Methods to Detect Deoxyribonucleic Acid Damage

Ravichandra Udupa[1], Kaveri Hallikeri[2]

[1]Assistant Professor, Department of Oral Pathology, Srinivas Institute of Dental Sciences, Mangalore, Karnataka, India, [2]Professor and Head, Department of Oral Pathology, Sri Dharmasthala Manjunatheshwara College of Dental Sciences, Dharwad, Karnataka, India

ABSTRACT

Deoxyribonucleic acid (DNA) is very stable or conservative in living organism and its primary structure is hardly changed. Several factors that form the living organism or the environment could cause an abnormal change in the DNA structure. As most of the DNA damage could be restored by the repair system present within the organism, the unrepaired DNA damage is very less. Mutation in the gene caused by DNA damage may lead to ageing of the organism, cancer, or some other diseases related to gene. Tobacco, environmental factors, pesticides used in plants, chemical exposure in the occupation, etc., is the possible causes which lead to DNA damage. There are a reliable number of methods detect DNA damage in various organisms. The main aim of this review is to present a brief account of the DNA damage and detection strategies.

Keywords: Ageing, Cancer, Damage, Deoxyribonucleic acid, Reactive oxygen species, Ultraviolet rays

Corresponding Author: Dr. Ravichandra Udupa, Department of Oral Pathology, Srinivas Institute of Dental Sciences, Mangalore - 574 146, Karnataka, India. Phone: +91-9731813443. E-mail: raviudupa23@gmail.com

INTRODUCTION

Deoxyribonucleic acid (DNA) is the main genetic material in living organism. DNA contains all the genetic information needed for the growth, development and reproduction of organism.[1]

The DNA is the prime genetic molecule, carrying all the hereditary information within chromosomes, immediately focused attention on its structure. The knowledge of the structure of DNA would reveal how the genetic messages that are replicated when chromosomes divide to produce two identical copies of them.[2]

CAUSES OF DNA DAMAGE

DNA damage plays a central role in numerous human diseases including cancer. These damages are caused by many factors including environmental factors (exposure to chemical and physical agents).[3]

DNA Damage Caused by Physical Factors

DNA damage can be caused by some physical factors such as ultraviolet rays and other ionizing radiation (IR).[1]

As climate change is raising global surface temperatures depletion of the stratospheric ozone layer is increasing levels of ultraviolet radiation (UVR). The UV-A (320-400 nm) penetrates deeper into the skin, reaching the basal layer of the epidermis inducing the DNA damage. In addition, endogenous factors such as free radicals generated during metabolic processes as well as IRs are known to interfere with genome integrity.[4]

DNA damage results in (i) misincorporation of bases during replication process, (ii) hydrolytic damage, which results in deamination of bases, depurination, and depyrimidination, (iii) oxidative damage, caused by direct interaction of IR with the DNA molecules, as well as mediated by UVR-induced free radicals or reactive oxygen species (ROS). Most of the DNA damage caused by IR is not direct damage, but first it induces a large number of free radicals in organism and then the free radical causes various types of DNA damage.[4]

ROS plays an important role in tumor development and it can be produced from endogenous sources such as from mitochondria, peroxisomes and exogenous sources, including environmental agents, pharmaceuticals, and industrial chemicals. This oxidative stress will cause the damage in DNA, protein, and/or lipid, which leads to

changes in chromosome instability, genetic mutation, and/or modulation of cell growth that may result in cancer.[5]

ROS-induced DNA damage can result in single or double-strand breakage, base modifications, deoxyribose modification, DNA cross-linking and if it is not repaired before DNA replication leads to cell death, DNA mutation, replication errors, and genomic instability.[5]

DNA Damage Caused by Chemical Factors

Many human cancers are associated with exposure to genotoxic chemicals. There is typically a long period (years) between early events that include initial carcinogen exposure, the onset of DNA damage and the fixation of mutations, and the subsequent appearance of a tumour.[6]

Chemical carcinogens can cause the formation of carcinogen-DNA adducts, or induce other modifications to DNA, such as oxidative damage and alterations to DNA ultrastructure (DNA-strand crosslinking, DNA-strand breakage, chromosomal rearrangements, and deletions). Although cells possess mechanisms to repair many types of DNA damage, these are not always completely effective, and residual DNA damage can lead to the insertion of an incorrect base during DNA replication, followed by transcription and translation of the mutated templates, ultimately leading to the synthesis of altered protein.[6]

DETECTION OF DNA DAMAGE

Determining the injure site and method is an important component of DNA damage study. A number of methods have been invented to detect DNA damage in various organisms.[7]

Various methods are commonly used for the detection and quantification of DNA damage.
I.   Polymerase chain reaction (PCR)
II.   Comet assay
III.   Halo assay
IV.   Terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay
V.   Gas chromatography-mass spectrometry (GS-MS)
VI.   Fluorescence *in situ* hybridization (FISH)
VII.   Annexin V labeling
VIII. Flow cytometry (FCM)
IX.   Immunological assay
X.   Radioimmunoassay (RIA)
XI.   Immunohistochemical assay
XII.   Enzyme-linked immunosorbent assay (ELISA)
XIII. Electrochemical methods
XIV.   Buccal micronucleus cytome assay.

PCR

PCR is one of the most reliably used techniques for detecting DNA damage. PCR is an ideal tool to amplify a small number of intact ancient DNA molecules present in vast excess of damaged molecules.[7]

PCR serves to copy DNA. It uses repeated cycles, each of which consists of three steps: Denaturing, hybridization and extension. Denaturation causes the two complementary strands to separate when DNA molecules heated up to 95°C. In hybridization lowering the temperature at 55°C causes the primers to bind to the DNA and the polymerases begin to attach additional complementary nucleotides at these sites, thus strengthening the bonding between the primers and the DNA. During extension stage, the temperature is increased to 72°C adds further nucleotides to the developing DNA strand. Each time these three steps are repeated and the number of copied DNA molecules doubles. After 20 cycles about a million molecules are cloned from a single segment of double-stranded DNA.[8]

An advantage of PCR is its ability to detect extremely small quantities of DNA and it is quick and repeatedly copied to produce a quantity sufficient to investigate using conventional laboratory methods.[8]

Comet Assay

The comet assay is a new procedure for evaluating DNA damage which involves the application of an electrical current to cells and results in the transport of DNA fragments out of the nucleus. The image of DNA migration obtained resembles a comet with a head and a tail, and hence, the term comet assay.[9]

Ostling and Johanson (1984) were the first to develop a micro gel electrophoresis technique for detecting DNA damage at the level of the single cell and later modified by Singh *et al.* (1988) by introducing a micro gel technique involving electrophoresis under alkaline (pH 13) condition0s for detecting DNA damage in single cells.[10]

The advantage of comet assay is its applicability to any eukaryotic organism and cell type. It is also inexpensive and gives results within a few hours. In addition to human peripheral blood lymphocytes exposed to different agents other cell types and organisms have also been tested with this assay.[9]

Procedure
The first step is to prepare homogenous slides and slides are sandwiched among three layers of low melting point agarose and dry at room temperature. The slides are lysed for approximately an hour using pre-chilled lysing solution. DNA unwinding using alkali (pH > 13)

buffer is completed before the electrophoresis. The cells are then electrophoresed under alkaline condition at 25 V and 300 mA. After the electrophoresis, the slides are neutralized using neutralization buffer, and stained by ethidium bromide which is a fluorescent agent. The slides are then ready to be analyzed (scored) using a fluorescent microscope.[3]

The most common measurements are tail length, intensity of head, and tail moment. Tail length, measured in microns, is only useful at a low damage level since the length of tail tends not to increase proportional to the damage to the DNA. The intensity of head and tail is given in the percentage of DNA in the tail of the comet. Tail moment is the measure of tail length multiply by tail intensity.[3]

Halo Assay

Halo assay was first described by Vinograd *et al.* (1965) and refined by Roti and Wright (1987). In this technique, propidium iodide (PI) a fluorescent dye, intercalates into the DNA helix and causes the change in the supercoiling status of the DNA. DNA can be seen as a fluorescent halo that changes diameter with PI concentration. Cells are lysed and individual nucleotides are visualized as "halos," and thereafter, halo area can be measured by an image analysis system.[7]

Advantage of halo assay is measures single cells and does not require radioactive labeling of DNA. Due to the limitation in its sensitivity, this tech is not used often. The assay was improved as alkaline-halo assay and fast halo assay with modification such as simplification of the lysis, denaturation, and staining procedures.[7]

TUNEL Assay

TUNEL staining relies on the ability of the enzyme terminal deoxynucleotidyl transferase to incorporate labeled 2'-deoxyuridine 5'-triphosphate into free 3'-hydroxyl termini generated by the fragmentation of genomic DNA into low molecular weight double-stranded DNA and high molecular weight single-stranded DNA. Therefore, TUNEL staining may be considered generally as a method for the detection of DNA damage (DNA fragmentation), and under the appropriate circumstances, more specifically as a method for identifying apoptotic cells.[11]

This main drawback for TUNEL is worsened by cell and tissue fixation. Therefore, chemical pre-treatment of the biological samples have been devised to improve TUNEL sensitivity.[12]

GC-MS

GC-MS is becoming widely used to quantify DNA damage because of its ability to identify a wide range of DNA base products. Baseline levels of DNA oxidation in healthy individuals have yet to be unequivocally established. Most study has been devoted to the quantification of 8-OH-guanine, either as the nucleoside (8-hydroxy-2-deoxyguanosine) after enzymic hydrolysis or as the base after acid hydrolysis.[13]

After DNA hydrolysis, GC-MS requires a derivatization procedure to convert the polar nucleosides/bases and internal standards to volatile, thermally stable derivatives which possess characteristic mass spectra. Trimethylsilylation is the most common derivatization reaction used. It is often carried out at a high temperature (90°C ± 140°C) and under nitrogen to prevent artifactual oxidation of samples.[13]

FISH

It is a non-isotopic labeling and detection method which determines the copy number or relative location of disturbed cellular DNA content in nuclei or chromosome. With this technique, visualization and estimation of DNA damage are carried out on a cell by cell basis. Chromosomes with numerical aberrations are detected efficiently by this method. FISH on touch preparations is an efficient method for the study of loss of heterozygosity testing and does not require normal DNA as control. A modification of FISH is interphase dual color and dual fusion FISH which detects minimal residual disease with chronic myelogenous leukemia after allogeneic hematopoietic stem cell transplantation.[7]

Computer-interpreted FISH assays are now sufficiently advanced to provide enormous amounts of data from a single cell, and even more from a tissue section.[14]

Annexin V Labeling

Annexin V is 35 kDa proteins a member of the Annexin family and has a high affinity for phosphatidylserine (PS) containing membranes after binding Ca21-ions. Probing for apoptotic cells with Annexin V *in vitro* has shown that the cell surface exposure of PS is an early event preceding nuclear changes, occurring while the integrity of the plasma membrane is still uncompromised. Furthermore, PS exposure appears to be ubiquitous among hematopoietic lineages, occurring irrespective of the apoptosis initiating stimulus. Recently, it has been demonstrated that apoptotic adherent cell types in culture and tissue embedded cells also expose PS at their cell surface.[15] Annexin V was first reported by Inaba *et al.* (1984). Because of its inability to penetrate the lipid bilayer, Annexin V does not bind to the vital cells.[7]

During apoptosis, PS is translocated to the outer surface of the membrane, with which annexin V binds with high affinity in the presence of Ca^{+2}. B lymphocytes

undergoing chromatin condensation were strongly stained with Annexin V because chromatin condensation coincides with PS exposure. DNA fragmentation is only detected in Annexin V-positive cells. Apoptosis in tumors cells caused by X-rays has been recently detected using (125) I-radiolabeled Annexin V.[7]

FCM

The nuclear DNA content of a cell can be quantitatively measured at high speed by FCM. Initially, a fluorescent dye that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei. The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Thereafter, such fluorescence data are considered a measurement of the cellular DNA content. The samples should be analyzed at rates below 1000 cells per second to yield a good signal of discrimination between singlets or doublets.[16]

This assay is useful in detecting chromosomal aberrations, sister-chromatid exchange, chemical adducts to DNA and DNA strand breakage. Recently, nucleotide excision repair has been also detected by alkaline unwinding FCM assay.[7]

Immunological Assay

This method is commonly used for the detection of oxidative DNA damage but it has limitations because of cross-reactivity of the antibodies with normal DNA bases. In this assay, DNA damage can be detected and quantified very efficiently by immunoslot-blot system utilizing chemiluminescent detection; secondary antibodies conjugated to alkaline phosphatase enzymes and secondary antibodies conjugated to radioactive iodine. Antibodies to modified nucleosides are also possible. Immuno-slot-blot assay is used to detect a very low levels of adduct in very small amount of DNA. It is a very sensitive and specific assay.[7]

RIA

Anti-carcinogen adducts or carcinogen modified-DNA antibodies were the first used in RIA, in which the antigen is synthesized in both a radiolabeled (tracer) and non-labeled (inhibitor) form. The standard curves are generated by mixing fixed amounts of antibody and tracer with increasing concentrations of inhibitor in a constant volume. With larger amounts of inhibitor, less radioactive material is bound by the antibody. A primary antibody is then precipitated, usually with a secondary antiserum, and radioactivity in the pellet or supernatant

is counted. Unknowns are similarly mixed with antibody and tracer, and antigen concentration is determined with the standard curve.[17]

RIAs are replaced by ELISAs, which do not require the use of radioactive material and the associated handling problems. In ELISA, the RIA tracer is replaced by a constant amount of immunogen bound to a microtiter plate.[17]

Immunohistochemical Assay

Immunohistochemically detection of DNA damage can be carried out on either fixed cells (e.g., lymphocytes or exfoliated oral or bladder cells) or tissue sections (frozen or paraffin).

There are two commonly used detection systems-immunofluorescences or immunoperoxidase. Cells can be counterstained with PI or ethyl green, respectively, to allow visualization of nuclei in adduct-negative cells. For immunohistochemical assays, it is important to run control experiments to demonstrate the specificity of cell staining. These controls should include cells treated in culture or tissues from animals treated *in vivo* with or without the chemical of interest.[17]

The major advantages of the immunohistochemical method are its ability to detect adducts in specific cell types within a tissue and its applicability to small amounts of sample. It is also applicable to stored paraffin sections, widely expanding the types of studies that can be carried out. The disadvantages of the immunohistochemical method are cross-reacting antibodies will result in errors in quantification.[17]

ELISA

ELISA is a powerful technique for detecting and quantifying a specific protein in a complex mixture. Originally described by Engvall and Perlmann (1971); this method enables analysis of protein samples immobilized in microplate wells using specific antibodies.[18] In this assay, the antigen is immobilized either by direct adsorption or via an antibody adsorbed to the wells of a microplate. The plate is blocked and the antigen is probed with a specific detection antibody. The detection antibody may be directly labeled with a signal generating enzyme or fluorophore or it may be secondarily probed with an enzyme or fluor labeled secondary antibody (or avidin-biotin chemistry).

ELISA has revolutionized immunology and is commonly used in a medical research laboratories and also has commercial applications including the detection of disease markers and allergens in the diagnostic and food industries.[18]

Electrochemical Methods

The electrochemical methods offer a sensitive, selective, low cost, and miniaturized device for the detection of DNA damage. DNA is an electroactive and surface-active substance yielding analytically valuable electrochemical signals. Adenine, cytosine, and guanine undergo redox processes at the mercury electrodes; guanine and adenine are oxidizable at carbon and some other solid electrodes. Some of these signals respond to the changes in DNA structure. 8-oxoguanine has been detected via its oxidation signal at carbon electrodes. The lesions such as thymine dimers could not be detected electrochemically until they are connected with distortions of DNA double helix. Electrochemical responses of native (double-stranded [ds]) and denatured (single-stranded [ss]) DNAs differ greatly at the mercury electrodes, allowing for determination of small amounts of ssDNA in dsDNA samples.[7]

Buccal Micronucleus Cytome Assay

The buccal micronucleus cytome assay is a minimally invasive technique for studying DNA damage, chromosomal instability, cell death, and the regenerative potential of human buccal mucosal tissue. Regeneration is dependent on the number and division rate of the proliferating (basal) cells, their genomic stability and their propensity for cell death. These events can be studied in the buccal mucosa (BM), which is an easily accessible tissue for sampling cells in a minimally invasive manner and does not cause undue stress to study subjects. This method is increasingly being used in molecular epidemiological studies to investigate the impact of nutrition, lifestyle factors, genotoxin exposure and genotype on DNA damage and cell death.[19]

Micronuclei are one of the biomarkers that are cytoplasmic chromatin masses with the appearance of small nuclei that arise from lagging chromosomes at anaphase or from acentric chromosome fragments. These are formed by chromosomal damage in the basal cells of the epithelium. When these cells divide, chromosomal fragments (or entire chromosomes which lack attachment to the spindle apparatus) lag behind and are excluded from the main nuclei in the daughter cells. These fragments form their own membranes and appear as feulgen-specific bodies, termed micronucleus in the cell cytoplasm. It is these cells that later mature and are exfoliated.[20]

In this assay cells derived from the BM are harvested from the inside of a patient's mouth using a small-headed toothbrush. The cells are washed to remove the debris and bacteria, and a single-cell suspension is prepared and applied to a clean slide using a cytocentrifuge. The cells are stained with feulgen and light green stain allowing both bright field and permanent fluorescence analysis that can be undertaken microscopically. Healthy normal cells can then be distinguished from those considered abnormal based on the nuclear to cytoplasmic ratio and nuclear morphology and texture.[19]

CONCLUSION

Currently, there are several methods available for detecting different kinds of DNA damage but with some or other limitations. There is a need to combine the features of different detection methods and to develop a unique strategy that can localize damage in genome, point out the nature of damage and quantify damage and repair processes. This will be helpful in developing repair strategies and will also provide better insight into the process of carcinogenesis and ageing.

REFERENCES

1. Jun S. DNA chemical damage and its detected. Int J Chem 2010;2:261-5.
2. Watson JD, Baker TA, Bell SP, Gann A, Levine M, Losick R. The structures of DNA and RNA. The Molecular Biology of Gene. 7th ed. San Francisco, CA: Pearson Education; 2008. p. 1-33.
3. Nossoni F. Single-cell gel electrophoresis (Comet Assay): Methodology, potential applications and limitations in cancer research. Basic Biotechnol 2008;4:30-5.
4. Rastogi RP, Richa, Kumar A, Tyagi MB, Sinha RP. Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. J Nucleic Acids 2010;2010:592980.
5. Wong VW, Szeto YT, Collins AR, Benzie IF. The comet assay: A bio monitoring tool for nutraceutical research. Curr Top Nutr Res 2005;3:1-14.
6. Poirier MC. Chemical-induced DNA damage and human cancer risk. Nat Rev Cancer 2004;4:630-7.
7. Kumari S, Rastogi RP, Singh KL, Singh SP, Sinha RP. DNA damage: Detection strategies. EXCLI J 2008;7:44-62.
8. Mullis KB, Faloona FA. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol 1987;155:335-50.
9. Da Silva J, De Freitas TR, Marinho JR, Speit G, Erdtmann B. An alkaline single-cell gel electrophoresis (Comet) assay for environmental bio monitoring with native rodents. Genet Mol Biol 2000;23:241-5.
10. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, *et al*. Single cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen 2000;35:206-21.
11. Loo DT. Tunel assay - An overview of techniques. Mol Biol 2002;203:21-30.
12. Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, *et al. In situ* apoptotic cell labeling by the TUNEL method: Improvement and evaluation on cell preparations. J Histochem Cytochem 1996;44:959-68.
13. Jenner A, England TG, Aruoma OI, Halliwell B. Measurement of oxidative DNA damage by gas chromatography-mass spectrometry: Ethanethiol prevents artifactual generation of oxidized DNA bases. Biochem J 1998;331:365-9.
14. Levsky JM, Singer RH. Fluorescence *in situ* hybridization: Past, present and future. J Cell Sci 2003;116:2833-8.

15. van den Eijnde SM, Luijsterburg AJ, Boshart L, De Zeeuw CI, van Dierendonck JH, Reutelingsperger CP, *et al. In situ* detection of apoptosis during embryogenesis with annexin V: From whole mount to ultrastructure. Cytometry 1997;29:313-20.

16. Nunez R. DNA measurement and cell cycle analysis by flow cytometry. Curr Issues Mol Biol 2001;3:67-70.

17. Santella RM. Immunological methods for detection of carcinogen-DNA damage in humans. Cancer Epidemiol Biomarkers Prev 1999;8:733-9.

18. Engvall E, Perlmann P. Enzyme linked immunosorbant assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry 1971;8:871-5.

19. Thomas P, Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, *et al.* Buccal micronucleus cytome assay. Nat Protoc 2009;4:825-37.

20. Jois HS, Kale AD, Mohan Kumar KP. Micronucleus as potential biomarker of oral carcinogenesis. IJDA 2010;2:197-202.