Chapter

The Importance of Molecular Diagnostic Techniques on Evaluation of Cancers

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Abstract

Cancer is caused by genetic changes controlling cell progression and differentiation. These changes are unregulated when tumours advance and acquire invasive and metastatic capacities due to the innate biologic characteristics of the cancer cell. In vivo and in vitro models show that these molecular changes are crucial for tumour development and survival. These molecular changes can be used to develop pristine cancer treatments. New methodological molecules are being developed to identify cancer-specific modifications in proteins, DNA, and RNA, as well as molecular distinctions between healthy and cancer cells. This approach enables effective early detection, precise diagnosis, and quick cancer therapy. DNA microarray techniques have been developed for identifying cancer-associated mutations and gene profiles. Molecular cancer diagnostics need improvement alongside advances in genomics, precision medicine, and immunotherapy. This chapter discusses different molecular diagnostics in the evaluation of cancers..

Keywords: cancer, molecular diagnosis, cancer detection, genetic modifications, prognosis of cancer

1. Introduction

Cancer therapies are secure and reliable. Each patient would only get a therapy if necessary, and it would be tailored to their cancer. The therapy would not impact other bodily tissues, minimising any potential adverse effects. In other words, the approach would be exact. While there is no perfect cure, molecular diagnostics (in conjunction with targeted medicines) bring us closer to this ideal. The "precision" or "personalised" medical revolution in healthcare includes molecular diagnostics. The capacity to provide personalised medicine, which has been a goal of physicians for centuries, is getting increasingly precise because to advancements in diagnostic and treatment equipment. Currently, the phrase "precision medicine" is used to describe a relatively young and developing discipline that offers crucial information that may be utilised to choose the most effective course of therapy for each patient. Precision medicine uses data from DNA, RNA, proteins, or other similar substances. Molecular diagnostics are essential to precision medicine since they are the tests that find these molecules [1]. There are aspects related to cancer therapy where molecular diagnostics shows significant contribution.

Molecular diagnostics may also be used in differential diagnosis to aid in the cancer diagnosis process. They may assist in distinguishing between cancer and benign tumours. Even classifying distinct cancer subtypes that affect the same tissue may be done with the use of molecular diagnostics. These findings might be used to calculate the aggressiveness of cancer. Blood malignancies, for instance, come in a variety of forms. Subtypes of blood cancer are often identified using molecular diagnostics [2]. AML, or acute myelogenous leukaemia, is one kind of blood cancer that is categorised into poor, moderate, or favourable risk groups based on chromosomal analysis. When an illness is deemed to be of intermediate risk, patients are subjected to molecular testing for a variety of mutations. Other molecular diagnostics are used to identify other AML subtypes, such as acute promyelocytic leukaemia. Treatment options may be affected by the cancer's molecular subtype. Prognosis A prognosis is a prediction of the consequences of medical therapy or the natural course of disease in the absence of treatment. Knowing that certain tumours are more aggressive by nature than others may assist patients and doctors in choosing the best course of action [3]. The gene FLT3 serves as an example. In persons with AML, changes in this gene are a sign of an aggressive malignancy. It is noteworthy that during the last several years, researchers have created drugs intended to block FLT3, and some are now undergoing clinical trials. If these drugs are effective, the existence of FLT3 changes, as identified by a molecular diagnostic, may help predict how well a patient would respond to therapy [4]. Another component of prognosis that molecular diagnostics may assess is the probability that cancer will return after therapy. For women with early-stage, node-negative, oestrogen receptor-positive, invasive breast cancer who will receive hormone treatment, several molecular diagnostics are available to forecast the chance of breast cancer recurrence. Multiple genes are examined by these procedures in cells taken from a breast tumour sample. Prediction of Treatment Response As previously mentioned, molecular diagnostics may assist in determining how patients will react to cancer therapy. A test for HER2/neu gene overexpression in a person's breast cancer tumour tissue is one such. Human epidermal growth factor receptor 2 (HER2) is a protein that is produced by cells when the HER2/neu gene is present [5]. This gene is overexpressed in around one-fourth of all breast tumours, resulting in an excessive amount of the protein being generated. Cells develop and divide more quickly due to the additional protein. Treatment for breast tumours with overexpression of HER2/neu may include the use of a medication called trastuzumab, which blocks the function of the HER2 protein. Because the findings of the test may also be used to evaluate the prognosis (i.e., the aggressiveness of the cancer), testing for HER2/neu is another example of a molecular diagnostic with several clinical applications.

2. Role of pharmacokinetics in cancer therapy

Pharmacokinetics is the study of how medications are absorbed, distributed, broken down, and eliminated. Pharmacokinetics When you ingest a tablet, the drug must be absorbed and dispersed throughout the body to reach the site of action—in this example, the malignant tissue—where it will have the desired effect. After some time, the drug is broken down and eliminated by the body, necessitating the need for further treatment. Genetics is only one of many variables that affect these processes' pace of progression. Genetic variations cause some individuals to metabolise medications more quickly than others, which has significant consequences for several cancer therapies [6]. One example of this is the drug irinotecan, which is used to treat colon cancer. Irinotecan is metabolised more slowly by individuals with

the genetic sequence UGT1A1*28 than by those without it. These people need to get a lesser dosage of the medicine than usual to avoid drug accumulation in the body. Monitoring therapy Response Naturally, both patients and medical professionals want to know right once if a therapy is effective. For colon and numerous other malignancies, simple blood tests and other molecular diagnostics have been developed to assess therapy response throughout the course of treatment [7, 8]. As in the instance of the blood cancer chronic myelogenous leukaemia, certain malignancies may become drug resistant. Imatinib, a drug that blocks a protein produced by an aberrant combination of genes, is often used to treat this malignancy. Years of imatinib treatment may be necessary for those with chronic myelogenous leukaemia, and in some individuals, the genetic makeup might alter with time. Reduced pharmaceutical efficacy might result from this adjustment. Therefore, molecular diagnostic testing may be used on individuals who no longer react to imatinib to evaluate whether the gene has altered. Patients with cancer who have had effective treatment are often followed up on a regular basis to look for symptoms of recurrence. When it comes to some malignancies, molecular diagnostics may help in figuring out if the disease has returned. It is debatable whether people without cancer symptoms should be monitored for recurrence of breast, prostate, and ovarian cancers using molecular diagnostics. Future research is anticipated to result in improved molecular diagnostics that can more precisely track disease recurrence in people who have had effective treatment [9].

3. Role of molecular biomarker in diagnosis

A cancer biomarker is a trait that may be used to predict a patient's prognosis, cancer risk, or incidence. These traits could be cellular, molecular, physiologic, or image based. The emphasis of the current review is on cellular and molecular cancer biomarkers. These biomolecules, which are present or created by cancer cells or healthy cells in response to cancer, may be discovered in tissues or bodily fluids. In order to find changes in nucleic acid, amino acids and other related components, or other biomolecules that may be used for cancer prognosis, diagnosis, cancer therapy, predicting drug response, or cancer monitoring, biomarker testing in cancer entails profiling tumours or bodily fluids. As opposed to cancer biomarker testing, genetic testing is used to find germline genetic changes linked to cancer susceptibility, hereditary cancer, or syndromes associated with cancer. Germline genetic markers may supply valuable information about therapy alternatives in addition to information on cancer risk. In a larger sense, they may also be thought of as cancer biomarkers [10].

Molecular Alterations Can be Inherited or Acquired. A genetic change's ability to be handed down from one generation to the next relies on the kind of cell it occurs. Germ cells and somatic cells both have the potential to experience genetic changes. The reproductive cells in humans are called germ cells. Somatic cells are all other cell types often known as nonreproductive cells. Mutations are often used to describe changes in DNA. DNA alterations that have detrimental effects on health though not always referred to as mutations [11]. However, occasionally DNA alterations that are "good" for the organism are referred to as mutations, such as when DNA alterations help plants or living things to adapt to their surroundings more effectively. Consequently, the meaning of the term "mutation" is ambiguous. Hereditary mutations or germline mutations refer to mutations that take place in germ cells. Only mutations in these cells can be passed on to the next generation since only eggs and sperm can produce embryos. Increased vulnerability to an illness or a change in how

the body metabolises a medicine are both consequences of germline mutations. On the other hand, mutations that take place in somatic cells are referred to as somatic mutations and may result from environmental exposure or spontaneous cellular processes. When cells do their regular tasks, including creating new cells and repairing DNA damage, spontaneous mutations might happen. Somatic mutations occur in healthy tissue, which subsequently progresses to cancer. Even though they happen in the same gene, somatic and germline mutations might have distinct effects on health. One such gene is P53, which has been linked to cancer [12].

3.1 Chromosomal modifications as cancer biomarkers

Biomarkers for cancer may be specific chromosomal abnormalities. Typically, the chromosomal mutation is both a biomarker and the primary driver of the malignancy. One instance of a targeted treatment is imatinib. Treatments for cancer that explicitly target chemicals involved in the development, spread, and advancement of cancer cells are known as targeted cancer treatments. These medications work to stop the reproduction or proliferation of cancer cells. Different chromosomal irregularities chromosomal translocations, such as the one that results in the Philadelphia chromosome, are typical in many different cancer types [13]. Most of the time, this translocation produces fusion proteins that are unable to turn off by themselves; in fact, more than 200 such proteins have been discovered. The anaplastic lymphoma kinase (ALK) gene is another example regulate the cell growth is often regulated by the ALK gene. About 3–5% of persons with non-small-cell lung cancer have this gene mutation in their tumour tissue, which plays a crucial role in various malignancies. People with non-small cell lung cancer whose tumour is positive for the ALK fusion gene may benefit from a targeted medication called crizotinib, which has been created to suppress the extra protein generated by ALK overactivity [14]. Chromosome inversion, or the end-to-end swapping of a section of DNA inside the same chromosome, is another chromosomal defect that sometimes manifests in cancer. Copy number variation is the name given to a third kind of chromosomal abnormality. This comprises chromosomal deletions (where a portion is lost) and amplifications (where a portion of the chromosome is duplicated). Gene duplication and gene amplification are additional terms used to describe chromosomal duplication. In this instance, a DNA fragment is improperly copied once or more. Overproduction of the protein may result from gene duplication in some regions, which may then lead to overactivity [15]. Human epidermal growth factor receptor 2 (HER2), a protein, is the case for certain breast tumours that are referred to as being "HER positive." Typically, this protein aids in cell growth. This gene causes malignancies to develop quickly and invade other tissues when they have an excessive number of copies. To check for this mutation, there are molecular diagnostic techniques available. Trastuzumab (Herceptin®), a medication that renders the HER2 protein inactive, was created because of the finding that HER2 was linked to breast tumours that grew more quickly [16]. Another medication called lapatinib (Tykerb®) prevents some proteins, including HER2, from doing their job. We all have genetic variances, in other words. Some of these variances are passed down via our families, while others are picked up during our lifetimes. Generic variation may take many distinct forms. Single nucleotide polymorphisms are variations in the DNA sequence that occur in at least 1% of the population yet vary from everyone else by one nucleotide base pair. SNPs are responsible for 90% of all DNA variation in humans. SNPs may be either good or negative; some can be helpful, while others might be detrimental, and yet others might have no discernible impact at all. There are several

distinct kinds of mutations that may take place. Point mutations are changes in a single nucleotide base to a different base, whereas insertions and deletions are changes in new nucleotide bases introduced into the sequence. In DNA, insertions and deletions are collectively known as indels. By counting the amount of DNA bases involved, they are arbitrarily separated from chromosomal deletions and amplifications. In accordance with current use, indel is used to describe deletions or insertions that include 1 to 50 nucleotides or less, while copy number variation is used to explain bigger deletions and insertions that generally involve more than 100 nucleotides [17].

3.2 Cancer biomarkers using proteins

The sort of biomarker that is being assessed by molecular diagnostics most often is protein biomarkers. Some proteins are released by tumours and reach the circulation. After that, these proteins may be assessed using a blood sample and a molecular diagnostic test. HER2 protein, which is the protein produced by the HER2/neu gene, and oestrogen receptor levels in breast tumours are two additional proteins that may be detected in tissue. An example of a protein biomarker is carcinoembryonic antigen (CEA) [18]. Patients who have previously been diagnosed with cancers of the rectum, thyroid, lung, breast, liver, pancreas, stomach, and ovaries have their blood levels of this protein monitored rather than utilised for diagnosis. In certain cases, a CEA test is carried out before to therapy and then repeatedly carried out during therapy to assess the efficacy of the treatment and look for cancer development or recurrence. The CEA levels may potentially affect the stage and prognosis of certain cancers. In certain cases of malignancies, the CEA test is only useful in individuals whose cancer has metastasised (spread outside of the initial area) and is used in combination with other biomarkers and clinical testing [19].

4. Partner diagnostics

There is a rising tendency towards employing molecular diagnostics to identify people with a specific biomarker that might be targeted with a specific treatment as our understanding of the biology of cancer advances. Since they enable focused therapy, molecular diagnostics are often referred to as companion diagnostics.

4.1 Genomics

The Human Genome Project, a major undertaking that resulted in the sequencing of all 3 billion nucleotide bases in human DNA, was completed in 2003. The study of how several genes interact to carry out a certain function is known as genomics, and this endeavour contributed to the emergence of this field. Because they lacked the tools to examine several genes at once in the past, researchers tended to concentrate on single genes. The laboratory techniques and advanced computer technologies needed for this kind of investigation have only recently become accessible [20]. It has become clear thanks to genomics that alterations in several genes and proteins are often linked to cancer. Consequently, molecular diagnostics, or assessments of several genes at once, have been developed to study gene profiles. For instance, OncotypeDX® Breast Cancer Assay is one of the most popular and well-researched gene profile or genomic assays. This investigation analyses a panel of 21 genes that provides information about breast cancer. It is designed to be used by female patients

with early-stage (Stage I or II), node-negative, invasive breast cancer that is positive for the oestrogen receptor and who will undergo hormone therapy. The result of this test is a score that represents the likelihood of a recurrence of breast cancer; the higher the score, the more likely the tumour will come back. This test may also determine if chemotherapy added to a woman's hormone treatment (also known as adjuvant therapy) will be beneficial in the case of early stages [21].

4.2 Proteomics

Multiple proteins may be found simultaneously using proteomic molecular diagnostics. It is challenging and hard for researchers to create proteomic tests that are effective for cancer since cells produce so many distinct proteins (one gene might produce many proteins). It is anticipated that breakthroughs would result from the collection of enormous datasets, or data listing protein levels in hundreds of thousands of individuals with varied health issues who participated in several research. Researchers will examine these datasets when more of them become accessible to identify protein patterns that could be connected to tumours in the hopes of developing helpful proteomic-based molecular diagnostics [22, 23].

5. Aspects of molecular diagnosis methods

Today, polymerase chain reaction (PCR) is a crucial technique in molecular diagnostics that allow medical professionals to "see" changes in our DNA that may be the underlying cause of medical issues and that may aid in the identification of novel biomarkers that can be specifically targeted by precision medications. They are often referred to as "diagnosis in vitro" or "in vitro diagnostics." The Latin phrase "in vitro," meaning "in glass," refers to the initial experiments conducted using glass test tubes. The distinction can be made between "in vivo diagnostics," which are conducted on a living human subject and provide visual information, and "in vitro diagnostics," which are presently conducted on bodily samples such as blood, saliva, or cancer cells. X-ray imaging, ultrasound imaging, and computed tomography (CT) scanning are often employed techniques for in vivo diagnostic purposes [24, 25]. Molecular diagnostics is sometimes referred to as "in vitro diagnostics" since all current molecular diagnostics are performed in vitro. Diagnostic techniques that combine in vitro and in vivo research are now widely used. For example, certain body molecules are marked with substances that make them visible using imaging technology when radioactive oestrogen is supplied to a person and imaging is utilised to examine oestrogen receptors in the living body. Obtaining a test specimen or a sample of tissue is the first step in a molecular diagnostic. According to the objective and kind of test, many procedures are employed to obtain tissue samples. Blood samples are often drawn from veins in the arm. Urine and mouth-saliva samples may both be examined. Skin samples may be taken after local anaesthesia [26]. When samples need to be collected from a solid tissue abnormality or tumour, a fine needle biopsy is the easiest and least invasive procedure. This includes inserting a thin needle into the tissue and aspirating cells. A core needle biopsy may be used if a larger amount of tissue is needed to extract cells and a small amount of surrounding tissue. Excisional and incisional biopsies, which each remove a portion of the anomaly or tumour, may also be used during surgery to remove more tissue. To collect cells, it is also possible to scrape tissues naturally exposed to the environment, such as the cervix and face. Another

method involves inserting an endoscope—a bendable, lighted device—through one of the body's natural holes. Small bits of tissue may be removed by the doctor using the endoscope to evaluate any anomalies on the organ's lining.

5.1 Maintaining tissues

Any tissue that is not immediately subjected to molecular diagnostic tests may be processed and kept for later use. Using formalin and wax to embed the tissue sample is one method of tissue preservation. Snap-freezing the tissue and keeping it at 80°C is another option. The vitality of the tissue for molecular diagnostic testing may be impacted by tissue type, collecting techniques, and storage techniques. Tissue banks are often used to store tissue that will likely not be used for many years, such as for research. DNA, protein, and RNA may all be preserved snap-frozen and kept at a temperature of 80°C for long periods of time [27].

5.2 DNA detection techniques

The direct identification of the nucleotide bases present in DNA is known as DNA sequencing. This technique, often called the Sanger method, was created for the first time by Fred Sanger. The DNA is initially split into two strands using the Sanger technique. One strand is then repeatedly replicated using chemicals that halt the copying process at various points along the DNA strand. Numerous shorter DNA strands of various lengths are produced because of this process. The chemicals employed to halt the replicating process have allowed the researchers to determine which nucleotide is at the end of each fragment. As a result, they can put together the DNA fragments to show the original DNA strand's sequence [28].

Today, it is possible to sequence DNA in a much quicker and less costly manner. Next-generation sequencing, or NGS, is the technique that is now most widely employed. Using a slide, the size of a Band-Aid®, this approach allows for the simultaneous operation of 500 million different sequencing processes. The slide is then placed into a device that examines each response independently and records the DNA sequences in a computer. It is a copying process comparable to the Sanger technique; however, it does not call for the use of modified nucleotide bases [29].

5.2.1 Probes of DNA

In many molecular diagnostic procedures, DNA probes are a crucial tool for identifying the presence of certain DNA sequences. The fact that each nucleotide base in DNA can only connect to one other base is used to the benefit of DNA probes. Building a DNA probe involves connecting nucleotide bases that complement the desired DNA sequence. For instance, you might create a probe using the sequence GGGAA if you were attempting to detect the sequence CCCTT. The appropriate circumstances would allow this probe to connect to CCCTT but no other DNA sequences [30]. DNA probes are much longer than just a few nucleotides; they are then combined with tissue samples that have been prepared for the test. After allowing the probes to bind to or hybridise with tissue DNA, they are removed by washing. The probe will adhere to a complimentary DNA sequence it detects if it matches one and will not wash away. Then, a fluorescent signal that indicates the existence of the sequence in a person's DNA may be seen. The DNA probes will wash off, and no fluorescence will be seen if the subject's DNA does not contain the problematic DNA sequence [31, 32].

5.2.2 Genomic microarrays

The capability of DNA microarrays to simultaneously identify hundreds of genes is crucial to the science of genomics. In DNA microarrays, DNA probes with specific DNA sequences are "arrayed" or "spotted" in a grid pattern on a very thin glass surface. Thousands of tiny dots aligned in exact rows and columns make up the DNA microarray. A single DNA probe, like the one previously mentioned, is included in each dot and is intended to hybridise with the corresponding DNA sequence present in the tissue sample. Numerous probe sites enable simultaneous detection of a wide variety of DNA sequences. Thus-called "high throughput," or the simultaneous examination of several DNA sequences, is made possible by this. An analysis sample comprising the subject's DNA is created once the DNA probes have been positioned in the microarray [33]. The sample's double-stranded DNA has undergone denaturement or been split into two complimentary single strands. After being divided into smaller pieces, the strands are then covered with fluorescent pigment. The chip is filled with the sample's labelled DNA, which is then given time to combine with the DNA probes. After washing the microarray, DNA that has not yet hybridised will come off, while DNA that has will not. Fluorescence is then used to identify bound and unbound DNA. If the DNA probe and the sample DNA have successfully hybridised, that area of the array will light up. Computers have information on which place corresponds to DNA sequence and can determine whether that sequence is present in the sample [34]. The array's detecting strategy may employ one to four colours, depending on the technology. Other names for DNA microarrays include genome chip, GeneChip® (a trademark for a particular device), and gene array. Bead, capillary, and well arrays all function similarly and may be used to mount microarrays on surfaces than glass. In other words, DNA probes are connected to the surface of the array (whose sequence is obviously known since you put them in), enabling thousands of genes or even the whole genome to be studied in a single experiment. Different gene variants are sometimes found through microarray analysis. The BRCA1 gene, for instance, has more than 800 mutations, which raises the risk of breast and several other cancers. These differences can all be discovered using a single microarray [35].

5.2.3 Cytogenetic evaluation

Examining the number and makeup of chromosomes is a component of cytogenetic testing. Dr. Janet Rowling, who made the important discovery that chromosomal aberrations might result in cancer in the 1970s, founded this discipline. While some do not classify cytogenetic testing as a "molecular diagnostic" since it does not include the detection of molecules like DNA, we will discuss it here so that it may be contrasted with more contemporary techniques [36]. Traditional cytogenetic tests include collecting cells from a specific region of the body and cultivating them for at least one day in a test tube. The cells that are dividing are then halted or, to put it another way, "frozen" in the act of dividing. Because only at this stage can the chromosomes be seen under a standard microscope, it is crucial that the cells actively dividing. On a microscope slide, the dividing cells are next put. A standard microscope is used to examine each chromosome in several cells (often at least 20). This kind of test is often used to identify Down syndrome, a condition in which the afflicted person has an extra copy of chromosome 21. By just counting the chromosomes, this genetic condition may be identified. Blood malignancies like leukaemia may also be classified using cytogenetics.

5.2.4 Fluorescence in situ hybridisation (FISH)

FISH, commonly referred to as molecular cytogenetic testing, is a technique for identifying and mapping genetic material, including genes or DNA sequences within genes. FISH is a technique for examining individual DNA segments under a fluorescence microscope to determine their existence, absence, relative location, and/or quantity. FISH is more adaptable than traditional cytogenetic methods since it may be used on cells that are not actively dividing. FISH is extremely useful for locating copy number alterations, notably translocations and amplifications, which typically occur with HER2 in breast and gastric malignancies. FISH makes use of fluorescent dye-coated DNA probes. The double strands of DNA in the chromosomes are split apart by special processing. For the DNA probe to connect to complementary DNA sequences in the chromosomal sample, it is then inserted. Following sufficient time for hybridisation, the probes are examined under a fluorescence microscope. Under the fluorescence microscope, hybridisation of the DNA probes with the chromosomal DNA may be seen [37, 38].

5.2.5 Primers

DNA primers are DNA sequences that act as the basis for DNA replication. DNA polymerases, the proteins that aid in DNA replication, are unable to start the process of creating new DNA from scratch. They are limited to adding nucleotide bases to an already-existing strand. Primers play an important role in this process since they start it off, while DNA polymerases finish it off. The laboratory technique known as polymerase chain reaction (PCR) amplifies little quantities of DNA to make it easier to detect and analyse. To separate the strands, heated double-stranded DNA is first used. After that, the temperature is decreased, and primers attach to the DNA's single strands. Then, the DNA copying enzyme (DNA polymerase, represented by the letter 'P' in PCR) replicates each individual strand. After heating the material, the procedure is repeated to separate the freshly formed double strands. The quantity of DNA strands doubles with each cycle of the procedure. The DNA sequence may be produced in a billion copies by PCR in a matter of hours [39, 40].

5.2.6 In-the-moment PCR

Like ordinary PCR, real-time PCR also detects or quantifies the DNA during the same process in addition to copying it. In typical PCR, the final product is examined once the reaction is finished. Real-time PCR is utilised in a variety of companion diagnostics to assess the likelihood that malignancies may react to certain therapy. Reverse-transcription PCR, or RT-PCR for short, is a PCR method that is covered in the next section. Most experts concur that real-time PCR, which is distinct from reverse-transcription PCR, should not be shortened to RT-PCR. To prevent misunderstanding, reverse-transcription PCR should only be referred to by the acronym RT-PCR.

5.3 RNA detection techniques

Many of the techniques just discussed for DNA detection have been modified to detect RNA. Since RNA tests can tell us if the DNA is actively being converted into proteins, they are helpful for assessing the level of gene expression.

5.3.1 Phosphorylation-dependent PCR

Reverse-transcription (RT)-PCR is comparable to PCR; however, it detects RNA rather than DNA. The chemical intermediary known as RNA is responsible for copying and translating the DNA code into proteins. In RT-PCR, the same procedures as in PCR are used, but instead of DNA, a form of RNA is used in the sample, which might be taken from a tumour, blood, urine, etc. A key advancement in research and medical technology is the capacity of RT-PCR to identify expressed genes as opposed to those that are just present. It is possible to utilise RT-PCR to evaluate if cancer has spread to other areas since it may identify even very low amounts of active genes.

5.3.2 MicroRNAs

MicroRNAs, which were first identified in 1993, are a class of RNA rather than a technique. Small, single-stranded RNA molecules called microRNAs, sometimes known as miRNAs, have a length of 19 to 25 nucleotides and attach to certain regions of bigger RNA molecules to stop them from synthesising proteins. MicroRNAs interfere with gene expression in this manner. Quantitative real-time, reverse-transcription polymerase chain reaction (PCR) is used in several microRNA testing [41].

5.4 Techniques for protein detection

Instead of the DNA that encodes them, proteins may be directly detected by molecular diagnostics. Immunohistochemistry is one of the most widely utilised strategies for protein detection. This procedure uses antibodies, a mechanism our immune systems use to get rid of foreign proteins from the body. In immunohistochemistry, a sample in which a target protein is to be identified is combined with antibodies that bind to the target protein. The antibodies are already marked with a marker of some kind, often a fluorescent one visible under a fluorescence microscope. In a test tube, the sample is combined with the antibodies, and they are given time to bind. Once removed, the sample is washed. The antibodies will bind to the protein of interest if it is present in the sample, which will result in a visible coloured label that can be viewed under a microscope. Antibodies not bound will wash away. Both qualitative and quantitative immunohistochemistry is possible. Oestrogen receptors are often found using immunohistochemistry [42, 43].

6. Other techniques in molecular diagnosis

6.1 Next-generation sequencing

NGS, technologies, and advances in sequencing technology are relatively affordable clinical testing platforms. These platforms enable multiple gene targets anywhere between a few and several hundred nanograms of DNA. Cost, projected test volume, intended genomic target breadth, and required sensitivity will all be taken into consideration when deciding which NGS platform to use. The depth of sequencing that is necessary depends on the latter variable. The price increases as the number of targets increases (e.g., entire genome > whole exome > tailored exome) [44].

Through operating additional instances concurrently (batching), it is possible to reduce the cost of reagents like chips for library preparation and flow cells for sequencing. Batching is made simpler by labelling individual samples with molecular

barcodes to enable sample deconvolution during bioinformatic processing. The sorts of modifications that may be identified depend on the platform that is used. Hybrid capture sequencing is the favoured method when breadth is required (whole exome, whole genome, focused panels), while amplicon sequencing is recommended for targeted panels that are optimised for read depth and test sensitivity. Hybrid capture sequencing is often used for this purpose, particularly when more comprehensive copy change information is sought. Furthermore, when translocation detection is required, the hybrid capture strategy should be used since DNA-based rearrangement detection necessitates the assay's ability to capture significant amounts of intronic sequence, which is where breakpoints are most often found. For the detection of translocations, hybrid capture sequencing is sensitive and specific when the breakpoints and partner genes for a given target are known with confidence. For specimens with a low tumour concentration in comparison to the typical contaminating stromal cells, the sensitivity of this method can occasionally be constrained. The ability of algorithms to distinguish between reads with poor mapping that indicate rearrangement and reads with poor sequencing quality determines their sensitivity, specificity, and, ultimately the dependability of the sequencing-based approach to rearrangement detection. False negative and positive findings may also occur with copy number calls, which are determined by comparing the read depth at a specific locus in the sample to a known diploid normal sample. The former happens when there is little tumour present, whereas the later might happen if there has been significant DNA degradation. Given these restrictions, parallel techniques like FISH should be accessible to validate or disprove unexpected or subpar results, or to be used in specimens with tumour content too low to reliably produce a translocation or copy number result by sequencing. When accessible, IHC for protein overexpression may also aid in validating the importance of new rearrangement breakpoints or partners. For translocation identification, laboratories may also think about using RNA-based sequencing techniques. The substantial technological advancements, such as the development of anchored multiplex PCR, it is now possible to identify fusion sequences from the short RNA fragments seen in formalin-fixed tissues.

The read "pileups" may be immediately visualised, and unusual occurrences can be readily noticed using any of the several publicly accessible genomes viewing tools (Integrated Genome Viewer; Genome Viewer, both from the Broad Institute, Cambridge, MA, USA). The potential of misinterpreting extremely low-level mutational events as clinically relevant when they may really be technical artefacts is evident when the data may be visualised. To optimise the assay sensitivity and specificity within the constraints of the specific sequencing technology, bioinformatics workflows should be used to create automated calls. Laboratories should use care while manually reviewing NGS data and refrain from enthusiastically endorsing low-level readings that are below the limit of assay detection established during validation [45].

6.2 Liquid biopsy

The term "liquid biopsy" describes the process of identifying tumour components in body fluids. These components might be tumour DNA or living cells (circulating tumour cells, or ctDNA), cerebrospinal fluid, blood, saliva, and urine specimens are among the fluids that may include these components. In contrast to invasive tissue biopsies, cell-free circulating tumour DNA (ctDNA) may serve as a non-invasive cancer biomarker. To identify ctDNA from tumours, translational cancer researchers are examining the use of liquid biopsies. In the future, ctDNA could be used as a non-invasive method for selecting therapeutic candidates and real-time monitoring of

treatment response. All people have measurable levels of cfDNA in their plasma, which is a by-product of normal cell death, which releases DNA fragments with a nucleosome-bound length of around 160 base pairs into the bloodstream. Additionally, tumour cells discharge their contents into the bloodstream, and the quantity of ctDNA that can be detected varies according to the severity of the illness. Most presently accessible technologies often fail to identify ctDNA in patients with early illness. In general, lung cancer-related ctDNA release may be seen at concentrations between 0.1% and 5% of total cfDNA. Therefore, to identify tumour-specific abnormalities in the plasma in most patients, extremely sensitive approaches are required. The ctDNA analysis focused on EGFR mutations and T790M mutations that confer TKI resistance [46]. Activating hotspot mutations and the T790M mutation that causes TKI resistance have received the most attention in published methodologies for ctDNA analysis in lung cancer. Techniques including real-time PCR, ddPCR, and NGS are often used. The patients who tested negative for these mutations in the plasma undertook routine biopsies and repeat testing on the tumour tissue. Detecting EGFR mutations in plasma similarly predicts how patients will react to EGFR TKIs as detecting mutations in tissue.

An unfavourable outcome is linked to the inability to eradicate the EGFR mutation in the blood after 8 weeks of combination platinum-based therapy and erlotinib treatment. Third-generation inhibitors can detect changes in the plasma levels of the T790M mutation in the relapse situation, and these changes often reflect the clinical state as determined by conventional radiographic staging. There have been cases when the plasma levels of T790M drop, yet the patient continues to advance radiographically. This might be an indication of the diversity of resistance mechanisms. Given that plasma-based testing is less sensitive than tissue-based testing, plasma genotyping assays should be designed to have the highest possible positive predictive value. This strategy will lessen the possibility of false positive outcomes, which might erode confidence in a finding and impair a clinician's ability to choose a course of treatment. However, if a plasma assay is negative, tissue biopsy or further plasma testing should be done to learn more about the tumour's genotype [47]. When performed on plasma samples, single gene tests are effective because they may quickly and confidently provide answers to clinical issues in a group with a high pre-test probability. Numerous interesting academic and commercial assays have been produced because of efforts to create NGS-based methods from plasma. To maximise test sensitivity and clinical actionability, these assays are often designed to maximise depth rather than breadth of sequencing. Improvements to NGS design for use in plasma specimens, focused bait design, adjustments to the library preparation chemistry, sequencing to thousands-fold depth of coverage, and molecular barcoding to identify and suppress PCR mistakes have all been proposed. Given the right setup, translocations and copy number changes may also be found in plasma [47, 48].

7. Validation on molecular diagnosis

This real-world instance highlights a significant flaw in molecular diagnostics: their inconsistency. The PSA test, which analyses PSA blood levels, is non-specific and often results in overdiagnosis and over-treatment [49].

7.1 Validity of analysis

For molecular diagnostics to be effective, it must have both analytical and clinical validity. Analytical validity is the main topic of this part, whereas clinical validity

is covered in the next section. A test's analytical validity relates to how effectively it captures the intended outcome. For instance, a test intended to find a mutation linked to melanoma should not provide a positive result for a completely unrelated mutation linked to diabetes.

7.2 Specificity

Specificity and sensitivity are two independent but related features of validity that excellent tests must demonstrate. These ideas may be used for both the clinical validity of the biomarker and the analytical validity of the molecular diagnostic test. Let us start with specificity. The test's specificity determines how well it can recognise people without the biomarker or disease. In other words, a particular test only returns a positive result when the biomarker or disease is present. Referring to our PSA example, 80% of men with positive PSA results do not have prostate cancer. This is since elevated PSA levels are not just related to prostate cancer; they are also linked to benign prostatic hypertrophy, or prostate enlargement, which is a rather common medical issue in older men. As a result, one of the issues with the PSA test as a tool for prostate cancer screening is that it lacks specificity. Analytical validity is a need for specificity. A specimen in which the true biomarker does not exist may test positive for an imperfectly repeatable or erroneous test. It also relies on the context of usage. It may have to do with the distinction between cancer and other diseases, between tumours, etc. The difficulty with a lack of specificity is that it may lead to emotional distress and force patients to seek risky follow-up tests and therapies. For instance, Men may get a needle biopsy if their PSA levels are high and/or they have abnormal results on a digital rectal exam. Such biopsies may be financially burdensome and might lead to worry and anxiety. Although prostate needle biopsies are generally safe, they may also lead to incontinence and erectile dysfunction in 1% of patients and serious bleeding or infections of the prostate gland or urinary system. Considering the dangers and downsides associated with these tests, it is important to limit the number of patients who undergo them needlessly, as is the case with any medical procedures. It is important to note that among men who have previously received a diagnosis for prostate cancer, the PSA test is still utilised to monitor recurrence. This demonstrates a crucial issue regarding molecular diagnostics: they may have several clinical applications, and the therapeutic value of those uses may change [50].

7.3 Sensitivity

One may think of sensitivity as specificity's antithesis. Sensitivity is the test's capacity to accurately identify patients who have the biomarker or disease; in other words, it should accurately identify everyone who has the biomarker or ailment. If the test is sensitive, you may reasonably expect to have a positive result if you have the biomarker or illness.

8. Positive and negative aspects

The outcomes of molecular diagnostics, like any testing, may be categorised as right or erroneous. A test is considered to have produced a genuine positive result when it accurately identifies a person as having a certain biomarker or disease. A

test is considered to have produced a genuine negative result when it accurately ascertains that a subject does not possess a certain biomarker or disease. We always want the test findings to be accurate or truthful; thus, this is the perfect scenario. The findings are considered false when a test yields inaccurate results. False positive results occur when a test declares someone to have a biomarker or ailment when they do not.

Tests that are reliable and effective have high rates of true positives and true negatives and low percentages of false positives and false negatives. In a perfect world, the test would not only show who has the biomarker or disease but would also show who does not have the biomarker or condition. Very few molecular diagnostics in practice come close to achieving this objective. The phrases positive and negative predictive values (PPV and NPV) are often used in molecular diagnostic testing. A highly specific test will have a high positive predictive value, meaning that if it comes back positive in a patient, it is almost certain that the patient has the ailment. In other words, if a patient's test results are negative, it is exceedingly improbable that the patient really has the ailment. A highly sensitive test will have a high negative predictive value. No exam, however, is flawless. It may be preferable to have a larger positive predictive value or negative predictive value depending on the situation.

Three factors determine the predictive values, both positive and negative:

- The condition's frequency in the general population
- The test's level of sensitivity
- The test's granularity

If the prevalence in the case were far greater, let us say 50%, happening in half of the 100,000 persons, we would have spotted 45,000 real positives but still had 10,000 false positives. The positive predictive value would have been 82% in this instance as 45,000 of the 55,000 positives were genuine positives. The significance of the usage context and analytical validity for a particular molecular diagnostic is brought out by these factors.

8.1 Reliable testing

Test reliability is an additional component of analytical validity. The capacity to repeat test findings is referred to as test reliability. If a molecular diagnostic is done on Monday and shows that a tumour is positive for a certain gene, it ought to show the same thing on Tuesday. Unreliable tests are obviously useless for determining diagnoses or appropriate treatments. HER2 Molecular Diagnostics Reliability There is a problem with the tests that are used to find HER2 overexpression in breast cancer. A gene called HER2/neu is overexpressed in around one-fourth of breast tumours, as was previously explained. A surplus of HER2 protein is produced by cells because of this overexpression. Because the HER2 protein is important in cell growth and replication, cells with excess HER2 experience an excess of signals directing them to proliferate and divide. Trastuzumab is a drug that prevents the HER2 protein from functioning. However, this drug can only be used to treat malignancies that have HER2/neu overexpression, which can only be identified with a test (a companion diagnostic). For this aim, two separate test types are available: one based on immunohistochemistry and the other based on FISH. Even though many women

get trustworthy and accurate findings from these tests, according to the American Society for Clinical Oncology (ASCO) and College of American Pathologists (CAP) recommendations, 20% of HER2 testing performed today may be false. These diagnostics may reveal overexpression of HER2/neu in certain tumours that first test negative and vice versa. These dependability issues are significant since the tests are used to guide therapy. A woman may not get the therapy she needs if a test result is falsely negative (i.e. if a tumour overexpresses HER2/neu, but the test results are negative). In contrast, a woman may get a therapy that is less likely to be helpful to her if a test result is false positive (that is, if the tumour does not overexpress HER2/neu but the test yields a positive result). Lack of standardisation in molecular diagnostics is one factor contributing to the sometimes-inaccurate nature of the tests for HER2/neu overexpression. In a perfect world, molecular diagnostics would be standardised, which would imply that every time they were done, they would be done in the same manner, on the same machinery, with the same chemicals. This is often not the case, however. Reliability may be challenging to get in molecular diagnostics since many of them need exact measurements, complex machinery, and/or various chemical mixes. The methods used to collect, prepare for analysis and keep tissue samples, or so-called pre-analytic variables, may all affect how consistently findings from a molecular diagnostic are obtained. The molecular makeup and consistency of the tissue may be significantly changed by these variables. Therefore, the collection, processing, and storage methods used for a tissue sample that is assessed using the same molecular diagnostic might affect the outcomes. This may be seen, for instance, in the difference between tissue samples that are fixed with formalin and embedded in paraffin and those that are frozen. Scientific investigations using molecular diagnostics may vary depending on pre-analytical conditions as well. Although standardisation of the pre-analytic parameters is preferred, it is still crucial that they be consistently reported. On Biospecimen Reporting for Improved Study Quality (BRISQ), experts released guidelines in 2011. When using human biospecimens, it is required to provide some pre-analytical information that is utilised to assess, interpret, compare, and repeat the findings of the experiment. These recommendations define these pre-analytical data.

ASCO-CAP advises labs to follow stringent tissue sample handling practises, among other things, to counteract the unreliability of molecular diagnoses. According to these standards, new HER-2 tests should also demonstrate 95% agreement with an existing HER-2 reference test that has been clinically validated (i.e., the reference test forecasts clinical outcome). Along with proficiency testing and competence evaluations, strict laboratory accreditation criteria are advised. It is crucial to standardise procedures so that findings from various patients and labs can be compared and so that anybody undergoing pathology testing may be sure that their results are correct. A test sample with a specific, known quantity of the biomarker being identified may sometimes be included in the kits of tests offered by the manufacturer. After that, the test may be calibrated using this standard. An internal standard, such as 100 micrograms of a protein, could be included in a test kit, as an example. They should also discover that the reference sample has 100 micrograms when they run it via other labs. By doing this, labs may confirm that their test produces accurate findings and that its results are like those of other laboratories. By mandating proficiency testing for labs, it may be possible to standardise laboratory tests. For instance, blood samples may be submitted to involved labs for the analysis of the relevant drug. The capacity of each participating laboratory to provide reliable findings determines whether it will get certification, which

is determined by the evaluation of all the participating labs' results at a single location. Some businesses have developed molecular diagnostics that need to send test samples to the business's own laboratory to solve the dependability issue. In this situation, the test may be conducted in the same manner each time, and the business has control over the accuracy of the findings. This is true for the test Oncotype® DX, which aids in determining the probability of benefit from further (adjuvant) treatment and the recurrence of breast cancer. Healthcare experts collect breast tumour samples for this test, which are then sent to the company's laboratory for examination. Clinical Validity The term "clinical validity" describes a test's capacity to provide data that is clinically relevant. The biomarker's tight relationship to a clinically significant outcome, such as a patient's reaction to treatment or the cancer's aggressiveness, is what determines the biomarker's clinical validity. According to our earlier illustration, the PSA test's lack of clinical validity—that is, the fact that regular PSA testing does not lengthen a man's life—is one reason it is ineffective as a prostate cancer screening tool. Clinical usefulness, which is discussed in greater detail in the section after this one, is connected to the idea of clinical validity. According to some experts, clinical utility is the capacity of a test to offer information that is clinically relevant—the same definition as clinical validity. Other specialists, on the other hand, think that clinical utility is a more comprehensive idea that includes a usefulness in the clinic's practical aspects. Clinical validity is seen by many experts, including a committee of the Institute of Medicine, as a measurement of whether the test accurately separates one population into two or more with various biological or clinical features or outcomes. Even though the difference is statistically significant, if it is not significant enough to warrant treating the two groups differently ot if understanding the difference is not linked to a therapeutic option for one group but not the other that enhances clinical outcomes, it is not clinically useful. It is noteworthy that several tests or assays may exist for a particular biomarker. They might vary in their therapeutic utility and analytical and clinical validity. Prior to being suggested to guide treatment in a particular use context, as indicated above, for patient care, each must be evaluated independently.

9. Conclusion

In conclusion, there has been a paradigm change in cancer diagnosis. Cancer is no longer simply identified based on morphological factors. Molecular changes at the DNA, mRNA, miRNA, and proteome levels and immunohistochemistry changes are increasingly supporting the diagnostic criteria. Advanced technology with high throughput and several platforms makes it possible to analyse all these as well as the whole genome more quickly and affordably. Precision medicine based on pharmacogenomics is being developed because of this, which is having a big influence on how medicine is now practised. It is now understood that a tumour may not be identified by a single gene modification but rather by a panel of genomic abnormalities that are "signature," allowing for focused treatment approaches and monitoring based on the tumour-specific alterations. The aim of cancer diagnosis in personalised medicine is to establish the proper prognosis and direct the course of treatment so that every patient receives precision medicine, which is the appropriate medication at the right dosage.

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