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Karyological Variation of Clonal Malignant Cell Lines

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Abstract Chromosomal analysis and DNA flow cytometry were performed on HeLa, malignant fibrous histiocytoma (MFH) and their clonal sublines. Two HeLa clones attained confluency in a 60-mm dish on the 45th day after cloning. In MFH two clonal sublines began to increase cell numbers at the first. One continued growth and became confluent on the 177th day but the other ceased to grow. The chromosome number in all parent and clonal sublines showed a wide range of distribution, mostly in the hypotriploid range. In spite of single cell origin, remarkable karyological variation were seen in all clonal sublines. DNA histogram of all tumor lines measured by flow cytometry displayed distinct G_1-peak which was narrower than expected from the distribution of chromosome numbers. All G_1-peaks as well as modal chromosome numbers were in hypotriploid region. From karyological variation in clonal sublines, chromosomal alteration may occur during growth. Furthermore, it is suggestive that the cells with near-triploid might be advantageous for proliferation, so that the cells with extremely small and large number of chromosomes can not progress.

Key Words : Clone, Malignant cell, Karyotype, Instability, Flow cytometry (FCM)

Introduction

Chromosomal abnomalities among the human malignancies have been reported by many authors[1-6] Heterogeneous subpopulations of malignant tumors have recently attracted a great deal of attention from the view point of the therapeutic management.[7,8] Whether chromosomal heterogeneity occurs during tumor development or it derives from originally heterogeneous tumor cells is one of long-standing questions in cancer research. Early idea favored a multicellular origin based on pathological observations.[9] Subsequently, the theories of unicellular origin gained prominence.[4,10,11] In order to determine the origin of heterogeneity, it is desirable to analyze karyotypes in the very early stage of tumor development. For this purpose, one must be able to diagnose the initial stage of tumorigenesis. Unfortunately, early diagnosis is usually not possible. Perhaps the most useful tool for such a purpose is to apply a chromosome analysis to clonal populations. The present investigation was undertaken to examine the chromosomal characteristics of cell lines derived from isolated single cells.

Materials and Methods

The parent cell lines examined were HeLa and malignant fibrous histiocytoma (MFH) which had been recently established in our laboratory. Cell cloning was performed by seeding single-cell suspensions in 0.2 ml of medium per well in 96 wel tissue culture plates (Falcon 3072 ; Becton Dick-

inson Co. Oxnard, Calf.) at a density of one cell per well. After incubation, we confirmed the clonality of each sample by microscopic observation. Wells containing two or more cells were excluded from the analysis. The clones were propagated and transferred to a 60-mm dish (Falcon 3002). When the cultures became confluent (10^6 cells / dish), cells were detached from the plastic dish by trypsinization and used for chromosome analysis, flow cytometry, and subculturing. All cell lines were cultured at 37℃ in 5% CO^2 in air in Dulbecco's modified essential medium (Nissui Seiyaku Co. Tokyo) supplemented with 10% heat-inactivated (56℃, 30 min) fetal bovine serum and antibiotics (penicillin 1000u% and streptomycin 1mg%) and the medium was changed twice a week. Chromosome analysis and flow cytometry were performed on these parent cell lines and established clones.

Chromosome study : The cultured cells were exposed to Colcemid (0.05 ug/ml) for 2.0-2.5 h, treated with 0.075M KCL hypotonic solution for 15 min at room temperature, and fixed with methanol acetic acid (3 : 1). After changing fixative twice, a few aliquots of the cell suspension in the fixative were spread on dry slide, air-dried without flaming and stained with Giemsa. Incidence of polyploid cells was determined by examining from 52 to 120 randomly selected metaphase plates from each line with high-power optics. Cells were photographed on Fuji minicopy film using Nikon photomicroscope.

Flow cytometry : Suspended cells were washed with PBS and filtered through nylon mesh (# 380). 10^6 tumor cells per 1 ml PBS were mixed with 0.1% Triton X100 for 1 min, and then stained with propidium iodide solution (final concentration 50 ug/ml) (Sigma) containing RNase (final concentration 1.0 mg/ml) (Sigma : RNase A). After 10 min incubation at 4℃ the cells were analysed by a FACS ANALYZER (Becton Dickinson). A control sample of normal fibroblasts which was newly established from the patient of pleuritis was used each time for channel number adjustment. DNA index was calculated as the ratio of the G_1-peak channel to that of diploid standard.

Results

Cell Clones

Several HeLa sublines were cloned from a parent line. Two rapidly growing sublines became confluent in a 60-mm dish on the 45th day. Two fast sublines were examined for chromosomal and FCM analysis. The fast two and parent lines were designated HeLa-C1, HeLa-C2, and HeLa-P, respectively. Two clones of MFH were proliferated at the beginning. One continued to proliferate and became confluent on the 177th day but the other ceased to grow at 1 month after cloning. The clone and parent lines of MFH were designated MFH-C, and MFH-P, respectively. The cell number of ceased line was not less than five hundreds at that time, then was on the decrease for 2 months. At last, the cells became flattened like normal epidermoid cells.

Chromosome analysis

HeLa-P

As shown in Table 1, the chromosome numbers of 109 cells examined varied greatly from 34 to 123 but they were mostly in the hypotriploid range with a mode at 60. Karyotypic variation was also marked. Many extra chromosomes were present mostly in C, D, and F groups, but their numbers were not constant. Some cells had two or three marker chromosomes (Fig 1).

HeLa-C1

The variation of chromosome number was less marked than in the HeLa-P. Figures 2 and 3 show the karyotypes of HeLa-C1 with the chromosome number of 62 and 61. In more than half of these cells, the number of group A chromosomes was 7 or 8. Many extra chromosomes were present in group C,

Table 1 Karyotypic Findings of 5 Malignant Cell Lines

Lines	n	Range	Mode	Marker
HeLa-P	109	34-123	60	2-3
HeLa-C1	55	32-68	62	1-2
HeLa-C2	52	36-112	61	1-2
MFH-P	120	45-222	57	2-3
MFH-C	52	33-112	56	3-4

Range= Range of chromosome numbers.
Mode= Mode of chromosome numbers.
Marker= Number of marker chromosome.

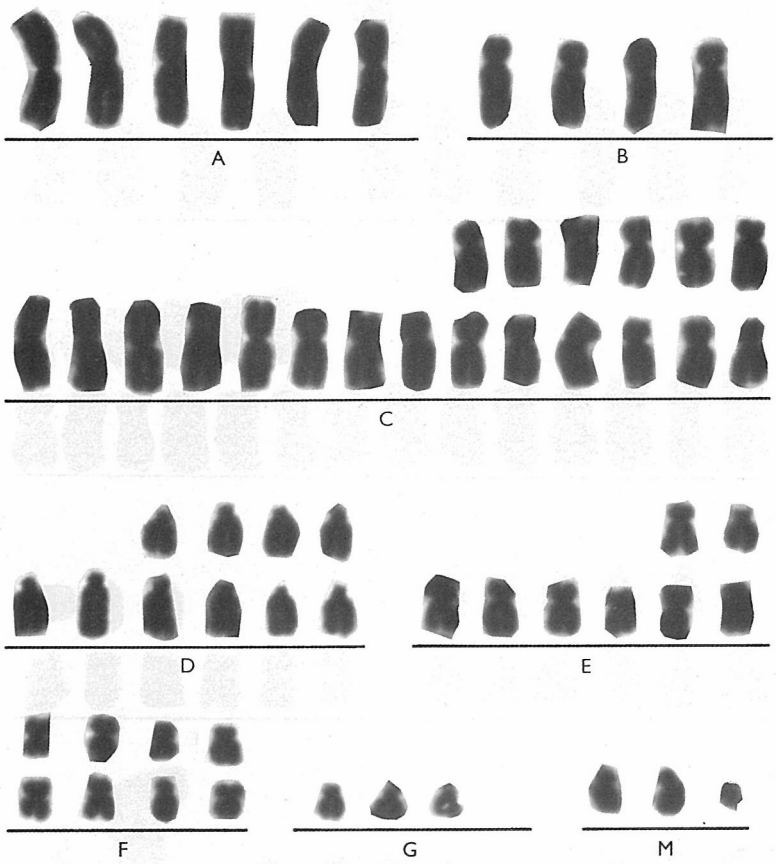

Fig. 1 Karyotype of One Metaphase Cell from Parental HeLa Cell Line (HeLa -P) with 62 Chromosomes (Mode 60). The majority of the cells in this line exhibits larg number of extra chromosomes in group C, D, and F. Some cells have two or more marker chromosomes.

D, and E. One or two marker chromosomes were often observed.

HeLa-C2

The range of variation was somewhat greater than in HeLa-C1. They often had 7 or 8 Group A chromosomes. The numbers of other groups were more variable.

MFH-P

Karyological variations were more marked in group C than in other groups. The cells often contained two or more long marker chomosomes.

MFH-C

Many karyological abnormalities were present as seen in the parental cells. There were several different types of marker chromosomes and the total number per cell was greater than that in the MFH-P cells.

Figure 4 shows the distribution of chromosome numbers of these five different lines ; distribution patterns were different.

Flow cytometry

Representative DNA histograms of six cell lines including normal fibroblasts were shown in Figure 5. All the malignant cell

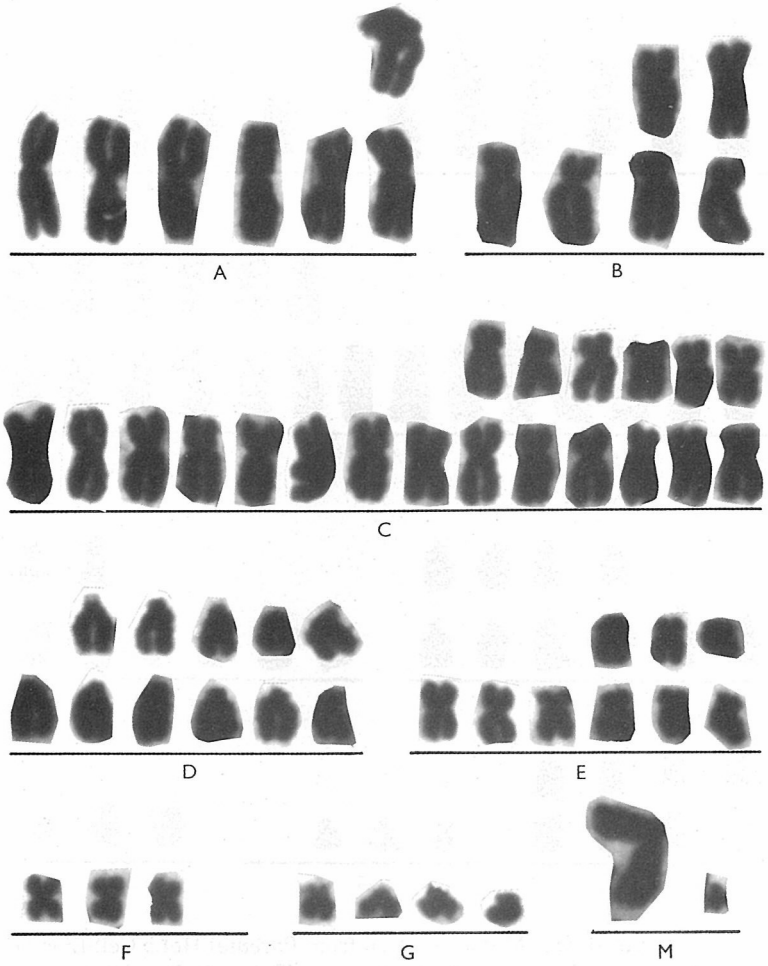

Fig. 2 Karyotype of a Cell from Clonal HeLa Subline (HeLa-C1) with 62 Chromosomes (Mode 62). Many extra chromosomes are present in group C, D, and E.

lines showed narrower G_1-peak than expected from the marked variation of chromosome numbers. G_2-peak was relatively wide as compared G_1 in all tumor cell lines. DNA index and CV (coefficient of variation) of the G_1-peak were shown in Table 2. The DNA indices of 5 malignant cell lines were in a narrow range from 1.36 to 1.41. MFH-C had the smallest modal chromosome number in malignant cell lines but the MFH-C showed an intermediate DI value.

Discussion

A stemline concept asserts existence of a predominant cell population with a characteristic modal number of chromosomes and particular pattern of chromosomal aberrations that are perpetuated on transplantaion. Such stemlines have been found in both animal and human tumors.[12-15] Using a property of stemline, Fidler et al.[16] recently demonstrated that the individual metastatic foci of the lung produced from a mouse melanoma can be of clonal origin. Available

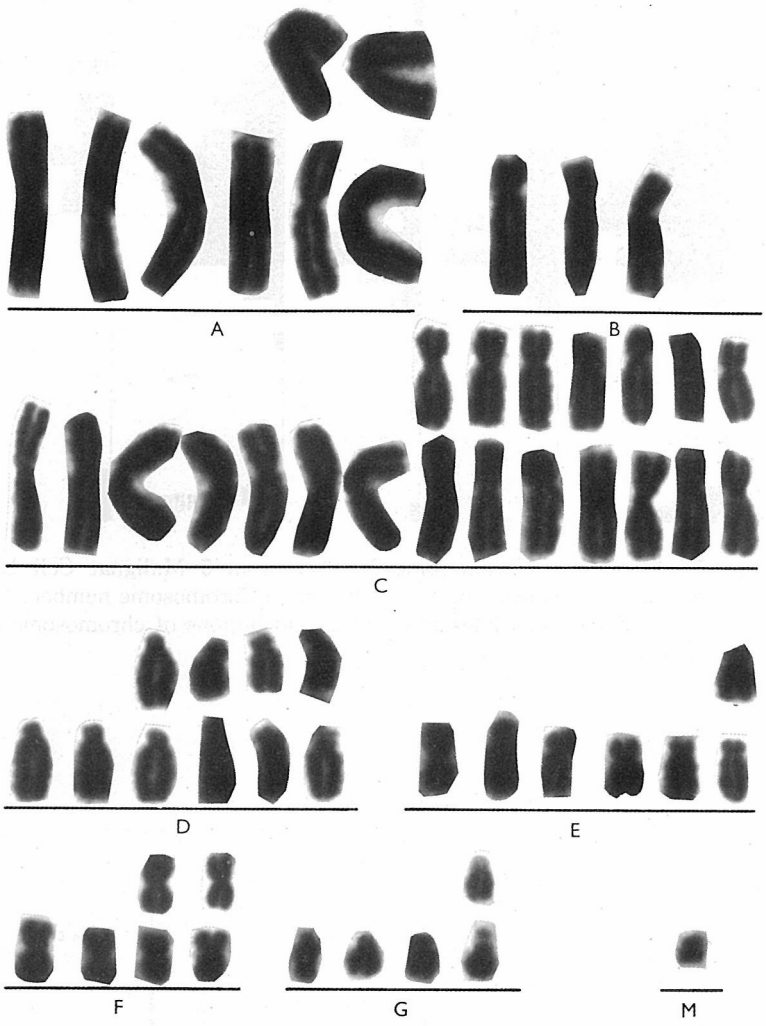

Fig. 3 Karyotype of a Cell from Same Subline in Fig.2 (HeLa-C1) with 61 Chromosomes. The Karyotype is slightly different from that seen in Fig.2. Many extra chromosomes are also present in group C, D, and F, but their numbers are not constant. One small marker chromosome is consistently observed.

data, however, indicate that the constancy of the stemline karyotype, though it is rather pronounced, is not always permanent, and that under certain circumstances or during serial transfers it undergoes numerical and structural changes.[1,17] Cytometric analysis demonstrated cellular heterogeneity of solid tumors consisting of different DNA content.[7,18–20] The results of our analysis seem to contradict with the stemline concept. In spite of clonal origin, karyotypic variations are generated. Chromosome number, karyotypes and marker chromosomes vary from cell to cell within the same clonal subline. Even if we restrict our attention to clonal cells having the same number of chromosomes, karyotypes were not the same. Someone may argue that HeLa cells might have acquired karyotypic instability in the long history of propagation. However, the

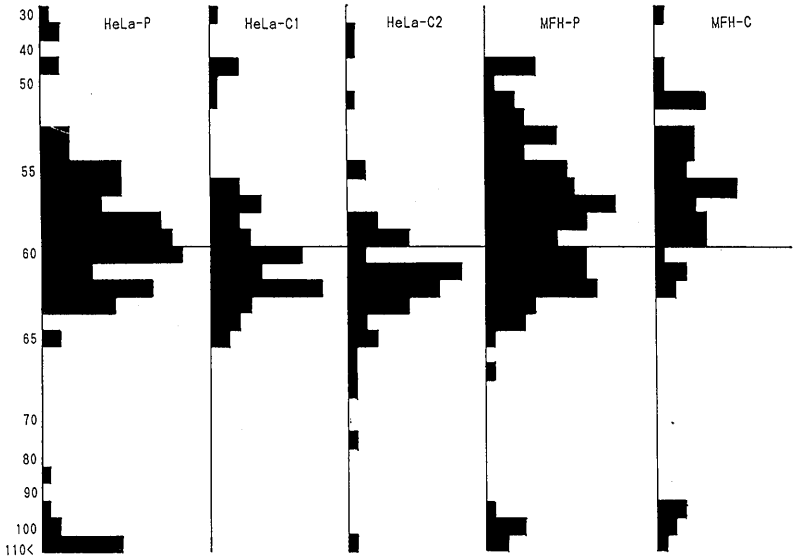

Fig. 4 Histograms of Chromosome Number from 5 Malignat Cell Lines. Abscissa : Frequency of cell. Ordinate : Chromosome number. All malignant cell lines show wide distributions of chromosome numbers.

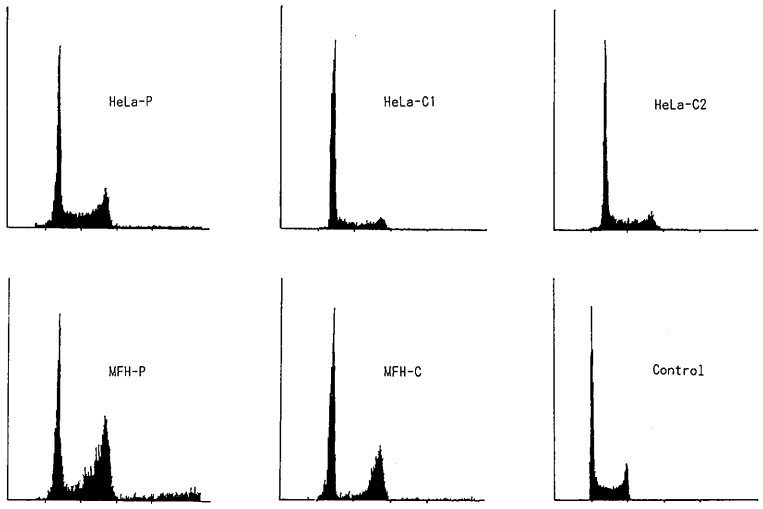

Fig. 5 DNA Histograms from 5 Malignant and Control Cell Lines. Abscissa : DNA content. Ordinate : Frequency of cell. All G_1-peaks in 5 malignant cell lines are narrower than expected from distributions of chromosome numbers.

Table 2 DNA Index and CV of 5 Malignant and Control Cell Lines.

Lines	DI	CV
HeLa-P	1.37	3.9
HeLa-C1	1.36	3.2
HeLa-C2	1.41	3.3
MFH-P	1.36	4.3
MFH-C	1.38	4.4
Control	1.0	2.7

CV : Coefficient of variation for the G_1 -peak.

MFH cells used in the present analysis were of recent origin and present results indicate that the chromosomal changes occurred rather quickly during a short interval of growth from a single cell. Genetic variability of malignant cells might underlie morphological atypism and abnormal cell growth. Nowell[10,11] has developed the concept of "clonal evolution of malignant tumor". It encompasses two major principles : 1) Tumors are unicellular in origin (clones) ; and 2) Tumors progress on the basis of genetic instability within the neoplastic population which leads to the sequential emergence of mutant subpopulations with increasingly "malignant" properties. Our results are basically in accord with the concept.

Very interestingly, however, DNA histogram measured by flow cytometry showed a sharp G_1-peak in contrast to the broad distribution of chromosome numbers. Similar findings have been observed by Kreamer et al.[21] The variability of G_1-DNA content is the same for both diploid and heteroploid cell populations despite marked variability of chromosome numbers in the latter populations. They suggested the causal reason that heteroploidy might include defects in the chromosomal condensation and kinetochore development systems. We speculate that many of the mitoses with variable DNA contents would not be able to divide successfully. Otherwise, a constancy of DNA content of G_1 cells cannot be explained. Accumulation of unsuccessful mitotic cells might account for the wide distribution of DNA content of G_2+M cells.

In all the malignant cell lines analyzed, modal chromosome numbers and G_1-DNA contents are hypotriploid. In a study of nearly 120 carcinomas, the modal chromosome numbers were distributed around either diploid or near triploid level.[22] This and our observation might suggest growth-advantage of cells with near-triploid chromosome numbers. As regard the emergence of near-triploids, Couturier-Turpin et al.[3] stressed the importance of two processes that could occur in the early stages of tumorigensis : 1) the very limited occurrence of structural and/or numerical anomalies primarily involving group A and F would lead to near-diploid cancers ; 2) the appearance of a diploid/hyperdiploid mosaic would subsequently lead to near-triploid cancers. Nowell and Couturier-Turpin et al. described that polyploid originates from diploid tumor cell. However, initial changes are not only from diploid but from polyploid which are found in some atrophic gastritis or dysplasia of uterine cervix.[20,23]

It suggests that the commonly observed polyploid cells in malignancies could be generated by a series of unequal cell divisions and selections. Chromosomal abnormality may be explained to be the result of incomplete formation of spindle fibers which required for equal cell division. Chromosomes devoid of spindle fibers would be randomly distributed into two daughters. Cells with more of the proliferation-associated genes would have greater opportunity of being selected during tumor cell progression. Without such genes cells will cease to grow. Assumption that a cell required a specific set of several chromosomes with proliferation -associated gene for growth would explain why a cell population continue or cease to grow as a result of unequal cell division. If one pair of genes is only necessary for proliferation, one of two daughter cells might not stop to grow at least.

Our cloning procedure of malignant cells should obtain useful information of tumorigenesis. Follow-up studies are presently under way in the attempt to compare with individual properties of cloned sublines.

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