

Abnormal Nuclear Shape in Solid Tumors Reflects Mitotic Instability

David Gisselsson,* Jonas Björk,[†]
Mattias Höglund,* Fredrik Mertens,*
Paola Dal Cin,^{‡§} Måns Åkerman,[¶] and
Nils Mandahl*

From the Departments of Clinical Genetics,* Occupational and Environmental Medicine,[†] and Pathology,[¶] University Hospital, Lund, Sweden; the Center for Human Genetics,[‡] Catholic University of Leuven, Leuven, Belgium; and the Department of Pathology,[§] Brigham and Women's Hospital, Boston, Massachusetts

Abnormalities in nuclear morphology are frequently observed in malignant tissues but the mechanisms behind these phenomena are still poorly understood. In this study, the relation between abnormal nuclear shape and chromosomal instability was explored in short-term tumor cell cultures. Mitotically unstable ring and dicentric chromosomes were identified by fluorescence *in situ* hybridization at metaphase and subsequently localized in interphase nuclei from five malignant soft tissue tumors. The vast majority (71 to 86%) of nuclear blebs, chromatin strings, and micronuclei contained material from the unstable chromosomes, whereas few (<11%) were positive for stable chromosomes. Nuclear morphology was also evaluated in fibroblasts and an osteosarcoma cell line exposed to irradiation. A linear correlation was found between the frequency of abnormalities in nuclear shape, on one hand, and cells with unstable chromosomes ($r = 0.87$) and anaphase bridge configurations ($r = 0.98$), on the other hand. The relation between nuclear shape and karyotypic pattern was investigated further in cultures from 58 tumors of bone, soft tissue, and epithelium. Blebs, strings, and micronuclei were significantly more frequent in tumors that contained rings, dicentrics, or telomeric associations than in those exhibiting only stable aberrations ($P < 0.001$) and a positive correlation ($r = 0.78$) was found between the frequency of such nuclear abnormalities and the intratumor heterogeneity of structural chromosome aberrations. These results indicate that the formation of nuclear blebs, chromatin strings, and micronuclei in malignant tissues is closely related to the breakage-fusion-bridge type of mitotic disturbances. Abnormalities in nuclear shape may thus primarily be regarded as an indicator of genetic instability and intratumor heterogeneity, independent of

cytogenetic complexity and the grade of malignancy.
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Malignant tissues frequently exhibit abnormal nuclear morphology including variability in nuclear size, abnormal chromatin structure, and irregularities in nuclear shape.^{1–4} In some tumor types, such as endometrial, ovarian, and mammary adenocarcinomas, the presence of nuclear atypia is associated with an unfavorable prognosis.^{5–7} In other neoplasms, however, such as soft tissue sarcomas, the presence of nuclear irregularities has little prognostic impact.^{8,9} Some investigations have indicated that nuclear irregularities may be associated with chromosomal aberrations.¹⁰ In experimental models of chemically induced neoplasia, atypia has been reported to increase with the degree of aneuploidy,¹¹ and a correlation between atypia and nuclear DNA content has been reported in ovarian and mammary carcinomas.⁵ Recently, the development of DNA fluorescence *in situ* hybridization (FISH) has allowed more detailed investigations of the relation between chromosome abnormalities and alterations of nuclear morphology. In neuroblastomas and mesenchymal tumors, amplified DNA sequences have been shown to preferentially aggregate in micronuclei, nuclear strings, or protrusions of the nuclear membrane, so-called nuclear blebs.^{12–15} In mesenchymal neoplasms, the amplified material is frequently carried in mitotically unstable dicentric, multicentric,^{16,17} or ring-shaped chromosomes.^{12,18} At anaphase, such rearranged chromosomes frequently fail to segregate in an orderly manner, instead forming bridges between the spindle poles. At the anaphase-telophase transition, these bridges may subsequently break, resulting in novel chromosomal variants in the daughter cells.^{19–21}

In this study, we further explored the relationship between such bridge-breakage instability and nuclear membrane irregularities in short-term cultures from musculoskeletal and epithelial tumors. FISH was used to identify mitotically unstable chromosome aberrations in metaphase cells from soft tissue tumors and to determine the positions of these chromosomes in abnormal inter-

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Address reprint requests to David Gisselsson, Department of Clinical Genetics, University Hospital, SE-221 85 Lund, Sweden. E-mail: david.gisselsson@klingen.lu.se.

phase nuclei. The correlation between anaphase bridging and abnormalities in nuclear shape was evaluated at different levels of chromosomal instability in irradiated fibroblasts and tumor cells. To delineate the relationship between nuclear abnormalities and the pattern of chromosomal aberrations in several histopathological entities, we also analyzed 58 tumors from ovary, lung, bone, and soft tissue.

Materials and Methods

Tumor Cell Culture, Chromosome Preparation, and Scoring of Abnormal Nuclei

Tumor material was obtained from the University Hospital, Lund, Sweden; the Karolinska Hospital, Stockholm, Sweden; and the Center for Human Genetics, Leuven, Belgium. The osteosarcoma cell line (OSA) has been previously described.²² The fibroblast lines GM498B and GM3349B were obtained from the National Institute of General Medical Sciences, Human Cell Repository, Camden, NJ. Culture, harvest, and chromosome preparation were according to standard procedures.²³ Cultured cells on chamber slides were treated with hypotonic 0.06 mol/L KCl for 30 minutes and then fixed in methanol:acetic acid, 3:1. Metaphase chromosomes and interphase nuclei were stained with Wright's stain and at least 25 metaphase cells and 1,000 interphase nuclei were analyzed per case. The total rate of abnormal nuclear structures (ANS) in each case was determined as the sum of the frequency of nuclei showing at least one nuclear bleb or nuclear string, and the frequency of micronuclei (MN).

A nuclear bleb was defined as a round or oval protrusion of the nuclear membrane connected to the main part of the nucleus by a thinner chromatin segment, a nuclear string as a chromatin thread connected to the membrane(s) of one or two nuclei, and a micronucleus as a rounded chromatin fragment located adjacent to a nucleus, with a diameter not exceeding one third of the diameter of that nucleus.²⁴ Cells exhibiting strings in connection with blebs were classified as nuclear strings. To exclude that artifacts in nuclear morphology were caused by the hypotonic treatment, cultures from a dedifferentiated liposarcoma were also stained by Giemsa after direct fixation with methanol:acetic acid. No significant difference in ANS (17% compared to 18%) was detected between these preparations and those exposed to hypotonic treatment.

Cytological Staining

To separately visualize cytoplasm and chromatin, cultured cells were fixed without Colcemid treatment, stained for 30 seconds with 0.2 mg/ml Evan's blue/phosphate-buffered saline, mounted with 1 μ g/ml diamidinophenylindole/2% 1,4-diazabicyclooctane/glycerol, and analyzed by epifluorescence microscopy on a ChromoFluor System (Applied Imaging International Inc.,

Newcastle, UK). At least 100 chromatin bridges were evaluated in each case.

FISH

Slides were prepared for FISH according to standard procedures.²⁵ Biotin-labeled whole chromosome painting probes for chromosomes 9, 10, and 15, and digoxigenin-labeled probes for 3, 9, and 12, were from Cambio (Cambridge, UK) and Oncor (Gaithersburg, MD), respectively. The multicolor karyotyping of the malignant fibrous histiocytomas MFH2 and MFH3 has been described previously.¹⁴ Morphological features of interphase nuclei were evaluated after diamidinophenylindole staining. To determine the chromosomal content of interphase chromatin, at least 100 abnormal nuclei were analyzed in each case.

Irradiation of Cell Cultures

Cell cultures from two normal fibroblast cell lines and the OSA tumor cell line were grown to confluence and then exposed to 6 Gy from ¹³⁷Cs at 0.54 Gy/minute. The culture medium was changed immediately after irradiation and the first passage was made after 24 hours. At least 50 metaphase cells and 1,000 interphase nuclei per passage were analyzed after Wright's staining. At least 50 anaphase figures were analyzed by Giemsa staining in parallel cultures.

Analysis of Tumor Cells in Primary Cultures

Archived Wright- or Giemsa-stained chromosome preparations from 70 solid tumors were selected for evaluation of nuclear morphology. Cases were compiled with the aim of achieving a high variability of karyotypic features within the sample, regarding both the number and the type of cytogenetic aberrations. After exclusion of cases with incomplete karyotypes or ambiguous diagnoses, 58 tumors remained for statistical analysis, including seven lung carcinomas, five ovarian carcinomas, one chondroblastoma, two chondromas, 11 chondrosarcomas, two aneurysmal bone cysts, six giant cell tumors of bone, nine osteosarcomas, two typical lipomas, five atypical lipomatous tumors, seven liposarcomas (two dedifferentiated, one round cell, two mixed-type, one myxoid, and one sclerosing), and one malignant fibrous histiocytoma. Karyotypes, described according to An International System for Human Cytogenetic Nomenclature,²⁶ were used to determine the highest number of structural and numerical aberrations in a clonal population for each case. Intratumor variability of numerical aberrations was assessed by the number of cells with chromosome counts deviating from the modal number, divided by the number of cells analyzed, whereas variability in chromosome structure was assessed by the number of structural rearrangements not included in the stemline, divided by the number of cells analyzed.

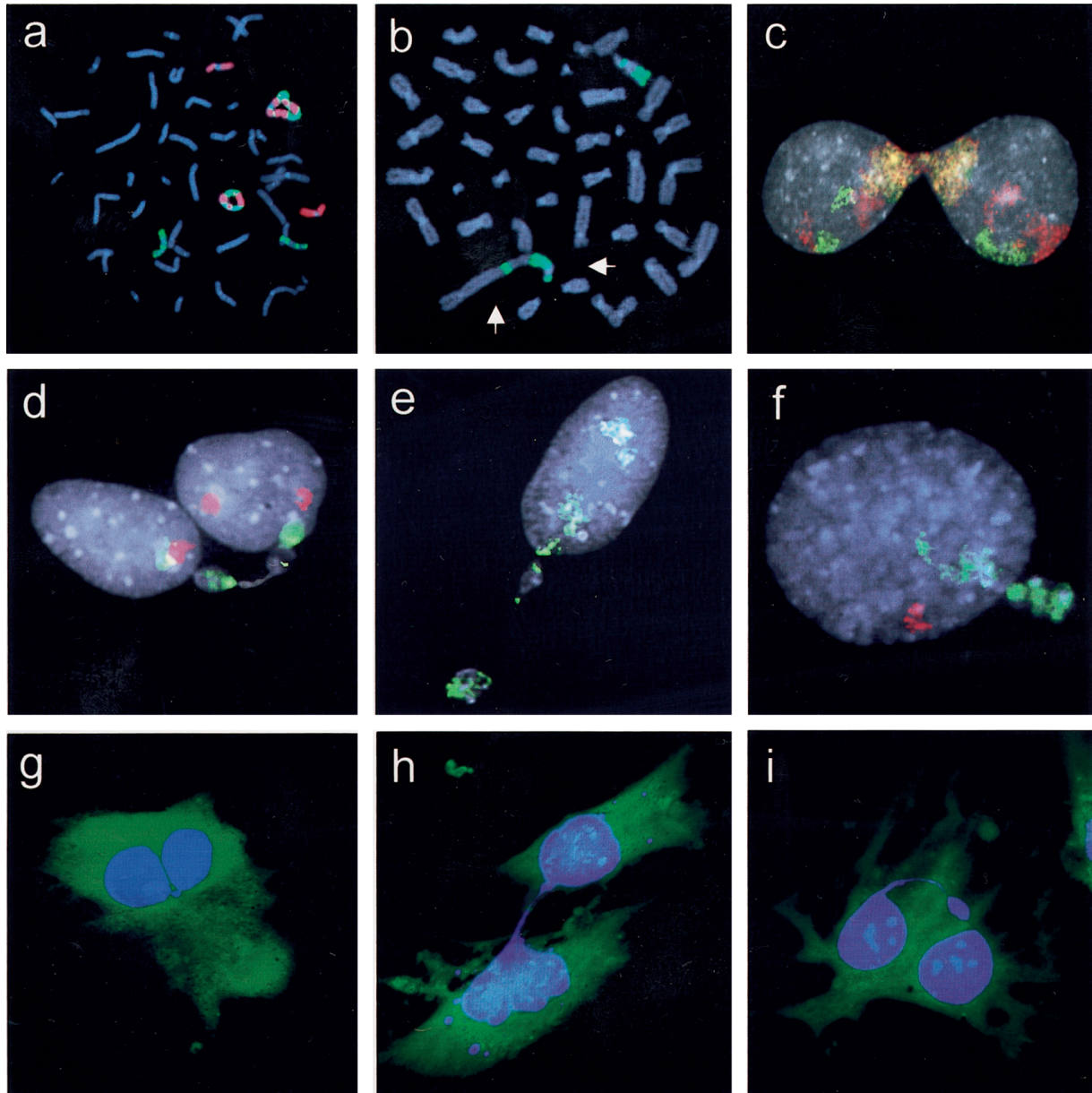


Figure 1. Whole chromosome painting. **a:** MFH1. Two ring chromosomes composed of material from chromosomes 9 (red) and 12 (green). **b:** MFH2. Two abnormal chromosomes, of which one is dicentric (arrows), are positive for chromosome 9 (green). **c:** MFH1. A chromatin bridge between nuclei is positive for chromosomes 9 (red) and 12 (green), resulting in a mixed red-yellow-green fluorescence; the chromatin of the normal homologues is also stained. **d-f:** MFH2. A chromatin string, micronuclei, and a nuclear bleb, respectively, containing sequences from chromosome 9 (green) but not from chromosome 10 (red). **g-i:** ALT1. Chromatin string connecting two lobes of a nucleus, connecting nuclei in two adjacent cells, and hanging freely in the cytoplasm, respectively.

Results

The Chromosomal Content of ANS

FISH analysis was performed to identify mitotically unstable chromosomes in five soft tissue tumors with abnormal nuclear morphology. Two atypical lipomatous tumors (ALT1 and ALT2) and a low-grade malignant fibrous histiocytoma (MFH1) exhibited simple karyotypic changes including supernumerary ring chromosomes, shown by whole chromosome painting to contain sequences from chromosome 12; the rings in MFH1 also carried material from chromosome 9 (Figure 1a). The two other tumors

(MFH2 and MFH3) were both high-grade lesions and had complex karyotypes including ring and dicentric chromosomes. Multicolor analysis showed that the majority of rings and dicentrics contained material from chromosomes 9 (Figure 1b) and 3, respectively. At analysis of interphase nuclei performed with whole chromosome paint for the unstable chromosomes, 71 to 86% of the blebs, strings, and micronuclei stained positive (Figures 1, c-f, and 2). As a control, whole chromosome painting was also performed for chromosomes not involved in mitotically unstable aberrations according to G-band analysis: chromosome 9 in ALT1 and ALT2, chromo-

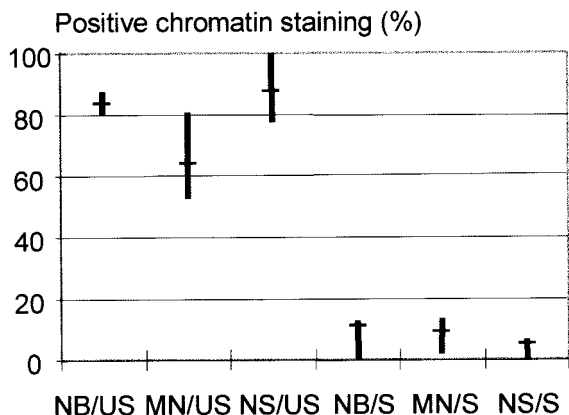


Figure 2. Results of FISH analysis of nuclear blebs (NB), micronuclei (MN), and nuclear strings (NS) in five soft tissue tumors. Whole chromosome painting for the mitotically unstable (US) chromosomes stained the majority of abnormal structures in all cases, whereas only a minor proportion stained positive for the selected stable (S) chromosomes used as a control. The fraction of abnormal nuclear structures with signals for the tested chromosome is indicated by median values (horizontal bars) and ranges (vertical bars).

somes 3 and 15 in MFH1, chromosome 10 in MFH2, and chromosome 1 in MFH 3. The proportion of ANS that stained positive for these chromosomes was 0 to 11%.

The Cellular Organization of Chromatin Strings

The arrangement of chromatin strings was studied further by epifluorescence microscopy in MFH1 and ALT1. Three different configurations were observed: chromatin bridges connecting two nuclear lobes within the same cell (41% of bridges in ALT1 and 20% in MFH1; Figure 1g); chromatin bridges connecting the nuclei of neighboring cells (35% in ALT1 and 37% in MFH1; Figure 1h); and chromatin strings protruding freely from a nucleus into the cytoplasm (24% in ALT1 and 43% in MFH1; Figure 1i). The strings connecting nuclei of different cells were invariably sheathed by cytoplasm.

Mitotically Unstable Chromosomes and Nuclear Abnormalities in Irradiated Cells

Nuclear morphology, metaphase chromosomes, and anaphase division figures were monitored during at least six passages after exposure to radiation. The results of the metaphase and anaphase analyses have been partly described previously.¹⁶ Before irradiation, the fibroblast cultures and OSA had ANS frequencies of 0.3% and 3%, respectively. In exposed cultures, the proportion of cells containing at least one ring chromosome, dicentric chromosome, or telomeric association (RDT), the frequency of anaphase bridge configurations (ABC), and ANS were determined after each passage. All cultures showed elevated RDT, ABC, and ANS levels at the first passage after irradiation. These parameters subsequently decreased in a parallel manner and reached the level of normal, nonirradiated cells at passages 3 to 4 in the fibroblasts and passage 7 in OSA. When RDT was compared to ANS at each passage, a linear correlation was observed ($r = 0.87$; Pearson correlation, $P < 0.001$,

two-tailed; Figure 3, a and b). An even stronger correlation was found between ANS and ABC ($r = 0.98$; $P < 0.001$; Figure 3, c and d). Similar results were obtained when nuclear blebs, NS, and MN were separately compared to RDT ($r = 0.88, 0.82, \text{ and } 0.84$, respectively; $P < 0.001$) and ABC ($r = 0.94, 0.93, \text{ and } 0.98$, respectively; $P < 0.001$). There was no significant correlation between MN and the frequency of metaphase cells with acentric fragments ($r = 0.22$; $P = 0.31$). The frequency of nuclear abnormalities was thus proportional to the mitotic instability of chromosomes in both fibroblasts and osteosarcoma cells.

Nuclear Aberrations and Karyotypic Patterns

Preparations from short-term cultures of 12 epithelial and 46 mesenchymal tumors were used for parallel investigation of the chromosomal aberration pattern and nuclear abnormalities. An extensive variation was observed in the frequency of nuclear aberrations within each tumor subgroup. The malignant tumors exhibited ANS between 0 and 30%, whereas the benign lesions all had ANS $< 2\%$. Tumors of borderline malignancy, including giant cell tumors of bone and atypical lipomatous tumors, had ANS between 4 and 20%. At metaphase analysis, 30 of the tumors contained ring chromosomes, dicentric chromosomes, or telomeric associations. ANS was significantly higher (14% versus 1.7%; $P < 0.001$, two-sided Z-test) in these tumors than in those with only stable types of chromosome aberrations, predominantly including gains and losses of chromosomes, translocations, inversions, deletions, insertions, and additions. ANS was found to correlate well to the intratumor variability of structural chromosome aberrations ($r = 0.78$; Pearson correlation, $P < 0.001$, two-tailed), but poorly to the variability in numerical aberrations ($r = 0.39$; $P = 0.002$), as well as to the total number of structural ($r = 0.37$; $P = 0.004$) and numerical ($r = 0.24$; $P = 0.075$) aberrations (Figure 4).

Discussion

Previous investigations of tumor cells have indicated that there might be an association between genetic aberrations and abnormal nuclear morphology.^{5,10,11} In the present analysis, we used cytogenetic and molecular cytogenetic techniques to study the relation between chromosomal instability at mitosis and nuclear shape. In the five soft tissue tumors analyzed by FISH, the vast majority (71 to 86%) of blebs, strings, and micronuclei contained material from mitotically unstable, potentially bridge-forming, ring, or dicentric chromosomes, whereas only a small proportion (0 to 11%) of the abnormal structures contained material from the stable chromosomes used as controls. Moreover, in irradiated cells, a strong correlation was found between the frequencies of nuclear irregularities, on the one hand, and the proportion of cells exhibiting mitotically unstable chromosomes and anaphase bridges, on the other. Finally, in preparations from short-term cultures of primary tumor material, cases with unstable chromosomes showed significantly higher

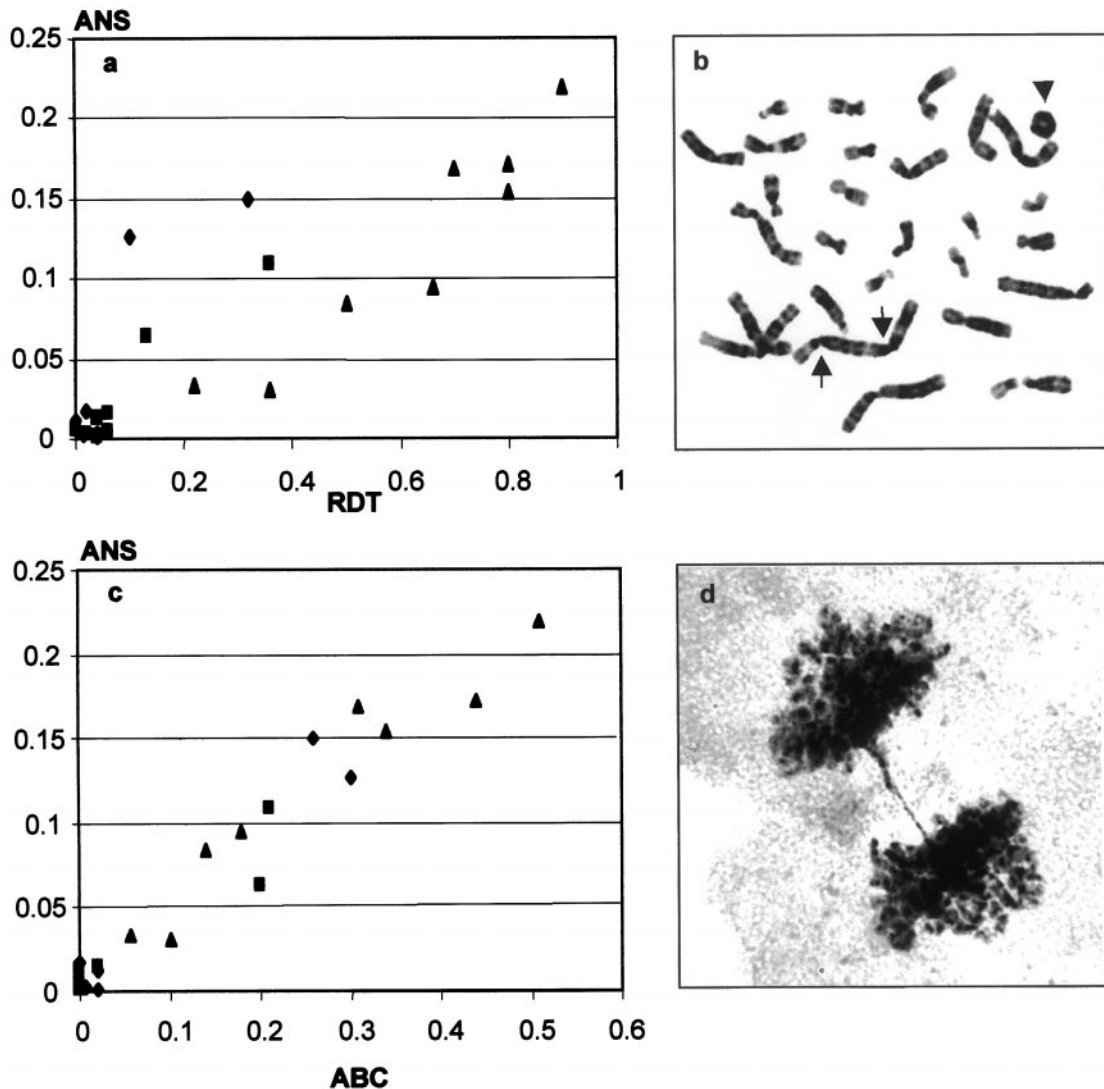


Figure 3. The relation of mitotic chromosomal instability and abnormal nuclear structures (ANS) in irradiated cultures of fibroblasts GM498B (squares) and GM3349B (diamonds), and OSA osteosarcoma cells (triangles). **a:** Positive correlation ($r = 0.87$) between the proportion of metaphase cells carrying unstable chromosomes (RDT) and ANS. **b:** A dicentric (arrows) and a ring (arrowhead) chromosome in an irradiated OSA cell. **c:** Positive correlation ($r = 0.98$) between the frequency of anaphase bridge configurations (ABC) and ANS. **d:** Anaphase bridge in an irradiated OSA culture.

frequencies of ANS than cases in which only stable aberrations were found. These results indicate a close association between the abnormalities in nuclear shape and chromosomal bridge-breakage events.

According to studies in several organisms, chromosomes participating in anaphase bridges may rupture and the broken ends will subsequently reunite to form novel structural aberrations in the daughter cells.^{19,20} Some of these newly formed abnormalities may also be dicentric or ring-shaped and, in their turn, participate in the formation of anaphase bridges. Such breakage-fusion-bridge cycles may lead to a considerable intercellular heterogeneity in the pattern of, in particular, structural chromosome aberrations.^{16,18} In this study, the frequency of nuclear abnormalities correlated strongly to intratumor variability of structural chromosome aberrations, whereas the correlation to numerical variability and the total number of chromosome aberrations was weak. It should be noted that most of the tumor preparations from

short-term cultures most probably contained fibroblasts or normal epithelial cells that could not be distinguished from the neoplastic cells at interphase analysis. Neither could the relative population sizes be determined with accuracy by cytogenetic analysis because a disproportionate mitotic activity among stromal and parenchymal tumor cells might give a false estimation. Cases with large proportions of normal cells might therefore exhibit lower ANS than what would have been obtained from pure tumor cell populations. However, the correlation between ANS and structural variability was still fairly strong ($r = 0.78$; $P < 0.001$) and all cases with ANS $>10\%$ exhibited $>50\%$ variability in structural aberrations, ie, more than half of the aberrant cells had rearrangements outside the stem line.

Little is known about the mechanisms leading to aberrant nuclear structure in pathological conditions. Some knowledge has been gained from the effects of mutagenic agents, such as radiation, on nuclear morphology.

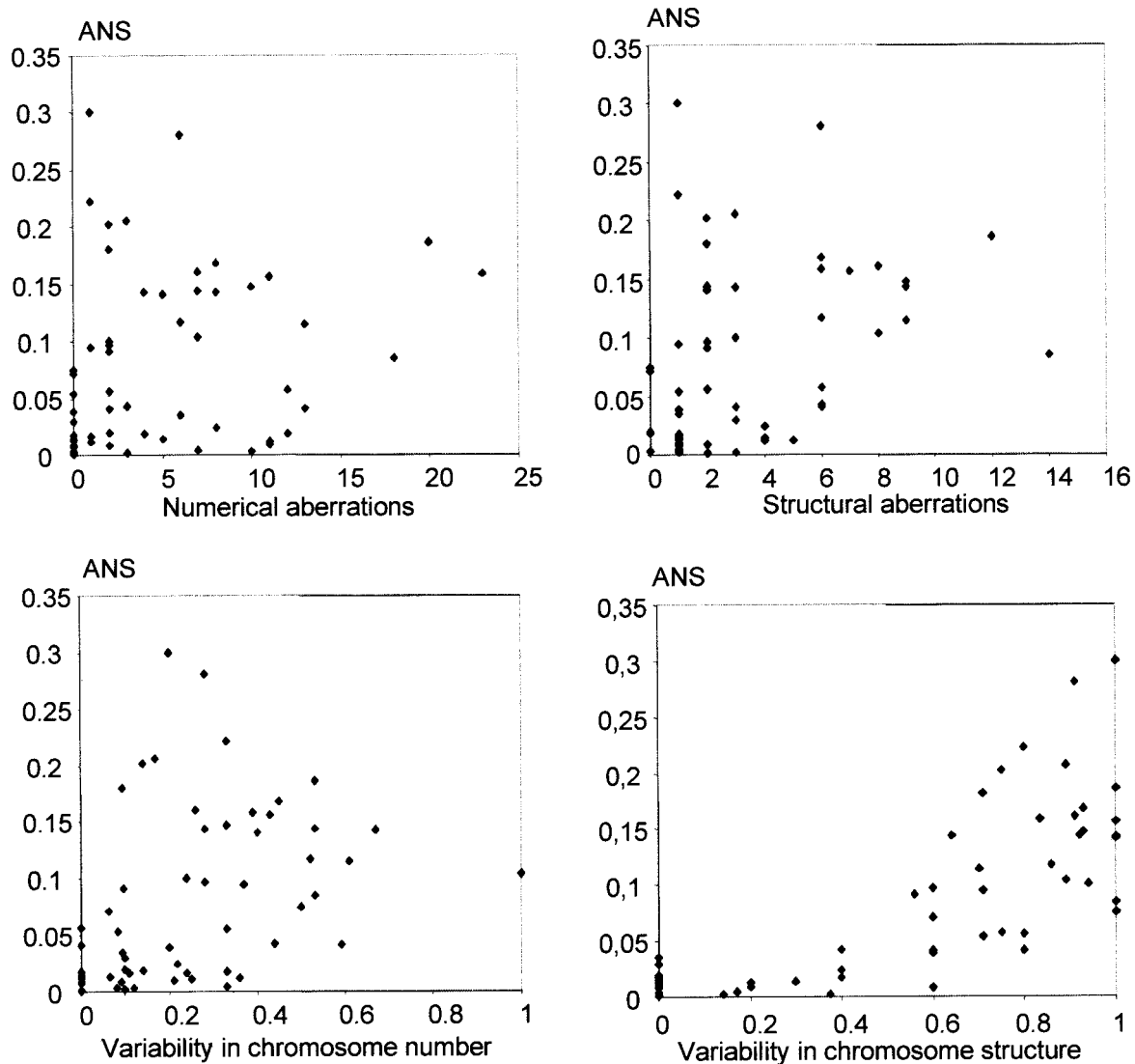


Figure 4. Nuclear abnormalities in relation to the karyotypic pattern. There were poor correlations ($r = 0.24$ to 0.39) between ANS and the number of both numerical and structural chromosome aberrations as well as between the intratumor variability in chromosome number, but a stronger correlation ($r = 0.78$; $P < 0.001$) between ANS and the variability in chromosome structure.

Irradiated fibroblasts are characterized by large atypical nuclei²⁷ and the frequency of micronuclei in exposed cells has long been used as a measure of the clastogenic insult.²⁸ It has been suggested that micronuclei may originate from acentric chromosome fragments, either resulting from double-strand DNA damage before cell division, or after the breakage of anaphase bridges.²⁹ In the irradiated cell populations in this study, an association was found between the frequency of micronuclei and chromosomal instability. MN showed a strong correlation to both RDT and ABC ($r = 0.84$ and 0.98 , respectively; $P < 0.001$), whereas there was no correlation to the frequency of acentric fragments. However, in the tumors studied by FISH, the proportion of micronuclei containing material from mitotically unstable chromosomes was lower than the corresponding values for blebs and strings. In the ALTs and MFH1 60 to 80% of MN were positive for sequences from unstable chromosomes, but in the highly malignant MFH2 and MFH3, the correspond-

ing values were only 52 and 54%, respectively (Figure 2). This indicates that, although the formation of micronuclei in irradiated cells and many tumors may frequently be associated with anaphase bridging, other processes may also contribute. This is in accordance with previous studies, showing that the origin of micronuclei may vary with the type of cytotoxic exposure. Substances affecting spindle function, such as colchicine, induce micronuclei containing whole chromosomes, whereas clastogenic agents, such as X-rays, mainly lead to the formation of micronuclei with acentric fragments.³⁰

In neoplastic disease, the relation between nuclear morphology and chromosomal aberrations has particularly been studied in tumors exhibiting genomic amplification. In neuroblastomas, micronuclei and nuclear blebs have been shown to carry amplified copies of the *MYCN* gene,¹³ and in low-grade mesenchymal tumors, amplified material from chromosome 12 frequently aggregate in nuclear blebs and strings.^{12,14,15} It has been sug-

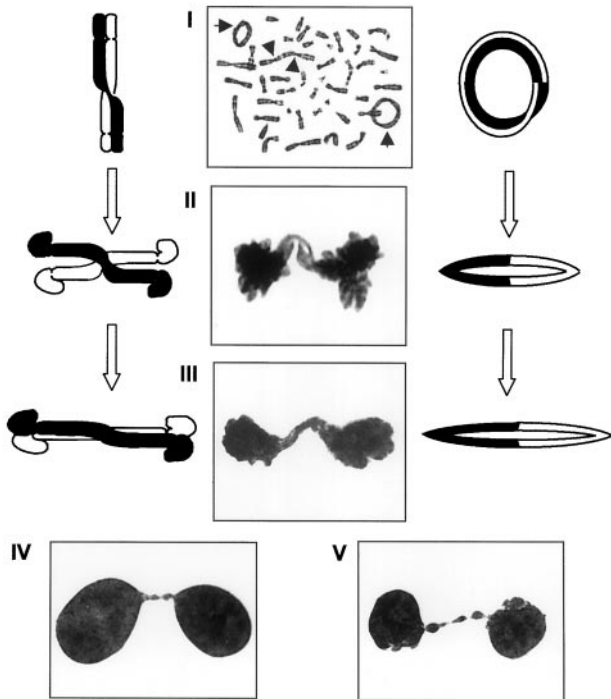


Figure 5. Model for the formation of nuclear abnormalities illustrated by cell division figures from a low-grade malignant fibrous histiocytoma: mitotically unstable chromosome aberrations such as rings (**I, arrows**) and dicentrics (**arrowheads**) may form bridges at anaphase (**II**) which, after telophase (**III**), may remain as nuclear strings (**IV**), or break up into nuclear blebs and micronuclei (**V**).

gested that nuclear irregularities are part of a mechanism by which amplified sequences may be eliminated from the main nucleus at interphase.^{13,31} Alternatively, the overrepresentation of amplified sequences in blebs, strings, and micronuclei could merely reflect their relative abundance in the genome, increasing the likelihood of detection at any location in the cell nucleus. However, in this study, a high ANS frequency was observed in several cell types where genomic amplification was not observed, including irradiated fibroblasts and three giant cell tumors of bone in which telomeric associations were the only cytogenetic abnormalities. Furthermore, aggregation of material from unstable chromosomes in ANS were seen also in the absence of intrachromosomal amplification, eg, in MFH2 and MFH3.¹⁶ Our data thus indicate a mechanistic link primarily between certain types of nuclear abnormalities at interphase, a heterogeneous pattern of chromosome aberrations at metaphase, and chromosome bridging at anaphase. This may be explained by a scenario in which chromosomes participating in anaphase bridges decondense into aberrant nuclear structures (Figure 5). According to this model, intact bridges would form chromatin strings, whereas fragments from broken bridges would form nuclear blebs or micronuclei. In malignant tissues, these ANS should thus be viewed primarily as an indicator of ongoing genomic reorganization. The correlation between disturbances of the chromosome segregation at mitosis and morphological parameters seems applicable to a number of different tumor types, mesenchymal as well as epithelial, re-

gardless of the level of cytogenetic complexity and the grade of malignancy. The observation that chromatin strings frequently connect neighboring cells through cytoplasmic channels offers intriguing possibilities for intercellular communication in malignant tissues and should be explored further.

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