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COMMENTARY

Etiology and chemoprevention of esophageal squamous cell carcinoma

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Squamous cell carcinoma (SCC) of the human esophagus has a multifactorial etiology involving several environmental and/or genetic factors. Current modalities of therapy for this disease offer poor survival and cure rates. Although a number of approaches could be undertaken to reduce the occurrence of esophageal SCC, including changes in lifestyle and improved nutrition, such approaches are not easily implemented. Chemoprevention offers a viable alternative that is likely to be effective against this disease. Clinical investigations in areas of high incidence of esophageal SCC have shown that primary chemoprevention of this disease is feasible, if potent inhibitors are identified. Studies in the Fischer 344 rat model of nitrosamine-induced tumorigenesis have proven valuable in understanding the biology of esophageal SCCs and help identify surrogate end-point biomarkers and putative agents that can be useful in human chemoprevention studies. Several compounds that inhibit tumor initiation by suspected human esophageal carcinogens have been identified using this model. These include diallyl sulfide, isothiocyanates and several polyphenolic compounds. Novel biomarkers, including nuclear/nucleolar morphometry using computer-assisted image analysis of preneoplastic lesions, have been developed to measure efficacy of chemopreventive agents against esophageal SCC. The identification of single agents that inhibit the progression of dysplastic lesions, however, has proven difficult. Results from a food-based approach suggest that the use of freeze-dried berry preparations can affect both initiation and promotion/progression of esophageal SCC in an animal model. These observations provide valuable information for future studies on chemoprevention of cancers of the esophagus in a clinical setting. Given the complex etiology of esophageal SCC, it is felt that the most effective chemoprevention strategies would include agents that reduce mutational events associated with carcinogen exposure in combination with agents that inhibit the progression of intraepithelial dysplasia to invasive cancer.

Abbreviations: ATB, antitumor-B; BITC, benzyl isothiocyanate; BRB, freeze-dried black raspberries; CAQITA computer-assisted quantitative image tile analysis; CYP2E1, cytochrome P450 2E1; EA, ellagic acid; 4-ECPR; 4-ethoxycarbophenylretinamide; EGCG, (–)-epigallocatechin 3-gallate; 4-HPR, *N*-(4-hydroxyphenyl)retinamide; HPV, human papilloma virus; MTG, mean tile grade; NMBA, *N*-nitrosomethylbenzylamine; NNN, N-nitrosonornicotine; PBITC, phenylbutyl isothiocyanate; PCNA proliferating cell nuclear antigen; PEITC, phenethyl isothiocyanate; PHITC, phenylhexyl isothiocyanate; PPITC, phenylpropyl isothiocyanate; SCC, squamous cell carcinoma; STRW, lyophilized strawberry preparation; %TG>4SD, percent tile grade >4 SD; TOC, tylosis esophageal cancer.

Introduction

Esophageal cancer in humans occurs worldwide with a variable geographic distribution and ranks eighth in order of cancer occurrence, combining both sexes (1,2). This malignancy exists in two main forms with distinct etiological and pathological characteristics, squamous cell carcinoma (SCC) and adenocarcinoma. More than 90% of esophageal cancers worldwide are SCCs (3,4), although adenocarcinomas are more prevalent in the USA (4–6). The principal precursor lesion of esophageal SCC is epithelial dysplasia (7). Microscopically these lesions represent an accumulation of atypical cells with nuclear hyperchromasia, abnormally clumped chromatin and loss of polarity. There is evidence from prospective studies that esophageal SCC probably develops through a progressive sequence from mild to severe dysplasia, carcinoma *in situ* and, finally, invasive carcinoma (8–10). These tumors frequently present as fungating, ulcerating or infiltrating lesions in the esophageal epithelium. Microscopically, esophageal SCCs range from well-differentiated tumors that exhibit keratinization, moderate nuclear atypia and minimal necrosis to poorly differentiated tumors with a high mitotic index and large areas of necrosis. A large majority of patients with cancer of the esophagus present with advanced metastatic disease. The prognosis for such cases is poor; the overall 5 year survival rate of patients with metastatic disease is <10% (1,2).

Epidemiology and etiology

The incidence of esophageal SCC shows marked variation in its geographic distribution and occurs at very high frequencies in certain parts of China, Iran, South Africa, Uruguay, France and Italy (7,11–14). In particular, areas located in the southern parts of the Taihang mountains on the borders of Henan, Shansi and Hopei provinces in China have among the highest incidence and mortality rates for esophageal SCC in the world. In Linxian county in Henan province the age-adjusted mortality rates for esophageal SCC have been reported as 151/100 000 for males and 115/100 000 for females (15). These observations point to specific environmental factors playing a significant role in the etiology of this disease.

Table I lists the known risk factors for SCC of the esophagus. Excessive use of tobacco has been implicated as a principal factor in the etiology of esophageal SCC. Several tobacco constituents, including nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, various aldehydes and phenols, may be causally related to esophageal cancer (16–18). Alcohol consumption has been shown to further increase the risk of SCC in the esophagus of tobacco smokers (19). Consumption of salt-pickled, salt-cured and moldy foods has also been implicated in the pathogenesis of this disease (20). Some of these products are frequently contaminated with *N*-nitrosamine

Table I. Risk factors for human esophageal SCC

Tobacco
Alcohol
Salt-pickled, salt-cured and moldy foods
N-nitrosamine carcinogens (from multiple sources)
Vitamin (A, C, E, etc.) and trace mineral (zinc, selenium) deficiencies
Hot beverages
Fungal invasion of esophageal tissues
HPV serotypes 16 and 18?
Heritable susceptibility genes?

carcinogens and/or fungal toxins. Extensive research in China and South Africa has suggested that *N*-nitroso compounds and their precursors are probable etiological factors for esophageal cancer in these high incidence areas (21,22). Several nitrosamines, including *N*-nitrosomethylbenzylamine (NMBA), have been isolated and identified in the diets and gastric juice collected from subjects in Linxian county in Henan province, China. The detection of O^6-methylguanine in the DNA of normal esophageal tissue taken from esophageal cancer patients in China further substantiates the role of methylating nitrosamines in the development of esophageal cancers (23,24). In addition, contaminated foods often contain nitrates, nitrites and secondary and tertiary amines, which act as precursors for nitrosamine formation *in vivo*. Under acidic conditions *N*-nitroso compounds can easily be formed in the stomach by the reaction of nitrites and amines (18).

Other factors associated with an increased risk of esophageal SCC include vitamin and trace mineral deficiencies. Plasma levels of vitamins A, C and E, among others, tend to be lower in patients with esophageal cancer. An inverse relationship has been noted between mortality caused by esophageal cancer and levels of zinc, selenium and other trace elements in foods from high risk areas for this disease. Consumption of hot beverages, such as tea, and fungal invasion in esophageal tissues leading to localized inflammation and irritation have been suggested as additional promoting factors for cancers of the esophagus (25). Finally, a role for human papilloma virus (HPV) has also been suggested in the etiology of SCC of the esophagus. A low frequency of HPV-16 or HPV-18 positivity has been reported in esophageal tumor samples (26). The exact role of HPV infections in esophageal carcinogenesis is yet to be elucidated.

Molecular alterations in human esophageal SCC

Molecular studies of human esophageal tumors have revealed frequent genetic abnormalities (Table II). Regardless of patient origin and suspected etiological factors, genetic changes that are consistently observed in esophageal SCC are: (i) alterations in tumor suppressor genes, specifically *p53*, leading to altered DNA replication and repair, cell proliferation and apoptosis; (ii) disruption of the G_1/S cell cycle checkpoint and loss of cell cycle control; (iii) alterations in oncogene function leading to deregulation of cell signaling cascades (27,28). Unlike tumors of the lung, skin and colon, mutational activation of *ras* genes is conspicuously absent from primary human tumors of the esophagus. However, some of the human cell lines established from esophageal SCCs have been shown to contain *ras* gene mutations (29). Moreover, when a mutated *ras* gene was transfected into a non-tumorigeneic cell line the transfectants became tumorigenic (29). These data suggest that

Table II. Molecular alterations in human esophageal SCC

	References
Loss of heterozygosity	
1p, 3p, 4, 5q, 9, 11q, 13q, 17q, 18q	28, 43
Loss of tumor suppressor gene function	
p53 mutation	30, 31
Methylation and/or loss of *p16MST1* and or *p15*	32
Reduced *Rb* expression	34
Gene amplification	
cyclin D1	34
HST-1	35
EGFR	35, 36
INT-2	37
Increased expression	
iNOS	38
hTERT	39
BMP-6	40
COX-2	41
c-*myc*	36
β-catenin	42

altered Ras function can contribute to human esophageal SCC development. Deregulation of Ras signaling, perhaps due to alterations in its upstream or downstream affectors, may be a potential mechanism. Some of the other genetic alterations that are commonly associated with clinical tumors include *p53* mutations (30,31), loss of p16MST1 and/or p15 (32) and/or RARβ (33) expression, amplification of cyclin D1, HST-1, EGFR and INT-2 (34–37), elevations in iNOS, hTERT, BMP-6, COX-2 and c-Myc expression (36,38–41) and cytoplasmic β-catenin levels (42). One or several of these alterations contribute to the growth and metastatic potential of these tumors (for an extensive review see references 27,28). In addition, loss of heterozygosity on chromosomes 1p, 3p, 4, 5q, 9, 11q 13q, 17q and 18q have also been frequently observed in tumors, supporting a loss of putative tumor suppression function (reviewed in references 28,43). However, the exact nature of these candidate genes, except for chromosome 17, has not been clearly defined. Chromosome region 17q25.2–25.3 carries the tylosis esophageal cancer (TOC) gene (44,45). Tylosis is an autosomal dominant trait characterized by hyperkeratosis palmaris et plantaris, which can be associated with a very high risk of esophageal cancer. In certain pedigrees with this syndrome >90% of the affected members will develop esophageal cancer by the time they reach the age of 70 (46). The exact function of TOC remains to be elucidated and awaits cloning and sequencing of the gene. Recently two other putative tumor suppressor genes, *FEZ-1* on chromosome 8q22 and *DLC1* on 3p21, have been identified as novel candidates that may play a role in esophageal carcinogenesis, since their expression is lost in some sporadic tumors (47,48). In contrast to an extensive literature on genetic alterations in frank tumors, very little is known regarding the genetic alterations in precancerous lesions of the esophagus. In a recent study focal accumulation of p53 protein has been observed in areas of esophagitis, suggesting that loss of suppressor function of this protein may be an early event during esophageal carcinogenesis (28). Molecular studies of tumors from high incidence areas in China have shown that alterations of oncogenes and tumor suppressor genes in clinical tumors are similar to changes seen with NMBA treatment of human fetal esophageal epithelium in culture (49). These

observations provide further evidence that exposure to nitrosamines is an important environmental risk factor in the pathogenesis of cancer of the esophagus.

Recent investigations into host susceptibility factors have led to the identification of polymorphisms in candidate genes that may determine an individual's risk for developing esophageal SCC. Evidence has accumulated to suggest that genetic polymorphisms in carcinogen-metabolizing enzymes may be of importance in determining an individual's susceptibility to cancer (50,51). Cytochrome P450 2E1 (CYP2E1) is one such enzyme that is involved in the metabolic activation of nitrosamines (52,53). In a large study involving residents of Linxian county in China, 146 cases with dysplasia, 150 cases with esophageal carcinoma and 150 controls were evaluated for polymorphisms in several Phase I and Phase II metabolizing enzymes (5A). A 3-fold increased risk of both dysplasia and cancer of the esophagus was observed among subjects with the *c*1/*c*1 genotype of CYP2E1. Additional correlations were made between esophageal cancer risk and allelic polymorphisms in the glutathione S-transferase M1 gene (54). Individuals with the *c*1 genotype are known to have a higher CYP2E1 enzyme activity as compared with other variants (55). However, the data on allelic polymorphisms as risk factors for esophageal cancer are relatively scant and need to be further evaluated before any conclusions can be drawn.

Approaches to prevention of SCC of the esophagus

In view of these studies it is clear that the occurrence and development of esophageal SCC is a result of interactions between environmental and genetic factors. Environmental carcinogens have repeatedly been shown to affect the genetic material of host cells, inducing uncontrolled growth and, ultimately malignant tumors. Hence, human esophageal carcinogenesis is a multifactorial, multistep process. In view of the exposures described, one approach to the prevention of esophageal SCC is through changes in lifestyle, including avoidance of alcohol and tobacco use. Additional benefits may be realized with the elimination of high salt foods that may be contaminated with microbial toxins, nitrosamines and their precursors. Significant educational efforts are necessary to inform populations at risk of the major role of these factors in development of the disease. Chemoprevention, to address factors associated with the etiology and progression of the disease, is another viable approach. It may have special relevance in high incidence areas of the world where carcinogen exposure is high. Animal models provide an excellent opportunity to evaluate chemoprevention strategies against cancer. The rat has been used almost exclusively as an animal model for studies of esophageal cancer. The remainder of this review discusses molecular alterations and chemoprevention approaches in the rat model and their relevance to human esophageal SCC.

Rat esophageal tumor model

Nitrosamine-induced tumorigenesis in the Fischer 344 rat has been found to be a useful model for molecular biology and chemoprevention studies of esophageal SCC (4,18). Several nitrosamines (Figure 1) act as fairly specific inducers of tumorigenesis in the rat esophagus, including the food contaminant NMBA and the tobacco-specific nitrosamine *N*-nitrosonornicotine (NNN). NMBA is by far the most potent inducer of tumors in the rat esophagus. As for other nitro-

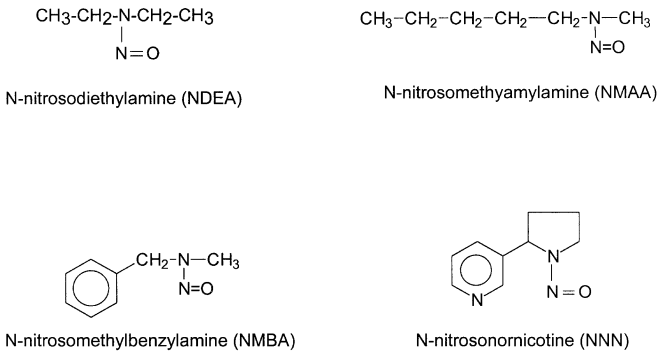

Fig. 1. Structures of some esophageal carcinogens.

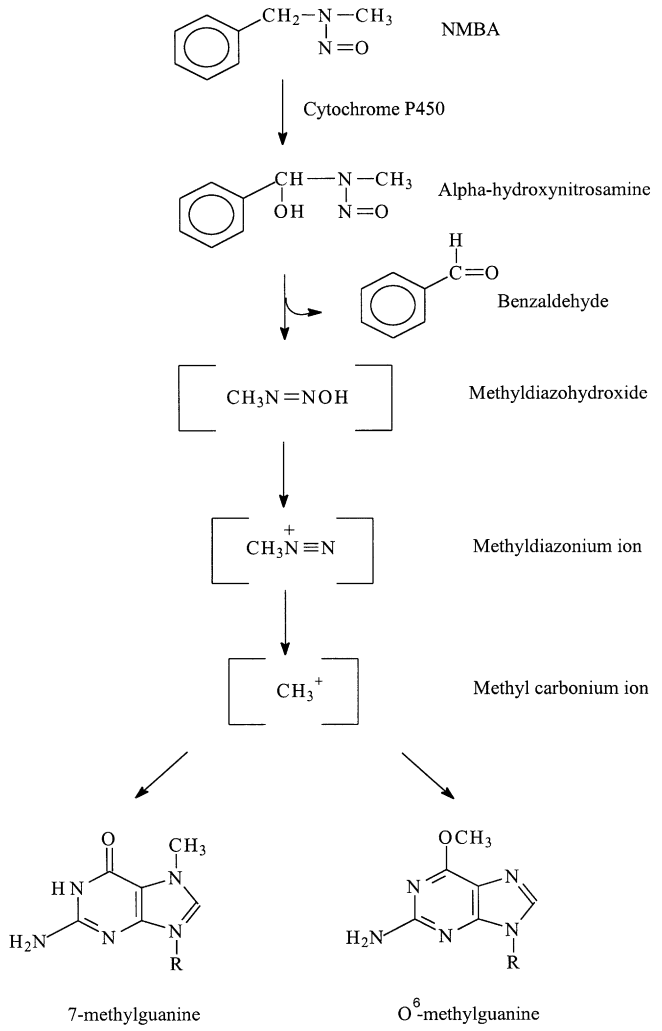

Fig. 2. Schema for metabolic activation of NMBA.

samines, the first step in the metabolism of NMBA involves hydroxylation of the methylene carbon by esophageal cytochrome P450 enzymes (Figure 2). This reaction produces the α-hydroxy derivative **1**, which spontaneously decomposes to methyldiazohydroxide and benzaldehyde. Methyldiazohydroxide leads to formation of the methylcarbonium ion, the ultimate electrophilic species that methylates guanine residues at the N^7 and O^6 positions (reviewed in reference 18). Irrespective of its route of administration, repeated dosing with NMBA

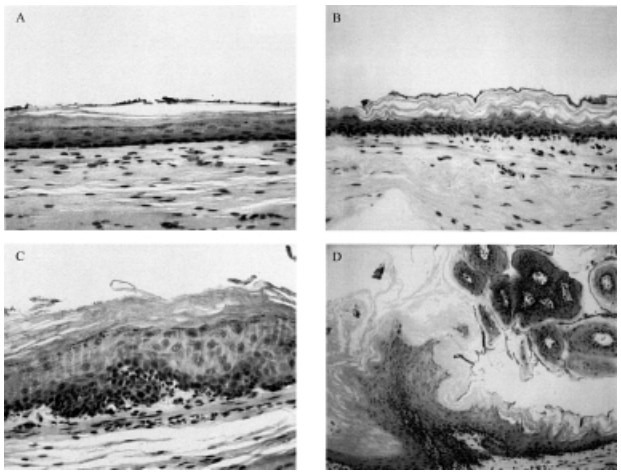

Fig. 3. Histological representation of NMBA-induced tumorigenesis in the rat esophagus. (**A**) Photomicrograph of an H&E stained normal rat esophagus (200× magnification). (**B**) Areas of hyperplasia with hyperkeratosis are among the earliest histological lesions seen following multiple treatments with NMBA (200× magnification). (**C**) Histological progression includes dysplasia, characterized by cellular atypia and disorganization of the basal cell layer of the epithelium (200× magnification). (**D**) At 15–20 weeks following NMBA treatment multiple papillomas can be seen in the esophagi of these animals (100× magnification).

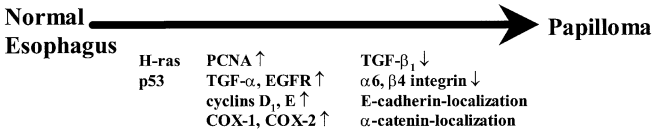

Fig. 4. Molecular events in papilloma development during NMBA-induced rat esophageal tumorigenesis.

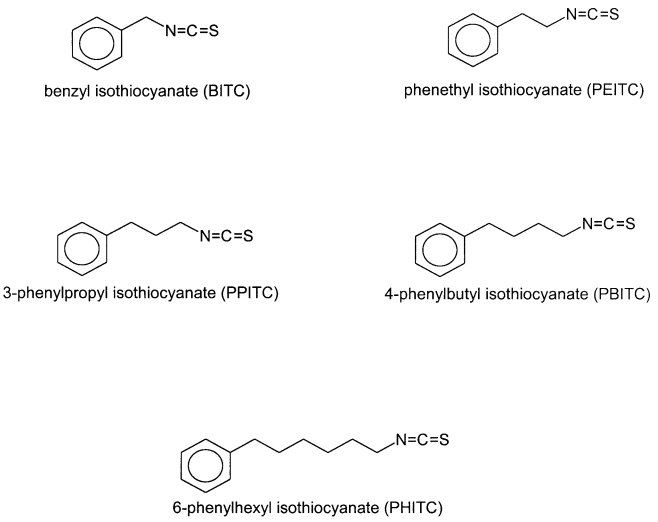

Fig. 5. Structures of isothiocyanates.

results in tumors within 15–20 weeks after initiation of exposure. NMBA-induced esophageal tumorigenesis in the rat closely mimics the human disease in that several preneoplastic lesions are also produced. These include simple hyperplasia, leukoplakia and epithelial dysplasia (Figure 3). Squamous papilloma is the predominant tumor histology in this model; the incidence of SCC is rather low since the animals often succumb to the occlusive effects of large papillomas in their esophagi before carcinomas can develop. In a typical tumor bioassay s.c. administration of NMBA at 0.5 mg/kg body wt three times a week for 5 weeks or once weekly for 15 weeks resulted in a 100% tumor incidence by 20 weeks (18). In the past several years our laboratory and others have used this model to develop surrogate end-point biomarkers, identify novel targets for intervention and therapy and evaluate putative chemoprevention agents against esophageal SCC.

Genetic analyses of rat esophageal tumors suggest that mutations in oncogenes and tumor suppressor genes are most likely due to formation of methylated guanine adducts in the DNA. As has been documented in human esophageal tumors, characteristic G:C→A:T transitions have been observed in the *p53* gene in ~30% of rat esophageal papillomas. These transitions have been found to be evenly distributed across the gene; no 'hot-spots' have been found for these mutations in the *p53* gene (56,57). In another study a 2.8-fold elevation in cyclin D1 mRNA levels was observed in papillomas. Cyclin E mRNA levels were also found to be elevated. Immunohistochemical staining revealed extensive nuclear staining for both G_1 cyclins (58,59). These observations suggest that cell cycle regulation is also altered during rat esophageal tumorigenesis. Increased expression of EGFR and proliferating cell nuclear antigen (PCNA), deregulated expression of transforming growth factor β1 and altered localization of E-cadherin and α-catenin have also been documented in these tumors (Figure 4) (18,60,61). A recent study from our laboratory has demonstrated elevated levels of COX-2 mRNA in papillomas and preneoplastic esophageal tissues. Additional studies have shown that tissue levels of prostaglandin E_2 are also elevated in papillomas (P.S.Carlton, unpublished data). Further studies will be necessary to investigate the significance of these findings. As has been observed in other animal models of chemical carcinogenesis, elevated COX-2 expression and function may play a role during the promotion/progression stages of esophageal tumorigenesis and provide an attractive target for future chemoprevention studies. In contrast to human esophageal tumors, a large majority, between 60 and 100%, of NMBA-induced papillomas in the rat esophagus carry a G:C→A:T mutation in codon 12 of the Ha-*ras* gene (57,62). The functional relevance of *ras* activation in this model is yet to be elucidated. Based on the evidence from chemically induced tumors in the mouse skin, where mutational activation of *ras* is a critical early event (63), and the fact that the G→A transition is the predicted mutation resulting from nitrosamine exposure, one would postulate that *ras* mutation occurs early and is mechanistically involved in NMBA-induced rat esophageal tumorigenesis. Results from a recent study, however, indicate that the G→A mutation in codon 12 of the *ras* gene is detected at very low frequency in premalignant lesions of the esophagus. A significantly higher number of papillomas have *ras* mutations. Thus, mutational activation of *ras* may play a role during the later stages of rat esophageal tumorigenesis (64).

Chemoprevention studies

Research in other animal models has shown that multiple compounds in foods have the ability to inhibit chemically induced cancer. Several pure compounds that function as anti-initiating agents inhibit NMBA-induced tumorigenesis in the rat esophagus. Ellagic acid (EA), a naturally occurring polyphenol, when given in the diet for the duration of the experiment at concentrations of 0.4 and 4.0 g/kg, significantly inhibits tumor development (65). EA was found to inhibit

Table III. Effects of arylalkyl isothiocyanates of different chain length on the induction of esophageal tumors in F344 rats by NMBA[a]

Group	Treatment	Tumor incidence (% inhibition)[b]	Tumor multiplicity (% inhibition)[c]
Experiment 1			
1	Vehicle control	0^{1}	0.0^{1}
2	NMBA control	100^{4}	6.7 ± 0.8^{3}
3	2.5 μmol/g BITC + NMBA	100^{4}	6.5 ± 0.6^{3} (4)
4	1.0 μmol/g BITC + NMBA	100^{4}	4.1 ± 0.6^{2} (38)
5	0.4 μmol/g BITC + NMBA	100^{4}	$5.6 \pm 0.7^{2,3}$ (17)
6	2.5 μmol/g PEITC + NMBA	$7^{1,2}$ (93)	0.1 ± 0.1^{1} (99)
7	1.0 μmol/g PEITC + NMBA	$40^{1,2,3}$ (60)	0.4 ± 0.1^{1} (94)
8	0.4 μmol/g PEITC + NMBA	$57^{2,3,4}$ (43)	1.1 ± 0.5^{1} (83)
9	2.5 μmol/g PPITC + NMBA	0^{1} (100)	0.0 ± 0.0^{1} (100)
10	1.0 μmol/g PPITC + NMBA	$7^{1,2}$ (93)	0.1 ± 0.1^{1} (99)
11	0.4 μmol/g PPITC + NMBA	$7^{1,2}$ (93)	0.1 ± 0.1^{1} (99)
12	2.5 μmol/g PBITC + NMBA	100^{4} (0)	4.0 ± 0.4^{2} (40)
13	1.0 μmol/g PBITC + NMBA	$93^{3,4}$ (7)	$5.1 \pm 0.7^{2,3}$ (24)
14	0.4 μmol/g PBITC + NMBA	$93^{3,4}$ (7)	3.9 ± 0.7^{2} (41)
15	2.5 μmol/g BITC	0	0.0
16	2.5 μmol/g PEITC	0	0.0
17	2.5 μmol/g PPITC	0	0.0
18	2.5 μmol/g PBITC	0	0.0
Experiment 2			
1	Vehicle control	0^{1}	0.0^{1}
2	NMBA control	100^{2}	7.2 ± 0.7^{2}
3	2.5 μmol/g PHITC + NMBA	100^{2} (0)	12.2 ± 1.1^{3} (869)
4	1.0 μmol/g PHITC + NMBA	100^{2} (0)	11.6 ± 1.2^{3} (861)
5	0.4 μmol/g PHITC + NMBA	100^{2} (0)	8.7 ± 0.9^{2} (821)
6	2.5 μmol/g PHITC	0	0.0

[a]Table adapted from Stoner *et al.* (80).
[b]Values with different individual numerical superscripts are statistically different from each other as determined by the χ^2 test (a Bonferroni adjustment was used to ensure an overall $P < 0.05$).
[c]Values are means ± SE. Values within this column that have no individual numerical superscripts in common are statistically different from each other as determined by ANOVA and the Newman–Keuls' ranges test ($P < 0.05$).

metabolic activation of NMBA into electrophillic species, as well as stimulate activity of Phase II enzymes involved in their detoxification (66,67). Addition of 13-*cis*-retinoic acid to the diet antagonized the preventive effects of EA (68). Diallyl sulfide, a component of garlic that acts principally by stimulation of Phase II enzymes, was also found to be an effective inhibitor of NMBA-induced tumorigenesis in the rat esophagus (69,70). The polyphenol fraction of black tea (theaflavins), as well as green tea (–)-epigallocatechin 3-gallate (EGCG), had a modest effect on tumor multiplicity when administered in the drinking water (71).

One of the most interesting groups of anti-initiating agents evaluated in the rat esophagus is the arylalkyl isothiocyanates (Figure 5). Phenethyl isothiocyanate (PEITC) is found as a glucosinolate in many cruciferous vegetables, such as cabbage, Brussels sprouts, cauliflower, etc., and is known to inhibit the metabolism of and DNA methylation by a series of nitrosamine carcinogens both *in vivo* and *in vitro* (72–74). Dietary administration of PEITC at concentrations of 3.0 mmol/kg diet or greater completely inhibits NMBA-induced esophageal tumorigenesis in the rat (74). At lower concentrations a significant reduction in tumor multiplicity was seen (75). Since isothiocyanates of longer alkyl chain length are more effective inhibitors of NNK tumorigenesis in strain A/J mouse lung, we also examined the effect of alkyl chain length as a function of inhibition of NMBA-induced tumorigenesis in the rat esophagus (Table III). We found the inhibitory activity of isothiocyanates to correlate with increasing side chain length. Phenylpropyl isothiocyanate (PPITC) was considerably more potent than PEITC, whereas benzyl isothiocyanate (BITC), a shorter chain length isothiocyanate, was less active. Reductions in NMBA-induced O^6-methylguanine levels in esophageal DNA following dietary administration of various isothiocyanates were found to correlate with the extent of inhibition of tumor incidence and multiplicity (76). Dietary PPITC was also found to effectively inhibit tumorigenesis from another important esophageal carcinogen, the tobacco-specific nitrosamine NNN (77). Interestingly, phenylbutyl isothiocyanate (PBITC) was found to be less potent than PPITC and phenylhexyl isothiocyanate (PHITC) actually enhanced the tumor response to NMBA (76,78–80). The mechanism of this enhancement does not appear to be due to either a stimulatory effect of PHITC on NMBA activation or an inhibitory effect of PHITC on DNA repair (81). Recent studies suggest that PHITC perhaps induces a cytotoxic effect in the rat esophagus and results in increased cell proliferation (82).

Whereas short alkyl chain isothiocyanates have proven effective agents against esophageal SCC in the animal model, their usefulness may be limited since they have no effects on NMBA tumorigenesis if administered post-initiation (83). Similarly, dietary sulindac and supplemental calcium and selenium were ineffective when administered post-initiation, while EA had only a modest effect on esophageal tumorigenesis (83,84). Decaffeinated green tea and black tea were found to be effective in the post-initiation period when given at very high concentrations (85). For a chemopreventive agent to be effective against human esophageal SCC it should possess significant inhibitory activity when administered subsequent to carcinogen exposure. To date, very few of the single compounds tested have been found to be effective inhibitors

Table IV. Effect of STRW on the induction of esophageal tumors and on formation of O^6-methylguanine in F-344 rats treated with NMBA[a]

Group	Treatment	Tumor incidence[b] (% inhibition)	Tumor multiplicity[c] (% inhibition)	O^6-mGua/DNA[c] (pmol) (% inhibition)
1	Vehicle control	0^1	0.0^1	0.0^1
2	NMBA control	100^2	4.1 ± 0.2^3	4.4 ± 0.9^3
3	NMBA+5% STRW	100^2 (0)	3.1 ± 1.0^2 (24)	1.4 ± 0.1^2 (68)
4	NMBA+10% STRW	80^2 (20)	1.8 ± 1.4^2 (56)	1.9 ± 0.7^2 (57)
5	10% STRW	0^1	0.0^1	0.0^1

[a]Table adapted from Stoner *et al.* (80).
[b]Values with different individual numerical superscripts are statistically different from each other as determined by the χ^2 test (a Bonferroni adjustment was used to ensure an overall $P < 0.05$).
[c]Values are means ± SE. Values within this column that have no individual numerical superscripts in common are statistically different from each other as determined by ANOVA and the Newman–Keuls' ranges test ($P < 0.05$).

of the promotion/progression stages of NMBA tumorigenesis in the rat esophagus. Furthermore, some of these agents can, in fact, enhance tumor development in the model. We have recently found evidence that a synthetic amide of all-*trans*-retinoic acid, *N*-(4-hydroxyphenyl)retinamide (4-HPR), significantly enhances esophageal tumorigenesis. At 0.8 gm/kg diet 4-HPR increased tumor multiplicity by 2.4- and 3.7-fold, respectively, in two independent tumor bioassays. Enhanced NMBA metabolism and DNA adduct formation, as well as increased average tumor size, contributed to 4-HPR effects on rat esophageal tissues (86; A.Gupta, unpublished data).

A 'food-based' approach to cancer chemoprevention is emerging as an alternative to the use of single compounds. Dietary supplementation with various freeze-dried vegetables has been found to be effective in a rodent model of colon carcinogenesis (87). Additional studies have documented the ability of whole foods, such as tomato juice, paprika juice, dry beans and soybeans, to inhibit carcinogenesis in animal model systems (88–93). The varied geographical distribution of human esophageal SCC in the world has been linked to diets deficient in fresh fruits and vegetables (20). Encouraged by these observations, we have followed a similar food-based approach to prevent NMBA-induced esophageal cancer. In an initial study we evaluated various fruits and nuts for their EA content. In particular, the strawberry (*Fragaria ananassa*) was found to contain high levels of EA, with most of the compound in the strawberry pulp (94). We initially evaluated a lyophilized preparation of strawberry (STRW) for its anti-initiation effects. At 10% concentration in the diet STRW administration resulted in a >50% reduction in tumor multiplicity in the rat esophagus (Table IV). This inhibition correlated with the ability of the berries to reduce the formation of O^6-methylguanine in esophageal DNA. In addition, 5 and 10% STRW significantly reduced dysplastic leukoplakia, a premalignant lesion, by 52 and 65%, respectively, when compared with NMBA controls. In contrast, the number of lesions classified as simple leukoplakia were found to be increased in these animals. At 5 and 10% in the diet STRW also reduced tumor multiplicity by >30% when administered only following NMBA treatment (80,95). In another set of studies, addition of freeze-dried black raspberries (BRB) to the diets in the post-initiation period also resulted in a significant reduction in tumor multiplicity (96,97). In these studies BRB preparations were also found to reduce PCNA positivity, a marker of cell proliferation, in esophageal tumors. While the reduction in DNA adduct levels points towards strong anti-initiating effects of berry preparations, their effects on premalignant lesions suggest a potential effect during the post-initiation phases of NMBA-induced esophageal carcinogenesis. It is possible that components of STRW and/or BRB act to arrest the progression of less advanced lesions, such as simple leukoplakia, to dysplastic lesions. Alternatively, berry components may induce cell differentiation in dysplastic epithelium, resulting in a reversal of these lesions to a less advanced phenotype. Although EA could, in part, account for the anti-initiation effects, additional compounds present in berries must contribute to their overall preventive effects, especially since EA is a poor anti-promotion/progression agent against NMBA-induced rat esophageal tumorigenesis (83). Some of these components, such as calcium and vitamin C, have been shown to influence cell proliferation and differentiation (98,99). Methanol and acetone/water extracts of strawberries also contain numerous compounds, several of which are at higher levels than EA (94). We have recently shown that certain methanol fractions have the ability to inhibit benzo[*a*]pyrene-induced Syrian hamster embryo cell transformation *in vitro* (100). Additional studies are now underway to identify compounds that are responsible for the ability of strawberries to inhibit cell transformation and both the initiation and progression stages of esophageal tumorigenesis.

Taken together, results from chemoprevention studies in the rat esophagus model have thus far identified several pure compounds that are potent anti-initiation agents (Table V). Their efficacy as anti-promotion/progression agents has been limited. Results from a food-based approach with berry preparations have been encouraging and warrant further investigation.

Biomarker studies

For screening of chemopreventive agents in clinical trials the use of cancer incidence reduction as an end-point is generally not feasible due to the exceptionally large numbers of subjects and long periods of observation necessary for such studies. Use of intermediate end-points based on morphological and/or molecular alterations in a given cancer is necessary for the successful conduct of clinical trials in cancer chemoprevention. Although several molecular alterations have been identified in rat esophageal papillomas, their utility remains limited. A majority of the studies have involved a simple quantitation of early (simple leukoplakia) and late (dysplasia) preneoplastic lesions, based on their histological features. Although such analyses are informative, they lack the sensitivity and objectivity necessary for a large-scale clinical study. Among the early markers in the rat model, DNA adduct levels in esophageal tissues soon after NMBA treatment have been found to be a useful marker to assess anti-initiation effects of

Table V. Preventative agents effective against NMBA-induced rat esophageal cancer

Agent	Proposed mechanism of action	References
Pure compounds		
Diallyl sulfide	Stimulation of Phase II enzyme activities	69, 70
Ellagic acid	Inhibition of Phase I enzyme activites, stimulation of Phase II enzyme activites	65–67
Isothiocyanates	Inhibition of Phase I enzyme activities	74–78, 80
Tea polyphenols	Inhibition of post-initiation events	71, 85
Food-based		
Freeze-dried strawberries	Inhibition of DNA adduct formation, inhibition of post-initiation events	80, 95
Freeze dried black raspberries	Inhibition of DNA adduct formation, inhibition of post-initiation events	96, 97

chemopreventive agents. Assessment of cell proliferation, by way of PCNA labeling index, is another marker of effect in this model (18).

Among others, computer-assisted quantitative image tile analysis (CAQITA) has been developed as a sensitive, quantitative and objective assessment of the degree of neoplastic change in premalignant lesions. The underlying assumption in grading intraepithelial neoplasia is that the greater the degree of abnormal deviation of nuclear and tissue architecture from normal, the further the lesion has progressed from its inception and the less time remains before it may progress to invasive cancer (101,102). We have evaluated the use of CAQITA as a surrogate marker for esophageal tumorigenesis in the rat model. In an initial validation assay changes in nuclear/nucleolar morphometry were analyzed in rat esophageal epithelium at different times subsequent to NMBA treatment. With a computerized imaging system sections of esophageal epithelium were divided into a contiguous row of image 'tiles' and a set of tissue features measured within each such image tile. These measurements were transformed into a *Z* score and compared between NMBA-treated and control tissues. The higher the score, the greater was the deviation from normal for a given 'tile'. Using two grading variables, mean tile grade (MTG) and percent tile grade >4 SD (%TG>4SD), these studies showed that %TG>4SD was a more sensitive indicator of neoplastic response to NMBA as compared with tumor incidence and tumor multiplicity. By week 10 following NMBA treatment %TG>4SD had already reached a relatively high value of 50%, even though no gross tumors were visible at this time. As a part of the validation the suppressive effects of PEITC on NMBA-induced esophageal tumorigenesis were quantitated by this technique. We found parallel reductions in MTG, tumor incidence and tumor multiplicity with PEITC administration (103). In another study the greater sensitivity and reproducibility of MTG in grading neoplastic changes and detecting a response to a chemopreventative agent (theaflavins) has been documented (104). These studies demonstrate the usefulness of MTG as an intermediate end-point in animal models and, possibly, human chemoprevention trials. Because of its increased sensitivity, MTG should be able to detect a greater difference in tissue biopsies and cytological smears before and after treatment of a given individual with a chemopreventive agent. This could allow for significant reductions in sample size without a loss of statistical power in future prevention studies.

Chemoprevention of human esophageal SCC: past, present and future

An important paradigm in clinical cancer chemoprevention studies must be to block the progression of premalignant lesions, such as epithelial dysplasia, to malignant SCC. With the availability of improved endoscopic and cytological screening methods, the identification and follow-up of esophageal dysplasia among high risk populations has become possible. The combined use of 'balloon cytology' coupled with endoscopic evaluation in China has been useful in identifying individuals with premalignant lesions and improving their survival by clinical intervention. These population cohorts have been a subject of limited intervention trials for primary chemoprevention of esophageal SCC.

As has been seen in the rat model of esophageal SCC, studies in human cancer have found a relationship between the pattern and rate of esophageal cell proliferation and risk for the disease. Individuals at a higher risk for esophageal cancer show a more rapid rate of cell proliferation in the superficial and intermediate layers of esophageal epithelium (105). Additional studies have found a positive correlation between increasing rates of cell proliferation and histological progression of premalignant lesions from hyperplasia to mild and moderate dysplasia (106). Consequently, clinical trials of efficacy have been conducted using candidate chemopreventive agents that may inhibit cell proliferation rates in the esophagus. However, these studies have met with limited success. In a study among residents of Linxian, China, daily supplementation with vitamins and minerals were evaluated over a period of 30 months. Esophageal lesions previously diagnosed as acanthosis, esophagitis, squamous dysplasia and SCC were followed for cell proliferation rates. At the end of the observation period no treatment effect on the overall amount of squamous epithelial proliferation was found in any of the histological categories (107). In another study supplementation of the diets of 200 subjects with calcium (1200 mg/day) for 11 months did not result in reduced rates of cell proliferation in the esophageal epithelium in either hyperplastic or dysplastic lesions (108). Although high levels of calcium have been shown to inhibit cell proliferation and stimulate cell differentiation in esophageal epithelial cells *in vitro* (98), these results are reminiscent of a lack of efficacy of this supplementation in the rat model of esophageal tumorigenesis. Even though cell proliferation rates were not measured, a study of the effects of antitumor-B (ATB; a mixture of Chinese herbs), a retinoid (4-ethoxycarbophenylretinamide; 4-ECPR) and riboflavin supplementation in the diet of subjects diagnosed with mild or marked esophageal dysplasia in Hunan, China, revealed a significant reduction in cancer development from pre-existing dysplasia. ATB treatment for 3–5 years reduced the cancer development rate by 52 and 47%, respectively. 4-ECPR lowered clinical cancer by 37–43%. The overall incidence of cancer was unaffected in subjects supplemented with riboflavin (109). The exact composition of ATB and its mechanism of

action against esophageal SCC remain to be elucidated. However, these results are encouraging and warrant additional experimentation.

It is clear that additional studies are needed to develop effective and practical chemopreventive strategies for human esophageal SCC. Results from animal studies offer several possibilities for future evaluation in a clinical setting. Dietary addition of inhibitors of the metabolic activation of nitrosamines and polycyclic aromatic hydrocarbons can be protective against this disease. Chemopreventive agents of potential use include isothiocyanates, such as PEITC and PPITC, and certain polyphenolic compounds, such as EA or the green tea polyphenol EGCG. Dietary addition of diallyl sulfide, sulforaphane or oltipraz, all of which promote carcinogen detoxification through stimulation of glutathione S-transferase and other phase II enzymes, could be protective. A food-based approach with freeze-dried strawberries or black raspberries is another possibility, especially given their effects on promotion/progression events in the rat model. A combination approach involving the supplementation of berry preparations with low amounts of isothiocyanates or other single agents that show efficacy could enhance the preventive effects of these regimens without increasing treatment toxicities. Use of novel end-point biomarkers, such as CAQITA, can greatly simplify the design and conduct of clinical trials in the future.

Special emphasis needs to be placed on the identification of critical molecular determinants in the development of esophageal SCC. Mechanistic studies in the Fischer 344 rat model can provide important clues as to new targets for intervention, as well as markers of effect for future human clinical trials, and will significantly contribute to the design of effective chemoprevention protocols for human esophageal SCC.

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