indicate a decrease in tumor lethality or delay in tumor onset. Using the standard (epidemiological) definition of incidence rate, the number of new events over the total rat-days of observation, the estimates of incidence rate are 0.2161, 0.2270, and 0.2144 tumors per 100 days of observation for ENU, NAC, and VA respectively.

The total number of tumors in each group was very similar when all animals whose tissues were available for histopathology were considered: 34.1% (14/41) of the ENU animals, 37.5% (18/48) of the NAC animals, and 41.1% (23/56) of the VA animals had histologically verified tumors. Of the 11 ENU animals which were euthanized (see Materials and methods) or died spontaneously, 6 had histologically confirmed tumors, 3 had proptosis and neurologic deficits prior to death but a central nervous system tumor was not found at autopsy, 3 had histopathologic slides judged to be uninterpretable due to poor fixation, and 2 were necrotic secondary to death several hours before discovery prohibiting full examination. Of the 8 NAC animals which were euthanized or died spontaneously, 4 had histologically verified tumors, 1 had neurologic deficits at death but no central nervous system tumor was found at autopsy, 2 died of trauma inflicted by cagemates, and 1 had necrosis at autopsy prohibiting full

Table 1. Number of animals with tumors and grading for each treatment group

	Low	Intermediate	High	Total
ENU	3	6	5	14
NAC	6	10	2	18
RA	9	7	7	23
Total	18	23	14	55

Each animals tumor(s) were examined and graded as described in the Materials and methods. In cases of multiple grades the highest grading was used. Using the Exact Kruskal-Wallis test, pairwise comparisons for ENU vs. NAC ($p = 0.1817$); for ENU versus RA ($p = 0.4752$).

examination. Of the 4 VA animals which were euthanized or died spontaneously, 3 had histologically verified tumors and 1 had neurologic deficits but no tumor found at autopsy.

Histologic slides of tumors were examined by a neuropathologist (MB) and graded as low, intermediate, or high grade as described in Materials and methods. Results are shown in Table 1. There was a trend toward a higher percentage of low grade tumors in the NAC and VA groups and higher percentage of intermediate and high grade tumors in the ENU group, but this did not reach statistical significance (Exact Kruskal-Wallis test, $p = 0.4088$).

Discussion

The ENU model has been studied for more than 30 years [35], but to our knowledge has only rarely been utilized in a chemoprevention experiment [36, 37]. In the early work characterizing this model in BD IX rats, brain tumors were not seen if the ENU exposure came before the 12th day of gestation, when teratogenic effects predominate over carcinogenesis [38]. Brain tumors increased in frequency the later in gestation the agent was given [25]. When the injection was given on the 18th day of gestation, the incidence of brain tumors approached 87% [26, 27]. The susceptibility period appears to correspond to the major period of gliogenesis in the rat brain [38]. The brain tumors induced by this carcinogen were reported to be mainly mixed gliomas, astrocytomas, oligodendrogliomas, and ependymomas [26]. The median survival time of affected animals was 240 days [27]. Similarly, the offspring of Sprague-Dawley rats given a single intravenous dose of ENU on the twentieth day of gestation induced neurogenic tumors in 100% of the offspring with an average survival time of 211 days [28, 39]. Non-neural tumors comprised only 6.4% of the tumors seen. In a number of reports, early histologic changes can be seen in the periventricular zones of ENU exposed animals as early as the second month of life [28, 35, 40–42].

The major mutagenic activity of ENU has been reported to be due to DNA adduct formation at the O-6 position of guanine, the misrepair of which results in a guanine to adenine alteration [38, 43–46]. A subsequent cell division prior to repair of the adduct is necessary for persistence of the misrepair [38]. The susceptibility of organs to ENU induced carcinogenesis appears to be inversely related to the organ's ability to repair this DNA damage, with the brain having a relatively poor ability to do so with persistence of O-alkylated bases in the brain for days to months [38, 46].

ENU has been reported to induce mutations in the human p53 gene at hotspots frequently seen in spontaneous human cancers and many of which are guanine to adenine transition mutations [47]. In a mouse model of in utero ENU exposure, homozygous deletion of the p53 gene resulted in the accelerated appearance of brain tumors over wild type or heterozygous mice, leading to the conclusion that p53 loss may be an early event in brain tumor induction by transplacental carcinogen exposure [48]. Rat type 1 astrocytes exposed to ENU *in vitro* become transformed when p53 mutant cells begin to appear, again implying that p53 mutation is important in ENU carcinogenesis [49]. Other work suggests involvement of the c-sis/PDGF system in transformation after *in utero* exposure to ENU [50].

Although the evidence of p53 mutation is strong, the *in vivo* ENU model has not been otherwise well characterized at the molecular level; therefore, it is not known if tumors produced in this way progress from low grade to high grade or if high grade tumors appear *de novo*, or if both are likely. Early work with this model found tumors at all stages of development [28]. Tissue culture studies of rat brains ex-

posed to ENU transplacentally has shown that 'the emergence of the malignant phenotype . . . is a stepwise process which culminates in the concomitant appearance of tumorogenicity and invasiveness' [35]. As one third of the tumors we characterized histologically were low grade, it is likely that at least some of the high grade tumors progressed from lower grade tumors in a process analogous to that observed in human patients. ENU-induced rat gliomas have been thought to be a good model for human gliomas in general because they have been noted to have many histologic and physiologic similarities to human brain tumors [38]. Although the ENU model is limited in its precise analogy to human low grade glioma progression, we are not aware of any other experimental brain tumor system which more closely models the human disease.

The total incidence of tumors in our experiment was below the 87% to 100% reported by previous authors [26–28, 39]. This is likely to have been due at least in part to the regular sacrifice of animals during the experiment which did not allow the ENU-treated animals to live long enough for the maximum number to develop tumors. In addition, rat strains have been shown to differ in the incidence of tumors in this model and the precise day of gestation and dose at which the ENU exposure is made also affect the tumor yield [28, 39]. We chose day 18 of gestation to be within the range previously reported to be effective for Sprague-Dawley rats and a relatively high dose of ENU (75 mg/kg) to shorten the latency to tumor appearance, but this may not be the optimal day or dose for this particular Sprague-Dawley rat.

Approximately 2000 compounds have shown some preclinical chemopreventative activity and at least 35 randomized human trials of potential agents have been conducted, most within the last 5 years [21]. A table of agents currently in preclinical or clinical trials can be found in Lippman 1994 [21]. Chemopreventative agents generally fall into three categories: 1) carcinogen blocking (antimutagenic), 2) antiproliferative, and 3) antioxidant [23], or 1) anti-initiation, 2) anti-promotion, or 3) anti-progression [21]. Because the ENU model mandates the use of agents given long after the initial carcinogenic insult unless the agent is administered to the pregnant female, agents of the first types (anti-mutagenic or anti-initiation) may not be effective in this model. We administered the agents after weaning in an effort to model the human low grade glioma in which case a tumor is already established and the goal is to prevent progression to a fully malignant phenotype. We chose NAC and VA for this experiment due to their low toxicity, ease of administration, and their differing putative mechanisms of action.

Retinoids and their receptors have been implicated in the development of cancer. Lack of vitamin A has been linked to stomach cancer in rats and to premalignant skin changes in humans [51]. Lower blood retinol levels and a lower average consumption of beta carotene have been reported in patients with cancer [51]. Rearrangement of a retinoic acid receptor (RAR) gene has been implicated in the development of some leukemias [52]. The oncogene v-erbA had been reported to interfere with retinoic acid receptor action as its possible mode of oncogenesis [53]. Abnormal patterns of RAR expression, particularly low levels of RAR beta transcripts, have been implicated in neoplastic progression of epithelial cancers of the aerodigestive tract [54]. Upregulation of RAR beta has been shown when premalignant lesions are treated with retinoids [55]. The rapid decrease in chemopreventative effect upon cessation of therapy in some trials suggests that retinoids are acting at a late stage in the carcinogenic process [23].

Studies of retinoids in various animal models have shown chemopreventative activity against mammary, bladder, oral, lung, pancreas, cervix, liver, colon, esophagus, skin, and prostate tumors (reviewed in Lippman 1994 [21]). The synthetic retinoid fenretinide suppresses mammary cancer in rats [56] and appears to be a particularly promising compound due to its potency and relatively low toxicity [20, 23, 57].

All trans-RA has been reported to produce remission in patients with acute promyelocytic leukemia [58, 59] and 13-cis-RA produced similar results in acute nonlymphocytic leukemia [60]. Another synthetic retinoid (isotretinoin) prevented new head and neck cancers, but not recurrence of the primary lesion, in patients who had already had

them once [61]. Large scale human trials are underway using vitamin A (retinol palmitate) for chemoprevention of second upper aerodigestive cancers [62]. Fenretinide has shown activity in human bladder cancer chemoprevention [63] and is being used in a larger, ongoing breast cancer trial [57]. However, two recent human trials have shown no protective effect of beta-carotene, and possibly a detrimental effect, in patients at high risk for lung cancer secondary to smoking or asbestos exposure [64].

Thiol compounds have been shown to have chemopreventative properties in multiple animal models [65]. N-acetylcysteine (NAC), a precursor of intracellular cysteine and reduced glutathione, is already in human use as a mucolytic, antioxidant, and antidote [66]. When administered in the diet, NAC decreased the incidence of lung tumors in mice given the carcinogen urethan [66] and has exhibited protective effects in rat models of colonic [30] and squamous cell [67] carcinogenesis. NAC has also protected rats from the cytogenetic damage caused by tobacco smoke [68]. The only significantly effective regimen in a mouse lung carcinogenesis model was administration of NAC both before and after the administration of carcinogen, consistent with a role in preventing initiation, but not promotion or progression of tumors [66]. Humans have been given NAC at doses as high as 500 mg/kg without apparent ill effect [69]. Large scale human trials are underway using NAC (600 mg/day for two years) for chemoprevention of second upper aerodigestive cancers [62].

The statistical analysis of animal carcinogenesis experiments is difficult, as has been discussed previously by McKnight and Crowley [33]. The problem in the ENU model comes from the occult nature of the tumors, which can only be detected at autopsy. The actual time to tumor onset is unknown; therefore, familiar statistical methods used to study competing risk do not apply [33]. Using time to death or sacrifice as a measure of incidence is confounded by tumor lethality and background mortality rate. We have analyzed tumor incidence using methods created to assist with this problem realizing that the methods are imperfect.

Given the statistical limitations of this type of experiment, our data indicate that VA increased the time to observation of tumors and prolonged survival, but did not reduce the total number of tumors. NAC prolonged survival but did not increase the time to observation of tumors or reduce the total number of tumors. The histologic data do not indicate a change in tumor grade being responsible for the delayed incidence or prolonged survival of treated animals. These data are consistent with the putative mechanisms of action of these agents with NAC acting early in carcinogenesis (anti intiation) and being relatively less effective when administered late in the carcinogenetic process, while retinoids are often effective later in the carcinogenic process (anti-promotion or anti-progression), as suggested by previous work [21, 23]. In the ENU mode, VA may delay progression, but did not appear to prevent it.

Our data indicate that 1) the ENU model reliably produces central nervous system meoplasms of varying histologic grade which are amenable to chemoprevention experiments; 2) retinoic acid shows promise as a chemopreventative agent in this model and that further studies of this agent, its mechanism of action, and other potential agents are warranted; and 3) human trials of agents active in animal models may eventually be indicated in patients with low grade gliomas in an effort to prevent the development of anaplasia. Such therapy may be especially useful in children who are so vulnerable to the side effects of radiation and cytotoxic chemotherapy and who have such a long at risk period for tumor progression. Glioma chemoprevention may also be indicated in patients without gliomas but at high risk for glioma development, such as patients with neurofibromatosis, or the Li-Fraumeni, Turcotte, or Lynch syndromes.

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