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Analysis of Research Methods for Heterogeneous Cellular Senescence Biomarkers

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Abstract:

Cellular senescence is a fundamental mechanism underlying organismal aging and the development of numerous age-related diseases. Accurate detection of senescent cells is key for understanding and intervening in the aging process. Molecules such as p16INK4a, p21, and SA-β-gal have been widely used in senescence detection. Traditional SA-β-gal staining is popular for its simplicity, but emerging high-throughput spatial omics techniques, such as seqFISH, now enable high-resolution and dynamic multi-marker tracking of senescent cells. Using multi-omics and spatial approaches, researchers are elucidating the distribution and molecular heterogeneity of senescent cells in tissues. However, existing detection methods still lack in specificity, throughput, and clinical utility, and a universal, standardized high-precision detection system remains elusive. This review systematically analyzes the molecular mechanisms and detection methods for p16INK4a, p21, and SA-β-gal, evaluates technological developments from classical biochemical methods to innovative spatial transcriptomics (ST), discusses the potential of single-cell multi-omics and novel biomarkers for improving detection accuracy and understanding senescence heterogeneity, and summarizes limitations and challenges faced by each approach. The results suggest that integrating multi-marker and spatial omics technology can significantly enhance the accuracy and resolution of senescent cell detection, providing a technical basis for developing innovative anti-aging drugs and disease interventions. This study provides reference for optimizing senescent cell detection and biomarker screening, emphasizing the importance of a standardized multi-omics system. Future directions should focus on improving clinical translatability, exploring molecular regulation and cellular heterogeneity within the senescent microenvironment, and advancing disease diagnosis and healthy aging.

Keywords:- Cellular senescence; senescence biomarkers; spatial transcriptomics; senescence heterogeneity.

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I. Introduction

Cellular senescence refers to a state in which cells are stably arrested in the cell cycle, having lost their ability to proliferate and no longer responding to mitogenic signals, while still maintaining metabolic activity and surviving for extended periods. The accumulation of senescent cells in tissues and organs contributes to the onset of age-related diseases. Therefore, the clearance of senescent cells is crucial for delaying aging and ameliorating age-associated diseases. As a core biomarker of cellular senescence, p16INK4a plays a vital role in elucidating the mechanism of senescence and developing interventions for aging.

The p16ink4a/Rb pathway and the p53/p21 pathway are the two principal routes mediating and maintaining cellular senescence [1]. p16INK4a functions as an inhibitor of CDK4/6, blocking the phosphorylation of RB protein, maintaining the repression of E2F by RB, and enforcing G1 cell cycle arrest. This mechanism constitutes the core of senescence-associated cell cycle arrest. Compared to p21, the expression of p16INK4a is more specifically associated with senescence rather than transient DNA damage responses.

Traditional markers of senescence include senescence-associated β-galactosidase (SA-β-gal), a lysosomal hydrolase encoded by the GLB1 gene. SA-β-gal specifically catalyzes the substrate X-gal to produce a blue product at pH 6.0 (due to weakened lysosomal acidification in senescent cells), whereas normal cell β-gal activity is optimal at pH 4.0 [2]. This pH-dependent enzymatic activity makes SA-β-gal a reliable marker for senescent cells. SA-β-gal staining is considered a gold standard due to its simplicity and direct results (blue granules under microscopy). It is widely used in basic research, disease modeling, drug screening, and studies of anti-aging interventions. However, SA-β-gal staining suffers from low throughput and specificity, potentially resulting in misidentification of non-senescent cells. In recent years, sequential fluorescence in situ hybridization (seqFISH) technology, with its high resolution and multiplex gene detection capability, has become an important tool in senescence research. Nonetheless, due to limitations in optical resolution and high cellular transcript density, seqFISH also faces challenges such as limited coverage and complex data processing.

This article focuses on optimizing seqFISH technology with respect to p16INK4a, aiming to overcome the low specificity and throughput limitations of traditional SA-β-gal staining. Ultimately, this enables high-precision detection and dynamic tracking of senescent cells, providing insights into senescence mechanisms and targeted clearance of senescent cells.

II. Principles of Biomarkers

A. P16 and P21

On a molecular level, cellular senescence is mainly achieved through two key pathways mediated by p16INK4a and p21, respectively [1]. Though both proteins induce cell cycle arrest, they differ significantly in their regulatory mechanisms, functional characteristics, and pathological implications.

P16, encoded by the CDKN2A gene, is a central regulator of stress-induced premature senescence (SIPS). By specifically inhibiting CDK4/6 kinase activity, p16 blocks Cyclin D–mediated phosphorylation of Rb, maintains Rb in its active form, suppresses the release of E2F transcription factors, and ultimately induces G1 arrest [3]. p16 activation is typically triggered by DNA damage, oncogenic signals (e.g., Ras mutations), or chronic inflammation (e.g., via the NF-κB pathway). Its sustained elevation in senescent cells is an important driver of the senescence-associated secretory phenotype (SASP) [4]. In cancer, loss or epigenetic silencing of p16 (such as promoter methylation) is common in various tumors (e.g., melanoma, glioma), making it a significant tumor suppressor. Serrano et al. demonstrated that, following induction of oncogenic K-Ras in mouse lung, pancreas, or skin, knock-out of p16INK4a circumvents senescence, leading to rapid progression to aggressive cancers (e.g., pancreatic ductal adenocarcinoma or lung cancer) [3] .

By contrast, p21, encoded by the CDKN1A gene, predominately participates in telomere-dependent replicative senescence. Its expression is highly dependent on p53: telomere shortening or DNA damage activates p53, which upregulates p21 and, in turn, broadly inhibits CDK2/4/6 kinase activity to block cell cycle progression. p21 has a dual role: transient expression can promote DNA repair, while sustained high levels result in cellular senescence. Unlike p16, p21 exhibits a transient increase in early senescence and may decline at late stages. Mutations in p21 are less frequent in cancer, but aberrant expression (e.g., overexpression in chemoresistant tumors) may promote tumor survival [5,6]. Abbas et al. showed that in replicative senescent human fibroblasts (e.g., IMR-90), early p21 elevation is accompanied by increased SA-β-gal activity and p53 phosphorylation [5].

Despite distinct pathways, p16 and p21 can act synergistically or antagonistically in some contexts. For example, in p53-deficient cells, p16 may be upregulated to maintain senescence; in precancerous lesions, excessive p21 may delay p16-mediated senescence and promote malignant transformation. The elucidation of these mechanisms supports the development of anti-aging drugs (e.g., senolyt-

ics) and cancer therapies (e.g., CDK4/6 inhibitors) [7].

B. SA-β-gal

Senescence-associated β-galactosidase (SA-β-gal) is one of the most widely used biomarkers of cellular senescence. It exhibits optimal enzymatic activity at pH 6.0, differing from conventional lysosomal β-galactosidase (pH optimum at 4.0), thus serving as a key indicator of senescent cells [2]. Elevated SA-β-gal activity is closely linked to the increased number and altered function of lysosomes in senescent cells, reflecting metabolic remodeling during senescence [8]. It is reported that activation of SA-β-gal is associated with the p16/Rb and p53/p21 pathways, and its levels rise during both replicative and stress-induced senescence [1].

In disease research, SA-β-gal–positive cells are markedly increased in various age-related conditions (e.g., atherosclerosis, osteoarthritis, pulmonary fibrosis) and in the tumor microenvironment, suggesting a role in disease development [9]. Notably, SA-β-gal–positive cells often display the SASP, secreting proinflammatory cytokines and matrix remodeling proteins that impact the microenvironment and nearby cells.

Nevertheless, SA-β-gal as a marker has limitations. Some terminally differentiated cells (e.g., macrophages) or those in special physiological states may also be SA-β-gal–positive, potentially causing false positives. Therefore, combined use with other markers (e.g., p16 expression, lamin B1 loss) is recommended [10]. Moreover, the exact biological function of SA-β-gal in the senescence process remains unclear—it is not yet determined whether it is a driver or merely a bystander in senescence, requiring further investigation.

Recent advances include the development of novel fluorescent probes (e.g., C12FDG) that improve detection sensitivity and specificity, and the combination of single-cell RNA-seq (scRNA-seq) with SA-β-gal staining for in-depth analysis of senescent cell heterogeneity [11].

In the future, research should further clarify the molecular mechanisms of SA-β-gal in senescence, develop more specific detection methods, and explore its clinical diagnostic and therapeutic value. With advancing knowledge, SA-β-gal will play an increasingly important role in both basic research and clinical applications.

III. Current Research Methods

A. Staining Methods

The principle of SA-β-gal staining is based on characteristic alterations in lysosomal function in senescent cells. The key difference is a shift in the optimal pH of β-galactosidase from 4.0 in normal cells to 6.0 in senescent cells. This pH-dependent activity allows senescent cells to specifically hydrolyze the X-gal substrate, generating an insoluble blue precipitate under weakly acidic conditions (pH 6.0), whereas normal cells display almost no activity under these conditions [2]. Mechanistically, this is closely related to changes in lysosomal membrane stability, increased lysosome numbers, and upregulation of GLB1 expression [1].

Typically, cells are fixed using a mixture of 2% formaldehyde and 0.2% glutaraldehyde, which preserves morphology and maximizes enzyme activity. Samples are incubated in X-gal–containing staining solution (pH 6.0) at 37°C without CO2 for 12–16 hours, then examined for blue precipitates by optical microscopy [11]. Note that while SA-β-gal activity is considered a reliable marker, its levels do not always correlate linearly with the degree of senescence, and false positives may occur in some premature senescence models .

In clinical research, SA-β-gal staining is used to analyze the distribution of senescent cells in diseases such as atherosclerosis and osteoarthritis. For example, up to 40% of cells in atherosclerotic plaques may be SA-β-gal–positive, with a positive correlation to disease severity [9]. With advances in single-cell technology, SA-β-gal staining is now often applied in combination with other markers (e.g., p16INK4a, γ-H2AX), offering new insights into the heterogeneity of senescence. However, practical challenges persist, including lack of standardized tissue processing and subjective interpretation of results [10], limiting its broader application in precision medicine.

B. In Situ Hybridization Technologies

Sequential fluorescence in situ hybridization (seqFISH) is a high-throughput, super-resolution RNA imaging technology that enables spatial localization and quantitative analysis of thousands of RNA species at single-cell and tissue levels. Its core principle employs combinatorial barcoding strategies, where target RNAs are repeatedly labeled and identified through cycles of hybridization and fluorophore removal [12]. For p16INK4a detection, specific probes (each containing 20–50 oligonucleotide pairs with unique fluorescent barcodes) are designed for p16INK4a mRNA. Each hybridization round uses different labeled probe combinations, and high-resolution microscopy is used to record fluorescent signatures. Fluorophores are then chemically or enzymatically removed, and subsequent rounds allow for signal stripping and reimaging. The barcode sequences are decoded to achieve precise recognition and localization of p16INK4a mRNA [12].

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seqFISH has revolutionized senescence research. Traditional methods such as immunohistochemistry or conventional FISH provide limited information, while seqFISH allows detection of p16INK4a at the single-molecule level in situ, revealing its spatial distribution [12]. For instance, in studies of the cerebellum, seqFISH has mapped the precise distribution of p16INK4a–positive cells, demonstrating that these cells tend to cluster in specific areas rather than being randomly distributed [13]. More importantly, concurrent detection of other senescence markers (e.g., p21, SA-β-gal) by seqFISH has revealed the presence of different senescent cell subpopulations, potentially with unique functions or pathological significance.

Looking forward, seqFISH for p16INK4a detection has significant developmental prospects. Combining novel probe designs with higher-sensitivity imaging systems may further improve detection efficiency and accuracy. Integrating seqFISH with scRNA-seq and other techniques provides comprehensive insights into the regulatory mechanisms of p16INK4a expression. Overall, as a powerful ST tool, seqFISH is paving new paths for senescence research, with continued development promising further insights into the precise role of p16INK4a in the aging process.

C. RNA-seq

scRNA-seq and single-nucleus RNA-seq (snRNA-seq) are powerful methods that dissociate complex cellular populations into individual cells, assigning unique cellular barcodes to each. High-throughput sequencing then yields transcriptomic or genomic data for each cell, making these techniques major tools for investigating the heterogeneity of senescence. They enable molecular-level interrogation of individual cells and characterization of their transcriptomic features [14]. Hernandez-Segura et al. utilized scRNA-seq to profile high expression of SASP factors such as IL-6 and MMP3 in senescent fibroblasts [10]. scRNA-seq is the most effective approach for single-cell transcriptome mapping, but increased cell size and fragility can limit sequencing depth and hinder detection of large or fragile cells [14]. snRNA-seq can access all cell types, but lower mRNA abundance in nuclei may reduce sensitivity. In contrast, ST utilizes RNA sequencing to deeply analyze spatially restricted cell clusters within tissue slices while preserving structural integrity. Currently, ST resolution is limited to 55 μm (Table 1), but future improvements are expected to yield single-cell resolution and comprehensive cell-type coverage, capabilities that surpass existing scRNA-seq and snRNA-seq methods. Thousands of transcripts can be detected simultaneously, providing opportunities to identify novel senescence-specific markers. However, present ST technology suffers from relatively shallow sequencing depth, covering only 50–60% of transcripts [15].

IV. Development of Emerging Biomarkers

Advances in single-cell sequencing, proteomics, and epigenomics have led to the discovery of senescence biomarkers beyond p16INK4a, p21CIP1, and SA-β-gal. These emerging markers reflect various complex aspects of senescent cells and provide more precise molecular targets for research and intervention.

On the epigenetic level, atypical senescence-associated heterochromatin foci (aSAHF) are enriched in H3K9me3 modifications, associated with LINE-1 element silencing. Schleich et al., studying a murine B-cell lymphoma model with in situ analysis and single-cell RNA-seq, observed high H3K9me3 accumulation in senescent cells. Manipulation of H3K9me3 regulators (e.g., SUV39H1/2) significantly affects tumor cell senescence, proliferation, and survival [16]. On the transcriptomic level, downregulation of circular RNA circCCNB1, accumulation of TERRA, and upregulation of miR-146a-5p have been shown to participate in senescence regulation [17]. Proteomics studies have found aberrant enrichment of the lysosomal membrane protein LGALS3 and a marked increase in oxidative modification of mitochondrial protein PARK7. Metabolically, α-ketoglutarate (α-KG) depletion and NAD+/NADH imbalance are key indicators. At the subcellular level, increased mitochondria-lysosome contacts (MLC) and abnormally enlarged lipid droplets (>2 μm) have diagnostic significance. In terms of extracellular vesicles, CD63+PD-L1+ exosomes and miR-21-5p–carrying SASPosomes are proposed as markers of senescence propagation.

The discovery of these new biomarkers has greatly expanded our understanding of senescent cell complexity, but translational challenges remain. Marker tissue specificity (e.g., SERPINE1 in liver senescence cells, MAP2 in neurons) necessitates context-specific detection schemes. Dynamic changes (e.g., early high CDKN1A, later CDKN2A expression) indicate a need for temporally resolved monitoring. Technically, multiplexed error-robust FISH (MERFISH) combined with AI image analysis is expected to realize multi-marker colocalization, while mass cytometry (CyTOF) can simultaneously detect dozens of protein markers.

V. Conclusion

This article systematically reviews mainstream cellular

senescence biomarkers (such as p16INK4a, p21, and SA-β-gal) and detection methods, providing detailed analysis from molecular mechanisms and detection principles to technical innovations. It is emphasized that precise detection of p16INK4a is conducive to understanding aging mechanisms and guiding disease intervention. While conventional SA-β-gal staining is widely used, it is limited in specificity and throughput. Newer technologies such as seqFISH enable high-resolution, dynamic tracking of senescent cells at single-cell and tissue levels. With advances in proteomics and epigenomics, novel biomarkers are continually discovered, advancing research on senescence heterogeneity and cell subpopulation function. The integration of multiple methods and spatial omics technologies is highlighted as the key to improving the accuracy of senescent cell detection, aiding the development of innovative senolytic drugs and disease intervention strategies. However, current technologies remain restricted by resolution, data processing, and clinical translatability. Looking ahead, establishing multi-omics biomarker databases, enhancing standardization, and leveraging artificial intelligence to probe the senescent microenvironment will greatly facilitate precise diagnosis and intervention of aging-related diseases.

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