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REVIEW ARTICLE

Genotoxicity Tests from Biomarker Studies to the Regulations: National Perspective

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Summary

Genotoxicity investigations have been in use for over 30 years. The usage facility of genotoxicity in various cell types, either in vitro or in vivo, application and evaluation easiness and more importantly predictive capability of cancer, strengthen its place among the range of available toxicity tests. Monitoring populations for disease and environmental exposures, determining the effects of occupational exposures, discovering the toxicity mechanisms of various agents are among the uses of it. Classification and labeling of every consumer product and their ingredients from food to medicine in the regulation system, requires mandatory genotoxicity data consultation. The genotoxicity tests with regards to regulation purposes have found acceptance in internationally validated guidelines as to be applied in tiered standard approach. Herewith, genotoxicity tests and their usage areas, validity, information on their position in the regulation system have been generally reviewed and also specifically the situation of Turkey has been evaluated for the occupational studies and national regulations.

***Key Words**: Genotoxicity, biomarkers of early effect, cancer predictivity, regulations, Turkey.*

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Biyogösterge Araştırmalarından Düzenlemelere Genotoksisite Yöntemleri: Ulusal Bakış

Özet

Genotoksisite araştırmaları 30 yılı aşkın süredir kullanılmaktadır. Genotoksisitenin pek çok hücre tipinde, in vitro ve in vivo kullanım olanağı, uygulama, değerlendirme kolaylığı ve en önemlisi kanseri öngörme yeteneği toksisite testleri arasındaki yerini güçlendirmektedir. Kullanım alanları arasında; populasyonları hastalık ve çevresel maruziyetler açısından izlemek, mesleksel maruziyetlerin etkilerini belirlemek, çeşitli etkenlerin toksisite mekanizmalarını ortaya çıkarmak bulunmaktadır. Gıdadan ilaca tüketiciye yönelik her türlü ürün ve bileşeninin; sınıflandırılması ve etiketlenmesi gibi düzenleyici (regülasyon) sistem içinde de genotoksisite verilerine başvuru zorunludur. Düzenleyici amaçlara yönelik genotoksisite yöntemlerinin uluslararası geçerliliği olan rehberlerde basamaklandırılmış standart bir yaklaşımla uygulanması kabul görmektedir. Burada, genotoksisite yöntemleri, genotoksisite yöntemlerinin kullanım alanları, geçerlilikleri ve düzenleme sistemindeki yerlerine ilişkin bilgiler genel olarak derlenmiş, aynı zamanda mesleksel araştırmalar ve ulusal düzenlemeler açısından ülkemizin durumu değerlendirilmiştir.

***Anahtar Kelimeler:** Genotoksisite, erken etki biyogöstergeleri, kanserin öngörülmesi, düzenlemeler, Türkiye.*

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1. GENOTOXICITY

Genetic toxicology test systems are widely used as components of carcinogenic and genetic risk assessment (1). The term "genotoxicity" refers either to a generic name regarding a chemical or a physical material's capability of inducing mutations (gene mutations, structural chromosome mutations, genome mutations) or indicator effects that have mechanistical relationship with the formation of mutations (for example, inducing DNA modifications, DNA repair or recombination) (2). The term "genotoxic" on the other hand, is generally used to identify the chemicals that could damage the DNA or chromosomes. Currently, routine genotoxicity methods were developed over the last 3 decades. Numerous tests have been developed and become widely used over this period (1). Evaluation of the probable genotoxicity and mutagenecity of a compound in the beginning of drug development is very important (3). It is acknowledged that in drug development, the genotoxicity data should be evaluated before the starting phase I studies and only if genotoxicity is absent, should the clinical research phase be commenced. Medications which can cause direct DNA damage can only be used in life threatening diseases (4).

Genotoxicity methods may be applied via bacteria, primitive eukaryote, mammalian cells, in test animals where somatic and germ cells are used, cell culture research where cell lines and primary cells are used in human biosurveillance studies. However, there is the possibility of in vitro and in vivo genotoxicity tests.

1.1 In vitro and In vivo Genotoxicity Applications

In vitro genotoxicity is applicable in primary cells and cell lines. Primary cells are cells isolated from the organ, kept only for a short period of time in cell culture, whereas cell lines are continuations of primary cells and have gained independent growth capability. However, in cell lines, there is a significant decrease in xenobiotic metabolism specific to the organ. The process of coculture is also used with in vitro implementations, thereby enabling interaction among cells (2).

Identifying mutations does not require the observation of pathological changes in the organ or in the whole organism due to the fact that they are cell dependent and so they can be easily determined in in vitro cell cultures. Therefore there exists the possibility of using in vitro genotoxicity methods as a guide amongst the alternative methods i.e. methods undertaken without using test animals (2, 5). In the European Union, in order to avoid using many standard tests where test animals are used, the 7th amendment (2003/15/EC) under the 76/768/EEC Directive of Cosmetics is now in place. There have been important developments regarding the establishment of alternative methods in some fields such as predicting local toxic effects and genotoxicity (5). Consequently, many in vitro tests have been adopted at OECD (Organisation for Economic Co-operation and Development) level.

In vitro genotoxicity research is important in screening and identifying the priorities. With in vitro tests, there should be outputs of change in the test design in order to achieve more meaningful results towards realistic in vivo conditions. In vitro genotoxicity is the result of a substance, having correlation with an organ specific carcinogenic effect in humans and prevention with an organ specific inhibitor may be considered the perfect proof of genotoxic carcinogenicity (6).

Mutagenecity tests involving in vivo methods are divided into two groups: while germ cell mutations in humans increase the risk of hereditary disease of the next generation, somatic mutations may cause cancer development. In general, in vivo genotoxicity tests are used to verify the in vitro ones of chromosomal tests (7). The utilisation of in vivo genotoxicity methods on test animals enables the identification of all the interactions in the organism as well. Also, it enables the identification of metabolic conditions regarding the chemical whose genotoxic effect is researched in its natural environment. With in vivo methods, as in human biomonitoring studies, genotoxic efficiency of the relevant chemical in exposed groups may be revealed as well.

2. USAGE OF GENOTOXICITY TESTS

In vivo and in vitro genotoxicity tests may be used to (2; 7-11);

1. Provide information regarding the efficiency of the compound in directly or indirectly inducing mutations and an alert about the probability of the compound's carcinogenicity,
2. Reveal the molecular mechanism underlying the chemicals genotoxic and carcinogenic effects,
3. Identify hazards in risk assessment within molecular epidemiologic research with regards to occupational and environmental chemicals,
4. Determine toxicity profiles of chemicals,
5. Monitor the diseases and effectiveness of clinical treatments,
6. Develop regulations concerning medical, cosmetic and industrial chemicals according to international and national guidelines.

2.1 The Use of Genotoxicity Methods as a Biomarker of Effect in the Field of Molecular Epidemiology

Increased efforts are being made to identify the effect of internal and external factors like environment, genetic, nutrition, life style and working conditions over the genomic stability in humans (8,9,12,13). The use of biomarkers in occupational toxicology and molecular epidemiology over the last 30-35 years has contributed significantly to this objective (14). The target of developing and validating biomarkers which are classified as effect, exposure and susceptibility, provides important information on reflecting specific exposures, to predict a disease risk in individual/populations, to understand the mechanisms in order to assess epidemiologic and cancer risk and to characterize the high risk groups (9,10,15). Amongst them, genotoxicity methods are included within the context of biomarkers of effect. In the carcinogenesis, the formation of DNA damage is considered to be the important starting point. However, due to the fact that the development of cancer can take many years, it is not practical to conduct prospective epidemiologic studies that cover such a period. Hence, genotoxicity biomarkers are widely used in molecular epidemiology research (16).

The use of these biomarker methods is based on their genotoxicity in short-term testing of many substances which have been identified as human carcinogens, and capable of inducing chromosomal damage (17). However, the significance of these indicators in predicting cancer is not completely known. Due to the widespread and prolonged use of genotoxicity tests, it is possible to evaluate whether they are successful or not in predicting cancer risk with international collaborations (18). Currently, there are two genotoxicity tests which are seen as fit for this purpose. Chromosomal aberration (CA) method and cytokinesis blocked micronucleus (CBMN) assay's application to human peripheral lymphocytes have been validated (19-21). The CA method, which determines the frequency of structural chromosome damage in peripheral blood lymphocytes, has been used as biomarker of early effects of genotoxic carcinogens in occupational and environmental exposures for over 30 years (22). As for the CBMN method, due to its capability of detecting both aneuploidy and clastogenicity (23) and because, compared to the CA method, it is easier and quickly applied and assessed, has gained wide usage. Moreover, it has become more comprehensive because of the evaluation possibility of chromosomal rearrangements within the CBMN assay, namely 'cytome assay' in lymphocytes (24). Another test which is being researched regarding the prediction of cancer, is the comet assay (single cell gel electrophoresis) (25). Besides the use of methods to identify genotoxicity in humans within the peripheral blood lymphocytes, there are also other applications via exfoliated cells and tissues which enable target cell response such as nasal, buccal, and bladder epithelial cells. It is possible to observe similarities or differences between the target tissue and the candidate tissues (26).

The exfoliated epithelial cells micronucleus (EMN) method, with its easy use and assessment features, is particularly widely used. Also, the necessity of conducting research regarding the human cancer risk prediction of the EMN method is emphasized (27).

Currently, for the micronucleus method in buccal cells, there are studies in order to validate the method using the research that has been conducted up until now as a database and the prediction of cancer risk in humans (28). Five studies from Turkey have also been conducted (29-33) and some of the first findings are already published (28).

Although molecular epidemiology studies on genotoxicity tests may include environmental, occupational and clinical investigations, the review concentrated on occupational ones from the Turkish perspective. In many occupations, there is exposure to genotoxic chemicals originating physically or chemically (12). Genotoxicity research within the working environment has potential benefits in terms of hazard assessment, industrial hygene, predicting probable carcinogenicity and biological (genetic) monitoring. In these environments, it is important to set up and use simple methods in order to monitor and identify genotoxicity (34). Additionaly, in Turkey, occupational biomonitoring with the use of genotoxicity tests is the most commonly used amongst the other molecular epidemiology studies. In Turkey, as a developing country, such investigations are valuable and crucial in terms of preventing additional cases of occupational diseases, developing future treatment approaches, maintaining health and safety of workers and to trigger the legal sanctions for work practices and to evaluate limit values. Thus, specific controls, work practices, and lowering the limit values could reduce exposures and prevent additional cases of occupational diseases (35). It is a fact that there are about 50 published genotoxicity researches within occupational environments in Turkey since 1991, according to the search on July 2011 in MEDLINE (PubMED) using the following keywords for the search; *genotoxicity, micronucleus, sister chromatid exchange, chromosomal aberration, Comet*, with combination of *occupational* or *workers* as the second key word and with *Turkey* as the third key word (30, 35-91). Since 1990s, the above mentioned studies have contributed to, both worker and workplace conditions in Turkey or improvements to and wider usage of the genotoxicity test procedures in other research areas within Turkey.

2.2 The Use of Genotoxicity Methods in Revealing Toxic Effect Mechanisms

Short term genotoxicity methods have an important role in predicting carcinogenic activity. In addition, their effectiveness in understanding and revealing the mechanisms which underly the biological activity has become well demonstrated and it is used mostly via in vitro methods (92). In the first stage of the determination of genotoxicity in cell cultures, there should be the formation of the dose-response curve. In determining the doses for the dose-response curve, the cytotoxicity must be already determined. The genotoxicity study is not recommended in doses exceeding the value determined as the limit value for the cytotoxicty (93). Thereby, cytotoxic effect 'handicap' can be prevented in this way while establishing the genotoxic effect. Also, the use of positive and negative control in the study of genotoxicity in cell culture increases the reliability of the study.

Developments in molecular biology provide new tools for the above mentioned mechanistic approaches (14). The genotoxicity mediated mechanisms regarding the chemical/physical agents and particle, nanoparticle and fiber structures can be evaluated with the help of in vitro genotoxicity methods. Particularly, it is possible to benefit from the cell lines representing the target tissues in order to elucidate the mechanisms of genotoxicity such as cell lines representing the lung for inhalation toxicants (94, 95) and human keratinocytes and reconstructed skin representing the skin for solar UV (96). Another example of the use of genotoxicity methods in mechanistic studies is the identification of genotoxic potentials of molecules which have clinical utilisation (97,98).

2.3 The Use of Genotoxicity Methods in Regulations

2.3.1. Regulatory Toxicology

Safety or toxicity tests are mandatory and very important for all types of products to be put onto the market. The function of regulatory toxicology is to conduct and interpret the results of the tests those described in previous sections.

The purpose of the regulations governing the use of chemicals is to protect humans against the probable dangers of drugs and medicals to ensure the safety of foods and beverages, to ensure the occupational safety of workers during chemical production and to protect people and the environment against possible damages from chemical residues like pesticides (11).

Since there are ethical reasons for testing products prior to human exposure, standardized testing requirements are systematized by the law (99). One of the issues that need addressing in future research is the development of standardized tests that could be used for regulatory purposes (100). The information acquired from the tests is based on the classification of each chemical by the regulators, and their labelling in the second phase. The first phase is undertaken according to internationally harmonized guidelines. In the classification, terms like hazard, toxic, irritant are used. Risk codes are used for the labelling in the second phase. The use of R-41 code in terms of severe damage to the eye or mentioning of R-49 code which specifies that when inhaled may cause cancer are some of the examples.

2.3.2. Harmonisation System

Amongst the toxicity tests, the genotoxicity tests in regulations are used with the purpose of predicting the carcinogenic potentials and measuring the chromosomal changes as a result of DNA damage induced by drugs, industrial chemicals, food additives, and cosmetic product compounds (6). The evaluation of genotoxicity in classification of chemicals is required for the labeling process. The labelling has a crucial role on the restrictions of the consumer product sales and especially on the workplace regulations (101).

There are national and international guidelines that enable testing the genotoxic potential of drugs or environmental factors (6). Genotoxicity methods are defined by the European Union (EU), OECD and International Conference on Harmonisation (ICH).

31 countries including Turkey have full membership of the OECD, which represents the three main economical regions of Japan, Europe and America. A test conducted according to OECD test guidelines is accepted by all regulatory authorities in OECD Member States (11). Under the heading of OECD Test Guidelines for the Testing of Chemicals, in the Section 4: Health Effects, the 'genotoxicity/mutagenicity tests' are involved (102).

ICH, another international harmonisation institute has published two guidelines regarding genotoxicity tests; S2A "Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals" and S2B "Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals". ICH S2A informs that; genotoxicity tests for drugs have been identified with regulations in the European Union and Japan and that the US Food and Drug Administration (FDA) have introduced test protocols as recommendations. In this guideline, it has been also concentrated on the need of ensuring coherence of the test protocols in different countries. ICH S2A, includes recommendations for test conditions, together with interpretation/evaluation of test results and an annex document that includes a glossary in order to develop the consistency of genotoxicity test implementations. ICH S2B has identified the standard test methods which will be used to research the genotoxicity potentials of pharmaceuticals. In March 2008, the S2A and S2B guides were merged by the ICH and has been submitted for comments under the heading "ICH S2 (R1): Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use" (103).

Thus under such guidelines, National guidelines and technical approaches evolve and gain international acceptance.

2.3.3 Regulatory Institutions

2.3.3.1 United States of America

Two main regulatory institutions in the USA deal with the regulation of almost all types of commercial chemicals and make recommendations on appropriate test methods. The relevant institutions are Environmental Protection Agency (EPA) and FDA.

The EPA is responsible for dealing with the framework of legislations such as the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Toxic Substances Control Act (TSCA), Resource Conservation and Recovery Act (RCRA) (104).

The EPA reviews the directives and guidelines which are drawn up for controlling chemicals in order to protect the environment under Title 40:Protection of Environment. In Part 158: Data Requirements for Registration there is subpart of 158.70: Satisfying data requirements which concerns pesticides. Accordingly, in the registration application for a pesticide, it is stated that the protocols presented by the OECD may be implemented for experimental purposes which included genotoxicity testing. In the subpart 158.230: Experimental use permit data requirements for toxicology, genotoxicity testing battery is also involved (105). The subpart of 40.798: Health Effects Testing Guidelines concerns registration and control of chemicals. Under this title, subpart F includes genotoxicity tests (106).

The FDA has provided recommendations on the necessity of using genotoxicity tests for food and drugs and these are elaborated on separately below:

In the Short Term Tests for Genetic Toxicology that is included within the Toxicological Principles for the Safety Assessment of Food Ingredients, also known as the Redbook 2000, there are genotoxicity method recommendations for food ingredients that have 1.5 µg or higher consumption (107).

As for drugs, the Center for Drug Evaluation and Research (CDER) of FDA, mentions the genotoxicity tests that could be used in evaluating drug safety and the requirements for the development phase under section number 7400 and under the section for Pharmacology and Toxicology and also under sub-section number 7400.4 (108).

2.3.3.2 European Union

The regulation of chemicals in the European Union includes the hazard classification and risk assessment need and approval system for drugs, pesticides, biocides and industrial chemicals (109). Each country, while using these directives as guidelines, either accepts the directive directly or prepares their own directives which are in compliance with EU regulations.

Drugs in the European Union are regulated by the European Medicines Agency (EMEA). The ICH S2A, S2B and S2 (R1) guildelines in the genotoxicity section under the Toxicology heading are recommended to medicine developers (103). The EMEA which follows a similar approach to the FDA, also defines the allowable genotoxic compound 'The EMEA which follows a similar approach to the FDA, also defines the allowable genotoxic compound residue limits in medicines.'

Dermatologic and sun blocking products are regulated according to the EU Cosmetics Directive. With the addition (2003/15/EC) to the European Cosmetic Directive (76/768/EEC), the producer is required to provide the toxicologic profile of the ingredient chemical. The cosmetic product can neither have chemicals with mutagenic and carcinogenic toxicity nor with reproductive toxicity as ingredients. Toxicologic information is evaluated by the Scientific Committee on Consumer Products (SCCP) (110).

The European Food Safety Authority (EFSA) in the European Union is the reference organization for food and feed safety, animal health and welfare, nutrition, plant protection and plant health, genetically modified organisms and pecticides. There are guidelines for each subject. For instance, for food additives, in the Guidance on Submissions for Food Additive Evaluations by the Scientific Committee on Food, under the toxicology data main studies of Section 3, there are genotoxicity tests (111).

2.3.3.3 Turkey

Regarding the regulations for chemicals, The Ministry of Health, Ministry of Agriculture and Rural Affairs and Ministry of Environment and Forestry have prepared directives according to the legislative implementations in line with the EU Directives and there are periodical updates.

The Ministry of Environment and Forestry has enacted the "Hazardous Chemicals Directive" on the regulation of industrial chemicals in 2001. The Directive covers all chemicals apart from medicines, food, cosmetic and pesticides. Within the framework of the Directive there is no reference to genotoxicity (112).

The regulation of pesticides and similar substances is undertaken through the "Directive on Registration of Plant Protection Products" by the Ministry of Agriculture and Rural Affairs. In the registration of a new plant protection product there are toxicologic data requirements. Among them there are genotoxicity tests as well (113).

As for cosmetic products, based on the 2005 Cosmetics Law by the Ministry of Health, a "Cosmetic Directive" has been prepared parallel to the Commission Decision numbered 96/335/EC and EU Cosmetics Legislation's Directive numbered 76/768/EEC. Within the context of the directive, the cosmetics preparations should present all types of toxicologic data regarding the ingredient substances during the permit application (114).

In Turkey, the registration, permission and control of medicines and other medical products are undertaken through the "Registration of Civil Medical Products" directive by the Ministry of Health. It was prepared in line with the directive on registration of civil medical products numbered 2001/83/EC (115). Within the framework of the Directive, under the heading addressing the toxicologic data required from all products applying for registration and production, there is a subheading for genotoxicity. It is also necessary to include the in vitro and in vivo (supporting toxicokinetic evaluations included) study results for genotoxicity.

The explanation for genotoxicity in the directive is as follows "the purpose of studies regarding mutagenic and clastogenic potential is to reveal the change that substances may do in the genetic substances of individuals and cells. Due to the fact that the exposure to mutagen, together with the risk of causing germline mutation and hereditary disorder bears the risk of somatic mutation which may cause cancer, mutagenic substances may be hazardous to health".

2.3.4 Utilisation System of Genotoxicity Tests in Regulations

Thus it can be seen that there are various studies of genotoxicity requested or recommended by the relevant authorities in the sale and registration of chemicals such as medicine, cosmetics, industrial chemicals and pesticides in the world in general. There is also a separate regulation for which methods of genotoxicity shall be applied to which type of chemicals and in which type of system. There is also the mention of a tiered system in the implementation genotoxicity methods. This tiered system includes;

i. Bacterial gene mutation test (Ames test)
ii. In vitro cytogenetic assessment of chromosomal damage in mammalian cells or the implementation of in vitro mouse lymphoma thymidine kinase (TK) assay
iii. Deriving on the achieved results, in vivo assessment of chromosomal damage in rodent hematopoietic cells

2.3.4.1 Approach in the assessment of safety

In the assessment of safety of compounds in terms of genotoxicity, a tiered system is held as the principle which is used to examine the end points of genetic damage (gene mutations, aneuploidy, and clastogenicity) related with human diseases (116). Research has demonstrated that not all genotoxic compounds may be determined with a single test (1). While some mutagens induce gene mutations via base pair substitutions or frameshift mutations, others may cause chromosome mutations. The probability of different mechanisms in genotoxicity has necessitated the regulatory institutions to define a standard test battery composed of complementary tests (Fig 1) (116, 117).

Before moving on to mutagenicity tests on a specific substance (or mixture) some aspects should be covered. These are;

i. Chemical structure and class of the substance (probable structure-activity relationships) and physicochemical features (solubility, stability, pH etc.)
ii. Expected metabolism pathways, chemical and biological reactivity/activity and known relations with genotoxic substances

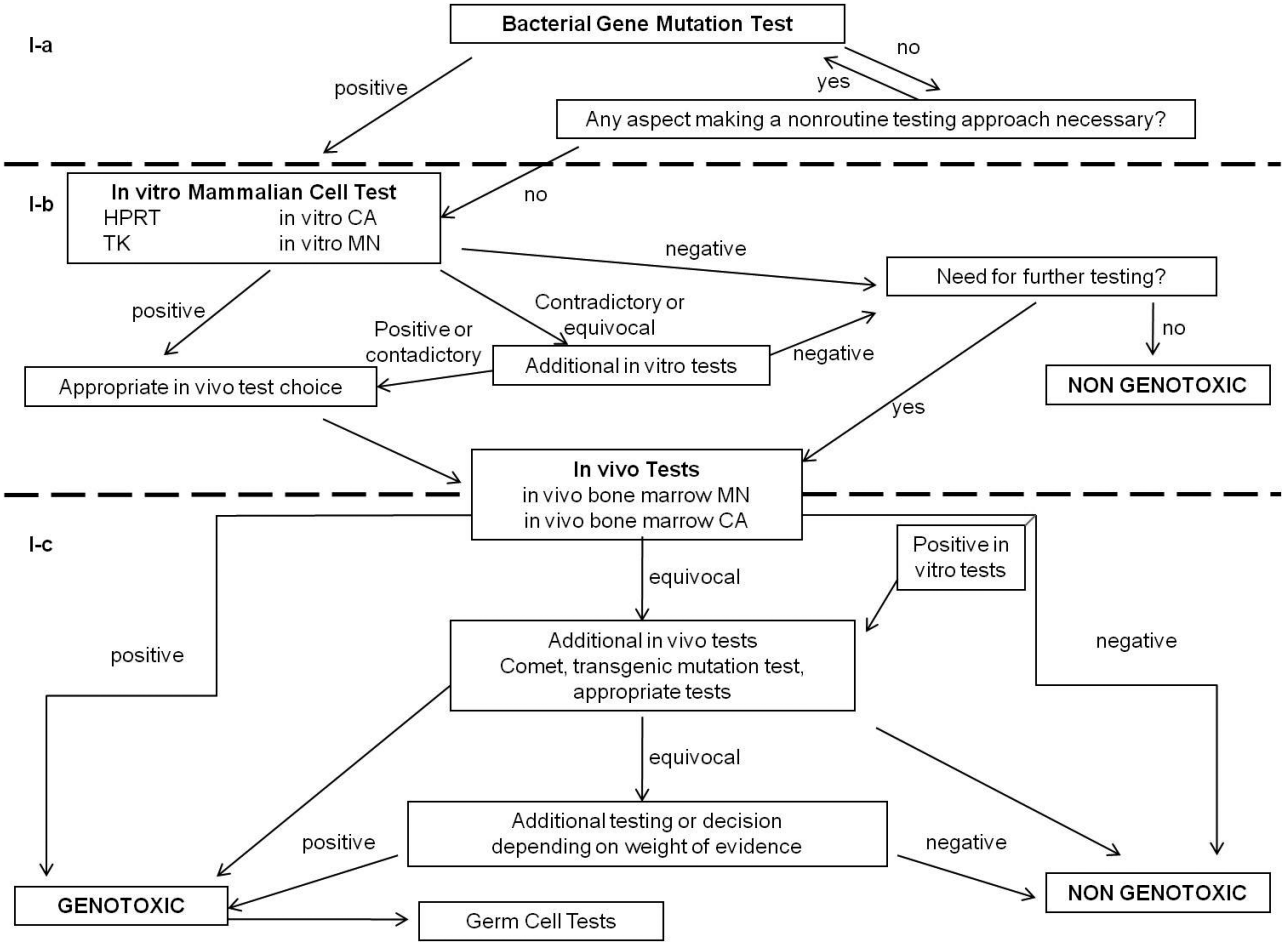

Figure 1. Test strategy in assessment of genotoxicity (modified from 116, 117)

CA: Chromosomal aberration test, MN: Micronucleus test, TK: Mouse lymphoma thymidine kinase gene mutation test, HPRT: Hypoxanthine-guanine phosphoribosyltransferase gene mutation test

iii. Exposure routes, bioavailability and target tissues (117,118)

Critical evaluation of previously existing data provides important information on the selection of appropriate in vitro method and even in vivo methods. After these preliminary evaluations, the in vitro tests are undertaken. The bacterial gene mutation test is conducted as a first stage test. Following the Fig I-a, in the bacterial gene mutation test:

i. Positive result: when a positive result is achieved from the test, the test substance is determined as "mutagen in bacteria" and in the next stage an in vitro mamalian cell test is conducted.
ii. Negative result: in the case of a negative result from the bacterial gene mutation test of the test substance, all data related to the test substance is reviewed in order to decide on whether a different method from the widely implemented methods is required or not. For example, considering the physicochemical features and metabolism data of the test substance, nonroutine strategies are configured in the test method. Following the reapplied methods with new strategies conforming to the negative result or achieving positive result, in vitro genotoxicity tests in mammalian cells are conducted (116).

Following the bacterial gene mutation test, in light of the acquired results, an in vitro mamalian cell gene mutation test is conducted (Fig I-b). Depending on the in vitro test results, a decision is then made on the need for further steps:

i. Positive result: substance demonstrates positive result with the application of one or more methods. The existence of conditions which could affect the reliability or the appropriateness of the positive result is checked. If necessary, additional in vitro studies may be conducted. Additional in vitro tests may be used to provide additional data related to the mechanism for the interpretation of positive results. The positive results acquired from the basic in vitro tests generally points to further analysis.
ii. Negative result: when the substance provides negative result in all test systems configures with in vitro test conditions, it may be definable as "in vitro non-genotoxic" and there may be no need for in vivo tests. Along with this, the need for additional tests is considered if (a) further genotoxicity testing would be helpful for assessing the mode of action of carcinogenesis by the chemical, (b) not all of the types of genetic damage (gene mutations, aneuploidy, and clastogenicity) are ascertained, (c) there are considerations of metabolic activation system alteration, and (d) it is mandatory for regulatory requirements. In these special conditions or "high" or "moderate and sustained" human exposure conditions, it is also recommended in vivo testing.
iii. Contradictory or equivocal result: Biological or statistical meaningfulness is at the limit. In this case, to clarify the positive or negative result, additional in vitro tests should be conducted. According to the result, the tiering system will be followed in terms of positive (or indecisive) or negative outcomes (116,117).

In the choice of an in vivo test, appropriate tissue options should be primarily considered. These choices are based on the exposure route (or routes), distribution of the substance in tissues, metabolic degradation and/or activation in different tissues and the target organ in the long term toxicity studies. As in vivo test, generally, a bone marrow micronucleus or clastogenicity test is recommended. However, in some special cases, selection of other tests may be the subject, even though they are not in standard battery (119). The results, with in vivo test, again are classified into three groups and dictate the further steps (Fig I-c):

i. Positive result: a positive finding about the existence of mutagenic effect of a substance in mammalian somatic cells in vivo, defines that the substance may be identifiable as "in vivo genotoxic". The existence of genotoxicity should bring about the thought that there could be a potential effect in the germ cells as well. In this case, testing of germ cell mutagenicity may also be considered (120-122).
ii. Negative result: Although a negative result is achieved at third stage (in vivo tests), if a positive result is acquired with in vitro tests, it is recommended to undertake additional in vivo tests. Also, in special cases particular to the substance, a secondary in vivo test may be conducted. However, in general, if an in vivo test results negative, it is concluded that there is no evidence that the substance is in vivo genotoxic and identifiable as "in vivo non-genotoxic".
iii. Equivocal result: These results may come about due to low statistical power. Therefore, increasing the number of animals used and/or scored cells may increase the statistical power. If the equivocal situation is not overcome, it is recommended to conduct a second in vivo test considering the substance. After that, according to the result, tiering system will be followed in terms of positive or negative outcomes (117).

When additional tests are needed, an appropriate test system is selected taking into account the entire information particular to the case. At present, many methods are utilised as additional tests. Unscheduled DNA synthesis (UDS) test with mammalian liver cells in vivo was developed in order to assess genotoxic activity in the liver and included in the OECD guidelines (123). With this method, DNA damage and the following repair in liver cells are determined. Generally, since the liver has an important role in the metabolism of the absorbed compounds, this method is appropriate to determine the genotoxic activity of the compounds that require metabolic activation. In the assessment of genotoxicity, comet assay, which is a simple procedure in most tissues, is utilised as well (118). While the standardization and validity studies are on going for the comet assay through inter-laboratory collaboration (124), currently it is only used as an indicator test in the assessment of

genotoxicity (117). Although in the second stage, in vitro TK test and CA test are frequently used, presently, the in vitro mammalian cell micronucleus test is considered for the evaluation of aneuploidy along with clastogenicity. It is also included by the OECD guideline (Test no: 487) due to its easy use but at the moment exists as a draft in the guideline.

3. CONCLUSION

Genotoxicity methods that enable the early prediction of diseases such as cancer which generally take a long time from exposure to appearence, is therefore very valuable. The information acquired from genotoxicity methods together with other toxicology data are among the most important tools for regulatory toxicology as well. In Turkey, genotoxicity tests are used mostly as occupational and environmental molecular epidemiology studies. The main purpose of these studies is to consider any effect leading in the pathway to cancer via determining probable genotoxicity and thus enabling early precautions to be taken. In Turkish directives regarding regulatory controls, there have been studies of compliance with the European Union and within this; the use of genotoxicity in a tiered system has also taken place. The need for this system, which utilises in vitro and in vivo genotoxicity methods to take place in continuous implementations, will enable the closure of the gap regarding the issue. Research capacity in Turkey should be suffice for undertaking implementations, interpreting and decision making towards regulatory purposes regarding medicine, food and cosmetic products etc. From epidemiology studies to regulations, the genotoxicity methods retain their importance and continue developing.

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