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Genome Based Cell Population Heterogeneity Promotes Tumorigenicity: The Evolutionary Mechanism of Cancer

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Abstract

Cancer progression represents an evolutionary process where overall genome level changes reflect system instability and serve as a driving force for evolving new systems. To illustrate this principle it must be demonstrated that karyotypic heterogeneity (population diversity) directly contributes to tumorigenicity. Five well characterized in vitro tumor progression models representing various types of cancers were selected for such an analysis. The tumorigenicity of each model has been linked to different molecular pathways, and there is no common molecular mechanism shared among them. According to our hypothesis that genome level heterogeneity is a key to cancer evolution, we expect to reveal that the common link of tumorigenicity between these diverse models is elevated genome diversity. Spectral karyotyping (SKY) was used to compare the degree of karyotypic heterogeneity displayed in various sublines of these five models. The cell population diversity was determined by scoring type and frequencies of clonal and non-clonal chromosome aberrations (CCAs and NCCAs). The tumorigenicity of these models has been separately analyzed. As expected, the highest level of NCCAs was detected coupled with the strongest tumorigenicity among all models analyzed. The karyotypic heterogeneity of both benign hyperplastic lesions and premalignant dysplastic tissues were further analyzed to support this conclusion. This common link between elevated NCCAs and increased tumorigenicity suggests an evolutionary causative relationship between system instability, population diversity, and cancer evolution. This study reconciles the difference between evolutionary and molecular mechanisms of cancer and suggests that NCCAs can serve as a biomarker to monitor the probability of cancer progression.

Increasing evidence illustrates that the somatic evolution of cancer is similar to natural evolution with system stability mediated genetic heterogeneity playing a key role (Nowell, 1976; Crespi and Summers, 2005; Heng et al., 2006b, 2008; Maley et al., 2006; Heng, 2007a,b,c; Goymer, 2008). This concept offers an explanation to many seemingly contradictory

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findings in the field including the recent unexpected failure to identify a handful of commonly shared cancer genes from initial attempts to sequence the cancer genome (Bielas et al., 2006; Heng, 2007a; Heng et al., 2006a; Greenman et al., 2007; Wood et al., 2007). An emerging genome-centric concept on cancer evolution states that overall genome level variation coupled with stochastic gene mutations serve as a driving force of cancer evolution by increasing the cell population diversity (Heng et al., 2006a,b,c). The importance of non-clonal chromosome aberrations (NCCAs) (both structural and numerical) and their dynamic interplay with clonal chromosome aberrations (CCAs) in the immortalization process has been recently demonstrated and supports the genome-centric concept of cancer evolution (Heng et al., 2004, 2006a,b,c, 2008; Ye et al., 2007). Similarly, the pattern of gene mutations within tumors occurs stochastically (Bielas et al., 2006). These data and the absence of universal gene mutations revealed by recent large scale sequencing efforts (Greenman et al., 2007; Wood et al., 2007; Heng, 2007b) suggests that the concept of genome dynamics and stochastic cancer evolution and its clinical implications should now be incorporated into our conceptual framework of cancer research (Heng, 2007a).

Studies on clonal diversity and subsequent clinical outcomes in Barrett's esophagus (Maley et al., 2006) reinforce the concept that cancer progression occurs through somatic evolution driven by genome instability coupled with an increase in or accumulation of clonal diversity. To date, however, most evolutionary analyses have focused on specific genetic loci rather than the overall genome level diversity. The impact of genetic variation at the genome level is much more profound than at the gene level, as the higher level of organization often constrains lower levels and displays more stable characteristics than lower levels (Heng, 2007a, 2008; Rubin, 2007; Ye et al., 2007). It is therefore expected that the major form of cellular population diversity is generated by karyotypic heterogeneity reflected as NCCA/CCA cycles (previously described as the waves of clonal expansion with the regeneration of genetic diversity in between) occurring during somatic evolution (Heng et al., 2006a,b,c; Heng, 2007a). It is thus more reliable and easier to measure the degree of diversity at the genome level than at the individual gene level. In addition, it has been a challenge to trace individual genes for most cancer types where there is a high level of genomic heterogeneity (Heppner, 1984; Heng et al., 2004; Bielas et al., 2006; Heng, 2007a,c; Wood et al., 2007).

Increased NCCAs are associated with multiple genetic and environmental factors including dysfunction of genes that maintain genome integrity, over-expression of onco-proteins, exposure to carcinogens, cells reaching crisis stages prior to immortalization, etc. (Heng et al., 2006b). We anticipate that for a given cell population, elevated NCCAs will directly promote tumorigenicity. This expected correlation, will support the biological significance of NCCAs in cancer formation. Previously, only the immortalization step was extensively shown to have such a correlation (Heng et al., 2006b). To further test the hypothesis that increased levels of NCCAs directly promote tumorigenicity, it is necessary to link the two events in a simple model system.

There are a number of in vitro tumorigenicity models available. Most however, focus on the link between tumorigenicity and specific pathways rather than the evolutionary mechanism of tumorigenicity. Accordingly, a large number of pathways have been linked to tumorigenicity without revealing common mechanisms (Vogelstein and Kinzler, 2004). In light of our concept that genome instability mediated somatic cell evolution is the common mechanism in cancer, we reexamined some of previously characterized systems and focused on overall genome diversity rather than specific pathways. We have selected five readily available in vitro models that represent various human and mouse cancer types, to confirm that the linkage between increased levels of NCCAs and tumorigenicity represents a common feature across drastically different models transcending previously characterized molecular pathways.

In this study, spectral karyotyping (SKY) was used to compare the degree of karyotypic heterogeneity displayed in various sublines of five in vitro systems, where the cell population diversity was determined by the frequency of NCCAs. The tumorigenicity of these models has been further analyzed to link elevated structural NCCAs and tumorigenicity. In addition, benign hyperplastic lesions (without evidence of carcinoma) were examined and displayed low levels of structural NCCAs. In contrast, premalignant dysplastic tissue of the c-myc transgenic mouse model displayed high levels of NCCAs. Based on the observations that there are many types of karyotypic aberrations, the distribution patterns of structural and numerical NCCAs as well as the contribution of various types of genome level variation to tumorigenicity have also been analyzed, suggesting the importance of using total frequencies of structural NCCAs when monitoring the potential tumorigenicity. Together, our analysis agrees with the proposed model that chromosomal instability produces genetic variation and the more variation there is, the more likely a favorable combination will be produced that will result in a lesion that will produce malignancy/tumorigenecity. Thus the identified common link between the elevated levels of NCCAs and increased tumorigenicity establishes a strong relationship between genome level diversity and tumorigenicity. Further, this information illustrates the relationship between the general evolutionary mechanism and large numbers of specific molecular mechanisms of cancer. In brief, the evolutionary mechanism of cancer is equal to the collection of total number of individual molecular mechanisms. As each individual case often involves different molecular mechanisms and the mechanisms are constantly changing during cancer evolution, it is difficult to predict the status of cancer and the response to treatment based only on tracing specific pathways. It is now necessary to study and monitor the probability of cancer through an evolutionary mechanism that would include the possibility of nearly unlimited combinations of molecular mechanisms. This study also suggests a use for NCCAs as a biomarker to evaluate the potential of tumorigenecity.

Materials and Methods

Cell culture and chromosome preparation

Various stages of cells representing the five models (Table 1) were briefly cultured. The original frozen cell passages used in the previous tumorigenicity studies (Miller et al., 2000;Karan et al., 2001;Roberts et al., 2005;Zhang et al., 2006) were short-term cultured. After 2–4 days culture, mitotic cells were harvested for chromosome preparation (Heng et al., 1992,2003;Heng and Tsui, 1993). Briefly, cells were grown to 70% confluence and treated with colcemid for 4–8 h. Trypsinized cells were harvested and treated with hypotonic solution (0.4% KCL, 10 min at 37°C), followed by Carnoy's fixation (3:1 of methanol and acetic acid) (three times at 20 min each) and air-dried. The chromosomal slides can be used for SKY immediately or stored at −70°C for future use.

Chromosome preparation from benign hyperplastic lesions

MCF10A-Rad6B clone 5 cells were derived by stable transfection of Rad6B, a fundamental component of postreplication DNA repair pathway as described in Shekhar et al. (2002). MCF10-Rad6B clone 5 cells (1×10^7) were suspended in Matrigel and injected into the mammary fat pads of female immunodeficient nude mice, and lesions from the injection sites were harvested at 70 days (Shekhar et al., 2006). Harvested xenografts were cultured in DMEM/F12 supplemented with 5% horse serum, 10 μg/ml insulin, hydrocortisone and 10 ng/ml EGF to derive MCl5. MCl5 cells were harvested and chromosomes prepared within 2–4 passages for SKY analysis (Heng et al., 2003).

Chromosome preparation from proliferating mammary glands of MMTV-c-myc transgenic mice

Proliferating mammary glands were collected from two virgin female MMTV-c-myc transgenic mice at age of 7 months. In our lab, virgin females of this transgenic line of mice spontaneously develop palpable mammary tumors at ages of 7–9 months, as described in more detail by Liao and Dickson (2000) and Liao et al. (2000). The proliferating mammary glands used in this study were collected from an area distant from a palpable tumor, and histology of the glands in the same area showed only proliferating glands without atypia. Proliferating glands were briefly cultured and chromosomes were prepared for SKY analysis.

SKY and data analysis

Following probe denaturation, hybridization and SKY detection (Heng et al., 2001, 2006b; Ye et al., 2001; Stevens et al., 2007), randomly selected mitotic figures were photographed and analyzed by SKY imaging software. Fifty to hundred SKY images were captured for each cell population to identify commonly shared karyotype features and to reveal the karyotypic diversity of these various cell populations. NCCAs were scored by identifying chromosomal numbers, chromosome translocations/large deletions or other types of abnormality detected within a given mitotic cell. There are two steps needed to score frequencies of NCCAs and CCAs. First, a 4% cutoff line is used to identify any specific recurrent karyotypes or CCAs. The frequency of a CCA is determined by calculating the number of cells displaying the same CCA divided by the total cells examined (50–100). Non-clonal karyotypes (NCCAs) are classified as having a frequency lower than 4%. The total frequencies of NCCAs of a given cell population is then calculated by using all cells displaying NCCAs divided by the total cells examined (Heng et al., 2006b,c; Ye et al., 2007). Both types of CCAs as well as frequencies and types of NCC As are listed in Tables 1 and 2.

In vivo tumorigenicity test

In earlier studies, we found that all cigarette smoke condensate (CSC)-treated MCF10A cells efficiently formed colonies in soft-agar (Narayan et al., 2004). We then re-established cell lines from the soft-agar colonies and further examined the persistence of their transforming characteristics. The re-established cell lines, when plated after 17 passages without CSC treatment, still formed colonies in soft-agar (Narayan et al., 2004). To determine whether the cell lines showing transformed characteristics in the anchorage-independent assay can grow in nude mice, we injected four selected CSC-transformed cell lines, MCF10A-CSC1, MCF10A-CSC2, MCF10A-CSC3, and MCF10A-CSC4 into female nude (nu/nu) mice (with 10^5 cells of each cell line suspended in Matrigel) (BD Biosciences, San Diego, CA). Palpable tumors appeared in 20 days and animals were sacrificed in 44 days.

Statistical analysis

Ninety-five percent confidence intervals were calculated by combining the lines with the highest and the lowest tumorigenicity for each model. A Student's *t*-test was then run on this data showing a significant difference in NCCA levels between cells with high and low tumorigenicity (P=0.01791) (Fig. 5A). Ninety-five percent confidence intervals of chromosome number were also calculated for each cell line studied (Fig. 5B–F).

According to our previous experience of scoring NCCAs, reproducibility of NCCA level is very high. Though many factors can influence NCCA frequency including culture conditions and genetic makeup of a given cell line, the frequency of NCCAs is reproducible for a similar group. For example, in the MCF10 breast disease model, duplicates of treated and untreated show a significant difference (P=0.00055) in NCCA frequency when the treated are compared to the untreated, however standard deviation within treatments is quite low (0.00212132 in

treated and 0 in untreated). Similarly, when comparing two stages of the immortalization process of the Li-Fraumeni model, duplicates of the earliest stage were similar (SD=0.008485281) and significantly different from the duplicates of later stages of the cell populations (P=0.0034). Similar results were reported regarding the frequencies of NCCAs in ATM−/− mice as well as various cancer cell lines with or without onco-protein expression (Heng et al., 2006b).

Results

Molecular characterizations and measured genome diversity for the five models

The molecular characterization of these five models has been accomplished by previous studies and the key points are briefly summarized (Table 3). To examine genome diversity, multiple color SKY was used to score the level of NCCAs and types of CCAs (Heng et al., 2001;Ye et al., 2001;Stevens et al., 2007). The following is detailed information on each model.

The LNCaP model—A unique prostate cancer model with three distinctive stages has been developed using sublines of LNCaP cells originally established from a human prostate adenocarcinoma (Lin et al., 1998). Within this model, C33 (passage number <33) represents the early stage that is androgen-responsive; C51 (passage 45–70) represents the middle stage with decreased androgen-responsiveness; and C81 (passage 81–120) represents the late stage with androgen-unresponsiveness and increased tumorigenicity, illustrated by a xenograft animal model, where C33 and C81 stage cells of the LNCaP cell model showed differential tumorigenicity when implanted subcutaneously in nude mice (Karan et al., 2001). In this model increased genetic aberrations, such as microsatellite instability and allelic loss were observed in later passages, but the karyotypes appeared to be stable throughout the progressive transformation (Karan et al., 2001). This illustrates the link between tumorigenicity and increased genetic alterations reflected by microsatellite instability and chromosomal allelic loss.

Three cell populations representing C33 (pd36), C51 (pd69), and C81 (pd125) were used for SKY analysis. The overall karyotypes of all cells at various passages shared the same set of five altered chromosomes demonstrating the overall stability at the karyotypic level as determined by the presence of stable CCAs (Fig. 1). At pd69, der(13;13) formed as a new transitional CCA, however, it was lost by pd125 (Table 1). Thus there were no specific late passage CCAs. Increased structural NCCAs, on the other hand, represent a significant feature of the transition between early and later passages.

Increasing level of NCCAs combined with progressing cell passages clearly correlates with increased tumorigenicity. The fact that C33, which exhibits delayed tumor formation (Karan et al., 2001) also has a relatively high degree of NCCAs (30%), further supporting the notion that increased levels of NCCAs promote tumorigenicity. From an evolutionary viewpoint, the higher the frequency of NCCAs increases the probability of cancer progression in shorter periods of time. Tumorigenicity, can be achieved with lower frequencies of NCCAs but requires longer timeframes for the selection process to occur.

The MCF10DCIS.com model—MCF10DCIS.com xenograft is a model of human comedo ductal carcinoma in situ. This cell line was cloned from a cell culture initiated from a xenograft lesion obtained after two successive trocar passages of a lesion formed by premalignant MCF10AT cells. Early passage cells display a less invasive capability while the late-passage cells have a more extensive invasive capability (Miller et al., 2000). We thus SKY analyzed various passages of this cell line to identify karyotype patterns as shown in Table 1.

The majority of the altered chromosomes were shared among the three passages examined. With passage progression, dynamic NCCAs and CCAs were evident with some CCAs being replaced by others. At passage pd46, in addition to increased NCCAs, even the retained CCAs were not evenly distributed throughout the population indicating a high degree of heterogeneity as the degree of homogeneity drops. At passage pd46, der(15)t(15;21) were newly formed and high levels of NCCAs observed, thus linking these changes to increasingly invasive phenotypes. The mechanism of highly aggressive phenotypes was recently linked to stromal–epithelial interaction (Tait et al., 2007; Shekhar et al., 2008).

The MCF10 model transformed by cigarette smoke condensate (CSC)—To exclude the possibility that a specific CCA such as der(15)t(15;21) play a major role in the increased tumorigenicity observed in the MCF10DCIS model, it would be ideal to use cell populations that display different degrees of tumorigenicity and yet share the same marker chromosomes (identical CCAs). Four transformed lines have been generated by treatment with CSC, independent of the MCF10DCIS.com model (Narayan et al., 2004). Even though all four lines displayed anchorage-independent growth in soft-agar, there was only one line that generated tumors in immunodeficient mice (see tumorigenicity session). Comparison of the karyotypic features of these four transformed lines showed they share six altered chromosomes in common (Fig. 2). Three of the alterations are shared in common with MCF10DCIS indicating the same origin for these two differently transformed systems (Table 1).

Although the four lines displayed the same sets of altered CCAs, NCCAs occurred at different levels in these lines. Various types of structural and numerical NCCAs are listed in Table 2. As illustrated by the tumorigenic assay of immunodeficient mouse xenografs, only CSC-MCF10A3 produced tumors in immunodeficient mice. In addition to elevated levels of NCCAs, the average chromosome number was also increased in CSC-MCF10A3. Therefore, in this system, increased ploidy and the frequency of NCCAs were linked to tumorigenicity.

The MCF10 model transformed by HOXA1—To exclude the possibility that ploidy rather than a high degree of diversity contribute to the tumorigenicity that is observed with CSC-MCF10A3, an additional subline was selected with identical karyotypes (and ploidy status) but these lines displayed a diversity of NCCAs. This subline was obtained by spontaneously transforming MCF10 cells by over-expression of HOXA1 (Zhang et al., 2003). Human growth hormone-regulated HOXA1 has been shown to be a mammary epithelial oncogene. HOXA1 stimulates the transcriptional activation of a number of pro-oncogenic molecules including cyclin D1 and Bcl-2 that promotes proliferation and survival. Over-expressed HOXA1 in human mammary carcinoma cells results in drastically increased tumorigenicity (Zhang et al., 2006). We compared the degree of genome diversity of the cell line over-expressing HOXA1 (stable transfected with HoxA1 expression plasmid) and the control cell line containing vector only (Table 1). Both the HOXA1 line and the control line shared identical marker chromosomes and the karyotypes were identical (Fig. 3). The major difference was the frequency of defective mitotic figures (DMFs), a new phenotype of chromosome condensation defects and G2-M checkpoint deficiencies (Heng et al., unpublished work). In addition, the frequency of errors in cell division that are related to DMFs was higher in the HOXA1 line (Fig. 3). DMFs represent an ignored karyotypic aberration. The key description of a DMF is its differential condensation among all chromosomes and its genetic consequences causing an increase in population diversity and possibly leading to typical chromosomal aberrations such as aneuploidy, deletion, or translocations. As DMFs are a typical form of NCCA (Heng et al., 2004, 2006a; Ye et al., 2007), the high frequencies of DMFs observed from the HOXA1 line indicates a high degree of genome diversity. Thus both the involvement of the HOXA1 oncogene and elevated NCCAs were co-linked to tumorigenicity.

Mouse ovarian cancer model—Mouse syngeneic ovarian cancer models have been established and have proven to be very useful in the study of temporal molecular and cellular events during neoplastic progression. Primary mouse ovarian surface epithelial cells were isolated and cultured for varying generations. It is known that tumorigenicity (tested in nude mice) rises with increasing passage number (Roberts et al., 2005). Three representative stages of a parallel experiment were selected for karyotype analysis representing pd9, pd45, and pd91 (Table 1).

Even at an early stage (passage 9), the karyotypes were clearly no longer normal as the population of cells contained 10% NCCAs and a CCA [der(10; 10)]. This initial CCA was replaced by two new CCAs der(1)t(1; 2), der(8)t(8; 16). Only der(1)t(1; 2) was detected during the later stages, illustrating karyotypic dynamics during in vitro culture (Heng et al., 2006c). Again, the most prominent feature linking the cell progression stages was the percentage of NCCAs. During early passages NCCAs were detected in only 10% of all cells analyzed. By passage 91, however, NCCAs were detected in almost all cells, even though these cells also contained a four CCAs. Thus, the elevated NCCAs and two clonal aberrations were linked to tumorigenicity. In a parallel experiment, the tumorigenicity of an independent cell culture series was linked to increased numerical NCCAs (aneuploidy) and no recurrent CCAs were detected and distinct remodeling of the actin cytoskeleton and focal adhesion complexes were coupled with down-regulation and/or aberrant subcellular location of E-cadherin and connexin-43 (Roberts et al., 2005).

Tumorigenicity analysis

To establish a strong relationship between the level of NCCAs and tumorigenicity, cells with different levels of NCCAs were injected into mice and then comparatively analyzed for tumorigenicity. In most of these models, the tumorigenicity of various stages of the cell populations was previously tested using this assay and the data are readily available (Miller et al., 2000; Karan et al., 2001; Roberts et al., 2005; Zhang et al., 2006). To reduce variation in our analysis, the original frozen cell passages used in the tumorigenicity studies were used in our SKY analysis. Since the relative levels of NCCAs detected should be similar among these cells including those used to test tumorigenicity, the detected occurrence of increased NCCA frequencies should take place prior to injection into animals. As illustrated in Figure 5 and Table 1, in each model, the highest tumorigenicity was always associated with the highest frequencies of structural NCCAs. Interestingly, in the LNCaP prostate cancer model, compared to early passage cells, the late stage cells with androgen-unresponsiveness, produced tumors two times faster, while the frequencies of NCCAs nearly doubled between early and late stage cells.

We then examined the tumorigenicity of the MCF10A-CSC model. As expected, the control MCF10A cells as well as three of the CSC-transformed cells lines (MCF10A-CSC1, CSC-2, and CSC-4) did not form tumors in the nude mice within 20 days, even though all CSC lines exhibit anchorage-independent growth. Only the MCF10A-CSC3 cell line grew and formed palpable tumors in the nude mice within 20 days (Fig. 4). Thus, tumorigenicity is linked to the highest level of genome diversity. In conclusion, for all five models, the highest levels of genome diversity were linked to tumorigenicity.

Examination of genome diversity in benign hyperplastic lesions

We previously demonstrated that stable transfection of Rad6B into MCF10A cells that have a stable pseudodiploid karyotype results in abnormal mitosis, severe aneuploidy, and the ability to form anchorage independent growth (Shekhar et al., 2002). In vivo implantation of MCF10A-Rad6B cells into nude mice generated benign hyperplastic lesions without evidence of carcinoma (Shekhar et al., 2006). These results suggest that despite severe aneuploidy,

MCF10A-Rad6B cells produced only hyperplastic lesions, an initial step of carcinogenesis. Based on the observation from all five models that the highest level of genome diversity (mainly reflected as structural NCCAs) was linked to tumorigenicity, we hypothesize that these hyperplastic lesions produced from MCF10A-Rad6B clone 5 cells will display low levels of genome diversity reflected as low levels of structural NCCAs. By performing SKY analysis of briefly cultured tissue of hyperplastic lesions, our data indeed show that the cells of hyperplastic lesions display a homogenous cell population with a very low level of structural NCCAs (4%), demonstrating the lack of genome diversity in the hyperplastic lesions of this particular system. Since MCF10A-Rad6B generated hyperplastic lesions represent an ideal control for other MCF10A series derived tumors, the inability to form carcinomas can be nicely explained as being the result of a lack of genome level heterogeneity. Thus, it further supports a positive correlation between elevated NCCAs and tumorigenicity.

Examination of genome diversity in premalignant dysplastic mammary tissue

If one considers the above benign hyperplastic lesions as examples of somatic evolution "dead ends" for the MCF10-Rad6B cells due to the lack of genome diversity, it would be interesting to investigate whether premalignant lesions with full tumorigenicity potential display elevated NCCAs, as contrasted from benign hyperplastic lesions, premalignant tissue has great potential for tumor progression. A transgenic mouse model (myc-transgenic mouse, Liao and Dickson, 2000; Liao et al., 2000) was chosen due to its high penetration producing mammary tumors in 7–9 months. When karotypes of these short-term cultured premalignant mammary tissues were examined, high levels of both numerical and structural NCCAs were observed (sNNCA=24%). This suggests that elevated levels of NCCAs existed prior to tumor formation, which represents an essential precondition for tumors to undergo the cancer evolutionary process.

Link various types of karyotypic variation with tumorigenicity

Due to the fact that there are many types of alterations at the karyotypic level, it is necessary to evaluate the relationship between them and to decide which types of variation are most useful in terms of serving as a biomarker to monitor tumorigenicity. We have divided NCCAs into structural and numerical NCCAs (Heng et al., 2006a,b). Structural NCCAs, have many subtypes, such as translocations (t-NCCAs), defective mitotic figures, or DMFs, which can generate further chromosome aberrations including breakages and translocations; chromosome fragmentation (Stevens et al., 2007), and other uncharacterized structures including "sticky chromosomes." All of these aberrations are capable of increasing the population diversity. Based on the MCF10-CSC-model and Rad6 model, it appears that the total frequency of structural NCCAs is a reliable index while chromosomal number changes are less reliable. Among structural NCCAs, the chromosomal translocation (t-NCCAs) rate seems to be the most reliable as only a proportion of other types of karyotypic aberrations can form translocations. Numerical NCCAs include polyploidy and aneuploidy. When both simple translocations and complex translocations exist, the complex types seem to be more dominant, and during later stages, there is a trend to accumulate complex types of chromosome aberrations. To further illustrate the complexity of this issue, Table 2 lists the distributions of various CCAs and NCCAs in the MCF10-CSC-model.

Further studies using additional independent models are needed to evaluate these potential biomarkers as a means to measure genome level heterogeneity and the consequences of tumorigenicity, as five in vitro model systems and two in vivo systems are not enough to draw definite conclusions on this important issue. However, it is clear that structural NCCAs are linked to tumorigenicity based on our observation and data available from the literature. Among all types of karyotypic aberrations, the frequencies of structural NCCAs represent the best biomarker. When five models were statistically analyzed, the five lines with the highest tumorigenicity had a significantly higher NCCA frequency than the five lines with the lowest

tumorigenicity (P=0.01791 Student's t-test) (Fig. 5A). We did not simply compare all lines with no tumorigenicity versus those that produce tumors as the presence or absence of tumorigenicity is a relative measurement especially since all models use different time scales. As illustrated by the LNCaP model, all lines will eventually generate tumors if a long enough time window is used.

Formulating a model that illustrates the relationship between evolutionary concept and molecular mechanisms

In summary of all models analyzed, it is clear that in each case examined (a given experimental model based on a selected cell line, individual animal lesion), a specific or combination of specific molecular pathways can be illustrated and thus linked by molecular analysis. However, there is no common molecular basis or mechanism leading to cancer evolution in general, since no specific form of genomic aberration is universally shared among diverse cancer cases. This is also true at the sequence level, as a recent large scale sequencing project indicated that there are many different genetic combinations or "hills" at the gene level in the context of the evolutionary adaptive landscape (Wood et al., 2007). If we abstract from these seemingly specific and unrelated causes, including a number of known molecular pathways, elevated DMFs, increased ploidy, simple or complex chromosomal translocations, and large scale stochastic changes at the gene level and epigenetic level, the picture of a common mechanism will emerge. That mechanism is karyotypic heterogeneity rather than a specific molecular pathway.

Our evolutionary explanation of why there is a correlation between elevated NCCAs, genome diversity and tumorigenicity is illustrated in the model shown in Figure 6. Based on the concept of cancer evolution and the realization that cancer is a disease of probability (Heng, 2007a), one can understand why elevated genome diversity will lead to the success of cancer evolution regardless of which molecular pathways or mechanisms are involved. This diagram links various molecular mechanisms with the evolutionary mechanism of cancer. It not only can explain the knowledge gaps between basic experiments and clinical findings (in experimental systems, many cancer genes can effectively cause a cancer phenotype, yet, these gene mutations only account for a small portion of the clinical cancer cases), but also focuses attention on the evolutionary mechanism rather than molecular mechanisms. There are large numbers of different molecular mechanisms that for all practical purposes cannot be predicted, in contrast, it would be much more useful to predict the increasing probability of cancer using the evolutionary mechanism. Such relationship between evolutionary mechanism and molecular mechanisms of cancer can simply be states as following:

$$\text{Evolutionary Mechanism} = \sum \text{Individual Molecular Mechanisms}$$

This formula offers insight into the relationship between system instability, karyotypic heterogeneity, individual molecular mechanisms and tumorigenicity.

Discussion

The evolutionary mechanism of cancer: System instability results in a higher probability of a new system becoming established

As illustrated by our model (Fig. 6), the linkage between the elevated degree of NCCAs and tumorigenicity explains the mechanism of cancer in simple evolutionary terms. A stable cell population, with lower degrees of change, translates into a lower probability of cancer formation. Increased system instability, in contrast, results in an increased probability of cancer

formation. Our experimental data illustrate the evolutionary mechanism of cancer formation and that system instability is the key causative factor. As we pointed out previously, many genetic, metabolic and environmental elements can contribute to genome system instability, including system dynamics (Heng et al., 2006b;Ye et al., 2007). When unstable, the genome system offers a higher probability of change or diversity, reflected as variable karyotypes that offer a greater number of different molecular pathways, which are the material for evolutionary selection as well as a precondition to establish new genome systems.

The seven examples described above involved both human and mouse cells of different cancer types and the malignant phenotypes have been linked to specific but different precipitating events. These events range from increased microsatellite instability and allelic loss, to chromosome ploidy, different chromosomal translocations and numerical aberrations, to HOXA1 gene and c-Myc expression, and to down-regulation of E-cadherin, as well as centrosome amplification caused by Rad6 and stromal–epithelial interaction (Table 3). For each characterized system, the linkage between a specific pathway or genetic event has been described as a given molecular mechanism. When considering all systems together, however, none of these events can be used to explain all cases. Significantly, the only common link to tumorigenicity is increased levels of NCCAs! Clearly, our correlative observation between increased levels of NCCAs and tumorigenicity supports the causal relationship between system instability reflected by elevated NCCA levels and tumorigenicity. Thus, such a correlation offers an evolutionary mechanism for cancer formation by generating cellular diversity.

It should be pointed out that the context of the term “mechanism” is very different among academic fields. In molecular biology, for example, mechanism typically refers to a change in a molecule that results in a specific phenotype or other molecular events. The evolutionary meaning of mechanism refers to the generation of cellular heterogeneity, which is the instrument or means of natural selection through population diversity. The evolutionary mechanism is therefore much broader than the molecular mechanism and can be achieved by many different molecular mechanisms or other mechanisms under specific circumstances (Heng, submitted). For example, different types of stress can trigger system instability. In molecular terms, the stress can be classified into specific molecular actions such as ER stress, metabolic stress, stress resulting from ineffective DNA repair, over-expression of certain oncogenes, etc. Regardless of the type of molecular stress, the system response is not stress specific but displays a common response increasing the level of system dynamics, confirmed by the elevation of NCCAs. Despite the common response of elevated NCCAs, a specific NCCA (or number of NCCAs) will be selected, however the associated molecular pathways will be more or less unpredictable and will continuously change. Each molecular mechanism that generates stress and the response to stress can contribute to or is even equal to the evolutionary mechanism of each specific case. However, the general evolutionary mechanism cannot be sufficiently explained or predicted by individual molecular mechanisms as there is no shared molecular mechanism in all cancer cases. Similarly, the term “causative relationship” has a different meaning when considering the difference between a single molecular pathway and a complex system. In the molecular sense, the causative relationship is defined within an isolated network where molecule A or event A (called cause) leads to B (called effect). In a complex system, however, cause and effect relationships might not be so narrowly defined nor maintain the same meaning as illustrated by experiments. An experimentally defined relationship setup between two parties can be easily changed when additional interactions are included. In fact, complicated interactions are always present in natural settings but are ignored in experimental analyses. To analyze complex systems, correlation studies are thus fundamentally important as causative studies among lower level parts of a system in an isolated setting may not be as reliable in the context of a complex system. In contrast, to study the mechanism of cancer evolution (and not individual molecular mechanisms), a general correlative relationship where system instability results in population diversity, and the

population diversity provides the necessary pre-condition for cancer evolution to proceed, in fact illustrates the causative relationship between system dynamics and cancer. It is likely that many different pathways are stochastically involved and selected when there is elevated instability and genetic diversity, based on the stochastic nature of karyotypic aberrations and the mechanism of cancer evolution. For example, some NCCAs may activate dominant oncogene defined pathways, while others may have various combinations of minor changes that eventually result in the final phenotypes of uncontrolled growth. The link between NCCAs and tumorigenicity in the majority of cancers supports our model. This concept predicts that the result of genomic instability (inherited or induced) is the generation of population diversity (evident though clonal diversity or non-clonal diversity or the combination of both) which drives the cancer evolutionary process. Interestingly, the cases we analyzed here represent the tip of the iceberg, as the often hidden link between population diversity and tumorigenicity can be easily found in cancer literature. Although most of these reports focus on specific molecular pathways, including specific oncogenes, tumor suppressor genes, epigenetic regulation, or genes responsible for tissue architecture, most of these aberrations can be linked to overall genome instability resulting in population diversity (Heppner, 1984; Vogelstein and Kinzler, 2004; Heng et al., 2006a,b,c). This fits well with the genome-centric concept of cancer (Heng, 2007c; Heng et al., 2008; Ye et al., 2007).

Advantages of using NCCAs/CCAs to monitor the cancer evolutionary process

Initially demonstrated in our in vitro immortalization model, the high level of NCCAs and dynamic interaction between NCCAs and CCAs plays an important role in cellular immortalization. The current study further provides solid evidence that elevated NCCAs are directly linked to tumorigenicity.

Recently, there has been an increased realization of the importance of applying evolutionary theory into cancer research (Goymer, 2008). A number of reports have either examined the evolutionary process of clinical samples or established computational cancer models of evolution (Merlo et al., 2006). Most studies have focused on tracing specific gene mutations or methylation patterns due to the available technologies (Kim and Shibata, 2004; Spencer et al., 2006; Galipeau et al., 2007; Jones et al., 2008; Vincent and Gatenby, 2008). However, there are some serious limitations regarding the strategies of gene based evolutionary analysis. First, the current technologies used in genetic analyses are based on a mixture of cell populations that only artificially profiles the most dominant clonal population and ignores the importance of heterogeneity. Second, as illustrated in previous publications, most solid cancers involve progression with high levels of stochastic change, where it is difficult to trace the genetic changes, and only during slow phases (prior to the blastic phase in CML, for example) of limited blood based cancers or solid tumors are some genetic changes traceable (Heng, unpublished data). Even in blood cancers, it is almost impossible to trace genetic changes in late stages. In addition, according to the theory of orderly heterogeneity and system complexity, it might be more meaningful to trace the higher levels of genetic organization (genome) than the lower gene levels (Heng, 2007a; Rubin, 2007; Heng et al., 2008). More importantly, in somatic evolution, macro-evolution is the main mechanism and replacement of various genomes is the driving force of somatic cell evolution. When the genome context changes, even when the gene state is the same, it often does not keep the same biological meaning. For example, in different human pancreatic cancer cell lines, the K-ras gene mutation was linked to very different pathways, possibly due to the different context of genomes. Interestingly, NCCAs and epigenetic programming responding to stimulation of the Ras-MAPK pathway may be a better marker for cancer progression than the upstream mutated oncogenes (Espino et al., 2008). Therefore, by focusing on genome diversity, the overall evolutionary potential can be measured based on the karyotypic heterogeneity. Indeed, monitoring the karyotypic level is more effective than monitoring the gene level, as focusing on karyotypic heterogeneity

is in fact studying the evolutionary mechanism while focusing on individual genes is studying a single specific molecular mechanism. Thus our current study offers a new direction that uses the degree of karyotypic heterogeneity to effectively monitor tumorigenicity.

One issue that needs further analysis is the contribution of specific CCAs in combination with elevated NCCAs. Traditionally, attention has focused on CCAs as only clonal expansion was thought to be important for the accumulation of additional gene mutations. Genome dynamics drive cancer evolution, therefore it would be interesting to study how key CCAs play a role in increasing the population diversity rather than just providing proliferation. In agreement with our previous findings, the current studies favor NCCAs rather than specific CCAs in monitoring genome system variance. However, it is still possible that for specific cases certain CCAs can contribute more to cancer evolution than others. For example, the mutation of p53, which can have many different functions, could be an example of a CCA that increases evolutionary dynamics, in addition to other functions. It is thus possible that some powerful CCAs when combined with a certain level of NCCAs, would be most effective in terms of cancer evolution. In fact, consistent with previous publications, we have observed that increased frequencies of complex CCAs (involving multiple translocations within one chromosome) are most frequently detected during the late stage of immortalization (Heng et al., 2006a) and during the formation of drug resistance (Heng et al., unpublished work).

It should be pointed out that, using a system approach to monitor NCCA/CCA dynamics is not contradictory to studying the function of various cancer genes, similar to not seeing the forest for the trees, these two approaches focus on two levels of genetic organization, and try to address different mechanisms (evolutionary and molecular) of cancer formation. Following decades of effort attempting to understand each molecular mechanism (including oncogenes, tumor suppressor genes, DNA repair genes, genes regulating transcription/RNA splicing/translation/protein modification and protein degradation, genes controlling cell cycle, cell death, cell proliferation and differentiation, cell communication as well as aneuploidy, micro-environments, and immuno-system responses) (Duesberg et al., 2005; Nelson and Bissell, 2006; Heng, 2007a; Heng et al., 2008), it seems that the complexity of cancer is too high and that just tracing individual pathways will not lead to understanding the nature of cancer due to the highly dynamic (stochastic and less predictable) features of this disease. It is time to focus more on the system's behavior and its patterns of evolution rather than mainly focusing on individual pathways alone (Heng, 2008b). Studying the dynamics of NCCAs/CCAs is just one such example of this approach.

Some technical clarifications of using NCCAs

The terminology non-clonal aberration is commonly used in the field of cancer cytogenetics (ISCN, 1995). There seems to be no disagreement on the use of this term, but there is a distinct disagreement on their biological significance. Prior to our publications (Mitelman, 2000; Albertson et al., 2003; Heng et al., 2004, 2006a,b,c; Heng, 2007a,b), the general rule in tumor cytogenetics is that only clonal chromosomal abnormalities found in tumors were considered significant and should be reported.

A clone is defined as a cell population derived from a single progenitor. It is common practice to infer a clonal origin when a number of cells have the same or closely related abnormal chromosome complements. In practice, there are two meanings when the clonal aberration is used in cancer cytogenetics: first, it means that they are derived from a common ancestor within a defined time frame; and it also means that they are karyotypically identical or similar to each other. This latter meaning is of importance to cancer research, as technically speaking, all different cancer cells as well as normal cells of one individual must come from a single progenitor cell of a fertilized egg. However, different tumor cells and normal cells of one individual are not considered clones when they display drastically different genetic profiles

(only when they share the same marker of abnormal chromosomes). The term non-clonal here is used to distinguish the clonal karyotypes rather than refer to cells not derived from a common ancestor. Another note of caution is that whether or not an aberration is clonal depends on the time frame of examination and the level at which the study takes place (karyotypic vs. gene). Within a given period, the clonal aberrations can further evolve making it hard to realize that they are derived from a common ancestor. In addition, the concept of clonality can be applied to different levels of genetic organization. Cell populations with the same p53−/− mutation can be referred to as clonal at a specific locus, but they might be considered non-clonal at the karyotypic level.

To establish a precise scoring system to monitor the level of genome instability is challenging, as there are many different types of genome level alterations. By comparing the type and distribution of aberration frequencies for these model lines, it appears that the proportion of structural NCCAs represents the best biomarker. When NCCAs are used to score the level of heterogeneity, the total frequency of structural and numerical NCCAs should all be included. At present, we have only focused on structural NCCAs, as numerical NCCAs more commonly exist among cell lines that might require a more sophisticated statistical model to quantify. According to our analysis, the structural NCCAs seem to play a more dominant role than numerical NCCAs, at least for the late stage of cancer progression (after transformation) that we examined in this study. Our on-going studies show that chromosomal number variation plays an important role prior to the formation of structural NCCAs during the immortalization process of the mouse ovarian model (Lawrenson et al., unpublished work). Further research is needed to incorporate other types of genome variation into the NCCA scoring process, such as including copy number variations.

As we discussed in previous papers, the 4% cutoff of clonal/non-clonal is based on the standard of practice in medical genetics. It would be ideal if we could examine more than 100 mitotic figures and use 1% as the cutoff line, but this is very time consuming and costly. In fact, a 4% cutoff is also reasonable as illustrated by our studies with large numbers of cell lines and clinical samples. For example, when studying the level of genome variations during the in vitro immortalization process, two additional cutoff lines were used (1% and 10%), the overall patterns of punctuated and stepwise phases of karyotypic evolution were the same as the 4% cutoff line (when the genome is unstable, the level of NCCAs often reaches over 20–50%). In our immortalization model, when the cell population reached the unstable phase, NCCA levels were 100%, regardless of which cut off line was used to separate CCAs and NCCAs (Heng et al., 2006b). In normal lymphocytes (based on both human and mouse data), the level of structural NCCAs is very low, in the range of 0.1–2%. For the purpose of establishing a baseline of structural and numerical NCCAs in normal individuals, we often score over 100 mitotic figures. Interestingly, as illustrated by a current study, the differential frequency of NCCAs is more important than the absolute level of NCCAs as for each system tested, there seems to be a baseline of instability. No matter which cutoff line is used; the elevated NCCAs can be easily scored.

The key point here is using NCCAs rather than a given CCA to measure the overall system status and determine how stable a genome system is within a population. The population behavior or stability can be monitored by the degree of population diversity. It is our belief that a new direction in cancer research will focus on controlling the process of system evolution, rather than focusing on specific drug targets, as there is no fixed target and just focusing on specific targets does not solve the issue of drug resistance in a dynamic evolving system. During the evolutionary selection process, any given pathway or specific target could become insignificant. Therefore, the apparent disadvantage of monitoring NCCAs in fact is an advantage in terms of monitoring the system status and its usefulness for system control.

One additional point needs to be clarified, the NCCA/CCA cycles we refer to could be described as clonal expansion and heterogeneity. The waves of dominant NCCAs or specific CCAs reflect the overall status of the stability of a population and the pattern of evolutionary dynamics. In contrast, using "clonal expansion" and "genetic diversity" to describe these two phases of population dynamics is not accurate. For example, during the clonal expansion phase, there is clearly genetic diversity. While, during the "genetically diverse" phase, all the new clones are still generated by clonal expansion. One of the key findings of our karyotypic evolutionary study is that there are two typical types of clonal expansion illustrated by the immortalization model: clonal expansion with a lower level of system instability where expanded clonals share the majority of karyotypic characteristics of the parental cells; and clonal expansion with high levels of system instability where expanded clonals share few or no key karyotypic characteristics. Interestingly, by just using a molecular profile such as tracing specific loci using a mixed cell population, drastically different evolutionary phases would not be appreciated. The partial reason that previous cytogenetic studies found the term "clonal expansion and genetic diversity" accurate is that the contribution of high levels of NCCAs were disregarded, resulting in easily identified marker chromosomes. From a molecular standpoint, it is easier to use the term clonal expansion in the molecular sense to study specific loci. When a specific locus is not an expansion, it can be called genetic diversity. However, if large numbers of loci were simultaneously monitored, it would be challenging to define the phase of clonal expansion. This is the exact situation when one studies karyotypic evolution based on a single cell within a dynamic cell population. In conclusion, it is useful to describe the change in frequency of the NCCAs or the amount of genetic diversity and also the phenomena of clonal expansion indicated by the types and frequency of CCAs.

Potential clinical implications

With an emphasis on the overall instability of the genome generating clonal diversity of cell populations as a major cause of cancer, this study favors a new approach to cancer research by focusing on the mechanism of cancer evolution rather than focusing on a specific molecular mechanism such as gene mutations or pathway. For the majority of cancer cases that involve multiple cycles of NCCA/CCA interaction, one specific pathway will likely not be successful. Thus more potential available pathways represented by high levels of NCCAs are necessary to develop a successful combination. It is likely that certain CCAs coupled with relatively powerful pathways can speed up the process of cancer evolution by drastically destabilizing the genome or by producing a high level of cell proliferation (such as specific powerful fusion gene mediated tumorigenesis). To complete the entire process of cancer formation, however, an overall high level of diversity is the key. Coupled with elevated levels of population diversity, there could be many pathways or great numbers of combinations of pathways that could lead to cancer through multiple steps. The combination of dominant pathways and high level genome dynamics create the most favorable conditions for cancer evolution. Therefore, reduction of factors leading to genome instability and reducing cell population diversity should become new areas of focus for clinical research. For example, the key to cancer prevention and treatment is stabilization of the genome system. When genomes are unstable, blocking one particular aberrant pathway will likely not be successful, as new pathways will eventually emerge.

It is true that stochastic gene mutations also contribute to population diversity and can be traced in evolutionary studies (Maley et al., 2006; Heng, 2007b). Similarly, epigenetic dynamics, as well as copy number variation all contribute to genome level alterations. It is very important to incorporate the degree of diversity at various levels. Our hypothesis that using the frequencies of NCCAs might be inclusive of most of the other types of genetic and epigenetic dynamics seems to be correct and needs to be explored further, as the vast majority of other levels of genetic alterations will lead to karyotypic changes if system evolution occurs. Based

on our viewpoint that the karyotype defines a genome system (both the overall expression pattern and the identity of a species), and that cancer evolution is driven by karyotypic mediated macro-evolution (Heng, 2007a,b; Ye et al., 2007; Heng et al., 2008), we anticipate that most cancer cases will have variable karyotypes. In fact, for many cases of leukemia, the seemingly normal karyotypes are only detected during the relatively stable phase of cancer progression. In the blastic phase, for example, karyotypic dynamics are overwhelming. Based on this consideration, this might be an advantage of using the highest level of genetic organization (the genome) to monitor genome system instability and evolution.

It should be pointed out that increased karyotypic diversity associated with various stages of cancer progression has been previously noted by others. The high level of karyotypic heterogeneity of NIH 3T3 cells has been linked to population diversity and transformation (Rubin, 1993). The literature has also provided ample evidence to support this viewpoint, though the evidence has been largely ignored. For example, many genes or pathways that are linked to genomic instability in fact generate increased karyotypic diversity (Akagi et al., 2003; Radisky et al., 2005; Heng, 2007a). Interestingly, the link between population diversity and tumorigenicity reconciles the gap between certain experimental findings and clinical data when considering how these powerful oncogenes contribute to cancer. Under experimental conditions, most oncogenes are capable of inducing tumors, as the conditions have been created that increase the probability of cancer progression by using strong promoters and artificial selection. In real clinical cases, these well characterized oncogenes have limited involvement. The combination of strong oncogenes and tumor suppressor genes can significantly increase the probability of cancer progression under experimental conditions further demonstrating the importance of diversity as over or under expression of many oncogenes and tumor suppressor genes are directly or indirectly caused by genome instability.

Lastly, our approach to monitoring genome diversity could also be a valuable concept to develop assays for clinical use. A study monitoring clonal diversity and subsequent clinical outcomes in Barrett's esophagus is one example (Maley, 2006). It is known that the lesions in Barrett's esophagus exhibit the unique feature of stasis that allows the establishment of a correlation between stages associated with some key genes (one of the possible reasons is that the pre-cancer phase could be relatively more stable where there are more opportunities for clonal expansion). However, different from Barrett's esophagus, most fast growing tumors exhibit high levels of diversity and dynamic karyotypic evolution, which is more typical of most progressive genomically unstable tumors. Monitoring the levels of non-recurrent genomic aberrations in these latter types of tumors rather than using the degree of clonal aberrations is a more accurate level of genomic instability and is a practical method of accessing the likelihood of cancer progression. In addition to the potential benefit of using the level of NCCAs to monitor cancer progression and to provide needed tools for early diagnosis, this concept will help us to refocus on overall genomic instability and the generation of population diversity, rather than continue to focus entirely on specific pathways alone.

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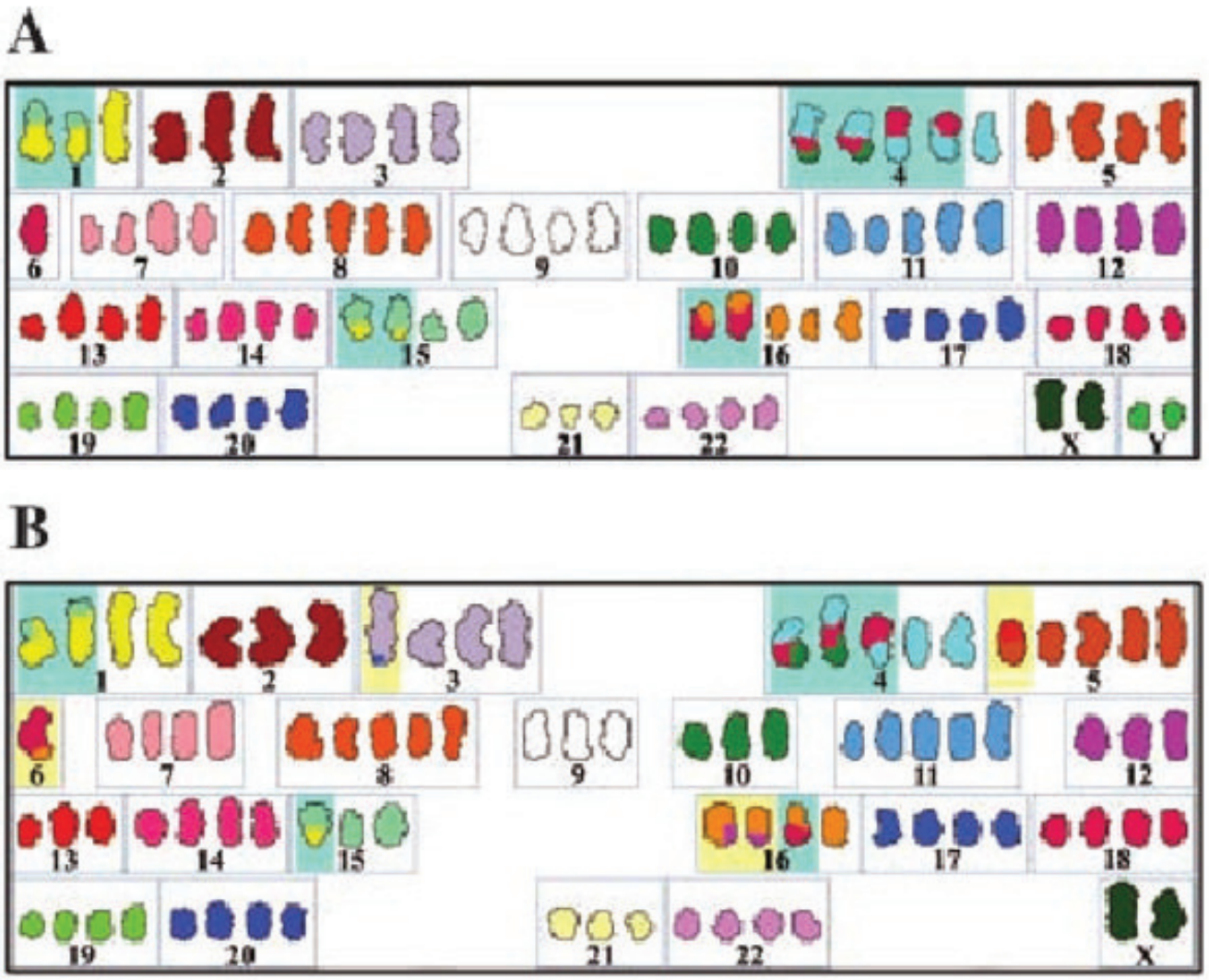

Fig. 1.

Example of increased levels of NCCAs detected from the late stages of in vitro models coupled with increased tumorigenicity. This figure shows a karyotype comparison between an early stage (p36) (A) and a late stage (p105) (B) of the LNCaP cell line. In addition to sharing all four types of CCAs as indicated by the blue colored boxes, there are more NCCAs detected as indicated by the yellow boxes coupled with increased tumorigenicity.

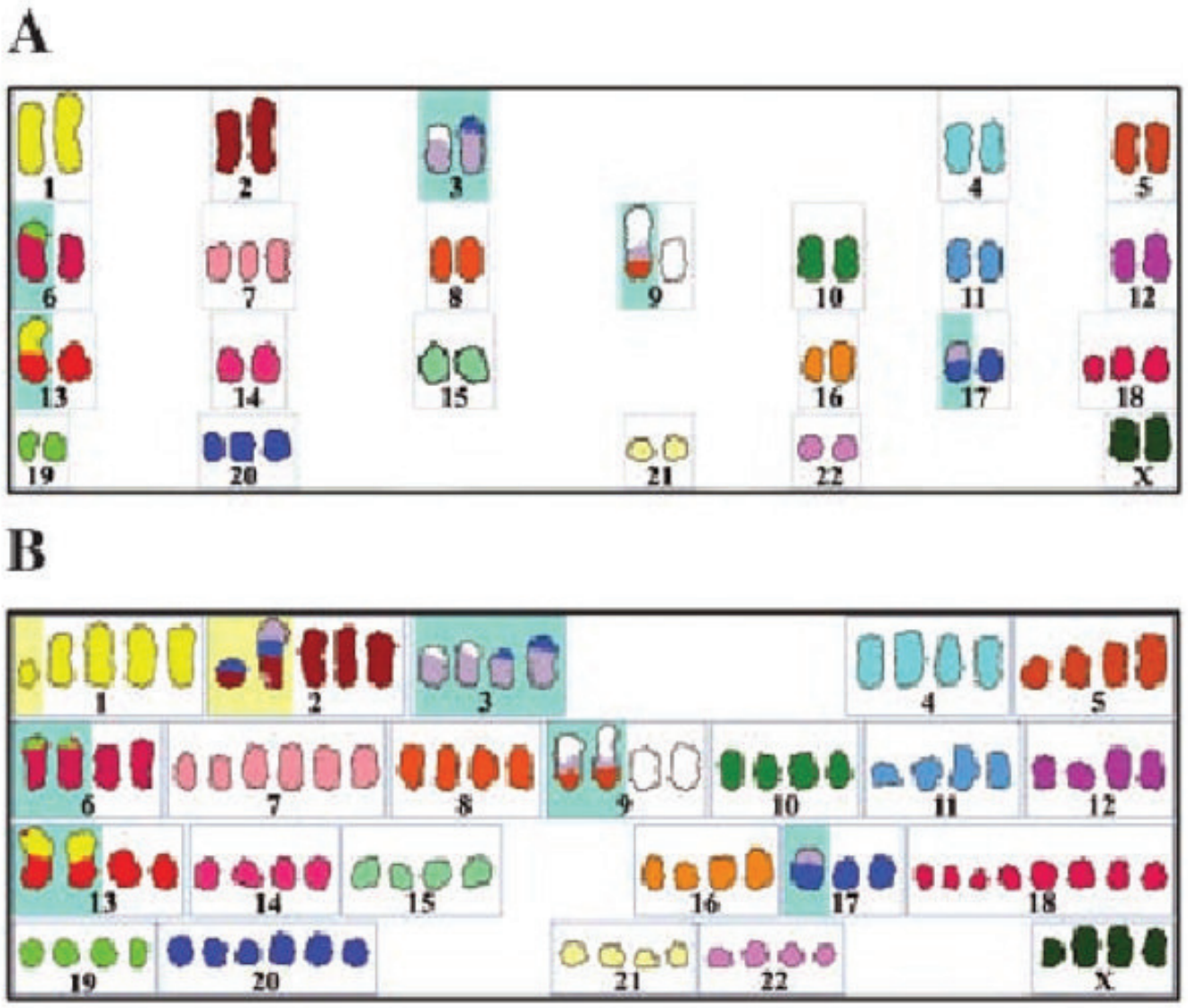

Fig. 2.

Example of increased levels of NCCAs detected from the late stages of in vitro models coupled with increased tumorigenicity. This figure shows the comparison between subline MCF10A-CSC-1 (A) and CSC-3 (B). Both lines share five common types of CCAs as indicated by the blue colored boxes. In line CSC-3 with increased tumorigenicity, in addition to ploidy changes, there were many NCCAs detected as indicated by the yellow colored boxes.

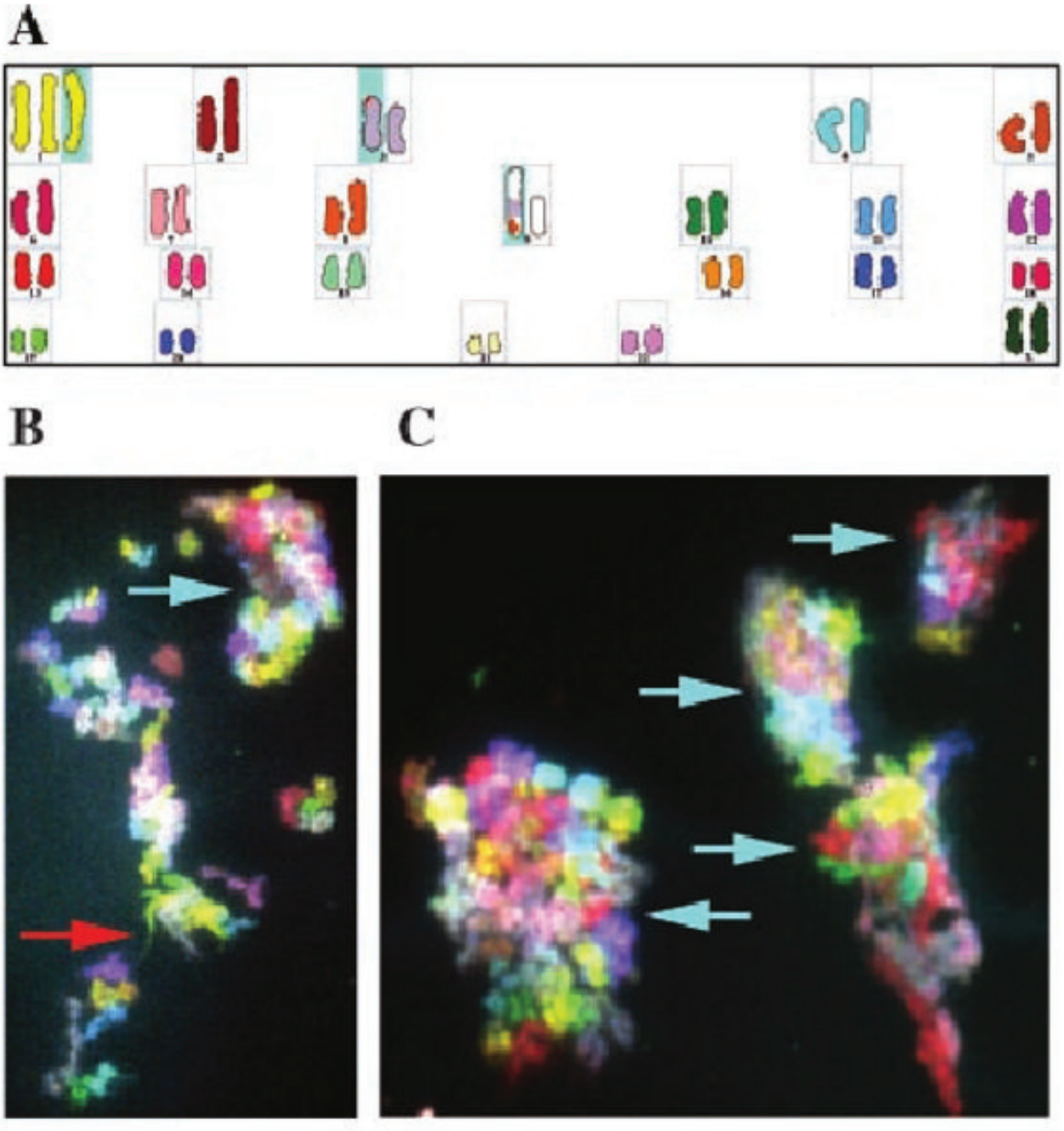

Fig. 3.

Example of increased levels of NCCAs detected from the late stages of in vitro models coupled with increased tumorigenicity. This figure shows the comparison between the HOXA1 expressed line and the control line generated from MCF10. Both lines displayed the same karyotypes with two identical CCAs indicated by the blue colored boxes (A). Interestingly, however, the HOXA1 line also displays a much higher level of abnormal mitotic figures (chromosomes) are not well condensed) (indicated by a red arrow) or separated (indicated by blue arrows) (B). These defective mitotic figures are types of NCCAs.

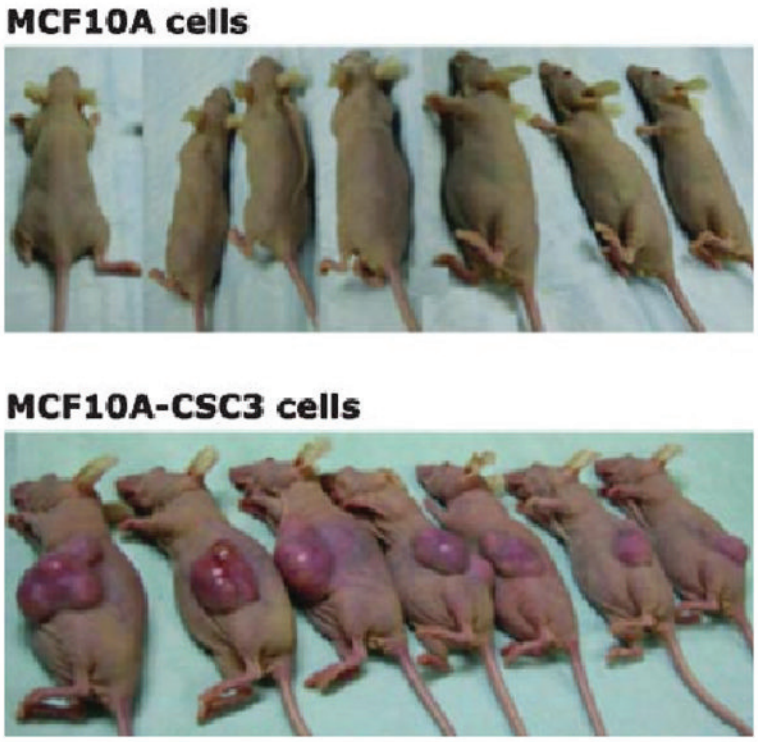

Fig. 4.

CSC3-transformed MCF10A cells form tumors in nude mice. The control MCF10A cells did not form tumors in nude mice within 20 days. Only the MCF10A-CSC3 cell line grew and formed palpable tumors in nude mice within 20 days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

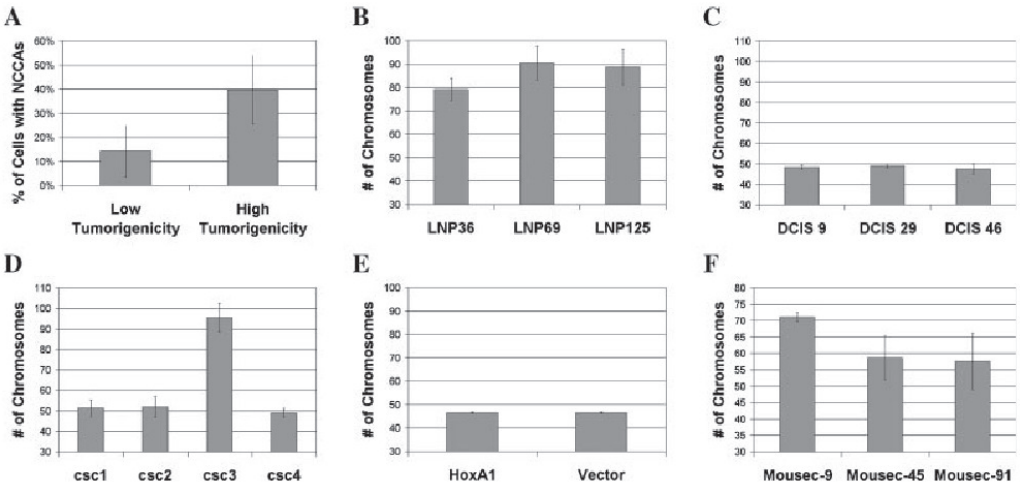

Fig. 5.

Distribution of structural and numerical NCCAs. A: Distribution of NCCAs across the five cell lines of five in vitro models with the highest tumorigenicity and the five cell lines with the lowest. Bars indicate 95% confidence intervals. The difference between high and low tumorigenicity is significant (*P*=0.01791, Student's t-test), illustrating the significant relationship between frequencies of NCCAs and tumorigenicity. B–F: Distribution of chromosome number across the five systems analyzed. Graphs represent average chromosome number, bars indicate 95% confidence intervals. Change in chromosome number does not associate with increased tumorigenicity in most lines except MCF10-CSC, possibly due to the ploidy. Passages/cell lines with higher tumorigenicity, however, do tend to show increased confidence interval widths indicating more variance in chromosome number.

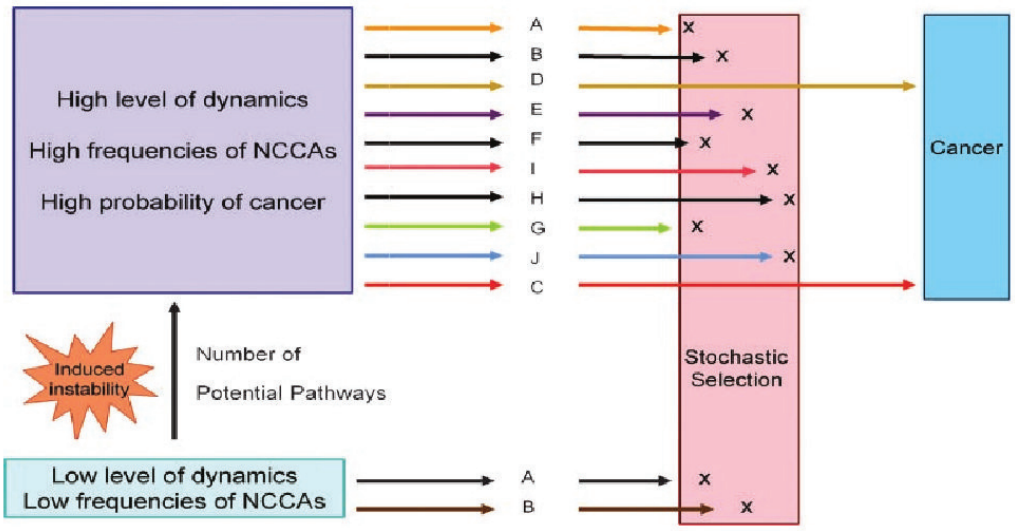

Fig. 6.

Illustrating the evolutionary mechanism of cancer and its relationship with molecular mechanisms. The evolutionary mechanism of cancer formation is summarized as three key components: 1, system instability; 2, increased system dynamics or population heterogeneity (reflected as an increased probability of a "hit" of a specific pathway or potential pathways); and 3, natural selection at the somatic cell level. There are many different molecular pathways that can trigger system instability, and it is the unstable system that activates different molecular pathways as the response to system instability. The somatic selection process stochastically favors different packages of genome alterations. The lower left box represents a normal stable state that typically generates infrequent NCCAs and when they do occur will likely go extinct. With increased instability, much higher levels of NCCAs occur representing an increasing number of potential genome systems coupled with specific molecular pathways. Each array represents a given molecular pathway, or the so called molecular mechanism. The increased number of pathways (represented by various colored arrows) increases the probability that evolution will proceed at a faster rate progressing much further in selected cell populations with some eventually achieving cancer status (the evolutionary mechanism).

TABLE 1

Types and frequencies of various CCAs and NCCAs of the seven models analyzed

Cell Lines tissue samples	Chromosomal number	CCAs	sNCCAs (%)	Tunorigenicity
The LNCap cell lines				
Pd36:	79.17± 14.35	der(1)t(1;15), der(6)t(4;6), der(4)t (4;6;10), der(15)t(15;1), der(16)t (16;6)	30%	Low
Pd69:	90.39 ± 22.43	der(1)t(1;15), der(6)t(4;6), der(4)t (4;6;10), der(15)t(15;1), der(16)t (16;6), der(13;13)	41.5%	
Pd125:	88.77 ± 19.97	der(1)t(1;15), der(6)t(4;6), der(4)t (4;6;10), der(15)t(15;1), der(16)t (16;6)	**53**%	High
MCF10DCIS.com model				
Pd9:	48.35 ± 2.18	der(1)t(1;2), t(3;17), t(17;3), der(6)t (6;19), der(9)t(9;3;5), der(21)t (21;17)	5.9%	Low
Pd29:	49 ± 2.03	der(1)t(1;2), t(3;17), t(17;3), der(6)t (6;19), der(9)t(9;3;5), der(21)t (21;17), der(15)t(15;21), der(3)t (3;9)	12%	
Pd46:	47.58 ± 4.54	der(1)t(1;2), der(6)t(6;19), der(9)t (9;3;5), der(15)t(15;21)	**42**%	High
MCF10-CSC model				
CSC-MCF10A1:	51.25 ± 14.29	der(1)t(1;13), der(3)t(3;9), t(3;17), t (17;3), der(6)t(6;19), der(9)t(9;3;5)	24.3%	No
CSC-MCF10A2:	51.82 ± 18.23	der(1)t(1;13), der(3)t(3;9), t(3;17), t (17;3), der(6)t(6;19), der(9)t(9;3;5)	30%	No
CSC-MCF10A3:	95.5 ± 22.00	der(1)t(1;13), der(3)t(3;9), t(3;17), t (17;3), der(6)t(6;19), der(9)t(9;3;5)	**42**%	Yes
CSC-MCF10A4:	48.96 ± 7.56	der(1)t(1;13), der(3)t(3;9), t(3;17), t (17;3), der(6)t(6;19), der(9)t(9;3;5)	34.8%	No
MCF10-HoxA1 model				
HOXA1:	46.55 ± 0.76	der(3)t(3;9), der(9)t(5;3;9) + 1 der (3)t(3;9), der(9)t(5;3;9) + 1	**15.3**%	Yes
Control:	46.5 ± 0.76		5.3%	No
Mouse ovarian cancer model				
Pd9:	71.08 ± 3.37	der(10;10)	9.1%	No
Pd45:	58.6 ± 13.21	der(1)t(1;2), der(8)t(8;16)	30%	
Pd91:	57.6 ± 13.89	t(1;2), t(8;9), t(5;3), t(3;2)	**50**%	Yes
MCF10-Rad6B (benign lesion)				
MC15	52.17 ± 16.18	der(1)t(1;2), der(1)t(1;5), t(3;17), t (17;3), der(6)t(6;19), der(9)t(5;3;9)	4.3%	No
Myc-transgenic mouse model (premalignant dysplastic tissue)				
MG2	40.41 ± 5.16	− 17	**24**%	Yes

Note: For each sample of these models, an average of 50 SKY images were analyzed. For the MCF10-HoxA1 model, in addition to the listed frequency of structural NCCAs, 78% of errors in segregation reflected by the sticking chromosomes were detected in the HoxA1 line, while 14% of errors were detected in the control cell line.

TABLE 2

Distribution of various types of structural NCCAs for MCF10-CSC model

Cell lines	Recorded abnormal structures	Frequencies (%)
MCF10A-CSC-1 (# of karyotypes=53)		
t-NCCA	1	1.9%
DMF	7	13%
Other abnormal images	5	9.4%
	Total	24.3
MCF10A-CSC-2 (# of karyotypes=60)		
New CCA	der(15;22)	6.7%
t-NCCAs	3	5%
Chr-F	3	5%
DMF	6	10%
Other abnormal images	6	10%
	Total	30%
MCF10A-CSC-3 (# of karyotypes=50)		
New CCA	der(15;22)	8%
t-NCCAs	5	10%
Chr-F	3	6%
DMF	10	20%
Other abnormal images	3	6%
	Total	42%
MCF10A-CSC-4 (# of karyotypes=60)		
New CCA	der(15;22)	10%
	der(13;22)	5%
t-NCCAs	5	8.3%
Chr-F	7	11.6%
DMF	7	11.6%
Other abnormal images	2	3.3%
	Total	34.8%

t-NCCA refers to translocated chromosomes. Chr-F refers to chromosome fragmentation. Other abnormal images refer to these previously uncharacterized mitotic aberrations.

TABLE 3

Various molecular mechanisms are linked to the increase in NCCAs, the common feature of the evolutionary mechanism of cancer

Cell model	Previous findings (molecular mechanisms: features or identified pathways)	Current common findings	Refs.
LNCaP	Increased microsatellite instability; gradually lost androgen response; Increased tumorigenicity	Increased frequencies of NCCAs; increased genome diversity	Karan et al. (2001)
MCFIODCIS.com	Stromal–epithelial interaction; increasingly invasive phenotypes	Increased frequencies of NCCAs; increased genome diversity	Miller et al. (2000), Tait et al. (2007), Shekhar et al. (2008)
MCF10-CSC	Increased ration of BCL-xL/Bax; increased expression of PCNA, gadd45; increased tumorigenicity	Increased frequencies of NCCAs ploidy; increased tumorigenicity in vivo	Narayan et al. (2004)
MCF10-HoxAl	Activation of cdD1 and Bcl-2; increased tumorigenicity	Increased frequencies of MDFs; increased genome diversity	Zhang et al. (2003, 2006), Heng et al. (2004)
Mouse Ovarian	Change: cytoskeleton and focal adhesion complex, down: E-cadherin and connexin-43, increased tumorigenicity	Increased frequencies of NCCAs; increased genome diversity	Roberts et al. (2005)
MCF10-Rad6	Centrosome amplification, aneuploidy and transformation; benign hyperplastic lesions	Low level of structural NCCAs; aneuploidy	Shekhar et al. (2002, 2006)
Myc-transgenic mice	Expression of A2 and E2FI; increased tumorigenicity	Increased frequencies of NCCAs	Liao et al. (2000), Liao and Dickson (2000)