



ARAŞTIRMA / RESEARCH

Cancerous cell lines alter their genomic organization and karyotype with increased passage number: a cytogenetic study

Kanserli hücre hatları, pasaj sayısı arttıkça genomik organizasyonunu ve karyotipini değiştirir: sitogenetik bir çalışma

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Abstract

Purpose: Here we cytogenetically investigated the chromosomal rearrangements in repeated cultures of six different cell lines over continuous passages.

Materials and Methods: MCF7, HCT116, A549, SHSY5Y, HEPG2, and NIH3T3 cell lines were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin. GTG banding procedure was used for the analysis of metaphase chromosomes, at least 20 metaphases were analyzed per cell line.

Results: We found chromosome number variations and structural changes in the all examined cell cultures as the passage numbers increase.

Conclusion: Cell lines have long been used in research to test drugs, to delineate molecular mechanisms, to understand the environmental effects and so on. The most important feature of a cell line is its genotype and karyotype similarities with their host organism. Cancer Cell lines, possess genomic/chromosomal instability that also lead them to change their phenotype along with their karyotype from one passage to next. Therefore, it is always best to verify karyotype before employing a specific cell line in a research project.

Key words: Cancerous Cell line, karyotyping, cytogenetic analysis, MCF7, HCT116, A549, SHSY5Y, HEPG2, NIH3T3

Öz

Amaç: İ Burada sitogenetik olarak, altı farklı hücre hattının tekrarlanan hücre kültürlerinde yeni kromozomal düzenlenmeleri araştırdık.

Gereç ve Yöntem: MCF7, HCT116, A549, SHSY5Y, HEPG2 ve NIH3T3 hücre hatları, %10 FBS ve %1 penisilin-streptomisin içeren DMEM besiyerinde kültüre edildi. Metafaz kromozomlarının analizi için GTG bantlama prosedürü kullanıldı ve en az 20 metafaz analiz edildi.

Bulgular: İncelenen hücre kültürlerinde pasaj sayısı artışıyla kromozom sayı varyasyonları ve yapısal değişiklikler tespit ettik.

Sonuç: İlaçları test etmek, moleküler mekanizmaları tanımlamak, çevresel etkileri anlamak için hücre hatları uzun süredir araştırmalarda kullanılmaktadır. Bir hücre dizisinin en önemli özelliği, konak organizmasıyla olan genotip ve karyotip benzerlikleridir. Kanser Hücreleri soyları, genomik/kromozomal dengesizliğe sahiptir ve bu da her pasajda fenotip değişimi ile birlikte karyotip değişimine neden olabilmektedir. Bu nedenle, hücre kültürlerinin kullanılacağı bir araştırma projesine başlamadan muhakkak kullanılan hücre hattının karyotipini doğrulamak gerekmektedir.

Anahtar kelimeler: Kanserli hücre hattı, karyotip, sitogenetik analiz, MCF7, HCT116, A549, SHSY5Y, HEPG2, NIH3T3

INTRODUCTION

Cancer is identified more than 277 different types and regarded as second leading cause of death worldwide. The studies showed that multiple gene

mutations are involved in cancer pathogenesis¹. Scientific studies that shed light on primer pathogenetic changes in cancer mainly arise from balanced rearrangements, as the second changes that occur during cancer progression are from unbalanced changes². Cancer cells generally gain

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multiple types of chromosomal aberrations during tumor progression, including rearrangements, deletions, and duplications. As a result, the genome becomes progressively more unstable³.

The development of cancer is generally believed to originate from the accumulation of multiple genetic aberrations. These aberrations range from single nucleotide mutations to cytogenetically detectable numerical and structural chromosomal alterations. The vast majority of cytogenetically studied neoplasms are hematologic malignancies, besides in tumor cells the morphological features associated with clinical outcomes are also in a strong relationship with karyotypic abnormalities⁴.

Cancer genes are often deregulated by genomic rearrangements. Therefore, analysis of the chromosomes involved, holds a key role in characterizing and identifying cancer cell lines. Cytogenetics may also be used to study the nature and extent of chromosome breakage induced by radiation or chemicals to distinguish individual cells or clones within a tumor cell population and to monitor the stability of chromosome rearrangements⁵.

The limited use of mammals in human health related scientific research has led to the development of new research strategies. One of those strategies involves mammalian cell culture technique. Cell culture is a procedure, in which individual living cell of an organism is sustained independent of its host under laboratory conditions. Cell culture has two aspects; hard to establish primary cell culture from healthy tissues with low passage numbers and a cancer cell line with high passage numbers.

In the late nineteenth century, Wilhelm Roux demonstrated that it is possible to maintain living cells outside the body in saline buffer for a few days, another researcher Leo Loeb developed a technique called "tissue culture within the body", and was able to culture cells from body tissues, then grafted them into adult animals. An American embryologist Ross Granville Harrison developed the first techniques of cell culture in vitro in the first decade of the twentieth century. The first cell line, the "L" cell line, was established by Earle in 1948 which was derived from subcutaneous mouse tissue and in 50s and 60s, another diploid cell lines HeLa (cervix), MRC-5 (lung) and WI-38 (lung) were developed from human tissue and Vero cell line obtained from

simian tissue. The primary cell cultures are obtained directly from the tissues or organs and are considered primary until the first passage (subculture). The cell lines established from normal tissues display finite growth but, cell lines obtained from tumor tissues were able to proliferate limitless⁶.

Because of being cost effective, providing unlimited supply of material and bypassing ethical concerns about using animal or human tissues, researchers prefer using immortal cell lines, besides cell lines provide pure population which makes results reproducible⁷.

In summary, utilization of commercially available cancerous cell lines that are well characterized by cytogenetics and biochemical markers allows comparison of results among different laboratories. However, as these cell lines tend to be maintained in culture over a long period of time, mutations can occur that may change characteristics and responses of cell lines that have initially been identified or non-existent at earlier passages⁸. Therefore here we cytogenetically investigated the possible chromosomal rearrangements in repeated cultures of six different cell lines over continuous passages.

MATERIALS AND METHODS

Cell Culture

MCF7 (Breast Ca) cell line was cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS) (Gibco, Grand Island NY, USA) and 1% penicillin-streptomycin (Gibco) at 37 °C and 5% CO₂. HCT116 (Colon Ca), A549 (Lung Ca), SHSY5Y (Neuroblastoma), HEPG2 (Hepatocellular Ca), and NIH3T3 (Mouse Fibroblast) murine cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium containing 10% FBS and 1% penicillin-streptomycin.

Adherent cell lines will grow in vitro until they have covered the surface area available or the medium is depleted of nutrients so the cell lines should be subcultured in order to prevent the cells dying. To subculture the cells they need to be brought into suspension and generally trypsin is used to release the cells from the flask⁹.

The subculturing method is as follows;

1. Before passaging the cultures by using an inverted microscope control the degree of confluency and confirm the absence contamination.
2. Remove the medium, wash the cell with **Phosphate-buffered saline (PBS)** using a half the volume of culture medium.
3. Use 1ml Trypsin per 25cm² of surface area, incubate flask to the incubator 2-10 minutes.
4. By using an inverted microscope, ensure that the cells are detached
5. Re-suspend the cells in a small volume of fresh serum-containing medium to inactivate the trypsin
6. Transfer the required number of cells to a new flask and add fresh medium.
7. Incubate as appropriate for the cell line.

Cytogenetic analysis

This study has been conducted in the laboratory of Medical biology and Genetics Department of Çukurova University. Cell lines were maintained in incubators rendering conditions of 5% CO₂, 37°C temperature and proper humidity. For harvesting process suitable flasks containing relevant cell lines were selected (six flask containing MCF7, HCT116, A549, SHSY5Y, HEPG2 ve NIH3T3 cell lines). For each flask, growth medium was depleted and instead, 2 ml of trypsin solution (Gibco) was added. After 5 min of waiting for optimal activity of trypsin solution, detached cells from substratum of flasks were observed under invert microscope. Then, for cessation of enzyme activity 4 ml basal or washing medium were added to each flask.

Mixed medium and trypsin solution containing resuspended cells in culture flasks were poured into centrifugation tubes and centrifuged at 2000 rpm for 10 minutes. After this first centrifugation, 5 ml of hypotonic solution (0,075M KCl) was added to each tube for swelling. After 15 min of waiting time for gaining maximal activity of hypotonic solution, 13-15 droplets of fixative solution (1 acetic acid/ 3 methanol, Merck) were added to each tube and then tubes are again centrifuged at 2000 rpm for 10 minutes. After centrifugation process, supernatant poured from tubes and pellet which contain cells are gently shaken by automatic shaker or manually, for gaining 1ml of cell suspension. Then 5 ml of fixative solution was added to each tube containing 1 ml pellet and tubes centrifuged at 2000 rpm for 10

minutes for washing process. After centrifugation, supernatant was depleted from each tube and again 5 ml of fixative was added. This process was repeated for three times. At the last repeat of washing, cells were waited overnight in fixative solution and then centrifugation process repeated and proper cell suspension was obtained. After that, final cell suspension of each sample were dropped on glass slides from an appropriate height, gaining optimal cell distribution on the surface of slides. Then, slides were rested to incubation at 60°C temperature for a night. Slides were stained using the Trypsin and Giemsa (GTG) banding method. Chromosome analysis was conducted using CytoVision software. For each case, at least 20 metaphases were analyzed. Results were evaluated according the international system for human cytogenetic nomenclature (ISCN 1981 and 1985) at 500–550 band resolution.

Statistical analysis

Since our study was a descriptive analysis, the results were compared with the literature, therefore no statistical analysis was performed.

RESULTS

MCF-7 (Breast Ca) cell line

MCF7 was derived from the pleural effusion of a 69 year old female suffering from a breast adenocarcinoma. It was named after the Michigan Cancer Foundation (MCF) and is the most studied human breast cancer cell line in the world¹⁰. Modal number of chromosome is 82¹¹.

In our study 20 metaphase plaques were examined using cytovision software. Chromosome numbers ranged 70-92 in different metaphases directly indicate the existence of triploidy/tetraploidy type of polyploidy with major deviations from normal triploidic/tetraploidic human chromosomes. All chromosomes were gone through drastic structural changes therefore they were not identifiable with cytovision karyotyping software. Those structural changes include various derivatives, big deletions, acentric fragments, and many other minor changes such as fragilities and gaps (Figure 1).

HCT116 (Colon Ca) cell line

HCT 116 is a colorectal carcinoma cell line. The stemline chromosome number is near diploid with the modal number at 45 (62%) and polyploids occurring around 6.8%¹².

In our study totally, 30 metaphases were examined. Chromosome numbers were ranged between 44-49, showing some deviations from normal diploidic number 46. Around 30% of chromosomes were undergone major structural changes in the forms of translocations, large deletions and new derivatives which could not thoroughly defined with cytovision software after standard cytogenetic and GTG banding procedures. It is noteworthy to mention that trisomy of chromosome 9 were observed in all metaphases examined. Our conclusion is that normal diploidic state impaired for considerable fraction of genes of this cell line (Figure 2).

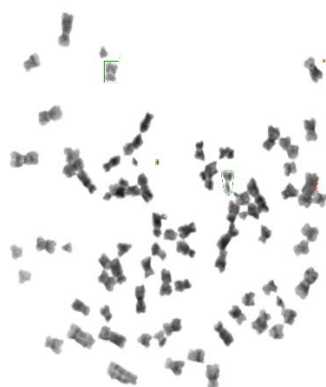


Figure 1. MCF7 cell karyotype

A549 (Lung Ca) cell line

The non-small cell lung cancer (NSCLC) cell line A549 was established in 1976 and has been very widely studied since then¹³. This is a hypotriploid human cell line with the modal chromosome number of 66, occurring in 24% of cells. Cells with 64 (22%), 65, and 67 chromosome counts also occurred at relatively high frequencies; the rate with higher ploidies was low at 0.4%¹⁴. In our study twenty metaphases were examined. Chromosome numbers were ranged between 55-66 indicating polyploidization in the form of triploidy with considerable deviations from triploid chromosome number, 69. Major and minor structural changes related to various chromosomes were observed

(Figure 3). Major numerical and structural aberrations observed implying that normal diploidic state impaired for many genes.

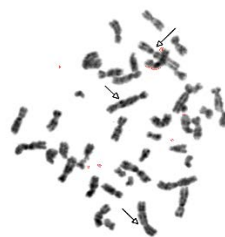


Figure 2. HCT116 cell karyotype.



Figure 3. A549 cell karyotype.

SHSY5Y (Neuroblastoma) cell line

SH-SY5Y was derived from the SH-SY subclone of the parental SK-N-SH human neuroblastoma cell line. The parental SK-N-SH cell line was established in 1970 from metastatic cells found in the bone marrow aspirate of a four-year-old female of unknown ethnicity¹⁵. Modal number of chromosome is 47¹⁶. In our study polyploidization, in the form of triploidy with considerable deviations from triploid chromosome number (69), were observed. Chromosome numbers ranged between 56 and 68 for different metaphases. All metaphases examined showed some kind of numerical and structural aberrations (Figure 4). There was consistency between many metaphase plaques in terms of observed structural aberrations. Predominantly encountered structural abnormalities were related to chromosomes 1, 3, 6, 13 and X,

together with some newly constituted chromosomes. This cell line is a female cell line which contain three X chromosomes with one X being rearranged. A bulk of chromosome material with unknown origin was added to qter region of this X chromosome.

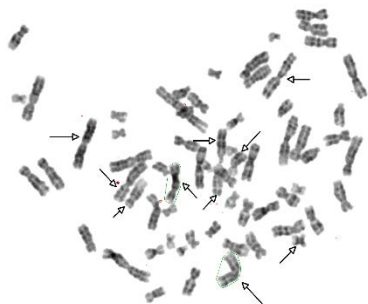


Figure 4. SHSY5Y cell karyotype

HEPG2 (Hepatocellular Ca) cell line

HepG2 was derived from a liver hepatocellular carcinoma of a 15 year old Caucasian male. Modal number of chromosome is 55¹⁷. Polyploidization was not observed. Chromosome number variations, which were mostly in the form of addition/lack of several chromosomes, of normal chromosome number (46 chromosomes) were found. Major structural changes related to almost all chromosomes were observed. Structurally unidentifiable chromosomes were subjected to various derivations, big deletions, translocations, acentric fragmentations, and other minor gains/losses (Figure 5).

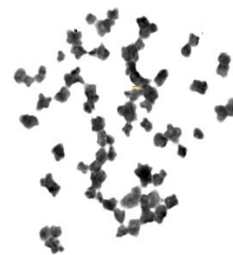


Figure 5. HEPG2 cell karyotype

NIH3T3 (Mouse Fibroblast) murine cell line

The murine cell line NIH 3T3 has been used as a model system in a multitude of different studies since its first description in 1963. NIH 3T3 immortalized spontaneously and became tetraploid shortly after its establishment. In a study, which molecular cytogenetic characterization of NIH 3T3 was done by using fluorescence in situ hybridization based multicolor banding (mcb), a complex rearranged karyotype presenting 16 breakpoints was characterized. Overall, only 1.8% of the NIH 3T3 genome is disome, 26.2% tri-, 60% tetra-, 10.8% quinta-, and 1.2% hexasome. Strikingly, the cell line gained only 4 derivative chromosomes since its first cytogenetic description in 1989¹⁸.

3T3 mouse cells are hypertriploid. The modal chromosome number is 68, which occurs in 30% of cells. Higher ploidies occur at a much lower rate of 2.4%¹⁹. In our study twenty metaphases were examined. Chromosome number variations which were in the forms of addition/lack of several chromosomes to/from usual chromosome number (40 chromosomes) were found. Polyploidization was not observed (Figure 6).

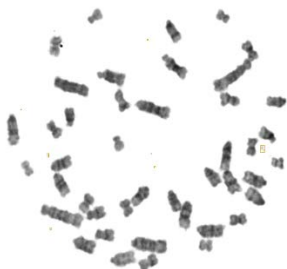


Figure 6. NIH3T3 cell karyotype.

DISCUSSION

Cell lines have long been used in research to test drugs, to delineate molecular mechanisms, to understand the environmental effects and so on. The most important feature of a cell line is its genotype and karyotype since its important to have the same or similar chromosome numbers as their host organism where they are stem from. This issue is very important due to the fact that chromosomal additions or deletions might affect gene loss, gene silencing and increased susceptibility to cellular stresses or the gene copy numbers, which may reside in the added or deleted chromosomal regions.

In our study we found that in MCF-7 cell culture, all chromosomes were gone through drastic structural changes therefore they were not identifiable and polyploidy was seen. Since, MCF is a commercial cell line which has been maintained since 1969 and was passaged many times. As being a tumor cell line and given the long maintenance time ~50 years, abovementioned major chromosomal changes were considered normal. It is understandable that those cells are not diploidic with regard to most genes, so molecular studies conducted on this commercial cell line may yield unexpected results. In accordance with our study, Rondon- Lados et al found that MCF7 cells demonstrated a modal number hypertriploid to hypotetraploid ($4n+/-$) (76 to 88 chromosomes). Each chromosome harbored either a numerical or structural aberration, which accounted for 58 different rearrangements (31 numerical and 27 structural). Polyploidy was also observed in 2% of the cells. Numerical alterations

were present in all chromosomes; losses were more frequent than gains. Structural aberrations (translocations, duplications and deletions) were found in all chromosomes except 4, 5, 13, 14 and 18²⁰. In a study of Kytola et al, isochromosomes pf 1q,5p,8q and 20q, loss of 1p,4p, 8p, 9p,11p, 11q, 13q, 18q, X and gain of 1q, 3p, 7q, 8q, 9q, 11q, 12q, 14q, 15q, 17q, 19, 20q were also found⁴.

In HCT116 cell line, we found that nearly one third of chromosomes were undergone major structural changes in the forms of translocations, large deletions and new derivatives, which could not thoroughly defined after standard cytogenetic and GTG banding procedures. It was found that trisomy of chromosome 9 were observed in all metaphases examined. Consistent with our study Knutsen et al., found that HCT166 cell line was near diploid. According to the study HCT116 contains chromosome 17, 18 translocations, 4q,16p,18p and 10q terminal band breaks²¹.

In A549 cell line, we found that chromosome numbers were ranged 55-66 and structural changes related to various chromosomes were observed. PENG et al., found that the chromosomal number of A549 varied from 62 to 66. There were 53 normal chromosomes and 13 derived chromosomes to be identified in karyotype analysis. Among 53 normal chromosomes, three chromosomes (1, 4, 12) were monosomic. Fourteen chromosomes (3, 6, 9,10, 11, 13, 15, 18 – 22, X and Y) were disomic. Six chromosomes (2, 5, 7, 8, 14 and 16) were trisomic. One chromosome (17) was tetrasomic²². Luk et al found that the A549 cell line was found to be hypotriploid. By GCH analyzes, A549 was characterized by more losses than gains, specifically the loss of all or most of chromosomes 4, 6, and 13. Gains were restricted to regions on 2p, 11p, 15q, and 17²³.

In SHSY5Y cell line, polyploidization in the form of triploidy with considerable deviations from triploid chromosome number (69) were observed. Chromosome numbers ranged between 56 and 68 for different metaphases. All metaphases examined had some kind of numerical and structural aberrations. Kim et al., found that and SH-SY5Y had near diploidic distribution with two large submetacentric chromosomes, which resulted from the insertion of 17q21 and HSR into 1p34. In the SH-SY5Y cell line, gains involved 1p34 _qter,2p13, 2p24, 15q21_qter, and 17q12 _q21. Losses involved 1p36, 11q21 _qter, 16q24, and X chromosomes²⁴.

In HEPG2 cell line chromosome number variations, which were mostly in the form of addition/lack of several chromosomes, of normal chromosome number (46 chromosomes) were found. According to the literature HepG2 cells are hyperdiploid karyotype – 52(47-54)<2n>XY, +2, +14, +17, +20, +2mar, t(1;21) (p22.2;p11-12), i(17q)/der(17)t(17;17)(p11;q11)²⁵.

In our study NIH3T3 cell line chromosome number variations, which were in the forms of addition/lack of several chromosomes to/from usual chromosome number (40 chromosomes) were found. Polyploidization was not observed. Leibiger et al, also found that 52 to 82 chromosomes were present in the studied NIH3T3 cell line¹⁸.

Cancer cell lines, unlike healthy cells, possess genomic/chromosomal instability that also caused them to change their phenotype along with their karyotype from one passage to next, which is possibly the reason for getting controversial results with the same experimental setup²⁶. In both early and late passages, a considerable cellular pleomorphy was present. However, there were also obvious morphologic differences between cells derived from early and late passages²⁷.

Even the cryostorage conditions can affect their karyotype as such cell lines with identical karyotypes gain genomic rearrangements and acquire different karyotypes after several passages and cryofreezing²⁸.

Another problem of studying cell culture is the misidentification of cell lines. It is an important problem in the biomedical sciences, contributing to the growing concerns about errors, false conclusions and irreproducible experiments. As a result of using misidentified cell lines due to cross-contaminations or inappropriate protocols, some research paper report results for a disease specific cell line turn out to be another disease, or human cell lines that turn out to be another species. In some cases, these errors may only affect results; in others they may make the results meaningless²⁹.

Short tandem repeats (STRs) are repeated segments of DNA that are typically 2-6 base pairs in length. These STRs are scattered throughout genome. The number of repeats of each STR at each genetic site varies within the human populations, and this variability in the number of repeats makes STR DNA testing extremely valuable as a human DNA identification tool³⁰. STRs have been routinely used

in human identification laboratories for applications such as paternity testing, forensic casework, and the identification of victims of mass disaster for more than two decades³¹. Tumor and transformed cell lines are more prone to genetic drift, which can accelerate with passage number, media content and other factors³². Cell line authentication procedures by using STRs will become relevant in source attribution for cultures in research laboratories and in cell based therapeutics. These procedures provide a standard methodology to assess the origin of a given cell and determine if contamination or clonotypic heterozygosity are an issue³¹. In our study because of non-authentication of cell lines, we do not know if there is a genetic differences between our cell lines and the original cell lines at a molecular level. In some metaphase plaques due to very complex rearrangements, some chromosomes also could not be defined.

Therefore, due to genomic/chromosomal instability which leads to different phenotype and make results unreproducible, it is always best to verify karyotype before employing a specific cell line in a research project and at the end of the project.

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