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Changes of heterogeneous cell populations in the Ishikawa cell line during long-term culture: Proposal for an *in vitro* clonal evolution model of tumor cells

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ABSTRACT

Genomic changes in tumor cell lines can occur during culture, leading to differences between cell lines carrying the same name. In this study, genome profiles between low and high passages were investigated in the Ishikawa 3-H-12 cell line (JCRB1505). Cells contained between 43 and 46 chromosomes and the modal number changed from 46 to 45 during culture. Cytogenetic analysis revealed that a translocation t(9;14), observed in all metaphases, is a robust marker for this cell line. Single-nucleotide polymorphism microarrays showed a heterogeneous copy number in the early passages and distinct profiles at late passages. These results demonstrate that cell culture can lead to elimination of ancestral clones by sequential selection, resulting in extensive replacement with a novel clone. Our observations on Ishikawa cells *in vitro* are different from the *in vivo* heterogeneity in which ancestral clones are often retained during tumor evolution and suggest a model for *in vitro* clonal evolution.

1. Introduction

In the clonal evolution of tumor cells a mutant cell with additional selective advantages can arise during expansion of the tumor cell population and become the precursor of a new predominant subpopulation [1]. It is suggested that clonal evolution in cancer is a parallel to Darwinian natural selection [2]. Thus cancer genomes can change during their progression, leading to a heterogeneous cell population. Serial passage *in vitro*, an essential process for maintaining established cell lines has, unlike cells *in vivo*, not been explicitly considered in the clonal evolution model.

Popular cell lines have been distributed to many laboratories worldwide and have been expanded extensively over while retaining the same name. This poses the question whether batches of authenticated lines with the same name remain stable. Significant variations have been observed in cell lines between low- and high- passage numbers [3]. Tumor cell lines are particularly prone to genome changes through cell culture [4], which can be missed because authentication based on short tandem repeat (STR) analysis identifies only the cell line of origin and disregards genetic drift [5,6]. Thus the genome of a cell line could be different between laboratories due to the passage of time. This possibility needs to be considered in experimental application.

The Ishikawa cell line originated from a well differentiated human endometrial adenocarcinoma and has been used for more than 35 years following its establishment in 1980 [7]. It has been reported that the karyotypes of two Ishikawa cell lines were different and STR analysis also showed different patterns [8]. One of them was subcultured 130 times after it had been obtained from the European Collection of Cell Cultures [8]. Another study had cultured and used the Ishikawa cell line without interruption for over 24 years [9]. Because of these long culture times, these data cannot be accepted as representative of other Ishikawa cell lines. In addition, it is advisable to confirm the identity of each cell line. Indeed, STR profiling in another case identified an ECC-1 cell line in a culture believed to be Ishikawa cells [10]. This indicates that misidentification or cross-contamination had occurred in the Ishikawa cell line and that the Ishikawa cell line had not been characterized correctly.

Although changes in cell lines during culture have been reported, the process has not been considered previously as an experimental *in vitro* model. We employed single-nucleotide polymorphism (SNP) microarray, single cell analysis at the chromosome level and mutation profiling at the sequence level to reveal genomic changes occurring during passage in the Ishikawa cell line. Our results demonstrate that the original genetic heterogeneity was largely lost and that cell populations are replaced by expansion of dominant clones after sequential passages.

2. Materials and methods

2.1. Cell lines and cell culture

The Ishikawa 3-H-12 cell line generated by cloning the original cell line at passage 25 [11] was deposited in our cell bank directly from

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the originator, Dr. Nishida, and registered as JCRB1505. Two batches of the frozen cell stocks were cultured independently, named as 12J and 12N. Because their passage numbers were unknown when deposited, we set up their culture from passage 1* using an asterisk to distinguish the number of passages after primary culture. MEM with 15% non-heat-inactivated fetal bovine serum without antibiotics was used as the culture medium. Cells were treated with 0.1% trypsin and 0.02% EDTA and split at 1/8 dilution once a week. The supernatant was inoculated onto Vero cells and nested-PCR designed for the detection of mycoplasma was performed following our standard protocol for quality control (http://cellbank.nibiohn.go.jp//legacy/cellbank/qualitycontrol/mycoplasmas/myco_detec_english.htm).

2.2. DNA preparation and cell line authentication

Genomic DNA was extracted from 12J cells at P3* and P20*, from 12N cells at P2* and P20* using the AllPrep DNA/RNA Mini Kit (Qiagen) and quantified using Nanodrop. Each sample was amplified by the PowerPlex® 16 STR System (Promega) and analyzed with the ABI 3500 Genetic Analyzer.

2.3. Metaphase chromosome analysis

Cells were harvested from 12J at P7* and P20*, 12N at P3* and P20* after incubation with 0.04 μg/ml Metaphase Arresting Solution (Procell) for 4 h, followed by treatment with a hypotonic solution (0.075 M KCl) and three successive changes of the fixative solution (methanol/acetic acid, 3:1). Chromosome numbers were counted on metaphases stained with Giemsa. G-banding karyotypes were based on conventional trypsin-Giemsa staining and analyzed using the Ikaros software (Metasystems). Multi-color fluorescence *in situ* hybridization (M-FISH) was performed according to the manufacturer's protocol (24xCyte kit MetaSystems). Signal detection and subsequent analysis of metaphases were carried out using the Metafer system and Isis software (Metasytems).

2.4. Whole genome analysis based on DNA microarray

DNA microarray analysis was performed using a high density chip, CytoScan HD array (Affymetrix). The data analysis was performed using the Chromosome Analysis Suit software (Affymetrix).

2.5. Cell population analysis based on FISH

Two probes, Vysis CEP12 (D12Z3) Spectrum Green and Vysis LSI MDM2 (12q15) SpectrumOrange, were used to detect the number of chromosomes 12 in low and high passages in the two batches. Hybridisation and detection were performed under the conditions recommended by the suppliers. Analysis of metaphases or interphase nuclei in 500 cells was performed in the 4 samples.

2.6. Sequencing analysis

Libraries were prepared using Ion AmpliSeq™ Cancer Hotspot Panel v2 (Life Technologies), consisting of 207 amplicons covering hotspot regions of 50 oncogenes and tumor suppressor genes. Approximately 10 ng of genomic DNA from each sample was used to construct barcoded libraries using IonXpress barcoded adapters (Life Technologies). Emulsion PCR of the combined libraries was performed using the Ion PGM™ Template OT2 200 Kit (Life Technologies) and sequencing was run on the Ion Torrent PGM using Ion 316™ chip. Reads were aligned to hg19 reference data and the analysis was carried out using the Ion Torrent Variant Caller Plugin (Life Technologies).

3. Results

In this study, samples at P2*, P3* and P7* are classified as low passage numbers (L), in contrast to P20* as high passage numbers (H).

3.1. Differences in STR

STR profiles of 16 loci are shown in Table S1, revealing identical profiles between 12J and 12N lines at the same passage numbers. However, changes were detected which occurred between low and high passage numbers (Table 1). Three different repeat lengths were detected for D18S51, PentaE and D13S317 at low passage number, which became 2 types at high passage number by loss of one type. At CSF1PO, the repeat number was changed in one of the alleles.

3.2. Chromosome number

In the 1000 metaphases analyzed, cells could be categorized into two groups based on ploidy. Near-diploid cells were detected in 89.0% of the 12J–L cells, 84.0% at the high passage number, 88.6% of the 12N–L cells and 83.5% at the high passage number. Because the remaining cells were all near-tetraploid, which could be due to a doubling of near-diploid complements, chromosome analysis was focused only on near-diploid cells. The chromosome number for 12J and 12N lines was counted in 50 and 100 cells, respectively, showing between 43 and 46 chromosomes with a mode of 45 chromosomes, indicating heterogeneous cell populations (Fig. 1). The fraction of cells with 46 chromosomes decreased during cell culture, and cells with 45 chromosomes became dominant at high passage number in both lines.

3.3. G-banding karyotype

Analysis of 20 metaphases at L passage number showed 11 and 16 types of karyotype in 12J and 12N lines, respectively, consisting of different combinations of abnormalities (Table S2). However, one homologue of each chromosome pair appeared normal (Fig. 2). Common features detected in all metaphases were monosomy X and additional material at 9p (Table S2). The main clone containing der(9), der(11) and der(13) occurred in 40% of the population (Fig. 2a, Table 2). Loss of the X chromosome caused a reduction in chromosome number, leading to a total of 45 chromosomes. In addition to this loss, trisomy 12 found in 5 of 20 cells in 12J–L and 6 of 20 cells in 12N–L appeared in cells with 46 chromosomes at low passage number. Other changes detected in more than 10% of cells at low passages were 3p+, 5p+, 11p+, 13p+ and 13q>− which varied between cells (Table S2). Karyotypes with 13p+ were always accompanied by 11p+ in both lines (Table 2, Fig. 3a).Abnormalities of 5p+ and 13q− were found mostly together with trisomy 12 (Fig. 2c).

SNP microarray profiles show that copy number change at high passage number is observed at 12q12 (Fig. S3). This indicates that the centromere of an abnormal chromosome composed of chromosomes 13, 12 and 8 is derived from chromosome 12. However, G-banding analysis cannot distinguish the centromere of origin and the banding pattern is mostly consistent with chromosome 13. Therefore, this derivative chromosome is assigned to der(13).

Table 1
Differences detected in STR profiles. Italics indicate changes through culture.

	CSF1PO	D13S317	Penta E	D18S51
	5q32	13q31.1	15q26.2	18q21.3
12J–L	11,*12*	9,12,*13*	*10*,11,19	12,*19*,20
12N–L	11,*12*	9,12,*13*	*10*,11,19	12,*19*,20
12J–H	11,*13*	9,12	11,19	12,20
12N–H	11,*13*	9,12	11,19	12,20