BIOLOGIJA. 2019. Vol. 65. No. 4. P. 251–261

Non-accidental structural chromosome aberrations in established monkey B-lymphocyte cell lines

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Established primate lymphocyte cell lines obtained from tumour samples and from EBV-positive monkeys served us as the model system for studying the role of genetic factors and chromosomal abnormalities in malignization. The investigation of chromosome regions and genes involved in chromosomal aberrations leading to malignization in these lines was the aim of our work. Cytogenetic analysis was performed at different stages of cultivation *in vitro*. To determine the oncogenes and tumour suppressor genes located on aberrant chromosomes, data on mapping rhesus macaque genes, and high similarity of human and monkey karyotypes were used. We found that, in the line obtained from lymphomatous baboon tissue, the inactivation of tumour suppressor gene RB1 on chromosome 17 after chromosomal rearrangement is one of the most probable causes of *in vivo* malignization. Chromosomal aberrations at the region of oncogene c-Ki-ras and tumour suppressor gene TP53 change the proliferative status and differentiation in established cell lines obtained from healthy but EBV-seropositive primates. The other cause of malignization in these lines is an increase in expression of the oncogene c-myc caused by trisomy of chromosome 8 where c-myc is located. Structural aberrations in established primate cell lines affecting several chromosomal loci were identified as: (1) causing the proto-oncogene activation – the central event in the tumour clone occurrence, and (2) deactivating tumour suppressor genes. The change in the chromosome number leads to increase in oncogenic products and to damage of regulatory functions associated with cell proliferation.

Keywords: cytogenetic analysis, karyotype, chromosome rearrangement, gene localization

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INTRODUCTION

The incidence of lymphoma increased at the end of the twentieth century. People of various ages suffer from this type of cancer. Their favourable prognoses depend on clinical and immunomorphological features of lymphoma (Peveling-Oberhag et al., 2013).

Infection with the Epstein-Barr virus (EBV) greatly increases the risk of lymphoma. The Epstein-Barr virus is a human herpesvirus 4, which targets B-lymphocytes and causes infectious mononucleosis in humans (Shannon-Lowe et al., 2017). After that, individuals become asymptomatic EBV carriers. The exact mechanism of EBV cancer induction is still unknown, though increased titers of EBV antibodies, high content of viral DNA in blood plasma of patients, and monoclonality of viral genome in giant Reed-Sternberg cells in Hodgkin's lymphoma shows the role of the virus in tumour development (Gurtsevitch et al., 2018). It was found, that in Burkitt lymphoma, which is non-Hodgkin's one, the viral protein Epstein–Barr nuclear antigen 1 (EBNA1) is expressed; proteins EBNA-1, LMP-1, and LMP-2 are expressed in diffuse large B-cell lymphoma, in NK/T cell lymphoma, and Hodgkin's lymphoma; nine EBV proteins are expressed in patients with heavy immunodeficiency (after organ transplantation or HIV infection) (Shannon-Lowe et al., 2017).

Lymphoma is characterized by enlargement of lymph nodes that accumulate malignant lymphocytes. By present time, the genes influencing on tumour development were described. They are known as proto-oncogenes and tumour suppressor genes. In normal cell, proto-oncogenes do not promote tumour growth, but under some influences these genes become oncogenic. The direct increase in gene expression causes uncontrolled levels of active protein in a cell, or the protein structure change makes the protein active and stable to degradation. In the presence of active oncogene, the cell does not undergo apoptosis; it grows and becomes malignant (Mugnaini, Ghosh, 2016).

Chromosomal translocations detected in lymphomas result in the transfer of proto-oncogene to the loci of immunoglobulin genes. It leads to permanently increased expression of the proto-oncogene. In acute leukaemia, translocations usually cause the merge of two genes and appearance of chimeric protein with oncogenic properties (Jung et al., 2006).

During the maturation of B-lymphocytes, many DNA rearrangements occur in their genome. Even in lymphocytes of healthy people, the oncogenic translocations in immunoglobulin loci were found. For example, in 50–60% of healthy adults, the translocation *bcl2*/IgH was registered, and in 2% – the translocation *c-myc*/IgH. The proto-oncogene product Bcl2 suppresses the apoptosis, and the protein Myc is a transcriptional factor regulating expression of 15% genes (Levy et al., 2017).

RAS (Retrovirus Associated DNA Sequences) is a family of other important genes. Proteins Ras are small GTPases possessing similar three-dimensional structure. When Ras proteins are bound to GTP before hydrolysis, they activate mitogen-activated protein kinase cascade causing cell growth and division. Ras mutations causing the loss of GTPase activity result in over-activation of cell growth and decrease of apoptosis that are favourable conditions for cancer. According to literature, Ras mutations were detected in 25% of human tumours (Gorfe, Cho, 2019).

In healthy cells, the growth is also controlled by tumour suppressor proteins. For example, the protein Rb prevents progression from G1 to S phase of the cell cycle. Rb binds transcriptional factors E2F and inhibits transcription of some cyclins and proteins necessary for DNA replication. However, if the Rb protein is phosphorylated or mutated, Rb loses inhibiting functions because the complex Rb-E2F dissociates (Uxa et al., 2019). The cyclins controlled by the mitogen-activated protein kinase cascade are responsible for Rb phosphorylation. Under normal conditions, Rb protein is phosphorylated in S, G2, and M phases. Then PP1 protein dephosphorylates Rb at M/G1 transition and Rb becomes the cell growth inhibitor again. The *RB* gene can be damaged or inactivated by mutations

or chromosomal rearrangements, and the cell uncontrollably goes to the S phase and through the cycle. The deletion of *RB* significantly increases the probability of cancer due to aneuploidy appearance (Di Fiore et al., 2013).

The other tumour suppressor is protein p53, which stops G1/S transition if DNA damage is present and initiates transcription of DNA repair proteins or apoptosis in the case of irreparable DNA damage (Draganov et al., 2019). Also, protein p53 prevents the formation of new blood vessels in a tumour. In normal cells, protein p53 binds to other protein Mdm2. Mdm2 marks p53 with ubiquitin and then p53 is degraded by the proteasome. In response to stress signals, ubiquitin is removed from p53 by the specific protease and p53 is not degraded. Mitogen-activated protein kinases phosphorylate the N-terminal end of p53, this prevents Mdm2 binding. According to the literature, in 50% of tumours, the *TP53* gene is mutated or inactivated (Aberg et al., 2017).

Determining proto-oncogenes located on aberrant chromosomes at lymphomas and leukaemias is of great importance in elucidating the role of specific chromosomal aberrations in malignization. According to PubMed database, there is no literature on chromosomal aberrations in monkey tumours and some translocations in human B-cell neoplasms are described. As we have already mentioned, these are translocations of *c-myc* or *bcl* genes (*bcl-2*, *bcl-6*) to loci of immunoglobulin genes coding heavy or light (kappa, lambda) chains, or translocations of immunoglobulin genes to the proximity of *c-myc* or *bcl* (Bende, 2007). *C-myc* is located on chromosome 8, *bcl-2* – on chromosome 18, *bcl-6* – on chromosome 3, *Ig heavy chain* – on chromosome 14, *Ig kappa* – on chromosome 2, and *Ig lambda* – on chromosome 22. Translocations t(8;14), t(3;14), t(8;22) and t(14;18), t(2;8) were found in human B-cell lymphomas. Also, the translocation of cyclin D1 gene t(11;14) and the translocation of *pax5* gene coding the protein important for B-cell differentiation t(9;14) were registered (Bende et al., 2007).

For studying the role of genetic factors and chromosomal abnormalities in the occurrence, development, and malignant progression of haemoblastosis, primate lymphocyte cell lines are used as the model system. We obtained a number of our established B-lymphocyte suspension lines in the process of hemopoietic tissue cultivation, using material from hamadryas baboon suffering from lymphoma and EBV-positive bear macaques (Araviashvili et al., 1994). We suggested that chromosomal rearrangements in these lines affected the genes involved in cell growth regulation.

By the present time, genomes of some monkeys (rhesus macaque, olive baboon, etc.) and humans are sequenced (Ensembl, 2019). Average human-macaque sequence identity is approximately 93% (Gibbs et al., 2007). The discovered regions of oncogene and tumour suppressor gene location (without precise localisation) in monkey chromosomes allowed us to identify the genes involved in chromosomal aberrations specific for the established lymphocyte cell lines.

With our work, we aimed to investigate the molecular reasons for primate lymphocyte cell malignization after some structural chromosomal rearrangements. This is new for monkey lines, the aberrations in which have not been investigated before; the comparison with data already obtained for humans is also interesting.

MATERIALS AND METHODS

Cell culture. Cell lines were cultivated in the nutritional medium RPMI-1640 containing 15% foetal bovine serum, L-glutamine 300 μg/ml. penicillin 100 μg/ml and streptomycin 50 μg/ml. The cultured lines were established B-cell suspension lines LUG-4 and its subclones E1-1, E5-1 and E9-1 obtained from baboon ill with haemoblastosis and lines MAL-1, MB-20 obtained from clinically healthy bear macaques (*Macaca arctoides*) seropositive against EBV-like virus, after cell stimulation with phytohemagglutinin (PHA).

Cytogenetic analysis. Cell cultures at the stage of logarithmic growth, i.e., 2–3 days after seeding in RPMI-1640 medium, without the addition of colchicine were used to obtain slides with metaphase plates. Cell suspensions were transferred into centrifuge tubes (5 ml per tube), then centrifuged for 5 min at 1000 rpm. After centrifugation, the most of supernatant was removed, with approximately 1–2 ml of supernatant left in a tube.

The cells were hypotonised in 0.56% KCl solution or its mixture with 1% sodium citrate (1:1). The volume of hypotonic solution used was 6 ml. Then the cells were incubated for 10 min at 37°C. The fixating mixture contained methanol and acetic acid in proportion 3:1. Two drops of the fixating mixture were added to hypotonised cells and then centrifuged for 5 min at 1000 rpm. After removal of the supernatant, the residue was resuspended and 5 ml of fresh cold fixating mixture was carefully added by dropping. Each fixation lasted for 1 hour. The fixating mixture was changed three times. The end volume of the fixating mixture depended on the number of cells and was equal to 2–3 ml. After that, the suspension of fixed cells was dropped on cold wet slides and then dried.

Staining. To determine the number of chromosomes, metaphase plates with remaining cytoplasm were stained by 1% Giemsa solution in phosphate buffer (pH = 6.8) for 5–6 min. Then 100 metaphase plates with remaining cytoplasm, moderate coiling of chromosomes and non-overlapping chromosomes were analysed. The percentage of the cells with modal chromosome number was found at analysis of 100 metaphase plates. The percentage of polyploidy cells was found during the analysis of 1000 metaphase plates from 3–4 slides.

For a detailed investigation of karyotypes, the chromosome G-banding method was used for differential staining. Unstained slides containing a large number of metaphase plates without cytoplasm and with good morphology and non-overlapping chromosomes were thermostated for 8–12 hours at 60°C. Then the slides were incubated for 1–5 min at the room temperature in 0.011% Trypsin solution, in which the solvent was 0.01% Versene solution, and D-glucose 0.4 mg/ml, KCL 0.17 mg/ml and NaCl 1.7 mg/ml were added. After that, the slides were washed for 15 s in the water mixture of D-glucose 1 mg/ml, KCL 0.4 mg/ml, and NaCl 8 mg/ml. The slides were stained in 2% Giemsa solved in phosphate buffer (pH = 6.8) for 5–6 min.

Search in the Ensembl database. To determine the localisation of oncogenes and tumour suppressor genes on chromosomes of the Old World monkeys, data on mapping macaque and baboon genes and high similarity of human and monkey karyotypes were used (Ensembl, 2019; Gibbs et al., 2007; Wienberg et al., 1992).

RESULTS

Six established primate B-lymphocyte cultures were investigated. Data of cytogenetic analysis are presented in Table 1 and Figs. 1–3.

Table 1. **Characterization of monkey lymphocyte cell line karyotypes**

Cell line	Diagnosis	Passage	Karyotype	Chromosomal rearrangements/localisation of genes
LUG-4	lymphoma	8	42,XY,del(3)(qter),der(17) t(3;17) (qter;pter)	(17p+)/RB1
		22	"	
		128	84,XX,YY,idemx2	
MAL-1	healthy	10	42,XX	M5-der(11)/c-Ki-ras
		166	42,XX,M1-M8	M7-der(16)/TP53
MB-20	healthy	36	42,XY,/45,XY,+2,+8,+15/	(+8)/c-myc
		76	45,XY,+2,8,+der(15)t(5;15) q21;pter)	

In tumour line LUG-4, cells had the initially pseudodiploid karyotype. Chromosomal marker 17p+ specific for LUG-4 was also found in all subclones E1-1, E5-1, and E9-1. By passage 128, LUG-4 became completely tetraploid.

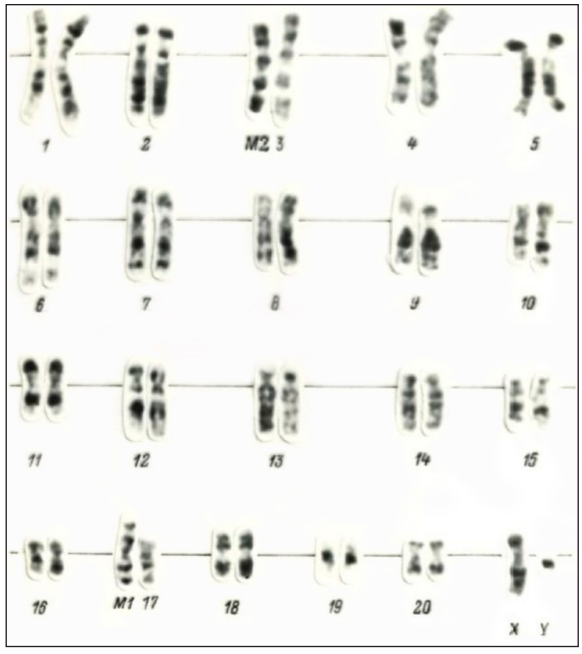


Fig. 1. Karyotype of parental cell line LUG-4 and its subclones (E1-1, E9-1, E5-1), 42, XY, del(3), der(17) t(3;17) (qter;pter) (G staining)

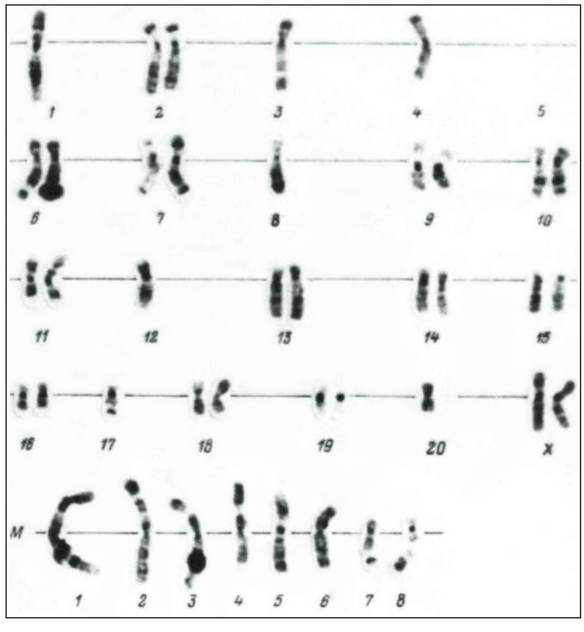


Fig. 2. Karyotype of B-cell line MAL-1, 42, XX, eight marker chromosomes (G staining)

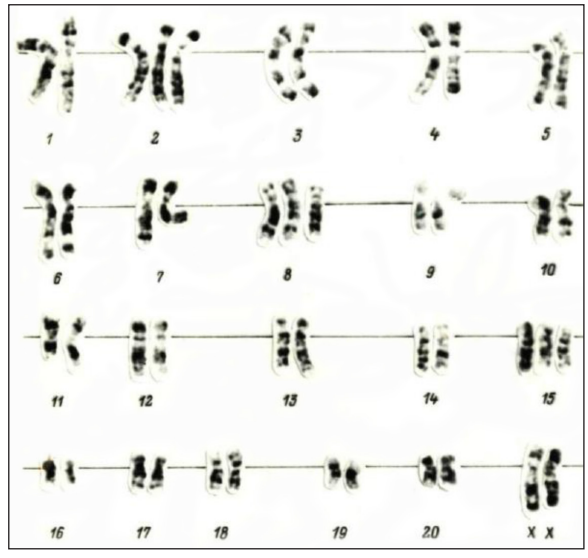


Fig. 3. Karyotype of the B-cell line MB-20, 45, XY, trisomy of 2, 8, and 15 chromosomes (G staining)

Analysed at different stages of cultivation, cell line MAL-1 was unstable. Karyotypical instability increased during the cultivation process. At initial stages (passage 10), we found cells with a normal karyotype 42, XX. By passage 26, some cells with chromosomal rearrangements appeared, and by passage 166, all cells with an initially normal karyotype had a pseudodiploid karyotype with eight marker chromosomes, in particular, der(11) and der(16).

In MB-20 cell line, three clones of cells with a different modal number of chromosomes were revealed: one clone with normal karyotype and two clones with abnormal karyotypes. In the process of cultivation, the same clones remained in the line, their ratio slightly changed.

Results of our search through the Ensembl database showed that in baboons and macaques, tumour suppressor gene *RB1* is located in chromosome 17, oncogene *c-Ki-ras* (KRAS) – in chromosome 11, tumour suppressor gene *TP53* – in chromosome 16, and the proto-oncogene *c-myc* – in chromosome 8 (Tables 2–5) (Chromosome 17. Olive baboon, 2019; Chromosome 11, Chromosome 16, Chromosome 8 Macaque, 2019). All results are presented in Table 6.

Table 2. **Localisation of RB1 oncogene in human and rhesus macaque chromosomes**

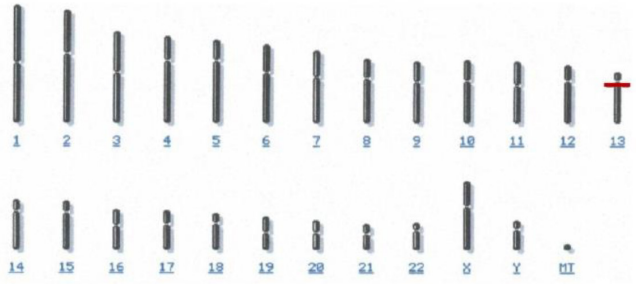
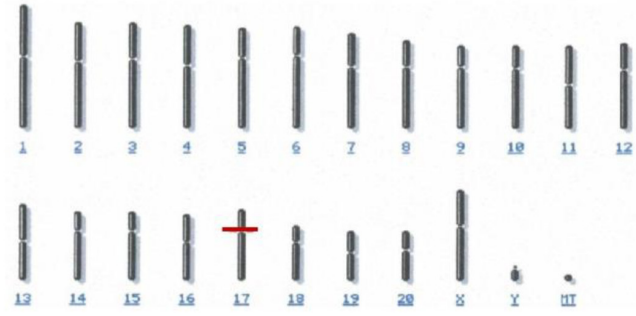
Gene	Species	Idiogram	Localization
RB1 (RB; OSRC)	Homo sapiens		13q14.2
	Macaca mulatta		17p1

Table 3. **Localisation of *c-Ki-ras* oncogene in human and rhesus macaque chromosomes**

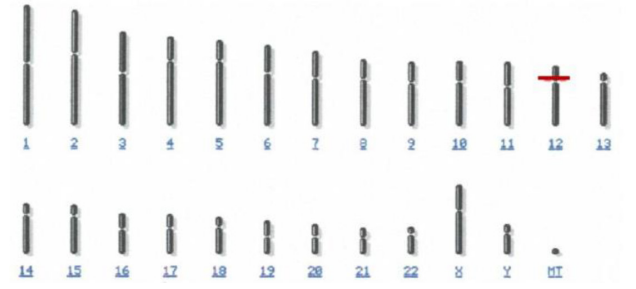
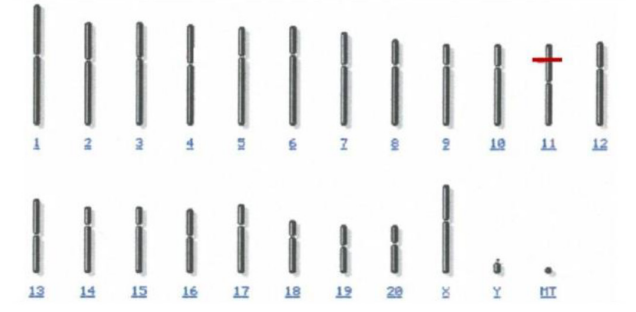
Gene	Species	Idiogram	Localization
c-Ki-ras Kirsten rat sarcoma (KRAS1; NS3; KRAS1; KRAS2; C-K-RAS; K-RAS2A; K-RAS2B; K-RAS4A; K-RAS4B)	Homo sapiens		12q12.1
	Macaca mulatta		11p

Table 4. **Localisation of tumour suppressor gene TP53 in human and rhesus macaque chromosomes**

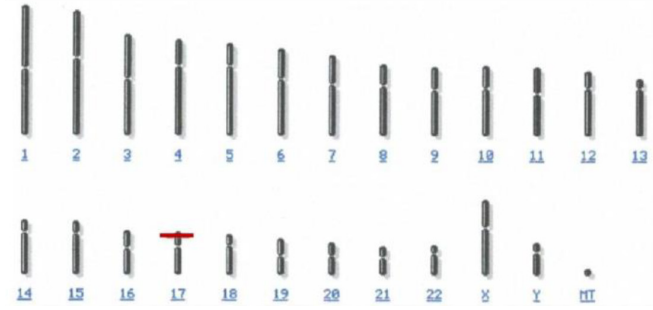
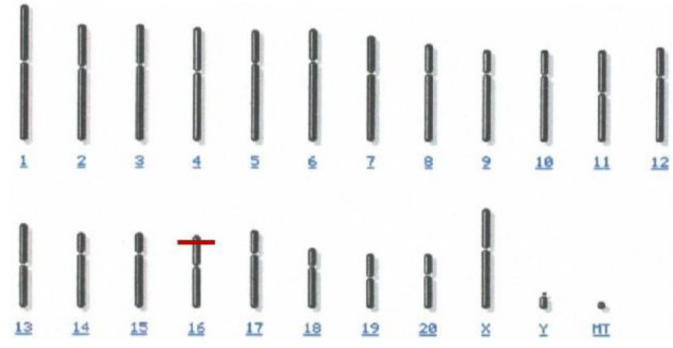
Gene	Species	Idiogram	Localization
TP53 (p53; LFS1; TRP53)	Homo sapiens		17q13.1
	Macaca mulatta		16p

Table 5. **Localisation of *c-myc* oncogene in human and rhesus macaque chromosomes**

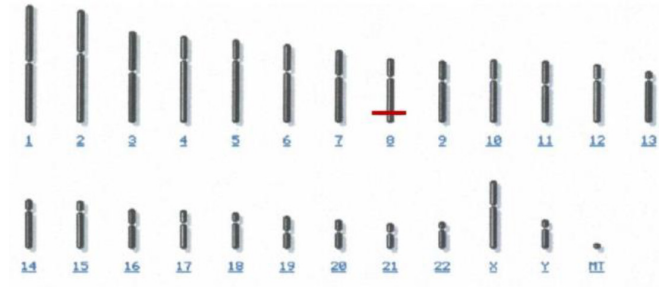
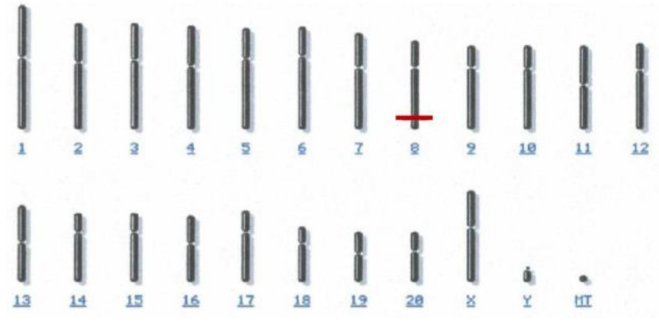
Gene	Species	Idiogram	Localization
c-myc (MYC)	Homo sapiens		8q24
	Macaca mulatta		8p

Table 6. **Localisation of important genes in human and monkey chromosomes**

Gene	Gene product function	Location (chromosome №)		
		Human	Macaque	Baboon
cdca7	cell division control	2	12	12
pdcd1	apoptosis regulation	2	12	12
erb B4	receptor tyrosine kinase 4	2	12	12
bcl6	oncogenic	3	2	2
met	oncogenic	7	3	3
ST7	tumour suppressor	7	3	3
c-myc	oncogenic	8	8	8
pax5	differentiation control	9	15	15
ccnd1	cyclin D1	11	14	14
c-Ki-ras	oncogenic	12	11	11
RB1	tumour suppressor	13	17	17
IgH	immunoglobulin heavy chain	14	7	7
nupr1	transcriptional regulator	16	20	20
TP53	tumour suppressor	17	16	16
c-erb B2	oncogenic	17	16	16
bcl2	tumour suppressor	18	18	18

DISCUSSION

We investigated the established B-cell suspension lines of baboons and macaques. From the hamadryas baboon ill with lymphoma, the cell line LUG-4 and its subclones E1-1, E5-1 and E9-1 were obtained. We found that the translocation t(3;17) was a structural chromosomal rearrangement specific for this line and was presented in the original lymphoma tissue cells *in vivo*. Subclones E1-1, E5-1 and E9-1 had the same karyotype.

It is well known that in the majority of cases the malignant growth is caused by activation of oncogenes and deactivation of tumour suppressor genes. Discussing our results from this point of view, we see that in the rhesus macaque and in the olive baboon, tumour suppressor gene *RB1* is located in the chromosome 17.

According to early data in the literature, monkey chromosomes 3 and 17 were considered to be homologous to the corresponding human chromosomes (Gibbs et al., 2007). In human chromosome 17, tumour suppressor gene *TP53* and proto-oncogenes *c-erb B2* (receptor tyrosine kinase 2), *c-erb A1*, and *c-erb A2* are located, but our search in the Ensemble database showed that in monkeys these genes are situated in chromosome 16, except *c-erb A2*, which was found in chromosome 2.

Considering chromosome 3, the human chromosome contains proto-oncogene *bcl6*, which is overexpressed in 40% of diffuse large B-cell lymphoma (Rosenthal, Younes, 2017). According to the newest data, this gene was found in chromosome 2 in monkeys (Ensembl, 2019). Instead, the monkey chromosome 3 contains the proto-oncogene *met* (receptor tyrosine kinase) and the gene suppressor of tumorigenicity 7 (*ST7*), which is not studied but its role in oncogenesis is proved.

Summing up the foregoing, it can be concluded that the most probable cause of malignization in monkeys due to translocation t(3;17) is the inactivation of tumour suppressor genes *RB1* and *ST7*, and activation of the proto-oncogene *met*.

From cells of clinically healthy bear macaques (*Macaca arctoides*) seropositive against EBV-like virus, lines MAL-1 and MB-20 were obtained after stimulation with phytohemagglutinin.

In line MAL-1 we found the following marker chromosomes: 1, 3, 4, 5, 11, 16, 17. The appearance of marker chromosomes was accompanied by loss of normal chromosomes 8, 12, 20.

Both in humans and monkeys, chromosome 8 contains the proto-oncogene *c-myc*.

Chromosome 12 of rhesus macaque includes gene *pdcd1* (programmed cell death 1) coding the surface membrane protein of the immunoglobulin superfamily. The product of this gene, protein Pdcd1, takes part in B-cell differentiation (Mishra, Verma, 2018).

Also, in chromosome 12, the gene *erb-b4*(receptor tyrosine kinase 4) is located. Mitogenesis and differentiation are among various mechanisms activated by this receptor. According to the literature, mutations in *erb-b4* are associated with malignancies (Longo et al., 2019). The second gene in this chromosome, *cdca7* encodes the protein associated with cell division. Protein Cdca7 is important for tumour growth because it mediates the effect of Myc in cancer cells (Jiménez et al., 2018).

In chromosome 20, gene *nupr1* encodes the transcriptional regulator nuclear protein 1. It was shown that this protein takes part in transcriptional misregulation in cancer and is also involved in the regulation of cell cycle, apoptosis, autophagy, and DNA repair responses (Emma et al., 2016).

Proto-oncogene *c-Ki-ras* is located in monkey chromosome 11, and tumour suppressor gene *TP53* is located in chromosome 16.

In cell line MAL-1, the appearance of marker chromosomes and the loss of normal chromosomes is a bad sign for normal karyotype. Favourable conditions for malignization are established, taking into account the above-listed genes located in these chromosomes and their importance for growth and apoptosis.

Cell line MB-20 is the only line which contained poplyploid clones (45 chromosomes) with a trisomy of chromosomes 2, 8 and 15. Chromosome 8 contains proto-oncogene *c-myc*. Monkey chromosome 15 includes gene *pax5* coding the protein important for B-cell differentiation (Okuyama et al., 2019). Apparently, the found trisomy of chromosomes 8 and 15 damages the regulatory gene mechanisms and leads to immortalization.

In conclusion, we may underline that detected chromosomal aberrations cause functional changes in DNA and result in abnormalities in cell differentiation and proliferation.

CONCLUSIONS

1. Translocations, inversions and partial deletions are the most characteristic types of chromosomal rearrangements not only in leukaemia and lymphomas, but also during long-term cultivation of haemopoetic primate tissues.

2. Structural aberrations in established primate cell lines affect several chromosomal loci and cause the proto-oncogene activation – the central event in the tumour clone occurrence – and deactivation of tumour suppressor genes.

3. The numerical change of chromosomes (trisomy) leads to an increase in oncogenic products, and as a consequence, a damage of regulatory functions associated with cell proliferation.

4. Our data contribute to the understanding of cell features in culture.

Received 18 June 2019
Accepted 25 October 2019

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NEATSITIKTINĖS STRUKTŪRINĖS CHROMOSOMOS ABERACIJOS BEŽDŽIONIŲ B LIMFOCITŲ LĄSTELIŲ LINIJOSE

Primatų limfocitų ląstelių linijos, išskirtos iš navikų ir bežd žionių, kurioms nustatytas EBV, buvo pavyzdinė sistema tiriant genetinių veiksnių ir chromosomų anomalijų įtaką piktybinių navikų susidarymui. Mūsų darbo tikslas – ištirti chromosomų regionų sritis ir genus, susijusius su chromosomų aberacijomis, sukeliančiomis šių linijų piktybinių navikų susidarymą. Citogenetinė analizė buvo atlikta skirtingais auginimo etapais *in vitro*. Onkogenų ir naviką slopinančių genų, esančių ant aberantinių chromosomų, nustatymui buvo naudojami rezus makakų genų žemėlapio bei didelio žmogaus ir bežd žionių kariotipų panašumo duomenys. Mes nustatėme, kad linijoje, gautoje iš babuino limfomos audinio, naviko slopintuvo RB1 geno inaktyvacija 17 chromosomoje po chromosomų persitvarkymo yra viena iš labiausiai tikėtinų piktybinių navikų susidarymo *in vivo* priežasčių. Chromosomų aberacijos *c-Ki-ras* onkogeno regione ir naviką slopinančio TP53 geno srityje keičia proliferacinę būklę ir diferenciaciją nustatytose ląstelių linijose, gautose iš sveikų, bet EBV seropozityvių primatų. Kita šių linijų piktybinių navikų susidarymo priežastis yra *c-myc* onkogeno ekspresijos padidėjimas, kurį sukelia 8 chromosomos trisomija ten, kur yra *c-myc*. Nustatytos struktūrinės tirtų primatų ląstelių linijų, turinčių įtakos keliems chromosomų lokusams, aberacijos: 1) sukeliančios protoonkogeno aktyvaciją – pagrindinę naviko klono susidarymo priežastį, 2) „išjungiančios“ naviką slopinančius genus. Dėl pasikeitusio chromosomų skaičiaus padidėja onkogeninių darinių kiekis ir pažeidžiamos reguliacinės funkcijos, susijusios su ląstelių dauginimusi.

Raktažodžiai: citogenetinė analizė, kariotipas, chromosomų persitvarkymas, genų lokalizacija