Sensors & Diagnostics

CRITICAL REVIEW

View Article Online
View Journal | View Issue

Cite this: *Sens. Diagn.*, 2024, **3**, 1807

Tumor diagnosis based on nucleolus labeling

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Received 29th June 2024,
Accepted 13th September 2024

DOI: 10.1039/d4sd00238e

rsc.li/sensors

The nucleolus is crucial for ribonucleoprotein particle assembly. Vital molecular regulators such as RB (retinoblastoma protein) and p53 (tumor suppressor protein) influence nucleolar function and tumorigenesis. The absence or inactivation of these proteins often leads to nucleolar dysfunction and alteration, which is a key indicator among the primary histopathological features of malignancy. These changes are closely related to the proliferation, differentiation, and survival of tumor cells, such as abnormalities in the number, size, and shape of nucleoli. In recent years, as the relationship between nucleoli and tumorigenesis has been further explored, various nucleolar labeling techniques have been developed for pathological analysis and tumor diagnosis, such as immunohistochemistry (IHC)/immunofluorescence (IF), and fluorescence labeling. These methods complement the traditional use of transmission electron microscopy (TEM) for observing nucleoli. In this review, we explore the relationship between the nucleolus and tumorigenesis and evaluate current methods for diagnosing tumors by examining nucleolar characteristics. We discuss the advantages, disadvantages, and applications of diagnostic techniques such as TEM, IHC/IF, and fluorescence labeling for analyzing the nucleolus.

1 Introduction

The nucleolus is an essential structure within the cell nucleus, primarily responsible for synthesizing ribosomal RNA (rRNA) and assembling ribosomal subunits. This process is crucial for cell growth and proliferation.[1–3] In tumor cells, the morphology and function of nucleoli often undergo significant changes.[4] These changes are mainly reflected in abnormalities in the number, size, and shape of the nucleoli.[5] Such alterations are closely related to the proliferation, differentiation, and survival of tumor cells,[6] and also with their metabolic activity and stress response.[7] Consequently, tumor diagnosis methods based on nucleolar markers have become a significant focus in oncology research in recent years.[8,9]

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Abnormal changes in the nucleolus primarily result from genetic and molecular alterations in tumor cells, abnormal cell cycle regulation, and cellular stress responses.[7] These changes are closely related to the upregulation of ribosome biosynthesis within the cells.[10] Studies have found that the enlargement of the nucleolus in most tumor cells is associated with active transcription of rRNA genes.[11] This is because tumor cells require substantial protein synthesis to support their rapid proliferation, with ribosomes serving as the protein synthesis factories.[12] Therefore, the enhanced function of the nucleolus is a manifestation of tumor cells adapting to their high proliferation state.[11]

RB (retinoblastoma protein) and p53 (tumor suppressor protein) play key roles in the molecular mechanisms regulating nucleolar function and tumorigenesis. RB protein is a cell cycle regulator that influences nucleolar function by regulating the expression of proteins related to nucleolar formation. p53 is widely involved in various cellular processes, such as cell cycle progression, DNA repair, and apoptosis. p53 regulates nucleolar function by controlling the nucleolar stress response and rRNA transcription. When cells experience stress or DNA damage, p53 inhibits rRNA synthesis, thereby reducing ribosome biosynthesis and preventing further cell proliferation.

Clinically, nucleoli have been used for tumor diagnosis.[13–16] For example, AgNOR polymorphism increased gradually according to the grade of histological lesions and could be used as a prognostic factor for squamous cell carcinoma progression.[13] The presence and number of large nucleoli in uveal melanoma are positively correlated with the maximum basal diameter of the tumor. Increased nucleolar counts in tumor cells were positively correlated with the primary tumor stage. The presence of prominent nucleoli and multiple nucleoli is associated with a significant decrease in overall survival and disease-free survival.[14]

TEM is the traditional method for observing nucleoli.[17,18] In recent years, as the link between nucleoli and tumorigenesis has been studied further, scientists have developed nucleolar labeling techniques for pathology and tumor diagnosis,[17] including immunohistochemistry (IHC)/immunofluorescence (IF), and fluorescence labeling.

TEM is a high-resolution microscopy technique that can observe ultra-fine details of the internal structure of cells.[19] Using TEM, the morphological changes of nucleoli, such as their size, number, and internal structure, can be observed.[20] While observing the nucleolus, TEM can also reveal changes in other ultrastructures within the cell, providing additional pathological information.[17] However, the operation of TEM is complex and costly, which limits its widespread application in clinical practice.[17,18]

Immunohistochemistry (IHC) and immunofluorescence (IF) are labeling techniques based on antigen–antibody reactions, used to detect specific proteins in tissue sections.[21] By employing specific antibodies against nucleolar-related proteins, IHC and IF can accurately locate and quantitatively analyze changes in nucleoli within tumor tissues.[22,23] Commonly used nucleolar marker proteins include nucleolar phosphorylation protein (NPM),[24] fibrillarin (FBL),[25] nucleolar organizing region protein (nucleolin, NCL),[26] and so on. IHC is easy to perform, highly sensitive, and can be observed under an optical microscope, making it suitable for clinical pathological diagnosis.[22] IF can simultaneously detect multiple nucleolar-related proteins, distinguishing them by the different colors of fluorescence signals.[27] However, the results of IHC and IF depend on the specificity and sensitivity of the antibodies and are also affected by the tissue sample processing procedures.[28]

Fluorescence labeling techniques use fluorescent probes to label specific nucleolar proteins or nucleic acids, enabling visualization and quantitative analysis of nucleoli.[29] Fluorescence *in situ* hybridization (FISH) is a commonly used fluorescence labeling method that detects rRNA genes in the nucleolus formation region through specific probes to observe the distribution and quantitative changes of nucleoli.[30] Additionally, fluorescence labeling techniques

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have the advantages of high sensitivity and high specificity.[31] They can monitor the dynamic changes of nucleoli in real-time in living cells, providing an important tool for tumor diagnosis.[32] However, fluorescence labeling technology requires professional operating techniques, and labeling and optimizing labeled probes are also challenging.

The nucleolus has 6 nucleolar sub-regions, including fibrillar centers (FC), dense fibrillar components (DFC), granular components (GC), nucleolar rim (NR), and peri-nucleolar compartment (PNC), and the periphery of the dense fibrillar component (PDFC)[33] (Fig. 1). The morphological and functional changes of nucleoli are of great significance in the occurrence and development of tumors, providing important tools for the diagnosis and pathological analysis of tumors.[34] In this review, we present the role of the nucleolus in tumor diagnosis, examine the relationship between the nucleolus and tumorigenesis, and introduce commonly used nucleolar labeling methods. We believe that this comprehensive review will be a valuable resource for researchers, physicians, and students interested in this field.

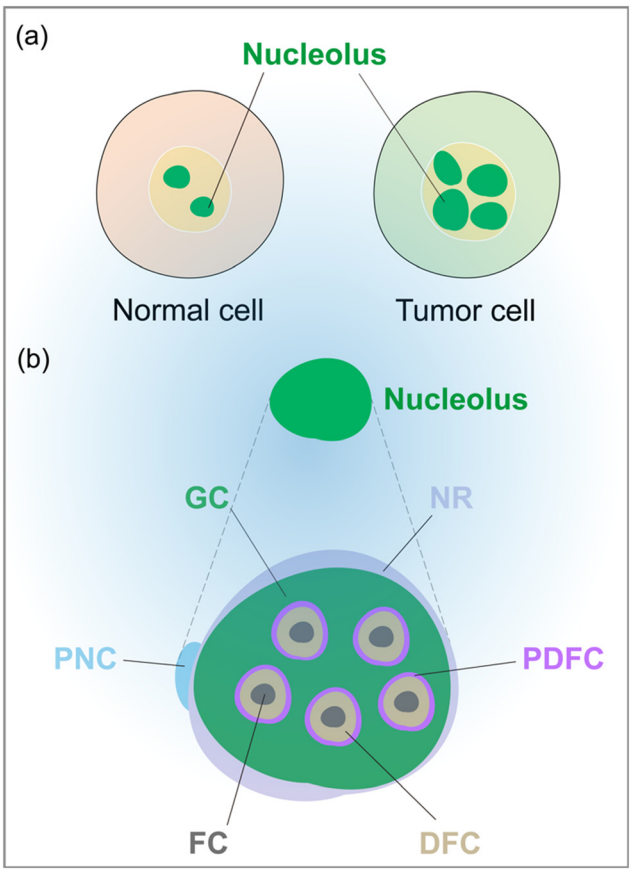

Fig. 1 (a) Compared to normal cells, the nucleoli of tumor cells mainly exhibit abnormalities in number, size, and shape. (b) Schematic showing different sub-regions of the nucleolus. FC, fibrillar centers; DFC, dense fibrillar components; GC, granular components; NR, nucleolar rim; PNC, peri-nucleolar compartment, PDFC, the periphery of the dense fibrillar component.

2 The relationship between nucleoli and tumorigenesis

2.1 Nucleolar changes in tumor cells: unraveling the complex causes

The nucleolus has long been a focal point of research in cancer biology due to its critical role in ribosome biogenesis and cellular stress response. Nucleolar changes in tumor cells indicate altered cellular processes, and understanding the causes behind these changes is crucial for unraveling the complexities of cancer development and progression. We explored the multifaceted factors contributing to nucleolar alterations in tumor cells, drawing on recent literature to provide a comprehensive overview.

2.1.1 Genetic mutations and oncogene activation. Genetic mutations form the bedrock of many complex mechanisms underlying the initiation and progression of cancer, serving as triggers in transforming normal cells into malignant ones.[35,36] These mutations disrupt the intricate balance of genetic instructions, often targeting genes responsible for maintaining cellular homeostasis, repair mechanisms, and regulatory pathways that govern cell division and death.[12,37]

Ribosomal proteins (RPs), fundamental components of the ribosome machinery, are encoded by a large family of genes. Mutations or dysregulation of these genes can lead to nucleolar stress, impaired ribosome assembly, and subsequent disruption of cellular processes.[38–41] This stress response can activate various signaling pathways, including p53, which usually protects against genomic instability and tumorigenesis. However, when overwhelmed or mutated, p53 dysfunction can facilitate cancer development.[42,43]

c-Myc is a prominent player in driving cellular transformation among the many oncogenes implicated in cancer.[44,45] Its aberrant activation, often through gene amplification or mutation, sets off a cascade of events that fuel tumor growth.[45,46] One of c-Myc's pivotal roles is in upregulating ribosomal RNA (rRNA) synthesis,[47,48] a fundamental step in ribosome biogenesis and protein production. Elevated rRNA synthesis supports the heightened biosynthetic demands of rapidly dividing cancer cells and contributes to nucleolar hypertrophy, a phenomenon observed in numerous cancer types.[49,50] This hypertrophy signifies a shift towards a more proliferative state, indicative of the cell's commitment to uncontrolled growth and division.[12]

Furthermore, c-Myc's impact extends beyond ribosome biogenesis, influencing multiple cellular processes such as metabolism, cell cycle progression, and angiogenesis, converging to create a tumor-permissive environment. For instance, the oncogene's capacity to reprogram cellular metabolism redirects resources toward biomass production and energy supply necessary for rapid cell multiplication.[51,52]

2.1.2 Dysregulation of tumor suppressor p53. The tumor suppressor protein p53, often referred to as "the guardian of the genome", is a critical regulator in maintaining genomic stability and preventing cancer.[53,54] It orchestrates responses to various cellular stresses, such as DNA damage and

oncogene activation, by balancing cell survival, growth arrest, and apoptosis.[39,55–61] When p53 function is compromised due to mutations or other factors, this equilibrium is disrupted, leading to uncontrolled cell proliferation, which is a hallmark of cancer.[62–64]

One significant consequence of p53 dysfunction is the hyperactivation of ribosomal biogenesis, which is closely associated with nucleolar function.[39,58] Normally, p53 ensures that ribosome production aligns with cellular needs, but in its absence, there is an excessive increase in rRNA synthesis and ribosome assembly, supporting the high metabolic demands of proliferating cancer cells.[65,66] This results in morphological changes within the nucleolus, indicating the substantial impact of p53 on nucleolar dynamics.[62,63,67,68]

Moreover, p53 dysfunction impairs the nucleolar stress response,[64] a protective mechanism that senses disturbances in ribosome biogenesis and activates compensatory pathways, including p53. Under normal conditions, when nucleolar function is disturbed, p53 is activated to halt the cell cycle, allowing time for repair or, if necessary, triggering cell death.[69] However, when p53 is non-functional, this safety net fails, allowing cells to continue proliferating despite accumulating damage, further potentiating tumor development.[68]

2.1.3 Imbalance in nucleolar proteostasis. Disturbances in nucleolar proteostasis caused by ribosomal proteins or chaperone imbalances contribute to nucleolar changes in tumor cells.[70–72] Aberrant protein synthesis and impaired ribosome biogenesis can result in nucleolar stress,[73,74] a cellular condition characterized by the accumulation of incomplete or dysfunctional ribosomal subunits,[75] triggering a cascade of events that support cancer cell survival and proliferation. Ribosomal subunit assembly is a process fundamental to protein synthesis and cellular metabolism.[70] Embedded within the nucleus, the nucleoli house a complex network of proteins, RNAs, and molecular machinery responsible for producing ribosomes—the cellular factories that translate genetic code into functional proteins. However, this intricate system of ribosomal biogenesis is not infallible and is susceptible to disruptions that can have profound implications, particularly in the context of cancer biology.[69,76]

One such disruption arises from disturbances in nucleolar proteostasis, the delicate balance of protein folding, trafficking, and degradation within the nucleolus. Perturbations to this balance, whether through mutations, misexpression of ribosomal proteins, or imbalances in chaperone proteins that assist in protein folding, can lead to significant nucleolar changes observable in tumor cells.[71,72] These alterations reflect an adaptation to the altered cellular environment and actively contribute to the transformation and maintenance of the cancerous phenotype.

Aberrations in protein synthesis, a direct consequence of disrupted ribosomal biogenesis, are particularly detrimental. They can induce a state of nucleolar stress.[73,74] Central to this response is the activation of tumor suppressor pathways, most notably the p53 pathway, which can halt cell cycle progression, induce DNA repair mechanisms, or initiate programmed cell death if the damage is irreparable.[77,78]

However, these protective mechanisms are themselves compromised in many cancer scenarios. Mutations or dysfunction of p53 and related pathways can convert the nucleolar stress response from a protective measure into a facilitator of cancer progression. Instead of inducing cell cycle arrest or apoptosis, the altered signaling promotes survival mechanisms that allow cancer cells to persist despite the ongoing stress.[79,80] This includes upregulation of pro-survival genes, enhancement of DNA repair capabilities, and modulation of metabolic pathways to sustain the high-energy demands of uncontrolled proliferation.[81,82]

Furthermore, nucleolar stress can contribute to chromatin remodeling, epigenetic modifications, and microRNA dysregulation, further skewing the cellular landscape in favor of cancer cell survival and proliferation.[83,84] It fosters an environment where cells can adapt and thrive under otherwise lethal conditions to normal cells, enabling them to evade apoptosis and continue their uncontrolled growth.

In essence, the disturbances in nucleolar proteostasis and the resulting nucleolar stress represent a critical node in the complex network of cancer pathogenesis.[85] Understanding the intricate interplay between ribosomal biogenesis, nucleolar function, and cancer cell survival mechanisms holds the promise of uncovering novel therapeutic targets and strategies for intervention.[86,87] By manipulating these pathways, researchers and clinicians aim to tip the balance back in favor of normal cellular homeostasis, halting or reversing cancer progression.

2.1.4 Epigenetic modifications. Epigenetic modifications, acting as the silent regulators of gene expression, play a pivotal role in shaping the landscape of cellular function and, critically, the dynamics of nucleoli.[88] These modifications, including adding or removing methyl groups to DNA and modifying histone proteins, can dramatically alter gene accessibility and, subsequently, protein production.[89–91] In cancer biology, such epigenetic alterations can take on a sinister twist, leading to the silencing of genes responsible for maintaining nucleolar homeostasis or the unwarranted activation of proto-oncogenes—a precursor to cancer-inducing genes.[92,93] This aberrant regulation is a crucial driver behind the transformative changes observed in the nucleoli of tumor cells,[94–96] underscoring the intricate link between epigenetics and cancer progression.

The nucleolus has increasingly been recognized as a sophisticated sensor and responder to cellular stress, adeptly integrating signals from genetic, epigenetic, and proteostasis networks.[27] Within this intricate cellular framework, the maintenance of protein homeostasis plays a pivotal role. Disruptions in ribosomal protein balance or dysfunctions in molecular chaperones can precipitate nucleolar abnormalities, thereby contributing to the complex and multifaceted interactions that promote a pro-tumorigenic environment.[97,98]

Technological innovations, such as next-generation sequencing and high-resolution imaging, empower

researchers to explore the nucleolus with unprecedented depth, uncovering its intricate complexities and hidden vulnerabilities. These breakthroughs are progressively illuminating the nucleolus's multifaceted role in cancer, from its involvement in cell cycle regulation to its emerging potential as a biomarker for early-stage tumor detection and disease monitoring. As these discoveries unfold, they are poised to redefine our understanding of the nucleolus in the oncological landscape.[99,100]

In conclusion, the nucleolar alterations characteristic of tumor cells serve as a vivid testament to the complex interplay of genetic, epigenetic, and proteomic influences. Deciphering this intricate web is vital for developing therapeutic and diagnostic strategies targeting disease causation's foundation. As scientific inquiry continues to shed light on the previously obscure realms of nucleolar biology, it paves the way for groundbreaking advancements in cancer treatment and diagnosis, heralding a future where therapies are more effective and finely tuned to each patient's unique molecular profile. The ongoing exploration of nucleolar dynamics offers hope in the relentless fight against cancer, underscoring the potential for transformative breakthroughs in personalized medicine.[101–103]

2.2 Rb and p53 influence the nucleolar function

The intricate dance of life within each cell is masterfully orchestrated by a cadre of molecular regulators, with the retinoblastoma protein (pRb) and p53 as central figures in this complex choreography. Central to the cell cycle's progression, pRb acts as a vigilant sentinel at the G1 phase's juncture. With meticulous precision, it evaluates whether the cell is sufficiently prepared for the rigorous process of DNA replication, ensuring that no premature steps disrupt the orderly progression of the cell cycle.[104,105] This crucial function of pRb is paramount in preserving the accuracy and integrity of cell division, much like a disciplined conductor leading a symphony of cellular events with unwavering precision.

Conversely, p53, revered as the guardian of the genome, vigilantly monitors the cellular environment for any signs of distress or damage.[79] Upon detecting DNA lesions or other stress signals, p53 rapidly activates a protective response. This can involve inducing programmed cell death (apoptosis) to eliminate cells bearing irreparable damage or arresting the cell cycle at the G2 checkpoint, thereby preventing the transmission of defective genetic material to daughter cells and safeguarding the fidelity of gene inheritance.[57,106–109] In essence, p53 functions as a crucial firewall, averting the propagation of genetic errors that could otherwise lead to oncogenesis. The lack of functional PTEN, or inappropriate activation of PI3K–AKT will ring from downstream target MDM2 of PTEN, which will decrease p53 activity and disable cancer cells to make a proper response to DNA damage[110] (Fig. 2).

Beyond their quintessential roles in orchestrating cell cycle checkpoints, pRb and p53 go into the nucleolus, where

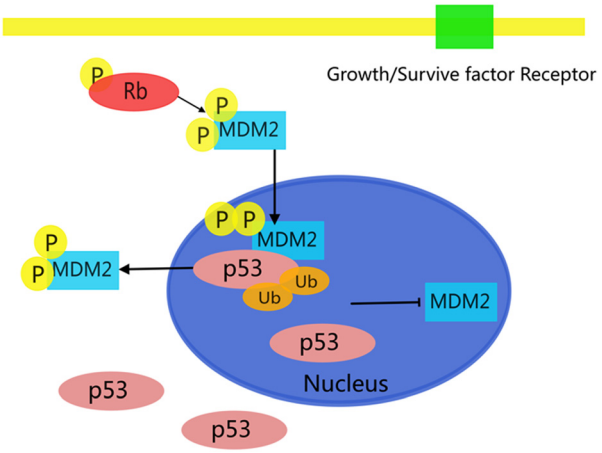

Fig. 2 Rb-driven entry of MDM2 into the nucleus prevents transcriptional activation of p53 and promotes p53 degradation.

the ribosomes, the intricate machinery of protein synthesis, are meticulously assembled.[111–114] Here, they take on a new role as transcriptional repressors, modulating the expression of ribosomal genes. pRb accomplishes this by engaging in a molecular tango with the upstream binding factor UBF,[115] while p53 interacts with the selectivity factor SL1,[116] both of which are integral components of the UBF–SL1 complex that recruits RNA polymerase I to the ribosomal RNA (rRNA) gene promoter.[117–119] By deftly hindering the formation of this vital complex, pRb and p53 can dampen rRNA transcription, exerting a powerful regulatory influence over ribosome biogenesis and protein synthesis—fundamental processes that are the bedrock of cell growth and proliferation.

However, pRb and p53 are frequently compromised in human malignancies, and their dysfunction dismantles the safeguards against uncontrolled cell division. It accelerates ribosomal RNA transcription, fueling the insatiable biosynthetic appetite of cancer cells and fostering their malignant transformation.[120–125] This impairment releases the brakes on cellular proliferation and leads to hyperactive rRNA transcription, fueling the heightened biosynthetic requirements of cancer cells and contributing to their malignant transformation.[120,123–125] This dysfunction manifests in an enlarged nucleolus, a telltale sign of the tumor suppressors' compromised status and a diagnostic clue to the presence of cancer.[126,127] The nucleolus's size thus becomes a diagnostic biomarker, revealing a disturbance in the cellular balance that often precedes a more aggressive cancer phenotype.

Unraveling the intricate interplay between nucleolar function, tumor suppressor proteins, and cancer progression is paramount. It enriches our understanding of the disease's molecular underpinnings and paves the way for the development of innovative diagnostic tools and therapeutic strategies. By manipulating the activities of pRb, p53, or their downstream effectors within the nucleolus, scientists aim to recalibrate the disrupted balance between cell cycle control and ribosome

biogenesis, inhibiting cancer's relentless progression.[128,129] This targeted approach represents a promising frontier in cancer therapy, offering the potential to intervene at the heart of the disease's machinery.

Furthermore, exploring the nucleolar dynamics associated with the dysfunction of pRb and p53 offers profound insights into broader biological themes such as cellular stress responses, senescence, and aging. This research frames the nucleolus as an active contributor rather than a passive observer within the complex network of signaling pathways that maintain cellular homeostasis.[57,130] This perspective accentuates the pivotal role of the nucleolus in preserving the delicate balance within cells. It underscores its significance as a central hub for elucidating and addressing the multifaceted nature of cancer.

As research in this field progresses, each discovery adds a piece to the puzzle, gradually elucidating the complex interplay between nucleolar function, tumor suppressors, and cancer biology. These advancements bring us closer to realizing the ambitious goal of transforming cancer from an ominous foe into a manageable and potentially curable condition through the application of molecular biology's vast arsenal. The ongoing exploration of the nucleolus and its regulators is not merely a scientific pursuit; it is a quest driven by the hope of alleviating human suffering and reclaiming lives from the clutches of this relentless disease. In this journey, every new insight illuminates a path toward a future where cancer is no longer a death sentence but a manageable chapter in the story of human health and resilience.

3 Nucleolus methods for tumor diagnosis

3.1 Transmission electron microscopy (TEM)

As a high-resolution imaging technique, TEM can observe the ultrastructural changes of the nucleolus.[131] The morphological abnormalities of tumor nucleoli can be directly observed through TEM, making it a valuable tool for tumor diagnosis (Fig. 3).[69]

3.1.1 Advantages of TEM in nucleolus research. Compared with optical microscopy, TEM has the following significant advantages: TEM displays supremely high resolution, allowing clear observation of the internal structure of the nucleolus and its subtle changes.[132] For example, using TEM, the formation of nano-ions approximately 100 nm in size (combined with linalool (SC) and doxorubicin (DOX)) can be clearly observed in U87 glioblastoma cells.[133] Their structures are distinctly visible in the nucleolus and mitochondrial membrane.[133] TEM reveals the fine structure of different regions in the nucleolus, including the specific morphology and distribution of FC, DFC, and GC.[134] These technical advantages make TEM an essential tool for studying the nucleolus's ultrastructure and diagnosing tumors.

3.1.2 Applications of nucleolus observation in tumor diagnosis by TEM. Observing the ultrastructural changes of nucleoli using TEM has been applied in diagnosing various tumors. For example, mesotheliomas with pleomorphic features are rare, and only a few studies on this mesothelioma variant have been published. The ultrastructure of the patient's tissue cells was observed using TEM. Mesotheliomas with pleomorphic features are characterized by large, often discohesive epithelioid cells that vary in size and shape. These cells have abundant dense eosinophilic cytoplasm and contain single or multiple irregular nuclei, often with one or several large nucleoli.[135]

Clinically, acute monocytic leukemia (AML-M5) can be classified into four subtypes: typical monoblast (TMB), atypical monoblast (AMB), atypical promonocyte (APM), and typical promonocyte (TPM).[136] The TMB subtype is poorly differentiated, while the TPM subtype is well differentiated. AMB and APM exhibit an intermediate level of differentiation. These differences in differentiation are characterized by features such as the larger size of AMB compared to TMB; numerous vacuoles and granules in TPM and APM compared to TMB and AMB; and larger nuclei and nucleoli in AMB and APM compared to TMB and TPM. These characteristics illustrate consecutive differentiation stages, aiding in the precise definition of monoblasts and promonocytes and refining the light microscopy criteria in M5.[136]

A study compared the ultrastructure of chronic gastritis, gastric cancer, and gastric precancer using TEM. Clinically, only a few chronic gastritis patients' gastric mucosal epithelial cells are likely to develop into cancer cells. Significant differences were found between CG and GC in terms of ultrastructure and molecular biology. Nuclear chromatins were either scattered, associated with nucleoli, or aligned along nuclear perimeters. The light-bright zones between heterochromatins in the nucleoli are euchromatins. The nucleoli exhibited high electron density without a capsule, and features such as nucleolar margination, multinucleoli, and nucleolar division were observed.[137]

3.1.3 Disadvantages of TEM in nucleolus research. TEM has achieved remarkable results in observing nucleolar structures and diagnosing tumors. However, its application still faces some challenges and limitations. For instance, TEM sample preparation is complex, the operation requires high technical skill, and the observation range is limited.

TEM requires ultrathin sectioning of samples, and the thickness of the samples is usually less than 100 nm.[138] This process is complicated and time-consuming, and it is easy to introduce human errors.[139] In addition, the preparation process of ultrathin sections may cause sample deformation or structural damage, thus affecting the accuracy of the results.[140] Since TEM uses high-energy electron beams to image samples, high-energy electron beams may cause damage to biological samples, especially to structures such as nucleoli that are rich in RNA and proteins.[141] Radiation damage from electron beams may change or destroy the structure of nucleoli and affect the observation results.[142] TEM provides only two-dimensional projection images of samples and cannot directly obtain three-dimensional

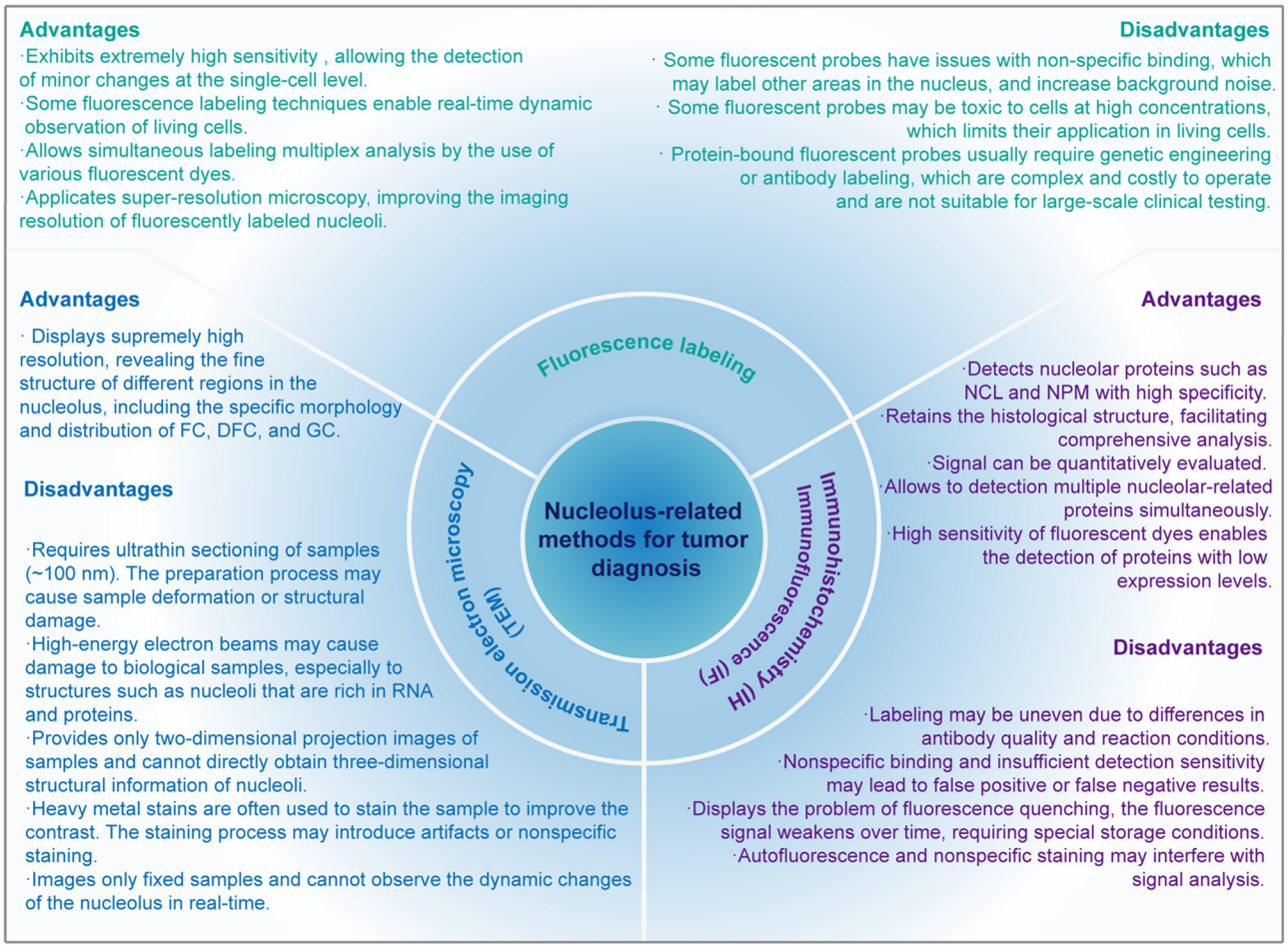

Fig. 3 Advantages and disadvantages of nucleolus methods for tumor diagnosis.

structural information of nucleoli.[143] Although three-dimensional reconstructed images can be obtained by electron tomography (ET) technology, this process requires more time and complex computational processing.[144,145] The nucleolus is an important structure in the cell nucleus, rich in RNA and proteins, and has a low natural contrast.[146] In order to improve the contrast, heavy metal stains (such as uranium, lead, *etc.*) are often used to stain the sample.[147] However, the staining process may introduce artifacts or nonspecific staining, thus affecting the observation results.[147] Although the resolution of TEM is very high, reaching the sub-nanometer level, the resolution may still not be enough to reveal the details of certain tiny structures when observing large-area samples.[148] In addition, TEM can only image fixed samples and cannot observe the dynamic changes of the nucleolus in real time.[149] The dynamic changes of the nucleolus during the cell cycle are very important for understanding its function and mechanism, which is difficult to achieve in TEM.

3.2 Immunohistochemistry (IHC)/immunofluorescence (IF)

IHC/IF have become core tools for tumor diagnosis.[150–152] Both immunohistochemistry and immunofluorescence are techniques that locate and quantify specific proteins in tissue sections through color development reactions based on antigen–antibody reactions.[153–158] IHC plays an important role in pathological diagnosis, especially in the classification and grading of tumors.[159] IF uses fluorescent dyes to label antibodies and observe the location and expression levels of markers through fluorescence microscopy.[160] IF provides high-resolution protein distribution maps at the cellular and tissue levels and is widely used in basic research and clinical diagnosis (Fig. 3).

When combined with other diagnostic methods such as gene sequencing and liquid biopsy, they could form a comprehensive diagnostic system to improve the accuracy and efficiency of diagnosis. Labeling nucleoli by IHC and IF can not only help diagnose tumors but also provide detailed molecular information about tumor cells, serving as a basis for the formulation of personalized treatment plans. For example, the expression levels of different nucleolar markers can indicate a patient's response to certain chemotherapy drugs or targeted therapies.[161]

3.2.1 Advantages of nucleolus labeling by IHC/IF. IHC can detect nucleolar proteins such as NCL and NPM with high specificity.[162] It retains the histological structure, facilitating

comprehensive analysis.[163] With the help of image analysis software, the labeling signal can be quantitatively evaluated.[164] IF can detect multiple nucleolar-related proteins simultaneously, distinguishing them by fluorescent signals of different colors.[27,165] Modified IF allows real-time observation of nucleolus changes during the cell cycle.[166] The high sensitivity of fluorescent dyes enables the detection of proteins with low expression levels.

3.2.2 Applications of nucleolus labeling by IHC/IF in tumor diagnosis. In breast cancer diagnosis, IHC is used to detect markers such as HER2, ER, and PR. Studies have found that overexpression of NCL is associated with the aggressiveness and poor prognosis of breast cancer. Labeling NCL by IHC can assist in assessing the malignancy of breast cancer.[167,168] NOLC1 is involved in nucleolar biosynthesis and has numerous phosphorylation sites, predominantly phosphorylated by casein kinase 2 (CK-2).[169] When the subcellular localization of NOLC1 was examined in human breast cancer MCF-7 cells, IF of NOLC1 was observed in the intracellular region.[169] Additionally, NOLC1 expression in the nucleoli increased after treatment with the anticancer drug doxorubicin.[169]

In prostate cancer, enlargement of the nucleolus is the key diagnostic feature of high-grade prostatic intraepithelial neoplasia (PIN), an early stage that appears to be the precursor to the majority of invasive prostate cancers. Several cancer genes implicated in PIN are known to augment ribosome production, including c-Myc, p27, retinoblastoma, p53, and growth factors that impact ERK signaling.[170] p120 nucleolar protein has indeed been used in immunohistochemical analysis, showing significantly elevated expression levels in prostate cancer. Moreover, p120 nucleolar protein is closely associated with cell proliferation, and its expression typically reflects the proliferative activity of cells.[171]

In lung cancer research, IHC/IF labeling of NPM and NCL can clarify nucleolar structural changes and functional abnormalities.[172–174] NPM1 overexpression correlates with ^{18}F-FDG PET/CT metabolic parameters and improves diagnostic accuracy in lung adenocarcinoma.[175] IHC on TMAs from 92 NSCLC samples and 42 non-cancerous lung tissues showed high nucleolin and phosphorylation of nucleolin (P-nucleolin) expression in the cytoplasm and nucleus of NSCLC tissues, while nucleolin was primarily nuclear and P-nucleolin was low in non-cancerous tissues.[176]

A proliferating cell nuclear antigen (PCNA) was detected in the nucleoli of human cell lines, including HeLa, Hep-2, and Namalwa, as well as in solid tumors from human renal and prostate carcinomas. Both strong and weak nucleolar fluorescence signals were observed in the renal and prostate carcinomas, indicating varying levels of proliferation among the tumor cells. Two human colon carcinoma cell lines with different growth rates were compared: Ω, an aggressive, fast-growing clone of the HCT 116 cell line, and CBS, a slower-growing cell line (group 3). The fast-growing Ω cells showed a higher percentage of nucleolar fluorescence (28.5%) compared to the slower-growing CBS cells (13.6%).[177]

3.2.3 Disadvantages of nucleolus labeling by IHC/IF. Due to differences in antibody quality and reaction conditions, IHC/IF labeling may be uneven, affecting the accuracy of the results.[178] Additionally, nonspecific binding and insufficient detection sensitivity may lead to false positive or false negative results.[178] IF has the problem of fluorescence quenching, and the fluorescence signal weakens over time, requiring special storage conditions. Furthermore, autofluorescence and nonspecific staining may interfere with signal analysis.

3.3 Fluorescence labeling techniques

Fluorescence labeling techniques have shown significant potential in tumor diagnosis, particularly in the study of nucleoli.[179] By utilizing these techniques, changes in nucleoli can be detected efficiently and accurately, providing crucial information for the diagnosis and treatment of tumors. Fluorescence labeling employs fluorescent dyes or fluorescent proteins to label nucleoli, allowing them to be clearly observed and analyzed under a microscope.[179] This offers an effective means for tumor diagnosis. Immunofluorescence has already been introduced in the previous section, so it will not be covered here. Instead, this section focuses on fluorescent probe labeling. Some fluorescent probe technologies can monitor the dynamic changes of nucleoli in real-time within living cells by labeling nucleoli, offering a novel approach for the early-stage diagnosis and treatment of tumors (Fig. 3). The markers used in fluorescence labeling methods can be DNA, RNA, or proteins. Since 2016, there has been a significant increase in the number of small-molecule fluorescent probes targeting nucleoli in published papers, with those targeting RNA being the most prevalent (Fig. 4).

3.3.1 DNA-binding fluorescent probes. Probes that directly target nucleolar DNA are rarely studied. DNA-binding fluorescent probes achieve nucleolus labeling by specifically binding to DNA sequences related to the nucleolus region.[180] These probes have unique advantages in identifying DNA

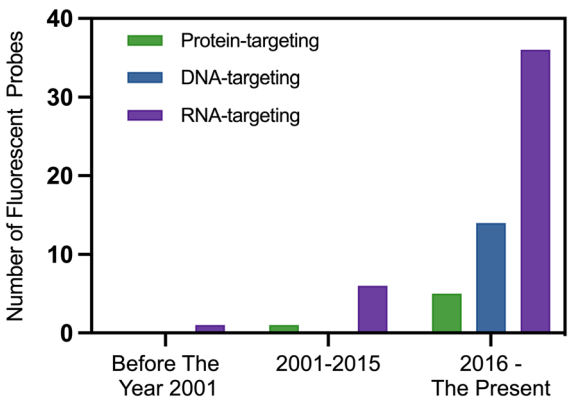

Fig. 4 Comparison of the number of small-molecule probes targeting nucleolar RNA, DNA, and proteins reported in the literature over the years. Statistics from Pubmed (**https://www.ncbi.nlm.nih.gov/**) published literature.

sequences within the nucleolus. The fluorescent probe PCV-1 has high sensitivity to mitochondrial membrane potential and high affinity for DNA, and its migration from mitochondria to nucleoli during cell damage can be dynamically visualized at the single-cell level. PCV-1 is the first probe that allows visualization of cell death and cell damage under super-resolution imaging and has the potential for diagnostic applications.[181]

3.3.2 RNA-binding fluorescent probes. RNA-binding fluorescent probes mainly achieve labeling of nucleoli by binding to rRNA enriched in nucleoli. This type of probe can specifically identify rRNA molecules. SYTO RNASelect is an RNA-specific fluorescent dye that can efficiently label RNA in some types of living cells, such as primary bovine turbinate cells.[182,183] This type of probe has high affinity and fluorescence intensity and is suitable for dynamic monitoring in some types of living cells. However, in practical applications, this probe may also label other RNA molecules in the cytoplasm, resulting in high background signals. Furthermore, SYTO RNASelect cannot label all types of living cells.[184] Eosin Y is a fluorescent dye commonly used for staining tissue sections and can label nucleoli by binding to RNA. It has high fluorescence intensity and is suitable for fluorescence microscopy observation, but it is less commonly used for live cell labeling. Eosin Y is usually used for staining fixed cells and tissues, and its application in living cells needs further exploration.[185]

Naphthalimide derivatives (NI-1 to NI-5) penetrate both the cell membrane and nuclear membrane, achieving clear nucleolar staining in live cells (Fig. 5). The presence of amino groups on the side chains of the naphthalimide backbone enhances their targeting specificity to the nucleolus. Molecular docking results indicate that NI-1 to NI-5 form hydrogen bonds and hydrophobic interactions with RNA, resulting in fluorescence enhancement upon RNA binding. These findings provide valuable support for the future diagnosis and treatment of nucleolar-related diseases.[186]

3.3.3 Protein-binding fluorescent probes. Protein-binding fluorescent probes can label nucleoli by binding to specific proteins in the nucleolus. This type of probe can specifically identify nucleolar proteins. UNC6934 is a chemical probe that targets the N-terminal PWWP (PWWP1) domain of NSD2. UNC6934 occupies the canonical H3K36me2-binding pocket of PWWP1, antagonizes the interaction of PWWP1 with nucleosomal H3K36me2, and selectively engages endogenous NSD2 in cells. UNC6934 induces the accumulation of endogenous NSD2 in the nucleolus, mimicking the localization defects seen in NSD2 protein isoforms lacking PWWP1, which result from translocations prevalent in multiple myeloma (MM). Mutations in other NSD2 chromatin reader domains also increase NSD2 nucleolar localization and enhance the effect of UNC6934.[187]

A novel chaperone@DNA molecular tool, the phenylboronic acid-modified avidin conjugated with an abasic site-containing DNA probe (PB-ACP), has been developed for the real-time observation of APE1 in the nucleus and nucleolus of living cells (Fig. 6). The phenylboronic acid-modified avidin not only serves as a chaperone to protect the AP-DNA from nonspecific degradation but also facilitates the targeted delivery of the probe to the nucleus. PB-ACP shows high specificity and sensitivity to APE1 due to the strong binding affinity of APE1 to both avidin and the AP site in DNA. The probe efficiently migrates from the cytoplasm to the nucleus, specifically displaying the distribution and *in vivo* activity of endogenous nuclear APE1. This tool offers a powerful method to investigate the cellular behavior of APE1 in living cells and improve cancer therapies targeting APE1.[188] These fluorescent probes targeting nucleolar proteins provide valuable support for the diagnosis of tumors.

3.3.4 Advantages of fluorescence labeling. Fluorescence labeling techniques exhibit extremely high sensitivity, allowing the detection of minor changes at the single-cell level.[189] Some fluorescence labeling techniques enable real-time dynamic observation of living cells.[190–194] Real-time dynamic observation aids in understanding tumor cell growth patterns and provides a basis for tumor diagnosis and treatment.[195,196] The use of various fluorescent dyes in different colors allows simultaneous labeling of multiple cellular structures for multiplex analysis.[197] The advancement of fluorescence microscopy technology, especially the application of super-resolution microscopy, has significantly improved the imaging resolution of fluorescently labeled

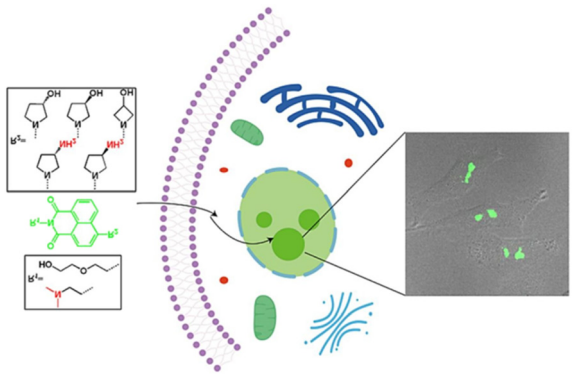

Fig. 5 Nucleolus imaging based on naphthalimide derivatives. Ref. 186 (Copyright is used with permission).

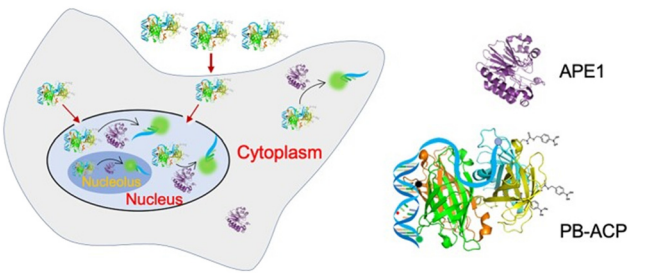

Fig. 6 Visualization of APE1 in the nucleus and nucleolus using the PB-ACP-based chaperone@DNA probe. Ref. 188 (Copyright is used with permission).

nucleoli.[198,199] Structural changes within the nucleolus can now be observed with greater clarity, providing more detailed information for the early-stage diagnosis of tumors.

3.3.5 Applications of nucleolus labeling by fluorescence labeling in tumor diagnosis. Nitrogen doped carbon dots (NCD) are used for nucleolar fluorescence imaging related to biological changes in cancer cells. Compared to the AgNOR method, the NCD method is faster and more sensitive in predicting tumor prognosis. The NCD method can accurately (detection limit: 50 nM) and quickly (within 5 minutes) assess cancer prognosis at the suborganelle level based on nucleolar characteristics. The NCD method visualizes and analyzes the results through captured fluorescence images. Due to the effects of chemotherapy, the nucleolar size in the treatment group is smaller than in the control group. The NCD fluorescence imaging results are similar to the changes measured by the AgNOR method, indicating that the NCD method evaluating tumor prognosis by nucleolar size is feasible and effective.[200]

Green-emitting carbon dots (m-CDs) have a significantly higher affinity for nucleolar RNA. Visualizing nucleoli in cells using m-CDs can accurately determine their number and morphology, distinguishing cancerous cells from normal cells (Fig. 7). Additionally, m-CDs can be used to monitor nucleolar dynamics during the apoptosis of malignant cells induced by DOX. m-CDs show great potential as nucleolar probes, with promising applications in cancer cell screening and therapeutic efficacy evaluation.[179]

3.3.6 Disadvantages of nucleolus labeling by fluorescent probes. Some fluorescent probes (such as Hoechst dye and DAPI) have issues with non-specific binding, which may label other areas in the nucleus, increase background noise, and affect the accuracy of labeling. Non-specific binding not only interferes with the detection of target signals but may also lead to misdiagnosis and missed diagnosis. Additionally, some fluorescent probes (such as SYTO RNASelect) may be toxic to cells at high concentrations, affecting the normal function and growth of cells, which limits their application in living cells and cannot label all types of living cells. Cytotoxicity issues restrict the use of these probes under long-term and high-concentration conditions, necessitating the development of low-toxic or non-toxic alternative probes.

Protein-bound fluorescent probes (such as fibrillarin-GFP fusion protein) usually require genetic engineering or antibody labeling, which are complex and costly to operate and are not suitable for large-scale clinical testing. These complex procedures and high costs limit the widespread application of these probes in clinical practice.[201]

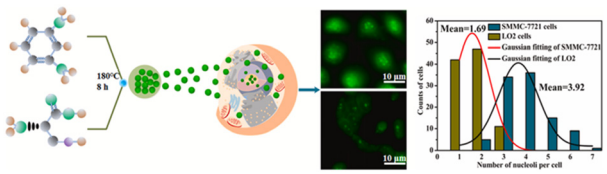

Fig. 7 Schematic illustration of the synthesis of m-CDs and the selective staining of nucleolus. Ref. 179 (Copyright is used with permission).

4 Perspectives and outlook

In the future, the efficiency and accuracy of TEM observations can be improved by enhancing sample preparation methods, allowing for a better revelation of fine structural changes in nucleoli. Developing automated image analysis tools using artificial intelligence and machine learning can increase the efficiency of identifying and analyzing changes in nucleolar ultrastructure. Integrating TEM with other imaging techniques, such as confocal microscopy and scanning electron microscopy, for multimodal imaging can provide more comprehensive cellular ultrastructural information, aiding in the comprehensive diagnosis of tumors. With the development of nanotechnology and molecular biology, new labeling methods such as nanoparticle labeling and the CRISPR/Cas9 system are expected to improve the specificity and sensitivity of labeling, reducing false positive and false negative results for IHC and IF. Low-toxicity fluorescently labeled nucleolar probes have been studied for dynamic observation of nucleoli. Combining fluorescent probes with other imaging techniques (such as super-resolution microscopy) can achieve multimodal and high-resolution imaging of nucleoli, and yield detailed tumor cell information at the single-cell level.[202] Multimodal imaging technology can integrate different types of signals, improving the resolution and information content of imaging to support accurate diagnosis. Combining big data analysis and artificial intelligence can lead to the development of automated analysis tools for fluorescence imaging data, improving diagnostic accuracy and efficiency. By training deep learning models, nucleolar features in fluorescence imaging can be automatically identified and analyzed, providing quantitative analysis results to assist doctors in making diagnostic decisions.

Data availability

All data created or analyzed in this study are available on request from the corresponding author.

Conflicts of interest

The authors declare that they don't have any competing financial interests or personal relationships that could have appeared to influence the work reported in this review.

Acknowledgements

This study was supported by the National Key R&D Program of China (SQ2024YFE0104607; 2019YFE0101200), the Science and Technology Commission Shanghai Municipality (13JC1403400, 18490740200), and the Plan of Jiaxing Innovation and Elites Leading.

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