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Properties of Intraepithelial Neoplasia Relevant to the Development of Cancer Chemopreventive Agents

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Abstract Cancer chemoprevention is concerned with the development of drugs or diet supplements that will avert the onset or stop the progression of the intraepithelial neoplasia which precedes invasive cancer. Two basic processes underlie the onset and development of intraepithelial neoplasia. First is genomic instability (often associated with chronic diffuse epithelial hyperplasia), which is the increased production of genomic structural variants due to unrepaired DNA breaks with secondary formation of abnormal structures, including ''mutator'' mutations in genes responsible for genomic stability, gene copy amplification or loss from DNA breakage-fusion-anaphase bridge cycles, unequal sister chromatid exchange, and accumulation of double minutes. Second is the development within an epithelium having genomic instability of multicentric neoplastic lesions that independently progress through each of the following processes at a continuously accelerating rate: clonal evolution, hyperproliferation, production of genomic structural variants, and apoptosis. Recommended chemoprevention strategies based on these mechanisms are (1) early diagnosis and treatment of genomic instability before the appearance of intraepithelial neoplasia, i.e., during the ''predysplastic'' or ''premorphologic'' phase, (2) development of multiple agents that block intralesional proliferation at steps along the ''command'' pathways of mitotic signal transduction and along the ''execute'' pathways of synthesis of daughter cell components, (3) development of nontoxic antiinflammatory agents, antioxidants, antimutagens, and proapoptotics, (4) avoidance of ''clonal escape'' through use of drug combinations, and (5) use of computer-assisted quantitative image analysis to assay modulation of surrogate endpoints in chemoprevention clinical trials. J. Cell. Biochem. Suppls. 28/29:1–20.

Key words: intraepithelial neoplasia; cancer chemoprevention; genomic instability; clonal evolution; gene amplification; computer-assisted image analysis; biomarkers

Cancer chemoprevention is the prevention of cancer by the administration of drugs or diet supplements. At the tissue level, this amounts to preventing the onset or progression of neoplasia while it is still confined to the intraepithelial compartment and has not yet become invasive, i.e., intraepithelial neoplasia. The NCI Chemoprevention Branch's multidisciplinary approach to chemopreventive drug development, and collaboration with the Food and Drug Administration to provide consensus guidance for applying this approach, have been described previously [1–6]. Briefly, chemoprevention drug development is an applied science effort beginning with the identification of candidate agents and the characterization of these agents in mechanistic assays and in vitro and animal chemopreventive efficacy screens. Promising agents are then further investigated in animal models to design regimens for clinical testing and to evaluate toxicity and pharmacokinetics. Effective agents with minimal preclinical toxicity and sufficient bioavailability to target organs of interest move into Phase I clinical safety and pharmacokinetics testing, and, as appropriate, into Phase II clinical chemoprevention efficacy trials. Agents may enter the development process at any point. For example, some drugs previously or currently under development for other uses may not require further safety testing before entering Phase II clinical chemoprevention studies. More than 40 agents are now being tested singly and in combination in over 80 clinical trials. Table I and II summarize in part, the chemopreventive

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drug development process now being administered by the Chemoprevention Branch.

Throughout the agent development process, a central aim of the chemoprevention program is to learn as much as possible about the molecular and cellular mechanisms of intraepithelial neoplasia, and to use this information to plan directions having the best chance of success. The contents of this strategy are described below.

ONSET OF INTRAEPITHELIAL NEOPLASIA

General Nature of Epithelia

It is useful to summarize in general terms the nature of the epithelial cell sheets from which human carcinomas derive. With dimensions resembling a sheet of writing paper, i.e., up to 0.1 mm in thickness (2–10 epithelial cells) and of broad surface area, epithelial cell sheets cover the external surfaces or line the cavities and ducts of the organ systems of the body, including: (1) skin and mucous membranes, (2) nasopharynx, larynx, and lung, (3) oral cavity, esophagus, and intestinal tract, (4) kidney pelvis, ureters, and bladder, and (5) reproductive system (fallopian tubes, uterus, and urethra). From these epithelial sheets are derived a variety of glandular epithelia with specialized secretory or absorptive functions, including in particular breast and prostate. Epithelial cell sheets, whether squamous or glandular, generally exhibit a self-renewing transition pathway through three cell layers of the following general types: a basal layer of proliferating stem cells, an intermediate layer of non-dividing differentiated cells, and a superficial layer of mature cells, which eventually undergo apoptosis and are shed to the external environment either directly or by way of a communicating lumen or tract. The estimated rate of self-renewal among squamous and glandular epithelia in rodents generally varies from 2 to 10 days, depending on their location and function [7].

DIAGNOSTIC TERMINOLOGY OF INTRAEPITHELIAL NEOPLASIA USED BY PATHOLOGISTS

Figures 1 and 2A,B illustrate the development of intraepithelial neoplasia in squamous epithelium such as cervix and in glandular epithelium such as colon, and also present the terminology used by pathologists to describe various diagnostic features. A most important point is that the morphology of neoplastic cells just prior to invasion, when they are described by relatively benign terms such as "severe atypia," "dysplasia," and "severe intraepithelial neoplasia," differs little if at all from their morphology just after invasion, although the terms describing them change dramatically to the dreaded diagnoses of "cancer" and "malignancy." As shown in Figures 1 and 2, the onset of neoplastic disease appears at least a decade before the phase of invasiveness. It is misleading if not potentially harmful to the patient to think that the condition of intraepithelial neoplasia is fundamentally different from that of "cancer," and requires some kind of "conversion" to become a "malignancy." This erroneous belief is abetted when intraepithelial neoplasia is called "dysplasia," "precancer," or especially by a name that is confounding as well as misleading, "preneoplasia." (To allude to intraepithelial neoplasia as preneoplasia is oxymoronic.) In 1969, before the term intraepithelial neoplasia came into use, Foulds [8] stated: "The most frustrating gap in the terminology of pathology of tumors in man is a lack of a satisfactory name for the so-called precancerous lesions. 'Preneoplastic'. . .can only mean that the lesions are not neoplastic whereas I maintain strongly that they are neoplastic and that this should be recognized in their designation."

The diagnosis of "carcinoma in situ" is frequently given to intraepithelial neoplastic lesions that show extensive replacement of the normal epithelium by neoplastic cells in a pattern that is judged by the pathologist to have a higher risk of early invasion than "severe intraepithelial neoplasia." The diagnosis of "carcinoma in situ" cannot be made with the same assurance and certainty as "invasive carcinoma" because it does not have an irrefutable marker as good as invasiveness. There is now well-reviewed evidence that "severe dysplasia" and "carcinoma in situ" are virtually identical and form a single spectrum of neoplastic change [9].

CHRONIC DIFFUSE EPITHELIAL HYPERPLASIA: A COMMON INITIAL PRECURSOR OF INTRAEPITHELIAL NEOPLASIA

Chronic diffuse epithelial hyperplasia is commonly seen as the precursor to intraepithelial neoplasia. Probably the most frequent cause is stimulation by growth factors and proliferation-inducing reactive oxygen species produced by the lymphocytes and macrophages of chronic inflammatory infiltrates in the subepithelial stroma. For example, in the oral mucosa, subepithelial chronic inflammatory cells were

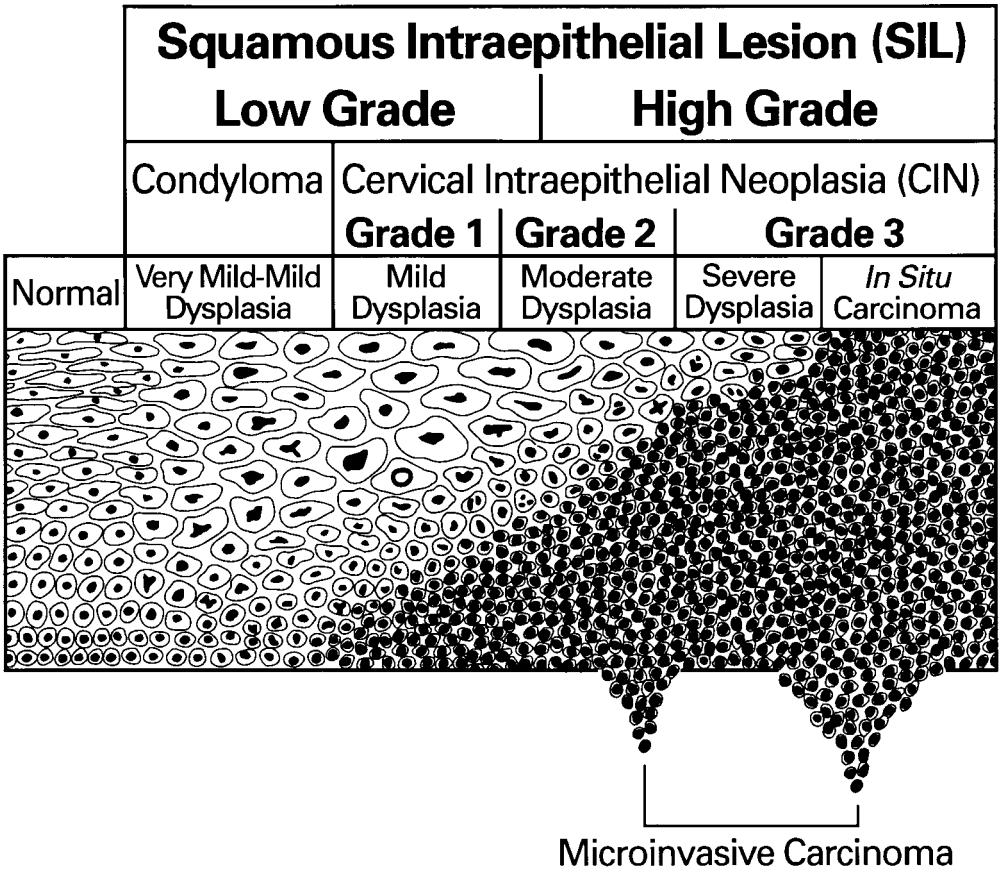


Fig. 1. Diagram of cervical intraepithelial neoplasia (CIN), with three grades, more recently reclassified as squamous intraepithelial lesion (SIL), with two grades. The borderline between inflammatory hyperplasia and early intraepithelial neoplasia is not clear cut. The diagnosis of Low Grade SIL may include some cases of "reactive" hyperplasia, but the diagnosis of High Grade SIL includes only cases of neoplastic change.

shown to induce epidermal growth factor (EGF) production and EGF receptors in the overlying squamous epithelium [10]. Examples of chronic inflammation associated with the neoplastic process are ulcerative colitis [11], urinary bladder inflammation associated with schistosomiasis [12], stones [13], long-term indwelling catheters [14], gall bladder inflammation secondary to stones [15], and "Barrett's esophagus," a condition in which esophageal inflammation secondary to gastric acid reflux leads to hyperproliferative metaplasia of the esophageal epithelium (from squamous type to hyperproliferating intestinal epithelial type), which leads to intraepithelial neoplasia [16]. In the skin, actinic (solar) keratosis, the commonest type of intraepithelial neoplasia, is practically always associated with chronic inflammation in the subepidermis [17]. In the larynx, subepithelial inflammation is a significant predictor of progression to carcinoma [18]. Cigarette smoke produces chronic inflammation of the respiratory mucosa, inducing metaplasia of the ciliated secretory epithelium to stratified squamous type, from which intraepithelial neoplasia develops [19].

Another cause of epithelial hyperproliferation is the regenerative hyperplasia associated with exposure to cytotoxic chemicals [20]. The importance of hyperproliferation to the neoplastic process has been reviewed [21, 22] and probably has never been better defended than in a recent series of lively and informative interchanges between Farber, 1995 [23] and Stemmermann et al. 1995 [24].

GENOMIC INSTABILITY: AN ESSENTIAL PRECURSOR OF INTRAEPITHELIAL NEOPLASIA

Genomic instability is the increased production of genomic structural variants due to unrepaired DNA breakage with secondary abnormal structural changes. According to long established usage, the term "mutation" refers

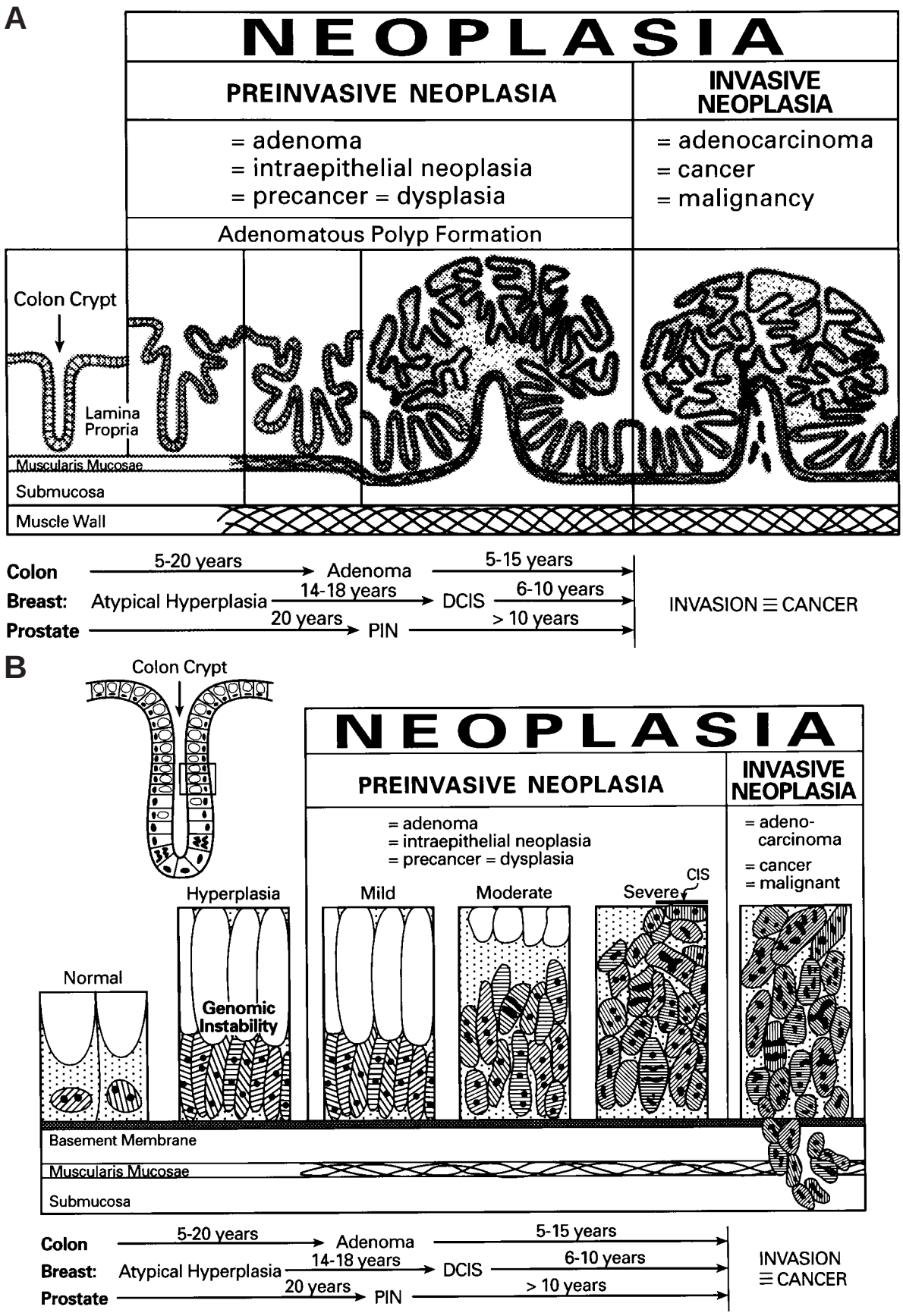


Fig. 2. **A:** Diagram of colorectal polyp formation at low magnification. Uncontrolled clonal expansion of a cell in the upper portion of the crypt leads to formation of a mass of multiple abnormal crypts that bulges up to form a polyp with a core made up of submucosal blood vessels. **B:** Diagram of colorectal polyp formation at high magnification. The cell nuclei of intraepithelial neoplasia of glandular epithelium on the surface of the polyp show the same aberrant variation in size, shape, and chromatin texture as the cell nuclei of intraepithelial neoplasia of squamous epithelium.

to alterations of the DNA sequence within a single gene, ranging in extent from a single nucleotide to a few kilobases. In this article, permanent and heritable structural changes of the genome of all sizes, from point mutations to loss of part of a chromosome arm containing thousands of genes, or even to stable loss or gain of an entire chromosome (e.g., Turner's syndrome and Down's syndrome, respectively), will be referred to as "genomic structural variations," including those that may be heritable only to the next cell generation before they become modified in S phase. Genomic structural variations occur at three levels of DNA organization: at the level of the primary DNA sequence (classic mutations involving single nucleotides and oligonucleotide sequences within a gene), at the level of DNA segments containing many genes (amplicons showing gain, loss, or recombination), and at the level of whole chromosomes (karyotypic aberrations of chromosome structure and number).

MECHANISMS OF GENOMIC INSTABILITY

Mutator Mutations in Genomic Stability Genes

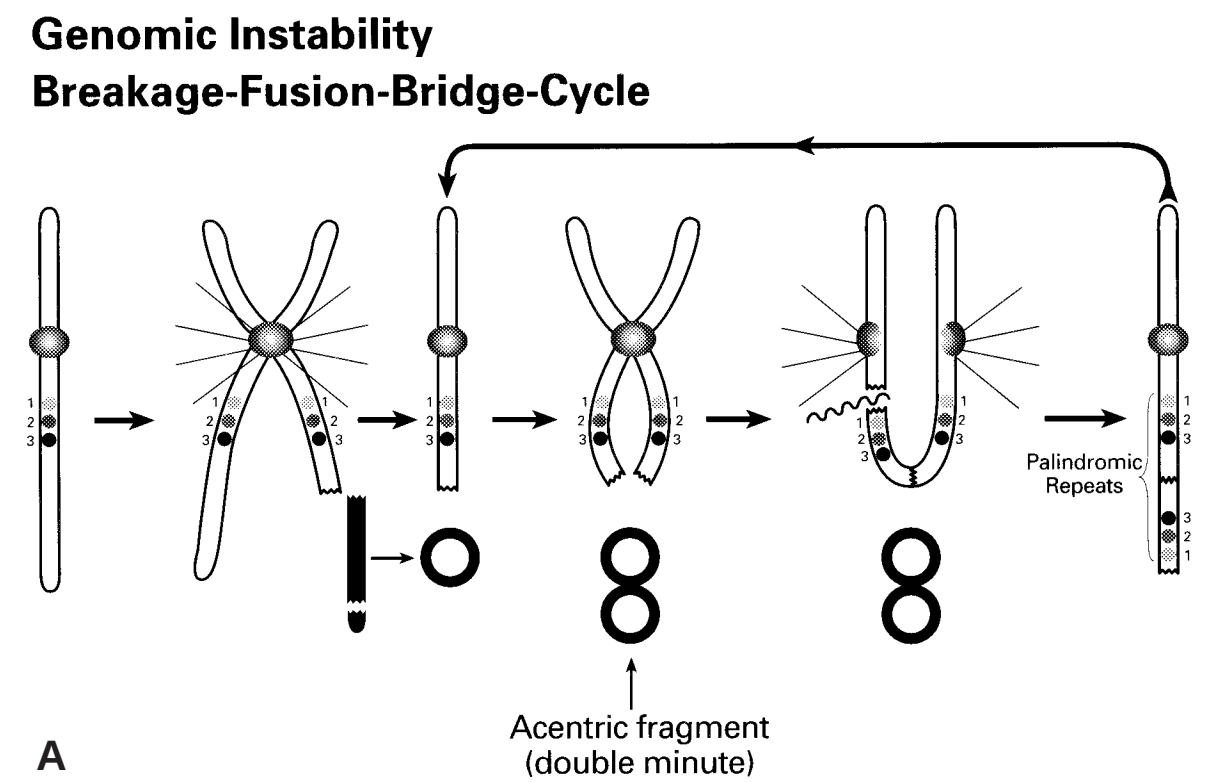
A paradigm to explain the generation of genomic instability has been offered by Cheng and Loeb [25]. Genes whose expression is required to maintain the fidelity of duplicating and segregating the genome are called "genomic stability genes." Mutations in one or more of these genes, aptly termed "mutator" mutations, lead to genomic instability. The list of genes whose functions help maintain genomic stability is long [25], including gene functions concerned with the following: (1) synthesis of balanced nucleotide pools [26,27], (2) DNA replication, (3) repair of damaged DNA, (4) cell cycle checkpoints preventing survival of cells with excessive DNA damage, (5) genes controlling the apparatus for synthesis and coordination of cell division and DNA segregation (DNA condensation, centriole synthesis and movement, spindle fiber and kinetochore synthesis, chromosome alignment at metaphase and anaphase, and cytokinesis), and (6) genes coding for xenobiotic metabolizing enzymes, which catalyze scavenging of activated carcinogens and reactive oxygen species before they can damage DNA. A conservative estimate of the minimum number of genomic stability genes would be in the low hundreds. Since the genomic instability produced by mutation of a genomic stability gene undeniably contributes to the risk of neoplasia, genomic stability genes are ready candidates to become tumor suppressor genes.

Gene Amplification and Loss of Heterozygosity Produced by DNA Strand Breakage With Aberrant Recombination

Figure 3A–C illustrates the principal mechanisms of gene amplification and loss of heterozygosity, which are as follows.

Gene amplification produced by the "Breakage-Fusion-Bridge Cycle" [28,29]. As shown in Figure 3A, a postreplication (late S or G2) double-stranded DNA break in one sister chromatid produces a proximal short arm and a distal acentric fragment having different destinies. At cell division, the shortened broken chromatid segregates to a daughter cell in G1 phase and is replicated in S phase up to the "sticky ends" of the break, which fuse together to produce a dicentric chromosome. "Sticky ends" refers to the fact that the two nucleotide sequences at the end of a double-stranded break in the DNA double helix overhang each other by a few nucleotides, and tend to fuse readily with other sticky ends that have complementary nucleotide sequences. Breaks in microsatellite DNA sequences are especially likely to fuse because they are made up of 50–200 dinucleotide repeats in tandem. When a dicentric produced by fused sister chromatids forms an anaphase bridge, breakage does not occur at the original fusion point, but randomly at any point, segregating a short chromosome with lost genes and a long chromosome with extra gene copies. The long chromosome segregates to a daughter cell and replicates in S phase to its sticky ends, which fuse as before to create a dicentric that forms another anaphase bridge. This "Breakage-Fusion-Bridge Cycle" continues indefinitely, producing a chromosome with many palindromic (nose to nose) gene repeats. The site of these many repeats can usually be visualized on metaphase chromosomes as a "homogeneous staining region," or HSR.

Gene amplification produced by unequal sister chromatid exchange [28]. As shown in Figure 3B, if multiple DNA double-stranded breaks occur in both sister chromatids being formed during S phase, unequal sister chromatid exchange may occur, such that the proximal sticky end of a break near the telomere may fuse with the distal sticky end of a break near the centromere, producing a very long chromatid with gene repeats in tandem (nose to tail). Many repetitions of such unequal sister chroma-



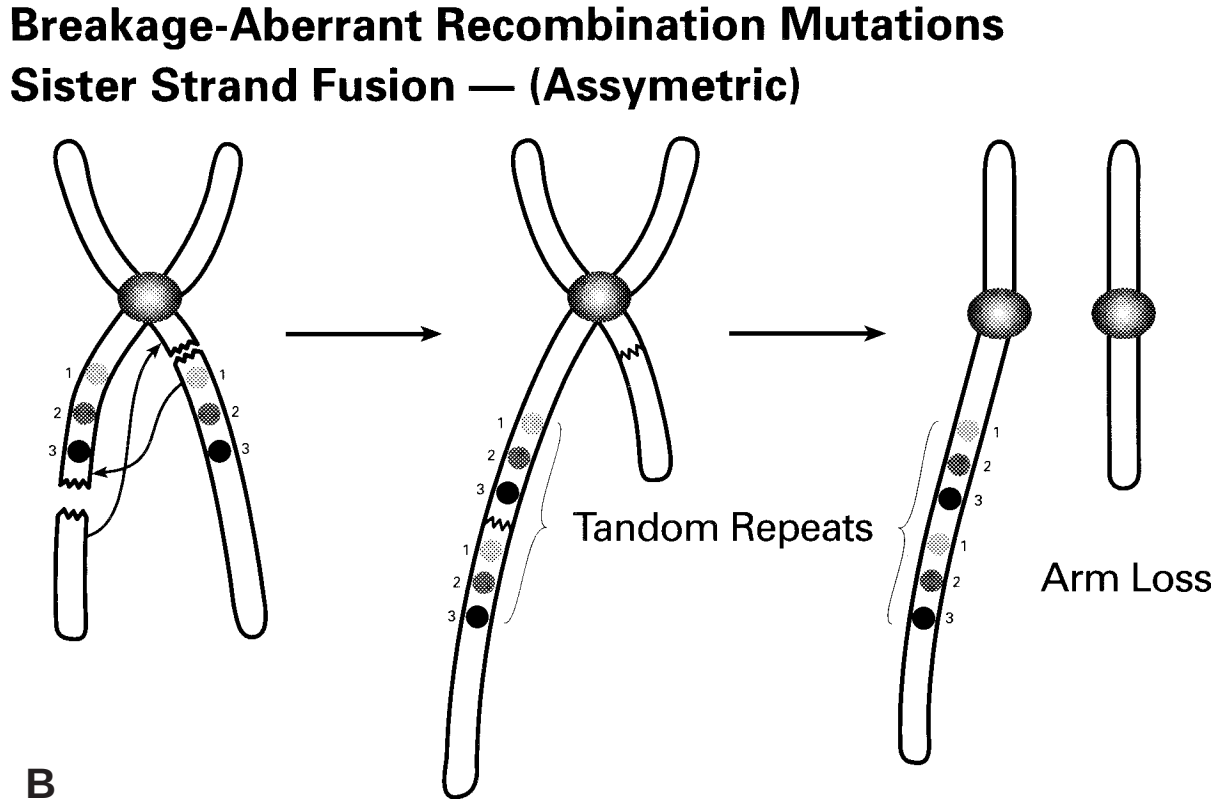


Fig. 3. **A**: The ''breakage-fusion-bridge'' cycle. A broken chromosome segment forms an acentric ring, leaving a short chromosome arm. During growth cycling, the broken ends repeatedly fuse and form an anaphase bridge, which breaks at different locations. The acentric ring replicates to form a double ring, and continues replicating with each cell cycle in this form. Microscopically in a metaphase spread the palindromic gene repeat segments appear as ''homogeneous staining regions,'' or HSR, and the double rings appear as ''double minutes.'' **B:** Asymmetric sister strand fusion. Sister chromatid breaks at different locations transpose to form a long chromatid with tandem gene segment repeats and a short chromatid with gene loss. With many repetitions of this process, the tandem gene segments appear as ''homogeneous staining regions,'' or HSR. **C:** Summary of mechanisms of gene amplification and loss of heterozygosity. If multiple gene copies in double minutes or on HSR favors escape from growth controls, these cells accumulate. If loss of an allele uncovers a mutated tumor suppressor gene, these cells will accumulate.

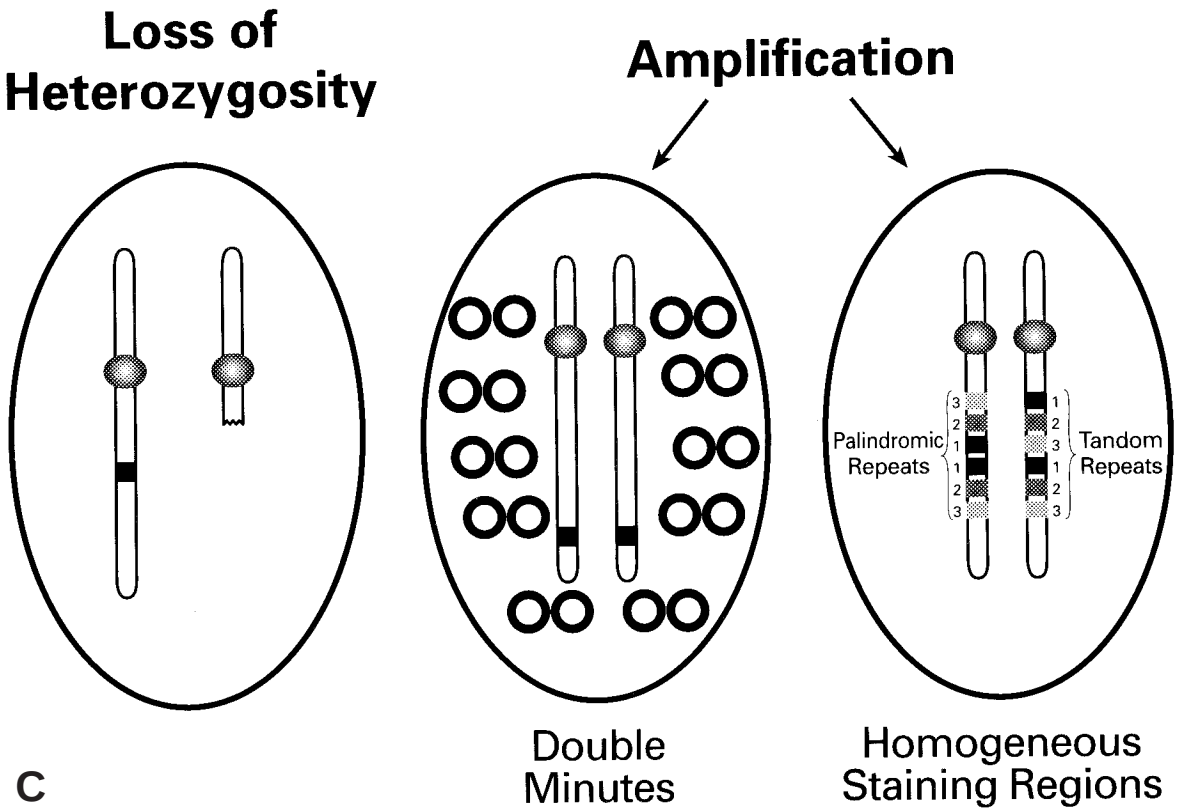


Figure 3. (Continued.)

tid exchanges produce regions of amplified gene dosage in which the gene repeats are arranged in tandem, as opposed to palindromic, sequence. The site of these many tandem chromatid segments also forms homogeneous staining regions, or HSR, on metaphase chromosomes.

Gene amplification produced by accumulation of distal chromosome fragments circularized into "double minutes" [29,30]. As shown in Figure 3C, after double-stranded DNA breaks occur, acentric fragments are produced. Such fragments may contain a telomere if the break occurred at the end of a chromosome arm, or not, if they derive from an interstitial region between two double-stranded breaks on the same chromatid arm. Acentric fragments tend to fuse their ends and circularize. During S phase they replicate synchronously with the rest of the genome to form "double minutes," so called because they appear as tiny paired structures within a metaphase spread of chromosomes. Because double minutes have no centromere, they segregate randomly and unequally at mitosis. If a double minute contains genes of selective advantage to the daughter cell because they permit escape from growth controls, cells will tend to accumulate that have many such double minutes, amplifying the number of growth-enhancing genes, as shown in Figure 4.

Loss of heterozygosity produced by accumulation of chromosomes with lost arm segments (allelic loss). With each of the three mechanisms of allelic amplification described above, chromosomes or chromatids with lost arm segments are produced that segregate to daughter cells. If the chromosome with a lost arm segment is of selective advantage to a daughter cell in terms of escape from growth controls, particularly if the lost segment contained a tumor suppressor gene, such cells will tend to accumulate to form a population exhibiting loss of heterozygosity.

CHARACTERISTICS OF INTRAEPITHELIAL NEOPLASIA IMPORTANT TO THE DEVELOPMENT OF CHEMOPREVENTION STRATEGIES

Multicentricity: Intraepithelial Neoplasia Develops at Multiple Sites That Progress Independently

Exposure of epithelial cell sheets to carcinogens usually occurs over a broad area, simultaneously exposing millions of proliferating basal

TABLE I. ABC's of Chemoprevention: Agents, Biomarkers, and Cohorts*

Agents classified by mechanism	Intermediate biomarkers	Clinical cohorts (Phase II)	Clinical cohorts (Phase III)
Colorectal			
Antiinflammatories (sulindac, piroxicam, aspirin, ibuprofen) Antiproliferatives (DFMO, calcium, curcumin)	Adenomas, proliferative indices, aberrant crypts, Lewis blood group antigens, sialyl-Tn antigen	Patients with previous adenomas or with adenomas <1 cm in diameter	Subjects at high risk (family history of adenomas or colorectal cancer, previously treated breast or endometrial cancer)
Prostate			
Testosterone 5α-reductase inhibitors (finasteride) Retinoids (4-HPR) Antiproliferatives (DFMO) *ras* farnesylation inhibitors (*d*-limonene)	PIN, PSA, PAP, cytokeratins (loss of 50–64 kDa), vimentin, nucleolar prominence, DNA content	Patients with PIN without prostatic adenocarcinoma; patients scheduled for radical prostatectomy	Patients with elevated serum PSA; subjects ≥60 years of age
Lung			
Retinoids/carotenoids (vitamin A, 13-*cis*-retinoic acid, β-carotene) Dithiolthiones and other organo-sulfur compounds (oltipraz, *N*-acetyl-l-cysteine)	Cellular atypia/dysplasia in sputum, bronchial atypical metaplasia/dysplasia, PCNA, blood group antigens, p53	Patients with recently resected Stage I lung or laryngeal cancer	Patients with previous lung, head, or neck cancers; subjects at high risk (smokers, occupational exposure to asbestos)
Breast			
Antiestrogens (tamoxifen, toremifene) Retinoids (4-HPR) *ras* farnesylation inhibitors (perillyl alcohol, *d*-limonene)	Atypical hyperplasia, DCIS, LCIS	Patients scheduled for breast cancer surgery	Patients with previously treated breast cancer
Bladder			
Antiinflammatories (sulindac, piroxicam, aspirin, ibuprofen) Antiproliferatives (DFMO) Retinoids (4-HPR)	TIS, dysplasia, DNA content, F- and G-actins, integrins, loss of heterozygosity (e.g., 9q), blood group antigens, *Rb*	Patients with previously resected TIS or T_a, T_1 disease without TIS	Subjects at high risk (occupational exposure to aromatic amines)
Oral			
Retinoids/carotenoids (vitamin A, 13-*cis*-retinoic acid, β-carotene) Antiinflammatories (carbenoxolone)	Dysplastic leukoplakia, keratin expression, GGT	Patients with dysplastic leukoplakia	Patients with previously treated head and neck cancers; subjects at high risk (smokers, tobacco chewers)
Cervix			
Retinoids (vitamin A, 4-HPR) Antiproliferatives (DFMO) Folic acid	CIN	HPV-negative patients with CIN III	Patients with CIN

*PIN, prostatic intraepithelial neoplasia; PSA, prostate-specific antigen; PAP, prostatic alkaline phosphatase; DCIS, ductal carcinoma in situ; LCIS, lobular carcinoma in situ; GGT, γ-glutamyl transpeptidase; CIN, cervical intraepithial neoplasia.

TABLE II. Chemoprevention Branch Testing Program: Results of In Vivo Screens*

	Lung			Colon			Mammary		Skin						
Agents	DEN	MNU	NNK	Crypts	Mouse	Rat	DMBA	MNU	Transg.	DMBA, B(a)P	Blad	Pros	Panc	Lymph	Esoph
N-Acetyl-*l*-cysteine		+	NE (M), NE (R)	NE		+	NE	+		NE, B(a)P	+				
Curcumin				+	+	+	NE	+			NE				
DFMO	NE	NE		+	+	−	+	−	+	−, B(a)P	+	NE	NE	NE	
DHEA				NE	+			+		NE	NE	+	NE		
4-HPR	+ (Lung)	NE	NE (M)	+	NE		−	+	NE	−	−	NE		+	NE
Ibuprofen		NE		+		−		+			+				
Indole-3-carbinol		NE		+	−		+	+		NE	NE				
Oltipraz	+	+		+	+	+	+	+		+	+	NE	NE	NE	
Piroxicam				+		+	NE	NE		+	+		NE		
13-*cis*-Retinoic acid				+											
9-*cis*-Retinoic acid				+		+		+				+			
d,*l*-Selenomethionine	NE (M)			NE											
l-Selenomethionine					NE							NE			
Sulindac				+		+		NE			+				

*DEN, diethylnitrosamine; MNU, methlnitrosourea; NNK, 4-(methylnitrosamino)-I-(3-pyridyl)-I-butanol; DMBA, 7,12-dimethylbenz(a)anthracene; B(*a*)P, benzo(*a*)pyrene; Blad, *N*-butyl-*N*-(hydroxybutyl)nitrosamine induced bladder cancer in mice); Pros, MNU-induced prostate cancer in rats; Panc, *N*-nitroso(2-oxypropyl)amine-induced pancreatic cancer in hamsters; Lymph, lymphoma in PIM-1 transgenic mice; Esoph, *N*-nitrosomethylbenzylamine-induced esophageal cancer in rats; NE, not effective; M, mice; R, rat; DFMO, difluoromethylornithine; 4-HPR, *N*-(4-hydroxyphenyl)retinamide; DHEA, dehydroandosterone.

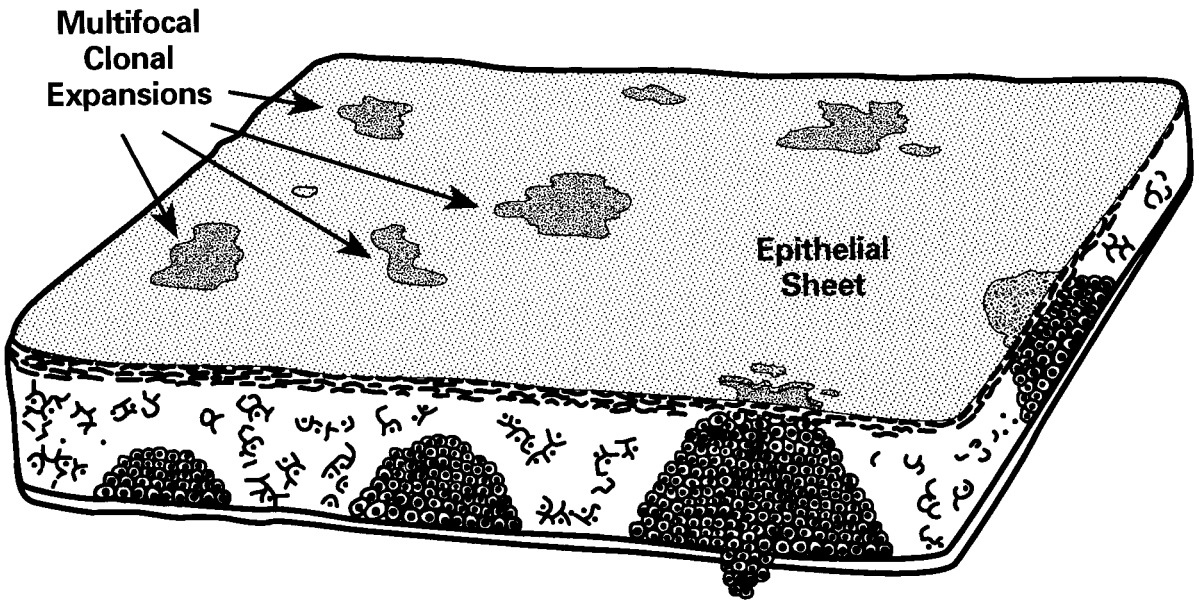


Fig. 4. Intraepithelial neoplasia develops at multiple sites, which progress independently, in an epithelium subject to diffuse genomic instability (called "field cancerization" by Slaughter et al. [33]).

cells to the risk of genotoxicity and postnecrotic regenerative proliferation. The skin, for example, has approximately one million basal cells per square centimeter; DNA-damaging solar radiation reaching an area of skin measuring ten by ten centimeters would simultaneously expose about a hundred million basal cells to the mutational and DNA breakage effects of pyrimidine dimerization and reactive oxygen species. Carcinogens absorbed into the body from the respiratory tract, such as those in cigarette smoke [31], or the digestive tract, such as nitrosamines derived from nitrite in unrefrigerated vegetables [32], are delivered via diffusion from subepithelial capillary networks to broad areas of epithelia, again simultaneously exposing millions of proliferating basal cells to DNA modification and mutation.

It is little wonder, then, that neoplastic clonal expansions start at more than one site in an epithelium that has developed diffuse genomic instability because of chronic exposure to carcinogens (shown in Fig. 4). Slaughter et al. [33], in a landmark paper describing observations on 783 patients with oral cancer, were the first to focus attention on the multicentric origin of in situ neoplasms of the oral cavity, each progressing independently to squamous cell carcinoma. They coined the term "field cancerization" to describe an epithelium "preconditioned" by a carcinogenic agent. More than 11% of Slaughter et al.'s patients simultaneously exhibited one or more other independent squamous cell carcinomas involving the esophagus or lung. Their concept of "field cancerization" of an epithelium may now be understood as the development of diffuse genomic instability after prolonged carcinogen exposure. Multiple actinic (solar) keratoses of the face is a common example of the multicentric development of intraepithelial neoplastic lesions. In one study [34], 8 of 15 actinic keratoses exhibited p53 gene mutations, and in another [35], 12 of 26 actinic keratoses showed overexpression of cyclin D and p53 protein. Adenomatous polyps of the colon are another example of multicentric intraepithelial neoplastic lesions, this time lifted up from the mucosal surface on fibrovascular stalks. As is now well known, adenomatous polyps exhibit different numbers and combinations of genetic lesions, not only among different polyps, but also at different sites within the same polyp [36].

As a final example, in the same prostate, multicentric lesions of prostatic intraepithelial neoplasia were each shown to contain different patterns of aneuploidy [37].

Accelerating Clonal Evolution

Clonal evolution of neoplasms, first enunciated by Nowell 1976, 1986 [38, 39], has been previously reviewed in relation to intraepithe-

lial neoplasia [19]. Briefly, it is the continuous occurrence within a neoplastic population of genomic structural variants that undergo clonal expansion at a more rapid rate of proliferation than surrounding cells. Further clonal expansions of variant cells may occur within the same original expanding clone, or at other sites in the neoplastic population within independent lines of clonal evolution. Thus, at any given time, multiple clonal expansions may occur at different sites in the same tumor. The "Gleason Score," constructed by the pathologist to indicate the aggressive potential of prostate cancers, is the sum of two numerical grades given to the least differentiated and most differentiated regional patterns seen. It is illuminating to appreciate that the two regional patterns represent the phenotypic expression of separate lines of clonal evolution occurring in practically every prostatic neoplasm.

Figure 5 illustrates the relationships between important kinetic properties of intraepithelial neoplasia. The rate of clonal evolution is defined as the rate of appearance in the neoplastic population of clonal variants that grow faster than surrounding cells ("CVGF"). The proliferation rate of neoplastic cells ("NC") in the population determines the production rate of genomic structural variants ("GSV"), because each turn through the cell cycle converts damaged DNA lesions into mutations and also subjects the genome to a tenfold greater mutagen sensitivity during S phase [40]. A fraction of the GSV will be clonal variants that can grow faster (CVGF). The focal expansions will add to the overall proliferation rate of NC. The final result is a continuously accelerating kinetic cycle involving increased production rates of NC, GSV, and CVGF, all of which are slowed by the increased production rate of apoptotic cells. The driving force of the cycle is entropic, i.e., a selection pressure exists toward increasing disorder and heterogeneity as controls for maintaining homeostasis are lost.

Accelerating Intralesional Proliferation Rate

The property of intraepithelial neoplastic lesions in which their rates of clonal evolution and cell proliferation mutually augment each other is a variant of the famous dictum of Ames et al., 1995, that "mitogenesis increases mutagenesis" [41].

Accelerating Intralesional Apoptosis

Figure 5 also shows the accelerating production of apoptotic cells (AC) that fail to pass DNA damage control checkpoints. The balance between neoplastic cell birth rate and death rate determines the net rate of growth in bulk of the intraepithelial neoplastic lesion.

The analysis illustrated in Figure 5 does not take into account complexities such as: (1) ischemic "oncosis" or anoxic death by swelling [42] due to inadequate capillary blood supply from mechanical compression or insufficient angiogenesis factor production, (2) augmentation of the intralesional cell proliferation rate by growth factors diffusing from adjacent hyperproliferating normal epithelium, or (3) the stimulation of mitosis and apoptotic cell death induced by reactive oxygen species from inflammatory cell infiltrates. Nevertheless, a mechanistic rationale is provided to explain some important kinetic features of neoplasia, that the rates of both clonal evolution and intralesional cell proliferation tend to accelerate with time, and are braked only by an accelerating rate of apoptosis. Accelerating proliferation rates for cervical intraepithelial neoplasia have been demonstrated by Richart [43].

Accelerating Intralesional Genomic Instability (Accelerating Production Rate of Genomic Structural Variants Prior to and During Intraepithelial Neoplasia, and After Progression to Invasive Carcinoma)

Prior to onset of intraepithelial neoplasia. The finding of allelic loss of 9q in microscopically normal-appearing hyperplastic epithelium adjacent to intraepithelial neoplastic lesions of the head and neck [44] is an example of a "predysplastic," or "premorphologic" change associated with genomic instability (see Fig. 1). Another example is the demonstration by fluorescence-labeled in situ hybridization (FISH), using chromosome-specific centromeric probes, of multiple clones with aneusomy/polysomy occurring in normal, non-hyperplastic epithelium adjacent to intraepithelial neoplasia of head and neck. The number and size of aneusomic clones increased with progression from normal non-hyperplastic to normal hyperplasia to dysplasia to cancer [45]. As a third example, in sputum smears containing dysplastic cells as observed by conventional light microscopy, there occur other cells that appear normal and non-

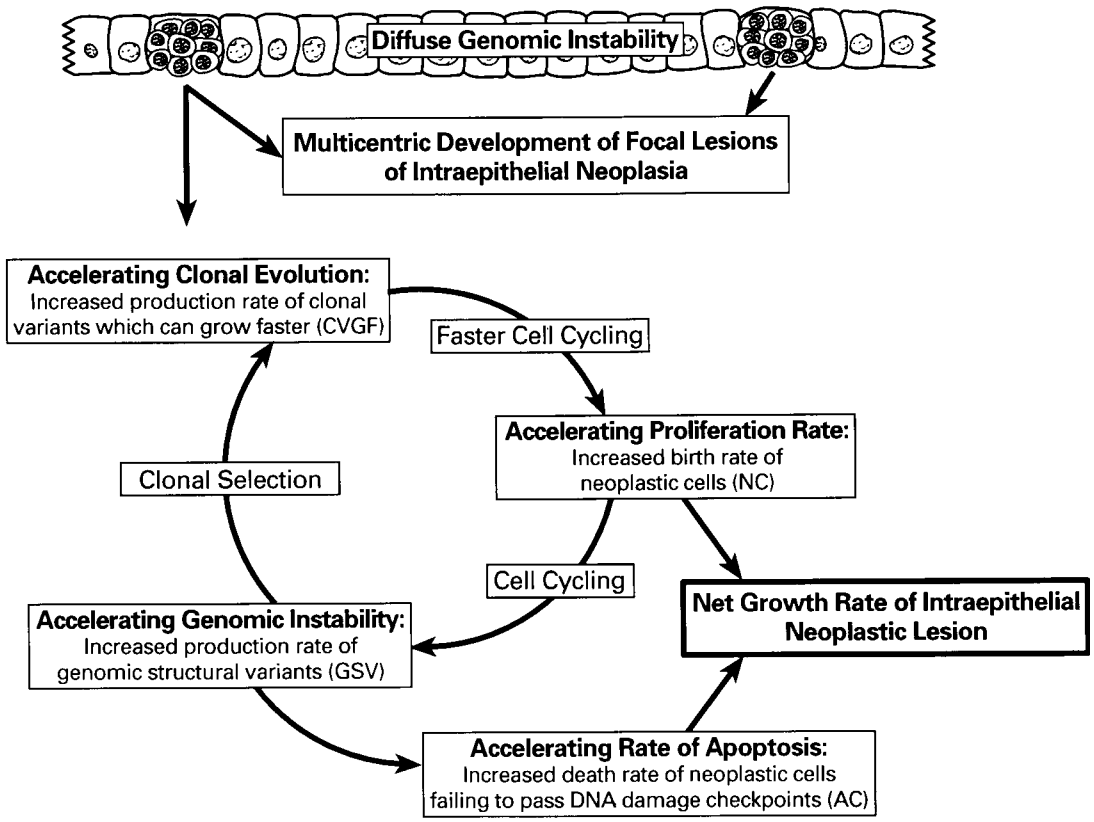


Fig. 5. The continuously accelerating kinetic cycle of intraepithelial neoplasia. Proliferating intraepithelial neoplastic cells (NC) produce genomic structural variants (GSV), a fraction of which form clonal variants that grow faster (CVGF). CVGF add to the average proliferation rate, which increases the production rate of GSV. This in turn increases the production rate of CVGF, and the cycle repeats. This accelerating cycle is slowed by concurrent cell death due to increased production of apoptotic cells that have failed to pass DNA damage checkpoints. The difference between birth and death rates determines the net rate of growth in bulk of the intraepithelial neoplasm.

dysplastic, but when these same cells are observed by computer-assisted image analysis, they show specific nuclear chromatin textural features that are only seen in smears containing dysplastic cells elsewhere. These "predysplastic" changes in the topographic distribution of DNA at the supramolecular level have been given the name of "malignancy associated changes," or "MACs" [46]. On the other hand, in one report the normal epithelium adjacent to colorectal polyps showed no allelic losses [47].

During intraepithelial neoplasia. Vogelstein's original model of genetic progression during intraepithelial neoplasia of colorectal polyps illustrates the accelerating pace of genomic instability, as does a model Sidransky has developed of genetic progression in intraepithelial neoplasia of head and neck using microsatellite analysis of allelic loss. Sidransky's model shows that the earliest losses are of 9p in squamous hyperplasia described above, followed by losses of 3p and 17p in dysplasia, then of 11q, 13q, 14q in carcinoma in situ, and finally of 5p and 4q after invasion [44]. In another example of progression of genomic instability in intraepithelial neoplasia, 61 cases of ductal carcinoma in situ (DCIS) of the breast exhibited a correlation between an increase in frequency of allelic loss (FAL) and increase in nuclear grade [48]. In a more detailed study, chromosomal losses of 16q and 17p occurred early in the genetic progression of DCIS, when it was low grade, followed by allelic losses of many more chromosome arms in lesions of intermediate and high grade (often including 1p, 1q, 6q, 11p, 11q, 13q, and 17q) [49].

During invasive cancer. Analyses in many laboratories of the allelotypes of many types of cancer have shown that the frequency of allelic loss, or FAL (fraction of the 39 non-acrocentric autosomal chromosome arms showing structural loss), or if fewer loci are selected, the

mean allelic loss, increases with time and tumor grade. A few recent examples have been published for head and neck [50], urinary bladder [51], and prostate [52].

Accelerating Phenotypic Heterogeneity

The propensity of neoplastic populations to show an abnormal increase in phenotypic heterogeneity of structure and function is well established [53]. Practically any geometric dimension, in particular nuclear area, shape, and chromatin texture, or any numerical count, such as number of nucleoli or number of mitosis, have a mean and variance that both increase with time. The variance here is a quantitative measure of phenotypic heterogeneity. Accelerating phenotypic heterogeneity in an intraepithelial neoplastic lesion is the immediate consequence of the accelerating genotypic heterogeneity just discussed.

NEOPLASTIC PROGRESSION

The term "neoplastic progression," illustrated for colorectal neoplasia in Figure 6, expands on the frequently expressed concept that "carcinogenesis is a multistep process" to encompass the complete panorama of evolving neoplastic change, from the first microscopic clonal expansion to the final terminal state of extensive bulk, invasiveness, disseminated metastasis, and death. Neoplastic progression may be defined as follows: "The propensity of neoplasms over time to increase in total bulk and extent of dissemination (as defined by Clinical Stage) and to increase in the extent of deviation from normal cell and tissue structure and function (as defined by Histopathological Grade)." The obvious clinical reality of neoplastic progression gives the simplest definition: "neoplasms increase in Stage and Grade with time." Neoplastic progression has kinetic properties; the time-dependent succession of states of increasing bulk and variability of structure and function have both a *rate* and an *extent*. For a given cancer patient, measuring the extent of neoplastic progression provides an estimate of the survival time remaining to him. Figure 6 shows how the extent of neoplastic progression in colorectal neoplasia can be estimated from corresponding changes at three different levels of magnitude: the clinical level, the tissue level (extent of invasion and lymph node metastasis), and the cytonuclear level (extent of variation of nuclear size, shape, and especially chromatin texture). A fourth, molecular, level may be added in the future, to include patterns of aneuploidy/aneusomy and other measures of the degree of genomic instability, such as extent of microsatellite instability and frequency of allelic loss. In the section on surrogate endpoint biomarkers below, it will be shown how chroma-

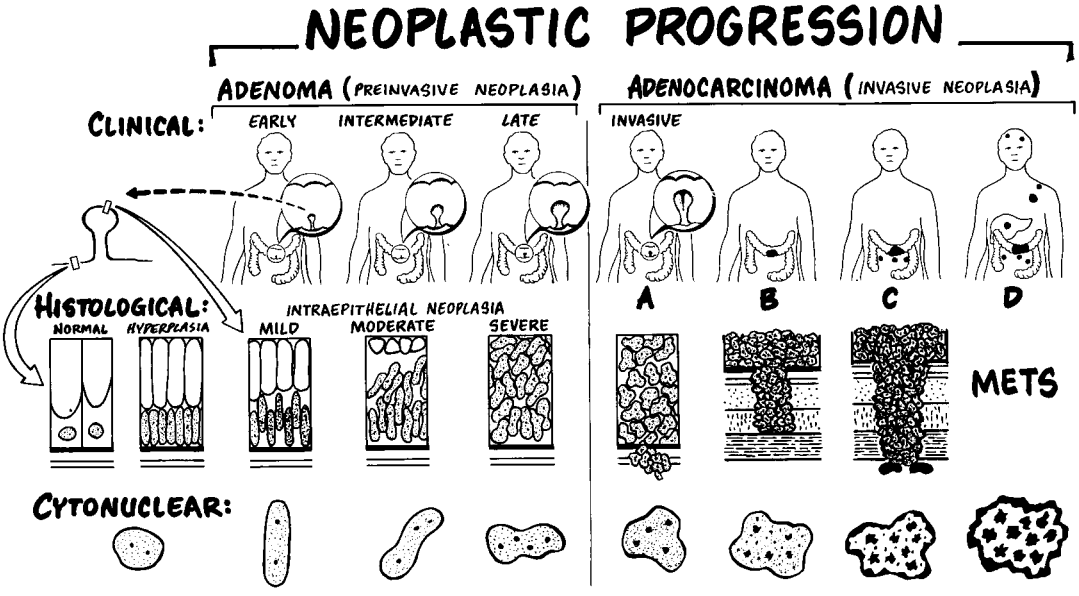


Fig. 6. The complete span of neoplastic progression in colorectal neoplasia. The extent and rate of neoplastic progression may be diagnosed at either the clinical, tissue, or cytonuclear levels and used to estimate the probability of survival.

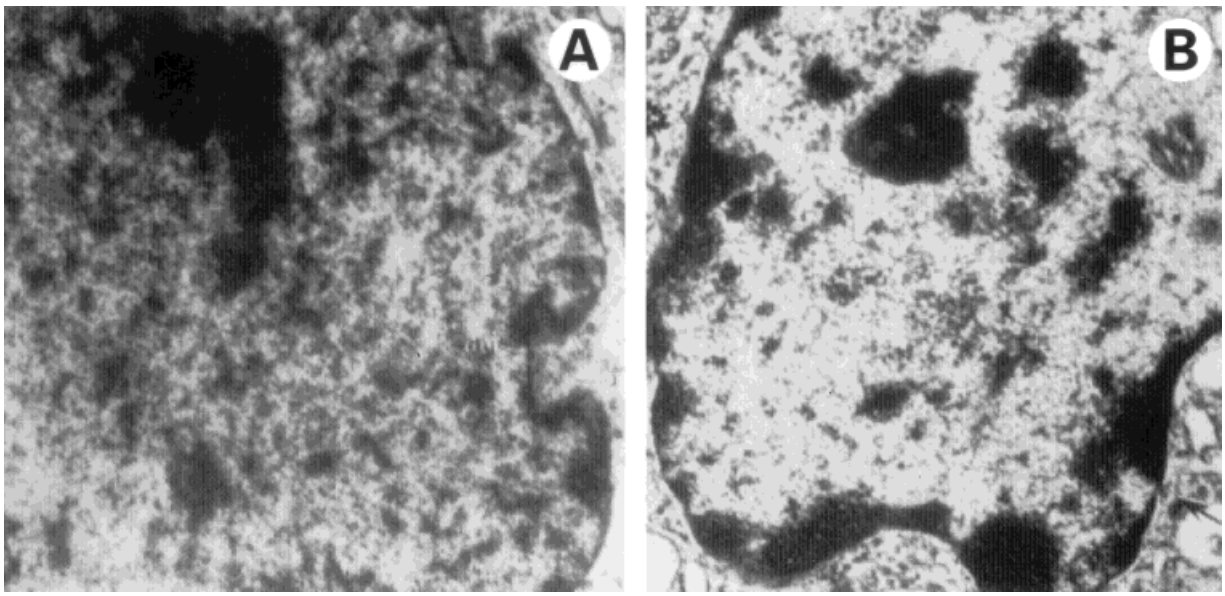


Fig. 7. Low-power electron micrographs of cell nuclei from the uterine cervix. **A:** Nucleus of a hyperplastic normal cell. **B:** Nucleus of an intraepithelial neoplastic cell of high grade. Note the dense chromatin clumps with sharp margins bordering intervening regions that stain more lightly (so-called "parachromatin clearing").

tin texture patterns specific for neoplasia, quantitatively measured by computer-assisted image analysis, can be used to estimate the extent of neoplastic progression, and also to monitor slowing of the rate of neoplastic progression produced by chemopreventive agents.

Rate of Neoplastic Progression Is Driven by the Rate of Clonal Evolution

From the above considerations, it is axiomatic that the characteristic clinical properties of neoplastic progression, namely, the constant increase in cell proliferative rates, in genotypic and phenotypic heterogeneity, and in total bulk and degree of dissemination, all derive from accelerating clonal evolution.

STRATEGIES OF CHEMOPREVENTION BASED ON THE PROPERTIES OF INTRAEPITHELIAL NEOPLASIA

Recognizing that intraepithelial neoplasia is a condition in which multiple neoplastic foci develop in an epithelium affected with genomic instability, and that the foci enlarge slowly over many years, the prime strategy of chemoprevention is to diagnose and treat intraepithelial neoplasia as early as possible, even during the predysplastic phase of diffuse genomic instability, with agents that maximally slow neoplastic progression, or, of course, abrogate it altogether. Chemopreventive agents under development by the chemoprevention program at the National Cancer Institute have been reviewed extensively [54–56].

Emphasis on Early Diagnosis and Therapy of Intraepithelial Neoplasia

FISH assays are now available to diagnose and monitor the treatment of field cancerization, i.e., of diffuse genomic instability, even before the onset of intraepithelial neoplasia in patients with proven high cancer risk (e.g., previous surgery for head and neck cancer). Diagnostic assays have been developed for microsatellite instability in urine [57], and sputum [58], and *ras* gene mutations in stool [59]. In a later section, computerized imaging methods will be described for diagnosing and monitoring treatment of neoplasia-specific changes in cytonuclear chromatin texture in early intraepithelial neoplasia. At the clinical level, a recent advance in early diagnosis is the LIFE system of fluorescent bronchoscopy [60], which induces natural autofluorescence in intraepithelial neoplastic lesions of the pulmonary bronchi so that they can be easily located for biopsy and analysis.

Emphasis on Developing Antiproliferative Agents

The central and powerful role played by hyperproliferation in driving the accelerating rate of progression of intraepithelial neoplastic le-

sions is undeniable, and justifies aggressive development of new and more powerful antiproliferative agents. Drugs need development that inhibits steps in "command" signal pathways related to initiation of cell proliferation (e.g., growth factors and their receptors, and the *ras*/*raf*/MAP kinase pathways) and steps in "execution" synthesis pathways that duplicate cell structure in preparation for cell division (e.g., enzyme-catalyzed steps in deoxyribonucleotide synthesis). Agents that are strictly proliferation suppressants, such as difluoromethylornithine, are cancer preventive in diverse animal models in the chemoprevention program [e.g., 61]. Since proliferative rates of advanced grades of intraepithelial neoplasia are generally 5–10-fold higher than those of normal epithelia, proliferation suppressants may tend to have more effect on neoplastic tissue. Interfering early enough during the development of intraepithelial neoplasia with proliferation-suppressant therapy has the potential of adding many more years of wellness and reduced cancer risk.

Emphasis on Developing Antiinflammatory Agents

Intervention with non-steroidal antiinflammatory agents should be considered in every subject whose intraepithelial neoplasia is likely to be associated with chronic inflammation, for example, lesions of the respiratory and digestive tracts and the uterine cervix. There are three enzyme activities associated with inflammation whose inhibition should be considered: the prostaglandin endoperoxide synthase and hydroperoxidase activities, which generate prostaglandins, and the lipoxygenase activity, which generates leukotrienes. The hydroperoxidase activity is important because it will "cooxidize" and activate carcinogens [62].

Agents that inhibit both prostaglandin endoperoxide synthase and the lipoxygenase, such as the plant phenolics curcumin and quercetin, appear to be of advantage.

Emphasis on Developing Antioxidants

Reactive oxygen species (singlet oxygen, superoxide, peroxide, hydroxy free radicals) and related endogenous free radicals (peroxynitrite, nitric oxide, hypochlorite) are both mutagenic and mitogenic [63]. They are of common occurrence in the environment, particularly in cigarette smoke and fossil fuel combustion products, and are endogenously produced extracellularly by chronic inflammatory infiltrates and intracellularly by "leaky flavoproteins" in the electron transport chain of mitochondria and in cytochrome P450 reductase of the endoplasmic reticulum [64]. Antioxidants appear to offer good potential for general chemopreventive action. Plant phenolics such as curcumin and flavonoids, in particular, have both antioxidant action by blocking lipid peroxidation, and anti-inflammatory action by blocking the enzyme lipoxygenase [65], which produces leukotrienes that are chemotaxic attractants to inflammatory cells.

Antimutagenic Agents

Preventing the mutagenic effects of chronic tobacco use is a common indication for intervention with antimutagenic agents. Antimutagens with different primary mechanisms of action are being tested, e.g., oltipraz, which induces Phase II xenobiotic metabolizing enzymes (glutathione synthetase, glutathione-*S*-transferase, epoxide hydrolase, UDP-glucuronyl transferase), and alkylaryl isothiocyanates, which suppress Phase I oxidizing enzymes (arylhydrocarbon hydroxylases). Since the availability of *l*-cysteine is rate-limiting to the activities of glutathione synthetase, glutathione-*S*-transferase, and glutathione peroxidase, antimutagens that induce these enzymes should be given in combination with *N*-acetyl-*l*-cysteine, which provides *l*-cysteine immediately on being transported into cells.

Emphasis on Developing Proapoptotic Agents

When a proapoptotic induces a rate of apoptosis that exceeds the rate of proliferation, it will cause the neoplasm to shrink and disappear. For example, sulindac causes apoptosis and shrinkage of established colonic polyps in patients with familial adenomatous polyposis [66], and the terpene perillyl alcohol produces massive apoptosis in established mammary tumors of rats, causing the tumors to completely disappear [67] (confirmed by the senior author who examined histological slides provided by Dr. Michael Gould). Animal models to screen for proapoptotic agents should be set up with protocols for late intervention that test for shrinkage of already established tumors.

Preventing "Clonal Escape" From the Effects of Chemopreventive Agents

Considering that intraepithelial neoplasia is characterized by multicentrically distributed

and independently progressing neoplastic lesions undergoing clonal evolution, when a chemopreventive agent is administered, the risk increases with time that clonal variants will arise which have switched to metabolic pathways other than those being affected by the chemopreventive agent, so that "clonal escape" ensues. The best chemopreventive strategy to combat clonal escape is to use combinations of chemopreventive agents with different mechanisms of action.

Use of Computer-Assisted Quantitative Image Analysis (CQIA) to Monitor Surrogate Endpoint Biomarkers for Clinical Trials of Chemopreventive Agents

In present-day histopathologic diagnosis, the established practice of estimating cancer risk by subjectively estimating nuclear grading, using relatively imprecise descriptive terms such as "nuclear pleomorphism and hyperchromasia" or "moderately increased number of mitoses," may now be supplemented by more objective and precise measurements offered by computer-assisted quantitative image analysis (CQIA).

Selection of the most useful SEBs. In searching for some property of early intraepithial neoplastic progression that correlates with high cancer risk, the morphological changes of intraepithelial neoplasia immediately suggest themselves, particularly if they can be measured quantitatively and objectively by means of CQIA. Such nuclear morphology-based SEBs have been critically reviewed previously [68, 69]. Briefly, they are increased nuclear size, altered nuclear shape, increased variance of nuclear size and shape (pleomorphism), altered chromatin texture, increased mitotic index, abnormal mitoses, and alteration or absence of differentiation and maturation. These SEBs have the advantage that they are not markers of intraepithelial neoplasia, they are intraepithelial neoplasia itself, by definition. Measuring morphonuclear changes and proliferative behavior of intraepithelial neoplastic lesions as predictors of later invasive neoplasia may be confounded by the fact that spontaneous regression of some of these lesions may occur, especially if the lesions are mild to moderate in extent. Therefore, in addition to quantitatively evaluating intraepithelial neoplastic lesions in biopsy samples from the same patient before and after intervention with a chemopreventive agent, comparison should also be made with lesions in control subjects given placebos.

The core endpoints now being used in chemoprevention clinical trials frequently include morphonuclear SEBs (nuclear and nucleolar size, shape, variance of size and shape, frequency of number of nucleoli per 100 cells, and particularly dozens of chromatin texture features), DNA ploidy, and proliferative index measured with antibody probes, all quantitated objectively with CQIA.

Design of computer software to detect nuclear chromatin texture features specific for neoplastic changes in epithelial cells. Dozens of nuclear chromatin texture features have been measured in neoplastic cells, based on analysis of patterns of change in optical density from one small pixel, 0.5 x 0.5 μ in size, to another in the digitized image of the nucleus. A new computer instrument with new software programs developed by Bacus Laboratories Imaging Systems (Elmhurst, IL), called the BLISS instrument, measures quantitatively and objectively many of the nuclear chromatin texture features used by histopathologists to make a diagnosis of intraepithelial neoplasia. An example of the measurement of one chromatin texture feature will be presented here: the "Deep Valley Detector."

"Deep Valley Detector," a Software Program That Detects Nuclear Features Specific for Neoplasia. The cell nuclei of intraepithelial neoplastic lesions are commonly described by the pathologist as showing "chromatin clumping." The edges of the chromatin clumps are said to be "sharply marginated." John Frost, the late Chief of Cytopathology at Johns Hopkins, coined the term "cookie cutter chromatin" to describe this characteristic sharp margination. He also emphasized that the space between chromatin clumps was lighter than normal, and called this space, as others have, "parachromatin clearing." Figure 7 compares the nuclear chromatin pattern of a normal hyperplastic cell of the uterine cervix with a neoplastic cell from the same tissue. The sharply marginated chromatin clumps and lighter area between the clumps of the neoplastic cell can easily be seen. A software program was designed that directs the computer to identify the number and location within the nucleus of a specific pixel pattern associated with sharp-edged chromatin clumps adjacent to areas of parachromatin clearing. The following com-

mand was given to the computer: "Find every set of three pixels in a row, the center pixel of which has an optical density which is less than the optical density of either end pixel by at least 0.05 OD units. Count the number of pixel triplets with this property and mark their location in an image of the nucleus." This program is called the "Deep Valley Detector" because it identifies the margins of chromatin clumps in neoplastic nuclei that have a steep and deep optical density "dropoff." The depth of the dropoff that is detected can be varied. Figure 8B shows the computer image of a nucleus from a neoplastic cell of high-grade cervical intraepithelial neoplasia, in which 111 "deep valley" sites were counted and their location shown (by a red mark in the original image). By contrast, Figure 8A is the nucleus of a non-neoplastic hyperplastic cell from adjacent cervical epithelium, which had only 16 sites. This large quantitative difference in number of "deep valley" sites in normal hyperplastic as compared to neoplastic nuclei was generally found for all nuclei in the specimen. The number of deep valley sites, including those of different depths, may be used to measure with precision the extent of neoplastic progression, and also modulating effects of chemopreventive agents on intraepithelial neoplasia may be quantitated by the change they produce in the number of "deep valley" sites per nucleus.

SUMMARY

A central aim of the chemoprevention program is to learn as much as possible about the molecular and cellular mechanisms of intraepithelial neoplasia, and to use this information to plan directions having the best chance of success [70]. In summary, intraepithelial neoplasia has two precursor conditions and four major properties. The first precursor condition, chronic hyperproliferation, is common but not essential. The second precursor condition, genomic instability, is essential. It is defined as the increased rate of unrepaired DNA breaks with secondary formation of abnormal genomic struc-

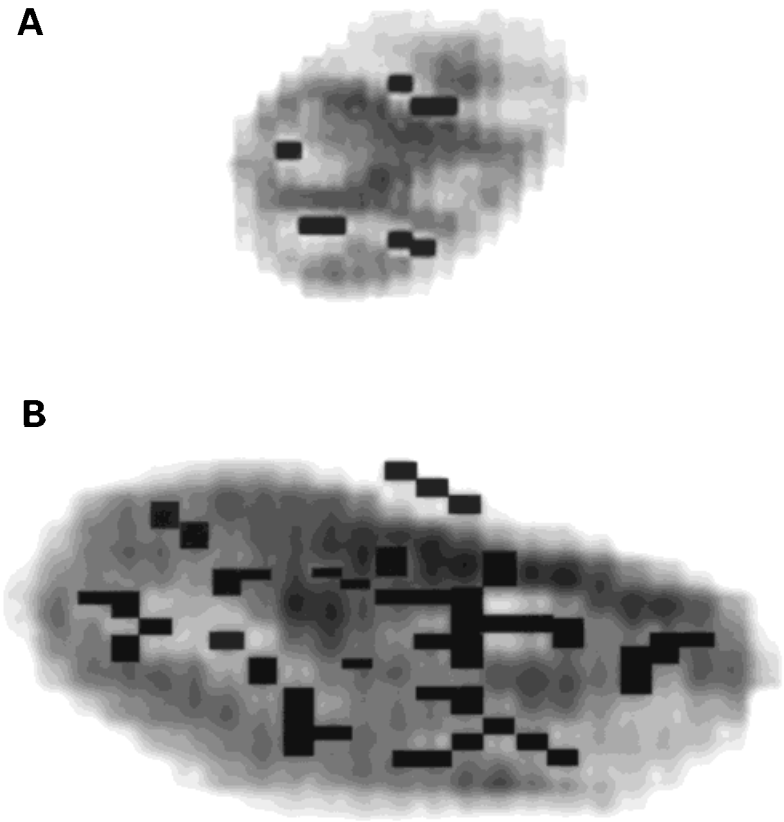


Fig. 8. Representative images of cell nuclei from the uterine cervix displayed by the computer showing the location of "deep valley" sites. **A:** Nucleus of a normal hyperplastic cell, with 16 sites. **B:** Nucleus of a neoplastic cell, with 111 "deep valley" sites.

tural variations, including oligonucleotide mutations, allelic loss and gain, and karyotypic (whole chromosomal) aberrations in structure and number. The four major properties of intraepithelial neoplastic lesions are (1) multifocality, (2) clonal evolution, (3) accelerating intralesional production of genomic structural variants cells (some of which form clones that grow faster and others which undergo apoptosis due to recognition by checkpoint controls of excessive DNA damage), and (4) increasing phenotypic heterogeneity. Efficient planning strategies for the chemoprevention program include the following emphasis: early diagnosis and therapy, development of more agents in the categories of antiproliferatives, antioxidants, antiinflammatories, and proapoptotics, the prevention of "clonal escape" by using combinations of chemopreventive agents, and the use of computer-assisted quantitative analysis to evaluate modulation of cell and tissue surrogate endpoint biomarkers in chemoprevention clinical trials.

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