The DNA-instability test as a specific marker of malignancy and its application to detect cancer clones in borderline malignancy

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Genomic instability

It is now widely accepted that cancer may be genetically unstable (Lengauer et al., 1998), and that the genomic instability is the engine of both tumor progression and heterogeneity (Nowell, 1976; Shen et al., 2000). The tumor instability is defined by a more rapid accumulation of genetic alterations in tumors compared with normal cells, and is classified into four different types, (1) subtle sequence changes; (2) alterations of chromosome numbers; (3) chromosome translocations; and (4) gene amplifications (Lengauer et al., 1998).

Subtle sequence changes

These changes involve base substitutions, deletions, and insertions of a few nucleotides. The repair machinery operates on sequence errors generated by polymerases or by mutagens.

Translesion bypass

Recently, a large number of new DNA polymerases have been identified, which although sharing significant amino acid sequence identity and similarity amongst themselves, exhibit little homology to any of the five previously identified polymerase families (Cordonnier and Fuchs, 1999; Frieberg and Gerlach, 1999; Johnson et al., 1999; Woodgate, 1999) and is referred to as the Y-family of DNA polymerases (Ohmori et al., 2001). Among them, the xeroderma pigmentosum variant (XPV) gene encodes human DNA polymerase η (poly η), which catalyzes efficient and accurate translesion synthesis past cis-syn-cyclobutane di-thymine lesions produced by ultraviolet light irradiation, not only by incorporating the correct nucleotide opposite a lesion, but also by elongating DNA chains that have a correctly incorporated nucleotide opposite a lesion (Masutani et al., 1999, 2000). Although these new DNA polymerases preferentially incorporate dCMP opposite acetylaminofluorene (AFF)-G.
and cytosine-guanosine-GG, other nucleotides are also incorporated opposite these lesions (Masutani et al., 2000). These mispaired incorporations of nucleotides or genomic alterations by poly η with its replacement by more error-prone bypass DNA polymerases may further cause a serious threat to genomic stability.

**Nucleotide-excision repair**

Nucleotide-excision repair (NER) genes are responsible for repairing damage caused by many exogenous mutagens, and skin tumors represent the major tumor type to which patients with NER defects are susceptible. In a heterozygote with one defective NER allele, however, inactivation of the normal allele does not always lead to a high mutation rate, because exposure to an additional environmental agent, ultraviolet light, is required to engender such mutations (Lengauer et al., 1998).

**Mismatch repair (MMR)**

A subset of sporadic colorectal cancers and most hereditary nonpolyposis colorectal cancer patients display frequent alterations in microsatellite sequences, (CA)n repeats, showing replication errors (RER+) or microsatellite instability (MIN or MMR) (Parson et al., 1993: Lengauer et al., 1998). Elements of microsatellites constitute one of the most abundant classes of repetitive DNA families in humans, and 50,000 to 100,000 (CA)n repeats are scattered throughout the human genome and may exhibit length polymorphisms (Thibodeau et al., 1993). Six human mismatch repair genes are now known which, when inactivated by mutation, lead to a MIN phenotype in cancer patients (Peltomaki and Chapelle, 1997). The tandem nature of elemental units within simple repeats is thought to render such sequences prone to slipped-strand mispairing and hence particularly prone to insertion or deletion mutagenesis during replication (Levinson and Gutman, 1987; Kunkel, 1993). Centromeres, which are essential chromosome structures for correct segregation of sister chromatids, are large, in the range of megabases, often containing repetitive DNA sequences in the condensed heterochromatin (Choo, 2001; Schueler et al., 2001; Goshima et al., 2003). Obuse et al. (2004) identified 40 genes including oncogenes in the interphase centromere complex (I-CEN-complex) which contains all proteins constituting centromeres and centromere chromatin. MIN introduced in centromeres, therefore, may induce defected checkpoint machinery for cell proliferation and differentiation.

**DNA methylation**

Cytosine-5-methyltransferase catalyzes the transfer of a methyl group from S-adenosylmethionine to the C5 position of cytosine (Santi et al., 1983). Abnormal methylations are very common in cancer cells and are capable of directly modifying carcinogenesis, causing point mutations in tumor suppressor genes in somatic and germ line cells (Jones, 1996). Hypomethylation preferentially occurs in DNA repeats contained in Alu and alpha-satellite and induces abnormalities of cell cycle checkpoints and genomic instability by alterations of the related genes (Cahill et al., 1994, 1998; Wong et al., 1999; Ferguson et al., 2000; Kikuchi et al., 2002; Satoh et al., 2003; Toyota et al., 2003).

**Alterations in chromosome number**

Chromosome number instability (CIN) involving gains and losses of whole chromosomes, aneuploidy, are likely to occur in most human malignancies (Mitelman et al., 1994; 1997). As reviewed by Lengauer et al. (1998) in detail, genes that, when mutated, can lead to CIN include those involved in chromosome condensation, sister-chromatid cohesion, kinetochore structure and function and centromosome-microtubule formation, as well as checkpoint genes that monitor the proper progression of the cell cycle (Hartwell and Smith, 1985; Hartwell, 1992; Murray, 1995; Elledge, 1996; Fukasawa et al., 1996; Nasmyth, 1996; Paulovich et al., 1997; Taylor and McKeon, 1997; Doxsey, 1998; Jin et al., 1998; Lane, 1998; Rotman and Shiloh, 1998; Zhang et al., 1998). The fact that genetic defects of so many genes can lead to CIN suggests a heterogeneous basis for CIN in cancers, with many genes each playing a role in a small proportion of the cases (Lengauer et al., 1998). In DNA-damage checkpoint deficiency, chromosomes containing damaged DNA could segregate inappropriately, resulting in CIN because sister chromatids are still connected by DNA or DNA-protein links and are also susceptible to gross structural alterations due to single-stranded gaps or double-stranded breaks. Genomic deletion caused by double-stranded breaks is reflected in the genomic mechanism of loss of heterozygosity (LOH), and losses of maternal or paternal alleles in a tumor are widespread and are often accompanied by a gain of the opposite allele (Lengauer et al., 1998; Shen et al., 2000).
Chromosome instability arising from failed DNA-damage checkpoints is often associated with enhanced mitotic recombination as well as with aberrant chromosome segregation (Hartwell and Smith, 1985). Thus, cancer progression is often associated with the accumulation of these gross chromosomal rearrangements (GCRs), such as translocations, deletions of chromosome arms, interstitial deletions or inversions (Gauwerky and Croce, 1993; Shikano et al., 1993; Mitelman, 1997; Chen and Kolodner, 1999). In many instances, GCRs inactivate tumor-suppressor genes or generate novel fusion protein that initiate carcinogenesis (Gauwerky and Croce, 1993; Barr, 1998). As described above, methylation deficiency (MET) often induces many genomic alterations and may finally form CIN. Jones and Gonzalgo (1997) suggested the existence of two different phenotypes of MMR- (MIN)-MET+, and MMR+-MET-, and this will explain an inverse relationship between CIN and MIN in colorectal and endometrial cancers (Lengauer et al., 1998). Cancers showing MMR deficiency are generally diploid, whereas MMR-proficient tumors are usually aneuploid showing CIN.

**Chromosome translocations**

These alterations can be detected cytogenetically as fusions of different chromosomes or of normally non-contiguous segments of single chromosomes. At the molecular level, such translocations give rise to fusions between two different genes, encoding fused transcript with tumorigenic properties (Lengauer et al., 1998). Large portions of chromosomal arms are often deleted during recombination that lead to translocations, and these deletions are seen as the losses of heterozygosity at the molecular level. Translocations will arise in cells that enter mitosis before recombination-promoting double-stranded DNA breaks are repaired. Alterations of genes involved in double-stranded breaks or DNA-damage checkpoints therefore, will induce the translocation instability in human cancers (Lengauer et al., 1998).

**Gene amplification**

Gene amplifications are seen at the cytogenetic level as homogeneously stained regions or double minutes. At the molecular level, multiple copies of an amplicon containing growth promoter genes can be seen (Lengauer et al., 1998). The mechanisms through which amplifications are generated are largely unknown, but amplifications occur more easily when p53 is inactivated (Yin et al., 1992).

**DNA-instability test**

As described above, recent progress in cytogenetic and biochemical mutator assay systems has enabled us to detect single gene alterations and gross chromosomal rearrangements (GCRs), from which it became clear that all cancers are genetically unstable. In order to detect the genome-wide instability of cancer cells, we developed a new method, the DNA-instability test.

**Background**

By quantitative analysis of the Feulgen hydrolysis curve, it was found that the extent to which nuclear DNA exposed to acid is denatured reflects the degree of DNA instability, which depends on the chromatin structure (Darzynkiewicz et al., 1979; Fukuda et al., 1985, 1986). The Bateman function was found to be appropriate for the Feulgen hydrolysis curve analysis (Böhm and Seibert, 1966; Pöppe et al., 1979; Madokoro et al., 1985). According to the Bateman function, the amount of apurinic acid or single-stranded DNA present at hydrolysis time t, is expressed by the equation $y(t) = y_0 k_1 / (k_2 - k_1)^*(e^{-k_1t} - e^{-k_2t})$, and the amount of apurinic acid or single-stranded DNA potentially present initially ($y_0$), as well as the rate constants for its production ($k_1$) and degradation ($k_2$), can be determined by fitting the function to the experimentally obtained hydrolysis curve. The hydrolysis curve is influenced by a number of factors, including the kinetics of the hydrolysis reaction and chromatin structure. In chicken erythrocytes, for instance, it was found that the depurination rate constant is decreased in compact form of chromatin (Duijndam and van Duijn, 1975). We also analyzed acridine orange (AO) hydrolysis curves obtained for various normal cell types, various cancer cell types, damaged cells induced by the administration of chemical carcinogens or X-ray irradiation, and neuronal cells in the process of aging, and it was found that the value of $1/k_1$ reflects the degree of chromatin condensation, while the values for $y_0$ and $k_2$ correlate with the degree of DNA instability or DNA damage (Fukuda et al., 1985; Miyoshi et al., 1986). It was found that the $k_1$ value, which reflects the degree of DNA instability, is much larger for
cancer cells than for normal cells (Fukuda et al., 1986). This finding led us to develop a method for differential fluorescent AO staining of cancer cells and non-cancerous cells utilizing the different degree of DNA instability at acid hydrolysis. The fluorochrome bound to the single-stranded polynucleotide, apurinic acid, emits metachromatic red-shifted fluorescence due to dye aggregation, while that intercalated in the intact double-stranded polynucleotide emits orthochromatic green fluorescence (Darzynkiewicz et al., 1979).

**Differential acridine orange-fluorescent staining specific for cancer cells**

AO staining after hydrolysis with 2N HCl at 30°C for 8.5 min was found to be the optimal condition (Fukuda et al., 1986). In human malignant tumors of epithelial and nonepithelial origin tested, all malignant tumor cells emitted metachromatic red fluorescence, while all of the non-malignant tumor cells and normal cells emitted orthochromatic green fluorescence when observed with a violet excitation light under a fluorescence microscopy (Figure 1a).

**Differential immunohistochemical staining specific for cancer cells using anti-single-stranded DNA antiserum**

The above described fluorescent AO staining for specific differentiation of cancer cells was also applied to the ordinary paraffin-embedded sections, but no differential fluorescent staining was obtained: possibly due to delicate alterations of nuclear DNA by the procedures of specimen preparation, and at present, this method is applicable only to the smeared specimens of isolated cells. Then, an improved method by using anti-single-stranded DNA antiserum which could bind more specifically to single-stranded nucleotide induced by acid hydrolysis was developed (Fukuda et al., 1993). The ordinary pathological sections were immunohistochemically stained with the antiserum after RNase digestion and DNA denaturation by hydrolysis with 2N HCl at 30°C for 20-30 min, and all cancer cells were specifically stained positive, in sharp contrast to the negative stainability of all non-cancerous cells (Figure 1b).

**Differential in situ-hybridization (ISH) method specific for cancer cells**

As for anti-single-stranded DNA antiserum, polyclonal anti-single-stranded DNA antiserum and monoclonal anti-cytidine antiserum were used in the immunohistochemical DNA-instability test described above. When the fixation of specimen was not appropriate, too long or insufficient, even non-cancerous cells sometimes showed weak but positive staining. Inappropriate fixation could induce partial denaturation of nuclear DNA which will render even non-cancerous cell nuclei positive for the immunohistochemical staining with anti-single-stranded DNA antiserum. In order to overcome this problem, an ISH method using a larger molecule as
a probe was developed, which bound more specifically to just sufficiently denatured DNA induced by the treatment with formamide solution (unpublished). The outline of this method is as follows:

(a) genomic DNA was extracted from frozen human liver stored at autopsy by homogenization and cell lysis, followed by RNase digestion and protein extraction;

(b) then the DNA was labeled with biotin by nick translation forming 500-2,000 kbp DNA fragments, followed by reaction with the avidin-biotin peroxidase complex;

(c) ordinary pathological sections and ethanol-fixed smeared specimens of isolated cells were treated with proteinase K, followed by the treatment with 70% formamide solution at 60°C, for 10 min or 50-60 min, respectively. Then the specimens were bound with the DNA probe, followed by the color reaction with DAB or Co-DAB to visualize the peroxidase reaction. By this method, the specificity for cancer cells was fairly improved even in inappropriately fixed specimens. When acid hydrolysis was used to denature nuclear DNA, even cancer cells apparently identified morphologically sometimes showed pseudo-negative stainability, presumably because of DNA-loss; and non-cancerous cells also sometimes stained positive, possibly due to partial denaturation of nuclear DNA artificially induced by inappropriate fixation. DNA denaturation with formamide solution, on the other hand, may not evoke DNA-loss because it is much milder than HCl hydrolysis. Furthermore, a DNA probe of large molecular weight will not bind to partially denatured nuclear DNA as artificially induced by inappropriate fixation. Since the first report of the DNA-instability test (Fukuda et al., 1986), numerous malignancy cases and so-called borderline malignancy cases were examined by this method, and all cancer cells were found to be positively stained, while comparative normal cells including vascular endothelial cells, fibroblasts and inflammatory cells contained in the same specimen were all completely negative. As described in this paper, a great number of reports concerning genomic instability of cancer cells have been presented. These reports, however, only demonstrated the presence of qualitative and quantitative alterations in certain specific genomic loci. In contrast to these commonly used methods to reveal genomic instability of cancer cells at certain specific DNA regions, the DNA-instability test above introduced revealed the presence of physical DNA instability in the entire DNA molecule of a cancer cell nucleus as demonstrated by increased liability to denature upon HCl hydrolysis or treatment with formamide solution. The mechanism of increased physical DNA instability of cancer cells against acid hydrolysis or treatment with formamide is not fully understood, but possible mechanisms have been proposed (Hatchoh et al., 1992; Fukuda et al., 1993; Nitta et al., 1993; Otaki et al., 1994). In addition to a variety of accumulated genomic alterations in cancer cell DNA, disturbed function of histone production due to certain genomic damage will impair the DNA packing function and may result in abnormal configuration that will also induce increased DNA instability upon HCl hydrolysis or treatment with formamide. Another possible mechanism of increased physical DNA instability of malignancy may be attributable to the production of reactive oxygen by cancer cells (Noriki et al., 1989; Fukuda et al., 1993). In the cell membrane of cancer cells, the amount of unsaturated fatty acids is known to be increased (Wood et al., 1985) with increased lipid peroxide (Noriki et al., 1989; Otamiri and Sjodhal, 1989), because unsaturated fatty acids are susceptible to peroxidation to produce lipid peroxide. The latter is included in the group of reactive oxygen, and may be the source of radical chain reactions producing further reactive oxygen. Reactive oxygen modifies enzymatic and structural proteins, rendering them susceptible to proteolytic attack (Levine et al., 1981; Dean, 1987), and gives rise to membrane damage through lipid peroxidation. This harmful chain reaction induces the inactivation of the enzymes which act as reactive oxygen scavengers. These finally induce serious DNA damage by modification, strand-break, and oxidative degeneration (van Hemmen and Meuling, 1975; Kasai and Nishimura, 1986; Peskin and Shlyanova, 1986; Noriki et al., 1989).

Applications of the DNA-instability test to detect cancer clones in borderline malignancy

Clonal evolution and progression of 20-methylcholanthrene-induced squamous cell carcinoma in mouse epidermis (Hirai et al., 2001)

In an attempt to understand the dynamic process of carcinogenesis and cancer progression, the clonal evolutions of squamous cell carcinoma (SCC) were examined in the lesions induced by repeated
Topical applications of 20-methylcholanthrene (20-MC) to the mouse skin, which induces hyperplastic epidermis, papillomatous lesion and invasive carcinoma. The lesions were examined histopathologically and immunohisto-chemically using anti-single-stranded DNA antiserum after HCl hydrolysis (DNA-instability test). Multiple clones with increased DNA instability at acid hydrolysis comparable to that of invasive carcinoma were noted already in early-stage (2-6 weeks) hyperplastic epidermis, and their number increased in middle (7-11 weeks), and late-stage (12-25 weeks) of hyperplastic epidermis showing clonal expansion, indicating that they belong to malignancy category (Figure 1c). All papillomatous lesions and invasive carcinomas showed positivity to the DNA-instability test. The analyses were carried out on the same lesions by means of the immunohistochemical labeling of p53 (a gene producing a protein which inhibits the entrance of abnormal cells to S-phase through G1-S checkpoint and push those to apoptosis even after slipping into S-phase: Finlay et al., 1988; Hollstein et al., 1991), of VEGF (vascular endothelial growth factor, a protein which induces vascular endothelial cell proliferation: Leung et al., 1989), of DFF45 (a protein which inhibits DNA fragmentation at the final step of apoptosis by forming dimer with DFF40, a DNase: Liu et al., 1997; Enari et al., 1998; Sabol et al., 1998; Sasaki et al., 1999), of PCNA (proliferating cell nuclear antigen, an auxiliary protein for DNA polymerase δ, playing an important role in the initiation of cell proliferation: Bravo et al., 1987; Wong et al., 1987; Jaskulski et al., 1988; Yang et al., 1993), and by staining AgNORs (chromosomal regions where genes for major ribosomal RNA are located, of which number may reflect the capability of cell proliferation and the degree of neoplastic malignancy: Goodpasture and Bloom, 1975; Howell et al., 1975; Reeves et al., 1984; Arden et al., 1985; Crocker et al., 1987; Ayres et al., 1988; Dervan et al., 1989; Muscare et al., 1991; Yu et al., 1992).

Cells with positive immunostaining for these cancer markers and with abnormal AgNORs parameters appeared (although with different percent values) in the clones with positive DNA-instability test in early- or middle-stage hyperplastic epidermis; and increased markedly in number in keeping pace with the clonal expansion in late-stage hyperplastic epidermis, papillomatous lesions and invasive carcinomas. The percentage of PCNA-positive vascular endothelial cells was significantly higher under the VEGF-positive clones showing positive DNA-instability test, and became higher toward the late-stage of cancer progression. Although the PCNA-positive cells were well defined in the basal layer in normal epidermis, the normal distribution was more or less disturbed already in the early- or middle-stage hyperplastic epidermis, especially in the clones with positive DNA-instability test, and markedly disturbed in the late-stage of hyperplastic epidermis, papillomatous lesions and invasive carcinomas. This clearly indicates the presence of disturbed cell proliferation and differentiation in these lesions, reflecting again the malignant characteristics. Although the foci with positive DNA-instability test were also often positive for these cancer markers, it was almost impossible to identify them as malignant based upon simple morphological criteria, because the degree of cellular and structural atypia in these foci was not highly pronounced, although the nuclei and nucleoli were only slightly enlarged even in the late-stage hyperplastic epidermis and papillomatous lesions. Based on these findings, we proposed a new concept of procancer (not pre-cancer as commonly used) as a cancer clone at an early-stage of cancer progression. A procancer clone already exhibits increased physical DNA instability as revealed by positive DNA-instability test with positivities for various cancer markers such as p53, VEGF, DFF45, PCNA and abnormal AgNORs parameters, but without remarkable morphological atypia to be used as the basis of malignancy diagnosis. We also defined these phenotypic abnormalities as functional atypia opposed to commonly used morphological atypia (Figure 2). We interpreted the dynamics of carcinogenesis and cancer progression demonstrated in this study as follows: procancer clones with positive DNA-instabili-
ty test appeared within 2 weeks after commencing topical applications of 20-MC. The number of these clones increased continuously toward the middle- and late-stage hyperplastic epidermis showing positivities for various cancer markers, reflecting clonal expansion. The number of papillomatous lesions derived from the procancer clones is much smaller than that of the latter. Furthermore, all cancer lesions identified by enhanced degree of morphologic atypia appeared only in the preceding papillomatous lesions, but they did not appear directly in the hyperplastic epidermis. The term papilloma seems to be inappropriate and should be replaced by papillomatous lesion, because all papillomatous lesions were positive for DNA-instability test and other cancer markers, indicating that all cells in the lesion are regarded already as cancer cells, and they must be referred to as procancer. The procancer clones with high DNA instability would produce multiple cancer sub-clones by enhanced proliferative activity as revealed by increased PCNA positivity. Mutations of the p53 gene would also enhance proliferation and reduce the likelihood of apoptotic cell death of procancer clones. Furthermore, paracrine secretion of VEGF by procancer clones in hyperplastic epidermis would induce dermal neovascularization, which in turn would enhance the growth and transition of flat hyperplastic epidermis to papillomatously elevated lesion by providing sufficient levels of oxygen and nutrients. A high percentage of DFF45-positive cells, especially in the late-stage of hyperplastic epidermis, papillomatous lesions and invasive carcinomas, could be interpreted as induction. The induced DFF45 binds to DFF40 and blocks its DNase activity, thereby allowing cancer cells to avoid the DNA-degradation machinery immediately before the final step of apoptosis. Taken together, these new features adopted by procancer and cancer clones should favor an increase in the number of descendants which could accept abundant nutrients, having a high chance to survive by escaping the apoptotic machineries driven by p53 and DFF40. Consequently, multiple cancer clones showing cancer progressions which would be suitable to invade or metastasize may be produced.

**Detection of procancer clones in human gastric adenoma (Otaki et al., 1994; Sun et al., 2003)**

Gastric adenoma is defined by the World Health Organization (WHO) as circumscribed benign lesions, composed of tubular and/or villous structures showing intra-epithelial neoplasia (Fenoglio-Preiser et al., 2000), and is widely considered to be a pre-malignant lesion (Correa et al., 1992). According to the degree of cellular and structural dysplasia, gastric adenoma is divided into two qualitative grades (low-grade and high-grade dysplasia) or three qualitative grades (mild, moderate and severe dysplasia) (Ming et al., 1984; Tosi et al., 1989: Goldstein and Lewin, 1997). Many authors have investigated the relationship between gastric adenoma and cancer, and showed that the frequency of malignant transformation from adenoma to carcinoma depends on size and histological grade of adenoma (Nakamura et al., 1988; Xuan et al., 1991; Tsujitani et al., 1992). Most investigators agree that severe, or high-grade dysplasia is the most important precursor of gastric cancer and strongly recommend gastrectomy, endoscopic mucosal resection (EMR) or polypectomy, particularly in light of the high percentage of early gastric cancer (Lansdown et al., 1990; Farinati et al., 1991; Di Gregorio et al., 1993). However, there remains disagreement regarding the risk of malignant potential for adenomas with mild and/or moderate dysplasia. Some authors believe that adenomas with mild and moderate dysplasia progress slowly or regress (Saraga et al., 1987; Farinati et al., 1989), while others have reported that the progression to severe dysplasia or evolution into gastric carcinoma ranged between 19-21% and 33-40% in mild and moderate dysplasia, respectively (Rugge et al., 1991; Feritta et al., 1993; Rugge et al., 1994). The morphological criteria for evaluating the potential malignancy, or probability of transition to overt cancer of gastric adenoma, however, are quite ambiguous and controversial, and the validity of them is confusing in everyday diagnosis. The DNA-instability test was applied to biopsy tissues of human gastric polyp assessed histopathologically as foveolar hyperplastic polyp, mild, moderate and severe dysplasia adenoma, and carcinoma lesions. The serial sections of the same tissues were also subjected to immunohistochemical staining for Ki67 (Schluter et al., 1993), p53, DFF45, and bFGF (basic fibroblast growth factor, a protein which has a similar effect as VEGF) (Folkman and Klagsbrun, 1987; Russel, 1989). The DNA-instability test was positive in 100% adenocarcinoma cases and 100% severe dysplasia cases, indicating their malignancy. All foveolar hyperplastic polyps were
negative to the DNA-instability testing. Furthermore, the percentage of glands positive for the DNA-instability test steadily increased in going from mild (10%), to moderate (40%), and to severe (100%) dysplasia (Figure 1d). All other cancer markers examined showed significantly higher values in the adenoma glands, being positive for DNA-instability testing, irrespective of the dysplasia grade as compared to those in the adenoma glands that were negative for DNA-instability testing. Furthermore, the former values were comparable to those in adenocarcinoma, indicating no difference of the values between adenoma and cancer in the areas with positive DNA-instability test. These results indicate that cancer cell clones are already present at the adenoma stage showing a positive DNA-instability test, enhanced proliferative activity, p53 mutation, and induction of DFF45 and bFGF. These factors, as above described, again allow cancer cell proliferation, producing heterogeneous subclones due to DNA instability, enhancing their survival rates by escaping apoptosis, and providing abundant nutrients during the early-stage of progression of gastric cancer. These cancer clones in the early-stage of cancer progression can be also referred to as procancer clones, as above described. While morphological criteria and existence of invasion seem to be the most reliable basis for malignancy diagnosis, it appears that a certain period is required before the degree of morphological atypia is manifested histopathologically to allow diagnosis of malignancy, even after the formation of procancer clones. During the extremely long incipient (1-4 years) and early (14-21 years) phases of tumor growth of gastric cancer (Fujita et al., 1978), the DNA alterations induced in cancer cells, which should be minute initially, will become intensified and exaggerated by repeated cell divisions (Hatchoh et al., 1992) during these long phases so as to make the diagnosis of cancer by the pathologist possible based upon the degree of morphological atypia. Further progression of procancer clones will produce the multiple subclones with the potency to invade and metastasize. Reflecting the malignant character of the procancer clones in adenomas, abnormal mitoses were encountered exclusively in those glands showing a positive DNA-instability test, indicating the presence of chromosomal abnormalities evoked by DNA instability (Steinbeck, 2001). Comparable results were also obtained in human colorectal adenoma (Nitta et al., 1993).

Detection of procancer clones in uterine cervical dysplasia (Khaled et al., 2000)

It is regarded that the majority of human uterine cervical carcinomas may arise through some pre-malignant changes, and that these changes are considered to have a greater or lesser degree of malignant potential. To present these stages as the spectrum of one single disease, it was initially introduced the concept of dysplasia/carcinoma in situ system, and later of CIN (cervical intra-epithelial neoplasia). The degree of potential malignancy, or the probability of transition to overt cancer of CIN is classified into 3 groups; CIN-I (mild dysplasia), CIN-2 (moderate dysplasia), and CIN-3(severe dysplasia) according to the cellular differentiation, nuclear abnormalities and mitotic activity in different layers of the mucosa. But the morphological criteria are quite ambiguous and controversial, and the validity of them is confusing in everyday diagnosis. The DNA-instability test was applied to mild, moderate, and severe dysplasia cases, squamous cell carcinoma in situ (CIS), invasive squamous cell carcinoma (SCC) cases, and normal human uterine cervix cases. The results showed that 100% SCC, 100% CIS, 77.8% severe dysplasia, 65.1% moderate dysplasia and 33.3% mild dysplasia cases were positively stained by the DNA-instability test diffusely or sporadically, indicating their malignancy (Figure 1e). Reflecting the malignant character, these cases showed a remarkable increase in the PCNA-index with the loss of polarity of PCNA-positive cell distribution and also increase in number, mean and largest sizes and maximum shape-irregularity of AgNORs dots, especially in the areas of positive DNA-instability test. The frequency of chromosome 17 polysomies, and the percentage of hyperdiploid cells measured by means of the chromosome index using interphase cytogenetics, and p53, and bcl-2-protein (a protein to extend cell survival by protecting the cell against apoptosis without affecting cell proliferation) (Bakhshi et al., 1985; Hockenbery et al., 1990) immunostaining positivities, were also found to be significantly increased in moderate and severe dysplasia and in cancer cases in comparison to normal and mild dysplasia cases as a whole; but the values showed no apparent statistical differences when counted exclusively in the areas with positive DNA-instability testing, irrespective of the dysplasia grade. This indicates that there is no differences between dysplasia and cancer lesions, where DNA-instability
testing shows positive staining. All these data clearly show that the clones with positive DNA-instability testing in the dysplasia can be again regarded as procancer clones with the functional atypia of enhanced potency of cell proliferation and protected survival capability, but without sufficient morphological atypia to make cancer diagnosis valid.

Detection of procancer clones in oral leukoplakia (Iwasa et al., 2001)

Oral leukoplakia is regarded as a precancerous or potentially malignant lesion (Kramer et al., 1978), and is defined as a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion (Axéll et al., 1996), and histopathological grade of dysplasia is thought to be the most important indicator of the malignant potential of leukoplakia (Burkhardt et al., 1985). The diagnosis of epithelial dysplasia, however, requires a considerable experience (Pindborg et al., 1985), and interobserver agreement rates are generally poor, ranging between 49% and 69% (Karabulut et al., 1995). The frequency of oral carcinoma in patients with oral leukoplakia ranges from 2.4% to 70.3% during a 6-20 year observation period (Einhorn and Wersäll, 1967; Silverman et al., 1984; Hansen et al., 1985; Silverman and Grosky, 1997; Schepman et al., 1998; Lee et al., 2000). Furthermore, Lee et al. (2000) reported that the cancer risk of oral leukoplakia with moderate to severe dysplasia was 2.3 times higher than that with hyperplasia or mild dysplasia. However, malignancy could also develop in non-dysplastic lesions such as hyperplasia (Silverman et al., 1984; Hansen et al., 1985; Murti et al., 1998; Schepman et al., 1998).

The DNA-instability test was applied to tissues of oral leukoplakia cases histopathologically assessed as hyperplasia, mild, moderate, and severe dysplasia, and invasive squamous cell carcinoma (SCC). The tissues were also subjected to immunohistochemical staining for p53, DFF45, VEGF, and analysis of AgNORs parameters. The DNA-instability test was positive in 100% SCC, 100% severe dysplasia, 72.7% moderate dysplasia, 50.0% mild dysplasia, and 23.7% hyperplasia cases, indicating their malignancy (Figure 1f). The proportion of lesions positive for PCNA, p53, DFF45, VEGF, and values of AgNORs parameters steadily increased from hyperplasia to mild, moderate, and severe dysplasia, and SCC, especially in those showing positive DNA-instability testing, also indicating their malignancy. But the percent numbers of the immunohistochemically-positive cells, and the mean values of AgNORs parameters in the areas with positive DNA-instability testing were not statistically changed in all lesions, showing no difference of them among the different stages of cancer progression, from hyperplasia to SCC where DNA-instability testing was positive. Furthermore, the proportion of PCNA-positive vascular endothelial cells in the vicinity of VEGF-positive epithelial lesion was significantly higher than that of negative DNA-instability testing, as revealed by triple immunohistochemical staining for VEGF, CD34 (a protein specific for vascular endothelial cells), and PCNA. Based on these results, 44.9% of oral leukoplakia were found to already contain procancer clones.

Detection of procancer clones in otorhinolaryngeal borderline neoplastic lesions (Tsuzuki et al., 1994)

Laryngeal leukoplakia is regarded as a precancerous mucosal change with great similarity to oral leukoplakia, and its malignant transformation rate varies from 1% to 40% (Bouquot and Gnepp, 1991). The inverted papilloma is a locally aggressive sinonasal tumor that has a high rate of recurrence, simultaneous and subsequent development of malignancy, and tendency to multicentricity. The reported incidence of malignancy ranges from less than 2% to 53% (Lawson et al., 1989). Despite the high association rate of malignancy, the inverted papilloma is not necessarily regarded as precancerous (Woodson et al., 1985; Segal et al., 1986; Weissler et al., 1986).

Pleomorphic adenoma (mixed tumor) is the most common neoplasm of the parotid gland and classified originally as benign (Batsakis, 1979), but the same author (Batsakis, 1982) defined malignant mixed tumor which exists in three clinical and histological forms, as carcinosarcoma (Batsakis, 1979), metastasizing mixed tumor (Chen, 1978), and carcinoma in mixed tumor (Moberger and Eneroth, 1968). Additional cases of carcinosarcoma (Tortoledo et al., 1984; Talmi et al., 1990; Cherian et al., 1992), metastasizing mixed tumor (Freeman et al., 1990; Sim et al., 1990; Pitman et al., 1992), and carcinoma in mixed tumor (Tortoledo et al., 1984) were further reported. The frequency of carcinosarcoma, and carcinoma in mixed tumor, was reported to be 0.2% (Tortoledo et al., 1994).
et al., 1984), and 2.1% (Moberger and Eneroth, 1968), respectively. Moberger and Eneroth (1968) further reported that distant metastases were present in 28.1% of all carcinoma in mixed tumor cases, but that no pleomorphic structures were demonstrable in the metastatic lesions. They further pointed out the difficulty of evaluating cellular atypia as a marker of malignancy in pleomorphic adenoma, and also that the lack of sharp delimitation and absence of a capsule may give a false impression of infiltration.

DNA-instability test was applied to the borderline malignancy cases, laryngeal leukoplakia, nasal inverted papilloma, and salivary gland pleomorphic adenoma cases, as well as to normal and SCC cases. The results showed that 100% SCC, 90.1% leukoplakia, 100% inverted papilloma, and 63.6% pleomorphic adenoma cases were positively stained by the DNA-instability test, diffusely or sporadically, indicating that they contain procancer clones. Reflecting the malignant character, these cases showed a remarkable increase in the PCNA-index with loss of polarity of PCNA-positive cell distribution, and also increased number, mean and largest sizes, and maximum shape irregularity of AgNORs, especially in the procancer clones with positive DNA-instability test.

These results indicate that all nasal inverted papillomas are malignant in nature, namely in situ carcinoma, and the majority of leukoplakia is also regarded as in situ carcinoma, although a certain percentage of simple hyperplasia may be also included among the in situ carcinoma. Furthermore, the pleomorphic adenoma of salivary glands is regarded as unstable tumor which often contains or predisposes to bear malignant subclones with occasional capsular or extra-capsular invasion, reflecting the progression of malignancy. Reflecting this (Steinbeck, 2001), abnormal mitoses were sometimes encountered among the clones in pleomorphic adenoma positively stained with DNA-instability testing. These cancer clones should be again referred to as procancer showing functional atypia with positive DNA-instability testing and abnormal expression of other cancer markers, but showing no sufficient morphological atypia.

**Detection of cancer clones in bone giant cell tumor (BGCT) (Azuchi et al., 1998)**

BGCT is one of the most difficult tumors to prospect the biological behavior merely on the basis of histological characteristics. Jaffe et al. (1940) regarded the smaller mononucleated fusiform or polyhedral stromal cells as the main component of the tumor rather than the multinucleated giant cells resembling osteoclasts, and classified BGCT into 3 histological gradings. They attempted to correlate the histological gradings of tumors with their prognosis. Willis (1949), and Russell (1949) were opposed to this concept that the tumor shows a range of structures and behaviors, and a few, initially indistinguishable from their benign fellows, invade and metastasize, without departing greatly from the structure of tumors of benign behavior. Williams et al., (1954) denied the significance of the normal mitotic counts proposed by King (1932) and the histological grading proposed by Jaffe et al. (1940) in evaluating the behavior of BGCT, stating that potentially aggressive or malignant BGCT are not initially distinguishable histologically as such.

Campannacci et al. (1975) used radiographic criteria for grading BGCT, and Tehranzadeh et al., (1989) appreciated the radiographic grading as critically important in planning the management of this tumor, but many other authors concluded that radiological assessment of BGCT is of limited and uncertain value in indicating prognosis. On the other hand, computed tomography (CT) is more powerful in determining thin cortical shells or invasion of the cortex by BGCT (Tehranzadeh et al., 1989), and Hudson et al. (1984) regarded CT as providing the most useful and complete evaluation of the exact extent of BGCT invasions which is essential to secure adequate surgical margins and prevent recurrence, especially when there is cortical penetration with invasions of soft tissue or joint cavities. So the histological and clinical criteria for malignancy of BGCT are quite ambiguous and controversial.

The DNA-instability test was applied to benign (osteochondroma, enchondroma), borderline (BGCT), and malignant (chondrosarcoma and osteosarcoma) mesenchymal tumors. As the results showed, 82.6% BGCT, and 100% malignant mesenchymal tumors were positive to the DNA-instability test, indicating their malignancy. All benign tumors were completely negative to DNA-instability testing. Reflecting the malignant character, all the DNA-instability test-positive malignant- and BGCT cases showed statistically higher values of the PCNA-index, and all the AgNORs parameters
in comparison to those of the benign mesenchymal tumor cases. C-myc immunohistochemistry was positive for 66.7% malignant cases, and 26.3% BGCT cases were positive to DNA-instability testing, especially in the areas with positive DNA-instability testing.

All BGCT cases negative to DNA-instability testing were also negative for c-myc expression. Among the BGCT cases positive to the DNA-instability test, 94.7% showed cortical bone destruction and 26.3% showed extra-osseous expansion or invasion, as revealed by CT. No such radiographic changes were detected in the BGCT cases negative to the DNA-instability test.

Furthermore, only stromal cells were positively stained in the BGCT cases that were positively stained with the DNA-instability test, and all giant cells were completely negative, indicating that the concept of Jaffe et al., (1940) seems to be true (Figure 1g). In such cases, abnormal mitoses (Steinbeck, 2001) were sometimes encountered among the stromal cells positive with DNA-instability testing, reflecting their malignancy. These results clearly show that the majority of BGCT are malignant, and the DNA-instability test and detection of the cortical bone destruction by CT may be regarded as the reliable histological and clinical markers, respectively, for distinguishing benign and malignant BGCT cases.

**Preserved normal basal cell layer in Bowen’s disease (Ishida et al., 2001)**

Bowen’s disease is an intraepithelial squamous cell carcinoma with potential progression to invasive squamous cell carcinoma (Sober and Burstein, 1995; Kirkham, 1997; Mackie, 1998). The percentage of cases with Bowen’s disease that progress to invasive squamous cell carcinoma is 4% (Thestrup-Pedersen et al., 1988) to 5% (Graham and Helwig, 1973), and 8% (Glogau, 2000), respectively. Bowen’s disease occurs mainly in sun-exposed skin areas (Thestrup-Pedersen et al., 1988; Kossard and Rosen, 1992) and is possibly caused by ultraviolet light (Reizner et al., 1994; Chuang et al., 1995), or on the lower extremities (Cox, 1994). A number of studies have demonstrated relatively high incidence of human papilloma virus (HPV) in Bowen’s disease affecting the genital region (38-80%) and other skin regions (19.4-40%), by using Southern blot analysis with HPV16 DNA (Ikenberg et al., 1983), immunohistochemical staining for viral antigens (Grussendorf-Conen and Giesen, 1990), and in situ hybridization (ISH) (Kettler et al., 1990). Bowen’s disease is also an important complication of chronic hyperarsenism (Yeh et al., 1968; Miki et al., 1982). Histologically highly atypical cancer cells with clumping lie in complete disorder throughout the entire epidermal thickness (Kirkham, 1997). The basal layer in Bowen’s disease may, however, appear free of dysplasia. Basal and suprabasal cells in Bowen’s disease appear almost normal on electron microscopy, except that the tonofilaments in these cells slightly aggregate in a fascicular form (Seije and Mizuno, 1969). Immunohistochemically, the basal cells are either weakly positive or completely negative for the expression of PCNA (Geary and Cooper, 1992; Takata and Matsui, 1994; Li and Lee, 1996), although the PCNA-index is generally high in other cell layers in Bowen’s disease (Tsuji et al., 1992). DNA-instability testing was applied to Bowen’s disease, squamous cell carcinoma (SCC), and seborrheic keratosis lesions. As the results showed, 100% Bowen’s disease and 100% SCC cases were positive, but all seborrheic keratosis cases were completely negative. In Bowen’s disease, all basal cells were completely negative to DNA-instability testing, although all cancer cells in upper layers were strongly positive diffusely (Figure 1h). They were also negative to p53 immunohistochemistry showing normal signals of chromosome 17 as revealed by interphase ISH with only a slightly enhanced PCNA-index, in sharp contrast to marked abnormalities of these cancer markers in all upper cell layers. Immunohistochemical staining with 34 β-E12 (monoclonal antibody against cytokeratins 1, 5, 10, and 14, which stains all normal epidermal keratinocytes including basal cells) showed that only basal cells of Bowen’s disease stained strongly and homogeneously, while all cancer cells in upper layers of Bowen’s disease were only sporadically stained.

These results clearly show that the basal cells in Bowen’s disease are normal in nature.

**Conclusions**

The newly developed DNA-instability testing demonstrated that a majority of so called borderline malignancies, such as gastric adenoma, colorectal adenoma. Uterine cervical dysplasia, oral and laryngeal leukoplaiki, nasal inverted papillo-
ma, salivary gland pleomorphic adenoma, and bone giant cell tumor, often contain cancer clones, and they should be dealt with as such to prevent further progression and recurrence by removing them as completely as possible. It also revealed that multinucleated giant cells in bone giant cell tumor and basal cells in Bowen’s disease are non-malignant or normal.

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