THE QUANTITY OF CELLS NECESSARY FOR THE ANALYSIS OF CHROMOSOMAL ABERRATIONS AT RESIDENTS OF KUZBASS PATIENTS WITH CANCER OF LUNG

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Received April 15, 2016;
Accepted in revised form June 01, 2016;
Published June 30, 2016

Abstract: The pilot research executed in 2014 in selection of patients with lung cancer (LC) and healthy residents of Kemerovo region (338 Caucasians: 159 having LC and 179 healthy) has shown statistically significant distinction between groups of level and range of chromosome aberrations in blood lymphocytes. Patients of cancer detection centre (before treatment) had increased chromosome aberration values, both of chromatid and chromosomal types. 250 metaphases were analyzed for each individual. For accuracy test of the received quantitative characteristics of mutational process and definition required and enough number of cells necessary for the analysis, at essential increase in further selection volume, 50 LC patients (selected randomly), comparison of cytogenetic data has been carried out in the analysis of 200 cells, then 400, 600, 800, 1000 and over 1000 metaphases. Totally, in this subgroup 50 000 cells which were at a mitosis metaphase stage have been studied. Statistical data processing was carried out with the use of a software package for Windows Statistica 8.0. Using Mann-Whitney U-criterion it is confirmed that frequency of aberrant metaphases doesn't change significantly for statistics in case of increase in analyzed cells quantity; the analysis of 200 cells gives no less reliable information about the individual CA level, than the analysis of 1000 and more cells. In this regard it is possible to make the conclusion that 200 cells is necessary and enough number of analysed metaphases at the assessment of individual level and chromosomal aberrations' range for Kemerovo region residents who have lung cancer.

Keywords: chromosomal aberrations, lung cancer, cytogenetic, industrial cities, environmental pollution

INTRODUCTION

Important task of modern ecological genetics is studying genome reactions to the impact of environmental carcinogens. Researches of many scientists are directed to identification of mutagens and assessment of their potential genetic danger [7, 8]. It is known that inhalation of the polluted atmospheric air significantly increases damageability of airways' cells and probability of their malignant transformation together with lung cancer (LC) formation.

LC is a common form of malignant neoplasms; it is also an important medical, social and economic problem [2]. The process analysis of cells' malignization and further neoplastic proliferation has shown that they are closely connected with genome reorganization which is often expressed by structural and numerical changes of chromosomes and their certain areas [25, 26]. Chromosomal Aberrations (CA) are violations of chromosomes' structure which arise spontaneously or as a result of exogenous (physical, chemical or biological) impacts [29]. In the researches conducted in Northern Europe [19], Italy [15, 16, 20], the Czech Republic [31], Taiwan [24], the USA [21] it was shown that increase in frequencies of chromosomal reorganizations reflects the higher risk of morbid growths origin. Increase of chromosomes restructurings level in blood lymphocytes is considered as a marker of additional risk for patients with solid tumors [32]. However, there are still a few cytogenic assays in blood cells of patients with LC.

Nowadays the large number of the methods which allow to obtain valuable information concerning cytogenetic effects of mutagen influence or directed to the description of the mutational process in organisms of cancer patients are developed. In this regard the wide range of the tests is used, including, for example, micronucleus analysis, TCR mutant lymphocytes, DNA comets, sister chromatid exchanges, microarray

Please cite this article in press as: Ryzhkova A.V., Minina V.I., Golovina T.A., Savchenko Ya.A., Bakanova M.L., Druzhinin V.G., Titov V.A. The quantity of cells necessary for the analysis of chromosomal aberrations at residents of Kuzbass patients with cancer of lung. *Science Evolution*, 2016, vol. 1, no. 1, pp. 113–119.

analysis technologies, NGS sequencing, M-FISH, SKY, a CGH, CNV analysis and others). Each of the used test systems has its own advantages. For example, micronuclear test allows to carry out the analysis of a large number of cells quickly enough (not less than 1000) and gives the chance to estimate not only clastogenic and aneugenic events, but also to characterize a ratio of the main vital processes – proliferation, destruction and the level of cytogenetic damages depending on cell kinetics [11]. More rarely researchers apply to using classical method genetic structure analysis – an assessment of chromosomal aberrations level in human somatic cells, which is one of the most available and descriptive methods of genetic structure research [12]. Meanwhile this method is well standardized, it allows to characterize all main types of damages, and on their range it is possible to judge the character of the operating mutagens. Obtained cytogenetic data can form a good basis for further deeper analysis with the involvement of expensive molecular methods.

Earlier we have carried out CA assessment on LC patients in the selection including 179 cancer-stricken and 159 healthy donors [1]. The analysis results show that on average, 250 cells from each examined were revealed statistically significant differences of CA cells frequency and aberrations' frequencies of chromatid and chromosomal types. In group of LC patients higher frequency of acentric fragment occurrence, chromatid interchange, dicentric and ring chromosomes is marked. When discussing the received results and further researches planning it is important to consider that spontaneous (not induced by any known mutagen) CA level does not represent any strictly determined value [5]. This value is influenced by: culture conditions, ways of fixing and preparation of slides, various approaches to the violations accounting and also the factors of statistical nature caused by the selection amount. Thus, in the CA analysis a researcher works not with true spontaneous level, but only with its estimates which can vary considerably. In this work methodical approaches to material collection, drug preparation and damaged chromosomes analysis have been standardized. Sufficiency of the used cells for the analysis is a matter of dispute.

In case of personal frequency of cytogenetic parameters determination the data obtained in the analysis of 100–1000 cells are traditionally used. More often there is pause on the lower bound of this range as the classical cytogenetic analysis is very time consuming. It is justified in case of all-group assessment receiving. Naturally, the more individuals and the more cells will be eventually included in group, the more precisely will be the result. N.P. Bochkov's calculations [3] showed that for 20% accuracy achievement in case 300 cells count for each individual and with the general frequency of 2% in aberrant metaphases it is necessary to examine at least 60 people. For the assessment of the individual CA level some researchers suggest to carry out the analysis of not less than 1000 cells in the conditions of spontaneous mutagenesis and of 500 cells in the conditions of radiation factor influence [10]and prove it by the need of estimates' accuracy increase. Other researchers consider 100–400 cells as optimum quantity for the CA analysis [17, 28]. Planning continuation of our researches on LC patients and increase in amount of selection up to 1000 people become actual determination of necessary and enough cells for the analysis; the aim of this research is frequency analysis and CA range during the estimating of different quantity of cells (the range from 200 to 1000 and more cells) in LC patients' blood lymphocytes of Kemerovo region residents.

MATERIALS AND METHODS

The collection of cell culture suspensions received from whole peripheral blood of 50 LC patients served as the material for research. The blood was sampled of entered (primarily) treatment in Kemerovo regional clinical oncologic dispensary, was processed and stored in the form of a ready collection in refrigerator at temperature of -20°C. Blood sampling and its further research was conducted according to the ethical standards designed in accordance with WMA Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" with the amendments of 2000 and "Rules of clinical practice in Russian Federation" approved by the Order of the Russian Federation Ministry of Health of 19.06.2003 no. 266. For each examined the memorandum of informed consent was issued. In total, 50 suspensions of the recorded blood cells were analyzed (each cell culture is received from one person chosen from the general selection of LC patients by a "blind" method). All suspensions were prepared of the same type in one laboratory by one group of researchers, observing unified approach to the CA accounting.

Conditions of cells cultivation and receiving of suspensions. Nutritious mix was prepared in an amount of: RPMI-1640 medium (4.5 ml), fetal calf serum (1 ml) and 0.1 ml of phytohemagglutinin (PanEco). Mixture was placed in sterile cultural matrasses and 0.5 ml of heparinized blood was added to it. Cultural bottles maintained temperature of 37°C during 48 h. In 2 hours before fixing colchicine (0.5 mkg/ml) was injected into cultures. Then hypotonization with 0.55% KCl solution and fixing by mixture of ethanol and glacial acetic acid in the ratio of 3 : 1 were carried out. Then suspension was dug out on the cooled fat-free glass slides and dried up over the spirit-lamp flame. Routine chromosomes coloring was carried out by 1% Giemsa stain prepared on the phosphate buffer. The chromosomal analysis was carried out with the help of ciphered drugs by means of Axioskop 2 plus microscope (Carl Zeiss, Germany) at low-powered (10 x 10) and high-power (10 x 100) magnification. In the Fig. 1 the metaphase plate containing exchange is presented. The analysis was carried out at first on 200 cells per person, then on 400, 600, 800, 1000 and over 1000 metaphases. Maximum was 2400 cells. Totally, 50 000 cells at mitosis metaphase stage have been studied.

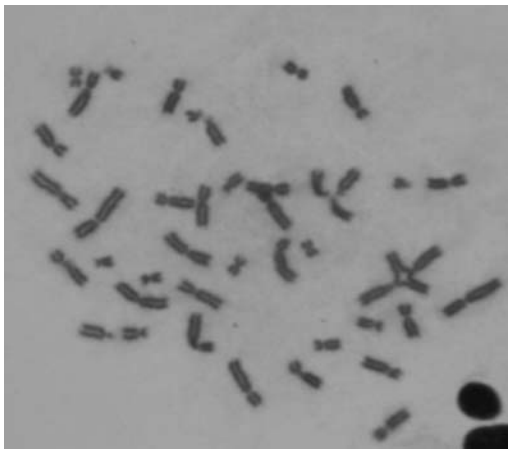

Fig. 1. The metaphase plate of lung cancer patient containing exchange (indicated by arrow).

Aberrations of chromatid and chromosomal types were registered in accordance with standard requirements [3–6]. The proportion of aberrant metaphases was defined by count of metaphases frequency with aberrations of chromosomes (percentage of the studied number of cells). For the purpose of the maximum research unification all the work on the cytogenetic analysis CA was carried out by one researcher and checked be the other through the selective analysis (in 90% of cases assessments have coincided, other 10% have been excluded from results).

Methods of statistical processing. Statistical data processing was carried out with the use of a software package for Windows Statistica 8.0. In case of chromosomal aberrations assessment descriptive statistics included value calculation: arithmetical mean (Mean) and its standard error (Std. Error), upper (Upper Bound) and lower (Lower Bound) of boundary of 95% of confidence interval for average, median (Me), coefficient of variation (Variance), standard deviation (Std. Dev.), minimum (Min) and maximum (Max) variable value, and also skewness (Skewness) and excess (Kurtosis) coefficients.

For each studied parameter distribution compliance of cytogenetic analysis data to the law of normal distribution was inspected. With use of Kolmogorov-Smirnov criteria with Lilliefors' correction authentically significant deviation of distributions from normal was shown (for example, for frequency of aberrant metaphases of chromosomal type: K-S $d = 0.17759$, $p < 0.01$; Lilliefors $p < 0.01$), therefore further, for groups comparison nonparametric rank tests were used, including Mann-Whitney rank test (Mann-Whitney U-test) for paired comparison.

RESULTS AND DISCUSSION

According to the formula suggested by N.P. Bochkov [3], for definition of cells number necessary for achieving 5% accuracy of measurement, it is necessary to consider the proportion of normal and aberrant metaphases:

$$N = 10\,000q/pW^2,$$

where q is the proportion of normal metaphases, p is the proportion of aberrant metaphases and W is relative accuracy (%).

That is, if the proportion of aberrant metaphases is 1% and the relative accuracy of 5% is required, it is necessary to analyze ~ 40 000 cells. With CA frequency of 5% and the same accuracy less cells will be required: ~ 8000. When using criterion in 1% of relative accuracy as it is accepted in exact sciences, it will be required to increase number of cells for the analysis by 25 times. In the conditions of the real routine cytogenetic analysis these volumes are elusive. That is why in the majority of research works it was accepted to use the accuracy of 100% and the analysis of ~ 100 cells was made. With development of interphase cytogenetics' methods allowing to analyze tens of thousands of cells without considerable labor costs, this approach was exposed to criticism more and more as obviously inaccurate. In this work we compared the CA frequencies received by the routine cytogenetic analysis of the same donors of different number of cells: 200, 400, 600, 800, 1000 and more than 1000 (to 1200). The descriptive statistics of the main index – the frequency of metaphases with CA, is provided in Table 1.

High average values of CA level (from 3.23% to 4.91%) among LC patients have been registered. Using Mann-Whitney U-criterion it was set that aberrant metaphases' frequency (the number of aberrations to the scanned cell) doesn't change statistically significantly in case of increase in quantity of the analyzed cells, i.e. the analysis of 200 cells provides no less than reliable information about the individual CA level (the amount of all possible types of chromosomes aberrations), than the analysis of 1000 and more cells. Nevertheless, increase in number of the scanned cells leads to median shift (from 4.50 to 3.00), variation coefficient reduction (from 6.83 to 0.55) and standard deviation (from 2.61 to 0.74) and "smoothing" of distribution peak sharpness (platykurtosis).

There is concern to overlook the mutation variant when scanning fewer cells. They include dicentric, ring chromosomes and different types of exchanges. During the analysis of a certain person in spontaneous mutagenesis conditions, really, the rare variant can be registered only after scanning, for example, 1000 cells. However in this case it is important to consider

conditions of genotoxic stress in which there is an individual.

Kemerovo region residents stricken with LC living under genotoxic effects of carcinogens caused by a lot of coal mining and processing plants have been examined. Structural integrity of chromosomes is an important indicator of functioning optimality of cell genetic apparatus and it depends on set of endogenous and exogenous impacts [23, 30]. Thus, CA frequency is the result of summary impact of various natural factors on a human body, which influence on genetic processes in a cell and, finally, lead to the structural damages observed at the chromosomal level [6, 14].

It drew attention that dicentric chromosomes, rings, translocations (influence of ionizing radiation and chemicals) [27], and also exchanges, (influence of chemical clastogens, ultraviolet radiation, alkylating agents) [13] are register already when scanning the first 200 cells and further with increasing of selection volume their frequency does not rise. The qualitative range of chromosomal aberrations is presented in Table 2.

From the table it is possible to see that frequency of polyploid cells, translocations, dicentrics, ring chromosomes and exchanges does not increase with raising number of the studied cells. On the contrary, significant frequency reduction of ring chromosomes, translocations, dicentrics, exchanges and polyploid cells is received statistically iwhen calculating 1000 and more cells. This occurs for several statistical reasons: the quantity of aberrant chromosomes is divided into bigger number of the analyzed metaphases and, as a result, decrease in average values can be observed.

With the aim of the maximum unification this research has been carried out taking into account a number of methodical conditions: identical technology of preparation and drug analysis in one laboratory conditions, rather large selection volume (50 000 studied metaphases), known genotoxic impact (dangerous industrial region, LC). These conditions' fulfillment has allowed to receive the most exact estimates not only of the total number of chromosomal aberrations, but also of particular types of unstable chromosomal abnormalities. According to the obtained data, the estimates received in this group during the analysis of 1000 and more cells statistically do not differ significantly from the results based on the accounting of 200 metaphases.

Table 1. Descriptive statistics of results of aberrant metaphases' frequency assessment (%), set during the analysis of different number of cells

n	Mean	Me	Min	Max	Std. Error	Variance	Std. Dev.	Skewness	Kurtosis	Lower Bound	Upper Bound
200	4.91	4.50	1.00	14.00	0.36	6.83	2.61	1.23	2.47	3.00	6.00
400	4.93	4.87	1.50	10.80	0.30	4.63	2.15	0.56	0.17	3.25	6.25
600	4.87	4.66	1.00	9.80	0.38	4.38	2.09	0.41	-0.30	3.45	6.23
800	4.61	4.60	1.80	9.30	0.27	3.36	1.83	0.40	-0.23	3.25	5.75
1000	4.67	4.35	1.70	8.90	0.30	3.45	1.85	0.27	-0.76	3.03	6.10
>1000	3.23	3.00	2.00	4.09	0.24	0.55	0.74	0.29	-1.13	2.90	3.80

Note. n – number of the studied cells.

Table 2. Different types of chromosomal aberrations' frequency set during different number of cells analysis

Types of chromosomal aberrations	Number of studied cells					
	200	400	600	800	1000	More than 1000
Single fragments	3.16 ± 0.27	3.13 ± 0.21	3.1 ± 0.21	2.92 ± 0.19	2.96 ± 0.21	2.03 ± 0.21
Chromatid interchanges	0.03 ± 0.01 *	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.008	0.02 ±0.008	0.01 ± 0.01
Chromatid-type aberrations	3.18 ± 0.27	3.16 ± 0.22	3.19 ± 0.22	2.95 ± 0.19	2.98 ± 0.21	2.05 ± 0.02
Pair fragments	1.47 ± 0.14	1.40 ± 0.11	1.36 ± 0.10	1.40 ± 0.12	0.02 ±0.008	0.01 ± 0.01
Dicentric chromosomes with fragments	0.12 ± 0.04	0.09 ± 0.02	0.06 ± 0.01	0.06 ±0.01	0.06 ±0.001	0.07 ± 0.04
Dicentric chromosomes without fragments	0.07 ± 0.02 *	0.10 ± 0.02	0.11 ± 0.02	0.12 ± 0.02	0.11 ±0.002	0.08 ±0.03
Ring chromosomes	0.08 ± 0.02 *	0.08 ± 0.01	0.07 ± 0.01	0.07 ±0.01	0.07 ± 0.01	0.07 ± 0.01
Chromosome-type aberrations	1.90 ± 0.17	1.88 ± 0.22	1.83 ± 0.13	1.83 ± 0.14	1.86 ± 0.14	1.14 ± 0.14
Polyploid cells	0.09 ± 0.02	0.09 ± 0.02	0.08 ± 0.02	0.09 ± 0.02	0.08 ± 0.01	0.07 ± 0.03
Translocations	0.09 ± 0.03 *	0.10 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.05± 0.02

Note. Average values are given with a mistake *(Mean ± Std. Error)*;
* differs from values in groups with studied 1000 ($p < 0.05$) and more than 1000 cells ($p < 0.01$).

Many authors have shown similar results when assessing the required number of cells for the CA analysis. In CA frequencies' researches among liquidators of Chernobyl disaster consequences (118 people), it was shown that in case of radioactive radiation influence as a result of radiation accidents it is enough to analyze 100 cells to reveal statistically significant effect [28]. In East Kazakhstan region 110 people have been examined and from 200 to 400 cells were analyzed. The analysis of cytogenetic disorders range has shown CA increase of chromosomal and chromatid types, at calculation both 200 and 400 cells, which can be connected to the complex radiation and chemical factors influence [17, 18]. Turkish researchers have examined 20 LC patients receiving X-ray therapy, their average age is 53.5 years, 75 cells have been scanned among each examined. As a result of analysis even small amount of cells received significant frequencies increase in particular CA types (dicentrics, rings, fragments) [22]. On the basis of Kemerovo state university the CA analysis among children (55 people) living in the conditions of above permitted radon dosage's effect in the city of Tashtagol has been carried out. It was set that CA frequency statistically does not change significantly in case of analyzed cells quantity increase (from 200 to 1000). Nevertheless, increase in number of the scanned cells has led to median shift (from 5.00 to 4.12), variation coefficient reduction (from 6.49 to 3.77) and standard deviation (from 2.55 to 1.94) [9]. The choice of such groups for research is quite reasonable as monitoring results allow to obtain information on various factors' influence on the organism and to define genome damage rate. As a result all this can be used as criteria during the formation of groups of people with high risk of various diseases developing, including oncology [16, 19].

CONSCLUSION

Thus, the received results indicate that 200 cells are enough for an adequate assessment of chromosomes damages' level in the group of lung cancer patients studied by us. At the same time, we do not extrapolate this result to other selections. For each studied group the situation can differ significantly and therefore it demands additional checks. It is necessary to carry out similar work (calculation of cells enough for the analysis) in comparison group (healthy individuals).

Calculation of the minimum number of cells necessary for receiving adequate CA estimates, will allow not just to optimize work of separately taken laboratory, but also can be useful to other groups of researchers. In our opinion, work on definition of minimum and enough cells number for the analysis has to precede any estimates of chromosomal mutagenesis.

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