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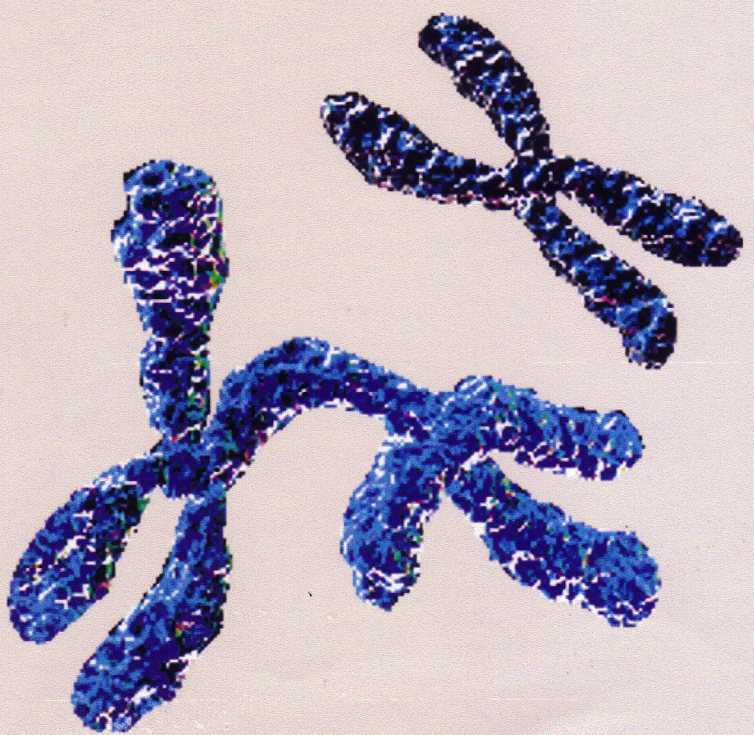

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HUMAN BREAST ADENOCARCINOMA: DNA CONTENT, CHROMOSOMES, GENE EXPRESSION AND PROGNOSIS

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Previous results from 409 patients with primary breast adenocarcinomas demonstrated a strong relationship between nuclear DNA content of breast cancer cells and prognosis. Tumors exhibiting DNA values within the limits of normal tissues (DNA euploidy) were found to be correlated with a favorable prognosis. In contrast, tumors with increased and non-modal DNA content values (DNA aneuploidy) were found indicative of poor prognosis. This was observed to be the case regardless of whether the percentage of cells above 2.5c or 5c, DNA index/modal value, or the histogram typing according to Auer et al. (type I–IV) were utilized to discriminate low-grade from high-grade malignant cases [1,2]. Multivariate Cox regression analysis showed that histogram typing provided significant ($p < 0.001$) prognostic information, independent of any other histopathological typing, and in cases of ductal carcinomas, histological grading. This prognostic significance was also independent of established survival determinants, such as tumor size and nodal status. Nevertheless, postoperative tumor size ($p = 0.04$) and nodal status ($p = 0.003$) showed also predictive significance, while histological typing and grading did not. Apart from this, an expected trend in relative risk rates for the various malignancy grades of ductal carcinomas could be observed ($p < 0.002$), however it did not prove to be independent of histogram typing of DNA profiles. Conflicting results have been obtained concerning the relation between axillary nodal status and the ploidy level of breast tumors. Our data from 980 patients [3,4] show no clear correlation between DNA histogram type and axillary node status, which is important and suggests that these two factors are independent prognostic variables. Thus, patients with histogram type I and node-negative tumors were found to have an excellent prognosis with 95% probability of 10-year survival. In contrast, patients with DNA histogram IV and node-positive tumors were shown to have an extremely bad prognosis with only 31% probability of 10-year survival [3].

Chromosome analysis in tissues from benign breast lesions, histogram type I and histogram type IV breast carcinomas, showed pronounced differences in chromosomal aberrations [5]. By means of comparative genomic hybridization (CGH) analysis, we observed a clear difference in the frequency of copy number changes, when benign tissue samples were compared with carcinomas. No copy number changes were observed in benign tissue, whereas varying frequencies of chromosomal aberrations were found in all carcinomas. When histogram type I tumors were compared with histogram type IV ones, the differences in both the frequency as well as the chromosomal distribution of numerical aberrations were obvious. Type I tumors revealed few copy number changes that involved virtually exclusively the gain or loss of entire chromosomes or chromosomal arms. Noteworthy is the observation that the cytogenetic correlate of the poor prognosis of patients suffering from type IV carcinomas was a significantly higher number of chromosomal aberrations, that also involve subchromosomal, regional, low and high copy number increases (amplifications). In line with these findings, southern blot hybridisation analysis showed an amplification of one or more oncogenes studied (c-erb-2, cyc-D1, int-2, c-myc, MDM 2) in 43 out of 98 (44%) histogram type IV carcinomas, but in only 1 out of 17 (6%) histogram type I tumors [6].

Our DNA content studies also show that simple determination of the stemline position is not the optimal DNA measure of intrinsic tumor malignancy potential. The fraction of cells scattered outside the modal peaks of the histograms are of utmost importance for adequate cytochemical malignancy grading in breast carcinomas. Thus, based on image cytometrical DNA content data we could clearly distinguish two subtypes of aneuploidy, strongly associated with high, respectively low clinical malignancy. These aneuploid subtypes could be defined by the percentage of non-modal DNA values as measured by the "Stemline Scatter Index" (SSI), which is defined as the sum of the percentage of cells in the S-phase region, the G2 exceeding rate and the coefficient of variation (CV) of the tumor stemline. Logistic regression analysis showed S-phase ($p = 5.3\text{E-}04$) as contributing most to the discriminative strength of the SSI, followed by CV and G2 Exc ($p = 0.003$ and $p = 0.03$ respectively), whereas none of the three summands was found to be selective on its own [7]. Using logistic regression, we could determine the cut-off value of SSI $= 8.8\%$ ($p = 0.03$), which enabled us to also subdivide diploid and tetraploid tumors into clinically low (SSI $\leqslant 8.8\%$) and high (SSI $> 8.8\%$) malignant variants.

One possible reason for stemline scattering is impaired distribution of chromosomes at mitosis, caused by numerical or structural centrosome aberrations. Cyclin A and E have been demonstrated to be involved in centrosome duplication. Real time quantitative PCR measurements of cyclin A and E transcript levels showed statistically significantly increased values in the tumors with a high SSI, compared to those with a low SSI. In addition centrosomal aberrations were observed in an average of 9.6% of the measured cells in aneuploid carcinomas with high SSI values and in an average of 2.5% of the cells in aneuploid and diploid tumors with a low SSI. CGH analysis indicated a clear difference of chromosomal aberrations between e.g. the two categories of aneuploid carcinomas. Highly scattered aneuploid variants were found to be characterized by increased numbers of chromosomal aberrations, especially subchromosomal, regional amplifications compared to aneuploid or diploid tumors with low SSI values.

Protein expression analysis by means of high resolution two-dimensional gelelectrophoresis (2-DE) exposed significant expression differences not only between benign breast tissue and cancer tissue, but also between tumors with high and low SSI. Thus, e.g. the high molecular weight tropomysins TM1, TM2 and TM3 were found to be highly expressed in ductal hyperplasia and fibroadenomas, but absent in all carcinomas [8]. Similarly, the levels of cytokeratins such as CK7, CK8, CK15 and CK18 were significantly lower in carcinomas compared to fibroadenomas [9,10]. In contrast, members of e.g. the stress protein family (pHSP60, calreticulin), oncoprotein 18 variant, elongation factor, glutathione S-transferase, superoxide dismutase, etc., were found to be upregulated in carcinomas. High levels of e.g. β-tubulin, vimentin, and HSP90 were observed in carcinomas with high SSI, but were weakly expressed in carcinomas with low SSI values.

In summary our data show that the DNA content distribution pattern of a given malignant epithelial cell population is closely related to the degree of centrosomal, chromosomal and gene aberrations, and in turn to altered gene expression patterns, both at the RNA and protein level, including specific posttranslational modifications. Our studies also clarify the superiority of the non-modal proportion of a tumor's DNA histogram over the modal DNA content value, in prediction of tumor aggressiveness.

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CHROMOSOMES, PLOIDY AND GENETIC IMBALANCES OF LUNG CANCER

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Lung cancer is a heterogeneous and highly aggressive disease which is reflected by a wealth of genetic alterations on the DNA and RNA level. In an attempt to understand this apparent chaos we used screening methods like Comparative Genomic Hybridization (CGH), Suppression Subtractive Hybridization (SSH) and cDNA microarrays. In addition, cytogenetic information on lung tumors were retrieved from the Mitelman Database of Chromosome Aberration and analyzed for chromosome numbers and alterations.

From the Mitelman database, in total 660 lung tumors were identified which 446 were histologically typed. The analysis was then mainly restricted to 160 adenocarcinomas (ADC), 145 squamous cell carcinomas (SCC), 14 ADC-SCC, 38 large cell lung carcinomas (LCLC) and 49 small cell lung carcinomas (SCLC). All showed at least subtle chromosomal abnormalities indicative of aneuploidy. About 30% and 22% of the near diploid ADC and SCC, respectively, carried only single chromosome change, in particular loss of chromosome Y and gain of chromosome 7, in contrast to only 8% of LCLC being generally highly aneuploid and carrying the highest chromosome numbers of all lung cancer subtypes. Except for 1 case (2%), all SCLC were highly aneuploid although 27% carried a near diploid chromosome number. DNA measurement of primary SCLC may indicate an even higher percentage of pseudodiploid cases in up to 90% of cases. Except for the near diploid cases, SCC were almost invariably hyperdiploid. In contrast, hypodiploid tumors were present in the ADC and SCLC subgroups, both being associated with a high degree of aneuploidy. Beside the near diploid cases, the histogram of the lung carcinomas according to their chromosome numbers showed a second peak in the near triploid range.

CGH revealed typical patterns of chromosomal imbalances in each lung cancer subtype and also specific alterations that were significantly associated with tumor progression and differentiation [1–6]. Amazingly, there were even chromosomal imbalances detectable that correlated with organ specific metastasis to the brain [5]. The highest prevalence of alterations were observed in SCLC. The data confirms that aneuploidy is a key factor in lung carcinogenesis being early detectable and also associated with tumor progression. Different chromosome numbers and imbalances are associated with lung cancer subtypes, their variation by chromosomal instability may cause transition in tumor morphology and differentiation.

The expression analysis is able to translate the genetic imbalances into disregulations of specific genes thus carrying the potential to identify candidates for diagnostic and therapeutic purposes [7–10]. Our cDNA microarray study [8] using a 24,000-element chip representing more than 17,000 unique genes on 67 lung cancer specimens including five SCLC from 56 patients showed that the major subtypes, i.e. squamous, adeno-, large cell and small cell carcinomas clustered into individual subgroups apart from normal lung. For the clustering a subset of 918 cDNA clones was chosen that discriminated best between the tumors form different patients (compared to tumor pairs from one individual). Doing so, the above mentioned tumor subgroups were associated with the up- or downregulation of a cluster of genes being most characteristic each tumor type. Squamous cell carcinoma (SCC) of the lung showed characteristics of a "true" squamous epithelium with expression of genes like p63, and cytokeratins 5, 13, and 17. Large cell carcinomas showed expression of genes involved in tissue remodeling. Adenocarcinomas, the largest subcollective, separated into three subgroups that were significantly different in survival. In group 3 adenocarcinomas with bad survival, genes involved in lung differentiation like TTF1 were downregulated. Together with large cell carcinomas the gene expression pattern suggested an epithelial–mesenchymal transition.

In summary, CGH and expression profiling are powerful tools for lung cancer characterisation and the identification of new diagnostic and therapeutic candidate genes. Microarrays may be used to supplement the conventional classification, however, the analysis of specific genes by RT-PCR or immunohistochemistry may prove to be an easier and cheaper alternative in this respect. Finally, our studies inspired two models for lung cancer progression, one being associated with small cell (neuroendocrine) dedifferentiation [11], the other with large cell (mesenchymal) dedifferentiation.

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DNA-ANEUPLOIDY AS A MARKER OF PROGRESSION IN ATYPICAL SQUAMOUS CELLS OF UNKNOWN SIGNIFICANCE (ASCUS), AND LOW GRADE SQUAMOUS INTRAEPITHELIAL LESIONS (LSIL), CONTAINING CERVICAL SMEARS

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Objectives: To compare positive (PPV) and negative predictive values (NPV) of conventional cervical cytology and of DNA-image cytometry (DNA-ICM) using DNA-aneuploidy as a marker for the prediction of progressive behaviour of cervical smears with Atypical Squamous Cells of Undetermined Significance (ASCUS) or Low-Grade Squamous Intraepithelial Lesions (LSIL) in a routine setting. Additionally interobserver reproducibility of DNA-ICM was tested remeasuring the slides by a second observer (V.Q.H.N.).

Study design: 197 patients with Pap smears, diagnosed as ASCUS or LSIL were included into a prospective cohort study. Slides were classified according to the Bethesda system. DNA-ICM was performed using an AUTOCYTE QUIC DNA-workstation after restaining the smears according to Feulgen, considering the four consensus reports of the European Society of Analytical Cellular Pathology (ESACP) on standardized diagnostic DNA-image-cytometry [4–7]. The mean interval between the initial cytological/DNA-cytometric diagnoses on routine smears and their histological verification was one month. Minimum cytological follow up interval was six months. DNA-aneuploidy was defined as either atypical stemlines ouside 2c, 4c or 8c $\pm 10\%$ or the detection of cells $>$9c (9c Exceeding Events, [3]).

Results: Using $\geqslant$CIN II as an output criterion, PPV of cytology was 34.9% and that of DNA-ICM 64.3%. NPV of DNA-euploidy for non-progression within six months was 85%. Using $\geqslant$CIN III as an output criterion PPV of cytology was 21.6% and of DNA-ICM 42.9%. NPV of DNA-euploidy for the prediction of non-progression within six months was 93.3%. Differences in PPVs between cytology and DNA-ICM were highly significant ($p < 0.001$). The overall proportion of agreement between two observers in DNA-ICM was 94.1%, $\kappa = 0.87$, CI = 0.74–0.99.

Discussion: We could demonstrate the high prognostic validity of DNA-ICM using stemline- or single-cell-aneuploidy as a marker for the differentiation between progressive and non-progressive, cytologically doubtful cervical lesions. Whereas euploid ASCUS or LSIL-lesions can be controlled cytologically after six months, aneuploid lesions should immediately be controlled histologically or removed. Our study additionally showed a good interobserver reproducibility of diagnostic DNA-ICM on cervical smears with ASCUS or LSILs in a routine setting.

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INDUCTION OF ANEUPLOIDY DURING CERVICAL CARCINOGENESIS: EVIDENCE FOR AN UNSTABLE TETRAPLOID INTERMEDIATE

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The mechanisms responsible for the development of aneuploidy during cervical carcinogenesis have not been clearly defined. We hypothesize that the majority of the observed numerical chromosomal aberrations follow a sequential pattern where the aneuploid cervical lesions characteristic of the advanced stages of the disease form via chromosomal loss from a transient tetraploid intermediate. To substantiate this mechanism, we are currently conducting a molecular epidemiological study to track the evolution of chromosomal alterations during the progression of cervical carcinogenesis. We have screened 1000 cervical cells from each of 128 different women exhibiting various stages of normal, dysplastic, and cancerous cells using multiple probe fluorescence *in situ* hybridization (FISH) for the presence of chromosome alterations affecting chromosomes 3 & 17. Nuclei containing four hybridization regions for both chromosomes 3 and 17 were considered to represent tetraploid cells whereas nuclei containing three hybridization regions for either chromosome were scored as aneuploid (hyperdiploid) cells. Significant increases in both tetraploid and aneuploid cells were seen with disease progression. The proportion of women exhibiting elevated frequencies of tetraploidy and aneuploidy increased from 1/26 and 0/26 among women with normal Pap smears to 20/39 and 22/39 for women with high-grade cervical lesions (HGSIL). Tetraploid cervical cells were often observed in the absence of aneuploid cells whereas the majority of aneuploid cells appeared to be near-tetraploid in chromosome number. Interestingly, in 39 of the 40 cases exhibiting elevated frequencies of near-tetraploid aneuploid cells, a preferential loss of 17 was seen. In only one case was the loss of chromosome 3 more common. Micronuclei, a biomarker of genomic instability, were found at increased frequencies in the cervical cells and were significantly associated with the presence of tetraploid cells. By using a pancentromeric DNA probe to identify the mechanism through which micronuclei were formed, we observed that there were significant increases in micronucleated cells formed through both chromosome loss and breakage. These results indicate that aneuploidy as well as genomic instability (as manifested by loss of entire chromosomes and chromosome fragments) often develops in cervical cells from a transient and unstable tetraploid intermediate.

ANEUPLOIDY IN LEUKEMIA DEVELOPMENT

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Aneuploidy is a common event in the development of leukemia. In childhood leukemia, hyperdiploidy is the most common type of cytogenetic abnormality. Approximately 30% of acute lymphoblastic leukemias (ALL) in children are hyperdiploid, and contain more than 50 chromosomes per cell. For example, in a study of childhood leukemia here in Northern California we and our collaborators found that 29% of the ALL cases were hyperdiploid [1]. The most common chromosome affected is 21 and to a slightly lesser extent X, 4, 6, 14 and 18 [2,3]. Interestingly, hyperdiploid childhood leukemias have recently been shown to arise *in utero* [4]. The clonal hyperdiploid cells are present at birth and can be detected in newborn blood spots called Guthrie cards. The hyperdiploid chromosomes come from both the mother and father and a recent study shows there is no evidence of imprinting [5]. Our laboratory and others have also shown that other forms of childhood leukemia arise *in utero* [6–9].

In acute myeloid leukemias in children the same clonal chromosomal changes are observed as in adults. The loss of chromosomes 5 and 7 (monosomy) and the gain of chromosome 8 (trisomy) are common clonal chromosomal abnormalities [10]. There is even a rare monosomy 7 syndrome in which many of the children develop a myelodysplastic syndrome that in many cases goes on to acute leukemia [11]. The current thinking as to how these selective aneuploidies arise is that random damage occurs to the DNA or the spindle apparatus and that selective advantage causes clones harboring these abnormalities to grow faster than surrounding cells. The initial damage that leads to the loss or gain of the chromosome, whether spontaneous or caused by chemicals, radiation or a virus, is considered to be random rather than selective to the specific chromosomes. Recently, we have tested an alternate idea, that metabolites of the leukemogenic chemical, benzene cause a higher rate of chromosome gain and loss on the chromosomes involved in leukemogenesis and that, as such are selective in their effects.

Occupational exposure to benzene has been shown to induce both numerical (aneuploidy) and structural chromosome aberrations in circulating blood lymphocytes [12]. Early studies showed that the loss or gain of C-group chromosomes (6–12, X) was often observed in benzene-associated leukemia patients. More recently, we have reported that the loss of chromosomes 5 and 7 (monosomy 5 and 7) and the gain of chromosomes 8 and 21 (trisomy 8 and 21), are significantly increased in benzene-exposed workers in comparison with controls [13,14]. We have expanded on these studies using a new OctoChrome device that was originally conceived in our laboratory and is currently manufactured by CytoCell (Banbury, UK). Using this method one can detect numerical and structural changes in all 24 chromosomes on a single slide.

The 8-square OctoChrome FISH technique was tested in a pilot study of 11 subjects (6 exposed and 5 matched controls) [15]. The long-term goal of this work is to determine if the damaging effects of benzene are greater in some chromosomes than in others. Initial analysis of this small group of 11 workers indicates that benzene exposure ($>$5 ppm TWA) caused increases in loss (monosomy) of some chromosomes but not others. The effects of benzene on each chromosome were assessed as the incidence rate ratio (IRR) from a Poisson regression model with the strongest effects being reflected by the highest IRR values. Monosomy of chromosomes 5, 6, 7 and 10 had the highest IRRs and statistical significance in this preliminary study (IRR > 2.5, $p < 0.005$). On the other hand, the monosomy levels of seven other chromosomes (1, 4, 9, 11, 15, 22 and Y) were unchanged in the exposed workers with IRRs close to 1.0, suggesting that benzene has the capability of producing selective effects on certain chromosomes. Similar selective effects were also observed on the induction of trisomy (gain of a chromosome). We are expanding these studies to a total of 88 subjects (31 controls, 31 exposed to $<$10 ppm and 26 to $\geqslant$10 ppm of benzene) in order to definitively test of our hypothesis that the damaging effects of benzene are greater in some chromosomes than in others.

In order to produce the chromosome-damaging effects described above, benzene must be metabolized to one or more genotoxic metabolites [16]. The most likely candidate toxic metabolites are 1,4-benzoquinone and 2-hydroxy-1,4-benzoquinone derived from the polyphenolic metabolites hydroquinone (HQ) and 1,2,4-benenetriol (BT), respectively. HQ and BT have previously been shown to induce micronuclei in hu-

man lymphocytes and HL60 cells and aneuploidy by disrupting microtubules. In addition, they have been shown induce the loss and long arm deletion of chromosomes 5 and 7 in human lymphocytes [17]. HQ has also been shown to increase the level of aneusomy of chromosomes 7 and 8 in human CD34-positive blood progenitor cells [18]. We have recently tested the ability of HQ and BT to produce selective chromosomal aneuploidy in human cells [19]. Human peripheral blood was exposed to HQ and BT, and the ploidy status of 9 different chromosomes (1, 5, 6, 7, 8, 9, 11, 12, 21) was examined using fluorescence *in situ* hybridization (FISH) of metaphase spreads. Poisson regression was used to provide interpretable incidence rate ratios (IRR) and corresponding p values for all 9 chromosomes. Statistically significant differences were found between the sensitivity of the 9 chromosomes to gain or loss. Chromosome 5 was highly sensitive to loss following HQ and BT exposure, whereas chromosomes 7, 8 and 21 were highly sensitive to gain in comparison to other chromosomes. Significant support for the "*a priori*" hypothesis that chromosomes 5 and 7 are more sensitive to loss induced by HQ and BT than the other 7 chromosomes was also obtained. These data support the notion that benzene metabolites affect the ploidy status of specific chromosomes more than others and can initiate or promote leukemia induction through these specific effects.

Our findings of selective aneuploidy in chromosomes related to leukemogenesis following chemical exposure suggest that leukemogenic chemicals, including benzene, produce non-random damage that is key to the cancer process. Thus, the thinking that chemicals, and for that matter radiation, induce leukemias by producing non-specific chromosome damage that only results in clonal chromosome changes as a result of selective advantage may be incorrect. Rather, it seems probable that chemicals may cause selective damage to certain chromosomes, which then initiates leukemia, or even promotes it, through the loss of heterozygosity of tumor suppressor genes or other genes related to growth and normal differentiation of white blood cells.

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USE OF DNA PLOIDY MEASUREMENTS IN SCREENING FOR EARLY CANCER AND PRE-NEOPLASTIC LESIONS OF THE UTERINE CERVIX

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Introduction: Population based screening of women from the onset of sexual activity based on regular intervals of 2–3 years has proven to reduce several fold the incidence of invasive cervical cancer as well as the mortality due to this disease [1]. However, such an undertaking requires significant resources as well as a very large number highly skilled cytotechnologists and cytopathologists. Therefore, to implement such programs in the conventional way may not be practical or even possible in many countries.

Materials and methods: We have developed a method to screen for early cancer and pre-neoplastic lesions (marked atypia) of uterine cervix that is based on the measurements of DNA ploidy of exfoliated cervical smears. The method is based on a simple, liquid based preparation whereby cells from the cervical brush are first suspended in a liquid fixative and then cyto-centrifuged onto a microscope slide [2]. The nuclei of the cells are then stained by a DNA specific and stoichiometric stain [3]. A fully automated high resolution image cytometer [4,5] is then used to measure the size and the DNA content of the cell nuclei on the slide. On average, 2,000 with the range of 1,000 to 8,000, cell nuclei are measured per slide consuming in less than 10 minutes (range 6–15 minutes) of the system's time. All samples with cells containing abnormal amount of DNA are examined by cytotechnologists (any cell with atypical amount of DNA could be brought under crosshair of the microscope for manual observation) for the conventional diagnosis. Samples without cells having abnormal DNA content are declared normal and no further observations are made by cytotechnologists.

Results: Using this approach we examined 6,000 women in rural area around Wuhan (Hubei Province, China) and compared this approach with conventional (manual) method using the best trained cytopathologists. The conventional method found 4 cancers and 7 cases of marked atypia, while the new method found 8 cancers and 27 marked atypia cases. All cancers found by the conventional method were also found by the new method.

Discussion: This approach is now being further tested on a much larger scale with the goal to implement it as the keystone for the population based screening for cervical cancer in Hubei Province and later in other provinces in China and elsewhere. The new method has a higher sensitivity (reduced false negative rate) at the same specificity, requires fewer skilled technologists and is more cost-effective than conventional Pap screening.

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CARCINOGENESIS BY ANEUPLOIDIZATION

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Despite over 100 years of cancer research, the cause of cancer is still a matter of debate [1,2]. Indeed, cancer has been hiding the secret of its genetic origin, like a magician hides the secrets of its trade. It has kept this secret by supporting many competing theories simultaneously with bits of evidence from its large repertoire of exotic genotypes and phenotypes. In addition cancer has kept researchers guessing on what is in that black box of the exceedingly long and phenotype-less lag periods from initiation of cancer with carcinogens to carcinogenesis [3].

Because carcinogenesis is irreversible, there is a consensus that cancer is caused by some kind of mutation, but there is no consensus on what kind of mutation it actually is. A majority of scientists currently think that specific gene mutations are the cause of cancer, but others think that specific rearrangements of the normal chromosome balance, alias, specific aneuploidies are the cause, and yet others think that mutations cause cancer via aneuploidy [1,2].

Here we try to determine, which of these theories provides a coherent explanation for all of the many odd features of cancer and carcinogenesis, focusing primarily on the prevailing mutation and the competing aneuploidy theories. For this purpose we first briefly define these theories.

1. The mutation-cancer theory

The mutation theory holds that cancer is the result of 4 to 7 gene mutations [4,5], which either generate dominant oncogenes or inactivate recessive tumor suppressor genes or both [6–10].

However, the mutation theory suffers from 3 unresolved problems:

(1) There are no consistent correlations between any particular gene mutations and cancer [11–13].

(2) Despite intensive efforts of over 2 decades there is as yet no functional proof that one or any combination of mutant genes from cancer cells can transform normal diploid cells into cancer cells [12,14–17]. Indeed, artificially mutated mice with mutant oncogenes or without tumor suppressor genes or even with combinations of both in their germ line are surprisingly procreative [16,18–22], and their cancer risk is "strain-dependent" [20] but within the known range of laboratory mice [3,23,24]. Although some of these studies point out that mutant mice have higher cancer risks than unaltered controls, e.g. Donehower et al. [20], the cellular cancer risk of these artificially mutated mice is extremely low. Since cancers originate from single cells [25–27] (see below) and mice consist of about 5×10^{10} cells, the cellular cancer risk of mice without tumor suppressor genes is only 5×10^{-10}. It is thus scarcely an argument for a role of such genes in carcinogenesis.

(3) Based on the normal, spontaneous gene mutation rate of about 10^{-6} per mitosis [28] only 1 in 10^{24} to 10^{42} human cells would ever become cancer cells [18, 29–31]. This number would be even lower, if the mutation rates of the recessive cancer genes, as for example the hypothetical tumor suppressor genes, are squared. Since humans consist of 10^{14} cells, only 1 in 10^{10} to 10^{28} humans would ever get cancer. In other words cancer would hardly exist. To reconcile spontaneous carcinogenesis with the spontaneous mutation rates, the proponents of the mutation theory have postulated that, prior to mutation of prospective cancer genes, another class of cellular genes must be mutated to mutator genes, which in turn would mutate prospective cancer genes to real cancer genes [8,29,32–35].

However, genes with this potential are only rarely found in cancer cells [1,10,13]. Moreover, since mutator genes and their mutations are escalating autocatalytically, their presence is eventually suicidal and thus hard to reconcile with the long latent periods of carcinogenesis and particularly with the immortality of cancer cells [13]. Tomlinson et al. expressed these reservations about the mutator-gene hypothesis as follows, "The scenarios for a role of a raised mutation rate assume that there is no selective disadvantage to a cell in having an increased number of mutations. This may not be the case: for example, a deleterious or lethal mutation may be much more likely than an advantageous mutation. More subtly, an accumulated mutational load might induce apoptosis" [36].

Thus there is no consistent correlative or functional proof for the mutation theory. In addition the postulated mutator genes are hard to reconcile with the long, preneoplastic lag periods of carcinogenesis and even harder with the immortality of cancer cells.

2. The aneuploidy-cancer theory

The aneuploidy theory holds that somatic evolution of the karyotype of a single cell causes random and cancer-specific aneuploidies, which encode cancer-specific phenotypes. The principle of generating new phenotypes from old genes – and thus independently of gene mutation – is also the basis of phylogenesis. Phylogenesis generates new species by rearranging old, phylogenetically conserved genes into new sets of chromosomes [37]. Thus cancer cells are new, semi-autonomous species of their own rather than mutants of their precursor cells.

According to the aneuploidy theory the somatic karyotype evolution is initiated by a random aneuploidy, which is either induced by carcinogens or spontaneously (see Fig. 1). Aneuploidy destabilizes chromosomes because it unbalances – and thus corrupts the normal functions of – numerous highly conserved teams of proteins including those, which segregate, synthesize and repair chromosomes. Aneuploidy also catalyzes gene mutations by corrupting protein teams that repair DNA and synthesize nucleotide pools. Thus aneuploid cells undergo chromosome non-disjunctions and gene mutations due to error-prone chromosome segregation and error-prone DNA repair and synthesis. The degrees of the resulting genomic destabilization would be proportional to the degree of aneuploidy [38,39].

The basis for the somatic evolution of neoplastic cells from randomly aneuploid precursors is selection of rare chromosome combinations with advantages for abnormal growth. Thus the rate-limiting step of carcinogenesis is the aneuploidy-catalyzed generation of new chromosome arrangements with neoplastic phenotypes by random karyotype variations – a process that is also analogous to phylogenesis. However, since the generation of a new, autonomous species is infinitely less likely than the generation of a parasitic cancer cell, phylogenesis is much slower than carcinogenesis.

According to the aneuploidy theory, immortality of cancer cells derives from the inherent heterogeneity of aneuploid cell populations. Indeed cancers are heterogeneous, "polyphyletic" [40] cell populations, which include sub-species that can survive otherwise fatal mutations, cytotoxic drugs, metastasis to heterologous locations, transplantation to heterologous hosts, etc. via sub-species-specific karyotypes. These karyotypes either activate alternative drug-resistant biochemical pathways or eliminate drug-specific receptors. It is because of this inherent genetic heterogeneity that populations of aneuploid cells are "immortal", although individual cells are not.

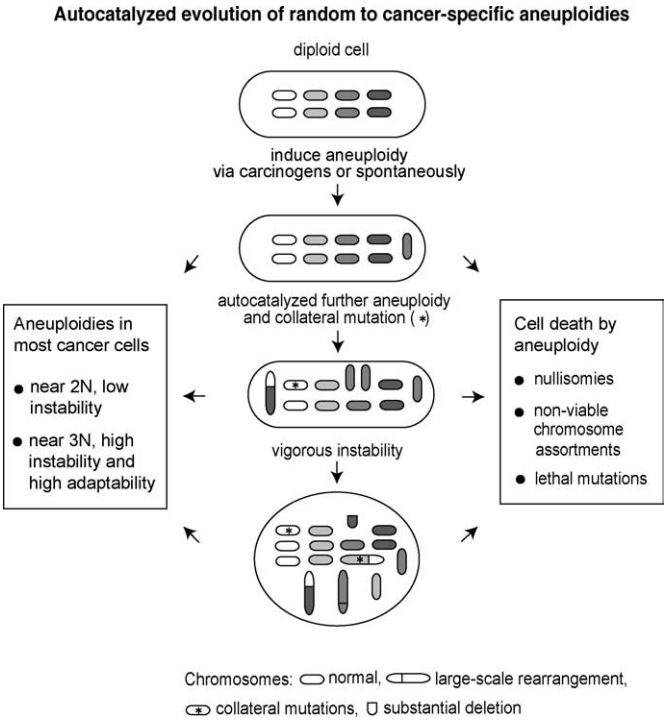

Fig. 1.

In sum, the aneuploidy theory proposes that the inherent instability of aneuploidy is sufficient to generate the multilateral genomic instability of neoplastic and preneoplastic cells, and is thus independent of, although not necessarily free of gene mutation (see Fig. 1). The majority, if not all, of the many heterogeneous gene mutations of cancer cells [1,2] may just be inevitable, but functionally irrelevant consequences of aneuploidy (see also below).

3. The abilities of the mutation and aneuploidy theories to explain 9 features of cancer and carcinogenesis

In the following we test the two genetic theories of cancer for their abilities to explain 9 features of cancer and carcinogenesis:

(1) *Cancers are clonal.* Nearly all cancers originate from single cells based on preneoplastic and neoplastic genetic markers [25–27]. This feature is compatible with both genetic theories of cancer.

(2) *Aneuploidy is ubiquitous in cancer*. Cancers are aneuploid [25,41–44]. By contrast, conventional gene mutations are independent of, and thus typically not associated with karyotype alterations. It follows that aneuploidy is necessary for carcinogenesis, as is postulated by the aneuploidy theory. By contrast, the mutation theory predicts diploid cancers.

(3) *Abnormal gene expression profiles of cancer cells correlate with aneuploidy*. According to Ruddon's *Cancer biology*, "Abnormal gene expression is the sine qua non of cancer cells" [27]. Recent analyses have shown that the abnormal gene expression profiles of cancer cells correlate very closely with the aneuploid doses of the corresponding chromosomes [45–47]. By contrast, the gene mutation theory predicts that the expression of only a few genes is altered, namely the 4 to 7 that are mutated and those that might be controlled by them.

(4) *No cancer-specific gene mutations.* About 50% of all cancers of a given kind contain various gene mutations of hypothetical oncogenes and tumor suppressor genes [11,30,48–51]. However, consistent correlations between such mutations and specific kinds of cancers have not been found [16,18,30,31]. For example, Little reports "While radiation-induced cancers show multiple unbalanced chromosomal rearrangements, few show specific translocations or deletions as would be associated with the activation of known oncogenes or tumor suppressor genes" [12]. Grosovsky et al. also find, "no consistent elevation of specific locus mutation rate has been reported" [52]. Further Gisselson et al. note, "the correlation coefficient between breakpoint frequency and telomere length [a potential mutator] was low in both osteosarcomas and pancreatic sarcomas" [53]. Moreover, mutations of oncogenes and tumor-suppressor genes of many clonal cancers are non-clonal, and thus not necessary for carcinogenesis [19]. A survey of genomic instability by Lengauer et al. states in 1998 (a) "no consistent pattern of defects in polymerases has been found in tumors" and (b) mismatch repair deficiencies are only in "13% of colorectal, ... endometrial and gastric cancers ... other types are rarely (<2%) MMR-deficient" [54]. In the words of a recent survey by *Scientific American*, "A few cancer-related genes, such as p53, do seem to be mutated in the majority of tumors. But many other cancer genes are changed in only a small fraction of cancer types, a minority of patients, or a sprinkling of cells within a tumor" [2]. Since specific gene mutations are not consistently associated with any kind of cancer, they cannot be necessary for carcinogenesis [19]. However, an abnormally high rate of gene mutations is a predictable consequence of aneuploidy.

(5) *Karyotypes and gene mutations of cancers are unstable*. Karyotypes of cancer cells may change from a few to 100% per mitosis, proportional to the degree of aneuploidy [38,39,55]. It is for this reason that the karyotypes of "clonal" cancers are heterogeneous, despite the clonal origin of cancers [1,13,16,25]. Likewise gene mutations of cancers are unstable, because they are also heterogeneous in clonal cancers [19]. However, conventional gene mutations are just as stable as the corresponding wild types, e.g. spontaneously mutating at less than 10^{-6} per mitosis per haploid gene (see above). By contrast, genomic instability is an inevitable consequence of aneuploidy. Indeed, clonality, aneuploidy and genomic instability define cancer.

(6) *Cancers have unique, exotic phenotypes, never observed in diploid biology*. The exotic phenotypes of cancers include "immortality", a phenotype that has never been achieved by any diploid cell, despite 3 billion years of mutations! In addition cancer cells can become readily resistant to cytotoxic drugs such as those used in chemotherapy, and can metastasize from one differentiated tissue to another [56]. None of these phenotypes has ever been observed in diploid animals. Thus cancer cells can generate phenotypes that are not

ever generated in normal diploid cells by gene mutations. However, the unique ability of aneuploid cells to generate variant sub-species by altering its karyotype provides a coherent explanation. Accordingly, the exotic phenotypes of cancers reflect variant sub-species that can evade toxins, otherwise lethal mutations and normal histologic barriers.

(7) *Mutagenic and non-mutagenic carcinogens induce cancers.* Besides mutagenic radiations and alkylating agents numerous non-mutagenic substances are carcinogenic including polycyclic aromatic hydrocarbons, hormones, metal ions, butter yellow, solid bodies, asbestos, etc. [18,57,58]. It follows that gene mutation is not necessary for the induction of carcinogenesis. By contrast, aneuploidy can be induced either by gene mutations that lead to direct or indirect chromosome breaks and rearrangements, or can be induced by agents that physically or chemically destroy the spindle apparatus – independent of gene mutation.

(8) *Long lag periods from carcinogen to cancer*. At carcinogenic doses carcinogens, e.g. radiations or chemicals, initiate carcinogenesis immediately, because no subsequent treatments are necessary [13]. Cancer appears only after lag periods of years in experimental animals and decades in accidentally exposed humans [13,26]. Take the late cancers after the Hiroshima bomb as an example [26]. Since mutagens and aneuploidogens act fast, initiation is compatible with the mutation and the aneuploidy theory. However, the long latent periods of carcinogenesis cannot be reconciled with gene mutation. But induction of a random aneuploidy followed by a somatic karyotype evolution with selection for autonomous growth, provides a coherent explanation for the long lag periods of carcinogenesis.

(9) *Age bias of cancer*. The cancer risk of animals and humans increases exponentially with age [4,26]. By contrast, the mutation theory predicts cancer at young age. According to the mutation theory all but one of the 4–7 genes postulated to cause cancer should be heritable. Thus newborns with all but one mutation missing from a carcinogenic combination should develop cancer at young age, as soon as the missing mutation has occurred in one cell of their body. But, there is virtually no cancer at young age. By contrast, the aneuploidy theory correctly predicts the age bias of cancer for two reasons: (i) Since aneuploidy is not heritable [59,60], carcinogenesis by aneuploidization has to be somatically initiated and completed, a process that appears to take decades for solid cancers in humans [13,26,61]. (ii) As more and more initiated cells are accumulated and able to multiply over a lifetime the risk of carcinogenesis would increase exponentially (as is also postulated by the mutation theory).

4. Conclusions

In sum aneuploidy is the genetic alteration that provides a coherent explanation for 9 of the 9 features of cancer and carcinogenesis described above and summarized in Table 1, but gene mutation only explains 3. In view of this we conclude that carcinogenesis is initiated by random aneuploidy and completed by specific aneuploidies, which evolve from random aneuploidy autocatalytically, but inefficiently and slowly over long periods of time. Thus carcinogenesis is analogous to evolution and cancer cells are new, albeit only semi-autonomous cell species, rather than mutants of their progenitor cells. According to the aneuploidy theory,

Table 1
Cancer and carcinogenesis in the light of the mutation and aneuploidy theories

	Features of cancer and carcinogenesis	Mutation theory	Aneuploidy theory
1	Cancers are clonal, based on neoplastic and preneoplastic markers	+	+
2	Aneuploidy is ubiquitous in cancer	−	+
3	Abnormal gene expression profiles of cancer cells correlate with aneuploidy	−	+
4	No cancer-specific gene mutations	−	+
5	Karyotypes and genotypes are unstable, and therefore heterogeneous or non-clonal	−	+
6	Cancers have unique, exotic phenotypes, never observed in diploid biology: immortality, resistance to cytotoxic drugs, metastasis	−	+
7	Cancers are induced by mutagenic and non-mutagenic carcinogens	+/−	+
8	Carcinogens initiate carcinogenesis immediately, but cancer follows only after lags of many years or many cell generations	+/−	+
9	Age bias of cancer	−	+

the many non-specific mutations of cancer cells are inevitable consequences of the inherent genomic instability of aneuploidy.

If confirmed, the aneuploidy theory offers a scientific basis for the identification and early treatment of preneoplastic lesions. In addition the theory proposes that cancer prevention will benefit from testing foods and drugs for their abilities to induce aneuploidy.

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THE INDUCTION OF CHROMOSOMAL INSTABILITY AS AN INDIRECT RESPONSE TO IONIZING RADIATION AND OTHER TOXIC AGENTS

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1. Inherited and inducible chromosome instability

The ability to maintain genome integrity in the face of endogenously and exogenously generated DNA damage is critical for healthy survival and complex homeostatic mechanisms have evolved to allow cellular adaptation to cellular stress and injury. In recent years there has been considerable progress in identifying the mechanisms by which eukaryotes respond to potentially harmful insults by initiating processes that either enhance cell survival or lead to the regulated loss of damaged or unwanted cells. Inherited or acquired deficiencies in genome maintenance systems contribute significantly to the development of malignant diseases and there are well-recognized chromosome instability/breakage syndromes that produce complex and often multi-system effects characterized by a significant predisposition to malignancy.

Since the discovery of the induction of mutations and chromosome aberrations by ionizing radiation in the early years of the twentieth century it has been accepted that these effects are due to DNA being irreversibly changed at the time of exposure, either during the processing and enzymatic repair of the radiation damage or during the first round of DNA replication immediately after exposure. As malignant transformation is generally regarded as being initiated by a gene mutation or a chromosomal aberration, the initiating lesion for malignant transformation has been similarly attributed to direct DNA damage. Accordingly, it has been widely accepted that most of these changes take place immediately following exposure. Thus, if the damage were repaired, the progeny of an irradiated cell would appear normal (Fig. 1a) but if misrepaired, the progeny would be expected to show any transmissible radiation-induced genetic change and all cells derived from such a cell would exhibit the same genetic change, i.e. the effect would be clonal (Fig. 1b).

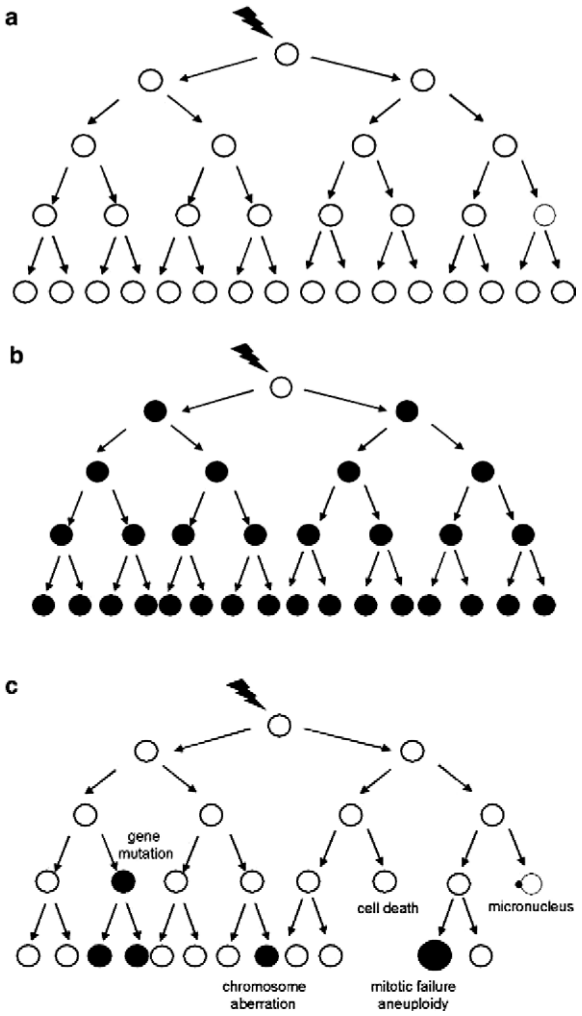

Fig. 1. Models of the responses of clonogenic cells to ionizing radiation with mutations and/or chromosomal aberrations shown as filled circles and apparently normal cells as open circles. (a) If a cell faithfully repairs DNA damage then its clonal descendants will appear normal. (b) If a cell is directly mutated by radiation then all its descendants will express the same mutation. (c) Radiation-induced genomic instability is characterized by non-clonal effects in descendant cells.

In recent years, many laboratory studies have demonstrated non-clonal chromosome aberrations and mutations in the clonal progeny of irradiated cells. In addition, the progeny of irradiated cells have been shown to exhibit an enhanced death rate and loss of reproductive potential that persists for many generations and possibly indefinitely in established cell lines. The terms lethal mutations and delayed reproductive death are used interchangeably for this delayed death phenotype. All the various effects in which delayed death, gene mutations and a variety of chromosomal abnor-

malities can be demonstrated in cells that are not themselves irradiated but are the progeny of cells exposed to ionizing radiation many cell divisions previously (Fig. 1c) have been interpreted as manifestations of a radiation-induced genomic instability [1–4]. Similar effects are now being reported for a range of chemical exposures [5–8]. Induced instability is a genome-wide process and the cellular phenotype is similar to that of the inherited chromosome instability syndromes, characterized by spontaneously high levels of chromosomal abnormalities and mutations. Despite the apparent similarities, radiation-induced genomic instability seems to reflect epigenetic processes rather than mutation of genome maintenance genes [9–13] but the induced instability phenotype in both haemopoietic tissue [14] and mammary epithelium [15] is strongly influenced by genetic factors with some genotypes being susceptible and others relatively resistant. Clearly, any process that increases the frequency with which genetic changes arise will increase the probability of potential malignant changes in target cells and potentially in tumour cells although it may be difficult to determine how an 'initiating event' arose and it may not be possible to distinguish between chromosomal instability as a delayed effect of exposure and chromosomal instability arising as a consequence of the malignant process.

2. Mechanisms underlying inducible instability

At present, the mechanism of induction of instability by ionizing radiation and other agents is not fully understood nor is it clear whether all endpoints reflect a common mechanism. In all the various studies, the frequency of induced instability is orders of magnitude greater than that of conventional gene mutation frequencies and although in some studies using established cell lines a large number of post-irradiation cell divisions before assay might have allowed for selection of a radiation-induced gene mutation that confers a mutator phenotype, overall the data indicate that the mechanism underlying induced instability is epigenetic. Typically, the spontaneous frequency of gene mutations in mammalian cells is of the order of 10^{-6} and this increases some 10-fold to $\sim 10^{-5}$ (0.001% of surviving clonogenic cells) after exposure to 1 Gy X-rays. However, approximately 10% of surviving cells produce clones that exhibit delayed hypoxanthine phosphoribosyl-transferase (*hprt)* mutations and a similar or much greater proportion exhibit chromosomal instability. In a comparative study of *hprt* mutations induced directly by irradiation or arising as a consequence of induced instability, 75% of those induced directly by X-rays ('conventional' mutations) involved partial or total gene deletions and 25% small scale or point mutations, whereas only 28% of the delayed mutations were associated with large deletions and the majority were small scale changes [16]. This observation of a mutation spectrum more like that of spontaneously arising mutations than conventional radiation-induced mutations is similar to the cytogenetic investigations in which the aberrations associated with radiation-induced chromosomal instability in primary cells are similar to those arising spontaneously in the cells. Unstable aberrations commonly result in cell lethality and this may account for a component of the delayed reproductive death/lethal mutation phenotype in some cell systems [10,17]. The association of radiation-induced chromosomal instability with increased intracellular reactive oxygen species (ROS), oxidative DNA base damage [9] and an association of increased ROS with radiation-induced delayed death caused by ongoing apoptosis or necrosis in CHO cells [10,18] provides a potential epigenetic mechanism for radiation-induced genomic instability. However, some death may also result from signal antonymy generating an apoptotic response to conflicting simultaneous signals for proliferation and cell cycle arrest [19].

3. Radiation-induced bystander effects and clastogenic factors

The paradigm of genetic alterations being restricted to direct DNA damage has also been challenged by observations in which cells that are not exposed to ionizing radiation exhibit responses typically associated with direct radiation exposure as a consequence of contact with irradiated cells or after receiving certain signals from irradiated cells. These phenomena are collectively known as radiation-induced bystander effects [3, 4,20]. Bystander effects may reflect at least two separate mechanisms for the transfer of a damaging signal from irradiated cells. One mechanism seems dependent on gap junction intercellular communication stimulating a damage-signalling pathway mediated by the tumour suppressor p53. Other studies implicate a second mechanism in which irradiated cells secrete cytokines such as TGF-β or IL-8 or other factors that

act to increase intracellular levels of reactive oxygen species in unirradiated cells.

Prior to these recent studies of bystander effects, there are numerous reports of clastogenic factors in the plasma of irradiated individuals that are capable of inducing chromosome breaks when added to cultures of unirradiated cells but with considerable inter-individual variation in both production and response. These factors are also produced by other cellular stresses and in patients with a variety of chromosome instability syndromes and inflammatory disorders [21,22]. These clastogenic factors are produced *via* superoxide and also induce the production of superoxide; this may be the explanation of their persistence over many years. The vicious circle of clastogenic factor formation and action shifts the pro-oxidant/anti-oxidant balance in cells towards the pro-oxidant state and clastogenic factors can be regarded as markers of oxidative stress. Their clastogenic activity may be related to the formation of lipid peroxidation products inosine nucleotides and cytotoxic cytokines; all candidates for mediating radiation-induced bystander effects.

4. Links between genotype-dependent chromosomal instability radiation-induced bystander effects, and inflammation

Investigations of radiation-induced genomic instability in haemopoietic cells have revealed that chromosomal instability may be bystander-mediated [23,24] and may also produce, bystander interactions (Fig. 2) involving inter-cellular signalling, production of cytokines and free radical generation [13,25,26]. The cells responsible for bystander-mediated chromosomal instability in unirradiated haemopoietic cells *in vivo* [25,26] are likely to be of the mononuclear phagocytic (monocyte/macrophage) lineage with characteristics in common with the activated phagocytes found in inflammatory conditions as such cells are able to produce gene mutations, DNA base modifications, DNA strand breaks and cytogenetic damage in neighbouring cells. *In vivo*, because of their migratory properties, it is possible that activated phagocytic cells generated as a consequence of induced instability may also contribute to genetic damage in non-haemopoietic cells. That instability-derived activated phagocytes may produce genetic lesions in neighbouring cells has similar implications to the mechanisms proposed to explain the relationship between inflammation and carcinogenesis. Studies *in vitro* of cells, other than haemopoietic cells, that implicate soluble factors and processes involving reactive oxygen species in non-targeted effects would be consistent with free radical/cytokine-mediated mechanisms comparable to an inflammatory reaction. It is of particular interest that a persistent sub-clinical inflammation among Japanese A-bomb survivors has recently been

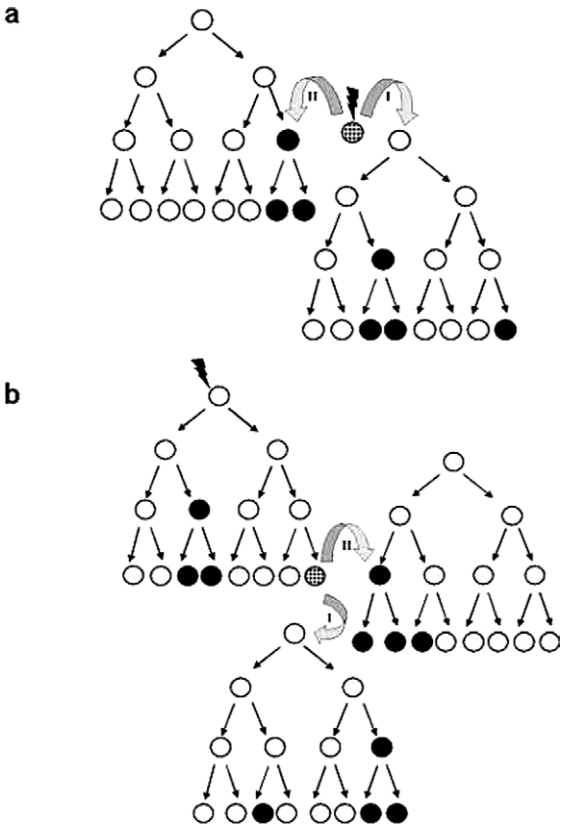

Fig. 2. Models of the inter-relationship between radiation-induced genomic instability in haemopoietic tissues [23,26] with mutations and/or chromosomal aberrations shown as filled circles and apparently normal cells as open circles and an 'activated cell' capable of producing a bystander signal as a hatched circle. (a) A bystander signal may induce a transmissible genomic instability or a damage response in an unirradiated clonogenic cell (I) or in a cell that has descended from an unirradiated clonogenic cell (II). In this model, instability may be a consequence of bystander signalling from an 'activated cell' and bystander effects may initiate and be responsible for radiation-induced genomic instability. (b) Radiation-induced genomic instability transmitted from an irradiated clonogenic cells may result in an 'activated cell' capable of inducing a bystander-mediated transmissible genomic instability or a damage response in an unirradiated clonogenic cell (I) or in a cell that has descended from an unirradiated clonogenic cell (II). In this model, radiation-induced genomic instability produces cells that provide a bystander signal able to induce instability; i.e. bystander effects can be both a cause (a) and/or a consequence (b) of inducible instability.

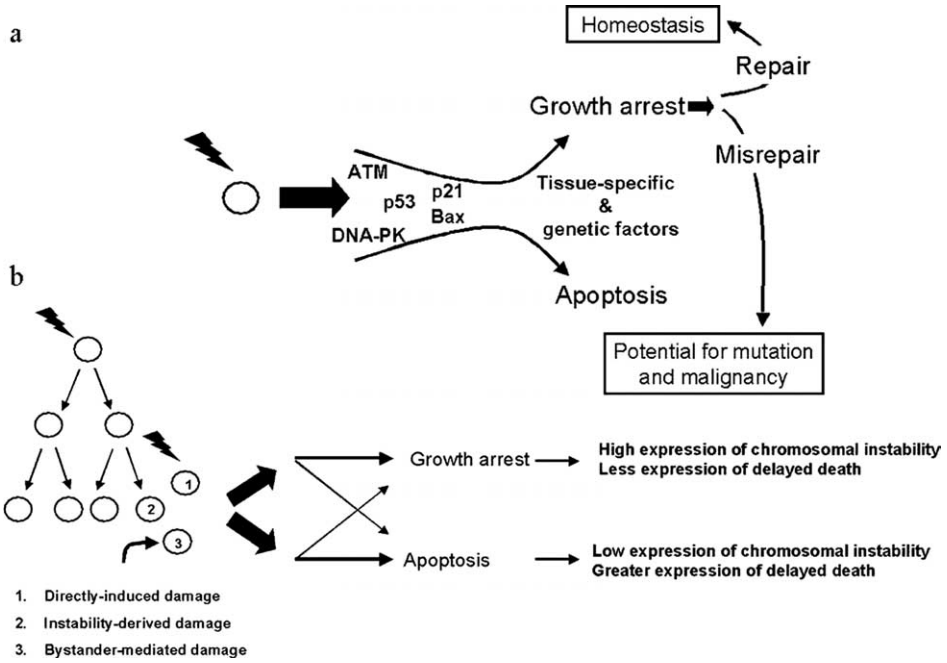

Fig. 3. (a) A schematic representation of how genetic modifiers influencing the p53 response pathway would reduce or reinforce the apoptotic response in a genotype-dependent manner. (b) The implications of genetic modification of responses in the p53 pathway for the expression of radiation-induced genomic instability and bystander effects in haemopoietic cells are shown schematically as a differential sectoring of response in genetic strains, susceptible to radiation-induced chromosomal instability and expressing low levels of delayed death and those relatively resistant to the expression of chromosomal instability expressing high levels of delayed death.

reported and it is suggested that radiation-induced enhancement of inflammatory reactions might contribute as an epigenetic and/or bystander effect to the development of several radiation-induced disorders, including the non-malignant conditions now reported in these individuals [27,28].

Taken together, a number of studies indicate that radiation-induced genomic instability and untargeted bystander effects may reflect inter-related aspects of inflammatory-type responses to radiation-induced stress and injury and contribute to the variety of the pathological consequences of radiation exposures. However, it is clear that whether a damaging signal is a consequence of direct radiation or arises as a consequence of an untargeted process, there are genotype-dependent and cell-type specific modifiers of signalling that influence the efficiency with which a damaged cell initiates an apoptotic or growth arrest response [13,29–31]. These genetically modified signalling processes (Fig. 3) may contribute to the underlying mechanisms for the probability of tumour development and the type of tumour induced by exposure to a given genotoxic agent being strongly dependent on genetic background. The genetic background that produces the more effective apoptotic response and phagocytic clearance would be less predisposed to adverse consequences of exposure due to a more effective elimination of unstable and potentially malignant cells. Thus, both the degree of inducible genomic instability and the potential consequences of this phenotype appear to represent a balance between the production of genotoxic/clastogenic factors and the response of the cell to such damaging agents. Both signal production and signal response may be significantly influenced by genetic and cell-type specific factors.

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GROSS GENOMIC ABERRATIONS, ANEUPLOIDY – A CAUSE RATHER THAN A CONSEQUENCE OF MALIGNANT TRANSFORMATION

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Numerical or gross genomic aberrations, also referred to as aneuploidy, are common features in early, precancerous lesions as well as cancerous lesions. The hypothesis that aneuploidy, a consequence of unequal chromosome segregation caused by abnormal centrosomes, plays a decisive role in the neoplastic process, was proposed a century ago by Boveri, and has recently made an impressive comeback. New evidence points to aneuploidy as a cause rather than a consequence of malignant transformation. Altered fidelity of chromosome segregation and centrosome alterations causing genomic instability, has been implicated in this process at an early stage.

We have recently demonstrated that patients with oral leukoplakia, a premalignacy of the oral mucosa, are at risk of developing cancer. Among the 150 patients with verified epithelial dysplasia, a carcinoma developed in 36 (24%). Of the 150 cases investigated, 103 (69%) were classified as diploid (normal), 20 (13%) tetraploid (intermediate) and 27 (18%) aneuploid (abnormal). Only 3 of 103 (3%) diploid cases, as opposed to 23 of 27 (84%) aneuploid cases, developed a carcinoma during a mean duration of 103 months follow-up. From the intermediate group of 20 tetraploid cases, 12 (60%) later developed a carcinoma. The malignant transformation rate of patients with DNA aneuploid oral leukoplakia was 70% within 3 years [1].

Among 45 patients with non-dysplastic oral leukoplakias, 4 of the 5 patients with aneuploid lesions developed an oral cell carcinoma [2].

Compared to those with leukoplakia, patients with erythroplakias have a higher tendency of malignant transformation. Of 25 patients with aneuploid lesions, 23 (92%) developed an oral carcinoma, compared to none of the patients with diploid lesions (0%). Furthermore, the survival rate for patients with diploid erythoplakia was 100%, whereas the mortality rate for patients with aneuploid lesions was quite high [3].

Recent evidence implies that the isoform of cyclooxygenase, (COX-2 may be involved in several important events throughout the tumorigenic process, and its overexpression has been related to genetic instability, apoptosis, angiogenesis and invasiveness. In oral carcinomas, COX-2 was expressed in 26 of 29 patients (88%) and aneuploidy was observed in 25 cases (94%). Notably, of the 22 patients with dysplastic leukoplakia lesions, COX-2 was exclusively expressed in a subgroup of nine patients (41%) identified to be at high risk of developing cancer by the aneuploidy of their lesions [4].

This studies clearly demonstrates that a subset of patients with oral premalignancies, as defined by the genomic instability marker aneuploidy (a global molecular marker), are at high risk of developing carcinomas. Thus, aneuploid leukoplakias and aneuploid erythoplakias are tantamount to carcinoma and patients with these lesions should be viewed and treated accordingly. Conceptually, these studies place gross genomic aberrations or aneuploidy as the cause of the malignant transformation at the beginning of this process, that is, at a time where chemopreventive measures are indicated [5].

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INSTABILITY OF CHROMOSOME STRUCTURE IN CANCER CELLS INCREASES EXPONENTIALLY WITH DEGREES OF ANEUPLOIDY

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Structurally altered or "marker" chromosomes are the cytogenetic hallmarks of cancer cells, but their origins are still debated. Here we propose that aneuploidy, an abnormal combination of chromosomes that is ubiquitous in cancer, catalyzes structural alteration of chromosomes via DNA breaks. Aneuploidy causes such breaks by unbalancing cooperative enzymes synthesizing and maintaining DNA and nucleotide pools, and even histones via the corresponding genes. DNA breaks then initiate deletions, amplifications and intra- and inter-chromosomal translocations. Our hypothesis predicts that the rate at which chromosomes are altered is proportional to the degree of aneuploidy: the more abnormal the number and balance of chromosomes the higher the rate of structural alterations. To test this prediction we have determined the rates at which clonal cultures of diploid and aneuploid Chinese hamster cells generate new, and thus non-clonal, structurally altered chromosomes per generation. Based on analyses of about 20 metaphases the number of new, structurally altered chromosomes were 0 per diploid, 0–0.23 per near-diploid/aneuploid, 0.2–1.4 per hypo-triploid, 3.25–4.8 per hyper-triploid and 0.4 per near-tetraploid cells. Thus instability of chromosome structure increased exponentially as aneuploidy deviated from the normal diploid and tetraploid chromosome balance. But, the particular chromosomes engaged in aneuploidy also affected the rates of alteration, particularly at low aneuploidy indices. We conclude that aneuploidy is sufficient to cause structural instability of chromosomes. We also provide evidence that aneuploidy is sufficient to cause numerical instability of chromosomes and the many genomic mutations of cancer cells that have been attributed to various mutator genes. Further, we suggest that certain structurally altered chromosomes encode cancer-specific phenotypes that cannot be generated by abnormal combinations of intact chromosomes.

ANEUPLOIDY THEORY PROVIDES THE "ALTERNATIVE PLAUSIBLE" EXPLANATION OF CANCER

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Abstract. The hundred-year-old aneuploidy theory provides a more comprehensive, productive, and satisfying explanation of carcinogenesis than gene mutation. Theodor Boveri was the first to point out that the normal cell is a product of the particular interaction of the balanced complement of chromosomes while malignant cells, on the other hand, resulted from aneuploidy, an uneven distribution of the qualitatively different chromosomes. The uneven and variable distribution of chromosomes produced a myriad of irreversible morphologically and metabolically abnormal phenotypes. Due to intrinsic error propagation, chromosomal imbalance was irreversible and progressive once generated, eventually leading to what Boveri described as tumors. Thus, a century ago Boveri understand that the autocatalyzed progression of aneuploidy *is* carcinogenesis. Modern experimental and analytical techniques such as phenotypic transformation analysis have confirmed Boveri's original aneuploidy theory of cancer. Independent of gene mutation, aneuploidy explains the gross biochemical abnormalities, abnormal cellular size and morphology of cancer cells, as well as the appearance of tumor-associated antigens, high levels of secreted proteins responsible for invasiveness and loss of contact inhibition, tumor formation, genetic instability leading to rapid appearance of drug resistance, the time-course of human cancers, carcinogen-induced tumors in mice, the absence of immune surveillance, and the failure of chemotherapy.

Keywords: aneuploidy, cancer, gene, mutation, chromosome, Boveri.

1. Introduction

The origin and nature of cancer has been one of the great enigmas since the time of the Egyptians and Greeks. The central paradox is that tumors are us and yet not us. The hundreds of different types of cancer are distinguishable in their details yet they all display the global or macroscopic characteristics that readily identify them as cancer. In an attempt to understand and explain carcinogenesis, cancer researchers have constructed elaborate roadmaps of metabolic pathways decorated with putative cancer genes. A striking feature of such maps is that they are static. They say nothing about the dynamics of the traffic along the pathways.

Of the numerous hallmarks of cancer, subtlety is not among them. To this day pathologist use the large, highly variable morphology of nuclei to diagnose cancer cells. The 60–90 chromosomes typically found in cancer cells is a much more satisfying explanation of these features than specific gene mutation [42]. A remarkable observation in virtually every type of mature solid cancer is the convergence of the average DNA index to around 1.7 [37]. In the ever-growing lists of pathways and cancer genes, where does one find the explanation of this phenomenon?

Another problem, often ignored, is the profound lack of functional evidence for the so-called cancer genes. In a recent census of cancer genes, Futreal et al. acknowledged that a mutant gene is typically convicted of causing cancer on little more than guilt by association. The authors state that, "Most cancer genes have been identified and initially reported on the basis of genetic evidence (that is, the presence of somatic or germline mutations) and without biological information supporting the oncogenic effects of the mutations. The underlying rationale for interpreting a mutated gene as causal in cancer development is that the number and pattern of mutations in the gene are highly unlikely to be attributable to chance. So, in the absence of alternative plausible explanations, the mutations are likely to have been selected because they confer a growth advantage on the cell population from which the cancer has developed" [19].

However, according to Cairns one of the problems with gene mutation causing cancer is that most mutations lead to loss of functions, rather than creation of new function [6]. Indeed, it is well documented that direct evidence for mutation in one or several genes transforming normal human cells into cancer is scant to non-existent [11,15–17,29]. Therefore, in the absence of functional evidence, it is not at all likely that mutations in cancer genes "have been selected because they confer a growth advantage". A more likely explanation for the mutations cataloged in the Futreal et al. census of 291 cancer genes is that they are innocuous, hence more readily accommodated as aneuploid cancer cells compete for survival in competition with normal, euploid cells.

In short, the most that can be said after a quarter-century of effort is that, "in the absence of alternative plausible explanations", gene mutation *may* confer a growth advantage on cells from which cancer has developed. Until convincing functional evidence is produced that one or more mutations can transform normal diploid human cells into cancer, then the 291 "cancer genes" remain hypothetical.

2. Aneuploidy theory provides the "alternative plausible" explanation of cancer

The hundred-year-old aneuploidy theory provides a more comprehensive, productive, and satisfying explanation of carcinogenesis than gene mutation. Over a century ago David Hansemann observed asymmetric mitoses in all of the epithelial cancers he examined [22]. This led him to the hypothesis that, "The cell of the malignant tumor is a cell with a certain abnormal chromatin content". Theodor Boveri agreed with Hansemann. Boveri said that while the normal cell is a product of the particular interaction of the balanced complement of chromosomes, malignant cells resulted from the uneven distribution of the qualitatively different chromosomes. The essence of cancer, he said, was not the abnormal mitosis itself, but rather "a certain abnormal chromatin constitution, the way in which it originates having no significance. Each process which brings about this chromatin constitution, would result in the origin of a malignant tumor" [5,33,34,43].

Boveri used non-mutagenic methods, such as dispermic sea urchin eggs or mechanical agitation of cells in culture, to produce multipolar mitoses [4] which led to an uneven and variable distribution of chromosomes, which in turn produced a myriad of irreversible morphologically and metabolically abnormal phenotypes. Due to intrinsic error propagation, chromosomal imbalance was irreversible and progressive once generated, eventually leading to what he described as tumors. Hansemann's asymmetric mitoses represent the visible manifestation of the autocatalyzed progression of aneuploidy during cell division [37]. Consistent with Hansemann and Boveri, our hypothesis is simply stated: the autocatalyzed progression of aneuploidy *is* carcinogenesis [10–12,30,37–39].

It is well documented that the normal human complement of exactly two copies each of 23 distinct chromosomes is very stable and does not readily become aneuploid *in vivo* or *in vitro* [1]. The initiation step of carcinogenesis is the production of near-diploid aneuploid cells that appear morphologically normal and almost always go undetected. Thinking only of "gatekeeper", "caretaker" and "checkpoint" genes [27,41], proponents of gene mutation ask: how is this aneuploidy initiated? Viable aneuploid cells are caused by exposure to radiation, chemical and physical (e.g. asbestos) carcinogens, and mitotic accidents. In other words, carcinogens are aneuploidogens [10,11,13–15, 29,30,44]. This explains why half of all carcinogens are not mutagenic – because it is the aneuploidogenic potential of these agents that counts. In general, substances and processes that interfere with normal cell division, which unfortunately includes many types of chemotherapy, are probably carcinogenic to some degree. The depressing regularity of relapse and the appearance of unrelated new cancers following the completion of chemotherapy and radiation are due in part to their power to cause aneuploidy [8,20,23].

As Boveri pointed out, aneuploidy interferes with normal cell division. The asymmetry caused by aneuploidy represents the real "check-point" of cell division by disrupting the balance of forces required for anaphase [9]. However, if enough near-diploid cells persist, eventually some will undergo tetraploidization by one of the mechanisms described by Oksala and Therman [36]. Tetraploidization produces the large, morphologically abnormal nuclei that pathologists can detect. Since doubling the number of chromosomes preserves the chromosomal imbalance of the near-diploid cells, the newly formed near-tetraploid cells retain the viability of their near-diploid precursors. However, with each cell division, the genetically unstable near-tetraploid cells lose chromosomes, undergo chromosomal breaks, fusions, and tend to die out in increasingly large numbers as the aneuploid cells chaotically march towards the equilibrium DNA index of 1.7 that is typical of mature cancer cells [38].

3. Phenotypic transformation analysis supports the aneuploidy theory of cancer

The commonly held view that cancer is caused by the mutation of a few specific genes [32] derives from the widespread assumption that complex pathways must have rate-determining, rate-controlling, or rate-limiting steps; a view that has dominated biochemical kinetics for nearly a century. The search for oncogenes and tumor suppressor genes, then, is simply the search for the rate-determining molecular steps in carcinogenesis. However, the past 25-years of applying metabolic

control analysis (MCA) to glycolysis, the tricarboxylic acid cycle, photosynthesis, and the syntheses of fatty acids, urea, nucleotides, and amino acids has conclusively shown that complex systems are not controlled by slow or rate-determining steps [18,24].

The results and insights of MCA make clear that in order to understand the genesis and evolution of complex phenotypes, one must go beyond simply constructing genetic roadmaps [7]. An analysis of the dynamics of the traffic along the pathways is essential. While mechanisms such as cooperative feedback inhibition are still addressed by MCA, they are given less emphasis than in classical studies of metabolic regulation. One of the fundamental discoveries of MCA is that even with a complete knowledge of the detailed properties of specific genes and gene products it is not possible to either predict or describe the phenotypes of complex biological systems in terms of a few individual genetic components [21,25]. This result applies with equal force to gene mutations. Therefore, alterations in a handful of "gatekeeper" or "caretaker" or "checkpoint" genes are likely insufficient – if not irrelevant – for the generation of cancer-specific phenotypes, since their numbers are too few to alter the normal phenotype, and since there is as yet no independent evidence that they are exceptionally pleiotropic [39].

While molecular biology focuses on specific genes, MCA, on the other hand, analyzes the connectedness of all metabolic components. MCA has been shown to accurately describe metabolic schemes of any complexity. In practice, however, MCA is limited to systems of 50 or so components. Phenotypic transformation analysis (PTA) is an adaptation that bypasses the practical limitations of MCA and allows for the global analysis of the whole cell, tissue or organ [39]. The results of PTA confirm the growing awareness that it is the fraction of the genome, ϕ, undergoing differential expression – not the magnitude, π, of the differential expression – that controls phenotypic transformation [3,39,40]. Furthermore, transforming the robust normal phenotype into cancer requires an average 2-fold increase in the expression of thousands of normal gene products [39]. Thus, the change in gene dose of large fractions of the genome caused by aneuploidy produces highly non-linear (i.e. qualitative) changes in the physiology and metabolism of cells and tissues [31]. There are three general scenarios of how a change in gene dose in decreasing fractions, ϕ, of the genome affects the phenotype of a cell [39]:

(1) For $\phi = 1$, the genome remains balanced regardless of changes in dose since there is a linear relationship between the total metabolic output, F, and the DNA content of a cell. This accounts for the rare occurrence of live births of triploid and tetraploid infants, in which every cell has exactly 3 or exactly 4 copies, respectively, of each of the 23 distinct chromosomes.
(2) For $\phi < 1$, the genome is aneuploid. The effect aneuploidy has on the metabolism of a cell becomes increasingly non-linear as the imbalance between the metabolic output, F_a, of an aneuploid cell and its DNA content increases.
(3) When $\phi \ll 1$, changes in gene dose have little discernable effect on the metabolism of a cell and have no effect at all on its morphology and other physical properties. Examples are Down's syndrome and the 7 mutant genes thought to cause colon cancer. PTA predicts that on average cells with a gain in gene dose should have a survival advantage over those experiencing a loss. This phenomenon, which has been experimentally confirmed [31], contributes to the autocatalyzed progression of aneuploidy.

The imbalance between the phenotype and genotype of aneuploid cells is quantifiable. A measure of this imbalance is the relative aneuploid flux, F_a, divided by the DNA index [39]. Since cancer-specific genetic instability has been shown to be proportional to the degree of genetic imbalance of aneuploid cells [12,28], the ratio of the aneuploid flux, F_a, to the DNA index defines the flux stability index, S_F [39]. A plot of S_F versus DNA index predicts that the most genetically unstable cells have a DNA index exactly halfway between the near diploid or pseudo-diploid and near tetraploid or pseudo-tetraploid values, i.e. DNA index $= 1.5$. The experimentally observed order of genetic instability for a series of colon cancer cell lines has confirmed the predictions of the flux stability index function [39].

To me, the most significant failure of the gene mutation hypothesis of cancer is its complete inability to explain the kinetics of carcinogenesis. The differential equation of PTA used to model the autocatalyzed progression of aneuploid cells during cell division [37] was integrated in order to analyze the time–course data of Armitage and Doll [2] for a number of cancers. The resulting sigmoidal equation produces a good fit to the sigmoidal time–course data for all the human cancers [37]. The 7-gene mutation hypothesis [26,35], on the other hand, produces a curve that is parabolic upwards and, more importantly, fails to explain the time course of human cancers.

4. Conclusion

Aneuploidy theory provides the "alternative plausible" explanation of cancer, independent of gene mutation. Aneuploidy theory and phenotypic transformation analysis explain the gross biochemical abnormalities, abnormal cellular size and morphology of cancer cells, as well as the appearance of tumor-associated antigens, high levels of secreted proteins responsible for invasiveness and loss of contact inhibition, tumor formation, genetic instability leading to rapid appearance of drug resistance, the Hayflick limit of cultured cells, the time-course of human cancers, carcinogen-induced tumors in mice, the absence of immune surveillance, and the failure of chemotherapy [11,37–39].

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CHROMOSOME ROSETTES, INTERCHROMOSOMAL TETHERS AND THE ORIGIN OF ANEUPLOIDY

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Abstract. Much evidence now suggests that aneuploidy is required for the initiation and progression of cancer. How cells acquire the additional chromosomes to achieve aneuploidy and how this new aneuploid state is successfully propagated in cancer cells in growing tumors are key questions. Previous studies have shown that, during mitosis of normal and cancer cells, all chromosomes are always incorporated into a single, wheel-like array called a rosette, raising the possibility that rosettes may play a role in transmission of the aneuploid state to subsequent cell generations through mitosis. Here, we have used time-lapse cinematography to track the movements of individual chromosomes in living cells as well as fluorescence *in situ* hybridization (FISH) and micromanipulation to study the dynamic aspects of rosette assembly. Results suggest that chromosomes are tandemly attached to one another throughout the cell cycle at the level of their centromeres by an elastic tether. We propose that this tether plays a major role in maintaining mitotic fidelity in normal cells and telomerase-positive, aneuploid cancer cells. It also provides a driving force for the observed microtubule-independent, organized chromosome congression as well as for the proper segregation of chromosomes that may not have successfully attached to spindle fibers via their kinetochores.

Keywords: mitosis, aneuploidy, cancer, micromanipulation, chromosome, segregation.

1. Introduction

1.1. Cancer and aneuploidy

Cancer remains a major cause of death worldwide. Despite advancements in diagnosis and treatment, the overall survival rate has not improved significantly in the last two decades. An obstacle to eventually eradicating this disease is the fact that we understand very little about how cancer cells originate from their normal counterparts. Current thought posits that cancer cells arise from the clonal evolution and natural selection of genetically unstable cells that acquire increasingly aggressive and proliferative behaviors [13]. Recent work has emphasized the key (possibly requisite) role that aneuploidy plays in (1) initiating cancer and (2) progressively destabilizing the karyotype, thereby facilitating the continued evolution of abnormal genotypes and phenotypes during cancer progression [1,2, 8,10,12]. The underlying mechanisms associated with the establishment of the initial aneuploidy, the temporal association of this event with aging, and the evolution and selective propagation of additional viable aneuploid cell types remain unresolved, but it is clear that these events are at the heart of cancer genesis and progression.

1.2. Chromosome rosettes in normal and cancer cells

For the past several years our interest in mechanisms associated with the initiation of cancer have focused on how cells acquire additional chromosomes to achieve aneuploidy and how this new state of aneuploidy is successfully propagated from one cell generation to the next. We believe that an important clue comes from the peculiar arrangement of chromosomes which becomes apparent during cell division. In cells from all organisms that have been examined thus far (human, rodent, avian, amphibian, fish, sea urchin and plants) chromosomes invariably arrange themselves by the end of prometaphase into a default, nearly planar, wheel-like configuration called a mitotic rosette (Fig. 1A–B) [7–10]. Within rosettes, centromeres of chromosomes are tightly packed into a circular array, forming a central "hub", while chromosome arms project radially from this hub (Fig. 1B). In cells with smaller chromosomes, such as human and rodent cells, the wheel-like chromosome arrangement is particularly striking. Cells with very large chromosomes (e.g., newt lung cells) also form rosettes but the ends of these unusually long chromosomes are often folded or intertwined, often masking their inherent arrangement into a rosette. In cultured cells, chromosome rosettes are oriented upright (i.e., perpendicular to the plane of the substratum), such that they appear as a bar or plate when viewed from above – hence the historical misnomer "metaphase plate". Assembly of the rosette occurs rapidly (within 1–2 min) following dissolution of the nuclear envelope occuring at the end of prometaphase, and this configuration persists throughout the remain-

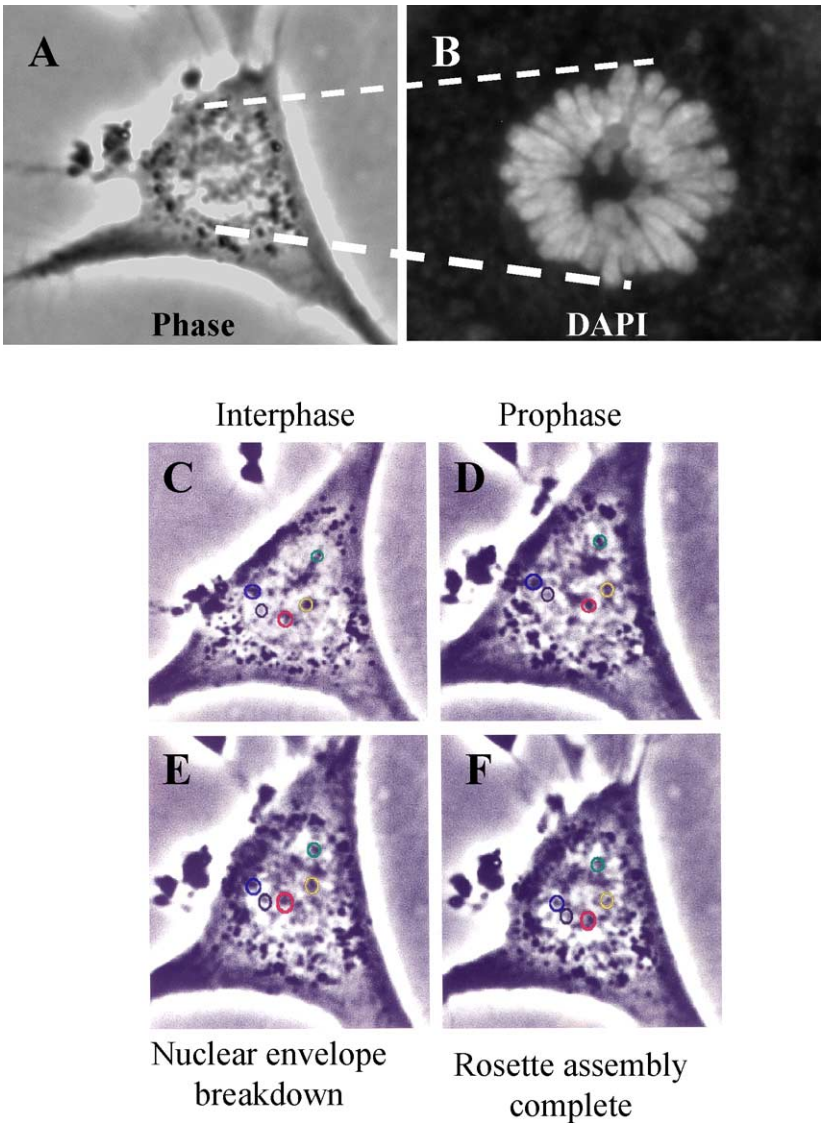

Fig. 1. (A) Phase-contrast micrograph of a normal human diploid fibroblast cell at the end of prometaphase. The cell is still somewhat flattened on the substratum, the nuclear envelope has recently disassembled and all chromosomes are arranged into a nearly planar, wheel-like chromosome rosette. (B) Staining of the chromosomes of the same cell shown in (A) with DAPI reveals the central hub of the rosette and the chromosome arms projecting radially from this hub. (C–D) Tracking of sequential movements of identified (color rings) centromeres within the nucleus of the same cell from S phase through completion of rosette assembly reveals that relative chromosome positions remain essentially unchanged during this interval.

der of mitosis and can still be observed in daughter cells immediately following cytokinesis, prior to assembly of the new nuclear envelope [7–9]. In mitotic normal diploid human cells, all chromosomes are always incorporated into a single rosette, and there is a remarkable tendency for chromosome homologs to be positioned on opposite sides of the rosettes and for heterologs to exhibit reproducible spatial interrelationships [7,11]. However, this relationship can be altered or even lost in some normal diploid cells in long-term cultures and in cancer cells. Analyses of diploid, triploid and tetraploid cells has shown that, regardless of how many chromosomes a cell possesses, all of them are always incorporated into a single rosette which appears to be composed of tandemly linked haploid sets [8]. The functional significance of the chromosome rosette and its remarkable chromosome distribution pattern is not known, but its apparent ubiq-

uity and conservation among human cells attests to its likely importance in the fidelity of chromosome congression and segregation, and possibly in the maintenance of proper relative positioning of chromosomes at interphase, at a time when they are transcriptionally active [9].

Importantly, as in normal cells, all chromosomes in mitotic cancer cells are always incorporated into a single aneuploid rosette, suggesting that the rosette may play a pivotal role in the successful transmission of this aneuploid state to succeeding cell generations through mitosis. In the aneuploid rosettes of mitotic cancer cells, the relative spatial positioning of chromosome homologs is often variable across the entire cell population, but tends to be similar among recently divided daughter cells. How and when additional chromosomes are inserted into rosettes to form these new aneuploid configurations, how chromosomes are so firmly held into the rosette configuration and the role of progressive telomere shortening and chromosome end fusion in driving this process are questions that lie at the foundation of cancer cell evolution. In this report, we have employed time-lapse cinematography, fluorescence *in situ* hybridization (FISH) and micromanipulation to study dynamic aspects of rosette assembly. Our results strongly suggest that chromosomes are tandemly attached to one another throughout the cell cycle at the level of their centromeres by a structure that appears to function as an elastic tether. We propose that this tether plays a key, failsafe role in maintaining mitotic fidelity of both normal cells and telomerase-positive, aneuploid cancer cells by providing the driving forces for microtubule-independent chromosome congression to the "metaphase plate" and by serving as a failsafe system for chromosome segregation in the event of occasional failed spindle fiber attachment to kinetochores.

2. Results

2.1. Tracking individual chromosome movements using time-lapse cinematography/FISH

We have carried out a correlated time-lapse cinematography/FISH study in an effort to reconstruct the sequence of chromosome movements associated with formation of the chromosome rosette. In phase-contrast images of living cells just prior to (or engaged in) mitosis, highly condensed regions of human chromosomes (e.g., subsequently identified using FISH as centromeric subdomains) appear as phase-dense (dark) spots (Fig. 1C–D). Sequential movements of centromeres within nuclei of living cultured human cells can be followed throughout interphase up to the end of prometaphase in successive photographs. Upon dissolution of the nuclear envelope, signaling the onset of prometaphase, centromeres begin to rapidly coalesce into a roughly circular array (Fig. 1E) which undergoes continued compaction to form the wheel-like chromosome rosette by the end of prometaphase (Fig. 1F). Tracking individual centromere (see colored circles) movements from S phase through completion of rosette assembly shows that relative chromosome positions remain essentially unchanged during this interval. This argues against the idea that chromosomes move individually and randomly during their congression and documents the maintenance of a close relationship between relative chromosome positions at interphase and their positions within mitotic rosettes (Figs 1C, 1F) [9,12]. The coordinated nature of these movements among adjacent centromeres strongly suggests the presence of centromeric interconnections during these movements. The fact that premitotic chromosome movements, as evidenced by aggregation of their centromeres into tightly packed linear centromere arrays, occur within nuclei prior to dissolution of the nuclear envelope, indicates that these movements are microtubule-independent. Indeed, none of the chromosome movements up to and including chromosome rosette assembly (i.e., premitotic movements of chromosomes prior to nuclear envelope breakdown, centromere coalescence into linear centromere arrays or assembly of chromosome rosettes) are inhibited by colcemid or nocodozole.

2.2. Micromanipulation reveals the presence of elastic centromeric interconnections

Following earlier reports by Maniotis et al. [4], we have used microsurgery and micromanipulation to test the nature and physical features of the connections that hold adjacent chromosomes together within chromosome rosettes (Fig. 2). Pulling on chromosomes situated within the rosettes of living human fibroblasts with a microneedle (★) results in the sequential removal of all remaining chromosomes (arrows) that appear to remain attached to one another in tandem by an elastic tether (Figs 2A–E). Surprisingly, once the last chromosome is released from the cell, the elastic tether between adjacent chromosomes apparently recoils and chromosomes quickly re-assemble themselves into a

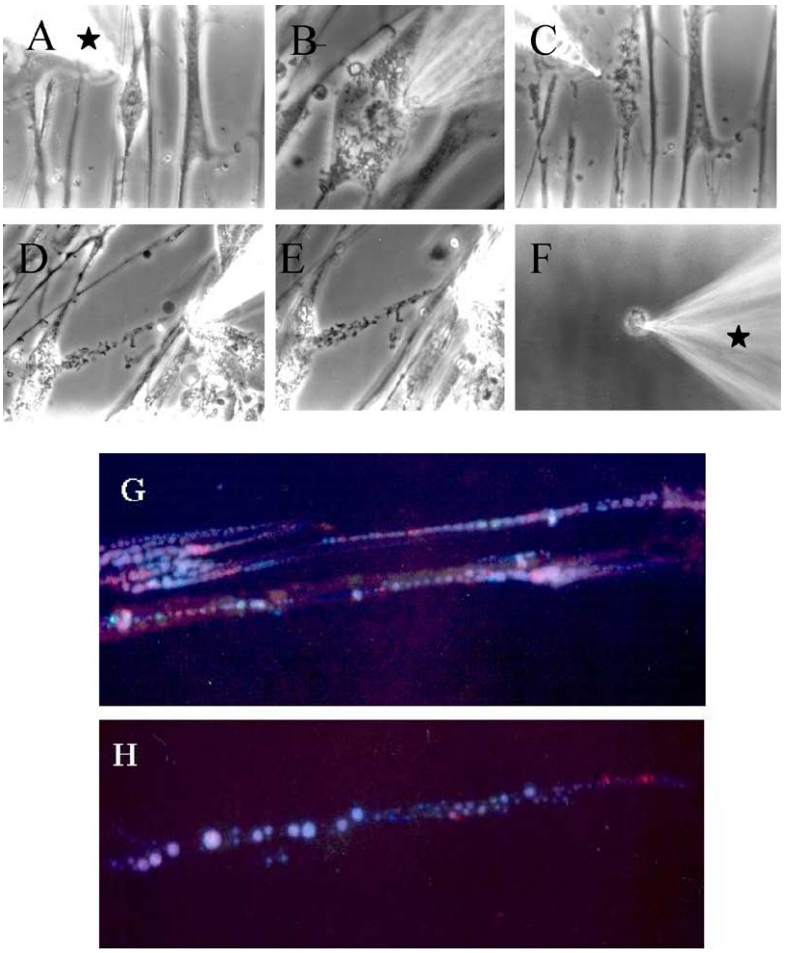

Fig. 2. (A–F) A series of photographs illustrating the steps involved in removal of chromosomes from a living human fibroblast. Pulling on individual chromosomes results in the sequential removal of all chromosomes that appear to be interconnected to one another in tandem by an elastic thread which we have called a tether. Release of the last chromosomes from the cell results in recoiling of the extracted chromsomes back into a "rosette configuration" at the tip of the microprobe. (G–H) Isolated mitotic rosettes were subjected to gentle homogenation, cytospun and exposed to unidirectional fluid shear forces. Under these conditions, ruptured chromosome rosettes formed long, linear arrays of tandemly interconnected chromosomes.

"rosette configuration" at the tip of the microprobe (Fig. 2F). This leads us to suggest that the driving forces for chromosome congression to form the rosette configuration may originate from the inherent elasticity and/or contraction of interchromosomal tethers that interconnect adjacent centromeric subdomains. As a further test for the presence of tethers, mitotic rosettes were isolated from cells and subjected to gentle homogenation with the aim of breaking interchromosomal tethers. When these rosette homogenates were cytospun and exposed to unidirectional fluid shear forces, long, linear arrays of attached chromosomes, corresponding to linearized rosettes were observed (Figs 2G–H). FISH using chromosome-specific probes confirmed that the "beads on the string" were individual chromosomes. Together, these results strongly suggest that (1) chromosomes are interconnected in tandem at the level of their centromeres to form a rosette configuration and (2) the physical basis for this association is an elastic thread which we refer to as an interchromosomal tether. Studies are currently underway to determine the structure, function and composition of these tethers.

3. Discussion

The functional significance of the chromosome rosette configuration and the observed conservation of chromosome spatial order within them is not known,

but several obvious possibilities present themselves. First, it is conceivable that an interconnected, ordered chromosome array in the form of a rosette helps to maintain the remarkably high degree of accuracy of chromosome segregation during mitosis. For example, if chromosomes are interconnected at metaphase via tethers, such interconnections could conceivably serve as a mitotic failsafe device for occasions when spindle fiber–kinetochore associations are deficient in one or more chromosomes. In this case, tethers would ensure that a chromosome not properly connected to spindle microtubules would still segregate successfully. Second, relative chromosome positioning may be critical for establishing the proper chromosome positional relationships necessary for maintaining normal patterns of gene expression in interphase cells [11,12]. Third, the chromosome rosette may be of clinical significance because mis-segregation of chromosomes during mitosis can cause incorporation of extra chromosomes into rosettes, resulting in a new state of aneuploidy, which appears to be a common, very early step in the multistage process of tumorigenesis [1,2, 13]. Additional indirect evidence supporting the existence of tethers includes the following: (1) the consistency of chromosome position in rosettes and the maintenance of this positional fidelity from one cell generation to the next [3,5,7]; (2) the tandem linkage of intact haploid sets to form single diploid, triploid and tetraploid rosettes [6–9]; (3) the close relationship between chromosome positions in mitotic rosettes and in their corresponding interphase nuclei [3,9]; (4) the fact that chromosome rosettes assemble very rapidly within 1 minute of disolution of the nuclear envelope; (5) the persistant segregation of maternal and paternal chromosomes in rosettes during early embryogenesis [5,7,8]; and (6) the existence of organized premitotic chromosome movements prior to dissolution of the nuclear envelope.

We have shown that, in immortalized cell lines, all chromosomes are always incorporated into a single aneuploid rosette. This observation favors the idea that, during the evolution of cancer cells, a state of stable aneuploidy evolves through natural selection, culminating in a new (but altered) chromosome spatial order. Studies described here strongly suggest that interchromosomal interconnections or tethers are of primary importance for the normal behavior of chromosomes during mitosis and, perhaps, for the creation and surprisingly stable propagation of abnormal karyotypes during proliferation of immortalized cancer cells. How additional chromosomes are inserted into rosettes during the development of aneuploidy is a question that lies at the basis of cancer cell evolution. In telomerase-negative cells approaching M2 crisis, relative chromosome positional order within mitotic rosettes appears to be extremely unstable, whereas it becomes much more stable in telomerase-positive immortalized cells. This observation favors the idea that a state of stable aneuploidy gradually evolves through repeated mitotic mis-segregations (aneuploidizations) and natural selection of telomerase-negative cells, culminating in a new, relatively stable, chromosome positional order in aneuploid cells that have become telomerase-positive and have managed to rejuvenate their telomeres. Relengthening of telomeres in the latter cells would tend to prevent further chromosome end-fusions and subsequent chromosome mis-segregations, thus allowing a more faithful propagation of the most recent aneuploid configuration in the newly immortalized cancer cell.

Acknowledgement

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THE CONTINUUM MODEL OF THE EUKARYOTIC CELL CYCLE: ORIGINS, APPLICATIONS, AND EXPERIMENTAL SUPPORTS

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The Continuum Model of the eukaryotic cell cycle proposes that the regulation of the cell cycle is determined by a continuous accumulation process occurring between the starts of S phases. In contrast, the current and consensus model of the cell cycle proposes that there are numerous sequentially arranged G1-phase controls that regulate passage through the cell cycle. For example, the restriction point, the decision to enter G0, decisions related to growth and division versus arrest and differentiation, as well as numerous G1-phase biochemical events are proposed to occur specifically in the G1-phase. The Continuum Model proposes that there are no unique G1-phase dependent controls.

The origins of the Continuum Model stems from studies on the bacterial cell cycle. Over three decades ago it was observed that the S and G2 phases in bacteria (termed the C and D periods) were invariant as growth rate for a particular cell varied. A similar phenomenon was observed in eukaryotic cells where S and G2 were relatively invariant while the G1 phase varied as growth rate (and interdivision times) varied. The finding of G1-phase-less eukaryotic cells set the stage for a unified view of the cell cycle. This unified view is termed the Continuum Model.

The Continuum Model explains a vast array of experimental data. Thus, the continuum model explains the existence of G1-less cells [1], the variation in interdivision times of exponentially growing cells [2], the pattern of c-myc expression during the cell cycle [3], the proposed G0 phase [4–6], the inability of whole-culture synchronization procedures to actually synchronize cells [6–11] the shortening of the G1 phase by induction of cyclins [12], the pattern of yeast cell growth during the cell cycle [13], the phosphorylation pattern of Rb protein (i.e., the finding of no G1-phase phosphorylation of Rb protein) [14,15], the finding of cells in differentiated tissues with a G1-phase amount of DNA [16], the problems with microarray analysis of gene expression during the cell cycle [17–20], how FACS analysis has affected our understanding of cell-cycle controls and parameters [21], and the determination of cell size as a function of growth rate [22].

The Continuum Model has been the subject of a number of reviews [6,9–11,15,18]. Many of the relevant papers can be read directly at www.umich.edu/∼cooper.

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GENETIC PATHWAYS AND DETERMINANTS OF CLINICAL OUTCOME IN COLORECTAL CANCER

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1. Introduction

Tumor progression is an evolutional process determined by the generation of heterogeneity and the selection of variants most suited to survival, growth and invasion [1]. Although colorectal cancer is one of the best examples of the multistep nature of tumorigenesis, the underlying mechanisms that sustain the evolution of these tumors are mostly unknown, with the exception of tumors with the microsatellite mutator phenotype (MSI) [2]. Since most colorectal cancers progress without MSI, alternative mechanisms have been postulated based on distinctive morphological, biological and genetic features of tumors [3–15]. However, the heterogeneous nature of the experimental approaches used to define the subtypes of colorectal tumors and the diversity of sample collections precludes the establishment of agreed models with potencial applicability.

To add some light to the identification of the genetic pathways of progression in colorectal tumorigenesis, we have explored the interrelationships between specific molecular alterations, genomic disruption profiles, and clinical features in a series of 129 carcinomas collected prospectively. Comprehensive approaches resolving chromosomal and subchromosomal alterations have been used to ascertain the type and degree of chromosomal instability. Multivariate correlates of molecular profiles have enabled us to classify colorectal carcinomas into five groups with distinctive features and determinants of clinical outcome. We also propose that the use of this classification may be useful to better reveal the factors governing the clinical outcome in each group.

2. Material and methods

The genomic damage of 129 sporadic colorectal tumors was analyzed using two different techniques: flow cytometry to measure aneuploidy and AP-PCR to measure allelic gains and losses. Aneuploidy mostly reveals gross numerical changes, whereas the genomic damage measured by AP-PCR (genomic damage fraction, GDF) includes both chromosome copy number variations and simple and complex unbalanced rearrangements [16,17]. To quantify tumor aneuploidy in a comprehensive way, we created a new index (Aneuploidy Index, AI) that considers both, the degree and the extension of aneuploidy in the tumor [18]. The relation between GDF and AI was analyzed as well as their prognostic value. These results together with data regarding microsatellite instability, p53 and k-ras mutations, and clinicopathological characteristics of the patients have been used to classify colorectal carcinomas.

3. Results

39 (30.2%) tumors were diploid and 90 (69.7%) were aneuploid. Both, AI and GDF were related to left location of the tumors; however, high values of AI were associated with advanced Dukes' stage whereas high values of GDF were associated with p53 mutations. For aneuploid tumors, both AI and GDF had prognostic value independent of Dukes' stage. However, there was no correlation between both parameters, what indicates that they are independent. Therefore, the combination of both variables was the best predictor of survival in aneuploid tumors. For diploid tumors it was surprising to find a subset of cases with very bad outcome: those in Dukes' stage C. This indicates that in diploid tumors the acquisition of malignity is related to the invasion of lymph nodes and suggests that diploid tumors may follow a pathway of progression independent of aneuploid tumors.

Taking all the results together, the following five groups (Fig. 1) can be defined based on the type and level of cumulated genomic damage: (1) tumors with microsatellite instability, right location and good prognosis; (2) diploid tumors lacking p53 mutations, left and right location, low subchromosomal damage and bad prognosis; (3) diploid tumors with p53 mutations, left location, high levels of subchromosomal damage and good prognosis; (4) high aneuploid tumors, p53 mutations, left location, high levels of numerical and

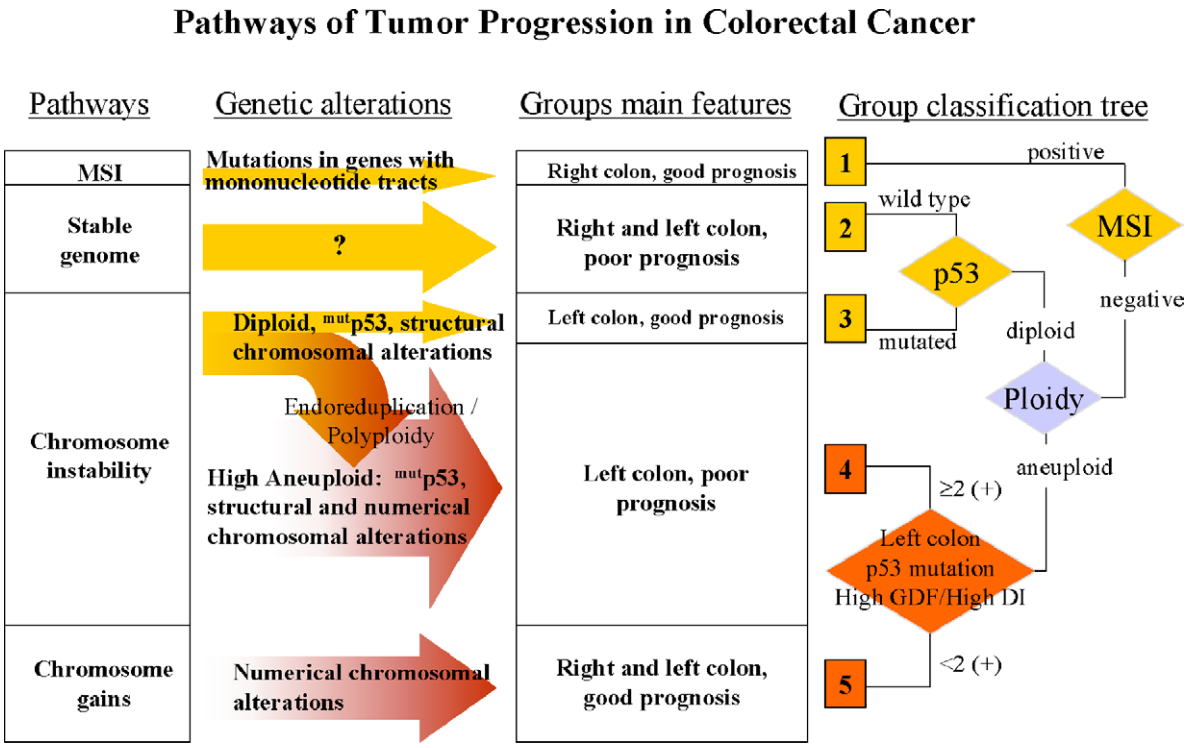

Fig. 1. Scheme of the hypothesized genetic pathways of tumor progression in colorectal cancer based on a model integrating different forms of genomic instability. Each pathway is depicted according to the approximate percentage of cases displaying a given type of genetic damage. The main features of each group and the proposed decision tree used to assign each case to the groups are shown.

structural chromosomal alterations and bad prognosis; and finally (5) low aneuploid tumors, no p53 mutations, left and right location, low levels of structural chromosomal alterations and good prognosis.

4. Discussion

Chromosomal instability has been proposed as the agent responsible for the genomic disruption observed in the majority of colorectal cancer cells [19,20]. Although this is an attractive hypothesis, the nature of this instability (or instabilities) and the underlying mechanism(s) are still a matter of debate. If chromosomal instability drives tumor progression, the type and degree of instability are likely to leave identity marks in the genome of the neoplastic cell. This will result in specific profiles of chromosomal disruption. The use of appropriate methods to detect and/or quantify chromosomal aberrations of a heterogeneous nature is therefore instrumental to the characterization and identification of distinct pathways of tumor progression.

In our setting, ploidy analysis by flow cytometry and detection of allelic imbalances by AP-PCR offer simple and comprehensive approaches to the global analysis of genomic damage resulting in the generation of two estimates of chromosomal instability (AI and GDF). Although they cannot be completely dissected to accurately reflect every type of chromosomal alteration separately and they coexist in some tumors, the statistical analysis of our data show that both types of genomic damage are independent.

Our results taken together with previous investigations advocate a classification of colorectal tumors according to their pattern of genomic disruption. These groups are likely to follow different pathways of tumor progression, which would be characterized by either different types of genomic instability or the accumulation of certain types of genetic alterations even in the absence of instability. Moreover, specific prognostic factors are revealed for each group. Although this classification must be refined with additional genetic analysis, the subtypes of colorectal cancers proposed here are likely to constitute the core of actual pathways of tumor progression. This indicates a need for different prognostic assessments depending on which group the tumor belongs to.

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GENOMIC INSTABILITY AND PROGRESSION TO CANCER IN TWO MODELS OF PRENEOPLASTIC DISEASE: BARRETT'S ESOPHAGUS AND ULCERATIVE COLITIS

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Barrett's esophagus (BE) and ulcerative colitis (UC) are acquired conditions in which chronic inflammation and/or epithelial damage is associated with an elevated risk of adenocarcinoma. It has therefore been suggested that these patients should be followed with endoscopic surveillance, however without knowing which subset of patients is at highest risk, this procedure is not time or cost-effective. The histopathological steps of the progression of BE and UC from metaplasia → indefinite for dysplasia → low grade dysplasia → high grade dysplasia → cancer are well known, however, inter-observer variability in a diagnosis of histology less than high grade dysplasia makes these grades unreliable predictors of disease progression. In order to more accurately assess risk of neoplastic progression, we have studied Ulcerative Colitis with our colleagues at the University of Washington, Drs. Teresa Brentnall and Mary Bronner, and have studied Barrett's esophagus with our colleagues at the Fred Hutchinson Cancer Research Center, Drs. Brian J. Reid and Patricia Blount, and Dr. Robert D. Odze at the Brigham and Women's Hospital. In the search for early and reliable intermediate biomarkers of cancer risk we have identified changes in DNA ploidy as particularly informative.

In Ulcerative Colitis aneuploid DNA contents are commonly found by flow cytometry in association with dysplasia, and multiple overlapping clones of aneuploid cells may occupy large fields of the precancerous colon [1]. The finding of DNA aneuploidy in patients with histologically negative or indefinite ulcerative colitis is predictive of increased risk of progression to cancer [2]. We hypothesized that chromosomal abnormalities that were too subtle to be detected as a DNA content abnormality by flow cytometry might occupy even larger fields in preneoplastic disease, and therefore we examined biopsies for the presence of increased levels of chromosomal instability (CIN) using fluorescence *in situ* hybridization (FISH). Increased levels of CIN were seen is all histologically negative biopsies examined in patients who had focal high grade dysplasia or cancer elsewhere in the colon, and thus, CIN appeared to be pancolonic [3]. Loss of chromosomal arm signals was the earliest change observed, with the highest level of alteration observed as chromosome 17p losses. These changes are, however, non-clonal in nature, and FISH performed even on single colon crypts shows a large heterogeneity of chromosomal abnormalities.

Barrett's esophagus presents a parallel model for study of neoplastic evolution. As in UC, genomic instability in the premalignant esophagus may be evidenced by large numbers of overlapping aneuploid clones [4]. In addition to aneuploidy, flow cytometry can detect elevated tetraploid fractions (% 4N $>$ 6%) in BE. Elevated 4N fractions are correlated with the presence of loss of heterozygosity at the p53 locus, and are predictive of subsequent progression to aneuploidy, which is observed, on average, 17 months after the initial observation of elevated 4N fractions [5]. Cells with elevated 4N fractions may be in either G2 or a tetraploid G1, however in both cases their gene expression profiles are similar and indicate a molecular phenotype of dysregulated G2/M functions and cell cycle checkpoints [6].

Because of the increased risk of cancer in BE patients, a program of endoscopic surveillance is usually recommended. Because most patients will never progress to cancer, it would be helpful to be able to tailor the frequency and intensity of surveillance to the magnitude of cancer risk in an individual patient. For this purpose, intermediate markers of cancer risk are needed [7]. To examine the predictive value of aneuploidy and elevated 4N fractions in BE, we followed a cohort of 322 patients for up to 15 years (1338 patient-years) [8,9]. The presence of aneuploid populations with greater than 1.35 times normal DNA content and the presence of elevated 4N fractions were both predictive of increased cancer risk (RR = 8–10), and the presence of both was highly predictive of cancer (RR = 23, CI = 10–50). Management of the subset of patients without high grade dysplasia is especially difficult, as very few will progress to cancer. In this subset of patients, the presence of either aneuploidy (DNA content greater than 1.35 times normal) or elevated 4N fractions is highly predictive of cancer risk (RR = 25, CI = 6.5–98) [8]. Among patients with negative, indefinite or low grade dysplasia, those with neither 4N fractions nor aneuploidy had a 0% 5-yr cumulative cancer incidence, compared with 28% for those with either of these findings [9].

These observations reinforce the belief that neoplastic progression in UC and BE is facilitated by an un-

derlying process of chromosomal and genetic instability, with aneuploidy as an overt manifestation. To determine whether telomere shortening might be a contributing factor in such instability, we examined telomere lengths by fluorescence *in situ* hybridization. In both BE and UC, chromosomal instability and telomere shortening are present in large fields of histologically non-dysplastic mucosa in early stages of disease, and the extent of telomere shortening is correlated with the degree of chromosomal instability detected by FISH. In UC, chromosomal arm and centromere losses, but not gains, are highly correlated with telomere shortening ($p \leqslant 0.001$ and $p = 0.001$, respectively) [10]. Frequencies of chromosomal losses are greater and telomeres are shorter in the nondysplastic mucosa from UC patients with dysplasia or cancer elsewhere in their colon, as compared to chronic UC patients who have never had dysplasia [10]. In UC the extent of chromosomal instability and telomere shortening is also related to the frequency of anaphase bridges, suggesting that telomere shortening contributes to chromosomal instability by promoting bridge-breakage-fusion cycles. Therefore, telomere attrition and chromosomal instability in these diseases may be early events in a cascade that leads to DNA content abnormalities, loss of tumor suppressors, and, ultimately, cancer.

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BIOLOGICAL AND CLINICAL SIGNIFICANCE OF NONRANDOM ANEUPLOIDY PATTERNS IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

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Childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) comprises a heterogeneous group of diseases with distinct clinically and biologically relevant genetic features, which were originally classified according to chromosome number and ploidy levels (Table 1). The vast majority of specific reciprocal rearrangements that were subsequently identified with cytogenetic and further characterized with molecular genetic means are typically found in the (pseudo)diploid category. The aneuploid groups, on the other hand, are characterized by mainly numerical changes and only few, less specific structural rearrangements [1–11].

Table 1
Classification of BCP-ALL according to the ploidy level

Ploidy level	Chromosome number	Frequency	Prognostic relevance
Near-haploid	23–29	<1%	bad
Hypodiploid	30–44	1%	bad
(Pseudo)diploid	46	65–70%	bad to very good*
Hyperdiploid	50–65	25–30%	very good
Near-triploid	66–73	<1%	good to moderate
Near-tetraploid	82–94	1%	moderate to bad

*The specific type of reciprocal rearrangement determines prognosis.

Despite their common occurrence, however, next to nothing is known about the mode of generation, the role and the contribution of whole chromosome changes to the neoplastic transformation process. This is mainly due to the lack of attractive and testable hypotheses as regards the potential deregulative effects of single or multiple chromosome copy number deviations on the genome-wide gene expression levels, but also on the difficulty to approach this problem experimentally. The development of novel investigative tools, such as various molecular genetic, FISH and gene array techniques, that enable the evaluation of tens of thousands of genes simultaneously, will eventually help to shed some light on the pathogenetic significance and biological consequences of these intriguing karyotype patterns [12]. My contribution will thus provide a brief overview about what is known so far about these genetic alterations in childhood ALL.

1. Near-haploid ALL

Near haploid cases are extremely rare and are prognostically unfavorable [2,8,10,13]. They contain at least the equivalent of a complete haploid set of chromosomes, two sex chromosomes and, in most instances, also two copies of chromosomes 10, 14, 18 and 21. Haploid clones commonly coexist with hyperdiploid ones, which then contain the duplicated chromosome complement [2,8,10,13]. Whether pure haploid leukemia exist, must therefore remain open.

2. Hyperdiploid ALL

With an incidence of approximately 25% to 30%, hyperdiploidy is one of the most common abnormalities in childhood ALL [7,11,14]. By definition, the chromosome number ranges from 50 to 65 with a mean peak at 55. The intriguing peculiarities of these leukemia are the nearly exclusive presence of nonrandom trisomies of chromosomes 4, 6, 10, 14, 17, 18, 20 and X, whereas chromosomes 1, 2, 3, 12 and 16 virtually always remain disomic [5,7,9,11]. Chromosome 21, on the other hand, is generally present in four and sometimes five copies.

Hyperdiploidy is generally considered as prognostically favorable, although up to 20% of affected children still relapse [1,4,9,11]. The statistical evaluation of a large number of such hyperdiploid karyotypes revealed that a combination of trisomies 4, 10 and 17 seems to be especially advantageous [9]. Based on the parameters age, sex, and the presence or absence of trisomy 4 and 18, Moorman et al. were able to further subdivide hyperdiploid cases into three different risk groups [11].

Occasionally, hyperdiploidy can also result from the duplication of a near-haploid clone [2,8,10,13]. Interestingly, these cases have a similarly poor prognostic outlook as their near-haploid counterparts. The two types of hyperdiploidy can be easily distinguished, however, because haploid and hyperdiploid clones usually coexist. Moreover, the duplicated hyperdiploid clones contain two or four copies of each chromosomes, but lack the characteristic trisomies that are present in the more common other form of hyperdiploidy.

3. Near-triploid and near-tetraploid ALL

With an incidence of approximately 1%, ALL cases in the near-triploid and near-tetraploid range are extremely rare. They seem to result from mitotic errors that differ from those, which generate the above described hyperdiploidy and, therefore, represent most likely a distinct biological subset with a poor outcome [3,7].

4. On the formation of hyperdiploid karyotypes

The mechanism, which leads to the increased number and the selection of particular chromosomes in hyperdiploid ALL, remains largely unknown. Theoretically, the following four possibilities could account for its formation [13,15–17]:

- The doubling of a near-haploid set of chromosomes, which would result in a widespread loss of heterozygosity (LOH) due to the generation of uniparental disomies (UPD);
- An initial tetraploidization with subsequent chromosome losses, which would result in LOH for approximately one third of the chromosomes and equal dosages on tetrasomic chromosomes;
- The sequential gain of individual chromosomes during consecutive cell divisions, which would result in unequal allele dosages for two thirds of the tetrasomic chromosomes;
- And, finally, the simultaneous gain of chromosomes in a single abnormal mitosis, which would result in equal dosages of tetrasomic chromosomes.

The currently available molecular genetic data indicate that hyperdiploidy usually arises from a diploid karyotype through a simultaneous nondisjunction error at a single abnormal cell division or, as already mentioned above, the duplication of a near-haploid karyotype [13,16,17]. This notion is further corroborated by the fact that, in the vast majority of cases, tetrasomy 21 results from a duplication of both homologs. Nevertheless, the possibility of sequential gains can also not be excluded with certainty so far [17].

In hyperdiploid forms of ALL, the chromosome number as well as their combinations may vary from patient to patient [1,4,5,7,9,11]. However, once formed, the abnormal karyotype is uniform and remarkable stable in the malignant cell population of an individual patient. Taking these observations into account, Heerema and coworkers investigated in which order the chromosome acquisition may occur by analyzing which chromosome are present in karyotypes with a particular modal chromosomes number [14]. They found that chromosomes 21 and X are present in virtually all cases. Karyotypes with a modal number of 52–54 chromosomes gained extra copies of chromosomes 14, 6, 4, 18, 17 and 10 in a consecutive order, whereas those with a modal number of 56–60 chromosomes had chromosomes 8, 5, 12 and 11 in addition. No other chromosomes are consistently present until a modal number of 68 chromosomes is reached, when nearly all chromosomes are encountered. Chromosomes 1, 2, 3, 7, 9, 13, 15, 16, 19, 20, 22 and Y become consistently trisomic only in karyotypes with a modal chromosome number of 68.

Based on a suggestion that imprinting phenomena may be instrumental in the chromosomal selection process [18], Paulsson et al. studied the parental origin of the supernumerary chromosomes in 10 hyperdiploid cases [17]. However, except for trisomy 8, which was of paternal origin in 8 of 8 patients, and trisomy 14, which was of maternal origin in 7 of 8 cases, they found no preferential duplication of maternally or paternally-derived alleles [17].

5. Genome-wide epigenetic deregulation rather than specific gene mutations as a possible pathogenetic factor in hyperdiploid ALL

The vast majority of childhood neoplasms, including various types of acute leukemia, are already initiated *in utero* during a period, when the delicate balance between growth, development and differentiation of the early fetal organogenesis is disrupted. In line with this notion, Wassermann et al. provided the first circumstantial evidence that ALL is initiated already very early during B-cell development [19]. More recently, it was then proven that the potentially leukemogenic nondisjunction event actually takes place also during the same period and, therefore, must be a very early leukemogenic event [20].

Epigenetic disturbances, such as those that result from abnormal DNA-methylation or chromosome missegregation, are the earliest and most ubiquitous changes, which precede and concur with malignant transformation. Considering the common occurrence of premalignant neoplasms in very young children that still can regress or differentiate, an epigenetic first step in these diseases is therefore a particularly appealing

one. In hyperdiploid and haploid ALL, this possibility is backed up by the general lack of gross structural chromosome abnormalities. In these cases, the tissue-specific growth, survival and differentiation control mechanisms may thus be disturbed solely by the unbalanced distribution of particular chromosomes. Trisomies of certain chromosomes may either enhance the proliferation capacity of early lymphoid cells through a change in dosage or relative dosage of a set of genes or in a similar process block differentiation [13,16,18]. The more benign behavior of hyperdiploid ALL versus the more aggressive one of haploid ALL may be explained by the lack of a trans-acting gene expression control mechanisms in the latter. In this context it should be noted, however, that almost 70% of the over-expressed genes in hyperdiploid ALL are located on the chromosome X or 21 [12]. Finally, the extraordinary genetic make-up of hyerdiploid leukemic cells could certainly also explain their rather unique *in vitro* behavior. Since they rapidly undergo apoptosis, it is virtually impossible to propagate them in culture [21, 22]. It therefore comes also of no surprise that not a single cell line has been yet established from such a hyperdiploid ALL.

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INDUCTION OF ANEUPLOIDY AND CHANGES IN REPLICATION PATTERN BY ELECTROMAGNETIC FIELDS

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1. Introduction

Maintenance of genomic stability is essential for the survival of any organism. Multiple mechanisms have evolved to insure homeostasis at the cellular level. A crucial stage in a cell's cycle, at which errors leading to loss of stability occur, is the S phase during which replication of the genetic complement happens (Lucas and Feng, 2003). In order to ensure genomic integrity, DNA replication has to occur in a very ordered manner at a specific portion of time during the S-phase, with the two allelic sequences replicating during the same time domain (Lin et al., 2003). Thus, it is evident that genomic stability depends on the fidelity of DNA replication, DNA repair mechanisms and chromosome segregation. Malfunctions in these processes have been reported to be connected with carcinogenesis and developmental pathologies (Biggins and Walczak, 2003; Nasmyth, 2002; Duesberg and Rasnick, 2001; Korenstein-Ilan et al., 2002).

The specific time interval during DNA synthesis at which a given DNA sequence is being replicated appears to be a reliable indicator of its transcriptional activity. Accordingly, expressed DNA loci undergo early replication, while unexpressed ones replicate late. Epigenetic alterations involving chromatin remodeling have also been implicated in gene activity status as well as in tumorigenesis (methylation: Estler, 2003; and acetylation: Sutherland and Costa, 2003). Thus, determining the replicating status of a sequence opens a window into the orchestration of mechanisms of gene activation and silencing.

The processes of carcinogenesis involve many steps in succession. Two hallmarks of the cancerous phenotype are aneuploidy and changes in gene expression. Thus an attempt has been made to estimate the risk of human exposure to different types of non-ionizing radiation in the environment, by following changes in aneuploidy and replication timing and asynchrony of centromeres following *in vitro* exposure of human lymphocytes.

2. Methods

Fluorescence *In Situ* Hybridization (FISH) for the detection of copy number – aneuploidy – has become an essential tool in the diagnosis and management of a variety of solid tumors and hematologic malignancies in the clinical setting, as well as an aid in the identification of particular genetic disorders and in prenatal diagnosis. After establishing the FISH assay as a reliable tool for the analysis of metaphases, interphase FISH has become the assay of choice for determining the copy number of a sequence in a cell. It affords screening of a large population of cells in a single preparation and enables to easily score various loci simultaneously. Determining aneuploidy using FISH is also amenable to automation, thus saving time as well as bias scoring.

The same cytogenetic preparations can be used to determine the temporal order of replication, based on the FISH replication assay, first described by Selig et al. (1992), which is by now accepted as reliable method for the determination of the mode of allelic replication (reviewed in Korenstein-Ilan et al., 2002). Accordingly, the replication status of a locus is inferred from the shape of the hybridization signal obtained at interphase following FISH with a locus-specific probe. This assay detects the chromatin conformation of the sequence studied. Prior to replication, each identified DNA locus shows a single dot like hybridization signal (singlet; S), while at the end of replication it assumes a doubled bipartite structure (doublet; D). Cells with one singlet and one doublet (SD – Fig. 1a) represent S-phase cells in which only one of the allelic sequences has replicated. Cells with two singlets (SS – Fig. 1b) represent those in which both sequences are unreplicated, and cells with two doublets (DD – Fig. 1c) represent those in which both sequences have replicated. Accordingly, a high frequency of SD cells shows asynchrony in replication timing of the two allelic counterparts; high frequency of DD cells indicates early replication of the identified locus; and high frequency of SS cells points to late replication.

Using two different exposure set ups, we exposed lymphocytes to CW non-ionizing radiation at two different regions of the electromagnetic spectrum, in two specially designed exposure systems. The first expo-

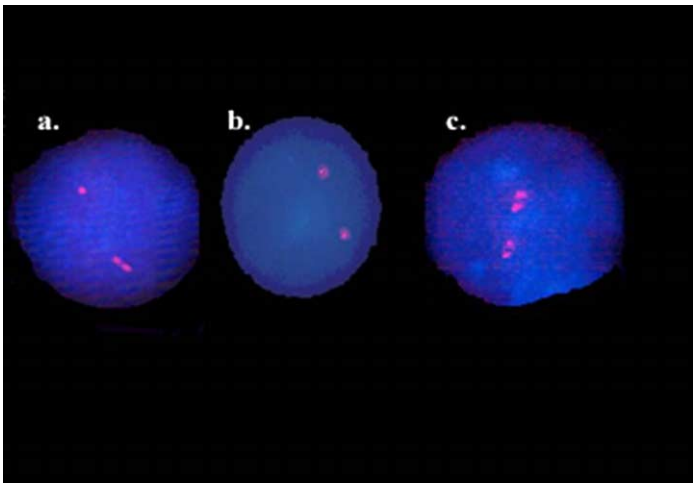

Fig. 1. Analysis of replication timing and asynchrony: (a) a cell in which one sequence has alredy replicated and one has not yet replicated (SD cell); (b) a cell in which neither sequence has replicated (SS cell) – indicating late replication; (c) a cell in which both sequences have already replicated (DD cell) – indicating early replication.

sure system (Fig. 2a) produced radiation of 100 GHz (THz radiation), while the second one (Fig. 2b) produced radiation characteristic for cellular phones of 800 MHz (RF radiation). Human lymphocyte cell cultures were set up and harvested according to standard cytogenetic techniques. For each exposed culture, we had a control sample which was grown in a different incubator and a sham exposed one, which was placed in the same incubator as the exposed sample, but on a different level so that it was not exposed. Cells in the THz set-up, were exposed for one, two or 24 hours. Cells in the RF exposure system were irradiated for either 24 or 72 hours. At the termination of the exposure cultures were removed to the "control incubator" till harvest time, after a total of 72 hours from culture set up.

We scanned slides of nuclei derived from cultures hybridized with probes specific for the centromeric regions of chromosomes 11 (orange labeled; Vysis, USA) and 17 (green labeled; Vysis, USA) using the Metafer platform for semi-automatic interphase FISH scoring. Cells were scored automatically; the gallery was then manually corrected by two independent technicians. Between 800 and 1100 cells were scored from each culture. The Metafer platform automatically presents the results obtained for the levels of chromosomal gains and losses for each locus plus a correlation between the two loci. The subset of cells which had two hybridization signals for both signals, were manually analyzed for the pattern of replication of 600 cells, as described above.

In addition, the scanned files of the slides which were acquired during the search were inspected for metaphases and the number of signal for each of the loci was noted. The proportion of aneuploidy in metaphases was compared with that determined for interphase nuclei.

3. Results

Exposure to radiations at the two different electromagnetic spectral regions conferred increased levels of asynchronous replication which were accompanied by increased levels of aneuploidy in a dose dependent manner. We determined that the THz radiation induced increase in both replication asynchrony and aneuploidy after two hours and 24 hours of exposure, but no effect was observed after one hour radiation. Exposure of cells for 72 hours to RF radiation revealed elevation in both replication asynchrony and aneuploidy. However, exposure for a shorter time of 24 hours showed changes in replication asynchrony and timing only, whereas no alteration in aneuploidy was observed.

4. Discussion

The results obtained demonstrate an increased aneuploidy and elevation of asynchronous replication of centromers following exposure to both THz and RF radiations. The observed effects were dependent on the length of the exposure time, showing a dose response

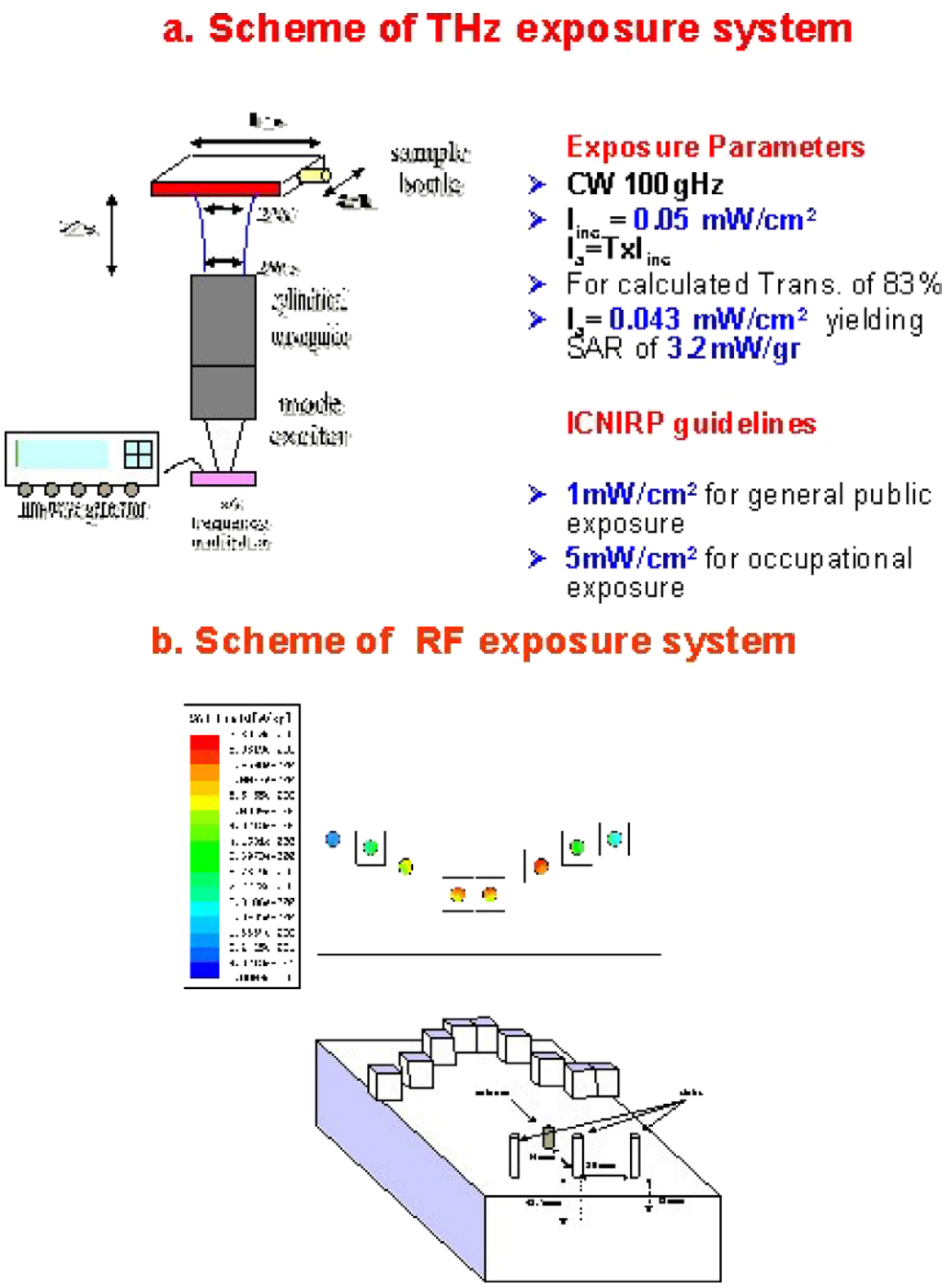

Fig. 2. Schemes of the exposure system used. (a) The exposure system used for the THz frequency and (b) the RF frequency radiation.

relationship. The increased asynchronous replication of centromers was found to be correlated with elevated aneuploidy. It may be suggested that coordinated replication of centromers is necessary for the proper function of the segregating machinery. Moreover it seems that the epigenetic changes involved in replication precede the onset of increase in aneuploidy. These findings suggest that prolonged exposure to non-ionizing radiation increases genomic instability, thus constituting a risk factor for cancer.

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CENTROSOME AMPLIFICATION, CHROMOSOME INSTABILITY, AND KARYOTYPIC CONVERGENCE

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Chromosome instability (CIN), usually associated with mitotic defects, plays a critical role in tumor progression via promoting mutations responsible for a series of malignant phenotypes. Abnormal amplification of centrosomes has been shown to be a major cause of mitotic defects and CIN in cancer cells [1]. The centrosome is a non-membranous organelle, composed of a pair of centrioles and a number of different proteins surrounding the centriole pair (PCM) (Fig. 1A). During mitosis, two centrosomes generated from semi-conservative duplication, form spindle poles and direct formation of bipolar mitotic spindles (Fig. 1B, a), which is an essential event for accurate chromosome transmission to daughter cells. The presence of more than two centrosomes (centrosome amplification) will disrupt formation of bipolar spindles (Fig. 1B, b & c), leading to an increased frequency of chromosome transmission errors [2]. There are several mechanisms responsible for centrosome amplification, including uncontrolled duplication of centrosomes, improper splitting of centriole pairs and cytokinesis block followed by progression into the next cell cycle [2]. Genotoxic insults such as irradiation and exposure to certain chemotherapeutic agents induce centrosome amplification both directly by uncoupling centrosome duplication and cell cycle progression and indirectly by inducing genetic alterations of certain genes whose products are involved in numeral homeostasis of centrosomes. One of such genes is p53: loss or mutational inactivation of p53 results in centrosome amplification [3]. Interestingly, many of advanced tumors as well as cultured tumor cells which lack functional p53 show extensive aneuploidy, yet the altered chromosome makeup is faithfully transmitted to daughter cells, hence they are chromosomally stable. Moreover, many of these cells contain normal number of centrosomes. It has been hypothesized that during tumor progression, cells acquire a CIN phenotype (i.e., centrosome amplification) in an early stage, resulting in a karyotypically heterogeneous population. Among these cells, one or few acquire chromosome compositions that promise the best growth properties under a given environment, which gradually dominate the population. For this population of cells, maintenance of this particular karyotype becomes a primary selection pressure, forcing the selection of the offspring which has acquired mutation(s) that counteracts the CIN phenotype (i.e., restoring normal centrosome profiles). We have simulated this "karyotypic convergence" model during tumor progression in culture [4]. Embryonic epithelial cells from 8-week-old male mice lacking p53 (p53$-/-$ MEEs) were passaged for 50 passages (p50). Every 5 passages, cells were examined for chromosome number by direct counting of metaphase spreads, and centrosome profiles by immunostaining γ-tubulin (Fig. 2). In early passages, the number of chromosomes extensively deviated from the norm ($4N = 80$), indicating that chromosomes were unstable in early passage p53$-/-$ MEEs. However, at $\sim$p30, the distribution of the chromosome number per cell became narrower. At p50, particular populations of cells were dominant in the culture: $\sim$60% of cells contain 90–94

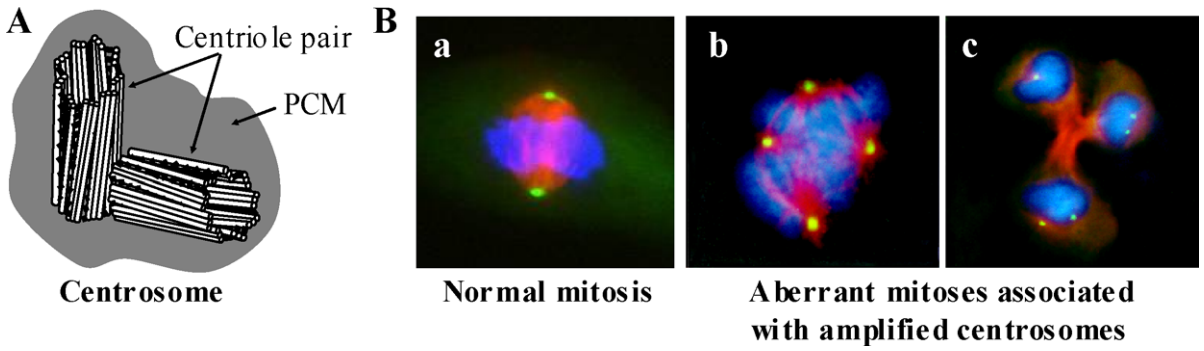

Fig. 1. Centrosome structure and abnormal mitosis caused by amplified centrosomes.

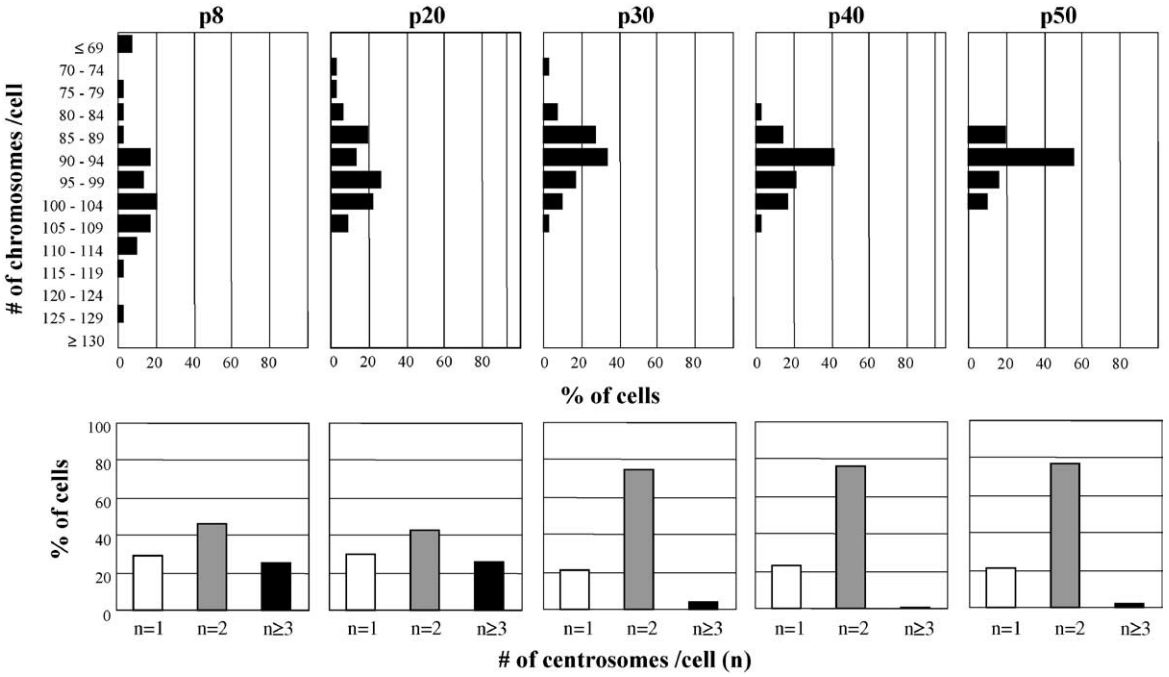

Fig. 2. Karyotypic convergence of p53−/− MEEs in culture is accompanied with restoration of normal centrosome profiles.

chromosomes. The changes in the frequency of centrosome amplification during passaging strikingly paralleled to destabilization/stabilization of chromosomes: high frequencies of centrosome amplification was observed up to p30. Thereafter, the extent of centrosome amplification gradually decreased, and at ∼p50, centrosomes behaved normally: cells contained either one or two centrosomes, and the frequency of abnormal mitosis due to amplified centrosomes was indistinguishable from wild-type cells (<1% of total mitotic cells). These findings strongly suggest that chromosome instability in p53−/− mouse cells is primarily attributed to centrosome amplification, and that centrosome amplification initially imposed by loss of p53 was suppressed in the late passage cells.

Our findings put forward a model of karyotypic convergence in p53−/− cells during prolonged culture, which may be applied to karyotypic convergence during tumor progression (Fig. 3). In early passages, p53−/− cells suffer extensive CIN due centrosome amplification, resulting in karyotypic heterogeneity. At a certain time point in culture, one or few cells within this karyotypically heterogeneous population acquire chromosome compositions that promise advantageous growth phenotypes (i.e., faster growth, serum-independent growth, anchorage-independent

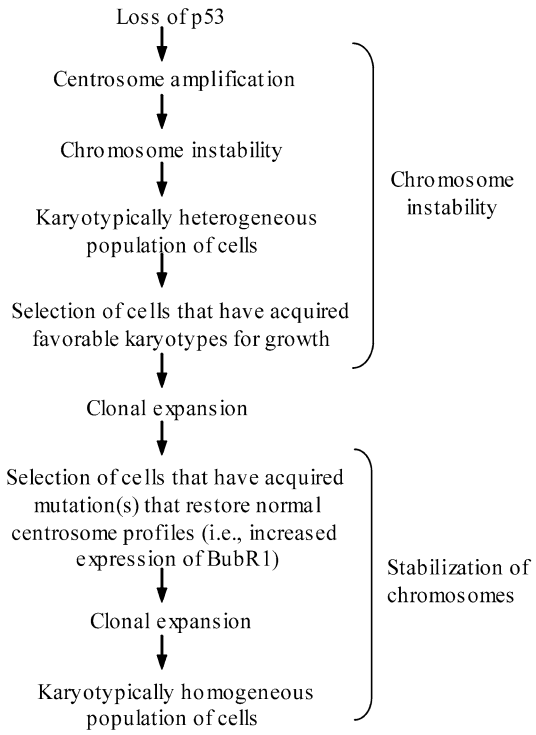

Fig. 3. Karyotypic convergence in cultured p53−/− cells.

growth, loss of cell–cell contact growth inhibition, minimal requirement of nutrients). Such cells gradually dominate the culture. For those cells, maintenance of this particular chromosome composition becomes priority, forcing the selection of cell(s) that counteract the cause of CIN (i.e., centrosome amplification in p53$-/-$ cells) to maintain the chromosome composition that allows the most efficient growth under a given environment.

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COLORECTAL CANCER PROGRESSION MODEL OF CHROMOSOMAL CHANGES DURING LYMPHATIC AND LIVER METASTASIS FORMATION

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Based on the analysis of colorectal cancer (CRC) by comparative genomic hybridization (CGH) we established a progression model of this tumor type [1]. In total, 63 tumor specimens from 40 patients were investigated comprising 30 primary tumors, 22 systemic metastases (12 liver, 6 brain, 4 abdominal wall metastases) and 11 lymph node tumors. The overall pattern of alterations was similar as previously described [2], tumor subgroups i.e. hematogenous metastases showed more alterations than lymph node tumors, particularly more deletions 1p, 3, 4, 5q, 10q, 14 and 21q21 and gains at 1q, 7p, 12qter, 13, 16 and 22q. Comparing liver metastases with their corresponding primary tumors particularly deletions at 2q, 5q, 8p, 9p, 10q and 21q21 and gains of 1q, 11, 12qter, 17q12–q21, 19, and 22q were more often observed.

Our data and the therof derived colorectal progression model indicate that there are independent pathways of colorectal tumor dissemination and that these are associated with a nonrandom accumulation of chromosomal alterations underlying the different characteristics of the metastatic phenotype. It also highlights two well known pathogenetic mechanisms: (1) Metastasis formation may occur immediately after invasion of the primary tumor. (2) Hematogenous dissemination may occur independently from lymphatic tumor spread. Obviously, there is an overlap between the genetic alterations of the tumor subgroups correlating with the fact that many tumors develop both lymph node or hematogenous filiae. Lymphatic spread, however, is not a prerequisite of systemic dissemination which may occur early after cancer initiation. Accordingly there is probably no stepwise acquisition, but a selection of tumor cell clones carrying the favorable metastasis-associated lesions that are initially generated randomly by the inherent chromosomal instability of most colorectal carcinomas.

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PATTERNS AND CONSEQUENCES OF CHROMOSOMAL ANEUPLOIDY IN CANCER CELLS

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Chromosomal aberrations are key events in the initiation and progression of cancer and can be detected in virtually all tumors. In the hematological malignancies, such aberrations frequently result in proto-oncogene dysregulation mediated by way of chromosomal translocations. These events can be triggered by deficiencies in proteins involved in double strand repair [1]. In cancers of epithelial origin, however, balanced chromosomal translocations are rare. In most instances, chromosomal aberrations result in genomic imbalances [2,3]. This has been firmly established by the complementary analysis of solid tumor genomes using spectral karyotyping (SKY) and comparative genomic hybridization (CGH). In addition, carcinomas reveal recurrent numerical chromosomal aberrations, which we refer to as chromosomal aneuploidies [4]. Using a combination of tissue microdissection and CGH, we analyzed genomic changes in the progression of cervical and colorectal neoplasia. Both tumor types are characterized by a strictly conserved distribution of genomic imbalances. For instance, the transition of cervical dysplasia to invasive carcinomas almost invariably required the acquisition of extra copies of chromosome 3q. In colorectal carcinomas, specific imbalances on chromosomes 7, 8q, 13, 18q and 20 were detected. Some of these changes occur at early stages. For instance, extra copies of chromosome 7 could be detected in colon polyps before mutational inactivation of p53. However, the acquisition of specific gains of chromosome 3q in cervical tumorigenesis was preceded by the presence of high risk HPV. We therefore conclude that compromising p53 and Rb-function via viral infection is a necessary, yet not sufficient, condition for cervical tumorigenesis. The average number of chromosomal copy number alterations (ANCA, measured by dividing the number of genomic imbalances by the number of cases studied) increased with increasing stages of cellular dysplasia [4]. Of note, specific chromosomal aneuploidies can occur in an otherwise diploid genome [5]. We have extended these analyses to mouse models of human carcinomas, including mammary gland adenocarcinomas induced by either over-expressing of erbB2 or by conditional, breast epithelium specific, knockout of BRCA1 [6,7]. Both cancer models revealed centrosome abnormalities that result in gross chromosomal and nuclear aneuploidy. Despite this chromosomal aneuploidy and intratumoral heterogeneity, we detected a non-random distribution of chromosomal gains and losses when the tumor genomes were analyzed by CGH. It therefore appears that the acquisition of tumor specific chromosomal imbalances renders a selective growth advantage to tumor cells and is conserved across species boundaries. The prevalent chromosomal instability can be interpreted as a mean to acquire these imbalances. We compared the average number of copy alterations (ANCA) in different mouse models. In general, mouse tumors induced by a strong oncogenic stimulus showed a lower ANCA index than those induced by deficiencies in tumor suppressor genes. This could indicate that a strong oncogenic promotor of cellular proliferation would alleviate the requirement of chromosomal imbalances to be acquired and maintained. Tumor models induced by conditional inactivation of tumor suppressor genes (such as BRCA1 in the mouse mammary gland) therefore appear to more faithfully recapitulate the sequential genome destabilization that we observe during human tumorigenesis. These data were corroborated using interphase cytogenetics with probe cocktails specific for the aberrant chromosomes in touch-preps from cancer specimens. In order to assess the consequences of chromosomal aneuploidies with respect to gene expression levels, we have conducted extensive comparisons between cytogenetic aberrations and expression profiles using cDNA arrays both in experimental cancer models as well as in primary tumor specimen.

Hypothetically, chromosomal aneuploidies could have two possible consequences: (i) the expression of all or most genes located on a chromosome is affected by chromosomal gain or loss, or (ii) the expression of only a few genes, whose reduced or increased expression is critical for tumorigenesis, is the target of chromosomal aneuploidy during tumorigenesis. Thus, whole chromosome copy number changes may simply reflect an economical and readily achievable mechanism for the cells to achieve and maintain such expression changes. However, the latter would implicate silencing mechanisms for all other genes located on the affected chromosome present in increased copy numbers. Data from experimental cancer models, from nor-

mal epithelial tissues that acquired chromosomal aneuploidies after immortalization and transformation, and from experimentally induced chromosomal trisomies after microcell mediated chromosome transfer suggest that only a subset of genes on those chromosomes that are subject to significant copy number increase as a consequence of genomic gain, however, the average expression values of all gene on a trisomic chromosomes increases as well [8,9]. The respective contribution of these changes (strong upregulation of only a few genes or low level increase of all genes on a chromosome) with respect to malignant transformation and tumorigenesis remains to be elucidated.

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SEQUENCE OF CENTROMERE SEPARATION – POSSIBLE LINK WITH ANEUPLOIDY AND CANCER

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At meta-anaphase junction the centromeres of eukaryotic genomes separate in non-random genetically determined sequence. The process appears to be governed by the composition and quantity of the pericentric heterochromatin. In mouse which harbors minor satellite of uniform composition in its pericentric region, the timing of separation is directly related to the quantity of the satellite DNA; the larger the quantity, the later the separation. In human, with several satellite fractions in the neighborhood of the centromere, the situation is complex.

The sequence of centromere separation is not affected by the application of spindle inhibitors (e.g., Colcemid, colchicine, vinblastine, etc.). Also, there appears to be no difference between the separated and unseparated centromeres in the pattern of their phosphorylation and acetylation. In contrast to the reports on biochemical studies on cell cycle, our studies show that the separated centromeres as well as the entire chromosome is highly phosphorylated [1] as well as acetylated at anaphase.

The 'inactive' centromeres in multicentric chromosomes separate ahead of the normal or functional centromeres in the entire genome. This might indicate that early separation of a normal centromere, ahead of its schedule, might lead to abnormal behavior and/or function of the centromere. More recently, it has been shown that the sequence of separation can be influenced by some chemicals belonging to various groups [2]. In conformity with the above statement, the drugs which induced alterations in the sequence of centromere separation also induce aneuploidy.

Currently, we are looking into the aberrations of centromere separation in individuals with Down syndrome and their parents. Our preliminary data show that chromosome 21 in the Downs' individuals, and apparently in their mothers, exhibit premature separation with higher than normal frequency. Some previous data from our lab and the literature show that errors in the sequence of centromere separation are associated with birth defects and certain cancers.

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ALTERATIONS IN NUCLEAR MORPHOLOGY, CENTROSOMES, AND CHROMOSOMAL STABILITY INDUCED BY EXPRESSION OF MUTANT P53

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1. Introduction

Our recent studies of p53 mutations in human breast cancer demonstrated that tumors with mutant p53 tend to have a significantly greater capacity to nucleate microtubules (MTs) than do tumors with wild type p53 [1]. MT nucleation is a major function of the centrosome during both interphase and mitosis. The interphase MT cytoskeleton, through interactions with the actin and intermediate filament cytoskeletons, affects cell polarity, vesicular trafficking, cell–cell adhesions, and cell migration. During mitosis, duplicated centrosomes nucleate the MTs of the bipolar mitotic spindle apparatus. Centrosome amplification can affect interphase and mitotic centrosome function by disrupting cell polarity and by increasing the frequency of multipolar mitoses [2,3]. In breast tumors, the extent of centrosome amplification correlates with the level of chromosomal instability (CIN), supporting the hypothesis that centrosome amplification can cause improper chromosome segregation through the formation of multipolar mitotic spindles [1]. In spite of this correlation between centrosome amplification and CIN, there has been no demonstration that centrosome amplification precedes CIN. In the work presented here, we explored the timing of changes in centrosomes, nuclear morphology, and CIN induced by expression of mutant p53 in cultures of mammary epithelial cells.

2. Methods

To further our understanding of the relationship between p53 and centrosome function, we have created two adenovirus vectors, M72 and M114, containing two different p53 mutations. We chose the p53 mutations based on the characteristics of the tumors in which they were found. Tumor BT0072, with p53 mutant M72, had 3 fold greater MT nucleating capacity, 10 fold greater centrosome number, and 3.4 fold greater CIN level than normal breast tissues. BT0114, with p53 mutant M114, had 11 fold greater MT nucleation capacity, 3 fold greater centrosome number, and 1.5 fold greater CIN than normal breast tissues. The M72 mutant is a truncation mutation that deletes the DNA binding domain, the nuclear localization signal, and the tetramerization domain, while the M114 has a point mutation in the DNA binding domain. We used the adenoviral vectors to deliver the mutant p53 to cultured human mammary epithelial cells previously immortalized with human telomerase. Infection rates of greater than 90% were achieved with the adenovirus vectors. We assayed infected cell cultures at days 2, 8, and 16 post-infection to determine the alterations induced by the 2 specific mutant p53s in nuclear morphology, centrosomes, MT kinetics, and chromosomal stability. Specifically, we measured the frequency of lobed, micro-, and large nuclei, centrosome characteristics, microtubule regrowth characteristics, and CIN of chromosomes 3, 7, and 17.

3. Results

3.1. p53 expression

As determined by Western blotting and immunhistochemistry, expression of mutant p53 was maximal on days 2 and 8, although cells continued to express mutant protein for more than 2 weeks. Results for protein expression and other parameters measured below are summarized in Table 1.

3.2. Nuclear morphology

On days 2 and 8, cells expressing M72 had significantly higher frequencies of cells with lobed nuclei and micronuclei than did uninfected control cells or control cells expressing GFP-adenovirus, with the frequency peaking on day 8. M72-expressing cells never had an increase in large nuclei. The frequency of lobed and micronuclei was increased in M114-expressing cells only on day 8. Also on day 8, cells expressing M114, but not M72, had significantly more large nuclei than control cells.

3.3. Centrosome characteristics

Cells expressing M72 had amplified centrosomes more frequently than did control cells on days 2 and 8,

Table 1
Timing of maximal effects of expression of p53 mutants

	p53 Mutant	M72			M114		
	Day	2	8	16	2	8	16
p53	Expression	++	++	+	++	++	+
Nuclei	Lobed	+	++	ND	≈	++	ND
	Micro	+	++	ND	≈	++	ND
	Large	≈	≈	ND	≈	++	ND
Centrosomes	Amplification	++	++	+	≈	++	+
	Separation	+	≈	ND	+	++	ND
Asters	Multiple	+	++	++	+	++	++
	Disorganized	≈	++	+	≈	++	++
MT Regrowth	Number	++	++	+	+	++	+
	Length	≈	≈	+	+	++	+
CIN	Chrom 3	≈	++	≈	≈	≈	++
	Chrom 7	≈	++	≈	≈	≈	++
	Chrom 17	≈	++	≈	≈	≈	++

but not day 16. The frequency of centrosome amplification peaked in M114-expressing cells on day 8, and remained greater than control cells through day 16. The frequency of non-adjacent centrosomes was also affected by the expression of mutant p53. The effect was relatively weak in M72-expressing cells, and was apparent only on day 2. The effect was more pronounced in M114-expressing cells, especially on day 8. Centrosome separation could be induced in control cells by MT depolymerization. This induction was largely mitigated by expression of both p53 mutants, except for day 8 of M114 when separation was hyperstimulated by MT depolymerization.

3.4. MT asters and regrowth

On days 2, 8, and 16 in MT regrowth experiments, cells expressing either p53 mutant had more frequent multiple and disorganized asters than did control cells. The effects were maximal for multiple asters on days 8 and 16 for both p53 mutants. M72 induced the greatest frequency of disorganized asters on day 8. The number of MTs was greater on all 3 days in M72- and M114-expressing cells than in control cells. However, the length of MTs was significantly greater in cells expressing M114 when compared to control cells only on day 8.

3.5. Chromosomal stability

Neither p53 mutant caused increased levels of CIN on day 2. The M72 mutant caused a 2 fold increase in CIN on day 8; but by day 16 CIN levels had returned to normal levels. In contrast, M114-expression cells had normal levels of CIN on days 2 and 8, but by day 16 had CIN levels approximately 3 fold greater than control cells.

4. Discussion

As seen in the timeline presented in Table 1, M72 and M114 have subtle differences in the timing of their effects on nuclear morphology, centrosomes, MTs, and CIN. The M72 p53 mutation maximally affects centrosomes and the number of MTs and begins to affect nuclear morphology, centrosome separation, and multiple asters as soon as day 2 post infection. The effect of M72 on CIN was apparent only transiently on day 8, with no detectable effect on either day 2 or day 16. In general, the effects of the M72 mutation on all of the parameters measured in this study appeared sooner than the effects of M114. M114 expression maximally effected nuclear morphology, centrosomes, and MTs

on day 8 with effects on centrosome separation, multiple asters, and MT regrowth first appearing on day 2. M114 effects on CIN were only detectable on day 16, which interestingly, is after the peak of expression of the mutant protein. This indicates that expression of the mutant p53 can cause a long-lasting change in the mechanisms responsible for the fidelity of chromosomal segregation. The differences in the effects of the two p53 mutants is likely due to the nature of the mutations; the M72 mutant is truncated and lacks the DNA binding domain, the nuclear localization signal, and the tetramerization domain. The M114 mutant is a point mutation located in the DNA binding domain. However, both mutants increased levels of CIN only after changes in centrosomes and MTs had occurred.

5. Conclusions

The experiments presented here are the first demonstration that centrosome amplification and changes in MT growth and organization precede chromosomal instability. These results also demonstrate that p53 mutants can directly affect the MT cytoskeleton. In the case of the truncated M72 mutant, these changes occur even in the absence of transcriptional regulation via DNA binding. Specific p53 mutations differ in the timing and degree of their effects on centrosome structure and chromosomal instability; *in vivo*, these mutants may have differential effects on tumor progression and metastasis. Identification of particular p53 mutations in the tumors of breast cancer patients and understanding the specific effects of these mutations on centrosomes, the cytoskeleton, and chromosomal instability may aid in developing custom tailored chemotherapeutic treatments.

Acknowledgement

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IS CENTROSOME AMPLIFICATION AND AURORA A KINASE OVEREXPRESSION A PRELUDE TO CHROMOSOME INSTABILITY AND ANEUPLOIDY?

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1. Introduction

The dual and opposing nature of centrosomes facilitate the assembly of a bipolar spindle apparatus needed for the orderly and equal partitioning of chromosomes during mitosis. In addition, centrosomes function as microtubule organizing centers to configure and maintain cell shape, polarity and morphogenesis. Therefore, the striking presence of centrosome anomalies in tumor cells, including amplification and impaired capacity to assemble and organize microtubules [1,2] has been linked to chromosome instability and aneuploidy in neoplastic cells. Although Boveri [3] identified the centrosome as the principle instigator of aneuploidy and cancer over a century ago, his hypothesis was subsequently abandoned. However, in light of new experimental results in our laboratory and elsewhere [4,5], Boveri's hypothesis has been resurrected. In addition, centrosome anomalies and cancer have also been linked with the discovery of a member of the aurora kinase gene family, STK15/aurora A, which is localized to the centrosome, and overexpressed in many human tumors (for review see [6]). Moreover, ectopic expression of STK15 was shown to induce centrosome amplification and aneuploidy *in vitro* [7] and was found to correlate with enhanced clinical aggressiveness, invasion and increased rate of metastasis in human bladder cancer [8].

2. Methods

We have investigated the correlation between centrosome amplification, aurora A expression and aneuploidy in a variety of experimental tumor models including human tumor cells *in vitro* and in an experimental animal model of *tumorigenesis* in rat mammary glands using quantitative fluorescence microscopy, Northern Analysis, and Fluorescence Recovery after Photobleaching (FRAP).

3. Results and discussion

To date, we have found a consistent and positive correlation among centrosome amplification, aurora A overexpression, and aneuploidy in each of the above models. Moreover, aurora A has been shown to target centrosomes with a rapid and dynamic exchange in and out of the centrosomal domain with a $t_{1/2}$ of 3 seconds, as determined in living HeLa cells using GFP-tagged aurora A and FRAP [9].

We analyzed aurora A kinase and centrosome amplification during tumorigenesis in a well-characterized rat mammary tumor model (Fig. 1) using mammary glands from virgin and parous animals exposed to the carcinogen methylnitrosourea (MNU). The rat model for mammary carcinogenesis closely approximates conditions in human mammary glands and facilitates studies of the sequence of molecular events leading to mammary tumorigenesis [10,11]. Our studies demonstrate that aurora A kinase overexpression, centrosome amplification and tumor progression are linked processes (Fig. 2). It is hypothesized that hormones induce a switch in the outgrowth of specific stem cell populations that result in progeny with persistent changes in the intracellular pathways governing proliferation and response to carcinogens [11–17]. Our studies indicate that these intrinsic differences between the parous and virgin gland may also influence the expression of aurora A kinase, thereby protecting mammary epithelial cells from centrosome amplification and associated events preceding tumor formation and progression. Based on these observations we conclude that aurora A expression and centrosome amplification play a key role in tumor development and progression, and both could provide a powerful tool for the assessment of prognostic factors in breast cancer.

An unusual feature of MNU-induced rat mammary tumors is that they are composed of predominantly diploid or near-diploid cells [18]. Since we have clearly shown that centrosome amplification and overexpression of aurora A to be early events in rat mammary carcinogenesis [10], we continue to evaluate how these events relate to the clonal origin and ultimate outgrowth of diploid tumor cell populations. Analysis of rat mammary epithelial cells by flow cytometry demonstrated an early manifestation of aneuploidy following MNU exposure, as predicted by our companion experiments. However, the aneuploid population was found to be transitory, dissipating within 40 days after exposure to the carcinogen. Ultimately, the surviving cells displayed a near diploid profile. We are still eval-

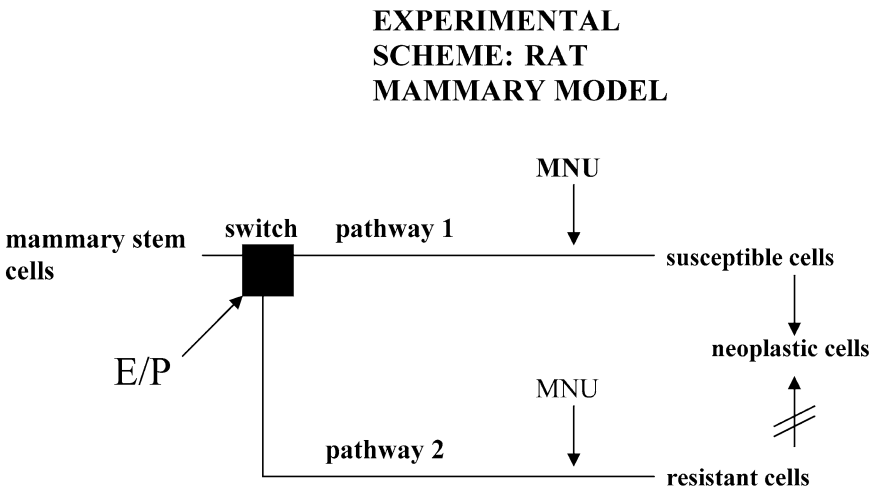

Fig. 1. Scheme illustrating proposed estrogen plus progesterone (E/P) switch (arrow). In pathway 1, post-pubescent virgin females exposed to MNU have high susceptibility mammary tumors, in pathway 2, Parous females, or those exposed to elevated E+P are generally resistant to MNU exposure.

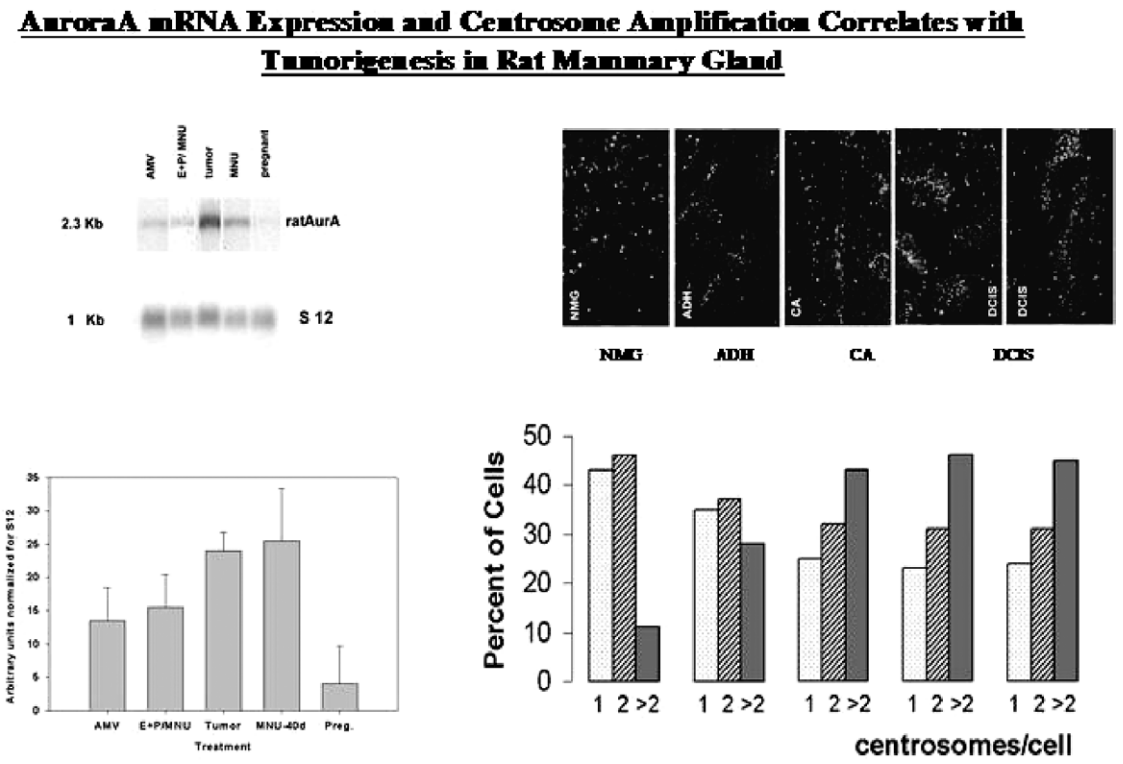

Fig. 2. Northern blot containing Poly A + RNA (5 μg) from rat mammary gland tissue representing different regimens with graph of arbitrary units (see [10]). Insets in upper right show confocal microscope images of centrosomes localization in rat mammary glands of normal (NMG), atypical ductal hyperplasia (ADH), mammary carcinoma (CA) and ductal carcinoma *in situ* (DCIS). Centrosome profiles for each of these stages are also shown. Cells with >2 centrosome/cell represent centrosome amplification (reproduced from Brinkley et al., in Hormonal Carcinogenesis, Li and Li, eds, Springer-Verlag (in press)).

uating these results, but they raise the interesting possibility that centrosome amplification, and subsequent aneuploidy, correlate with chromosome instability, but may not always be a prelude to the clonal outgrowth of tumor cells.

4. Conclusions

Centrosome amplification and elevated expression of the serine/threonine kinase aurora A are consistent and early events in the onset and progression of cells to aneuploidy. Although the mechanism providing a direct link between defects in the mitotic apparatus and aneuploidy has yet to be fully elucidated in tumor cells, there is a growing list of molecular components and regulatory processes that make the mitotic machinery a prime target for tumorigenesis In all model systems studied thus far, with the possible exception of MNU-induced mammary tumorigenesis, centrosome amplification appears to correlate directly with aurora A overexpression, aneuploidy and cancer.

Acknowledgements

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PATHOLOGIC TELOPHASES: SIGNIFICANT SOURCE OF INTERPHASE ANEUPLOIDY

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1. Introduction

The abnormal distribution of DNA values in interphase nuclei and the corresponding chromosome aneuploidy was recognized being a problem in cancer research, when slide based microphotometry appeared. The proposal was raised that it would be of interest to measure precancerous lesions [1]. Indeed, such quantitative cytochemical studies on normal and malignant, but most important also on premalignant cells from the human uterine cervix [2] were performed at Stockholm's Karolinska in 1964.

The correlation between "DNA aneuploidy" in interphase nuclei and cytogenetic aneuploidy on countable chromosomes was a crucial approach for further investigations. Nils Atkin (1962) has pointed out that the ploidy-level indicated by the modal interphase DNA value was in agreement with that he had obtained from chromosome counts [3]. Furthermore, he concluded that only those metaphases having the modal (stem-line) chromosome number regularly went on to complete division. Consequently, DNA measurements on anaphases and telophases are a more reliable indicator of the stem-line than are measurements on prophases and metaphases. The comparison of the DNA content and chromosome number was a pivotal approach to realize genomic instability occurring in human tumorigenesis [4].

Here is to remember the year 1890, when David Hansemann, then third assistant to Rudolf Virchow in Berlin, described asymmetric cell divisions in *Epithelkrebsen* [5]. Chromatin bridges in anaphase segregation, lagging and sticky chromosomes caused obviously unbalanced telophases. Twenty-four years later, Theodor Boveri has incorporated Hansemann's findings into a general hypothesis on the origin of malignant tumors [6].

The focus of the present essay is to quantify the mitotic failure in tumorigenesis. Not only the frequency of karyokinesis is correlated to tumor pathology, but the nuclear divisions also do not obey the equational rule of mitosis. Instead, asymmetric anaphases and telophases characterize dysplasia as well as cancer.

2. Methods

A variety of human tissues were diagnosed according to standards in pathology. Histological specimens were from breast, uterine cervix, skin, oral mucosa, stomach and colon mucosa. Nuclear DNA contents were determined by slide-based microphotometry on Feulgen stained sections of 8 μm and 15 μm for interphase nuclei and division figures, respectively. In any case, the reliability of microphotometry depends on the thickness of the specimen (Figs 1, 2). We thoroughly investigated the bias caused by inappropriate microtome adjustment [7].

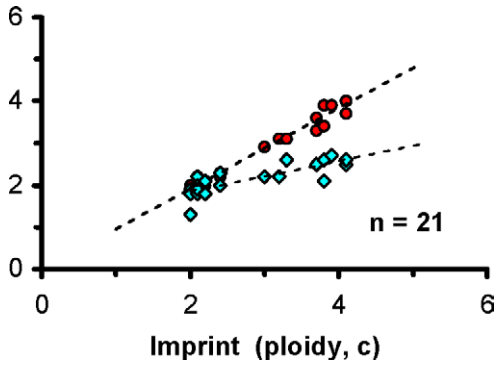

Fig. 1. Feulgen DNA from interphase nuclei in 8 μm (●) and 4 μm (◆) sections, each compared with replica imprints. Mean ploidy of 150 nuclei was recorded from 21 breast cancers.

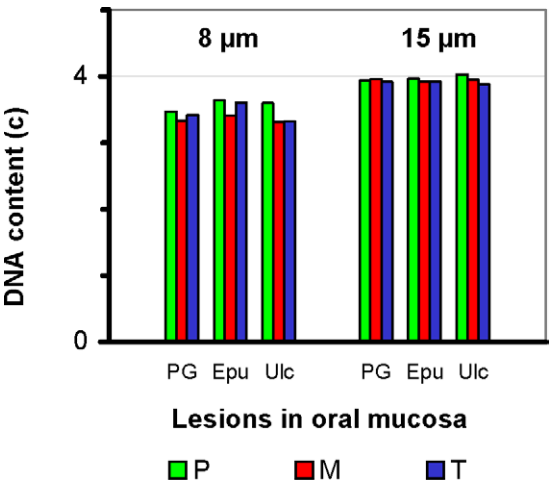

Fig. 2. Depth requirements for mitotic divisions as seen from 8 μm and 15 μm sections. Feulgen stained specimens from oral mucosa: Epu – epulis, PG – pyo-genic granuloma, Ulc – common ulcus; P – prophase, M – metaphase, T – both halves of telophase.

3. Results

Premalignant and malignant lesions exhibit a wealth of nuclear divisions. Their morphology might be close to normal mitoses. However, microphotometry reveals that these figures possess abnormal DNA contents being therefore pathologic entities (Fig. 3). Multipolar division figures might be much more striking, but occur only in the range of 10^{-3} and do not make a deciding influence upon tumor growth.

Tumor progression was documented in nuclear division figures by their quantitative aberrations from a regular 4.0c DNA content. True mitoses were used as internal standard either from normal mucosae or from inflamed tissues. Table 1 shows exemplary records from oral and colon mucosae [8]. The significance of the results was high-lightened by the mitotic activity in fetal liver, whereas many nuclei of the breast cancer cell-line MDA231 contained more than 4.0c DNA.

According to the Hansemann–Boveri hypothesis, a tumor originates from mutated nuclei that segregate their chromosomal aberrations into the daughter products. The injury may occur anywhere during the cell

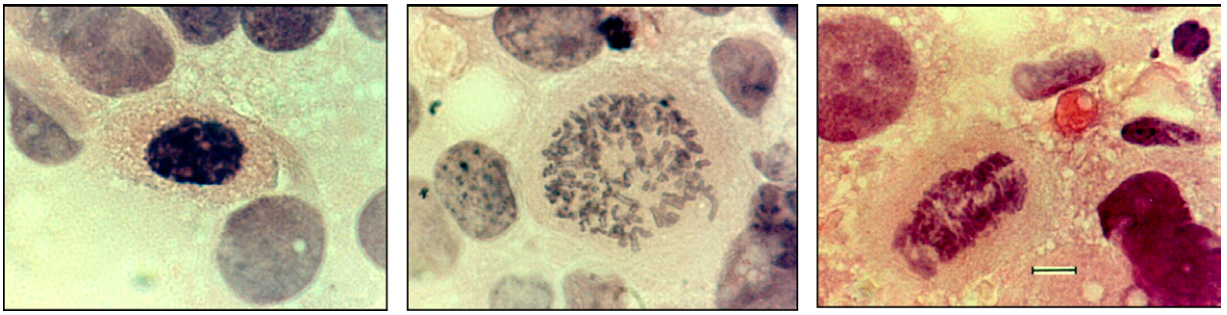

Fig. 3. Imprint cytology from breast cancer, HE stained. Specimens were destained and subject to Feulgen procedure for microphotometry. Prophase 6.1c (left), metaphase 7.2c, anaphase 5.2c. Bar: 10 μm.

Table 1
Frequency distribution of DNA content in nuclear divisions

Histology	(n_1/n_2)	P (%)	M (%)	T (%)	4.5c ER (%)	5.0c ER (%)
Oral mucosa						
Pyogenic granuloma	18/457	42.7	46.2	11.1	0.8 ± 0.5	0
LGD	14/125	30.4	56.0	13.6	4.2 ± 2.4	0
HGD	15/341	35.2	60.1	4.7	56.6 ± 7.6	40.6 ± 6.8
Ca	25/940	33.9	60.8	5.3	73.3 ± 4.4	57.9 ± 4.6
Colon mucosa						
Normal mucosa	71/5	52.1	42.3	5.3	0	0
Bacterial colitis	34/322	37.3	46.6	16.1	1.4 ± 0.8	0.2 ± 0.2
Ulcerative colitis						
LGD	93/850	51.0	36.4	12.6	23.6 ± 1.8	5.5 ± 0.9
HGD	22/246	44.3	39.8	15.9	43.2 ± 5.5	22.0 ± 6.0
Colon adenoma						
LGD	14/425	46.6	39.1	14.3	7.9 ± 2.0	2.8 ± 1.3
HGD	12/498	44.8	42.4	13.0	25.8 ± 4.4	16.2 ± 4.1
Ca	16/538	52.4	39.2	8.4	62.1 ± 4.4	46.3 ± 7.0
Control						
Fetal liver	18/456	42.6	43.6	13.8	0	0
MDA231	2/167	34.1	46.1	19.8	41.4 ± 5.6	21.6 ± 3.7

P – prophase, M – metaphase, T – both halves of telophase. ER – exceeding rate, c – genome equivalent; n_1 – number of cases; n_2 – divisions. LGD – low-grade, HGD – high-grade dysplasia. Scatter was standard error of the mean.

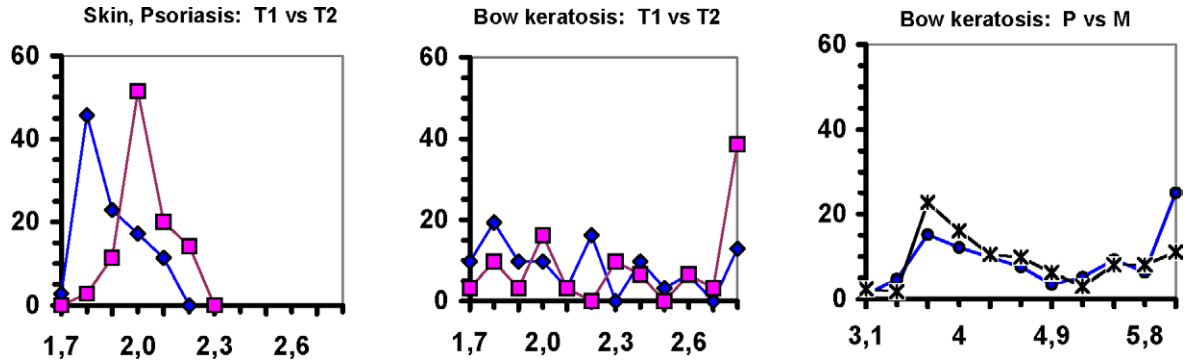

Fig. 4. DNA profiles from divisions in human skin. Psoriasis (left) with minor differences in telophase hemispheres (T1, T2), whereas premalignant Bowenoid keratosis shows severe aberrations between T1 and T2 as well as prophases (P) and metaphases (M) from regular 4.0c. Sections: 15 μm; Feulgen.

cycle, but becomes only effective if every checkpoint can be avoided up to anaphase and through telophase. Therefore, it was most important to show that pathologic mitoses do occur prior to aneuploidy in interphase nuclei [8].

Further investigations addressed bipolar telophases in an environment of other aberrant division figures [9]. The mean difference between two corresponding "halves" was 0.2c in high-grade dysplasia of colon adenoma ($n = 65$) telophases, whereas 0.4c differences were recorded from Bowenoid keratosis ($n = 31$) and in high-grade dysplasia of oral mucosa ($n = 16$). Figure 4 shows that unbalanced telophases may be detected already in premalignant lesions in human skin.

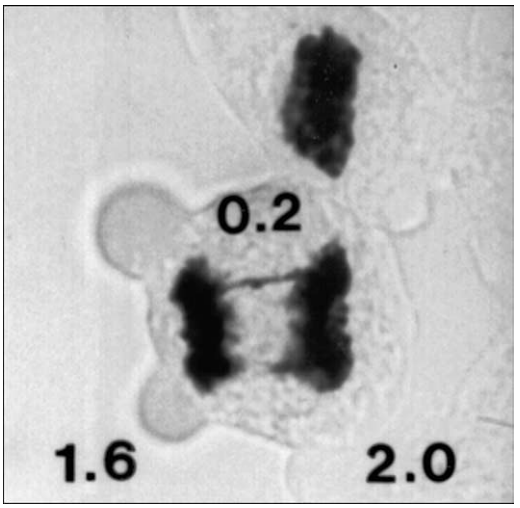

Fig. 5. Source of aneuploidy: successful telophase. Errant, lagging and sticky chromosomes are most frequent (values DNA content c).

4. Discussion

The cascade of multiple checkpoints should exert a tight control over the entire cell cycle. However, aneuploidy becomes constitutive when nuclear defects remain undetected or not repaired. Two different modes of control slippage are proposed: (i) The complete cascade is somewhat leaky, and a few aneuploid nuclei successfully enter telophase. The gedanken experiment precludes that a single checkpoint might be responsible, because downstream instances would detect such defects. The remaining possibility appears highly unlikely that the ultimate checkpoint (in anaphase) could be the only gate for tumorigenesis. (ii) Genome instability creates a peculiar chromosome constitution that cannot be detected by the cascade of checkpoints. The shaken nucleus generates an aneuploid telophase that allows continuation. This model follows Boveri's chromosome lottery [6].

In any case, each type of error passing the anaphase control must do so repeatedly if a tumor shall establish. One cannot discriminate in the microscope, whether pathological complements will be stopped at any checkpoint or whether they are just running through the cell cycle, anaphase included. This problem, however, does not cut down the diagnostic rule saying that nuclear defects represent a measure for tumorous lesions and their progression.

Pathology has not only to judge upon the pattern, the texture and the diameter of cell nuclei, but has to measure DNA contents to evaluate the genomic status. Aneuploidy of interphase nuclei alone, without apparent pathologic mitoses, characterizes but a lesion with interrupted or hampered progress in tumorigenesis. Regarding aneuploidy, only telophases, successful

in clonal selection, drive tumor development and the switch to malignancy.

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THE CLINICAL CONSEQUENCES OF MASSIVE GENOMIC IMBALANCES IN CANCERS

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We examined genomes of cancerous cells in terms of microarray data, DNA methylation, multidrug resistance and renewed attempts at generating cancer cells by oncogenic transformation [1]. Most cancerous genomes are non-diploid, rearranged and variably methylated [2–14]. The data highlight the importance of the fraction of any cancerous genome undergoing differential RNA expression [4], the importance of genetic background on the cancerous phenotype [15] and the importance of stochastic gene expression in network fluxes [16–18]. The data provide insights into, (i) cancer data transfer from mouse to human, (ii) methylation data transfer from cancerous cell lines to clinical samples, and (iii) the concept that mutations in a handful of oncogenes, tumor suppressor genes, repair genes and cell cycle genes are the prime movers in the genesis of cancer [5,14,19]. Given the exceedingly poor record of new cancer drug developments based on gene selection from functional genomics approaches, future therapeutic avenues will need to move to *methylation-based gene regulatory networks operating from grossly imbalanced genomes*.

1. Genomic technologies

Recent technological advances are inundating us with data [20]. At the genomic level, Representational Oligonucleotide Microarray Analysis is providing data on genomic regions with altered copy number [21]. At the level of the methylated genome (the methylome) emerging technologies report on the methylation status of selected CpG islands, promoters, repetitive elements and histone modifications [9]. At the transcriptional level, microarrays, Serial Analysis of Gene Expression, Massively Parallel Signature Sequencing, BeadArray^{TM} and Mass Spectrometry-based approaches generate avalanches of data. At the level of proteomes, automated multidimensional liquid chromatography coupled to mass spectrometry, (LC-LC-MS-MS) and 2D gel platforms interfaced with multicompartment electrolysers provide data on protein profiles. At the immunohistochemical level, high throughput robotic imaging technology, integrated with bioinformatic deconvolution, is allowing insights into cellular aspects of the cancerous state [22].

Genome-wide data are fed into bioinformatics pipelines in which an ever increasing number of algorithmic protocols produce Molecular Portraits of Cancers [23,24]. Further analysis, however, quickly concentrates on only a handful of gene products which are significantly increased or decreased in tumors, as these are believed to drive the etiology and pathogenesis of cancers [25]. The most important of these are thought to be oncogenes, tumor suppressors, repair genes and cell cycle genes [1,14,19,25,26], which are, without exception, all prioritized as potential targets for the therapeutic pipeline.

2. Genomic status of cancer cells; cytogenetics

While genomic imbalances have always been diagnostic for solid tumors, recent data on multiple myelomas extends this near ubiquity of aneuploidy and nonrandom recurrent chromosomal abnormalities to hematological neoplasms [27]. Each row of this figure is the chromosomal constitution of an individual patient, and each column represents a departure from the diploid condition, with trisomies being shown in yellow and monosomies in red. In addition, a large proportion of patients with hypodiploidy and near-tetraploidy also carry a translocation involving the immunoglobulin heavy chain locus, IgH and these translocations are frequently unbalanced and associated with monosomy elsewhere in the genome [28]. Thus massive changes in gene dosage are a hallmark of these hematologic neoplasms.

3. Genomic status of cancer cells; microarrays

Microarray analysis of cancerous tissue reveals multiple regions of the genome with altered copy number. Primary breast tumors analyzed with a combination of genomic and mRNA expression microarrays allow simultaneous detection of imbalances at both the DNA

and RNA levels. Amplifications of chromosomal regions are found in *every* breast cancer tumor and on *every* human chromosome in at least one sample. Most importantly, a significant fraction of amplified genes are correspondingly more highly expressed [8]. Thus residents of amplicons and deletions have their RNA outputs determined by their nondiploid status, partly by the altered regulatory networks of the chromosomally corrupted cell and partly by the altered methylome. These RNA outputs impact upon proteomic networks in a nonlinear manner [29]. Given the genomic variation between cancer cell populations and the nonlinear nature of transcriptional output to phenotype, it is hardly surprising that microarray studies of 300 breast cancers reveal that tumors with increased HER2 levels are as aggressive as those with normal HER2 levels [30].

4. Methylation

Single locus analyses reveal that certain regions of cancerous genomes can be hypermethylated, hypomethylated or show no change [9]. The hypermethylation of CpG islands, for example, is believed to lead to the development and progression of all common forms of human cancer, with the regulatory inactivation of tumor suppressors claimed to be an important component. However, the data differ between mouse and human sources. There can be completely different organizations of CpG island promoters between homologous mouse and human genes [31] and methylation in cell lines and primary malignancies are radically different. At least 60% of loci methylated in cell lines are never methylated in primary tumors. Thus cancerous cell lines are a poor resource for identifying novel targets of DNA hypermethylation involved in oncogenesis [6]. The unresolved issue from the methylation characteristics of any genome is whether gene silencing is a causal event in the *initiation* of cancer, or whether it is a by-product of the ongoing evolution of the tumor cell population.

5. Mouse to human

Although mice with a knockout of the catalytic gamma subunit of phosphatidylinositol-3-OH were originally found to have a high incidence of colorectal carcinomas, three subsequent independent knockouts of the same gene, in different genetic backgrounds, never developed colorectal carcinomas [15]. Similarly a mutant tumor suppressor (APC) in the C57BL/6 strain has many polyps in the colon, whereas the same mutant tumor suppressor in the AKR strain has very few polyps. The universal finding is that the phenotype depends on the background in which it is evaluated. In addition, knockouts of cancer-related genes in mice do not necessarily reproduce similar phenotypes to those from mutations in homologous human genes. Knockout of the mouse Wt1 gene does not predispose to cancer whereas human WT1 does. Inherited mutations in mouse p53 do not predispose to breast cancers, whereas human p53 does. Transgenic mice that model Ras in humans do not give rise to the same tumors as in humans. Lastly, even though the Ras system is thought to be highly conserved between different species, microarray data reveal substantial differences between rodents and humans in the signaling pathway downstream of Ras [1,32]. Conservation at the single gene level between elements of a network is no guarantee of conservation at the level of network fluxes and outputs.

The fragility of classification schemes such as oncogenes and tumor suppressors is also exposed in different organisms and in different genetic backgrounds. The prototypic oncogene, p53, was reclassified as a tumor suppressor after a decade of being an oncogene. Similarly, another oncogene of many years standing, Kras2, has now been shown to have tumor suppressor properties [33]. Mouse tumor suppressors such as Smad3, E2f1 and Mom1 have not been found to be mutated in any human cancer [26]. Even more importantly, however, recent data fail to replicate earlier work on oncogenic transformation [1] and cast considerable doubt on the original mutational underpinnings of such transformation [34]. The notion that a small number of key genes can produce permanent oncogenic transformation in normal diploid human cells once again rests on very insecure foundations. In contrast, gross genomic imbalances and transformation are correlated in these newer data.

Finally, recent data on cyclin E and cyclin-dependent kinase 2 (CDK2) which for a decade have been held to be pivotal in driving cancer cell proliferation, have revealed that knockouts and various inhibitory methods such as RNAi, antisense and dominant-negatives, all fail to stop mitosis in colon cancers, cervical cancers and osteosarcomas. Five recent papers totally demolish the long held view. McCormick, a leading cancer researcher involved in generating some of these data, concludes that *there's no real evidence that CDK2 does*

anything in somatic cells [35]. Clearly, researchers and drug companies need to reevaluate drug development efforts aimed at any single cell cycle protein [35].

6. Genomic imbalances

The dominant paradigm that cancer cells arise from normal diploid cells through the acquisition of a series of mutations in a handful of oncogenes, tumor suppressor genes, repair genes and genes impinging on the cell cycle, is in need of serious revision. The cleanest test of this is afforded by the data showing that cancerous cells and aneuploid cell lines, but not diploid cells, rapidly become resistant to chemotherapeutic drugs. The additional finding that such cells spontaneously and rapidly *revert* their phenotype is galvanic in its implications [36]. Even if standard gene mutation or methylation reprogramming were able to account for the acquisition of multidrug resistance, either by inactivation of drug-metabolizing genes, or by activation of multidrug transporters, for example, the *rapid spontaneous reversion* of such phenotypes in diploid contexts is near impossible. However understanding the rapidity of this reversion in terms of chromosomally unstable aneuploid genomes is straightforward [36].

The available data make it highly probable that the *initial event* predisposing to a cancer is one that generates a massive genomic imbalance involving hundreds of genes. In its simplest forms this is a spontaneous nondisjunction event or a translocation. A single autosomal nondisjunction event generates aneuploid daughter cells and will be a huge shock to most regulatory networks [2,4,7,11,13]. The resultant genomes will now need to instantaneously cope with hundreds of extra copies of genes in either a trisomic or monosomic condition. Similarly, a balanced translocation, while initially only effecting genes at its breakpoints, eventually becomes unbalanced [28,37], so hundreds of genes are again in a haploid state. Networks in these aneuploid and unbalanced translocation situations are initially pushed to either haplo- or triplo-thresholds. Compensatory nondisjunctions of nonhomologs will partially alleviate such imbalances, a phenomenon well described in yeast [38]. In addition, further mutational and methylational changes occur in the chromosomally corrupted genomes, but these are all *secondary* consequences of unbalanced systems attempting to reach some form of stability. The clinical outcomes of genomic imbalances at any given time will depend on the *initial* event (intrinsic or extrinsic), that perturbed the cellular circuitry, the *order* in which subsequent amplifications, deletions and translocations occur, the genome-wide *methylation profile*, and the various *polymorphisms* (both SNP and larger rearrangements) that are unique to each individual and whose totality influences the phenotypic outcome.

Huge changes in gene dosages involving hundreds of genes are of such enormous consequence that they swamp most piecemeal gene-by-gene alterations. In the long term, aneuploid, segmentally aneuploid and unbalanced translocation-carrying cell populations become inexorably trapped in devastating transitions from one unbalanced state to another [39]. Their genomes become so fundamentally flawed in a gross chromosomal sense that there is no possibility of a return to the initial diploid condition. It is self evident that drugs targeted against single genes will have vanishingly low probabilities of rectifying such deranged regulatory circuitry.

The results of continuing genomic imbalances will manifest clinically as extensive heterogeneity within the same tumor, heterogeneity between different tumors from the same patient and heterogeneity between patients. Available data corroborate this. Microarray data from three *different areas of the same kidney tumor* are instructive; the correlation among the expression profiles for the three areas of the same tumor only exceeds 0.5 in two of the six patients sampled [40]. Prostate cancer often has many distinct foci, only one of which may be invasive and have a deleterious effect on the patient [41]. The tumor variation between patients requires no comment; a huge literature documents the extensive between-individual variation.

These data on variation mean that *no two cancers will have exactly the same genomic imbalances and rearrangements, nor the same methylome, even in monozygotic twins*. It is this variation that is pivotal to any cancerous cell population as it evolves along its unique genomic trajectory, sometimes achieving a metastatic status and sometimes simply remaining benign. Recognizing the types of trajectories is the key diagnostic on which therapeutic decisions can be based for the individual. Attempting to redirect trajectories from becoming potentially devastating to remaining benign is a challenge in *understanding networks*.

In conclusion, with very few exceptions such as Gleevec and hematological neoplasms, the approach of the last 30 years of focusing on single genes has been manifestly unproductive at delivering drug targets in the solid tumors, which, after all, make up 90% of cancers. It's rarely the single gene that is the culprit, it's the

imbalanced genome that is the problem, with network perturbations changing from tumor to tumor. It would be prudent for high priority therapeutic strategies to focus on preventing somatic genomic imbalances and attempting to shunt cancerous cell populations to benign cul-de-sacs.

Three decades of cancer *data* reinforce one conclusion; the prioritized single gene approach is assuredly almost always doomed, both diagnostically and therapeutically. It's legacy is continuing therapeutic failures and unexpected complications, such as the increase in brain cancers with women treated with Trastuzamab (Herceptin) for breast cancer [42]. The *genomic imbalance-network* approach, by contrast, faces up to the reality. The choice of approaches is stark indeed.

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DRUG RESISTANCE-SPECIFIC ANEUSOMIES OF HUMAN COLON AND BREAST CANCER CELLS

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We have observed previously that only aneuploid and tumorigenic, but not diploid rodent cells acquire and lose drug-resistance at high rates [1,2]. In view of this we have proposed that specific assortments and rearrangements of chromosomes, altering the dosage of thousands of regulatory and structural genes, may generate drug-resistance. The proposal is based on the fact that aneuploidy destabilizes the karyotype autocatalytically and thus generates numerous new chromosome combinations and reassortments, of which some may confer resistance to cytotoxic drugs [3]. But the predicted resistance-specific aneusomies were not found in the drug-resistant rodent cells, because the resistant cells were not karyotyped and because their chromosomes were too unstable to detect specific aneuploidies numerically. In the meantime we have found that the karyotypes of several human colon and breast cancer cell lines are much more stable than those o equally aneuploid rodent lines, a feature that would facilitate the detection of specific aneusomies. Therefore, we have now selected several puromycin-resistant clones from these human cell lines in an effort to find the predicted drug resistance-specific aneusomies. Here we report that, indeed, 3 out of 3 puromycin-resistant clones of colon cancer lines, and that 1 out of 1 puromycin-resistant clone of a breast cancer line contained each 2 to 3 resistance-specific aneusomies. We conclude that specific aneusomies are necessary, if not sufficient to generate drug-resistant phenotypes.

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IN SITU QUANTIFICATION OF GENOME INSTABILITY IN BREAST TUMORS

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1. Introduction

Genome instability is one of key features in breast and other solid tumors, contributes to the accumulation and the selection needed for their progression. However, the little is known about the onset and actual level of instability during cancer progression. To address this, we assessed copy number variation of two different target loci in genome using dual-color fluorescence *in situ* hybridization (FISH) and three-dimensional (3D) image analysis. We quantified the level of genome instability by enumerating actual copy number of FISH signals in $\sim$40 μm thick sections at various stages of breast cancers. Our analysis exhibited low instability in normal skin, normal duct and usual ductal hyperplasia (UDH) in breast. However, instability was remarkably high in ductal carcinoma *in situ* (DCIS) and somewhat lower in invasive cancer (IC). DNA content in DCIS and IC was also increased by measuring nuclei in 3D images. To confirm this process *in vitro*, we quantified copy number of same loci using dual-color FISH and measured average of chromosome composition by array based comparative genomic hybridization (CGH) at several passages of *ZNF217*-mediated immortalized human mammary epithelial cells (HMEC). We also assessed telomere events to reveal the correlation between instability and telomere dysfunction (crisis). The *in vitro* analysis suggested that instability sharply increased at the transition from UDH to DCIS by passing through telomere crisis and was reduced in IC by telomerase reactivation.

2. Methods

2.1. Tissue processing and microscope imaging

Frozen tissues were obtained from UCSF Breast Oncology Tissue Repository. The tissues were evaluated in H&E sections and cut in 20 $\sim$ 40 μm. Dual-color FISH to sections were performed for the centromere of chromosome 1 (1c) and chromosome 20q13.2 (20q) with DNA counterstaining by YO-PRO-1. The probes were labeled with Alexa568 and Cy5, respectively. Several areas per section were imaged using a confocal microscope (Zeiss LSM410) with a 63$\times$ objective. Three consecutive scans were executed per imaging with 488, 568 and 633nm lasers, corresponding to YO-PRO-1, Alexa568 and Cy5. The images typically carry $512 \times 512 \times 100$ voxels with $0.2 \times 0.2 \times 0.3$ μm resolution.

2.2. Image analysis and statistics

A nuclear segmentation and FISH signal detection in 3D images were semiautomatically executed using a custom program developed in house. Briefly, nuclei were delineated as high intensity regions against low (background) intensity regions and visually assessed to detect clusters of nuclei. The clustered nuclei were subdivided and the voxels comprising nucleus were summed as DNA content. FISH signals of 1c and 20q in each segmented nucleus were detected similarly. The analytical accuracy was established by a statistical model that assumed random loss of true signals and random detection of spurious signals in all normal areas (a total of 537 nuclei). Linear regression analysis was used to assess correlations between copy number of FISH signals and nuclear volume.

2.3. HMEC analysis

ZNF217-mediated immortalized HMECs were used for genomic analysis at several passages through telomere crisis. The chromosome composition was assessed using array CGH and the copy number of 1c and 20q was measured using dual-color FISH as described above. Telomere length and telomerase activity at each passage were measured by Southern blot and TRAP assay analyses, respectively.

3. Results

The statistical analysis in normal tissue indicated that $93 \pm 4\%$ of genuine FISH signals were correctly detected, with a $4 \pm 4\%$ probability of an spurious signal being incorrectly detected. The results are similar to those previously reported for disaggregated nuclei. Therefore our segmentation is accurate enough for the analysis of copy number changes. The level of instability in normal ductal epithelium, UDH, DCIS and IC was calculated as the standard deviation of the copy number enumerated in several images of each tissue. The level of instability in the HMEC cells was similarly estimated at several passages before, during and after immortalization. One UDH sample presented a unimodal bivariant distribution which however, a significant number of randomly distributed nuclei (22%, $p < 0.001$) with a single copy of chromosome. All three DCIS cases exhibited an extremely high level of instability. One DCIS case showed strong correlation between copy number and nuclear volume, suggesting concomitant polyploidy. Spatial statistical analysis of all three samples exhibited that nuclei with significantly different chromosomal composition were randomly mixed, suggesting that the variation of gene copy was caused by genome instability, but not by stem cell like clonal expansion. Analysis of IC showed somewhat lower level of instability in all cases with areas of both normal and abnormal ploidy. Cells were again randomly mixed. The analysis of HMEC using FISH and CGH revealed increased instability during telomere dysfunction (crisis) at passages 21–22 followed by slightly decreased instability after telomerase reactivation.

4. Discussion

Our study showed a significant increase in the level of instability at the transition from hyperplasia to DCIS carrying malignant phenotype. Instability slightly decreased in IC, may suggest that some of IC are composed of a clonal subpopulation selected from cells in DCIS with a proliferative advantage. HMEC analysis showed a similar increasing of instability during telomeric crisis, suggesting critical telomere shortening caused genomic instability. Probably the similar process occurs *in vivo* at the transition from UDH to DCIS. IC would be formed by further selection of cells with strong survival and/or proliferative factors and by clonal expansion of selected cells with telomerase reactivation. Therefore deranged cancer genome in IC could be little stabilized and could retain a lethal malignancy.

5. Conclusion

We have demonstrated how FISH to thick sections can be effectively used for *in situ* analysis of genome instability. By using our system, we were able to quantify the level of genome instability at the accumulation of chromosomal aberration in intact cells. Our study suggested the mechanism of genomic instability in DCIS and slightly reduced instability in IC although further assessment is required to confirm these. By measuring telomere repeat and telomerase activity *in vivo*, we would demonstrate our hypothesis that the transition to DCIS in fact coincides with telomere dysfunction.

CENTROSOME GENES INVOLVED IN CELL CYCLE PROGRESSION, CYTOKINESIS, SPINDLE FUNCTION AND THE FIDELITY OF CHROMOSOME SEGREGATION

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Centrosomes were named by Theodor Boveri over 100 years ago because they were discrete structures found at the center of the cell (hence the term *centrosome or central body*) (see [1]). Centrosomes are the only nonmembranous organelles in vertebrate cells. They are 1–2 microns in diameter and lie in close proximity to the nucleus. Centrosomes are composed of centrioles and pericentriolar material (PCM, Fig. 1). Centrioles are barrel-shaped structures ~0.5 microns long and ~0.2 microns in diameter. Nine sets of triplet microtubules make up the barrel. Other protein structures are found both inside and outside centrioles (see [2]). Each centrosome has two centrioles that are oriented at right angles to one another. The older maternal centriole has additional appendages that distinguish it from the younger daughter centriole (see below).

The best-known function of the centrosome is its ability to nucleate the growth of new microtubules. Microtubules are organized into astral arrays in interphase cells and form mitotic spindles in mitosis (Fig. 1, left). Microtubules nucleated from centrosomes at the poles of the mitotic spindle bind to chromosomes and position them at the spindle center. As cells exit mitosis, chromosomes move toward the spindle poles along tracks formed by microtubules. This results in segregation of chromosomes into two daughter cells following cell division (cytokinesis).

Like chromosomes, centrosomes duplicate precisely once every cell cycle. The fidelity and timing of centrosome duplication is essential for ensuring that this process is effectively coupled to other events such as cell cycle progression and DNA replication. Uncoupling of these events can lead to excess centrosomes that organize multipolar spindles (Fig. 1, right) or single centrosomes that organize monopolar spindles.

If centrosome number or function is perturbed, spindle abnormalities can result (see [2]). For example, supernumerary centrosomes can lead to the formation of spindles with multiple poles (Fig. 1, right). In turn, multipolar spindles can segregate chromosomes into more than two cell progeny (Fig. 2). Moreover, multidirectional forces exerted on a single chromosome in a multipolar spindle can create chromosome breaks (Fig. 2, bottom right). In other cases, failure to duplicate or separate centrosomes can lead to the forma-

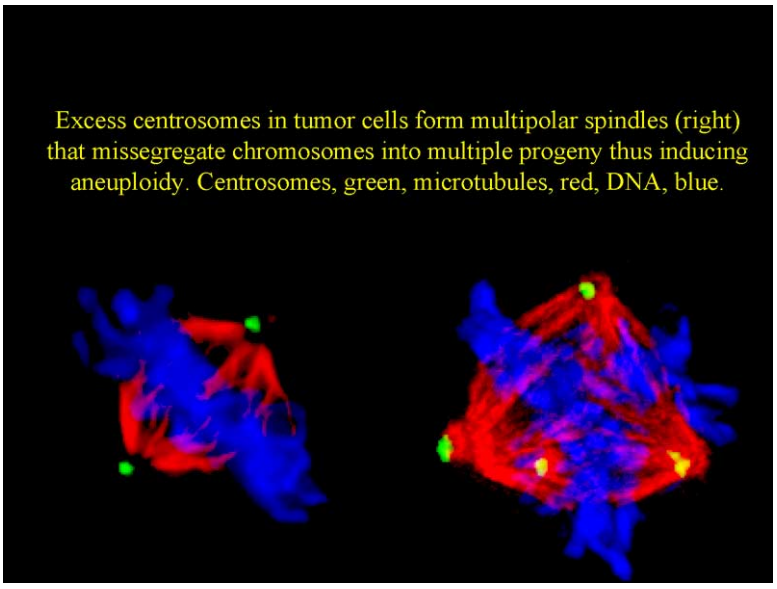

Fig. 1. Centrosomes and mitotic spindle organization in normal and tumor cells. Immunofluorescent image of centrosomes in a nontumor cell that organize a normal bipolar spindle in mitotis (left). A cell from a breast tumor showing excess centrosomes that organize a multipolar spindle (right) that induces chromosome missegregation aneuploidy. Images: Keith Mikule and Steve Doxsey.

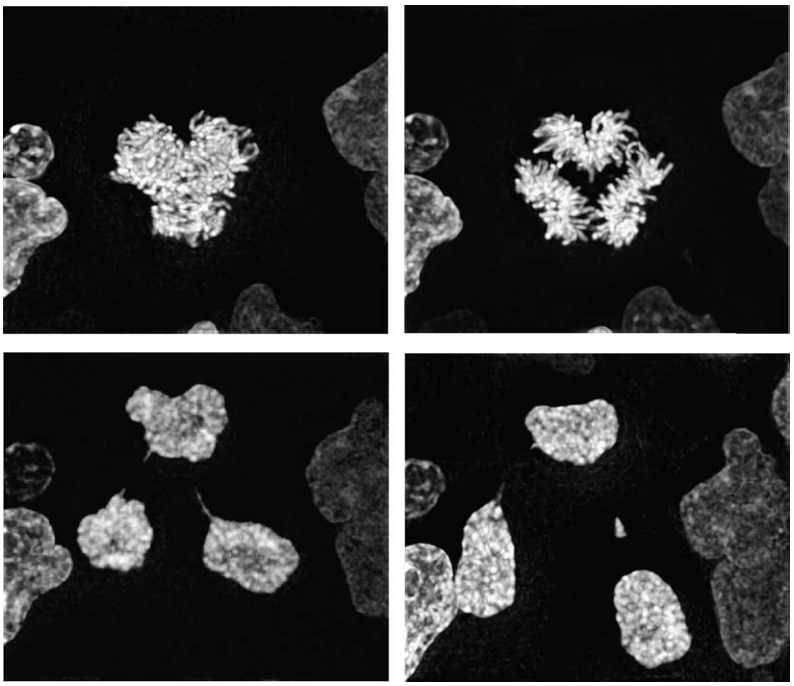

Fig. 2. Chromosome missegregation in a human prostate tumor cell. PC-3 cell line constitutively expressing histone H2B fused to green fluorescent protein (GFP) to label chromosomes. Cells were examined by fluorescence time lapse imaging using optical sectioning and deconvolution to obtain three-dimensional images. Three-dimensional images are displayed as two-dimensional projections. Images: Agata Jurczyk and Steve Doxsey.

tion of monopolar spindles that are unable to segregate chromosomes resulting in cell division failure. In all of these scenarios, daughter cells would receive abnormal numbers of chromosomes and become aneuploid.

Recently, we and others demonstrated that centrosomes are abnormal in nearly all human malignant carcinomas (Figs 3, 4) [3,4]. We next addressed whether centrosome defects were a secondary result of tumorigenesis or if they contributed to the process, perhaps through the induction of genetic instability. We first asked whether centrosome defects were present in precancerous lesions. To our surprise, we found that centrosome defects were present in 20–55% of precancerous lesions of the breast, cervix and prostate [5]. Of great interest, was data showing that aberrant centrosomes were present in precisely the same lesions that were aneuploid. We also found that the centrosome protein pericentrin was elevated in many human carcinomas [6].

A second approach that we used to address the role of centrosome proteins in genetic instability was to test whether pericentrin could induce tumor-like features. We constructed cell lines stably overexpressing the protein and found that they showed centrosome defects, genetic instability, enhanced proliferation, growth in soft agar, loss of mitotic checkpoint control, changes in cell and nuclear morphology and abrogation of the mitotic checkpoint [6,7]. We recently demonstrated that pericentrin binds PKA, PKC, cytoplasmic dynein and the gamma tubulin ring complex, and that specific disruption of pericentrin's interaction with some of these proteins induced a tumor-like phenotype [7–11].

The third approach we took to address the role of centrosome proteins in genetic instability was to screen for cancer-like features following centrosome gene silencing. We found that silencing of many centrosome genes affected the fidelity of chromosome segregation either through spindle dysfunction or cytokinesis failure [12]. We unexpectedly found that silencing of nearly all centrosome genes induced cell cycle arrest in normal cells but not in cells with abrogated checkpoint function [12].

In our current model, altered centrosome protein levels cause centrosome defects that may contribute to tumorigenesis through the induction of genetic and cellular disorganization. Centrosome defects could act as epigenetic modifiers of the genome and together with genetic mutations, provide a powerful driving force for increased genetic instability [13]. This condi-

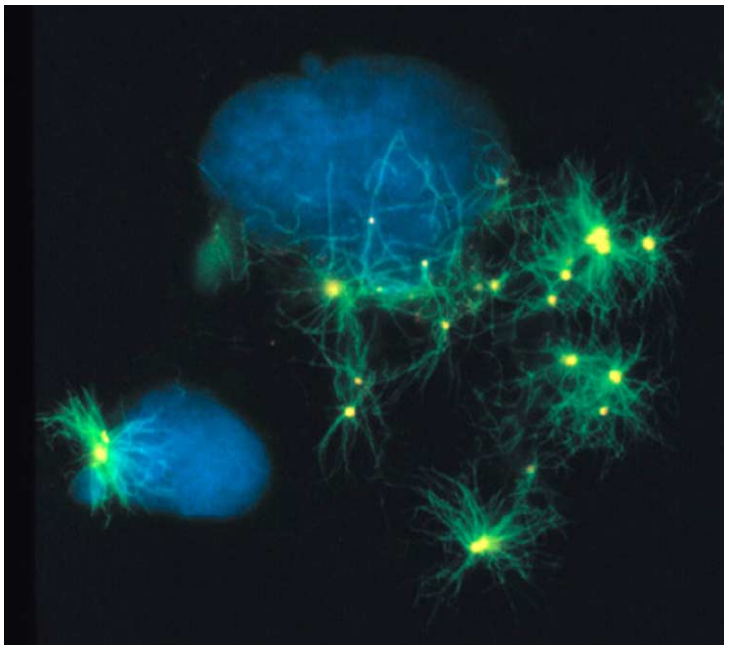

Fig. 3. Centrosomes are abnormal in size, shape and number in human tumor cells. Abnormal centrosomes in a breast tumor cell (right, yellow) compared with a normal diploid cell (lower left). Newly nucleated microtubules (green) arise from a single centrosome in the nontumor cell (yellow, bottom left). In the tumor cell, multiple centrosomes of hetergeneous size, shape and number, nucleate microtubules (right) and contribute to the formation of multipolar spindles and chromosome missegregation (not shown). Blue, nuclei. Images: Steve Doxsey.

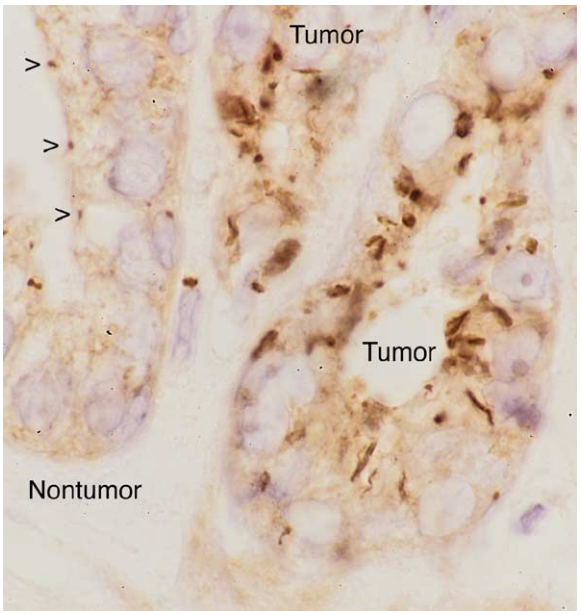

Fig. 4. Centrosomes are abnormal in size, shape and number in human prostate tumors. Abnormal centrosomes in a prostate tumor (right, brown) compared with normal centrosomes in nontumor cells (left, arrowheads). Tissue section stained by immunoperoxidase for the centrosome protein pericentrin and for hematoxylin and eosin. Note consistent size of centrosome in nontumor cells and abnormal centrosome numbers, shapes and sizes in tumor tissue.

tion could accelerate accumulation of alleles carrying pro-oncogenic mutations and loss of alleles containing wild-type tumor suppressor genes, features characteristic of the most prevalent human cancers. The consequence of these events would be to create a larger pool of genetically altered cells from which to spawn clonal populations with greater survival potential. This genomic plasticity could ultimately facilitate emergence of gene dosage changes that are pro-tumorigenic [14].

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TELOMERASE REACTIVATION AND GENOMIC INSTABILITY DURING IMMORTAL TRANSFORMATION OF CULTURED HUMAN MAMMARY EPITHELIAL CELL

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1. Introduction

Recent studies utilizing genetically engineered mice [1,2] and human tissues and cells [3–8] have led to new models for the mechanisms underlying early stage human carcinogenesis and genomic instability [1,7,9,10]. These models propose that progressive telomere erosion with DNA replication, in the absence of sufficient telomerase activity, eventually leads to telomere dysfunction. The eroded telomeres are viewed as DNA damage, and if ligated by the non-homologous end-joining (NHEJ) pathway, can create end-to-end fusions and ongoing cycles of breakage/fusion/bridge (BFB). The resulting genomic instability may inhibit or promote transformation, depending upon the context (e.g., pre-existing mutations such as in p53, cell type, and environmental conditions). This type of genomic instability can then be ameliorated by re-expression of telomerase activity, which maintains telomeres at short, stable lengths, thereby conferring both an indefinite proliferative potential and "capped" telomeres no longer subject to NHEJ. The immortality conferred by telomerase reactivation is considered crucial for human carcinogenesis, since unlimited proliferative potential may be required to accumulate the multiple rare errors necessary for malignancy and metastasis. The amelioration of genomic instability likely also contributes to carcinogenesis by maintaining the viability of cells that have acquired malignancy-associated derangements.

Cells derived from normal somatic tissues of long-lived organisms such as humans exhibit extremely stringent repression of telomerase activity and cellular senescence *in vitro*. In contrast, most human cancers express telomerase activity. The errors that allow telomerase reactivation to occur during human carcinogenesis have not been well defined. In order to understand the mechanisms underlying immortal transformation and telomerase reactivation during human breast carcinogenesis, we have undertaken a long-term program to generate pathologically relevant models of human mammary epithelial cell (HMEC) immortalization. Our *in vitro* studies support the model described above [8,11–17].

2. Methods and results

HMEC have been obtained from reduction mammoplasty tissues showing no epithelial cell pathology. Cultured HMEC encounter two distinct proliferation barriers that must be compromised for finite lifespan HMEC to achieve immortality. A first barrier (stasis) is RB-mediated in response to various stresses, can be frequently overcome by alterations in pathways governing RB, and is not a response to critically short telomere lengths. Cells at stasis do not exhibit chromosomal aberrations and artificial introduction of hTERT, the catalytic subunit of telomerase, does not provide a growth advantage to these cell populations. In HMEC, stasis is associated with up-regulated expression of the cyclin-dependent kinase inhibitor $p16^{INK4}$, a low labeling index (LI), and cell arrest in G1. HMEC cultured in a serum-free medium can spontaneously down-regulate p16 expression, associated with methylation of the p16 promoter. The resulting p16(−) post-selection HMEC can grow for an additional 30–70 population doublings (PD), with ongoing telomere erosion due to the absence of telomerase activity, before encountering a second proliferation barrier.

As they approach this second proliferation barrier (agonescence or crisis), post-selection HMEC show evidence of telomere dysfunction. Gross chromosomal aberrations are seen 10–20 population doublings before cessation of net proliferation and include abundant telomeric associations, and chromosome fusion and breakage events. Cytogenetic analysis shows that 100% of metaphases eventually exhibit structural abnormalities. If p53 is functional, these aberrations induce a mostly viable arrest in all phases of the cell cycle, with a mean TRF (telomere restriction fragment)

length of ∼5 kb and a LI of ∼15%. Some mitotic failures and cell death also occurs. This molecular phenotype is distinct from stasis or crisis, prompting the use of a new term "agonescence", to distinguish it from the viable stasis arrest and the non-viable crisis. Agonescence is telomere length-dependent, and can be efficiently circumvented by exogenous introduction of hTERT. HMEC arrested at both stasis and agonescence exhibit a senescent morphology (large, flat, vacuolated cells) and express senescence-associated β-galactosidase activity, so these markers cannot be used to distinguish between these two distinct types of senescence arrest. Crisis occurs in cells that have lost p53 function and thus p53-dependent checkpoints, and is characterized by massive cell death and a LI of ∼40%.

Spontaneous transformation to immortality has not been observed in cells derived from normal human breast tissues. We postulate that at least two alterations are required to activate endogenous telomerase activity in cells that have overcome stasis. In cultured HMEC, the likelihood that all the necessary errors would occur in the same cell, even under conditions where widespread genomic errors are generated, is exceedingly small. However, if the telomere dysfunction barrier is approached in cells already harboring one error predisposing to immortality, the genomic instability resulting from telomere dysfunction can give rise to rare additional complementary errors that allow reactivation of endogenous telomerase activity and immortalization. We have overcome this stringent barrier and obtained immortally transformed HMEC lines by exposing cells cultured from normal tissues to a variety of pathologically relevant immortalizing agents, e.g., chemical carcinogens, oncogenes known to be overexpressed in human breast cancers (c-Myc, ZNF217), and inhibitors of p53 function. The rarity of immortal transformation in cells exposed to these agents, and the clonal origins of the immortal lines, suggests that the pre-existing error needs to be complemented by additional error(s) generated during the time of genomic instability.

Overcoming the telomere-length barrier in cultured HMEC appears to involve reactivation of telomerase, and amelioration of the prior genomic instability. However, newly immortal p53(+) HMEC lines initially display little or no detectable telomerase activity using a TRAP assay, due to the ability of p53 to repress telomerase in newly immortal HMEC. When telomeres become extremely short (mean TRF <3 kb) an extended process termed conversion ensues, wherein the p53-mediated repression of telomerase is gradually relieved and telomerase activity gradually increases. Immortal HMEC lacking functional p53 show initial telomerase activity. Fully immortal HMEC maintain telomeres at short, stable lengths.

The genomic stability of p53(+) and p53(−) HMEC transitioning through telomere dysfunction and telomerase reactivation has been examined by karyology, fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH). Instability was maximal during the period of telomere dysfunction, and was reduced thereafter in p53(+) lines. We have obtained one carcinogen-treated p53(+) HMEC line that immortalized prior to the onset of widespread genomic instability (initial mean TRF >5 kb). Newly immortal populations of this line show continued telomere erosion until a mean TRF of 2–3 kb, without evidence of genomic instability as assessed by karyotype analysis and CGH. Recent studies suggest that very low levels of telomerase activity are present in the newly immortal cells and may preferentially maintain the shortest telomeres. These short telomeres may consequently be present in a capped form not seen as damaged DNA, and thus not subject to NHEJ and the resulting BFB cycles.

3. Discussion

Our studies provide support for the model that a senescence barrier based upon telomere dysfunction drives chromosomal aberrations that can either restrain or promote further transformation, depending upon the cellular context. In most cells, telomere dysfunction provides a stringent limit to the number of replications a single lineage can undergo, and thus limits the opportunity for deleterious mutations to accumulate within that lineage. However, rare mutations predisposing to telomerase reactivation that do arise may be complemented by chromosomal aberrations generated by the telomere dysfunction occurring when telomeres become severely eroded. Our data suggest that overcoming telomere dysfunction involves reactivation of telomerase, which maintains telomeric ends and prevents them from making telomeric associations.

The generation of widespread chromosomal aberration at telomere-length dependent senescence may account for some of the genomic instability seen in human carcinomas, even those like breast cancer that are largely p53(+). We hypothesize that the degree of aneuploidy in transformed cell lines or tumors may depend upon the timing of the errors that allow telomerase re-

activation, rather than just on p53 status or other mutations that might specifically promote genomic instability. Cells that accumulate all the errors necessary for immortalization *after* the onset of telomere dysfunction may harbor karyotypic derangements that get perpetuated by ongoing BFB cycles. Cells that acquire all the necessary errors *prior* to the onset of telomere dysfunction could avoid the resultant gross genomic instability and become immortal with near-diploid karyotypes. This model is consistent with our small sample of immortally transformed HMEC lines.

The relevance of our *in vitro* model to human breast carcinogenesis is supported by recent *in vivo* observations of early stages of cancer progression. Studies using FISH to examine telomere length in human tumor tissues and pre-malignant lesions, such as ductal carcinomas *in situ* (DCIS), have shown that telomere shortening is one of the earliest and most prevalent molecular changes in tumorigenesis [4,5,18]. Evidence of genomic instability is also first observed during early stages of malignant progression of many organ types, including in breast DCIS [6,19,20]. While some further instability may occur during progression to primary and metastatic disease, the major emergence of instability occurs at pre-malignant stages. Breast cancers, like most epithelial-derived tumors, exhibit a high degree of karyotypic complexity. Although the manner in which genomic stability is compromised is presently not well defined, the type of instability shown, gross chromosomal rearrangements, is consistent with errors generated by double strand breaks, or NHEJ "repair" of uncapped telomeres. Assays for telomerase activity show that it too first becomes detected at the stage of DCIS (in $\sim$50%), and then increases to nearly 100% of primary tumors [21–25]. These data, especially their temporal order in carcinogenesis, are all consistent with the model that short telomeres induce telomere dysfunction, leading to genomic instability, which is then partially resolved by the reactivation of telomerase.

Our data has been generated utilizing our unique collection of HMEC lines immortalized using oncogenic agents/molecular derangements associated with breast cancer *in vivo*. We believe this is a critically important consideration if *in vitro* studies are to be used as models for clinical interventions. Most *in vitro* immortalization systems employ agents (e.g., SV40-T, HPV-E6/E7, H-ras) that have not been linked to breast cancer pathogenesis. Additionally, determining the actual derangements that occur during carcinogenesis to reactivate telomerase and thus ameliorate genomic instability can not be done in systems that bypass this question by artificial introduction of hTERT. Furthermore, short-lived animals do not exhibit stringent repression of telomerase even in normal tissues. Consequently, they normally express RB-mediated, non-telomere length dependent senescence, but not senescence associated with telomere dysfunction. We postulate that overcoming telomere dysfunction may be a rate-limiting step in human carcinogenesis. Understanding the mechanisms responsible for reactivation of endogenous telomerase during human carcinogenesis, as well as assaying potential therapeutic agents that target telomerase activity, may require examination of human cells *in vitro* and *in vivo*.

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MECHANISMS OF CHROMOSOME INSTABILITY RESULTING FROM TELOMERE LOSS

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Telomere loss can result in chromosome rearrangements associated with tumorigenesis. Human tumor cells commonly demonstrate spontaneous telomere loss despite the expression of telomerase, suggesting fundamental deficiencies in the ability to properly maintain telomeres. We have utilized selectable marker genes located adjacent to a telomere to investigate the nature of the chromosome rearrangements resulting from telomere loss in both mouse embryonic stem (ES) cells (MCB 22:4836) and human tumor cells (Neoplasia 2:540; Neoplasia 4:531). Mouse ES cells have a very low rate of spontanesous telomere loss, although telomere loss can be induced by transfection of expression vectors containing the I-SceI endonuclease, which specifically introduces a double-strand break at an 18-bp site located within the plasmid sequences. In contrast, the human tumor cell line has a high rate of spontaneous telomere loss despite the presence of telomerase activity, which is typical of many tumor cells due to their inability to properly maintain telomeres. Telomere loss induced by the I-SceI endonuclease in the mouse ES cells resulted primarily in the addition of new telomeres at the site of the break, while telomere addition resulting from spontaneous telomere loss was relatively infrequent event in the human tumor cells. In both cell types, chromosomes that did not acquire a new telomere underwent sister chromatid fusion and subsequent breakage/fusion/bridge (B/F/B) cycles. B/F/B cycles occur when the fused sister chromatids break during anaphase as the two centromeres are pulled in opposite directions, resulting in a chromosome without a telomere in the next cell cycle. Following DNA replication the sister chromatids fuse again, repeating the cycle. The B/F/B cycles in mouse ES cells were relatively short, lasting only a few cell cycles, while B/F/B cycles in human tumor cells continued for more than 20 cell generations. In both cell types, B/F/B cycles resulted in amplification of subtelomeric DNA sequences, and continued until the chromosome acquired a new telomere. The most common mechanism for telomere acquisition during B/F/B cycles in both cell types involved nonreciprocal translocations of the ends of other chromosomes. Although these translocations result in the acquisition of a new telomere and the stabilization of the marker chromosome, they transfer the instability to the chromosome that donated the telomere since it is now missing a telomere. Thus, chromosome instability resulting from the loss of a telomere can be passed sequentially from one chromosome to another. Telomeres can also be acquired by duplication, possibly involving break-induced replication, which unlike nonreciprocal translocation results in the net gain of a telomere and therefore results in stabilization of the genome. Direct addition of telomeres is a relatively rare event during B/F/B cycles even in the mouse ES cell line, and therefore direct addition appears to occur primarily when a break occurs in close proximity to a telomere. These results demonstrate that telomere loss can result in amplification and nonreciprocal translocations commonly associated with human cancer. Telomere loss can also lead to other forms of chromosome instability, including formation of double-minute chromosomes, complex rearrangements involving other chromosomes, and aneuploidy. B/F/B cycles have been shown to lead to the formation of double-minute chromosomes by looping out the amplified regions, which appear to be highly unstable, apparently due to the presence of the inverted repeat structures. Chromosomes involved in B/F/B cycles have were also found to fuse with other chromosomes, transferring the amplified regions to these chromosomes following breakage of the dicentric chromosomes. Finally, B/F/B cycles may lead to aneuploidy, since dicentric chromosomes may cause a failure of cytokinesis resulting in tetraploid cells that can progress to aneuploidy. B/F/B cycles can therefore result in a wide variety of chromosome changes in cancer cells in addition to the typical structures commonly expected for B/F/B cycles involving amplification of a gene on the end of the chromosome on which it was originally found.

ARE THERE DIFFERENT TYPES OF CANCER ANEUPLOIDY?

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1. Introduction

Aneuploidy is produced by an initial (2C) to (4C) polyploidization followed by chromosome loses and rearrangements to widely varying numbers and types of chromosomes. Aneuploidy formation has been modeled as a "random process" [1,2], implying that with continued division all cancers will eventually converge to similar chromosome compositions [3]. Such random models, however, do not easily explain the widely varying clinical behavior of aneuploid human cancers: For example, aneuploid neuroblastomas often spontaneously regress [4] while aneuploid pancreatic carcinomas are almost invariably fatal [5]. A wide spectrum of clinical outcomes is found for other aneuploid tumors [6–33].

We hypothesize that this heterogeneity of clinical outcomes for patients with aneuploid tumors may be in part due to the existence of different types of aneuploidy, some of which favor destabilization of the cancer genome and promote malignant progression and other types of aneuploidy which are more genetically stable and produce relatively indolent tumors. In support of this possibility, it has become recently apparent that all of the steps involved in the formation of aneuploid cells are controlled by diverse sets of gene networks controlling DNA replication and repair and transit through mitosis (reviewed in [34,35]). These gene networks may provide a complex genetic substrate for the production of different types of cancer aneuploidy: For example, the initial (2C) to (4C) tetraploidization step, felt to precede the formation of most aneuploid cells, could be either due to aberrations in the DNA synthesis complete-checkpoint, the S/G_2 DNA-damage checkpoint, the wait anaphase-checkpoint, the telophase exit pathway, and other, as of yet unidentified, genes responsible for mitotic transit in different aneuploid precursor cells. The particular pathway utilized for producing tetraploidy in a given cancer might exert a profound effect on its subsequent rate of chromosome loss and genetic stability. The chromosome loss or non-disjunction rate in a given tumor could also be influenced by various genetic defects in the mechanisms responsible for chromosome attachment to the mitotic spindle, the wait anaphase checkpoint, and so on. Finally, the number of recombinant and broken chromosomes in a given aneuploid cancer may be largely a function of its specific combination of genetic defects in the G_1/S-DNA damage checkpoint, the p53-controlled and/or other DNA-repair mechanisms, and abnormal activations, or sloppiness, of the chromosome recombination pathways, etc.

2. Methods

To test this hypothesis, karyotypes from the diploid CCD-34Lu fibroblast and the aneuploid A549 and SUIT-2 cancer lines underwent fluorescence *in situ* hybridization (FISH) and DAPI-counterstaining. Intact metaphase- (M), anaphase- (A) and telophase- (T) cells of these lines were also Feulgen-stained and individual DNA contents measured by image-analysis microscopy. The number of DAPI-stained and FISH-identified chromosomes, 1–22, X, Y, the M-, A-, and T-cell DNA measurements, and chromosome structural abnormalities were compared using the chi-square, Mann–Whitney rank sum, and the Levene's equality of variance tests.

3. Results

Virtually all of the evaluable diploid CCD-34Lu karyotypes had 46 chromosomes with two, normal appearing homologs (Figs 1A, D). The aneuploid chromosome numbers per karyotype were highly variable, averaging 62 and 72 for the A549 and SUIT-2 lines, respectively ($p < 0.01$, Figs 1B, C). However, the A549 chromosome numbers were more narrowly distributed than the SUIT-2 karyotypes chromosome numbers ($p < 0.01$). The averages and spreads of the intact M-cell DNA distributions exactly paralleled those of the DAPI-chromosome number distributions for the three lines, ruling out inadvertent selection bias being responsible for the differences in the aneuploid chromosome counts. Measurement of the DNA differences between intact A/T-cell chromosomal masses revealed that the diploid CCD-34Lu cells always had balanced mitoses. However, the aneuploid A549 and SUIT-2 cells often had uneven mitoses, with unequal partition-

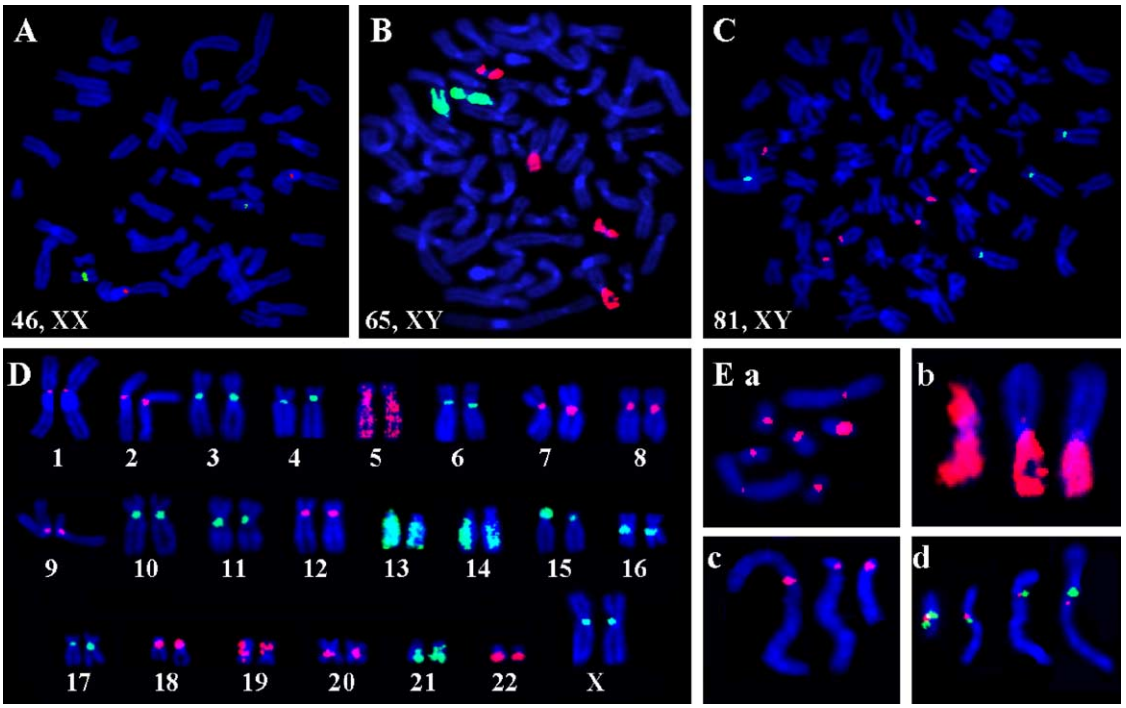

Fig. 1. Localization of chromosome specific FISH probes in DAPI-stained karyotypes. The diploid CCD-34Lu (A), and aneuploid A549 (B) and SUIT-2 (C) cell lines were hybridized with specific centromeric and paint DNA probes specific for chromosomes (1 red, 16 green), (19 red, 13 green) and (17 red, 4 green) respectively. (D) A composite karyotype of FISH-identified chromosomes of the diploid CCD-34Lu line. (E) FISH-localization's of (a) extrachromosomal fragments (ECF's) found in the aneuploid SUIT-2 line showing two normal centromeric localization's of the FISH probes for chromosome 1 and in five ECF's from the same karyotype. (b) Normal chromosome 19 paint localization next to chromosome 19 breaks and refusions to another chromosomes(s) found in the same karyotype of the aneuploid A549 line. (c) Centromeric localization of the FISH probe in a normal chromosome 2 and in several abnormally placed, telomeric regions classified as END's in the same karyotype of the A549 line. (d) Fusion's found in both aneuploid cell lines with centromeric chromosome probes labeled in red (r) and green (g) from left to right: 18r/16g, 7r/10g, 20r/9g, and 18r/10g. (Reproduced, with permission from Isaka, T., Nestor, A.L., Takada, T. and Allison, D.C. Chromosomal variations within aneuploid cancer lines. *J Histochem Cytochem*, **51**, 1343–53, 2003.)

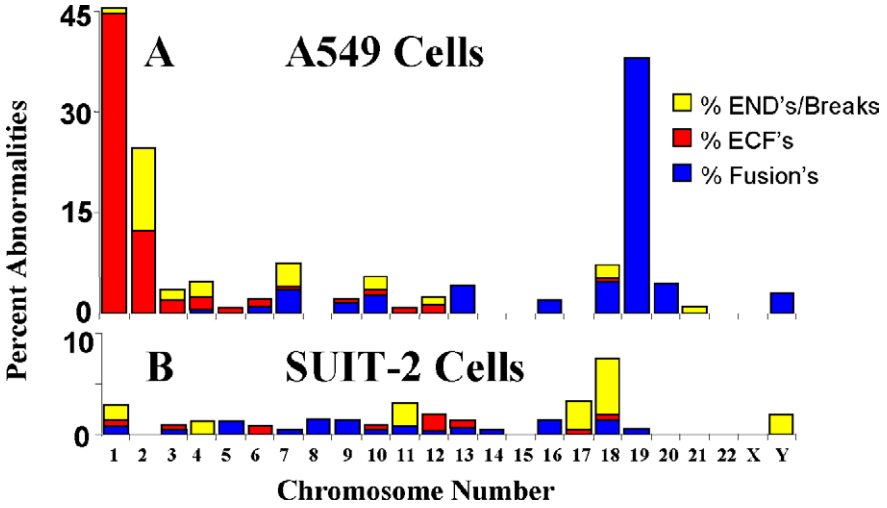

Fig. 2. The average percentages of FISH-identified chromosome abnormalities per individual chromosome in the (A) A549 and (B) SUIT-2 lines. (Reproduced, with permission from Isaka, T., Nestor, A.L., Takada, T. and Allison, D.C. Chromosomal variations within aneuploid cancer lines. *J Histochem Cytochem*, **51**, 1343–53, 2003.)

ing of DNA into the daughter chromosomal masses. However, uneven mitoses were more frequent and of a greater magnitude in the SUIT-2 line than the A549 line ($p < 0.01$). There were no FISH-identified structural abnormalities in the diploid CCD-34Lu chromosomes (Fig. 1D). Surprisingly, 25% of the A549 chromosomes had FISH-detected, structural abnormalities (Fig. 1E), compared to only 7% of the SUIT-2 chromosomes (Figs 2A, B).

4. Conclusions

It is apparent that the chromosomal compositions and mitotic abnormalities of the long term, aneuploid A549 and SUIT-2 cancer lines are widely divergent. These results suggest that differing genetic changes in the cell cycle, DNA repair, recombination, and mitotic transit gene networks of these lines, rather than chance, govern their chromosome makeups. If this turns out to be the general case, it may prove that varying genetic defects in these gene networks between different tumors also contribute to the wide spectrum of clinical aggressiveness of aneuploid human cancers.

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EXPLOITING MICROARRAY MEASUREMENTS TO UNDERSTAND THE GLOBAL EFFECTS OF ANEUPLOIDY IN CANCER

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Aneuploidy involves the rearrangement of the entire genetic information of the cell. This is expected to translate into global changes responsible for malignancies at various levels of the intracellular regulatory network, including the level of gene expression patterns. Understanding this connection, however, is not an obvious task [1]. Chromosomal gains, losses and rearrangements will obviously have a direct effect on gene expression levels but these directly induced changes need to retain the self-consistent nature of the entire genetic network of the cell. In other words, the expression level of each gene needs to be consistent with the expression level of its regulatory inputs. In order to understand the effect of aneuploidy on global gene expression patterns we need to develop our understanding on the ploidy regulation of gene expression and develop methods that take into account the various feedback loops influencing this direct effect.

Microarray technology allows us to measure the expression levels of thousands of genes but this information needs to be reassembled in a fashion that would produce testable biological hypotheses. We are currently developing computational approaches that would allow us to determine the relevant gene expression modules in cancer and their inferred regulatory network in a probabilistic fashion. We will review our initial results on two human cancer related models.

In our first set of experiments we aimed to correlate aneuploidy and gene expression patterns with the phenotype of normal, immortal and neoplastic breast epithelial cells. We have measured gene expression patterns in normal primary breast epithelium, immortalized non-malignant cells, *in vitro* transformed cells and breast cancer cell lines. We have also analyzed these cells by complementary genomic hybridization or SKY analysis when appropriate. Immortalized cells harbor several chromosomal translocations with a (near) normal chromosomal number and with already a significant number of differentially expressed genes relative to normal cells. During malignant transformation the cell loses its ability to maintain a normal "centromere-count" and a new burst in gene expression changes appear. Our initial analysis was aimed at understanding the source of this second wave of gene expression changes.

One of the ultimate goals of our approach is to study cancer cells as a continuously mutating genetic network under evolutionary pressure.

We will also present microarray based results on a recently established model system in which aneuploidy was effectively reversed in human cancer [2]. The reversal of aneuploidy was due to the selective apoptosis of cells with abnormal chromosome numbers. In other words the cells regained their ability to "count centromeres". The more normal karyotype of the cells was accompanied by more normal phenotypic behavior. Microarray measurements revealed that the expression levels of significant number of genes returned to a more normal level, thus the reversal of aneuploidy showed a very strong correlation with reversal to more normal gene expression patterns.

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ELEVATION IN GENOMIC INSTABILITY FOLLOWING EXPOSURE OF CELLS TO NON-IONIZING THZ RADIATION

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1. Introduction

Recent technological break-through in electromagnetic radiation sources, components and devices, in the THz region (which spans the spectral interval between the microwave- and the infrared regions of the electromagnetic spectrum) has triggered new applications in the field of material science, biology and biomedicine. Particularly, biological applications are based on the specific spectroscopic fingerprints of biological matter in the far infrared and tera-hertz (THz) spectral regions, due to the high density of electronic, vibrational and rotational states. In addition, the different values of the absorption coefficient and index of refraction of the water and the tissue carbonated proteins at such frequencies, provide a unique contrast mechanism for biomedical imaging applications. In order to examine the safety of being exposed to THz radiation we have studied genetic and epigenetic changes induced in lymphocytes following exposure to radiation at 100 GHz.

2. Methods

The exposure to 100 GHz radiation was carried out in a specially designed exposure system, maintaining a temperature of 37°C. Since the penetration depth of radiation at 100 GHz into a water suspension of lymphocytes is very short (∼0.13 mm), the most efficient way to illuminate uniformly the lymphocyte cells lying, due to gravity, at the bottom of the culture flasks was by illuminating them from the bottom. The power density of the 100 GHz radiation at the bottom of the flask was 0.05 mW/cm^2 which corresponds to specific absorption rate of 3.2 mW/gr (where the international guidlines limit exposure to a value of 1 mW/cm^2 for the general population and to 5 mW/cm^2 for occupational exposure). Lymphocytes isolated from peripheral blood were irradiated for 1, 2 and 24 hours and were harvested by common cytogenetic procedures 69 to 72 hours after the onset of exposure. The genetic and epigenetic markers for genomic instability were the increase of the levels of aneuploidy and replication synchrony, respectively. These parameters were evaluated by interphase FISH based cytogenetics. We scanned slides of nuclei, derived from the exposed and appropriate control cultures, hybridized with probes specific for the centromeric regions of chromosomes 11 (orange labeled; Vysis, USA) and 17 (green labeled; Vysis, USA) using the Metafer platform for semi-automatic interphase FISH scoring. Cells were scored automatically; the gallery was then manually corrected by two independent technicians. Between 800 and 1100 cells were scored from each culture. The Metafer platform automatically presents the results obtained for the levels of chromosomal gains and losses for each locus plus a correlation between the two loci. The subset of cells which had two hybridization signals for both signals, were manually analyzed for the pattern of replication of 600 cells.

3. Results

The exposure of lymphocytes to THz radiation induced increase in both replication asynchrony and aneuploidy after two hours and 24 hours of exposure, but no effect was observed after one hour radiation. The aneuploidy level, in the exposed cultures, increased by 37% for centromere of chromosome 17 (CEN17) and by 50% for centromere of chromosome 11 (CEN11) compared to the control cultures. The level of aneuploidy observed in the control cultures was 9.4 $\pm$ 2.5% and 7.2 $\pm$ 2.5% for CEN17 and CEN11, respectively. After two hours of exposure the levels of aneuploidy levels increased to 12.8 $\pm$ 3.7% and 9.5 $\pm$ 2.9%, respectively ($p < 10^{-3}$). These levels increased slightly more after 24 hours of exposure to 13.1 $\pm$ 3.6% and 11.8 $\pm$ 2.5%, respectively ($p < 10^{-2}$). When analyzing the frequency of asynchronous replication in these cultures we observed an even more pronounced effect. While two hours of exposure led to an increase of about 56% in the frequency of asynchronous replication of each of the two loci tested, 24 hours of exposure led to a 108%

and 71% increase for CEN17 and CEN11, respectively. The levels of asynchronous replication in the control cultures were $14.7 \pm 3.3\%$ and $14.4 \pm 3.5\%$ for CEN17 and CEN11, respectively. After exposure these levels reached $24.7 \pm 5.3\%$ after two hours and $28.5 \pm 4.7\%$ after 24 hours for CEN17 ($p < 10^{-5}$). For CEN11, after two hours of exposure asynchronous replication was observed in $22.4 \pm 4.9\%$ of cells and was $24.6 \pm 5.0\%$ after 24 hours of exposure ($p < 10^{-4}$).

4. Discussion

Both genotoxic and epigenetic effects are induced in lymphocytes following exposure to CW 100 GHz radiation of 0.05 mW/cm^2 when exposure period exceeds one hour. The induced effects seem to saturate already for short exposures and occur at power density much lower than those set by the international guidelines for exposure limits.

REVISITING G1-PHASE ARREST AND SYNCHRONIZATION BY LOVASTATIN USING VIDEOGRAPHIC TIME-LAPSE ANALYSIS OF INTERDIVISION TIMES

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It has been proposed that lovastatin arrests cells in the G1-phase of the division cycle, and that release from lovastatin inhibition produces a synchronized culture [1]. A new method of methocel-time-lapse-videography is introduced to analyze cell division patterns following lovastatin treatment. Time-lapse videomicroscopic analysis of the cell division pattern following release of L1210 cells from lovastatin inhibition indicates that the released cells are not synchronized. Moreover, lovastatin does not appear to arrest cells with a G1-phase amount of DNA as has been proposed. Analysis of previously published synchronization and growth-arrest experiments supports these experimental results. It is concluded that lovastatin neither synchronizes cells, nor arrests cells in the G1-phase of the division cycle [2].

A reconsideration of whole-culture approaches to synchronization indicates that such methods, in theory, cannot synchronize cells. This is because whole culture methods do not lead to a narrowing of the size distribution of cells. Thus, cells may be arrested with a particular DNA content (e.g., a G1-phase amount of DNA) but this uniform DNA content does not mean the cells represent cells at a point during the division cycle because the cells do not have a size representative of the normal cell size at a particular time during the cell cycle. Consideration of the three most common methods of whole-culture synchronization – arrest with a G1-phase amount of DNA, inhibition of DNA synthesis, and inhibition of mitosis – reveals that all of these methods do not work to produce cells representative of cells at any particular point during the normal cell division cycle [3,4].

These results are consistent with proposal that batch methods of synchronization (i.e., those that treat all cells equally) cannot, in theory, synchronize cells.

A general analysis of cell-cycle controls have been presented in recent reviews [5,6].

Additional relevant results may be seen at www.umich.edu/∼cooper.

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STATISTICAL ANALYSIS OF PROPOSED HUMAN CELL-CYCLE-SPECIFIC AND YEAST CELL-CYCLE-SPECIFIC GENE EXPRESSION PATTERNS BASED ON MICROARRAYS

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Using a statistical approach, we re-examine the proposal that numerous genes in human cells are expressed with a periodicity consistent with cell-cycle-specific expression [1]. The postulation of these cyclically expressed genes was based on microarray analysis of thousands of genes on Affymetrix chips. The degree to which periodic patterns are present in the published data is consistent with random fluctuations in the experimental points, with no underlying biologically-based temporal periodicity [2]. There is no need to postulate cell-cycle-specific expression patterns to explain the microarray data. Among our observations is evidence that there is no periodic appearance of peaks over the proposed two generations of synchrony that cannot be accounted for by random noise and experimental variation as randomized data exhibit periodic patterns similar to the actual experimental data, peak amplitude does not decay in the second period as expected if cells were actually synchronized, and a comparison of two replicate experiments shows that data for genes identified as having periodic expression is not reproducible either in peak height or phase location. We conclude that the published microarray data does not support the proposal that in human cells there are numerous cell-cycle-specifically-expressed genes [2].

Microarray analysis of gene expression during the yeast division cycle has led to the proposal that a significant number of genes in *Saccharomyces cerevisiae* are expressed in a cell-cycle specific manner [3]. Four different methods of synchronization were used for cell-cycle analysis. Randomized data exhibit periodic patterns of lesser strength than the experimental data. Thus the cyclicities in the expression measurements in the four experiments presented do not arise from chance fluctuations or noise in the data. However, when the degree of cyclicity for genes in different experiments are compared, a large degree of non-reproducibility is found [4]. Re-examining the phase timing of peak expression, we find that three of the experiments (those using α-factor, CDC28, and CDC15 synchronization) show consistent patterns of phasing, but the elutriation synchrony results demonstrate a different pattern than the other arrest-release synchronization methods. Specific genes can show a wide range of cyclical behavior between different experiments; a gene with high cyclicity in one experiment can show essentially no cyclicity in another experiment. The elutriation experiment, possibly being the least perturbing of the four synchronization methods, may give the most accurate characterization of the state of gene expression during the normal, unperturbed cell cycle. Under this alternative explanation, the observed cyclicities in the other three experiments are a stress response to synchronization, and may not reproduce in unperturbed cells [4].

A complete review of a large number of microarray analyses of gene expression during the cell cycle of different eukaryotic cell types has been published [5].

Additional relevant results may be seen at www.umich.edu/∼cooper. Also, the papers listed here can be read directly at this site.

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PHOSPHORYLATION–DEPHOSPORYLATION OF RETINOBLASTOMA PROTEIN IS NOT NECESSARY FOR PASSAGE THROUGH THE MAMMALIAN CELL DIVISION CYCLE

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Phosphorylation of the Retinoblastoma protein (Rb) during the G1 phase of the mammalian cell division cycle is currently believed to be a controlling element regulating the passage of cells into S phase [1–8]. We find, however, that the suspension-grown cell lines U937, L1210, and MOLT-4 contain exclusively hyperphosphorylated Rb. Rb protein is phosphorylated even in the newborn cells just produced by division. Furthermore, when adherent NIH3T3 cells are grown at very low densities to avoid overgrowth and contact inhibition, these adherent cells also contain only hyperphosphorylated Rb. NIH3T3 cells exhibit hypophosphorylation when the cells are grown at moderate to high cell densities. Non-adherent cell lines grown to high densities also dephosphorylate the Rb protein. We propose that cultures of adherent cells such as NIH3T3, when grown to moderate cell densities, are made up of two populations of cells: (a) cells that are relatively isolated and therefore growing exponentially without contact inhibition; these cells have phosphorylated Rb protein, and (b) cells that are growth-inhibited by local cell density or contact inhibition; these cells have dephosphorylated Rb protein. The observation in adherent cell lines that Rb is both hyper- and hypophosphorylated in the G1 phase and only hyperphosphorylated in the S- and G2-phases, is thus explained by the effects of cell density and contact inhibition. When adherent cells are grown to moderate densities there are two populations of cells. Growth inhibited cells have a G1-phase amount of DNA and dephosphorylated Rb protein while growing cells exhibit a range of DNA contents (G1-, S-, and G2/M-phase DNA contents). It thus appears that there is a phosphorylation event occurring in the G1 phase because two types of Rb protein are present in cells with a G1-phase amount of DNA while only phosphorylated Rb is found in cells with S- and G2/M-phase DNA contents. Phosphorylation–dephosphorylation of Rb protein during the G1 phase is not a necessary process during the NIH3T3, L1210, MOLT-4, and U937 division cycles. We propose that phosphorylation–dephosphorylation of Rb is independent of the division cycle and is primarily determined by growth conditions throughout the division cycle [1–3].

It may be argued that the cells studied in these experiments, non-adherent, leukemic cells of mouse and human origin, may be mutated in such a way as to have lost their "normal" control system for Rb phosphorylation. In answer to this critique it should be noted that all of the cells studied have a normal cell cycle, grow normally, and do not appear aberrant in any aspect of growth. The point of the experiments performed on Rb protein is that this is a case of normal cell growth without any need or requirement for cyclic phosphorylation/dephosphorylation. More to the point, the experiments on the effect of growth density on NIH3T3 cells provides a simple explanation of why many researchers have obtained results that have been interpreted as supporting a cycle-specific pattern of Rb phosphorylation. If cells are not grown so as to avoid all growth inhibition, one can get a mixture of cells that are growing and that are growth arrested. The growing cells would have Rb phosphorylated in all phases of the cell cycle. The non-growing cells would have a G1-phase amount of DNA and coincidentally (i.e., independent of the DNA content) have dephosphorylated retinoblastoma protein. Analysis of cells by phase would indicate two types of Rb protein, phosphorylated and dephosphorylated in the cells with a G1-phase amount of DNA while there would be only phosphorylated Rb in cells with S and G2/M phase amounts of DNA. Other experiments on cells "synchronized" (i.e., treated in such a way as to have a particular DNA content) by whole-culture synchronization methods have been analyzed. These results are consistent with the proposals made by the continuum model.

These results suggest that the postulation of Rb phosphorylation as an archetypal G1-phase event needs to be reevaluated.

Additional relevant results may be seen at www.umich.edu/∼cooper.

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SPECIFIC ANEUSOMIES IN CHINESE HAMSTER CELLS AT DIFFERENT STAGE OF NEOPLASTIC TRANSFORMATION, INITIATED BY NITROSOMETHYLUREA

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We have recently proposed that carcinogens initiate carcinogenesis by inducing random aneuploidy which autocatalytically evolves cancer-specific karyotypes. Here, three predictions of this hypothesis were tested: *Prediction 1*, Carcinogens generate aneuploidy. Indeed, up to 90% of Chinese hamster embryo (CHE) cells were rendered aneuploid by Nitrosomethylurea (NMU) prior to neoplastic transformation, although NMU is thought to cause cancer by "direct mutagenesis". *Prediction 2*, Aneuploidy generates neoplastic karyotypes autocatalytically, because it renders mitosis error-prone, by unbalancing spindle proteins, and chromosomes unstable, by unbalancing cooperating repair enzymes. This evolution is accelerated by supplemental carcinogens. Accordingly 90 foci of transformed cells appeared 10 weeks after the first of six NMU-treatments of 2 million CHE cells. After latencies of 7 to 21 weeks these transformed cells generated tumors in syngeneic hamsters. *Prediction 3*, Specific aneuploidies generate the multiple stages of neoplastic transformation, e.g. transformation *in vitro* versus tumors in our system. Surely, 79% of the cells transformed *in vitro* were trisomic for chromosome 3, and 59% were monosomic for chromosome 10, compared to 8% expected for random distribution of any aneusomy among the 12 CH chromosomes. Moreover, 52% shared both trisomy 3 and monosomy 10, compared to 0.6% expected for random distribution of any two aneusomies. Among the tumor cells, 65% were trisomic for chromosome 3, 51% were trisomic for chromosome 5, and 30% shared both trisomies. The specific aneusomies of tumors included structurally altered chromosomes with segmental aneuploidy. Thus the two stages of transformation have common and distinct, specific aneusomies. Surprisingly the same aneusomies have been observed by others in tumorigenic CH cells, decades ago, but have been abandoned as causes of transformation. We conclude that transformation-specific aneuploidy is either necessary, or necessary and sufficient for neoplastic transformation.

ELEVATED LEVELS OF TETRAPLOID CERVICAL CELLS IN ASCUS HPV-POSITIVE PAP SMEARS

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1. Introduction

The diagnosis of an Atypical Squamous Cells of Undetermined Significance (ASCUS) Pap smear is by definition vague and ambiguous. It has no histologic counterpart and conveys only that the patient has cytologic findings that may or may not be associated with a cervical lesion [1]. Because clear diagnostic information is not obtained from an ASCUS Pap smear, the proper follow-up and management of women diagnosed with ASCUS cells is controversial [2]. A conservative "wait and see" approach is often recommended as cervical cancer is a relatively slow developing disease [3], and only a small proportion of women diagnosed as ASCUS are determined to harbor advanced high grade lesions upon subsequent colposcopy or biopsy [4]. Conversely many women often undergo additional costly and invasive procedures (colposcopy and biopsy) in an attempt to ensure that the quasi-normal, quasi-abnormal cells detected on an ASCUS smear are not representative of a cancerous lesion. The ramifications of over-treatment are enormous as approximately 2.5 million women are diagnosed with an ASCUS Pap smear annually in the United States [1]. Recent efforts have attempted to use the presence of Human Papilloma Virus (HPV) DNA to identify women with elevated risks of developing cervical lesions. Epidemiological evidence clearly indicates that persistent HPV infection is the number one etiological agent associated with the development of cervical cancer [5]. Several studies have demonstrated that women originally diagnosed with an ASCUS Pap smear and who are subsequently shown by colposcopy and biopsy to have a high-grade lesion are almost always infected with HPV [6], suggesting that HPV triage is a very sensitive method to detect high-grade lesions. However, the discrimination between women who have a high grade lesion and those who do not using solely an ASCUS HPV-positive Pap smear result becomes problematic as almost 20% of all normal asymptomatic women are HPV positive [7]. Some have questioned the value of using HPV testing as a secondary diagnostic tool to the ASCUS Pap smear [8] due to its low specificity in identifying women who will develop neoplastic lesions [9]. Considering that most HPV infections regress spontaneously [10], even among the majority of women diagnosed with equivocal or abnormal cervical smears [11], the triage of women based upon infection with HPV may not be the best option. A biomarker of an additional molecular event further along the pathway to cervical carcinogenesis may provide more specificity in the diagnosis and treatment of the disease [12]. Numerical chromosomal abnormalities such as tetraploidy and aneuploidy frequently accompany cervical cancer development and are believed to represent early and important genetic events during cervical carcinogenesis. We have previously shown that tetraploidy is a transient and genetically unstable intermediate which through chromosomal loss can lead to the aneuploid lesions characteristic of neoplastic cervical lesions [13]. The identification of elevated levels of numerical chromosomal aberrations in women diagnosed with ASCUS Pap smears may therefore be of prognostic value.

2. Methods

We used multiple probe fluorescence *in situ* hybridization (FISH) to simultaneously analyze chromosomes 3 & 17 in 1000 cervical epithelial cells from each of 257 different women for the presence of numerical chromosomal aberrations. HPV status was determined using either Digene's Hybrid Capture II test or using polymerase chain reaction (PCR) to simultaneously amplify the HPV L1 and human β-globin genes. The MY09 and MY11 primers were used to amplify HPV DNA and enable the identification of at least 20 different HPV types.

3. Results

A statistically significant increase in the proportion of women diagnosed as ASCUS HPV-positive were determined to have elevated levels of tetraploid cervical cells (5/69) as compared to the Normal HPV-negative women (0/75; p-value of 0.02). A significant association also exists between the presence of elevated levels of numerical chromosomal aberrations

(both tetraploidy and aneuploidy) and HPV infection when the analysis is performed irrespective of diagnostic categories. 10/127 HPV-positive patients exhibited elevated levels as compared to 1/130 HPV-negative patients (p-value of 0.005). Similar results were also observed for aneuploidy (6/127 for HPV-positive and 0/130 for HPV-negative; p-value of 0.014).

4. Discussion and conclusions

Consistent with expectations, the frequencies of numerical chromosomal aberrations in cervical cells obtained from the majority of women diagnosed with an ASCUS Pap smear do not differ significantly from those diagnosed as Normal. A modest but statistically significant proportion of women diagnosed as ASCUS HPV-positive, however, did have elevated levels of tetraploid cervical cells. The incidence of ASCUS diagnosed women exhibiting elevated frequencies of tetraploid cells is similar to the proportion of ASCUS diagnosed women who are believed to harbor more serious lesions. Furthermore, our results suggest that these numerical chromosomal aberrations are the consequence of HPV infection and as such may provide a more specific diagnostic tool to identify women with elevated risks of developing cervical lesions. These results are consistent with our hypothesis that tetraploidy is a transient and genetically unstable intermediate in the development of cervical cancer that may be used to identify women diagnosed with ASCUS Pap smears that have an elevated risk of developing cervical cancer.

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CHROMOSOME TRANSFER INDUCED ANEUPLOIDY RESULTS IN COMPLEX DYSREGULATION OF THE CELLULAR TRANSCRIPTOME IN NORMAL IMMORTALIZED AND DIPLOID CANCER CELLS

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1. Introduction

Aneuploidy is a consistent genetic alteration of the cancer genome [1–4]. When the first quantitative measurements of the DNA content of cancer cells were performed, aneuploidy was defined as a variation in nuclear DNA content in the population of cancer cells within a tumor [5]. With increased resolution of cytogenetic techniques, such as chromosome banding comparative genomic hybridization [6], and spectral karyotyping and m-FISH [7,8] it has become clear that in addition to nuclear aneuploidy, specific non-random chromosomal imbalances exist. Indeed, despite genetic instability in cancer genomes, cancer cell populations as a whole display a surprisingly conserved, tumor specific pattern of genomic imbalances [4,9]. At early steps in the sequence of malignant transformation during human tumorigenesis, i.e. in pre-invasive polyps or dysplastic lesions, such chromosomal aneuploidies can be the first detectable genetic aberration [1,4,10]. This suggests that there is both an initial requirement for the acquisition of specific chromosomal aneuploidies, and a requirement for the maintenance of these imbalances despite genomic and chromosomal instability. This would be consistent with continuous selective pressure to retain a specific pattern of chromosomal copy number changes in the majority of tumor cells.

In cervical carcinomas, for instance, the gain of chromosome 3q is present in more than 85% of all cases [10]. This specific chromosomal imbalance occurs already in premalignant lesions [11]. When using interphase cytogenetics with BAC clones for chromosome 3q it has become clear that a considerable percentage of moderately and severely dysplastic lesions contain clonal cell clusters that have acquired additional copies of this chromosome. This aneuploidy can occur in either diploid or tetraploid cells [12]. Similarly, one of the first genomic alterations observed in colonic polyps is trisomy of chromosome 7. In invasive carcinomas, this aberration is maintained, however, additional copy number imbalances of chromosomes 8, 13, and 20 occur [1,4].

The conservation of these tumor and tumor-stage specific patterns of chromosomal aneuploidies suggests that they play a fundamental biological role in tumorigenesis. It remains, however, unresolved how such genomic imbalances affect global gene expression patterns. One could postulate that expression levels of all transcriptionally active genes on trisomic chromosomes would increase in accordance with the chromosome copy number. Alternatively, changing the expression level of only one or a few genes residing on that chromosome through tumor specific chromosomal aneuploidies may be the selective advantage necessary for tumorigenesis. That would require the permanent transcriptional silencing of most of the resident genes.

2. Methods

Methodology to analyze the consequences of chromosomal imbalances in tumor genomes has become available through the development of microarray based gene expression profiling, yet the few reports that specifically address this problem come to quite different conclusions [13–16]. Due to the large number of chromosomal aberrations usually found in cancer cells, it is difficult, if not impossible, to identify the consequences of specific trisomies, independent from other co-existing genomic imbalances and gene mutations. In order to develop a model system that allows direct correlation of acquired chromosome copy number alterations with transcriptional activity in genetically identical cells, we have used microcell mediated chromosome transfer. The introduction of three different chromosomes into karyotypically diploid, mismatch repair deficient colorectal cancer cells and into immortalized normal breast epithelial cells allowed an assessment of the consequences of specific aneuploidies on global gene expression levels relative to their diploid parental cells.

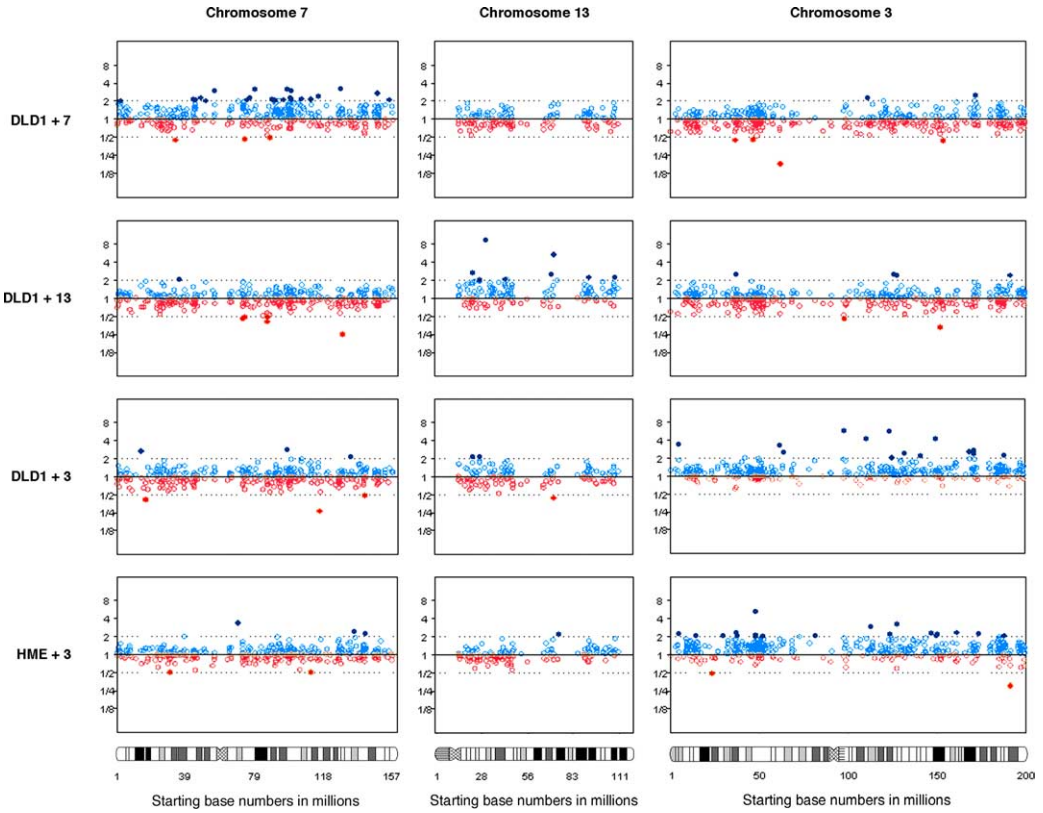

Fig. 3. Global gene expression profiles.

3. Results

Our results show that, regardless of chromosome or cell type, chromosomal trisomies result in a significant increase in the average transcriptional activity of the trisomic chromosome. Figure 1 shows the distribution and expression levels of all genes mapped on each of the three introduced chromosomes. In all four clones, the expression of all genes on the trisomic chromosome is slightly increased and very few genes were found to have very high expression ratios. Table 1 shows the expression ratio values averaged across all genes on a particular chromosome. The introduction of chromosome 3, 7 or 13 in the DLD1 cell line or chromosome 3 in immortalized mammary epithelial cell line resulted in a significant increase in average gene expression on only the trisomic chromosome (Ratio.3vs.0 $= 1.17$, $p < 0.0001$; Ratio.7vs0 $= 1.19$, $p < 0.0001$; Ratio.13vs0 $= 1.26$, $p = 0.0009$; Ratio3vs.0 $= 1.20$, $p < 0.0001$, respectively). This increase is accompanied by an increase in the expression of genes on other chromosomes as well, with trisomy of chromosome 7 in the diploid colorectal cancer cell having the most pronounced effect.

4. Conclusions

First, alterations in the copy number of whole chromosomes results on average in an increased expression of transcriptionally active genes residing on that chromosome. Second, chromosomes not observed to be aneuploid in particular tumor types (i.e. chromosome 3 in colorectal tumors) also have increased transcriptional activity when placed into that cellular environment. Thus, their presence is not neutral with respect to the transcriptome, and in fact the resulting increased expression of certain genes residing on those chromosomes (such as tumor suppressor genes) may explain why they are selected against. Third, aneuploidy not only affects gene expression levels on the chromosomes present in increased copy numbers, but a substantial number of genes residing on chromosomes other than that which has been introduced are significantly increased or decreased. We therefore postulate that the genomic imbalances observed in cancer cells exert their affect through a complex pattern of transcriptional dysregulation.

Table 2
Average gene expression profiles by chromosome

Chr.	Ratio.0	Ratio.7vs0	p.7vs0	Ratio.13vs0	p.13vs0	Ratio.3vs0	p.3vs0	Ratio.0	Ratio.3vs0	p.3vs0
1	0.96	1.03	0.0242	1.01	–	0.99	–	0.98	1.02	–
2	0.98	1.03	–	1.00	–	1.00	–	1.00	0.98	–
3	0.92	0.98	–	1.00	–	1.17	**<0.0001**	0.98	1.20	**<0.0001**
4	1.04	0.98	–	0.98	–	0.98	–	1.11	0.96	–
5	0.93	1.00	–	0.99	–	1.02	–	0.99	0.99	–
6	0.93	1.05	0.0183	1.02	–	0.99	–	0.94	0.96	–
7	0.86	1.19	**<0.0001**	0.98	–	0.99	–	0.88	1.02	–
8	0.93	1.02	–	1.02	–	1.01	–	1.00	0.98	–
9	0.96	1.00	–	1.00	–	0.98	–	0.96	1.00	–
10	1.01	1.01	–	0.99	–	1.00	–	1.03	1.01	–
11	0.92	1.01	–	0.97	–	1.00	–	0.95	0.99	–
12	0.92	1.05	0.0147	0.99	–	1.00	–	0.93	0.99	–
13	0.99	1.01	–	1.26	**0.0009**	1.04	–	1.11	0.99	–
14	0.92	1.02	–	1.01	–	1.02	–	0.93	1.00	–
15	0.90	1.03	–	1.02	–	1.01	–	0.97	1.01	–
16	0.92	1.05	–	1.01	–	0.99	–	0.87	1.00	–
17	1.00	1.06	–	1.02	–	0.99	–	0.98	0.98	–
18	1.03	1.01	–	1.04	–	1.01	–	1.19	0.94	–
19	0.94	1.02	–	0.95	–	0.99	–	0.94	1.01	–
20	0.87	1.01	–	1.01	–	0.99	–	0.93	1.00	–
21	0.89	1.04	–	1.03	–	0.99	–	0.87	1.00	–
22	0.94	1.04	–	0.98	–	0.97	–	0.91	1.00	–
X	0.79	1.03	–	0.99	–	0.99	–	0.85	1.02	–
Y	1.10	1.00	–	0.99	–	0.74	0.0002	0.99	0.99	–

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Chromosomal instability, aneuploidy, and gene mutations in human sporadic colorectal adenomas

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Abstract. Whether *in vivo* specific gene mutations lead to chromosomal instability (CIN) and aneuploidy or viceversa is so far not proven. We hypothesized that aneuploidy among human sporadic colorectal adenomas and *KRAS2* and *APC* mutations were not independent. Additionally, we investigated if 1p34–36 deletions by dual target FISH were associated with aneuploidy. Among 116 adenomas, 29 were DNA aneuploid by flow cytometry (25%) and 29 were *KRAS2* mutated (25%). *KRAS2* mutations were associated with aneuploidy ($P = 0.02$). However, while G–C and G–T transversions were strongly associated with DNA aneuploidy ($P = 0.007$), G–A transitions were not. Within a second series of 61 adenomas, we found, instead, that *APC* mutational status and aneuploidy by flow cytometry were not associated. However, a statistically significant association was found with specific *APC* mutations, i.e., occurring in the mutation cluster region (MCR, codons 1200–1500) or downstream ($P = 0.016$). Finally, the correlation of 1p34–36 deletions with flow cytometric and FISH detected aneuploidy was also significant ($P = 0.01$). Specific *KRAS2* and *APC* mutations and loss of genes in the 1p34–36 region appear associated with aneuploidy suggesting that these events are not independent and may cooperate in inducing human sporadic colorectal adenomas. A cause effect relationship between gene mutations and aneuploidy remains, however, to be demonstrated.

Keywords: Aneuploidy, chromosomal instability, oncogenes, tumor suppressor genes

1. Introduction

Most human solid tumors show a plethora of recurrent numerical and structural chromosomal aberrations [13,19] (see also The Mitelman Database of Chromosome Aberrations in Cancer at http://cgap.nci.nih.gov/Chromosomes/Mitelman), which revealed distinct and converging pathways of karyotypic evolution by multivariate analyses [16]. Losses or gains of defined chromosomal regions or loss of heterozygosity (LOH) were observed in human sporadic colorectal adenomas of very small size (range 1–3 mm) [28]. These findings are in agreement with other studies performed with independent techniques such as G-banding karyotyping [2], DNA content flow cytometry [9], interphase fluorescence in situ hybridization (FISH) [3,6,14], and gain/loss of DNA by comparative genomic hybridization (CGH) [15,25], that allowed detection of both numerical and structural chromosome aberrations. Gains of chromosomes 7, 13 and 20, loss of chromosome 18, and deletion of 1p among human sporadic colorectal adenomas are well documented [2,3,14,17]. Among 70 cases examined in a study by Bomme et al. [3] using FISH with pericentromeric region probes, gains of chromosomes 7, 13 and 20 were present respectively at 34%, 44%, and 32%. The median proportion of cells with trisomy was larger than 50%. Parallel G-banding karyotyping of 64 cases was in good agreement with these data. In particular, trisomy 7 was detected in non-neoplastic cells [17] and was present alone in 5 adenomas [2], suggesting a pathogenetic role of trisomy 7 in colorectal tumorigenesis. FISH was also used by Herbergs and colleagues [14] to detect trisomy 7 as the most frequently occurring chromosome numerical aberration in sporadic colorectal adenomas (13/35 cases, 37%). Ried and collaborators [25] found trisomy 7 by CGH in 5/26 (19%) cases of sporadic colorectal adenomas, while the incidence of 7+ in the study of Hermsen et al. by CGH [15] was more than 40%, but limited to the adenomatous components of adenomas which progressed into early cancer. Several investiga-

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tions by G-banding and FISH reported rearrangements of chromosome 1 and, in particular, deletions of its short arm, as one of the most common early structural changes in colorectal adenomas [3,6]. Allelic imbalances at *APC* locus also represent an early event since they were observed in adenomas with an average size of 2 mm at a frequency of 55% [28].

Aneuploidy in colorectal adenomas was also extensively evaluated by DNA content flow cytometry which provides the degree of DNA aneuploidy or DNA Index (DI) and unique information on the presence of multiple DNA abnormal subpopulations or heteroploidy [9,10]. DI values were highly heterogenous and characterized by a non-random distribution with modal values at DI = 0.9, 1.15, 1.50, 1.80, 2.0 and 2.2. A valley was clearly evident at DI = 1.3–1.4 which separated near-diploid subpopulations from near-triploid ones. DNA aneuploid adenomas were characterized, in the 72% of cases, by DI values in the near-diploid region (DI $\leqslant$ 1.4), while, on the opposite, the majority of aneuploid adenocarcinomas (about 72%) were with DI $>$ 1.4.

KRAS2 and *APC* mutations were also commonly observed in colorectal adenomas and in their hypothetical precursor non-dysplastic and dysplastic aberrant crypt foci [30]. Specific *KRAS2* mutations and DNA aneuploidy were reported to be associated in human colorectal adenomas [10,11] and in cell lines [23,24,26]. That the RAS pathway and spindle assembly may collide in yeast was proposed [27].

APC mutations, on the other hand, would have a direct role in CIN and subsequent karyotypic abnormalities [8,18]. According to experiments using mouse embryonic stem cells carrying *APC* mutated alleles, APC truncated proteins, which miss the carboxyl-terminal sequences, would loose their interaction with the elongating spindle microtubules and the kinetochores, respectively via hBUB1 and EB1, and generate aneuploidy. Mutations in *hBUB1* were also implicated in CIN, but were rarely found in colorectal cancer [5,20].

2. Aneuploidy and *KRAS2* mutations

Among 116 examined human sporadic colorectal adenomas, 29 were aneuploid (25%) and 29 were *KRAS2* mutated (25%). *KRAS2* mutations were analyzed using sorted epithelia nuclei as previously described [10]. *KRAS2* G–C and G–T transversions, but not G–A transitions in codons 12 and 13 of exon 1, were strongly associated with DNA aneuploidy by logistic regression analysis which provided an Odd Ratio = 6.1 [11] and by contingency table analysis (Table 1). DNA aneuploidy was also detected in limiting dilution experiments after about twenty doubling times for murine cells transfected with the human G–C *KRAS2* mutated oncogene [23] in association with

Table 1

KRAS2 and *APC* mutations and deletions at 1p36 versus DNA Index among human sporadic colorectal adenomas

	DI = 1	DI $\neq$ 1	P
KRAS2			
wild type	70 (80%)	17 (20%)	
mutated	17 (59%)	12 (41%)	$P_{12} = 0.02$
G $\rightarrow$ A transitions	12 (70%)	5 (30%)	$P_{13} = 0.35$
G $\rightarrow$ C/T transversions	5 (42%)	7 (58%)	$P_{14} = 0.007$
APC			
wild type	30 (71%)	12 (29%)	
mutated	10 (53%)	9 (47%)	$P_{12} = 0.24$
<MCR	6 (86%)	1 (14%)	$P_{13} = 0.66$
$\geqslant$MCR	4 (33%)	8 (67%)	$P_{14} = 0.04$
			$P_{134} = 0.024$
			$P_{(1+3)4} = 0.016$
1p36 deletions			
absent	18 (86%)	3 (14%)	
present	1 (20%)	4 (80%)	$P_{12} = 0.01$

DI, DNA Index; P, P-values obtained by the Fisher's exact test; MCR, mutation cluster region (codons 1200–1500).

inhibition of apoptosis and loss of check-points in the G2M cell cycle phases [24]. New experiments with cell lines derived from human colorectal adenomas and carcinomas and permanently transfected with specific *KRAS2* G–C and G–T transversions and G–A transitions are in progress.

3. Aneuploidy and *APC* mutations

A role of APC in the origin of CIN and aneuploidy in an *in vitro* model was suggested [8,18]. We aimed to verify this hypothesis *in vivo* among human sporadic colorectal adenomas. Aneuploidy was associated with an abnormal nuclear DNA Index (DI $\neq$ 1) as evaluated by flow cytometry. With this technique we also sorted epithelial nuclei, as previously detailed [10], in which analysis of *APC* mutation spectrum by DNA sequencing was performed. Amongst 61 adenomas, 33% exhibited aneuploidy and 31% *APC* mutations. Microsatellite instability, investigated in a subset of 15 adenomas, was present in 1 case. Among the examined 14 *APC* mutated adenomas, LOH was detected in 4 cases and a double *APC* mutation in 1 case. The incidence of aneuploidy among *APC* wild type and mutated adenomas was respectively 29% and 47% ($P = 0.24$), suggesting that *APC* mutational status and aneuploidy were not associated (Table 1). Of the 7 *APC* mutations occurring upstream MCR, 6 were associated with diploid adenomas. Of the 12 mutations occurring in the MCR or downstream, 8 were associated with aneuploid adenomas (67%). A statistically significant association between *APC* mutation type and aneuploidy was observed (Table 1), suggesting that the specific *APC* mutations within and downstream MCR may be associated with aneuploidy.

4. Aneuploidy and 1p deletions

We investigated the numerical aberrations of chromosomes 1, 7, 17, 18, the 1p deletions and the nuclear DNA content as obtained by flow cytometry, in a series of 34 human sporadic colorectal adenomas. From these adenomas, 51 intra-adenoma regions were microdissected according to two degrees of dysplasia and presence of foci of early cancer. Isolated epithelial nuclei were analyzed by FISH using centromeric probes for chromosomes 7, 17 and 18 and, in a double-target analysis, a centromeric probe for chromosome 1 simultaneously with a telomeric probe mapping to the 1p36 band [6]. Considering the presence of numerical aberrations for at least one among the investigated chromosomes and/or abnormal DNA content, aneuploidy incidence was 35%, while 1p deletion incidence was 38%. The correlation of 1p deletions, mainly at 1p36, with aneuploidy was highly significant (Table 1), suggesting that loss of genes in this region may be implicated in CIN *in vivo*.

5. Conclusion

The significance of aneuploidy in cancer and the knowledge on the mechanisms causing CIN and aneuploidy still remain very limited.

In the present study, based on our previous investigations among human sporadic colorectal adenomas [10,11], we report further evidence for the association of *KRAS2* G–T/C transversions, but not G–A transitions, with DNA near-diploid aneuploidy, suggesting a possible involvement of specific *KRAS2* mutations in CIN *in vivo*. A link of *KRAS2* mutations with aneuploidy *in vitro* was also shown using a mouse cell line transfected with a *KRAS2* G–C transversion [23]. Similar results were also obtained using human transfected cell lines [26]. The mechanisms of KRAS2-mediated CIN and aneuploidy, however, are still not well understood. A study using mouse cells suggests the importance of G2M checkpoints and inhibition of apoptosis [24]. Other observations in yeast suggest the interaction of RAS-dependent specific proteins with the cytoskeleton and the mitotic spindle [27].

Using a limited series of 61 human sporadic colorectal adenomas, we also reported that *APC* mutations *in vivo* were not significantly associated with aneuploidy. However, subset group analysis, so far limited to small sample sizes, suggested that the specific *APC* mutations occurring within and downstream MCR might be associated with aneuploidy and have eventually a role in CIN *in vivo.* This last observation would be partly in agreement with previous studies using mouse embryonic stem cells carrying *APC* mutated alleles, suggesting that *APC* mutational status could be directly linked with CIN and aneuploidy [8,18].

Additionally, based on previous investigations, we also reported that 1p34–36 deletions were strongly associated with aneuploidy, suggesting that loss of genes in this region may be implicated in CIN *in vivo*. No gene level investigations are so far available linking gene mutations in this chromosomal region with aneuploidy [7,29].

Many studies are presently conducted to attempt a better understanding of the mechanisms causing CIN and aneuploidy. An interesting view is that aneuploidy, proposed to be a primary cause of cancer, is due to an abnormal dosage of normal genes [21]. Alternatively, in a recent comprehensive review, more than 70 genes have to date been reported that monitor genome integrity and CIN and coordinate cell cycle progression with DNA repair [1]. Among these, p53 inactivation in association with the dysfunction of telomeres was suggested as one of the most important driving forces of CIN [22]. Unfortunately, inactivation of p53 is quite rare in colorectal adenomas with moderate dysplasia while aneuploidy is already quite common. Additionally, other studies failed to prove the involvement of p53 in CIN both *in vitro* [4] and *in vivo* [12]. Other CIN driving mechanisms may include microtubule dynamic instability, kinetochore structure and function, chromosome condensation and sister-chromatid cohesion [5,20]. Cell cycle checkpoints and apoptosis were also postulated to play a role in CIN, though the relative importance of the various mechanisms is so far unknown.

The understanding of CIN mechanisms in association with specific gene mutations need additional work with the use of *in vitro* and *in vivo* models. Whether specific gene mutations lead to aneuploidy or viceversa is so far not proven, and the hypothesis that specific carcinogens in the human large intestine induce aneuploidy in parallel with specific gene mutations cannot be ruled out. It is likely that both subtle gene mutations and large scale chromosomal alterations cooperate to tumor genesis and progression in an evolutionary process characterized by divergence factors generating heterogeneity and convergence factors generating selection. An initial altered gene expression state, due to an abnormal dosage of normal and mutated genes, may lead to an equilibrium gene expression state which represents a specific tumor phenotype.

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