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Review Article

Lung Cancer Biomarker Detection Techniques by Using Salivary Sample; A Comprehensive Review

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Abstract

Lung cancer poses one of the most significant challenges to modern medicine, it killing thousands every year. Salivary biomarkers will benefit us by being cost-effective and non-invasive, as well as by allowing the simple detection of lung cancer detection.

Saliva collection is more practical and comfortable compared with other invasive methods, and saliva can be a desirable body fluid for biomarker detection in clinical applications. The integration of omit methods has allowed accurate detection and quantification of transcripts found in saliva and their biomarkers has been discovered and validated in a series of studies.

Here we review recent developments in salivary diagnostics that have been accomplished using salivaomics, the mechanisms of saliva diagnostics, as well as the translational and clinical application of saliva biomarkers.

Keywords: Lung Cancer; Salivary Biomarker; Lung Cancer Detection Technique

Abbreviations

CEA: Carcinoembryonic Antigen; CYFRA-21: Cytokeratin-19 Fragments; PKM2: M2-Pyruvate Kinase; KLKB1: Plasma Kallikrein; SAA: Serum Amyloid A; IGFBP-2: Insulin-Like Growth Factor-Binding Protein-2; Upar: Urokinase Plasminogen Activator Receptor; Pro-GRP: Pro-Gastrin-Releasing Peptide; PRX1-Peroxiredoxin 1; Ciz1: p21(Cip1)-Interacting Zinc Finger Protein; MMP-1: Matrix Metalloproteinase-1; miRNA: MicroRNA; Pgrmc1: Pgrmc1; NSE: Neuron Specific Enolase; CCN-I: Cyclin: I; EGFR-Epidermal Growth Factor Receptor; EGF-19: Epidermal Growth Factor-19; FGR: Fibroblast Growth Factor; FRS-2: Factor Receptor Substrate-2; GREB-1: Growth Regulation by Estrogen.

Introduction

Lung cancer is characterized by uncontrolled cellular growth in the tissue of the lung that may metastasize and spread into other tissue in the lung or in another place in the body [1]. There are two main forms of lung cancer– small cell lung carcinomas (SCLC) and non-small cell lung carcinomas (NSCLC), from these, the majority of lung cancers fall into the NSCLC category (80%) with a significant proportion of the reminder falling into the category of SCLC [1,2]. NSCLC consists of 3 major subtypes: Adenocarcinoma (ADC), Squamous Cell Lung Carcinoma (SQLC), and Large Cell Carcinoma (LCC) [3].

Lung cancer is a leading cause of death in the world, 1.69 million deaths in 2015 according to WHO. Currently used diagnostic methods for lung cancer such as X-rays and computed tomography (CT) scans, Positron emission tomography scan (PET), Magnetic resonance imaging (MRI) [4]. Besides this, high cost, a risk of radiation exposure, and poor sensitivity and specificity are problems with current screening techniques. A better diagnostic measure for lung cancer that gives the early detection of the disease, therefore allowing for effective involvement, it is necessary to lower lung cancer mortality rates [3,5].

The detection of cancer biomarkers molecules helps to differentiate between normal and cancerous conditions, may be used to develop a more effective diagnostic tool for lung cancer. Cancer biomarkers consist of genetic materials or proteins because cancer is a heterogeneous disease that reflects gene and protein changes within a cancer cell. Proteins are main functional units of biological processes. The recent advances in proteomics technologies, including mass spectrometry, protein labeling, protein array-based approaches, imaging, and protein bioinformatics, have enabled to rapidly discover more biomarkers and to better understand their roles. Various biomarkers have been identified from blood, serum, plasma and saliva samples for detection of lung cancer. From above sample salivary diagnostics is a noninvasive, easily usable tool for patient specimen collection [6].

Biomarkers for Lung Cancer have the potential to improve early detection beyond the use of computed tomography scans with the 2D-DIGE proteomic analysis of saliva samples from Lung Cancer patients; three biomarkers for lung cancer(calprotectin, zinc-α-2-glycoprotein, and haptoglobin) achieved good sensitivity and excellent specificity and accuracy for detection. Transcriptomic biomarker profile including the B-Raf gene (BRAF, which is involved in directing cell growth), cyclin I (CCNI, which binds activated cyclin-dependent kinase 5), the epidermal growth factor receptor (EGFR), fibroblast growth factor 19 (FGF19), fibroblast growth fac-

tor receptor substrate 2 (FRS2), growth regulation by estrogen in breast cancer 1 (GREB1), and leucine zipper putative tumor suppressor 1 (LZTS1) has been identified, and a section consisting of five of these markers is able to differentiate Lung Cancer patients from cancer-free subjects [7,8].

Biomarker

A biomarker can be defined as a assessable and quantifiable biological parameter that can give either as an sign for health, disease status, environmental exposure or pharmacological responses to a therapeutic intervention. Prognostic biomarkers are used as indicator of a benign or a malignant condition, whereas diagnostic biomarkers show the development of a cancer [9].

Biomarkers are molecules that can be measured in blood, saliva, plasma, serum, tissue etc. to assess the presence of state of disease. Biomarkers provide a active and influential approach to accepting the spectrum of disease with applications in observational and analytic epidemiology, randomized clinical trials, screening and diagnosis and prognosis. A clinical use of biomarker is elucidation of the specific indication, standardization of analytical methods, characterization of analytical features, and incremental yield of different markers for given clinical indications.

Biomarkers can replicate the entire spectrum of disease from the earliest manifestations to the incurable stages. A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

Characteristics of biomarker [10]

i) An ideal biomarker should be harmless and easy to assess.

ii) The cost of follow-up tests should be relatively low, there should be proven treatment to modify the biomarker.

iii) It should be consistent across genders and ethnic groups.

iv) If the biomarker is to be used as a diagnostic test, it should be sensitive and specific and have a high predictive value.

v) A highly sensitive test will be positive in nearly all patients with the disease, but it may also be positive in many patients without the disease. selected of clinical value, a test with high sensitivity also have high specificity, in additional terms, most patients without the disease should have negative test result.

Development of biomarker

Figure 1: Development phases of biomarker.

Lung cancer biomarker

Biomarkers for lung cancer have numerous potential clinical uses. They can be used for danger stratification, early lung cancer detection, most advantageous treatment selection, prognostication and observe for recurrence. All of these area could benefit from the use of sensitive and specific, noninvasive, cost-effective biomarkers. Different markers, reflecting the biology of lung cancer development from premalignancy to invasive lung cancer, may confirm more useful for each of these areas [11].

Different Lung cancer biomarker

Figure 2: Different lung cancer biomarker.

Saliva and its scope in lung cancer detection

Saliva is a biological fluid composed of more than 99% water and less than 1% proteins, electrolytes and other low molecular weight components. It originates mainly from three pairs of major salivary glands (the parotid, submandibular, and sublingual glands) as well as from 300 to 400 minor salivary glands present in the oral cavity. The gingival crevicular fluid containing bacteria, epithelial cells, erythrocytes, leukocytes and food debris contributes only in small part to the formation of oral fluids. Thus, saliva acting a part in the lubrication, mastication, Swallowing and digestion. It protects the reliability of the oral tissues, but also provides clues for local and systemic diseases and conditions [6].

Molecules likewise DNAs, RNAs, proteins, metabolites, and microbiota, there in blood, might be also present in saliva. Thus, their concentration changes can be used as biomarkers to detect early stage cancer or to monitor the response to therapeutic management. Salivary diagnostics is a noninvasive, easy to use tool for

patient specimen collection. Saliva testing potentially allows the patient to gather their own saliva samples, even at home thus savings healthcare costs, enabling convenient and multiple sampling as well as having a positive impact on patient compliance [6].

Constituents in saliva

Genome and epigenome

The salivary genome shows human and microbial DNA. Both, the amount and the value of salivary DNA are quite good: The entire DNA in saliva is about 24 lg, ranging from 0.2 to 52 lg. while it is about 10 times lower in blood, genotyping requires as low as 5 ng/mL of DNA to work efficiently. Saliva samples also yield sufficient DNA for sequencing arrays and polymerase chain reaction (PCR) assays. The ratio of absorbance at 260 nm and 280 nm (A260/A280) able to calculated for purity of the DNA. The mean value of A260/A280 is 1.56 for saliva and 1.71, which shows that the quality of salivary DNA is comparable to that in blood. Tumorigenesis is a multistep process, involving both genetic and epigenetic changes in its pathology. Aberrant DNA methylation was the first epigenetic mark to be associated with cancer; as a consequence of the alteration, it causes in normal gene regulation.

Salivary genome and epigenome are assayable by a various group of biomolecular techniques, counting methylation arrays, PCR and quantitative PCR (qPCR)- based genotyping [6].

Proteome

The salivary proteome comprises the entire protein content of the oral cavity [6]. Saliva contains more than 2,000 proteins that are involved in many biological functions to maintain oral homeostasis. Unlike the relatively stable status in serum, proteins in saliva appear to be more susceptible to biochemical processes and degradation Salivary protein degradation happens rapidly, and even happens during saliva collection and handling, which may compromise its clinical usefulness. Our laboratory has developed methods to stabilize the salivary proteome with protease inhibitors; as a result, we can keep salivary proteins constant for 2 weeks when stored at 4˚C without significant degradation and without affecting downstream applications [7].

Presently, mass spectrometry (MS) is the central technology for salivary protein identification. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) can obtain stable profile of salivary proteome of healthy controls. It could be also used in detecting the differences between pre- and post-orthodontic treatment or in high-throughput breast cancer biomarker discovery. Two-dimensional gel electrophoresis (2DE) combined with MS performed well in lung cancer and breast cancer biomarker detection with high sensitivity and specificity [6].

Transcriptome

Transcriptome studies have focused mainly on mRNA and miRNA, which are secreted from cells and enter the oral cavity through various sources, including salivary glands, gingival crevice fluid, and desquamated oral epithelial cells. The transcription of specific mRNA and miRNA is altered in disease states. The human salivary transcriptome was first discovered in laboratory using microarray technology, allowing high-throughput analysis. Then developed Direct-Saliva-Transcriptomic-Analysis (DSTA) to permit simple stabilization of salivary RNA and direct analysis without further processing.

Compared with salivary mRNA, salivary miRNA are more stable, and the fold change in miRNA between cancer and normal cells is fairly large [7]. Noncoding RNAs (ncRNAs) are emerging as new regulators of diverse biological functions, playing an important role in oncogenesis and tumor progression. Because of the small size of these molecules, they are very stable in different body fluids and not as susceptible as messenger RNAs (mRNAs) to degradation by ribonucleases (RNases). Based on gene microarray and quantitative real-time PCR (qRT-PCR) technology, Several mRNA and miRNA candidates were discovered in lung cancer with good sensitivity and specificity [6].

Metabolome

Metabolome, a global comprehensive overview of the metabolic status, provides a new insight into pathophysiologic mechanisms of various diseases. It allows measuring the levels of endogenous metabolites, thus enabling biomarker discovery. The endogenous metabolites, including nucleic acids, lipids, amino acids, peptides, vitamins, organic acids, thiols and carbohydrates, represent a valuable tool for detection of biomarkers for various diseases and monitoring disease progression. In 2010, Sugimoto., *et al.* identified that cancer-specific signatures are embedded in saliva metabolites by capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS). Fifty-seven principal metabolites were found to accurately predict the probability of being affected by a specific disease, yielding large area under the receiver operating characteristic curves (AUCs). Other salivary metabolites were established to differentiate oral squamous cell carcinoma and neurodegenerative dementia patients from controls based on MS.

Biology of lung Carcinogenesis

Progress in accepting the series of molecular changes original the progression from preneoplasia to invasive lung cancer has spurred research into biomarkers for early detection and into the possibility of personalizing treatment based on biomarker profiles. The known preneoplastic lesions of the bronchial epithelium are: squamous dysplasia and carcinoma in situ, which progress to squamous cell carcinoma; atypical adenomatous hyperplasia, which may precede adenocarcinoma; and distribute idiopathic pulmonary neuroendocrine cell hyperplasia, which may develop to carcinoid. No preneoplastic lesion has been identified for small cell carcinoma. Alterations in gene expression and chromosome structure have been demonstrated in these preneoplastic lesions and the changes appear to be sequential – their frequency and number increase with increasing atypia. several alteration create in preneoplastic lesions include: hyperproliferation and loss of cell cycle control; abnormalities in the p53 pathway, the ras genes and genes in the genomic region; aberrant gene promoter methylation; increased vascular growth; altered extracellular matrix; decreased retinoic acid and retinoid receptor expression; and altered expression of multiple proteins [11].

The mechanism of salivary diagnostic

Recent translational salivary biomarker development studies have supported that salivary biomarkers can discriminate oral and systemic disease patients from non-disease subjects. However, the mechanisms of how diseases distal from the oral cavity would lead to the appearance of discriminatory biomarkers in saliva are largely unclear. Investigating the origin of salivary biomarkers will

be a significant goal in the development of salivary diagnostics, and the mechanisms of salivary diagnostics need to be elucidated. Studies have increasingly demonstrated that some salivary biomarkers might derive from systemic sources. Gao., *et al.* used mouse models of cancer to determine whether salivary biomarker profiles are affected by distal tumor development. Their data analysis of nerve growth factor production and the transcription factor Egr-1 suggests that the production of growth factors in tumor tissue represents one mechanism by which a distant tumor can alter the transcriptome of the salivary glands, and hence of saliva. While their report did not demonstrate the mechanistic connection between systemic disease development and salivary biomarker alterations, it did begin to paint a picture of the concept that systemic networks exist in the human body that allow communication between distal diseases and the salivary glands. Signals transmitted through such networks might induce related signaling pathways that result in altered gene expression and protein translation, and thereby produce disease-induced salivary biomarker profiles. Therefore, the salivary transcriptomic profile might be composed of transcripts that originate in distant diseased tissues as well as transcripts that originate in salivary glands, and transcription factors that originate in distant tissues might alter the expression levels of these transcripts.

Diagnostic tools for detection of lung cancer

Figure 3: Lung cancer detection system.

Technique for lung cancer detection

Detection of EGFR mutation

Electric Field–Induced Release and Measurement (EFIRM) [12]

Epidermal growth factor receptor is a glycoprotein with an extracellular epidermal growth factor binding domain and an intracellular tyrosine kinase domain that control signaling pathways to regulate cellular proliferation. Epidermal growth factor receptor binding to its ligand consequences in autophosphorylation by intrinsic tyrosine/kinase activity, triggering several signal transduction cascades. Sustained activation of these sequences of downstream targets is considering to yield more aggressive tumor phenotypes. Mutations in epidermal growth factor receptor have exposed in involvement with some lung cancers. Lung carcinomas with mutated epidermal growth factor receptor have significant responses to tyrosine kinase inhibitors, although for unselected patients, it does not appear to have a survival benefit. conversely, in a division of patients, there appears to be a significant survival advantage. Both EGFR mutation and gene amplification status may be significant in influential which tumors will respond to tyrosine kinase inhibitors [13].

EGFR belongs to the erbB family of directly linked receptor tyrosine kinases, which include erbB1 (also known as EGFR), erbB2 (HER2), erbB3, and erbB4. Even though their basic structures are similar, everyone has different property, counting variation in tyrosine kinase activity. They have extracellular ligand binding domain, a transmembrane portion, and intracellular tyrosine kinase and regulatory domains. Upon binding of a specific ligand (Epidermal growth factor), the normally working of EGFR undergoes conformational alteration and phosphorylation of the intracellular domain occurs which leads to downstream signal transduction by different pathways. They consist of the Raf1-extracellular signal-regulated kinase, PI3K/Akt, and signal transducer and activator of transcription (STAT) factors. Depending on the pathway, the end result is cell proliferation or cell maintenance by inhibition of apoptosis [13].

Principle

Electric Field-Induced Release And Measurement (EFIRM), which relies on a multiplexible electrochemical sensor that can detect EGFR mutations directly in bodily fluids like saliva and plasma.

In non-small cell lung cancer (NSCLC), epidermal growth factor receptor (EGFR) mutations have emerged as important biomarkers in predicting the response to the EGFR tyrosine kinase inhibitors. The detection of these mutations is based on invasively obtained biopsy samples, which is repeatedly not acceptable in a clinical setting. The analysis of circulating tumor DNA or circulating tumor cells in the blood is an alternative approach but is often complicated, technique dependent, and time consuming. A noninvasive, readily available, diagnostic procedure with minimal preparation that Provides immediate information on EGFR mutation status is desirable [12].

Saliva-based epidermal growth factor receptor (EGFR) mutation detection in Saliva sample

Epidermal growth factor receptor mutations in serum and saliva were detected by EFIRM in blinded samples by trained laboratory personnel. Each saliva sample was measured in duplicate. Paired probes (capture and detector; TsingKe, Beijing, China) specific to the two TKI sensitive mutations were used: for the exon 19 deletion (19 del), a capture probe 50-TGT TGC TTC CTTGAT AGC GAC G-30 and a detector probe 50-GGA ATT TTA ACT TTC TCA CCT-FITC-30; for the L858R point mutation, a capture probe: 50-CAG TTT GGC CCG CCC AAA ATC- 30and detector probe: 50-TTG ACA TGC TGC GGT GTT TTC A-FITC-30. The detector probes were labeled with fluorescein isothiocyanate. The EFIRM detection method involves four primary steps. Firstly, copolymerization of capture probes with pyrrole on the bare

gold electrodes by applying a cyclic square wave electric. Secondly, hybridization of the sample with detector and capture probes. Thirdly, the combination of anti-fluorescein antibody conjugated to horseradish peroxidase (1:1000 dilution). Fourthly, chromogenesis by 3,30, 5,50 - tetramethylbenzidine substrate for horseradish peroxidase and measurement of the amperometric signal. The total exposure time of the protocol was less than 10 minutes, and the process required 20 to 40 µL of the biological sample.

Statistical Analysis

To evaluate the performance of EFIRM in detecting EGFR mutations, the receiver Operating characteristic curve for each probe was plotted, and the area under the Curve (AUC) and its 95% confidence interval (CI) were calculated. All analyses were Performed using SAS 9.3 TS Level 1M1. We used the G*power program to estimate the sample size needed for validation in a blinded group using one-way analysis of variance with a power of 0.95 at a = 0.05.

Optimization of EFIRM for EGFR Mutation Detection Using Lung Cancer Cell Lines

We optimized EFIRM to detect two EGFR mutations: p.L858R (point mutation c.2573T.G in exon 21) and p.E746-A750del (c.2236_2250del15 in exon 19, a 15-base pair deletion). Genomic DNA samples from the human lung cancer cell lines NCI-H1975 and HCC827, which harbored the respective mutations, were used for the EFIRM optimizations. We first optimized the electrical field profile for the appropriate number of hybridization cycles. The hybridization signal increased rapidly after two cycles of electrical waves. After five cycles, perfect match signals reached a plateau, whereas the mismatch sequences generated only background signal levels. We therefore defined the optimized hybridization cycle as five for all subsequent studies. The specificity and sensitivity of EFIRM detection for the respective EGFR mutations were investigated by decreasing the ratio of mutant EGFR DNA to wild type EGFR DNA. For the p.E746-A750del, as little as 0.1% mutant DNA was detected in the presence of wild type DNA. For the p.L858R point mutation, as little as 1% mutant DNA was detected when a control sample was used. We used 10 ml of 2 ng/ml DNA for these experiments. These data demonstrated that EFIRM was able to detect EGFR mutations with high sensitivity and specificity.

Applications of EFIRM

- EFIRM detect EGFR mutations in the saliva of patients with NSCLC.
- It is a noninvasive, rapid, and cost-effective way [12].

Scope of salivary lung cancer biomarker

- Lung cancer biomarkers may allow us to identify populations who would benefit from computed tomography screening, may differentiate individuals with benign pulmonary tumors from those with early malignancies and may allow for personalization of lung cancer treatment based on tumor characteristics.
- Salivary lung cancer biomarker report high sensitivity and specificity [11].
- The biomarker is used to available diagnostic methods for many lung cancers is based on patient's symptoms and clinical information.
- Salivary diagnostics can be performed in the clinic without specially trained professionals, using point-of-care technology to allow the detection of cancer without any preparation.
- Saliva collection is more practical and safe compared with invasive methods of sample collection, because of the infection risk from contaminated needles during, for example, blood sampling [14].

Future prospects for development of ideal biomarker

- The development of biomarkers for prognostication and personalization of therapy after diagnosis seems happened, and there is hope for the development of practical biomarkers for the early detection of lung cancer in the expected future [11].
- Further research studies will reveal which method will be the most suitable to be applied in a clinical practice [15-20].

Conclusion

The recent advances developed in technology specifically improved techniques for the identification, detection, and verification of biomarkers and have thus improved our understanding of lung cancer. Earlier diagnosis of lung cancer is urgently needed to decrease the lung cancer mortality rate. A noninvasive technique that integrates an understanding of lung cancer biomarkers and identifies lung cancer patients from hemo-analysis would be a better alternative to the current diagnostic methods. From our experience studying lung cancer biomarkers, we have found that many protein biomarkers discovered in the serum and/or plasma seem to overlap with other diseases, especially other cancers and inflammatory diseases. so we have used salivary sample for lung cancer detection.

The best method to verify a lung cancer diagnosis would be one that involves a multiple biomarker approach rather than a single biomarker approach. cancer detection via a single marker remains difficult due to the low sensitivity, specificity and reproducibility of the identified lung cancer biomarkers. Some biomarkers show high sensitivity and specificity, it should be noted that small-scale verification in retrospectively selected samples from one institution may not represent a general test with the samples prospectively collected from multiple institutions. With plenty of lung cancer biomarkers mentioned above and many more that are published, now is the time to widely validate the markers in large-scale clinical samples and to assess their effectiveness in various combinations to develop an optimized multi-marker approach. Therefore, the combination of multiple biomarkers that best distinguishes cancer samples from controls should be utilized in the development of cancer detection technology for clinical applications.

Conflict of Interest

No conflict of interest.

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