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GENETIC VARIABILITY OF THE MOUSE HEPATOMA CELLS MH–22a REVEALED BY RAPD–PCR–FINGERPRINTING UNDER DIFFERENT CONDITIONS OF CULTIVATION

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Aim: **to study genetic variability in clonal lines of the mouse hepatoma MH-22a and the dependence of the capability of different clonal line cells for differentiation on the amount of genetic disturbances revealed by the RAPD-PCR method.** ***Methods:*** **The hepatoma MH-22a and its ten clonal lines were transplanted into subcutaneous connective tissue (SCT) and the eye anterior chamber (EAC) by injection of the cells to syngenic C3HA mice. The growth of transplants was checked in 15–20 days after transplantation. The tumors were fixed in neutral paraformaldehyde (10%), passed through ethanol of increasing concentrations, embedded in paraffin and stained by hematoxiline and eosine and Van Gisone. The genetic heterogeneity in the hepatoma cell population MH-22a and its clonal lines *in vitro* and *in vivo* was revealed by random amplified polymorphic DNA (RAPD-PCR). For the estimation of genetic variability revealed in the fingerprints was used the genetic variability index (GVI), based on the Bootstrep and Mikulinskaya's statistic programme.** ***Results:*** **The comparative analysis of the genetic structure of the clonal line populations *in vitro* and *in vivo* has revealed that the amount of clones with the high, intermediate, and low variability is approximately the same in both cases. It was also shown that GVI in various clonal lines *in vitro* correlated with their vital ability: the clones yielding clone lines had the lowest GVI. The same GVI value was found in the clonal lines proliferated in the EAC regardless of their capability for differentiation. Intraclonal analysis has shown that the highest values of changes revealed on fingerprints of the amplification of DNA products do not prevent from differentiation of tumor hepatoma cells in the EAC.** ***Conclusion:*** **These data allow concluding that the mouse hepatoma cells MH-22a can preserve ability for differentiation in spite of significant changes in their genome.**

Key Words: **genetic variability, RAPD-PCR, tumor, differentiation.**

It is of a special interest to find out to what degree the capability of tumor cells for differentiation and loss of malignancy depends on genetic variability taking place at cell malignant transformation and tumor progression. The experiments with cells of mouse teratocarcinoma [20] have shown that normal development could continue after introduction of tumor cells into mouse blastocytes, a part of produced pedigrees being chimeric. Cariological study has revealed that the used teratocarcinoma cells have aneuploid set of chromosomes and do not have Y chromosome, which means that the aneuploidy does not prevent the tumor cells from the loss of malignancy and from differentiation into normal tissues. It was also found that reversion to normal state could be accompanied by the loss of certain chromosomes by the cells [4] and by transformation of the diploid–tetraploid cell population into the diploid one [26] and that this reversion could occur at preservation in cells of the polyploid modal class [9]. Study of sarcomas has shown that malignant cells can acquire signs of benign cells after segregation of certain chromosomes [23], while in other types of malignant cells this can occur due to specific chromosomal rearrangements as a result of hybridization of different cell types [11, 14].

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Abbreviations used: AF DNA — amplified fragments of DNA; EAC — eye anterior chamber; GVI — genetic variability index; RAPD–PCR — random amplified polymorphic DNA PCR; SCT — subcutaneous connective tissue.

Unlike the methods used earlier in the studies of genome instability, the random amplified polymorphic DNA (RAPD–PCR)–fingerprint method allows detecting the most variable DNA fragments and revealing genes or their fragments in ampliphicated DNA fragments. The method is based on amplification of random DNA segments with single primers of arbitrary nucleotide sequence. This reaction reveals polymorphous DNA sites that are inherited in a Mendelian fashion. To perform RAPD–PCR, those decanucleotides that provide discrete products of amplification are empirically chosen. This method usually allows the acquisition of 20 to 40 fragments of different lengths with molecular masses from 50 to 2200 bp, that at the electrophoregramms leave special patters named fingerprints. This method was successfully used in studying colorectal and pancreatic carcinomas [1], gliomas [21], Willms' tumor and breast carcinomas [25], etc. By this method, homologous deletions were demonstrated in the 2q33 region of chromosomes in human lung carcinoma and in the 6p21.3 region in astrocytomas [15]. It was also found that the degree of genetic changes revealed by the RAPD–PCR method correlated with genotypic, phenotypic, and clinic characteristics of human colorectal and non–small cell lung carcinomas [3, 5].

The aim of the present work was to study the genetic variability in clonal lines of the mouse hepatoma MH–22a and the dependence of the capability of different clonal line cells for differentiation on the amount of genetic disturbances revealed by the RAPD–PCR method.

For induction of differentiation in hepatoma cells, the tumor cells were transplanted into the eye anterior chamber (EAC) of syngenic mice. The EAC is known to be a relatively immunologically privileged place in the body; besides, it contains a good nutritional medium for cell proliferation. As found earlier [24], tumor cells of mice and rats of different histogenesis, which proliferate in the EAC, have a higher level of differentiation than when proliferate in the subcutaneous connective tissue (SCT).

MATERIALS AND METHODS

The cell line of the mouse hepatoma MH–22a was obtained from the Cell Cultures Museum of Institute of Cytology of the Russian Academy of Sciences. The cultivation of the hepatoma cell line was carried out under standard conditions. To obtain clones, hepatoma was spread on Petri dishes (35 mm), 250–300 cells on each, and cultured under the same conditions as the basic cell population. Ten clone lines were used in the work.

The hepatoma MH–22a and its ten clonal lines were transplanted into SCT by injection of 1 mln cells to syngenic C3HA mice. As a rule the tumors, 1–1.5 cm in diameter, were detected in two weeks. The transplantation of the tumor cells into the EAC for revealing their potential for differentiation was performed using intraperitoneal Nembutal narcosis (concentration — 8 mg/ml, 100 μl per mouse). The eye was washed with a penicillin solution, then one drop of atropine (1%) and one drop of dicain (0.25%) were put after dilatation of pupil in several minutes. The suspension of hepatoma cells (25–50 μl) was injected into the EAC with a syringe. The growth of transplants was checked in 15–20 days after transplantation. The tumors were fixed in neutral paraformaldehyde (10%), passed through ethanol of increasing concentrations, embedded in paraffin and stained by hematoxiline and eosine and Van Gisone.

All animal procedures were carried out according to the rules of local ethic committee.

The genetic heterogeneity in the hepatoma cell population MH–22a and its clonal lines *in vitro* and *in vivo* was revealed by RAPD–PCR. As primers for the RAPD–PCR, three synthetic random decanucleotides were used: 447 (5′–AACGGTCACG–3′), 452 (5′–CCGGC–TACGG–3′), and 453 (5′–AGCTGCCGGG–3′) [13]. The total DNA of the hepatoma cells was isolated by the standard procedure [12]. The cell DNA (0.05 μg) was amplified with each of the primers (50 pm), Taq–polymerase (1.25 unit), dNTP (100 μM), and $MgCl_2$ (2.5 μl) in reaction buffer (50 μl) during 40 cycles on the PCR–amplificator (Omni–E Hybaid, USA). The thermal cycling was performed as follows: denaturation at 94 °C for 30 s, annealing at 40 °C for 1 min, and synthesis of DNA at 72 °C for 1 min. The time of denaturation in the first cycle and the time of elongation in the last one was 5 min. The products of the RAPD–PCR were separated electrophoretically on 2% agarose gel in Tris–borate buffer, and the DNA fragments were stained with ethidium bromide. The RAPD–PCR carried out with primer but without DNA and with DNA without primer were used as controls. The pattern of amplificated DNA obtained by means of electrophoresis present themselves as fingerprints that could be used for judging about genetic changes in each examined case.

For the estimation of genetic variability revealed in the fingerprints the genetic variability index (GVI) was used, calculated by deletion of the total number of changes on obtained fingerprints on the number of examinated tumors in the each group. For this the statistic method was used based on the Bootstrep and Mikulinskaya's statistic programme [10].

RESULTS

Ten clonal lines of MH–22a were examined at their transplantation into the SCT (9 tumors) and into the EAC (41 tumors) morphologically and genetically by means of the RAPD–PCR method. The subcutaneously proliferated tumors were mainly incapsulated and not fused with surrounding normal tissues. The tumors were uneven, of an intermediate density, with the cut section surface of a white–pink color. Three tumors (22/6, 525, and 544) metastasized to various tissues. The most part of tumors grown in the SCT consisted of poorly differentiated cells with big hyperchromic nuclei and a thin ring of cytoplasm. Such cells had a sufficiently high nucleus–cytoplasm ratio and no histotypical structure. Peripheral parts of tumor nodules consisted of cells of larger sizes and with numerous vacuoles in their cytoplasm. The tumor nodules of 22/5, 22/8, 525, 543, and 544 clonal lines MH–22a contained separated small areas of sufficiently large cells with well–distinct nuclei. Mitoses in the tumors were arranged irregularly, their number decreasing in hypertrophic tumor cells located near areas of necrosis.

Forty one transplants of MH–22a from EAC could be divided into two groups according to their morphological characteristics — in undifferentiated tumors and in tumors with elements of differentiation. Twenty undifferentiated transplants from EAC consisted mostly of cells with polymorphic nuclei; chromatin in such cells was either non–structured or low structured. The nuclei were surrounded by a thin ring of cytoplasm, mitosis occurred very rarely. Sometimes there can be found interweaving bundles of cells with small hyperchromic nuclei. Poorly differentiated transplants as a rule had necrosis areas. Such areas were of two types: one was homogeneous and eosinophilic, the other contained a number of nuclear fragments on the eosinophilic background. Cells formed long parallel bundles and had poorly structured nuclei and non–distinct cytoplasm. Cells between such bundles were separated with thin collagen fibers.

The elements of cytotypic differentiation were found in 21 studied transplants from the EAC. The areas of cytotypic differentiation consisted of large tumor cells with structured nuclei and distinct nucleoli. The cytoplasm in such cells is light, eosinophilic, abundant; the nucleus–cytoplasm ratio is usually low. There are a sufficiently great number of mitoses. Not infrequently, the transplants contain structures that can be considered as signs of histotypic differentiation. In such areas of tumors, cells are arranged as pseudolobuli surrounded by thin collagen fibers. The small groups of small cells (8–10 in group) with angular hyperchromic nuclei are surrounded by collagen fibers. The bundles of small spindle–shaped cells with non–

structured nuclei are surrounded by collagen fibers and resemble bundles of connective tissue.

Tumors obtained after their proliferation in the SCT and AEC were examined by the RAPD–PCR–fingerprinting with three primers (Fig.1). The obtained amplificated DNA fragments (AF DNA) were in the range of lengths from 300 to 2800 bp. The differences of fingerprints were observed by 23 AF DNA. A comparison of fingerprints of AF DNA of clonal lines MH–22a *in vivo* with fingerprints of AF DNA of the same clonal lines *in vitro* [2] has revealed some changes. They consisted in the appearance of new AF DNA, the disappearance of the AF DNA peculiar to the basic population, and their shifts in location and molecular weight, as well as an increase or decrease of intensity of fluorescence of the AF DNA. Interestingly, the total amount of such changes in fingerprints as the appearance of a new AF DNA and an increase of the fluorescence level was practically the same as the total amount of such changes as the disappearance of AF DNA and a decrease of the fluorescence intensity. In general, the changes of AF DNA were revealed in 80% transplants and most of them were found at application of PCR with one out of three primers, 447. The other two primers did not show any significant variations on fingerprints of different tumors. According to the obtained data, the most variable were the AF DNA containing 850, 900, and 950 bp.

GVI was calculated from the data obtained by RAPD–PCR in groups of clones differing by proliferation conditions *in vivo* and their capability for differentiation (Table 1). The lowest GVI — 2.18, was found in non–differentiated tumor from the SCT. The tumors proliferated in the EAC were characterized by a higher GVI, the undifferentiated tumors having practically the same GVI (2.60) as the tumors with elements of morphological differentiation (2.47).

Analysis of genetic structure of the hepatoma population MH–22a *in vivo* revealed fractions of tumors characterized by the low, intermediate, and high genetic variability separately in groups of undifferentiated tumors and of tumors with elements of cytodifferentiation (Table 2). As seen in this Table 2, most tumors, regardless of areas of proliferation and the differentiation level, consist predominantly of cells with the low genetic variability. About $^1/_5$–$^1/_3$ of the tumors had an intermediate amount of genetic changes, and only occasional clones had the high genetic variability.

Interclonal genetic variability under different conditions of proliferation and different degree of the transplant differentiation was studied in 8 clonal lines in comparison with genetic variability of the general cell population of MH–22a (Fig. 2). Only one poorly differentiated tumor from the SCT, transplanted from the general hepatoma population, had the genetic variability that did not differ from that *in vitro*. All the clones proliferat–

Table 1. GVI in clones MH–22a under different conditions of proliferation *in vivo*

Place of transplantation	Number of clones	Level of cell differentiation	GVI	*p*
SCT	8	Undifferentiated tumors	1.5	0.75
EAC	15	Undifferentiated tumors	2.60	0.86
	17	Tumors with elements of cytodifferentiation	2.53	0.88

Table 2. Genetic structure of clonal populations differing by the level of differentiation

Clones of MH–22a	The part of clones MH–22a with different number of genetic changes (%)		
	0–3	4–7	8–12
In vitro	56	32	12
In vivo			
Undifferentiated clones	74	17	9
Clones with elements of differentiation	65	29	6

Note: The data in the first line are taken from the paper of Alexandrova S.A. and Shvemberger I.N. (2003).

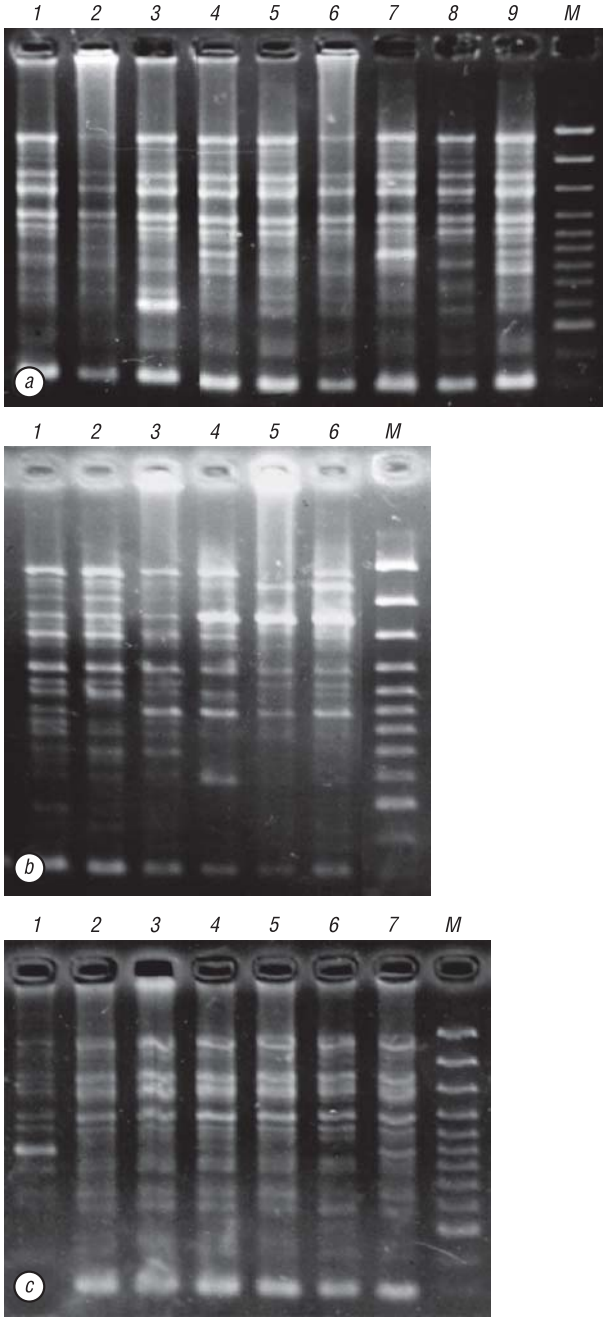
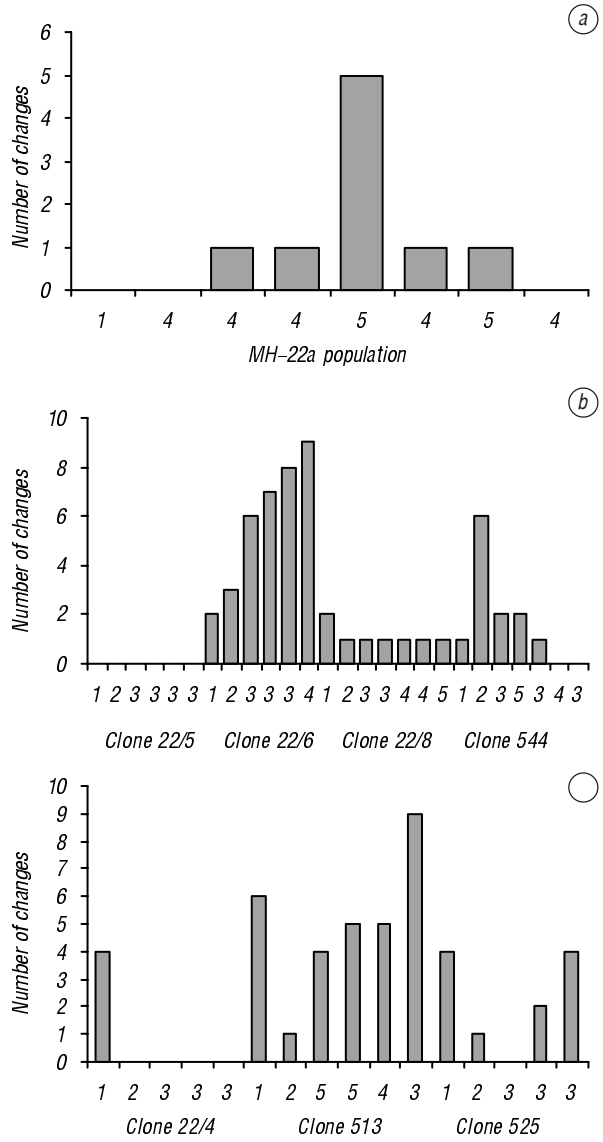


Fig. 1. Electrophoregrams of amplified DNA fragments obtained from cells of the basic population of tumor hepatocytes of MH–22a and its clonal lines after their growth *in vivo* in SCT and EAC with primer 447:
a — basic population of MH–22a; *1* — basic population of MH–22a *in vitro*, *2* — SCT, *3–9* — EAC; *b* — clonal line 22/6; *1* — clonal line 22/6 *in vitro, 2* — SCT, 3–6 — EAC; *c* — clonal line 22/8; *1* — clonal line 22/8 *in vitro, 2* — SCT, *3–6* — EAC. *M* — DNA Ladder 100 bp (Fermentas)

ing in the EAC had the signs of the cytotypic or histotypic differentiation but differed by the number of genetic changes from 0 to 5 (Fig. 2, *a*).

The significant differences in the amount of genetic changes in AF DNA were revealed at transplantation *in vivo* of the clonal lines that had a low genetic variability *in vitro* (Fig. 2, *b*). In these clones both the low number of genetic changes (22/5, 22/8) and their high level (22/6, 544) were observed. Both the low and the high levels of genetic variability could be observed in transplants with or without signs of differentiation, The same interclonal genetic variability was also found in the clonal lines characterized by an intermediate level of genetic variability *in vitro* (Fig. 2, *c*).



1 — clonal lines in vitro
2 — transplants from SCT
transplants from EAC:
3 — nondifferentiated transplants
4 — transplants shown an elements of cytotypic differentiation
5 — transplants shown an elements both of cytotypic and histotypic differentiation

Fig. 2. Interclonal genetic variability of tumor hepatocytes MH–22a proliferating in SCT and EAC:
a) general cell population of hepatoma MH–22a;
b) clonal lines of hepatoma MH–22a with low genetic variability *in vitro;*
c) clonal lines of hepatoma MH–22a with average genetic variability *in vitro*

Table 3. Genetic variability in clones MH–22a differing by vitality

Group of clones	The number of clones	GVI	*p*
Clones lost after the first passage	5	5.08	0.6
Clones stopped growing after several passages	20	3.51	0.9
Clones giving clonal lines	9	2.85	0.77

DISCUSSION

Earlier, we studied genetic heterogeneity of cell population of mouse tumor hepatoma MH–22a by the RAPD–PCR *in vitro* [2]. The genetic structure of the population was examined in 34 clones, using three random primers. As the result the genetic structure of the population based on number of changes in fingerprints of clones was revealed.

Based on such approach, genetic structure of hepatoma cells was studied *in vivo*. It turned out that all clones of MH–22a hepatoma, regardless of their degree of differentiation *in vivo*, could be divided into three groups, with the low, intermediate, and high genetic variability (see Table 2). It is interesting to note that the clonal populations of tumor cells proliferated *in vivo* contained clones with low, average, and high genetic variability in approximately the same ratios as the clones *in vitro*.

The importance of genetic instability level for prognosis of disease was shown in a study of the human non–small cell lung carcinoma, using PCR with arbitrary primers [5]. Thus, analysis of 65 samples of such tumors has shown that patients had the poorer prognosis of diseases at high genetic variability in tumor cells than at their intermediate and low variability.

The study of MH–22a clone vitality was carried out in parallel with detection of their genetic variability *in vitro* according to their GVI [2]. There was compared the amount of clones that were lost soon after their first passage, clones that were cultivated after defreezing during only several passages, and clones that produced stable cell lines. The clones that lost their vitality soon after the first passage had the highest GVI. The clones that yielded stable clonal lines had the lowest GVI. The intermediate GVI was found in clones that were cultivated during only several passages (Table 3). From these data, it may be concluded that the vitality of clonal lines MH–22a depends on the level of genetic variability in their cells.

The GVI was also determined for each group of transplants MH–22a *in vivo* under different conditions of proliferation (SCT or EAC) and with different degree of cytotypic differentiation (see Table 1). The comparison of data obtained in clones of hepatoma cells at their proliferation *in vitro* and *in vivo* has shown the GVI in clonal lines *in vivo* to be close to the GVI values in clonal lines characterized by vitality *in vitro*. These data allow concluding that clones with the higher GVI values found in the first and second groups of clones (see Table 3) practically lost their ability to proliferate *in vivo*, i.e. they are non–vital. It can be suggested that the high genetic variability prevents from long cultivation of clonal lines both *in vitro* and *in vivo*. Hence, the successful growth of the clonal lines MH–22a *in vitro* and *in vivo* is optimal at the GVI values in the range 0–3. At the same time, use of

GVI allowed the conclusion to be made that the hepatoma cells differentiation in the EAC was not directly connected with the genetic variability level. As seen from Table 1, only a tendency for the reduction of GVI can be revealed in highly differentiated tumors.

Comparison of MH–22a clones has shown that capability for differentiation can be observed in clones both with the low and with the highest GVI values obtained by using the RAPD–PCR method.

The RAPD–PCR method was used to study genetic variability of 10 clonal lines Balb/c–3T3 [13]. Interclonal analysis of non–transformed fibroblasts revealed no changes in fingerprints during four passages of cultivation. After transformation of these lines by silicon or cadmium chloride, genetic changes were observed in all transformed clonal lines, but these changes themselves and their intensity were different in all studied samples. Besides, the silicon–transformed clonal lines had shown a higher variability than the clonal lines transformed by cadmium chloride. After the transformed lines had been transplanted into the SCT of nude mice, the amount of genetic changes increased in most samples, in some cases very significantly, up to 13 changes. From these results, the authors have made a conclusion that the observed changes, indeed, are connected with the tumor cell transformation and that each tumor has its own way of progression.

The capability of malignant myeloid cells for differentiation and loss of malignancy were studied by Lotem and Sachs [17, 18] in myeloid clonal lines both *in vitro* and *in vivo*. They have found that the cells of myeloid leukemia could be transformed into mature granulocytes or macrophages by induction of differentiation by various cytokines. These clones were also shown to be heterogeneous in their capability for induction of differentiation. The leukemic cells reverted to normal ones could be introduced into embryos and take part in normal hemopoiesis. It is to be emphasized that the revealed capability of leukemia cells for normalization did not correlate with the amount of chromosomal disturbances. This might mean that genomes of malignant cells could be reprogrammed epigenetically by the corresponding cytokines.

Different chemical substances can act as differentiation inductors in myeloid cells. At present, for this purpose, cytosine arabinoside, methotrexate, radiation, and glucocorticoid hormones are used [8, 19]. Application of such inductors at high doses can cause cell death by induction of apoptosis in these cells, whereas low doses can induce differentiation and loss of malignancy. However, differentiation of different leukemia cell clones requires different combinations of these agents. Insulin, bacterial polysaccharides, forbol ether, retinoids, vitamin D3, and their various combinations also can induce differentiation in leukemia cells [7, 22]. At present, suitable inductors of differentiation were also found for other types of tumors. Thus, induction of melanoma cells could be produced by combination of interpherone β and meserine [16], while cells of human prostate tumor, by interleukine 6 and cATF [6], etc. Thereby, these data show that there are different ways of gene expression for the differentiation induction and whereas certain genetic changes prevent differentiation in one way, there can exist alternative ways leading cells to differentiation.

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ГЕНЕТИЧЕСКАЯ ВАРИАБЕЛЬНОСТЬ КЛЕТОК ГЕПАТОМЫ МЫШИ МН–22а, ВЫЯВЛЕННАЯ МЕТОДОМ RAPD–PCR–ФИНГЕРПРИНТА, ПРИ РАЗЛИЧНЫХ УСЛОВИЯХ КУЛЬТИВИРОВАНИЯ

Целью **настоящей работы являлось изучение генетической вариабельности клоновых линий гепатомы мыши МН-22а методом RAPD-PCR и зависимости способности различных клоновых линий к дифференцировке от количества генетических перестроек.** ***Методы*****: клетки гепатомы МН-22а мыши и десяти ее клоновых линий были трансплантированы в подкожную соединительную ткань (ПСТ) и переднюю камеру глаза (ПКГ) сингенных мышей линии С3НА. Через 15–20 дней после трансплантации клетки опухоли фиксировали в нейтральном параформальдегиде (10%), пропускали через спирт возрастающей концентрации, помещали в парафин и окрашивали гематоксилином и эозином и по Ван Гизону. Генетическую гетерогенность клеток гепатомы МН-22а и ее клоновых линий *in vitro* и *in vivo* выявляли методом RAPD-PCR-фингерпринта. Для оценки выраженности генетических изменений был вычислен индекс генетической вариабельности (ИГВ) на основе полученных фингерпринтов с использованием методов Бутстреп и статистической программы С. Микулинской.** ***Результаты*****: сопоставление признаков клоновых линий *in vitro* и *in vivo* показало, что доля клонов с высокой, средней и низкой изменчивостью в обоих случаях примерно одинакова. Также было показано, что ИГВ, полученные для разных групп клонов *in vitro*, коррелировали с их жизнеспособностью — клоны, давшие начало клоновым линиям, имели наименьший ИГВ. ИГВ такого же порядка был определен для клоновых линий, пролиферировавших в ПКГ независимо от их способности к дифференцировке. Внутриклональное исследование показало, что наиболее высокие значения показателей изменений, выявленных на фингерпринтах продуктов амплификации ДНК, не являются препятствием к дифференцировке клеток гепатомы в ПКГ.** ***Выводы*****: клетки гепатомы МН-22а могут сохранять способность к дифференцировке несмотря на наличие в их генетической структуре значительных изменений.**

Ключевые слова**: генетическая вариабельность, RAPD-PCR, опухоль, дифференцировка.**