

# Quantitative Grading of Rat Esophageal Carcinogenesis Using Computer-assisted Image Tile Analysis

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## Abstract

**Our objective was to grade, by computer-assisted quantitative image tile analysis, the intraepithelial neoplasia (also called dysplasia) that develops in esophagi of rats given *N*-nitrosomethylbenzylamine (NMBA) for 5 weeks. To perform image tile analysis, the computer divides the video image of the neoplastic epithelium into a row of contiguous small rectangular images, or “tiles,” 84 × 292 μm in size, and quantitatively measures four selected tissue features within each image tile. The computer then calculates a tile grade for each image tile as the weighted sum of the four feature measurements, transformed into statistical Z-scores, the weights being determined by Fisher linear discriminant analysis of 300 tile grades of the neoplastic epithelium referenced to the mean tile grade (MTG) of 300 image tiles of normal epithelium. The two grading parameters, MTG and the percentage of tile grades exceeding the MTG of normal epithelium by >4 SD units (%TG>4SD), were validated as endpoints for screening chemopreventive agents in the rat NMBA-induced esophageal carcinogenesis model in two ways: (a) after NMBA treatment, %TG>4SD developed in parallel with tumor incidence and tumor multiplicity (number of papillomas/tumor-bearing rat); and (b) placing the chemopreventive phenethylisothiocyanate in the food of NMBA-treated rats produced parallel reductions in MTG, tumor incidence, and tumor multiplicity. Both MTG and %TG>4SD, measured by quantitative image tile analysis, are sensitive and objective continuous parametric response variables expressed to three significant figures, with wide dynamic range, that may be evaluated by *t* tests to compare tissue neoplastic changes before and after treatment with a chemopreventive agent.**

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## Introduction

When pathologists make the diagnosis of intraepithelial neoplasia (or equivalently, dysplasia), it is established practice to estimate the degree of cancer risk by assigning a grade to the lesion, such as “mild,” “moderate,” “severe,” or “carcinoma in situ,” based on the degree of abnormal morphological aberration of individual cell nuclei and on the presence of focal regions of increased cell density attributable to clonal expansions of proliferating basal cells (1, 2). The underlying assumption in grading intraepithelial neoplasia, generally born out in practice, 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. In this study, the use of CAQIA<sup>2</sup> to grade the degree of neoplastic change in the rat NMBA-induced esophageal carcinogenesis model (3) is validated as a method for screening cancer chemopreventive agents. The validation is carried out in two ways: (a) the CAQIA grading system is shown to correlate over time with two universally acknowledged measures of response to a carcinogen, tumor incidence and tumor multiplicity (the number of esophageal papillomas/rat); and (b) the reduction in CAQIA grade produced by placing the chemopreventive agent, PEITC (4), in the diet is shown to parallel closely the reduction produced in esophageal tumor incidence and multiplicity.

For screening cancer chemopreventive agents in clinical trials, the use of cancer incidence reduction as an endpoint is generally not feasible because it usually requires unacceptably large numbers of subjects and too many years of observation. In the Chemoprevention Program of the National Cancer Institute, many chemoprevention trials are being conducted in <2 years, with fewer than 100 subjects, through the use of intermediate endpoints based on morphological changes in the preinvasive lesion, intraepithelial neoplasia (also called dysplasia). In the Chemoprevention Program, the changes in intraepithelial neoplasia produced by a chemopreventive agent, in addition to being graded by a pathologist, are commonly measured by CAQIA, which supplements the grade assigned by the pathologist (1, 2) with a sensitive, quantitative, and objective assessment of the degree of neoplastic change. The details of grading intraepithelial neoplasia by CAQIA are described below.

## Materials and Methods

**Chemicals.** NMBA was obtained from Ash Stevens (Detroit, MI). PEITC was obtained from Aldrich Chemical Co. (Milwaukee, WI). NMBA dosing solutions (in 20% DMSO) were

<sup>2</sup> The abbreviations used are: CAQIA, computer-assisted quantitative image analysis; NMBA, *N*-nitrosomethylbenzylamine; PEITC, phenethylisothiocyanate; %TG>4SD, percentage of tile grades of neoplastic esophageal epithelium exceeding the mean tile grade of normal esophageal epithelium by >4 SD units; MTG, mean tile grade.

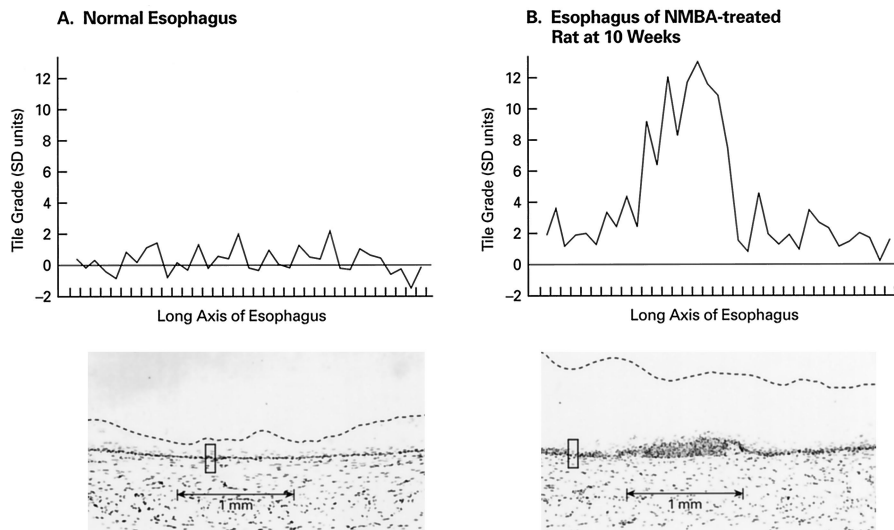


Fig. 1. Tile grades measured along the long axis of portions of esophagus from an untreated rat (A) and an NMBA-treated rat (B). Each tic on the *abscissa* of A and B represents the width of one tile, 84  $\mu\text{m}$ . Below each graph is a histological section of a segment of esophagus in which the size of one tile is illustrated. In the histological section below B, a plaque of high-grade intraepithelial neoplasia is shown.

stored at 4°C in amber bottles. NMBA and PEITC were assayed for purity by high-performance liquid chromatography, and their purity was >97 and >99%, respectively. NMBA was measured in a fume hood under subdued light. All other chemicals used were of the purest grade available.

**Preparation of Diets and Drinking Water.** Powdered AIN-76A control and experimental diets were prepared every 2 weeks. We have demonstrated previously that PEITC is stable in the AIN-76A diet for at least 2 weeks. The control diet consisted of 20% casein, 0.3% D,L-methionine, 52% cornstarch, 13% dextrose, 5% corn oil, 5% Alphacel, 3.5% AIN mineral mixture, 1% AIN vitamin mixture, and 0.2% choline bitartrate. Initially, all dry ingredients were mixed together for 5 min in a Hobart D-300 food mixer set on medium speed. Corn oil, or PEITC (3.0 micromol/g diet) in corn oil, was added, and the mixture was blended at medium speed for 15 min.

**Animals.** Male F344 rats were obtained from Harlan Sprague Dawley (Indianapolis, IN) at 5–6 weeks of age. Rats were kept in quarantine for 2 weeks prior to use. Animals were housed in groups of three in plastic cages with hardwood bedding (Beta Chips; Northeastern Products Corp., Warrenburg, NY). Rats were fed modified AIN-76A diet (Dyets, Bethlehem, PA), given water *ad libitum*, and were maintained under standard conditions (20  $\pm$  2°C, 50  $\pm$  10% relative humidity; 12-h cycles of light and darkness). Hygienic conditions were maintained by twice-weekly changes of the animal cages and water bottles; cages were sterilized before use. The floor of the animal room was sanitized once weekly with a detergent disinfectant (Roccal II; National Laboratories, Montvale, NJ). At the time of sacrifice, rats were euthanized by CO<sub>2</sub> inhalation and subjected to a complete gross necropsy examination. Esophagi were excised, opened longitudinally, affixed to white index cards mucosa side up, and preserved in 10% neutral buffered formalin. Esophageal tumors >0.5 mm were counted and mapped, and their sizes were measured.

**Sectioning and Staining.** Formalin-fixed esophagi were cut into four equal segments and oriented in paraffin blocks so that histological sections showed a cross-section of the entire esophageal wall. For each rat, one histological slide of the esophagus was prepared and stained with the DNA-Feulgen stain. No counterstain was used.

**CAQIA.** Image analysis equipment was the CAS 200 system with an attached x,y microscope stage Multiscan image recorder. All calculations described below were automatically performed by the computer. As illustrated in Fig. 1, in image tile analysis the computer first defines a row of contiguous small images, or “tiles,” overlying the esophageal epithelium, each tile measuring 84  $\mu\text{m}$  parallel to the long axis of the esophagus and 292  $\mu\text{m}$  at a right angle across the submucosa, basal cell layer, and part of the overlying keratin layer.

**Measurement of Four Tissue Features of Intraepithelial Neoplasia within Each Image Tile.** The computer was set to automatically perform measurements of four selected tissue features within each tile along the neoplastic esophageal epithelium at  $\times 40$ . For this study, the four tissue features selected for measurement within each image tile are closely related to the nuclear morphological features used by pathologists to make a diagnosis of intraepithelial neoplasia. Three of the tile features are related to the tendency of neoplastic epithelia to show an abnormal focal “piling up” of cells and a diffuse increase in cell density. Recalling that the histological sections have been Feulgen-stained for DNA, the first tile feature, “Sum OD per Tile,” in absorbance units, is proportional to the total DNA within a tile and becomes increased in tiles containing piled-up neoplastic nuclei, hypertetraploid nuclei, or both. The second tile feature is the “Fraction of Tile Area Covered by Nuclei,” in  $\mu\text{m}^2$ . The area covered by nuclei is defined by the border profiles of groups of overlapping neoplastic nuclei. The third tile feature is “Configureable Run Length with Step Length of 14.38 Micrometers,” in hole counts per tile. At  $\times 40$ , there are >20,000 pixels within a tile. In relation to a given pixel (the index pixel), the computer scans along contiguous rows of pixels in the vertical and horizontal directions and measures the absorbance of a second pixel 14.38  $\mu\text{m}$  away from the index pixel in each direction. If the second pixel has an absorbance less than that of the index pixel by 0.05 A units or greater, it is counted as a “hole.” Crowded and overlapping neoplastic nuclei within a tile tend to exhibit these steep absorbance dropoffs, or “holes,” between their intersecting profiles. The fourth tile feature, “Deep Valley Detector” (part of the software package named Valley Slope Peak produced by BLISS, Inc), in hole counts per tile, measures the tendency of

neoplastic nuclei to exhibit increasingly broken up and clumped chromatin texture. Pathologists describe this neoplastic property as “increased chromatin clumping,” “sharply marginated chromatin clumps,” and “irregular thickening and sharp margination of nuclear borders.” The computer reviews all pixel triplets in the horizontal, vertical, and two diagonal directions and counts as triplet holes those triplets whose center pixel has an absorbance less than that of either end pixel by 0.05 *A* units or greater. Triplet holes are found in neoplastic nuclei at sites of sharply marginated chromatin clumps and along the sharply marginated inner border of irregularly thickened nuclear membranes.

**Z-Score Transformation of the Four Feature Measurements within Tiles.** The raw measurement of each tissue feature within a tile is transformed into a statistically standard normal deviate, or Z-score, according to the equation,

$$Z = (x - X)/S,$$

where *x* is the raw measurement of a feature within a tile image from an NMBA-treated rat, *X* is the mean value of the same feature measured in tiles from normal rats, and *S* is the corresponding SD measured in tiles from normal rats. The four features/tile described above were measured in at least 300 tiles of neoplastic esophageal epithelium from NMBA-treated rats and 300 tiles of normal epithelium from untreated age-matched normal rats.

**Calculation of the MTG of NMBA-induced Esophageal Intraepithelial Neoplasia.** The grade of an individual tile is defined as the weighted sum of four tissue features measured within the tile, in Z-score units, where each weight is determined by Fisher linear discriminant analysis (5). The MTG is calculated from the grades of 300 tile images of neoplastic esophageal epithelium in a given tissue section; it is a single number expressed to three significant figures characteristic of the intraepithelial neoplastic epithelium, analogous to a grade given to the epithelium by a pathologist (such as “mild,” “moderate,” or “severe”). The MTG is a continuous parametric response variable scaled in SD units. When it is measured in sections of normal esophageal epithelium, values centered around zero result; when it is measured in esophageal epithelium from NMBA-treated rats, the MTG shifts to higher values. In the normal esophagi from 12 untreated rats at the 10th week after starting studies 1 and 2 below, the grand mean and SD of the 12 MTGs was  $0.025 \pm 0.723$  SD units; in 12 normal rats at the 20th week it was  $0.050 \pm 0.626$  SD units; and in 6 normal rats at the 25th week, it was  $0.057 \pm 0.570$  SD units. These data confirm that, as expected, the MTGs of normal esophageal epithelium cluster around zero.

**Calculation of the Percentage of Tile Grades Exceeding the MTG of Normal Epithelium by >4 SD Units (%TG>4SD).** The parameter %TG>4SD is defined as the percentage of tile grades of neoplastic esophageal epithelium that exceed the mean tile grade of normal esophageal epithelium (confirmed to be zero above) by >4 SD units. Like the MTG, the %TG>4SD is a continuous parametric variable scaled in SD units, which provides a single number characteristic of neoplastic esophageal epithelium analogous to a pathological grade given to the epithelium by a pathologist.

**Studies.** Male F344 rats were used in all experiments. Two studies were performed. Study 1 compared the development of the parameter %TG>4SD with the variables “tumor incidence” and “tumor multiplicity” (number of esophageal papillomas/tumor-bearing animal) after treatment with the regimen of 0.5 mg/kg NMBA s.c. three times a week for 5 weeks (4). Groups

of 22 NMBA-treated rats were sacrificed at 10, 15, and 20 weeks, and a larger group of 33 rats was sacrificed at 25 weeks.

Study 2 compared, in NMBA-treated animals, the concurrent reduction in Mean Tile Grade (MTG), tumor incidence, and tumor multiplicity produced by giving the chemopreventive agent, phenethylisothiocyanate (PEITC), in the diet of AIN 76A at a dose of 3 micromoles PEITC per gram of diet. A set of four groups, 6 rats per group, was sacrificed at 10, 20, and 25 weeks. The first group in each set consisted of normal untreated rats. The second group contained rats treated with PEITC only, the third group received NMBA only, and the fourth group received both NMBA and PEITC.

In both Study 1 and Study 2, the rats were acclimated on AIN-76A diet for two weeks, then randomly assigned to the different study groups. Body weights were recorded weekly throughout both studies.

## Results

**Pathological Changes in Rat Esophageal Epithelium after the Administration of NMBA.** Fig. 2 illustrates changes in the esophageal epithelium occurring 10 weeks after initiating the NMBA regimen of s.c. injections three times weekly for 5 weeks. These histopathological changes have been described in detail in previous reports by other laboratories and by our laboratory (6, 7). The keratin layer of the squamous epithelium had increased ~2-fold in thickness, an effect routinely seen after administration of NMBA (6, 7). Compared with normal esophageal epithelium (Fig. 2A), the epithelia of NMBA-treated rats exhibited changes consistent with the diagnosis of diffuse-moderate to high-grade intraepithelial neoplasia (1, 2), as illustrated in Fig. 2B. The nuclei of basal cells and overlying intermediate cells had increased in number and size and were more variable in both size and in shape (pleomorphism). Individual basal cell nuclei showed increased granulation of their chromatin and occasional coarse chromatin clumps. Nuclear borders were variably increased in thickness.

**Development of Multiple Plaques of High-Grade Intraepithelial Neoplasia.** As shown at low magnification in Fig. 1B and at high magnification in Fig. 2C, by the 10th week after starting NMBA treatment, there occurred every few mm along the epithelium sharply demarcated plaques of densely packed epithelial cells exhibiting the classic morphological features of high-grade intraepithelial neoplasia (5). The plaques varied from a fraction of a mm to 2 mm in length (Fig. 1B) and extended beneath the keratin layer from 5 to 15 nuclear diameters in thickness. As illustrated in Fig. 2C, cells in the plaques exhibited markedly increased nuclear size, abnormally altered nuclear shape, abnormal variability of nuclear size and shape, granulation and coarse clumping of nuclear chromatin, and nuclear borders that were variably thickened with focal regions of sharp margination of the inner nuclear border. The epithelial plaques appeared to represent early microscopic phases of papilloma development because their cell pattern progressively merged with the pattern of developing papillomas that became grossly visible by the 15th week. Visible papillomas all showed high-grade intraepithelial neoplasia. At the 20th and 25th weeks after initiation of NMBA treatment, the number of microscopic epithelial plaques with high-grade intraepithelial neoplasia had increased by more than 50%, and the intervening epithelium continued to exhibit diffuse moderate- to high-grade intraepithelial neoplasia. Wargovich *et al.* (8) reported that when the s.c. dose of NMBA was increased 7-fold over the dose used in this study, *i.e.*, from 0.5 mg/kg triweekly to 3.5 mg/kg triweekly, fewer papillomas appeared, and there was rapid pro-

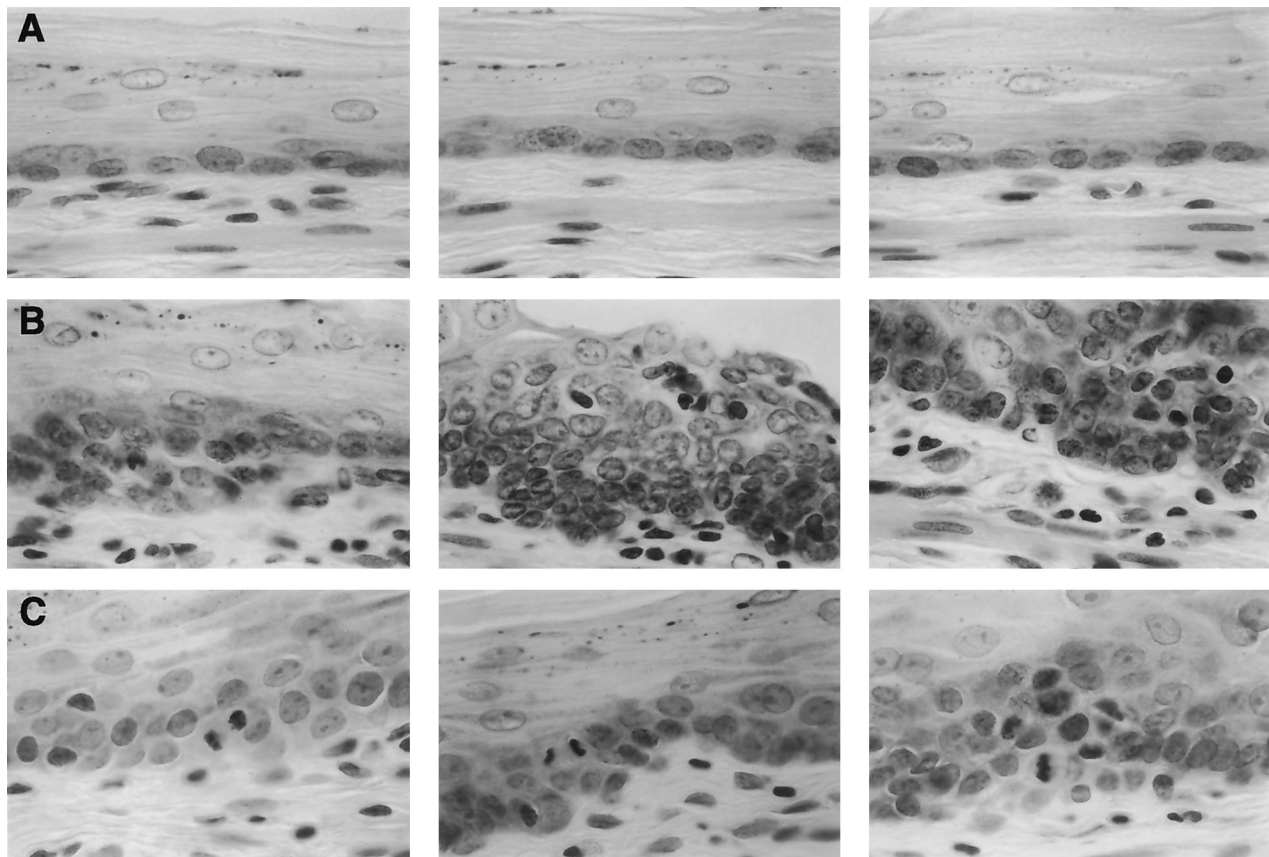


Fig. 2. Histological sections of rat esophageal epithelium at  $\times 1000$  stained with H&E. A, portion of normal esophageal epithelium from an age-matched untreated rat at 10 weeks. B, portion of esophageal epithelium from an NMBA-treated rat at 10 weeks showing moderate- to high-grade intraepithelial neoplasia. C, portion of an epithelial plaque from an NMBA-treated rat at 25 weeks showing high-grade intraepithelial neoplasia.

gression of the papillomas to multiple invasive squamous cell carcinomas by the 15th week (see the “Discussion” for details).

**Correlation between the Tile Grade Measured by CAQIA and the Classic Pathological Grade Assigned by Pathologists.** Fig. 1 shows that the tile grades of esophageal epithelium exhibiting diffuse-moderate to high-grade intraepithelial neoplasia measured between 2 and 4 SD units, whereas tile grades measured in the plaques, all of which exhibited high-grade intraepithelial neoplasia, exceeded 4 SD units and extended as high as 12 SD units. Thus, a useful correlation was shown between the MTG of intraepithelial neoplasia measured by CAQIA and the pathological grade of the same lesion made by a pathologist. Because the tile grades of esophageal epithelium between the plaques were  $<4$  SD units, and the tile grades of the plaques were always  $>4$  SD units, the value of the variable  $\%TG>4SD$  measured over the entire epithelium was correlated with the number of plaques present.

**Study 1: Comparison of an Increase in the Variable  $\%TG>4SD$ , Measured by CAQIA, with the Development of Tumor Incidence and Tumor Multiplicity.** Fig. 3 shows the time course of the neoplastic response of rat esophageal epithelium to treatment with NMBA, measured by the three variables of  $\%TG>4SD$ , tumor incidence, and tumor multiplicity. By the 10th week, the  $\%TG>4SD$  had already increased to  $>50$  percentage points, whereas tumors had not yet begun to develop, demonstrating that  $\%TG>4SD$  was the most sensitive

variable. The linearly increasing value of  $\%TG>4SD$  from 10 to 25 weeks paralleled the increase in tumor incidence to 100% at 15 weeks and increase in tumor multiplicity from 15 to 25 weeks.

**Study 2: Comparison Over Time of the Suppressive Effect of the Chemopreventive Agent PEITC on the Variable MTG with the Variables “Tumor Incidence” and “Tumor Multiplicity.”** Fig. 4 compares the suppression by PEITC of the MTG of esophageal intraepithelial neoplasia, measured by CAQIA, with the concurrent suppression by PEITC of Tumor Incidence and Tumor Multiplicity, at the 10th, 20th, and 25th week after start of the NMBA regimen. There was correlation between the degree of suppression of all three variables over time. Fig. 3 shows that between the 20th and 25th weeks, the MTG decreased  $\sim 25\%$ , whereas the  $\%TG>4SD$  continued to increase. An explanation for this observation is offered in the “Discussion.”

## Discussion

This study demonstrates the effective use of CAQIA to grade intraepithelial neoplasia developing in esophagi of rats given NMBA. The two grading variables, MTG and  $\%TG>4SD$ , defined in “Materials and Methods,” were validated as endpoints for screening of chemopreventive agents in the rat esophageal carcinogenesis model in two ways: (a) after NMBA

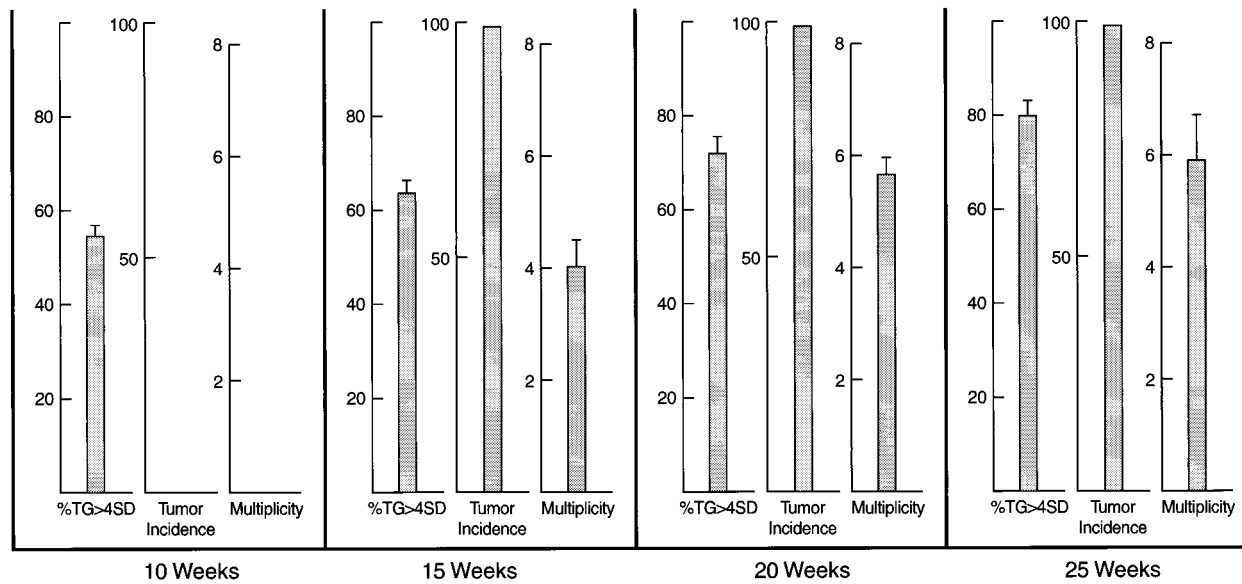
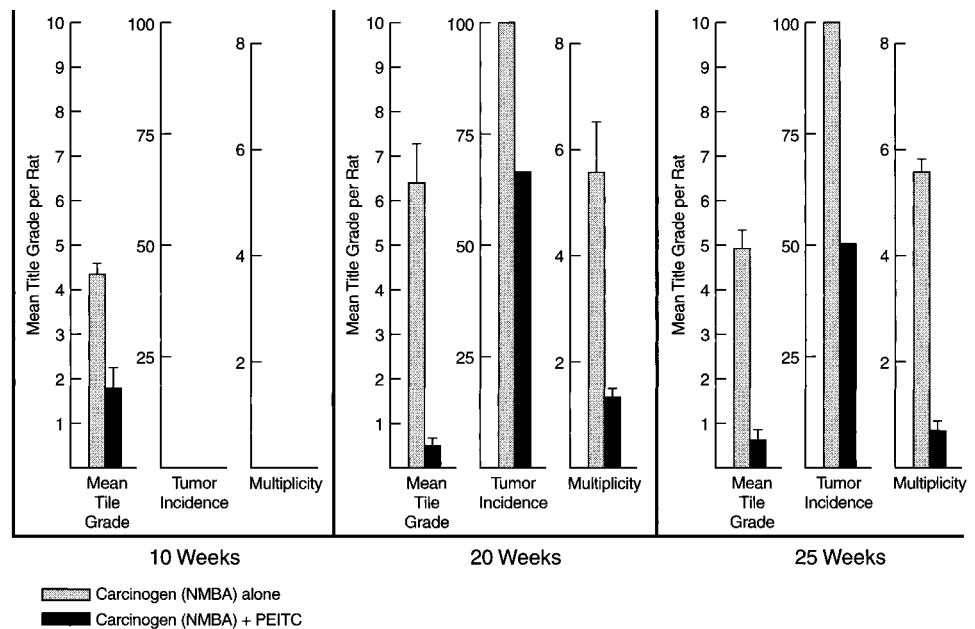


Fig. 3. Histogram panels comparing the image tile parameter, %TG>4SD, with Tumor Incidence and Tumor Multiplicity (number of papillomas/tumor-bearing animal) from the 10th to 25th week after initiation of the NMBA regimen of 0.5 mg/kg s.c. three times per week for 5 weeks. Twenty-two rats were sacrificed at each time point for each measurement of tumor incidence, tumor multiplicity, and %TG>4SD. At 10 weeks, no grossly visible papillomas had yet begun to develop, whereas the %TG>4SD has already reached the relatively high value of 52 percentage points.

Fig. 4. Histograms comparing the tumor-suppressive effect of placing the chemopreventive PEITC in the diet on the MTG, Tumor Incidence, and Tumor Multiplicity. □, rats treated with NMBA alone (six rats/time point). ■, effect of PEITC.



treatment, the appearance and increase over time of the variable %TG>4SD developed in parallel with esophageal tumor incidence and multiplicity; and (b) placing the chemopreventive agent PEITC in the food of NMBA-treated rats produced parallel reductions in the MTG, Tumor Incidence, and Tumor Multiplicity.

Of interest was the unique observation, as early as 10 weeks after initiation of the NMBA regimen, of the appearance of multiple microscopic plaques of high-grade intraepithelial neoplasia in the esophageal epithelium, which appeared to represent early phases of papilloma development. Histological

sections of all of the grossly visible papillomas showed very high-grade intraepithelial neoplasia. In prior experiments (3), the s.c. dose of NMBA of 0.5 mg/kg three times weekly for 5 weeks was chosen to produce the maximal number of papillomas for experimental chemoprevention studies. At this dose, so many esophageal papillomas are produced that they block passage of food, and the rats die before progression of the papillomas to squamous cell carcinomas can occur. Wargovich *et al.* (8) reported that a 7-fold higher s.c. dose of NMBA, *i.e.*, a dose increased from 0.5 to 3.5 mg/kg three times weekly for 5 weeks, induces fewer papillomas and more rapid progression to mul-

tiple invasive squamous cell carcinomas. In this report, by the 15th week after start of NMBA, only 66% of 29 rats had papillomas, whereas in 17% of rats, the papillomas had already progressed to invasive squamous cell carcinomas.

The variable %TG>4SD was a more sensitive indicator of the neoplastic response to NMBA than were Tumor Incidence and Tumor Multiplicity. By the 10th week after starting the NMBA regimen, %TG>4SD had already reached the relatively high value of 50 percentage points, whereas grossly visible papillomas had not yet begun to develop.

The high value of 50 percentage points for %TG>4SD at the 10th week suggests that with %TG>4SD as the endpoint, a chemoprevention screening assay could be completed in 15 and possibly in 10 weeks, compared with the usual observation time of 25 weeks required when endpoints of tumor incidence and multiplicity are used. The potential savings in time and number of animals would appear to be considerable.

Of note was the observation that between the 20th and 25th week, a 25% decrease in the MTG occurred, while the %TG>4SD continued to increase. A plausible explanation is that by the 25th week, the morphologically most extremely aberrant tumor cells, and therefore the ones with extremely high tile grades, had begun to undergo apoptosis and disappear, leaving behind a population of cells with tile grades still >4 SD units, but with a lower MTG. In studies subsequent to those reported here, a similar decrease in MTG after the 20th week was again seen.

Use of the continuous response variables %TG>4SD and MTG to measure development of NMBA-induced rat esophageal carcinogenesis has statistical advantages in that both variables are robust estimators scaled in SD units, with wide dynamic range, that may be evaluated with *t*-tests to compare tissue morphological changes before and after treatment with a chemopreventive agent. By contrast, Tumor Multiplicity is a small-number discrete variable with narrow dynamic range, and Tumor Incidence is a small-number frequency variable that usually must be evaluated by less robust procedures requiring larger sample sizes (8).

The variables %TG>4SD and MTG have obvious potential as intermediate endpoints in human clinical trials of chemopreventive agents (5). If they were used to quantitatively evaluate tissue biopsies and cytological smears before and after administration of a chemopreventive agent to the same individual, application of the paired sample *t*-test statistic might well allow up to 50% reduction in cohort size without significant decrease in study power (9). In addition, because of their increased sensitivity, %TG>4SD and MTG might allow earlier

detection, and therefore earlier treatment, of intraepithelial neoplasia in cytological smears from exfoliated epithelia, such as cervix and lung, and from fine-needle aspirates such as breast (10).

In summary, the continuous variables %TG>4SD and MTG, measured by CAQIA are more sensitive response variables for grading NMBA-induced esophageal intraepithelial neoplasia than are the classic response variables of Tumor Incidence and Tumor Multiplicity. In humans, grading of intraepithelial neoplasia using the variables %TG>4SD and MTG promises to provide a sensitive and objective supplement to the subjective microscopic grading of intraepithelial neoplasia as performed by the pathologist.

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