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Review Article

Use of Saliva Biomarkers and DNA Epigenetics in Forensic Identification: A Narrative Review

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ABSTRACT

Forensic identification plays a pivotal role in criminal investigations and mass disaster scenarios. Traditional methods, such as dental records, fingerprints, and DNA profiling using short tandem repeats (STRs), have limitations in cases involving degraded samples, partial remains, or the need for investigative leads like age estimation. This review explores the emerging potential of saliva as a non-invasive, information-rich biological fluid and DNA epigenetics as a transformative tool in forensic science. Saliva is easily collectible and contains a diverse array of biomarkers, including genomic DNA, proteins, microbes, and cell-free DNA. Concurrently, DNA methylation, a stable epigenetic mark, provides a novel layer of information beyond the genetic code. This narrative review synthesizes current literature on the application of salivary biomarkers for body fluid identification, sex determination, and recent advances in age estimation. It further delves into the principles of DNA methylation, highlighting its forensic utility for constructing epigenetic clocks to predict chronological age and for differentiating body fluids through tissue-specific methylation patterns. The integration of salivary analysis with epigenetic profiling presents a powerful, complementary approach to traditional forensics. Key findings indicate that while these methodologies show immense promise, challenges regarding standardization, validation, and the influence of confounding factors remain. Future research should focus on developing robust, portable assays and expanding reference databases to facilitate the routine adoption of these tools in forensic practice.

KEYWORDS

• Saliva • Forensic Identification • Biomarkers • DNA Methylation • Epigenetics • Human Identification • Body Fluid Identification.

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INTRODUCTION

Accurate and rapid human identification is a cornerstone of forensic science, essential for criminal justice, resolving missing persons cases, and identifying victims of mass disasters.[1] For decades, the field has relied on conventional techniques. Dental record comparison and fingerprint analysis are established methods but require pre-existing records and intact physical structures.[2] DNA profiling, particularly using autosomal Short Tandem Repeats (STRs), represents the gold standard for individualization due to its high discriminatory power.[3] However, this method has inherent limitations. It struggles with highly degraded DNA, mixed samples, and, crucially, it cannot provide investigative leads about a donor's physical characteristics or age unless compared directly to a reference profile in a database.[4]

These limitations have catalyzed the search for novel biomarkers and analytical approaches. Saliva has emerged as a forensically relevant biological fluid, frequently encountered at crime scenes on items like cigarette butts, envelopes, and drinking containers.[5] Its collection is non-invasive, it yields abundant DNA from buccal epithelial cells and leukocytes, and its composition includes a rich repertoire of proteomic and epigenetic markers that can offer additional intelligence.[6]

Epigenetics, the study of heritable changes in gene expression that do not alter the DNA sequence itself, provides a new dimension for forensic analysis.[7] DNA methylation, the addition of a methyl group to cytosine bases in CpG dinucleotides, is the most studied epigenetic mechanism in forensics. These patterns are stable, tissue-specific, and change predictably with age, making them ideal forensic markers.[8] The purpose of this narrative review is to comprehensively explore the current evidence and potential of integrating saliva-based biomarkers with DNA epigenetic analysis to advance forensic identification. We will examine the composition of saliva, its specific biomarkers, the principles of DNA methylation, and how their confluence can address critical gaps in contemporary forensic practice.[9,10]

Saliva as a Forensic Tool

1. Composition and Characteristics of Saliva

Saliva is a complex mixture secreted by major and minor salivary glands, comprising approximately 99% water and 1% electrolytes, proteins, enzymes, mucins, and cellular components.[11] The cellular fraction primarily consists of exfoliated buccal epithelial cells and leukocytes, which are a robust source of genomic DNA for STR profiling. Saliva also contains cell-free DNA (cfDNA), which is increasingly recognized for its utility in epigenetic and metagenomic analyses.[12] Biochemically, saliva is rich in proteins like α-amylase, mucins, statherin, and histatins, which can serve as specific biomarkers for body fluid identification. Compared to blood, saliva offers easier, non-invasive collection and a lower risk of blood-borne pathogens. While seminal fluid has high DNA content, its presence is context-specific. Saliva's ubiquity at many crime scenes gives it a distinct practical advantage.[13]

2. Collection, Preservation, and Analysis of Saliva Samples

The non-invasive nature of saliva collection allows for easy sampling from suspects and victims using swabs or specialized kits like the Oragene® DNA Self-Collection Kit.[14] However, forensic samples recovered from scenes pose challenges, including contamination with other DNA, degradation due to environmental factors (heat, humidity, UV light), and the presence of PCR inhibitors from food or bacteria.[15] Proper preservation is critical; air-drying swabs and storage at low temperatures can mitigate DNA degradation. Modern forensic collection kits often include stabilizing agents that protect DNA and RNA from nucleases, preserving the sample's integrity for subsequent molecular analyses, including epigenetic assays that require high-quality DNA.[16,17]

Saliva Biomarkers in Forensic Identification

1. Definition and Classification

Salivary biomarkers can be categorized into several classes. Genetic biomarkers include STRs and Single Nucleotide Polymorphisms (SNPs) used for standard DNA profiling and ancestry inference. Proteomic biomarkers encompass specific proteins and enzymes,

with α-amylase being a classical marker for saliva identification.[18] Other proteins like statherin and histatins offer higher specificity. Furthermore, the salivary microbiome, comprising a unique community of bacteria, and the metabolome, the collection of small-molecule metabolites, provide additional layers of individualizing information.[19]

2. Applications in Forensic Science

The primary application of salivary biomarkers is body fluid identification. mRNA profiling can detect saliva-specific transcripts such as HTN3 (for histatin 3) and STATH (for statherin), which are more stable and specific than protein-based assays.[20] For sex determination, the Amelogenin gene, routinely co-amplified in STR kits, can be effectively analyzed from salivary DNA. A rapidly advancing application is age estimation. While proteomic changes have been explored, recent focus has shifted to salivary microRNA (miRNA) expression profiles, which show age-correlated patterns.[21] Research is also exploring the use of salivary metabolite degradation rates as a potential tool for estimating the postmortem interval.[22]

3. Advantages and Limitations

The foremost advantage is the non-invasive and easy collection of saliva, facilitating repeated sampling. It is a rich source of diverse biomarkers. However, limitations include susceptibility to contamination, intra and inter-individual variability in composition due to diet, health, and circadian rhythm, and significant environmental degradation when samples are exposed to the elements.[23]

DNA EPIGENETICS IN FORENSIC SCIENCE

1. Overview of Epigenetic Mechanisms

Epigenetic mechanisms regulate gene expression and include DNA methylation, histone modifications, and non-coding RNAs. For forensic purposes, DNA methylation is the most widely applied due to its chemical stability, well-defined patterns, and analyzability with DNA-based platforms.[24] Methylation patterns are established during development and can be influenced by age, environment, and lifestyle, yet they remain sufficiently stable in post-mortem samples for analysis.

2. DNA Methylation as a Forensic Tool

DNA methylation serves three key forensic functions. First, for age estimation, highly accurate models (the "epigenetic clock") have been developed based on the methylation levels of specific CpG sites in genes like *ELOVL2, FHL2, and PDE4C*.[25] Second, for tissue identification, body fluids including saliva, blood, semen, and vaginal fluid exhibit distinct methylation profiles at certain genomic loci, allowing their differentiation.[26] Third, it shows potential for inferring lifestyle factors, such as smoking habits, through exposure-specific methylation signatures.[27]

3. Epigenetic Clock and Forensic Age Prediction

Forensic epigenetic age prediction models use quantitative methylation analysis of a small panel of age-informative CpG sites. These models can predict a person's chronological age with a mean absolute error (MAE) of approximately 3-4 years, a level of accuracy highly valuable for generating investigative leads in cases involving unknown suspects or victims.[28]

Integration of Saliva and Epigenetic Analysis

Saliva is an ideal matrix for epigenetic studies in forensics. It provides a sufficient yield of high-quality DNA from buccal cells, which itself carries a tissue-specific methylation signature.[29] This allows for a dual-purpose analysis from a single sample: standard STR profiling for identification and DNA methylation analysis for intelligence purposes. Researchers have identified CpG sites that are not only age-predictive but also exhibit minimal variation between blood and saliva, enhancing the applicability of blood-derived epigenetic clocks to saliva samples.[30] Recent studies have successfully developed saliva-specific age prediction models, demonstrating the direct utility of this fluid.[31] The combined workflow involves extracting DNA from a saliva stain, quantifying it, and then performing targeted bisulfite sequencing or pyrosequencing of the informative CpG loci. This integrated approach can confirm the sample's origin as saliva (via tissue-specific methylation) and simultaneously provide an age estimate for the donor, all from a minimal sample.[32]

Emerging Technologies and Future Directions

The future of this field lies in leveraging high-throughput technologies. Next-Generation Sequencing (NGS) enables epigenome-wide association studies (EWAS) to discover novel forensic methylation markers.[33] The integration of Artificial Intelligence (AI) and machine learning can improve the accuracy of multivariate age prediction models and pattern recognition in complex datasets.[34] There is a pressing need to develop standardized, forensic-grade databases for salivary biomarker and methylation frequencies across diverse populations. Finally, a major goal is the translation of this research into rapid, on-site forensic kits, perhaps based on CRISPR-based detection or portable sequencing technologies, that can provide real-time intelligence from saliva evidence at a crime scene.[35]

Ethical and Legal Considerations

The ability to infer age, tissue source, and potentially lifestyle traits from epigenetic data raises significant privacy and informed consent issues.[36] The admissibility of such biomarker-based evidence in court requires establishing rigorous standards for reliability, reproducibility, and error rate, as per the Daubert standard.[37] Developing international guidelines and regulatory frameworks is essential to govern the ethical collection, analysis, and storage of epigenetic data in forensic contexts to prevent misuse and protect individual rights.[38]

CHALLENGES AND LIMITATIONS

Several challenges impede the routine implementation of these methods. Saliva samples are prone to degradation and bacterial contamination, which can compromise downstream epigenetic assays that require intact DNA.[39] There is a lack of large, standardized, and population-specific reference databases for salivary methylation markers, leading to potential interpretation biases.[40] Furthermore, methylation patterns can be influenced by population ancestry, health status, and environmental factors, introducing variability that must be accounted for in predictive models to ensure accurate and unbiased results.[41]

CONCLUSION

The convergence of salivary biomarker analysis and DNA epigenetics marks a significant advancement in forensic science. Saliva provides a readily accessible and information-rich source of evidence, while DNA methylation offers a stable and informative metric for predicting age and identifying body fluids. Together, they complement traditional DNA profiling by providing valuable investigative leads when reference profiles are unavailable. Despite existing challenges related to standardization and validation, the continued refinement of analytical techniques and the expansion of reference databases hold the promise of integrating these powerful tools into mainstream forensic practice, ultimately enhancing the resolution and efficiency of human identification.

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