Genetic instability and divergence of clonal populations in colon cancer cells in vitro

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Summary

The accumulation of multiple chromosomal abnormalities is a characteristic of the majority of colorectal cancers and has been attributed to an underlying chromosomal instability. Genetic instability is considered to have a key role in the generation of genetic and phenotypic heterogeneity in cancer cells. To shed light on the dynamics of chromosomal instability in colon cancer cells, we have analyzed genetic divergence in clonal and subclonal derivates of chromosomally unstable (SW480) and stable (HCT116, LoVo) cell lines. Conventional G-banding karyotyping and arbitrarily primed PCR (AP-PCR) fingerprinting were used to calculate genetic distances among clones and parental cells, and to trace tree-type phylogenies among individual cells and clonal cell populations. SW480 cells showed enhanced karyotypic heterogeneity in clones as compared with parental cells. Moreover, genetic clonal divergence was also increased after two consecutive episodes of single-cell cloning, demonstrating that the homogeneity induced by the bottleneck of cloning is disrupted by genetic instability during clonal expansion and, as a consequence, heterogeneity is restored. These results demonstrate genetic drift in clonal populations originated from isolated cells. The generated cell heterogeneity coupled with selection provides the grounds for the reported feasibility of pre-neoplastic and neoplastic cells to generate new phenotypic variants with increased evolutionary potential.

Key words: Colorectal cancer, Genomic instability, Tumor progression

Introduction

Tumor progression is an evolutionary process determined by two main factors: the generation of heterogeneity and the selection of the variants most suited to survival, growth and invasion (Leith and Dexter, 1986). Different theoretical and experimental studies have postulated that the heterogeneity results from a mutator phenotype (reviewed by Beckman and Loeb, 2005). As a paradigm of this model, a defective DNA repair machinery has been linked to ubiquitous genetic instability in a subset of colorectal cancers (Perucho et al., 1994). Other mechanisms of DNA repair have not been clearly demonstrated in colorectal cancers. Nevertheless, the underlying mechanisms that sustain tumor evolution in most tumors have been only partially elucidated. Classical studies have revealed genetic and phenotypic instability in cell lines (Cifone and Fidler, 1981; Cram et al., 1983; Kraemer et al., 1983) that results in heterogeneous cell populations. This heterogenity is the basis of malignant potential and contributes to the development of variant cells with different abilities (Chow and Rubin, 1999b; Leith and Dexter, 1986; Poste et al., 1981).

The accumulation of multiple structural and numerical chromosomal abnormalities is a characteristic of the majority of colorectal cancers (Dutrillaux, 1995). Most studies have inferred the presence of chromosomal instability from the genomic damage detected at an end-point (Anderson et al., 2001; Giaretti et al., 2003; Hermsen et al., 2002; Rabinovitch et al., 1999; Risques et al., 2003a; Sieber et al., 2002). Nevertheless, it has also been noted that a correct assessment of instability (considered as a matter of rate) is only feasible in a time-course study (Lengauer et al., 1998). Few studies investigating genetic instability in cancer cells have made an actual assessment of mutation rates (Gorringe et al., 2005; Lengauer et al., 1997; Ribas et al., 2003; Roschke et al., 2002). Therefore, little is known of the dynamics of chromosomal instability, even in those cell lines that have been thoroughly investigated and considered as archetypes of the chromosomal instability pathway (e.g. SW480 colon cancer cells) (Lengauer et al., 1997). In a dynamic setting, and using G-banding and molecular cytogenetic techniques, we have previously demonstrated the presence of high rates of structural chromosomal instability in the SW480 cell line (Camps et al., 2005; Ribas et al., 2003). These experiments also revealed higher instability rates in derived clones as compared with parental cells. This result was unexpected because cloning implies a bottleneck reduction of genetic heterogeneity. To achieve insights into the nature of this process and how it might condition genetic drift in cell populations derived from a single isolated cell, we analyzed pre-existing and de novo chromosomal heterogeneity of single-cell clones derived from

SW480 cells and compared it with that of the chromosomally stable HCT116 and LoVo cells. Single-cell G-banding karyotyping and DNA fingerprinting allelotyping by arbitrarily primed PCR (AP-PCR) of cloned cell populations was applied to trace phylogenetic trees and to calculate genetic distances within and among clones.

Results and Discussion

Enhanced genetic diversity after clonal episodes

In previous studies, we demonstrated that cell cloning did not abrogate genetic heterogeneity of the parental cell populations; rather, it resulted in higher genetic heterogeneity (Camps et al., 2005; Ribas et al., 2003). To illustrate the magnitude of this increase better, we analyzed genetic divergence in metaphase spreads from parental cells and single-cell clones. A phylogenetic analysis of the G-band karyotypes revealed a higher heterogeneity among cloned rather than SW480 parental cells (Fig. 1). Diversity indices were calculated using Hamming distances (Table 1). It is of note that genetic distances among SW480 cells, and particularly in derived clones, were underestimated because uncharacterized reorganizations were considered as a single change. This was not the case in LoVo and HCT116 cells, in which all de novo structural and numerical alterations could be characterized. As expected, LoVo clones showed lower diversity indices compared with parental cells, suggesting that pre-existing heterogeneity was largely reduced after single-cell cloning. Likewise, HCT116 cells showed the lowest heterogeneity among metaphases and, as expected, the generated clones exhibited similar or lower levels of diversity (Table 1).

Genetic drift in cloned cell populations

Cytogenetic analysis provides a detailed map of chromosomal alteration data at the single-cell level but such analysis is impractical for populational analysis when hundreds or thousands of cells must be considered. Therefore, we applied the AP-PCR DNA fingerprinting technique, which provides a genome-wide screening of markers that are randomly distributed (Welsh and McClelland, 1990) and allows the simultaneous and independent genome-wide scoring of multiple types of genomic alterations, including losses and gains at chromosomal and subchromosomal level (reviewed by Risques et al., 2003b). Genetic clonal divergence was investigated in first- and second-generation single-cell clones. The second round of cloning was performed from the initial clones to distinguish between pre-existing and de novo cell heterogeneity. To have comparable data array among all clones, AP-PCR genetic profiles were adjusted and normalized in reference to parental cells. More than 180 loci were analyzed in six AP-PCR experiments (Fig. 2). Higher genetic divergence (determined as the index of differences between the fingerprints of the clones and the parental cells) was observed in SW480 clones (first cloning: 0.181±0.032; second cloning: 0.250±0.043). LoVo clones also showed high levels of genetic divergence (first cloning: 0.148±0.046; second cloning: 0.164±0.046); by contrast, in HCT116 clones, the rates of heterogeneity were negligible (first cloning: 0.067±0.019; second cloning: 0.053±0.022) because they were slightly above the sensitivity of the technique (0.05). Genetic heterogeneity of SW480 clones is in agreement with the structural instability observed in cytogenetic studies, whereas the nature of the differences detected in LoVo clones probably represents pre-existing heterogeneity (Kleivi et al., 2004; Masramon et al., 2000) and, to a less extent, the presence of de novo subchromosomal alterations (Abdel-Rahman et al., 2001; Soulie et al., 1999).

The indices of genetic divergence determined by AP-PCR were calculated in the same way as the genomic damage fraction (GDF) (corresponding to the differences between the DNA fingerprints of the tumor and the normal colonic mucosas) in

Table 1. Diversity index* between parental and derived clones in colon cancer cell lines

Cell line	No. of metaphases analyzed	Mean (95% CI)	*P*
HCT116 parental	15	0.033 (0.027-0.039)	<0.001†
clone H1	15	0.021 (0.017-0.026)	
clone H2	15	0.037 (0.033-0.040)	
clone H3	15	0.004 (0.003-0.006)	
clone H4	12	0.033 (0.026-0.040)	
clone H5	15	0.011 (0.007-0.014)	
all HCT116 clones	72	0.028 (0.027-0.029)	0.469‡
LoVo parental	9	0.080 (0.063-0.097)	<0.001†
clone L1	22	0.006 (0.005-0.008)	
clone L3	15	0.028 (0.019-0.038)	
clone L6	13	0.006 (0.003-0.009)	
all LoVo clones	55	0.013 (0.011-0.014)	<0.001‡
SW480 parental	18	0.083 (0.079-0.086)	<0.001†
clone S1	6	0.206 (0.188-0.225)	
clone S2	8	0.169 (0.155-0.183)	
clone S4	5	0.205 (0.187-0.222)	
all SW480 clones	19	0.209 (0.203-0.215)	<0.001‡

*Diversity index represents Hamming distances.
†Analysis of variance between individual clones and parental cells.
‡Student's *t*-test of parental cells compared with all clones considered as a single group.

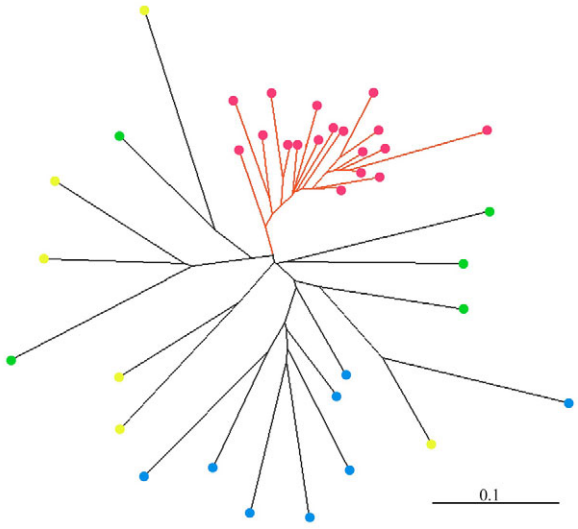


Fig. 1. Phylogenetic tree of metaphases analyzed in parental and derived clones of SW480 cells. Each dot represents a cell. Parental cells are represented in red and show the smaller genetic distances. Cells of clones S1, S2 and S4 are represented in yellow, blue and green, respectively. The scale of the diversity index (Table 1) is shown at the bottom right.

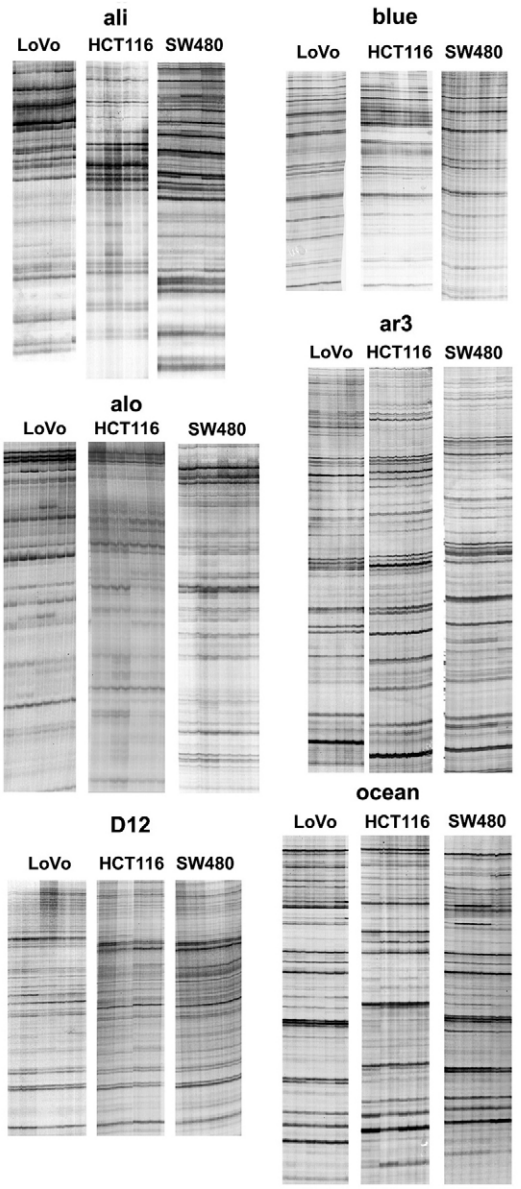


Fig. 2. Illustrative examples of the DNA fingerprinting analysis of the three cell lines with six different primers. Each panel comprises the parental cells (left-most lane) and different clones and subclones. The six different primers used are given above each panel (described further in Materials and Methods). Differences between parental and derived cells were analyzed densitometrically (see Materials and Methods).

human primary early (Tarafa et al., 2003) and advanced colorectal tumors (Risques et al., 2003a). Therefore, both measurements can be directly compared. Interestingly, the magnitude of the divergence between clones compared with the parental cells was in a similar range to the GDF in primary colorectal carcinomas compared with normal tissue (GDF=0.174±0.085). In consequence, we can conclude that genetic drift after clonal expansion (for 30-50 cell generations) might be equivalent to the genetic divergence observed in an advanced tumor with respect to normal tissue. This figure demonstrates the enormous evolutionary potential of tumor cells.

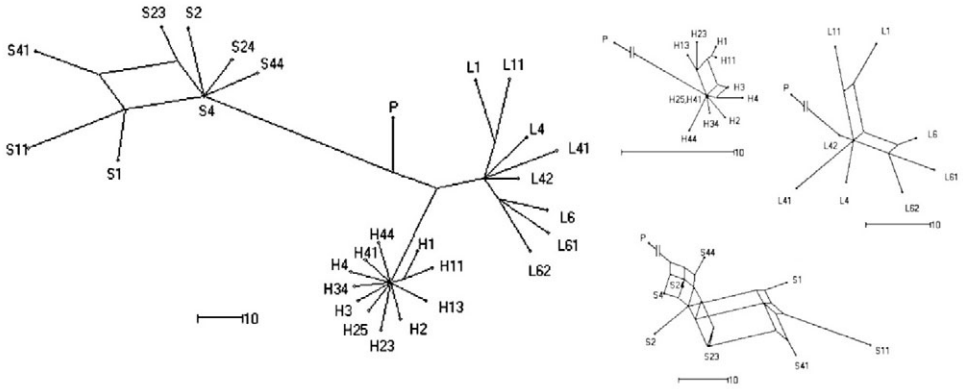
The pattern of genetic divergence is characteristic of each cell line

AP-PCR fingerprints also provide a powerful tool to gain insights into the relationships among clones by using phylogenetic approaches (Tarafa et al., 2003). Split decomposition methods and principal component analysis (PCA) were used to compare the evolutionary paths and genetic distances of the clones of the three cell lines, always relative to the respective parental cell fingerprint. In consequence, all representations show the divergence of the clones in regard to a common reference point (P) representing the parental cell line. Phylogenetic analysis confirmed that the pattern of alterations detected in the clones of each cell line was specific and resulted in grouping of clones of the same cell line (Fig. 3). LoVo clones grouped according to the rationale of the cloning process; that is, original and derived subclones were always branched, suggesting that initial cell line heterogeneity conditioned tree formation. The low number of alterations observed in HCT116 clones precluded the drawing of evolutionary patterns. SW480 clones were arranged in a complex network showing higher diversity and multiple relationships owing to the high level of de novo alterations. Maximum likelihood cluster analysis produced identical figures (data not shown). Noteworthy, subclones derived in the second round displayed similar levels of genetic divergence as those obtained in the first round (Fig. 4), demonstrating once again that the loss of heterogeneity induced by the bottleneck of the first cloning is abrogated by genetic instability during clonal expansion. This result is in agreement with the complexity of the trees generated with cytogenetic data (Fig. 1).

Implications in tumor progression and biological behavior

We show here that the SW480 cell line maintains a relatively stable karyotype at the population level despite high rates of ongoing chromosomal instability, as has been previously demonstrated in another colon cancer cell line (HT-29) by Roschke and colleagues (Roschke et al., 2002). Moreover, we also show that cell isolation and clonal expansion is accompanied by an increase of instability rates. The importance of this change has been substantiated by previous studies demonstrating the feasibility of pre-neoplastic and neoplastic cells to disrupt the homogenization process of clone isolation by favoring the generation of new phenotypic variants that restore and sometimes surpass the original heterogeneity of the cells (Poste et al., 1981). In this regard, Chow and Rubin have reported an increased transformation capacity in NIH 3T3 cells after clonal isolation (Chow and Rubin, 1999a), which might be related to the accumulation of genomic damage and contribute to the progressive neoplastic development (Bielas and Loeb, 2005; Chow and Rubin, 1999b). In addition to instability, clonal instability selection plays an important role in driving the accumulation of chromosomal alterations (Chow and Rubin, 2000; Gorringe et al., 2005; Tomlinson and Bodmer, 1999).

The maintenance of the phenotypic diversity profile might play a significant role in stabilizing the biological behavior of an otherwise heterogeneous population (Leith and Dexter, 1986) and is determinant in conferring the properties and malignant potential of the tumor (Fidler, 2003; Minn et al.,



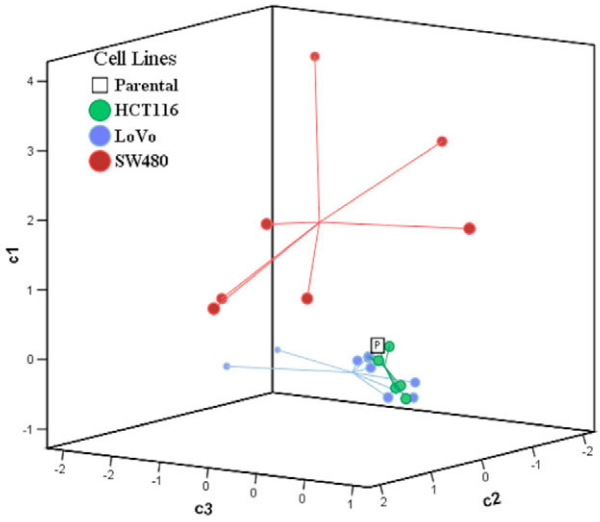


Fig. 3. Split decomposition tree (upper panel) and principal component analysis (PCA) (lower panel) constructed using AP-PCR data of clones and subclones compared with the respective parental cell. Parental cell lines were represented as a single and common point (marked P) defined by a vector (0,0,0,...). Each AP-PCR band corresponds to a component of the vector and the values indicate loss (–1), gain (+1), or no change (0) in the clone in regard to the fingerprint of the parental cell. Clones of the SW480 cell line are labeled 'S', clones of LoVo are labeled 'L' and clones of HCT116 are labeled 'H'. Tree and space arrangement revealed the differential nature of genetic alterations characterizing the divergence in the clones derived from each cell line. In PCA analysis, the spikes are traced to the centroid point of the clones for each cell line.

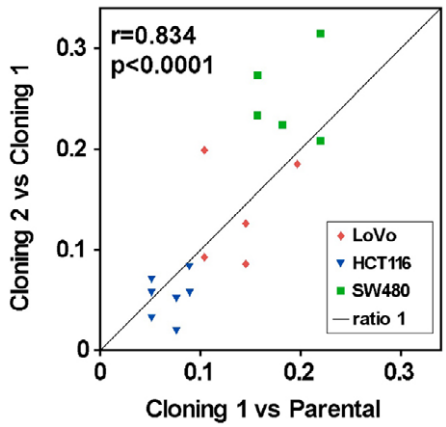


Fig. 4. Genetic divergence of clonal populations was of the same order in two successive cloning processes. The index of genetic divergence was assessed by AP-PCR (see Materials and Methods). The X-axis represents the divergence between each expanded cell population cloned directly from the parental cells (Cloning 1) versus (vs) the corresponding parental cells. The Y-axis represents the divergence between each of the second-generation clones (Cloning 2, expanded from a clone from the first cloning) versus the clone from which they were isolated. The diagonal represents ratio 1, corresponding to equal levels of genetic divergence in both cloning processes.

2005). The disruption of this equilibrium in the clone isolation process would lead to an enhanced instability in the emerging cells. In natural tumor evolution, the reduction of cellular complexity is likely to occur at different progression levels, including those related to disease dissemination and response to therapy. Adaptation to new selection pressures might be facilitated by dynamic modulation of chromosomal instability. It is of note that the benefit of enhanced mutation rates in adaptative evolution systems has been demonstrated in prokaryotic (Denamur et al., 2005; Giraud et al., 2001) and eukaryotic models (Rutherford and Lindquist, 1998). Here, we demonstrate for the first time in mammalian cells that ongoing genetic instability generates divergent heterogeneous cell populations when growth isolated. The degree of divergence among cell populations is maintained in two successive processes of cell cloning, indicating that the roots of the genetic drift lie in de novo alterations and not in the pre-existing heterogeneity.

In summary, these results provide genetic grounds for the reported feasibility of isolated pre-neoplastic and neoplastic cells to generate new phenotypic variants that restore and sometimes surpass the original heterogeneity. Understanding the genetic dynamics of these cell lines is of great relevance not only in investigations addressing the role of genetic instability in tumor progression, but also in studies involving cloning procedures that compare their molecular and biological properties.

Materials and Methods

Cell lines

HCT116, LoVo and SW480 were obtained from the American Type Cell Collection and grown in standard culture conditions. Clones (five or more per cell line) were obtained by limiting dilution and grown for 30-60 cell doublings (Ribas et al., 2003). Thereafter, a second round of cloning was performed in some of these clones in identical conditions to the first procedure. Second-round clones were grown for 40-50 doublings. DNA from parental cells and clones was prepared by standard methods. Analysis of G-bands was performed as described. The clonality criteria and the description of the tumor karyotypes followed the recommendations of the ISCN (ISCN, 1995).

DNA fingerprinting by arbitrarily primed PCR (AP-PCR)

Parental cells and clones (first and second cloning) were analyzed concomitantly in six independent AP-PCR experiments. Primers were selected based on reproducibility and pattern readability according to previous studies performed in a distinct set of samples (Risques et al., 2003a; Tarafa et al., 2003) and were: ali (5′-AGCCACCACGCCCGGCCGTA-3′), alo (5′-ATCCACCCACCTCGGCC-GTA-3′), ar3 (5′-GCGAATTCATGTACGTCAGG-3′), blue (5′-CCGAATTCG-CAAAGCTCTCGA-3′), d12s77 (D12) (5′-GAAGGGCAACAACAGTGAA-3′, 5′-CTTTTTTTTCTCCCCCACTC-3′), and ocean (5′-AATTCTCAGCAGATCCG-3′). Assay conditions and details of the analysis are essentially as previously described (Risques et al., 2003a; Tarafa et al., 2003). The reaction mix contained [^{33}P]dATP to visualize the bands. PCR products were analyzed in a 6% polyacrylamide 8 M urea denaturing sequencing gel. The gels were dried and exposed to X-ray film at room temperature without an intensifier screen for 2-4 days. Films were scanned and the intensity of the bands was densitometrically measured using Phoretix 1D Advanced v 3 (Nonlinear Dynamics). Band intensity values were normalized in each lane to compensate for differences in sample loading, labeling and exposure. Fingerprints of matched clones were always run next to each other and were compared to determine differences in band intensity. The threshold of variability was defined from a set of reproducibility experiments and adjusted by band intensity. Changes of band intensity between normal and tumor tissue were considered significant when the difference, measured as a ratio, was above the 95% confidence interval of reproducibility determinations. AP-PCR genetic profiles were adjusted and normalized in reference to parental cells in order to have comparable data array among all clones. 180 different anonymous DNA markers were included in the analysis. Owing to the polymorphic appearance of some markers, the total number of informative tags per sample ranged 98-160. Differential display for every tagged band was assessed according to reproducibility assays and scored as losses (negative values) or gains (positive values) based on the relative intensities of the clones in regard to parental cells. The summary of differences (the number of bands denoting changes in clones divided by the number of informative bands) was considered as an index of genetic divergence and was comparable with the genomic damage fraction (GDF) defined previously by assessing differences between normal and tumor tissue fingerprints (Risques et al., 2003a; Tarafa et al., 2003). The theoretical range of the divergence index is from 0 (identical fingerprints) to 1 (not a single band in common). As assessed in reproducibility experiments (Risques et al., 2003a; Tarafa et al., 2003), divergence indices of up to 0.05 might be attributed to assay variability. Changes of mobility attributable to microdeletions or microinsertions in microsatellite sequences were observed in a few AP-PCR bands in HCT116 and LoVo cells (both showing microsatellite instability), but were not considered for genetic divergence assessments. AP-PCR bands containing a microsatellite sequence often show a distinctive signature appearing as three or four regularly spaced bands displaying parallel behavior.

Statistical analysis

Contingency tables were analyzed by Fisher's exact test or χ^2 test. Statistical differences between means of quantitative variables were analyzed with unpaired Student's *t* tests or analysis of variance (ANOVA), as appropriate. All reported *P* values are derived from two-sided statistical tests. Phylogenetic analysis was performed using Maximum Likelihood methods implemented in the PHYLIP package (Phylogeny Inference Package version 3.5c; distributed by J. Felstein, University of Washington, Seattle, WA). Genetic distances were calculated using Hamming measures implemented in the PAST software (Hammer et al., 2001) (available at http://folk.uio.no/ohammer/past). The Hamming distance is calculated as the number of differences between two vectors and attributes identical weight to each change. It is considered a natural measure and is the simplest way to calculate a distance (Durbin et al., 1998; Pinheiro et al., 2004). Tree drawing was performed with the TreeView program (version 1.6.1; available from R. D. M. Page, University of Glasgow, UK; http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

In AP-PCR experiments, all cell clones were compared against the parental cell. Hence, for each clone, we obtained a vector comprising all AP-PCR bands with values indicating if each band is unchanged (0), gained (+1) or lost (–1) in the clone compared with the parental cell. Parental cells show very different fingerprints, although comprise essentially the same bands; because they have been used as reference, the vector representing each parental cell will be the same and composed by 0 (0,0,0,0,...). In consequence, all three parental cell lines can be represented as a single point that corresponds to the origin or root. This was carried out to abolish pre-existing differences to determine if the type of instability in each cell line was similar or not. Since evolutionary data may not conform to a single tree-like structure in our experimental setting, split decomposition methods were also applied to AP-PCR data after qualitative transformation using the SplitsTree version 3 program (http://www.splitstree.org) (Huson, 1998). This approach does not assume a rooted tree and therefore allows the tracing of complex networks reflecting multiple phylogenies. PCA was performed with the PAST software to illustrate the differential nature of genetic divergence in each cell line.

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