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Precancerous Lesions of the Human Esophagus: Multiparametric Study of Esophageal Biopsies from a High-Risk Population in Linxian, China

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Abstract Histopathology, morphometry, tritiated thymidine incorporation and immunohistochemistry were studied in 221 esophageal biopsies from subjects with cytologica hyperplasia in Linxian, China. A spectrum of 7 morphologic entities were found: (1) normal/near normal (NN); (2) basal cell hyperplasia 0 (BHO); (3) simple hyperplasia (SH); (4) mixed basal and spinous cell hyperplasia (MBS); (5) basal cell hyperplasia 1 (BH1); (6) dysplasia (D); and (7) non-proliferative lesion (NP). Forty percent of the biopsies had combinations of histologic types. The thickness of the epithelium was increased in SH, MBS, and BH1, but not in BHO and NP. Elongation of papillae was frequently seen in SH, MBS, BH1, and D. Papillary bleeding was very prevalent in the esophageal specimens studied. A variety of cellular changes were found in peripapillary areas especially when bleeding occurred. [^{3}H]-thymidine labeling index was dramatically increased in the entire epithelium in dysplasia, and also increased in cell layer 3 of MBS, BH1 and D. Blood group antigen Le^Y and lectin WGA showed consistent positivity in cellular membranes of the squamous cells, and these changes occurred before gross morphologic alterations. These findings provide a hypothesis for the sequence of pathogenetic events leading to esophageal carcinoma, and define each step with corresponding biomarkers for cancer prevention studies.

Key words: chemoprevention, esophagus, intermediate biomarker, Le^Y, pathology, precancerous lesions, WGA, [^{3}H]-dThd

Linxian, China is one of the high-risk regions of esophageal cancer, and has attracted the attention of investigators in studying the epidemiology, etiology, pathology and hereditary characteristics of esophageal cancer [1-5]. With the development and application of balloon cytology techniques [6], not only have many patients with esophageal cancer been found, but also populations with epithelial hyperplasia; the latter group has been regarded as being at high risk for esophageal cancer development and a main resource for studies on the prevention of esophageal cancer [7,8].

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This report is based on the analysis of biopsy specimens of esophageal mucosa which were taken in an endoscopic survey in Linxian in 1984. Findings of histopathology, microautoradiography (MAR) and immunohistochemistry (IMH) are reported.

MATERIALS AND METHODS

Biopsied Materials:

Two hundred and twenty-one esophageal biopsies were studied from 215 individuals over 30 years of age who showed cytologic evidence of severe hyperplasia. One set of specimens was fixed by 10% buffered formalin for morphology and IMH study. Another set of specimens was similarly fixed after incubation with [^{3}H]dThd for MAR study. The

specimens were routinely processed and embedded in paraffin. All studies were carried out on four-micron tissue sections.

Histologic Criteria of Normal Epithelium of the Esophagus (Fig.1A):

The human esophagus is normally covered by a non-keratinized, stratified squamous epithelium, composed of basal, spinous and superficial cells. The basal cells are a single layer of cuboidal cells with a dark-stained round nucleus. The spinous cells are polyhedral with abundant cytoplasm. The nucleus is round with a small nucleolus. There are 7-14 layers of the spinous cells. While moving upward spinous cells gain more cytoplasm. The thickness of the normal epithelium was defined as 100-120 grid-units under magnification 400. Epithelium with normal morphology having a thickness of more than 120 units and less than 140 units was defined as near normal epithelium (NN). The height of papillae was less than 1/2 the full thickness of the epithelium.

Techniques:

1. MAR: This method has been given in detail in other recent publications [9]. Four slides were prepared and each slide had no less than 5 tissue sections.
2. IMH: The avidin-biotin-peroxidase technique was used. A panel of Lewis blood group antibodies Lea, b, X, and Y (Signet Inc. Dedham, MA) were applied to the specimens. Another panel of lectins included Dolichos biflorus agglutinin (DBA), Ulex europaeus agglutinin (UEA), Peanut agglutinin (PNA), Soybean agglutinin (SBA), Sophora japonica agglutinin (SJA), Ricinus communis agglutinin I (RCA), and Wheat germ agglutinin (WGA) (Vector, Burlingame CA). The procedure for this study has been given in previous publications [10,11]. ABC kit was purchased from Vector.
3. Morphometric study: Measurements were taken of the following: Thickness of the epithelium; density of papillae (total number of papillae/length of biopsied tissue); height of the highest papilla; prevalence of branched papilla; frequency of bleeding papillae (number of bleeding papillae/total number of papillae); cellular changes; inflammation of the epithelium, papilla and lamina propria.
4. Scoring system for MAR specimens:

A total of 200 basal cells in flat areas of esophageal epithelium were counted. The number and position of [^{3}H]dThd-labelled cells were recorded in the first 5 layers and the layer above the fifth layer starting from the basal layer. In order to count 200 basal cells, 8 fields (ranging from 3 to 12) were usually counted from 20-25 tissue sections.

5. Evaluation of IMH:

The biomarkers regularly present in the epithelial cells were selected as structural markers for further evaluating positivity and normal morphology.

RESULTS

Histopathologic study:

1. Classification of esophageal lesions (Table 1):

Based on the criteria of normal esophageal epithelium described above, only 10% of the biopsies were identified as normal and 7.2% as dysplastic. The vast majority of the biopsies were shown to have a variety of morphologic changes. These changes could be grouped into a total of seven categories:

Normal/near normal epithelium (NN).
Basal cell hyperplasia 0 (BH0).
Simple hyperplasia (SH).
Mixed basal and spinous cell hyperplasia (MBS).
Basal cell hyperplasia 1 (BH1).
Dysplasia (D).
Non-proliferative lesions (NP).

The main morphologic features of each category with abnormal changes were as follows:

BH0: Basal cells showed enlargement both of nucleus and cytoplasm (Fig.1B). Basically only one or two layers of basal cells were involved. The enlarged basal cells had a round nucleus with a single prominent nucleolus, but no obvious variations in staining and shape of the cells. BH0 was also seen in the papillae.

SH: The most striking feature of SH was an increase in the thickness of the squamous epithelium without obvious morphologic variation (Fig.1C). The thickness was defined as no less than 140 grid units. The increased thickness was mostly due to an increased number of mature spinous cells with no remarkable evidence of cytologic abnormality. A few cases had superficial cells increased more than spinous cells. Polarity of the cells was normal.

MBS: Proliferative cells appeared in the basal layer and the lower part of spinous layer (Fig.1D). The nuclear/cytoplasmic ratio was increased. Binuclear cells or even giant cells with multiple nuclei were occasionally seen. However, in general no obvious variations in shape and staining of nuclei were revealed.

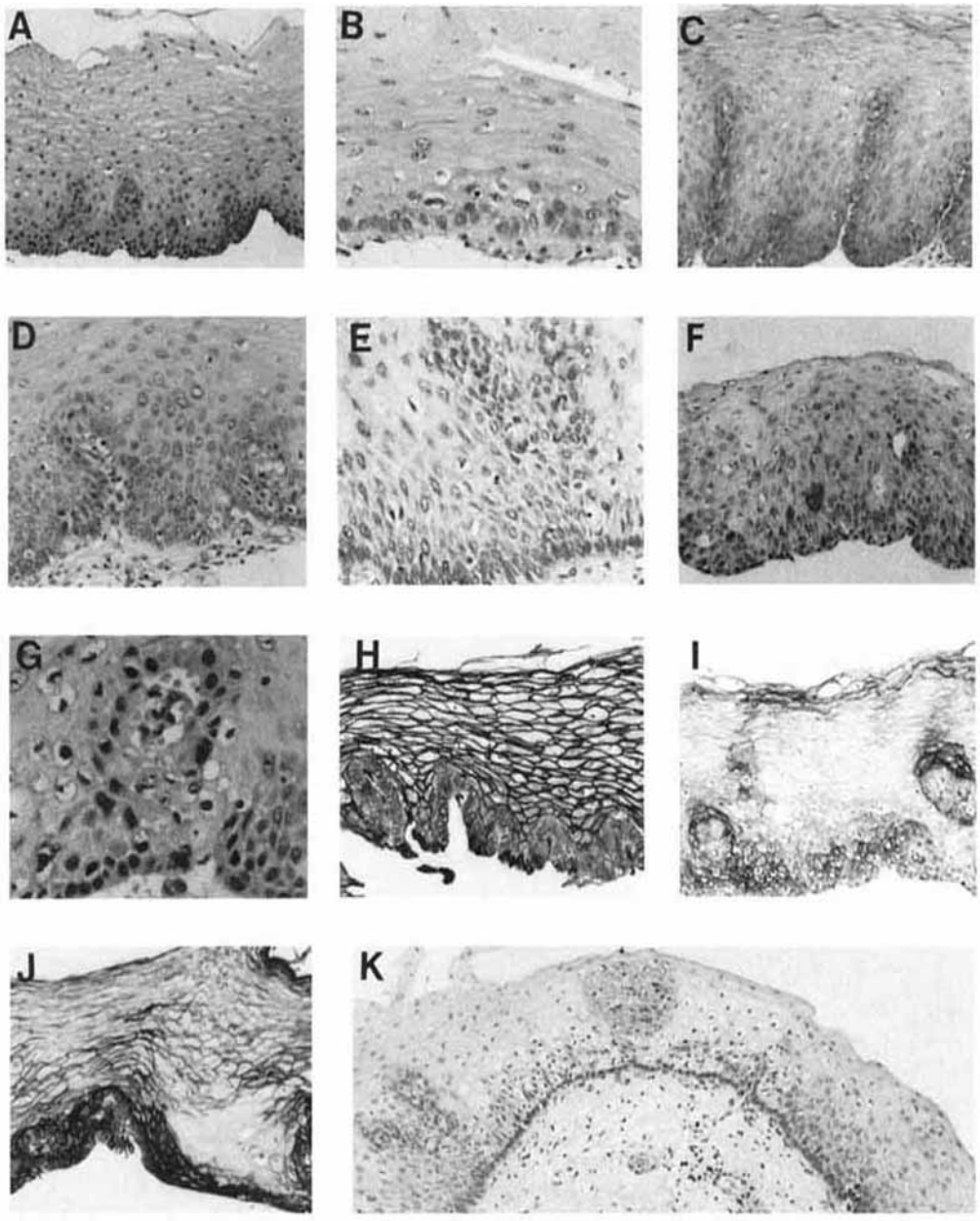


Fig. 1. Characteristic features of the histopathologic categories of esophagus and IMH studies of LeY and WGA. Normal epithelium, H.E. 100X **(A)**; Basal cell hyperplasia 0, H.E. 200X **(B)**; Simple hyperplasia, H.E. 100X **(C)**; Mixed basal and spinous cell hyperplasia, H.E. 200X **(D)**; Basal cell hyperplasia 1, H.E. 200X **(E)**; Dysplasia, H.E. 100X **(F)**; Cells degenerated in PP area, H.E. 200X **(G)**; Normal pattern of WGA binding to cellular membrane, IMH. 100X **(H)**; LeY antigen preserved in PP areas and the lower part of spinous cell layer, IMH. 100X **(I,J)**; Multiple lesions appearing in a specimen, the center showing papillary bleeding surrounded by NP with hyperplasia at the two sides, H.E. 100X **(K)**.

Table 1. Summary of Findings of 221 Biopsies Studies

Group	No. of Biopsies(%)	Thickness of Epithelium[1]	Height of the Highest P[1]	Occurence Rate of Bleeding P (%)	[^{3}H] LI
NN	22 (10.0)	118.1	38.5	37.5	0.064
BH0	32 (14.5)	123.8	48.5	24.7	0.072
SH	38 (17.2)	171.4*	52.0#	25.0	0.070
MBS	64 (29.0)	126.6#	53.2*	26.5	0.070
BH1	35 (15.8)	106.6#	39.6	31.6	0.073
D	16 (7.2)	115.0	64.3	43.6	0.111#
NP	14 (6.3)	102.5	35.8	43.0	0.070

t test: * p value <0.001; # p value <0.05 as compared to NN.
1: Average values measured by unit-grid under magnification of 400X.

BH1: More than one layer of basal cells were seen with proliferation, usually up to 5 layers or more. The proliferative cells were crowded and uniform with a spindle shaped nucleus (Fig.1E). No obvious variations in shape and staining of the cells were seen.

D: The proliferative cells increased greatly in number and size, with a remarkable variety of shapes and staining. Giant cells might appear. Mitotic activity was increased. The polarity of the cells was abnormal. Only part of the epithelium was involved. The basal membrane was intact. No infiltration of dysplastic cells could be seen in the lamina propria or other part of the tissue (Fig.1F).

NP: Degeneration of the squamous cells was the main feature including karyorrhexis (Kr), karyopyknosis (Kp), dyskeratosis (Dk), parakeratosis (Pk), acanthosis/clear cell (C), keratin granulation (G) and koilocytosis (K). These changes were usually found in spinous cells around papillae accompanied by bleeding and/or inflammation (Fig.1G).

The division of 221 biopsies into histopathologic groups is given in Table 1. Each group could be further divided into two subgroups, i.e., single-lesion and multiple-lesion. The latter subgroup was defined as a major lesion plus one or more milder proliferative lesions. Overall, 60.2% of the biopsies had single-lesions, and 39.8% contained more than one category of lesions. In order to simplify the analyses, the single-lesion subgroup is the major focus of this report.

Morphometric study:

1. The thickness of epithelium in each of the histologic categories:

The measurements were taken under magnification of 400X from an area between two open-papillae or close to one open-papilla. Table 1 gives the average thickness of each group, which varied between groups of proliferative lesions. SH was (171.4 grid-units) 69% thicker than NN (118.1). MBS was second thickest. However, BH1 and NP were thinner than NN. BH0 and D were not different from NN in this measurement.

2. Papillae: There were no significant differences in the density of the papillae between the histopathologic groups. However, the height of the highest papilla was significantly greater in SH (52.0 grid-units) and MBS (53.2) as compared with NN (38.5) (Table 1). Branching papillae were more prevalent in MBS (65.7%), BH0 (55.5%) and SH (50.0%) than in NN (33.3%). Papillary bleeding was a common phenomenon in the esophageal biopsies studied, with a high occurrence rate in D (0.44) and NP (0.43). This should not be considered as a biopsy artifact because bleeding papilla were not confined to the margins of the tissue; a papilla without bleeding was often seen next to a bleeding one. Furthermore, a variety of cellular degenerations were observed around bleeding papillae, and were considered to be caused by bleeding or inflammation.

3. Cellular changes in the epithelium: The most frequently seen cellular change was Kr (34.1%), followed by Kp (21.3%), Dk (10.4%), C (9.6%) and G (7.7%) and Gn (3.2%). All kinds of cellular changes might be seen in each histologic group except for Gn which appeared only in D and MBS. These changes were frequently distributed to the outer spinous layer and PP area. It was noted that a cluster of cells with Kr appeared in the spinous layer

just above the top of bleeding papillae and formed a band of dying cells parallel to the lumen of the esophagus. Occasionally a sheet of cells above the band separated from the mucosa beneath.

4. Inflammation: Chronic non-specific inflammation was observed in lamina propria (86.1% of total specimens studied), in papillae (60.9%) and inside the epithelium (33.3%). Small lymphocytes were the major type of inflammatory cells. The intraepithelial lymphocytes always had a convoluted nucleus.

Immunohistochemical Study:

1. Lewis antigens a, b, X and Y: There were a total of 75 biopsies studied for Lewis antigens. LeY was the only one of this group of antigens expressed regularly in esophageal epithelium of all biopsies studied. The LeY-positive material was normally present in spinous cell membranes, forming a delicate, well-defined network. The biopsies with over 2/3 LeY-positive spinous cells were considered a normal pattern; positive cells were seen in 33.3%, 42.9%, 50.0% and 50.0% of D, BH1, MBS and NP biopsies, respectively. The rest of the groups retained better LeY-positivity, i.e., 87.5% of BH0, 78.6% of SH and 61.5% of NN. However, the LeY-positive spinous cells remaining in good shape were only seen in a small number of the biopsies, even in the NN group. The cellular membrane decorated by LeY was weak, expanded, fuzzy or broken into granules. The well-preserved LeY-positive cells only appeared in the PP area or close to the basal layer (Fig.1I,J). Dysplastic cells and hyperplastic basal cells in BH1 were LeY-negative.

2. Lectins

All of the lectins used in the study reacted with cellular membranes but binding sites differed. WGA and RCA bound to both spinous and basal cells. UEA bound spinous cells, glandular cells and endothelial cells of blood vessels. Occasionally UEA was predominantly seen in cytoplasm. PNA was constantly present only in glandular cells; SBA also reacted with glandular cells. SJA and DBA were negative in a majority of cases, although occasionally a staining pattern similar to RCA was observed. In the present report, we will concentrate on the findings of WGA.

Seventy-four specimens were studied for WGA. Each group had a high percentage of WGA-positive biopsies (NN 75.0%; BH0 62.5%; SH 85.5%; MBS 90.0%; BH1 85.7%; D 100.0% and NP 70.0%). But the percentage of WGA positive cells in well-defined structures (Fig.1H) was low (less than 50.0%) in every group except for D (remained 100.0%). Some cases without morphologic changes had obvious damage to WGA binding components in the cellular membrane; as seen in LeY preparation the cellular membranes stained by WGA became expanded, fuzzy, weakly stained or completely negative. In the lesions with mild damage the change in WGA binding seemed to start from the PP area.

[^{3}H]dThd labeling study:

The labeling indices (LI) of [^{3}H]dThd are shown in Table 1. NN had the lowest LI in the first 5 layers. Significant differences were found between dysplasia and NN in single-lesions or multiple-lesions ($P < 0.05$ and < 0.001 respectively). When comparing the LIs of individual cell layers of each category, significant differences in cell layer 3 were found between NN and MBS ($p < 0.05$), BH1 ($p < 0.01$), and D ($p < 0.01$).

DISCUSSION

1.Histopathologic background of the individuals with cytologic hyperplasia:

Dysplasia is the well-accepted precancerous lesion of the esophagus; however, research in high-risk areas of esophageal cancer has expanded the concept of precancerous lesions. Hyperplasia, esophagitis and elongation of the papillae have also been considered to be important precancerous lesions [12,13]. It is obviously important to understand the histopathologic criteria of cytologic hyperplasia in the design, monitoring and evaluation of a prevention trial.

The present study of 221 biopsies provides evidence that people with cytologic hyperplasia from the high-risk area displays a broad spectrum of seven histologic entities from near-normal epithelium to dysplasia. Both D and NN were rare (Table 1). The vast majority of specimens (76.5%) contained different types of epithelial cell proliferation. A small percentage of biopsies (6.3%) were defined NP with degeneration of epithelial cells only. The presence of seven histopathologic groups indicated that this high risk population contained different stages of cancer development.

NN is not a proliferative lesion, but cannot be regarded as normal epithelium. According to findings derived from the multiparametric studies, NN had normal-appearing cells in all parts of the

Hypothesis on Pathogenesis of Neoplasia of the Esophagus

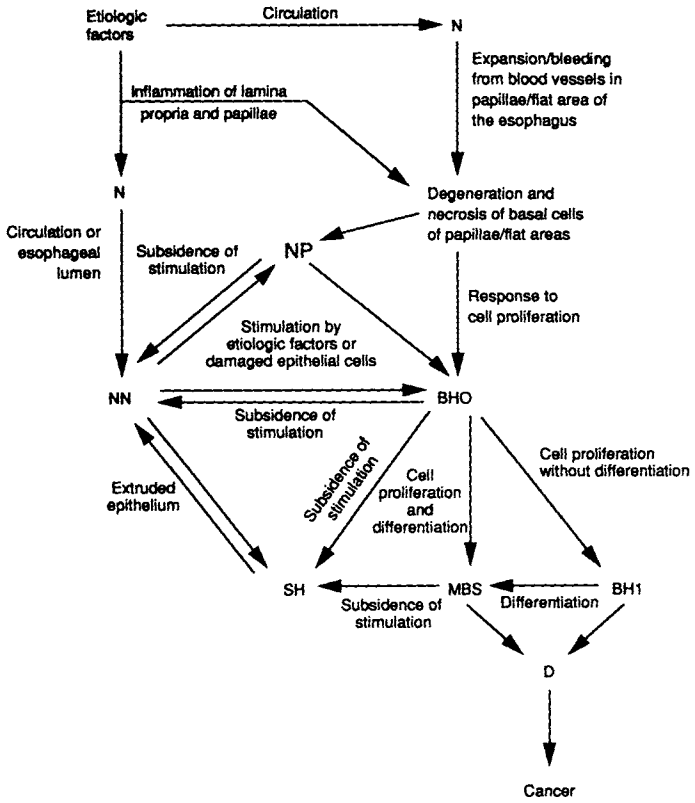


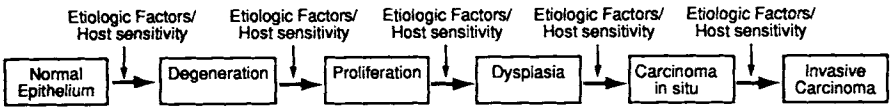
Diagram 1. See Discussion.

epithelium, but other abnormalities had already appeared. Up to 77% of the NN cases had papillary bleeding, and in 37.5% papillae bleeding was accompanied by cellular damage in PP areas. This was especially clear in LeY and WGA preparations. All these findings indicated that some subtle changes within the cells of the epithelium of NN had occurred before gross morphologic changes.

Basal cell enlargement was the main morphologic feature for BH0 that may not only serve an indicator for discriminating BH0 from NN, but may also be a first morphologic signal of active response to stimulation. Since BH0 displayed a wide range of epithelium thickness (60-208 grid-units), it is possible to postulate that BH0 may be derived from proliferative stimulation of a rather quiescent state, i.e., NN or SH. If stimulation continues, BHO may develop into further stages, i.e., MBS, BH1 or even D. It is reasonable to speculate that these proliferative lesions may also reverse to a normal or quiescent state when stimulation subsides or under intervention with nutrients or chemopreventive agents (Diagram 1).

The morphometric studies also provided further evidence that papillae play an important role in the pathogenesis of proliferation. Striking papillary changes, including bleeding, branching, elongation, inflammation, accompanied proliferation of basal cells and cellular degeneration in PP area. BH0 was sometimes more severe in PP than in flat areas. In addition, the LI of PCNA (proliferating cell nuclear antigen) (0.268) was found to be almost 3 times higher in papillary areas as compared with flat areas (0.090) in the first three layers of a group of 40 biopsies. Branching and elongation of papillae represent a later stage after inflammation and bleeding with a greatly increased capacity for overall cell proliferation in the esophagus. It was not uncommon that multiple foci with different proliferation states appeared on the two sides of a

Summary of Biomarkers During the Process of Cancer Development of the Esophagus



Morphology	NN	NP	BHO SH MBS BH1	Dysplasia	Neoplasia without invasion	Neoplasia with invasion
Cellular Membrane						
LeY (spinous cells)	3+	(-)~2+	(-)~2+	(-)~2+	(-)~2+	
WGA (basal & spinous cells)	3+	(+)~2+	(+)~2+	(-)~3+	(-)~3+	
Cytokeratin AE1	Basal cell (+) Spinous cell (-)	Basal cell (+)	Basal cell (+/-) Spinous cell (2+)	+/(-)	+/(-)	Four patterns
Overall ^{3}H dThd labeling index	Normal	Normal	Increased (significant differences in cell layer 3)	Increased with significant differences		

Diagram 2. See Discussion. Four AE1 patterns of esophageal carcinoma were described in reference 11. A 4-grade scoring system was used for lectin evaluation: (3+) The fraction of positive epithelial cells immunostained was greater than 2/3 of all epithelial cells normally stained; (2+) The fraction of positive epithelial cells was more than 1/3 and less than 2/3; (+) Less than 1/3 of the cells were positive; (-) No epithelial cells were positive.

flat area with a bleeding papilla surrounded by degenerated spinous cell in the center (Fig. 1K). This phenomenon suggests a close relationship between bleeding/inflammation, basal and spinous cell degeneration/damage and proliferation starting from basal cells. NP is not a proliferative lesion but is a good indicator of the potential for development of a series of subsequent lesions. A multiple-lesion may imply a more active or unstable state in the pathogenetic process. The comparisons of [^{3}H]dThd labeling indices between single-lesions and corresponding multiple-lesions did not show significant differences. It may be convenient to make a surgical pathologic diagnosis based on single-lesions even with other mild proliferative lesions present.

2. A hypothesis of the evolution of carcinogenesis in the esophagus:

Based on the findings of multiparametric studies on 221 esophageal biopsies, a hypothesis of the evolution of carcinogenesis in the esophagus is proposed here (Diagram 1), and each step has accompanying biomarkers (Diagram 2). The initial stage would be degeneration/necrosis of basal and inner spinous cells induced by direct pathogenic factors, carcinogens or by bleeding and/or inflammation of papillae. The main features include changes of morphology as seen in NP and alterations in components of cellular membranes (carbohydrates, glycoprotein or glycolipid) as defined by lectin WGA and LeY antigen.

The second stage is repair of damaged cells or reaction to stimulation. Basal cells first show enlargement and proliferation (BH0). When the cells increase in number they move upward from the first one or two layers into upper levels of the inner spinous layer. If the cells show an ability to differentiate during migration, the MBS lesions are formed. Otherwise BH1 will appear. If the pathogenetic factor/carcinogen effect stops, the proliferative cells in BH0, MBS and BH1 may return to normal through differentiation, and SH may remain. If the top part of SH sloughs off, the

thickened epithelium may regain a normal appearance. During this stage the cell population in S phase is increased in layers 3, 4 and 5, but the overall labeling index of the first five layers is not significantly different from normal.

In the third stage the proliferative lesions continue to be affected by carcinogens. The cells may acquire the morphology of dysplasia, then carcinoma in situ and invasive carcinoma. At this stage the cells in S-phase increase dramatically as demonstrated by a high overall [^{3}H]dThd labeling index.

This hypothesis may help us understand the process of carcinogenesis in the human esophagus, and may serve as a pathologic basis to quantitate strategies for esophageal cancer prevention. The first two stages would be the best candidates for reversion of lesions in cancer prevention.

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