Chapter

RNA Sequencing in Potentially Malignant Disorders

Ramya Ramadoss, Rajkumar Krishnan, Lekshmy Jayan and Priyadharini Shankaran

Abstract

RNA sequencing is a molecular technique which utilizes next generation sequencing to identify and quantify ribonucleic acid (RNA) in a given sample. This technique is utilized in the detection of changes in gene expression. Potentially malignant oral disorders are one of the most troublesome lesions seen in the oral cavity which predisposes to the development of oral cancer. Though there are many methods employed in the diagnosis of these disorders, biopsy followed by histological examination is the gold standard procedure followed in the diagnosis. RNA sequencing has been receiving attention among researchers. Many studies have been conducted to analyze the application of RNA sequencing in the diagnosis of PMODs as well as in the malignant transformation to oral squamous cell carcinoma. The article attempts to summarize the progress in RNA sequencing pertaining to Potentially malignant disorders.

Keywords: RNA sequencing, Potentially Malignant Disorders, Diagnostic Markers, Molecular Diagnosis, Oral cancer

1. Introduction

RNA sequencing is a molecular technique which utilizes next generation sequencing to identify and quantify ribonucleic acid (RNA) in a given sample. This technique is utilized in the detection of changes in gene expression [1]. It also detects mutations or single nucleotide polymorphisms etc. RNA sequencing has greatly replaced cDNA microarray owing to more precise reproduction of using lanes and flow cells. Another added bonus is that this method allows de novo reconstruction of the transcriptome i.e., unknown material can be analyzed [2].

Potentially malignant oral disorders are one of the most troublesome lesions seen in the oral cavity which predisposes to the development of oral cancer. As the saying goes, "prevention is better than cure" it is better to identify and tackle the lesion in the premalignant stage rather than once cancer has developed. Sarode et al. (2014) defined OSCC prone disorders as 'It is a group of disorders of varying etiologies, usually tobacco; characterized by mutagen-associated, spontaneous or hereditary alterations or mutations in the genetic material of oral epithelial cells with or without clinical and histomorphological alterations that may lead to oral squamous cell carcinoma transformation' [3].

Though there are many methods employed in the diagnosis of these disorders, biopsy followed by histological examination is the gold standard procedure followed

in the diagnosis [4]. Molecular techniques like PCR, ELISA are attempted in identifying a sensitive and specific marker. A handful of markers (salivary and serum) are attempted but none are identified to lack both specificity and sensitivity ideally required by a diagnostic marker. Microarray has emerged as a promising method as it helped in comparison and analysis of multiple samples at the same time. RNA sequencing has been receiving attention among researchers [3, 4]. Many studies have been conducted to analyze the application of RNA sequencing in the diagnosis of PMODs as well as in the malignant transformation to oral squamous cell carcinoma [5, 6].

2. RNA sequencing

RNA sequencing is the molecular technique in which the quantity as well as the sequence of RNA in a biological sample tissue of choice is determined using next generation sequencing. Here, the coding and noncoding RNAs or in short the transcriptome of the gene is analyzed. It was first described a decade ago. During the infant period of this technique, the Sanger sequencing technology was used in RNA sequencing which is greatly replaced in the present by more accurate next generation sequencing technology. RNA sequencing is found to be superior to many other techniques especially tissue microarray hybridisation [7]. The proposed advantages of the former over the later are:

- Microarray hybridisation requires use of species specific probe. It cannot be used to identify a novel or unknown sequence. RNA sequencing on the other hand can be successfully employed in the identification as well as quantification of an unknown sequence.

- Background signal or non-specific binding Is less in RNA sequencing as compared to microarray.

- Quantification of the transcriptome is more reliable in RNA sequencing as compared to microarray.

- RNA sequencing has greatly replaced cDNA microarray owing to more precise reproduction of using lanes and flow cells.

3. Steps in RNA sequencing

The procedure of RNA sequencing is performed by three steps:

1. RNA isolation

2. RNA selection or depletion

3. cDNA synthesis

4. Preparing sequencing library

5. Next generation sequencing

The first step in RNA sequencing is the isolation of RNA from the sample provided. The main requirement is that the sample should possess RNA of sufficient

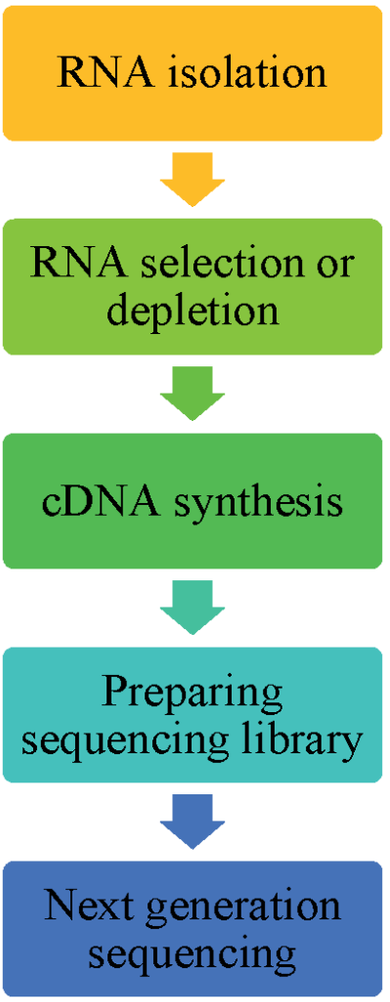

Figure 1.
Steps in RNA sequencing.

quality to enable library sequencing. Based on the quality of the RNA, measured by a bioanalyser an RNA integrity number is given ranging from 1 to 10. The number 10 shows least degradation of RNA with highest quality. RNA of low quality may result in erroneous sequencing.

The next step is the selection of RNA species from the pool of total RNA including mRNA, tRNA, rRNA etc. in this pool, around 95% is contributed by rRNA and are removed before sequencing as it may have the tendency to overshadow the read of other types of RNAs. This can be achieved by many techniques, like selecting poly A RNAs by targeted reaction with poly-T- oligos in magnetic beads. There are also commercially available kits which deplete the rRNA like Ribozero or Ribominus. In another method, the sample is treated with the enzyme, DNAse to reduce the quantity of the genetic material (DNA) in it. The quantity of RNA is determined by either capillary or gel electrophoresis. In the final step, the isolated RNA is then transcribed to DNA. DNA is more stable than RNA and thereby facilitates techniques of amplification without undergoing damage. Also, most of the sequencing libraries require DNA. This cDNA is then utilized in next generation sequencing (**Figure 1**) [8].

4. Advantages and disadvantages of RNA sequencing

Advantages of RNA sequencing are

1. We can do genome wide analysis as well as targeted analysis

2. It can analyze both novel sequences as well as known sequences

3. It has very low background noise

4. It is cheaper in comparison to Sanger sequencing

The drawback of RNA sequencing is that the depth of coverage depends on the sequenceability [9].

5. RNA sequencing in potentially malignant oral disorder

Since the scope of this current chapter is the role of RNA sequencing in the detection of potentially malignant oral disorders. This particular technique has gained attention in the identification of these disorders. The studies have focused mainly on leukoplakia, oral submucous fibrosis and also in lichen planus.

6. RNA sequencing in leukoplakia

Oral leukoplakia is the most commonly reported potentially malignant oral disorder. World Health Organization (WHO) defined oral Leukoplakia as "a white patch or plaque that cannot be characterized clinically or pathologically as any other disease" [9].

Leukoplakia is at present defined as "A white plaque of questionable risk having excluded (other) known diseases or disorders that carries no increased risk for cancer" (WHO 2005).

Diagnosis of Leukoplakia is predominantly based on clinical appearance as histological appearance seems varied. Microscopical architecture presents with a non-specific pattern of atrophy or hyperplasia. Histological evaluation is mainly done to delineate the presence and absence of epithelial dysplasia as malignant transformation rate is about 2–3% [9].

Numerous metabolic and molecular pathways are altered in leukoplakia and the disease is manifested as a culmination of all the altered metabolic pathways.

Numerous studies are conducted in analyzing the role of RNA sequencing leukoplakia. Philipone et al. in 2016 conducted a study which utilized deep RNA sequencing in the role of miRNA in both dysplastic and non-dysplastic leukoplakia. The predictive value of these markers were analyzed in both the groups. miRNA shows possible predictive value in the progression of dysplastic leukoplakia [10].

Another study by Chang et al. in 2019 conducted a study to analyze the role of potential miRNAs in the malignant transformation of leukoplakia to oral squamous cell carcinoma. They used small RNA sequencing to screening these markers in patients with leukoplakia and normal subjects. Further bioinformatics study revealed that miRNA-423-5p and miRNA-222-3p were found to have significance in the diagnosis of oral leukoplakia. RNA sequencing helped in revealing the role of these markers as potential diagnostic markers in leukoplakia as well as in the detection of malignant transformation [11].

Simming Zu et al. in 2020, analyzed the role of circular RNAs in the development of leukoplakia and identified circHLA-C has role in the progression of the disease using Sanger sequencing. They reported that levels of circHLA-C increases

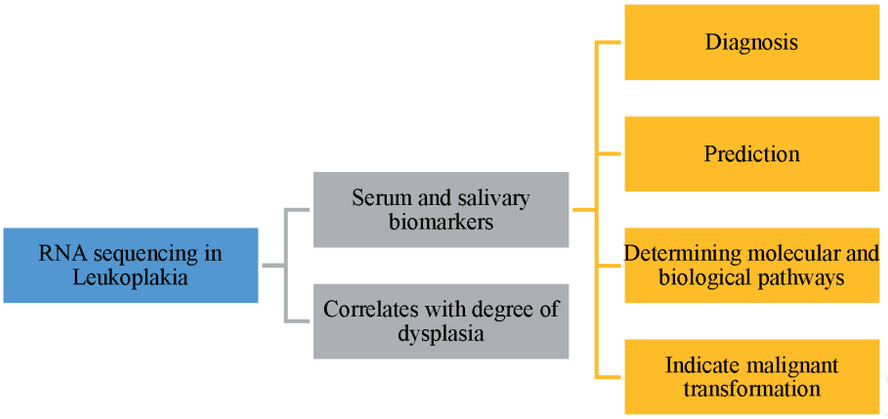

Figure 2.
Applications of RNA sequencing in leukoplakia.

with degree of dysplasia. It is a potential diagnostic marker and a genetic marker in oral leukoplakia [12].

Transcriptome analysis was conducted using RNA sequencing, differential expression in the study reported by Farah et al. (2019) which evaluated leukoplakia cases with or without dysplasia. They concluded from their study that reactive changes in the connective tissue of the lesion is an early manifestation of development of dysplasia in leukoplakia. Utilization of RNA sequencing in detection of molecular changes in oral leukoplakia will help in understanding the evasive process of development of the disease [9].

The studies conducted evaluated the role of various markers in diagnosis, prediction of the disease. The studies also revealed that the molecular pathways of the disease can be determined by RNA sequencing. It is also suggested that as the degree of dysplasia increases the progression of disease also advances. RNA sequencing may be helpful in filling the blanks in understanding the molecular and biological pathways in the development of leukoplakia (**Figure 2**).

7. RNA sequencing in oral submucous fibrosis

Oral submucous fibrosis may be defined as "an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it, is always associated with a juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat" [13]. 'Slowly progressive disease characterised by the fibrous bands in the oral mucosa, ultimately leading to severe restriction of mouth movement including the tongue' World Health Organization (1978).Oral submucous fibrosis is highly prevalent in South east Asia owing to the increased consumption of arecanut. Arecoline was identified as the single most important etiological agent in the development and the progression of the disease. It has a high malignant transformation rate of 7–30% [13].

Several studies analyzed the molecular profile of Oral sub mucous fibrosis and revealed that the rna profile was altered significantly in OSMF. Tsai et al. reported that role of the prime etiological agent areca nut lead to consistent elevation of miRNAs. Research evidences have substantiated the role of miRNAs in development of oral potentially malignant disorders [14].

Shangui Zhou et al. in 2019 conducted a study to determine the role of Intergenic/intertwining long RNA (IncRNAs) expression in OSMF. They used RNA

Figure 3.
Applications of RNA sequencing in oral submucous fibrosis.

sequencing to transcript the samples and found that 231 IncRNAs were upregulated and 456 were downregulated. IncRNAs were found to be associated with the regulation of progression of OSMF. These markers also plays a role in the inflammatory signaling associated with this disorder. This study was considered the first study to evaluate the role of IncRNA expression in the progression of oral submucous fibrosis [15].

Xiaohuan Zhong et al. studied the role of oral microflora in the development of oral submucous fibrosis and in the malignant transformation with continued use of arecanut. The genra of bacteria varied with site as well as conditions like alcohol or smoking. In patients with alcoholism and arecanut chewing, Prevotella was increased but at the same time Actinobacillus was reduced. But they suggested that since the sample size was small it was difficult to analyze the role of confounding factors in the oral bacterial dysbiosis [16].

The studies conducted in oral submucous fibrosis using RNA sequencing suggest the possible role in analyzing the rate of progression of the condition and also in the malignant transformation to oral squamous cell carcinoma. Also, one of the reasons for the poor prognosis of OSMF was because of lack of proper understanding of the molecular pathogenesis and the pathway of progression to oral squamous cell carcinoma (**Figure 3**).

8. RNA sequencing in oral lichen planus

Lichen planus is an inflammatory mucocutaneous disease involving skin, hair, nails and mucosal surfaces- esophageal, genital, oral, ocular, optic and less commonly bladder, nasal, laryngeal and anal mucosa. It is derived from the Greek word "*leichen*" means tree moss and Latin word "*planus*" means flat [17].

It is a T cell mediated autoimmune disorder in which cytotoxic CD8 + T cells trigger apoptosis of the basal cells of the oral epithelium. Associated with other autoimmune disorders like myasthenia gravis, alopecia, vitiligo, ulcerative colitis. The disease has been implicated to be caused by exogenous trigger also. One of the common difficulties in studying tis disorder because of the overlap between features of oral lichen planus and other oral mucosal conditions, to the highly variable application of diagnostic criteria and the potential co-existence of additional non-OLP inflammatory conditions in same patients [17].

It can be defined as "Lichen planus is a chronic immunological mucocutaneous disorder that varies in appearance from keratotic to ulcerative (Wilson)" [17].

"Oral lichen planus is a non-infectious, cytotoxic T-cell mediated, chronic inflammatory autoimmune disease affecting oral cavity, involves the oral mucosal stratified squamous epithelium and underlying lamina propria which may be accompanied by skin lesions" [17].

In a study conducted by Ku Wang et al. in 2016, the role of oral microbial flora in oral lichen planus. MiSeq sequencing was done to detect the species present in the saliva of the patients and then compared the results with that of normal patients. There was an upsurge in Porphyromonas and Solobacterium and reduced numbers

of Hemophilus, Corrynebacterium, Cellulosimicrobium and Camplyobacter. In patients erosive lichen planus it was found that was a significant reduction in Streptococcus. The levels of Porphyromonas correlated with both disease progression and the immune dysregulation which is considered as the main culprit in the development of the disease [18].

Qiaozhen Yang et al. (2017) conducted a study utilizing RNA sequencing in the detection of genes responsible for malignant transformation of oral lichen planus to oral squamous cell carcinoma. Around 19 common differently expressed genes associated with oral lichen planus and OSCC were detected. Further analysis using polymerase chain reaction test revealed that among these 19 genes BCL9L, GMPS, HES1, PER2 and TSPAN33 were associated with the malignant transformation of oral lichen planus [19].

In another study conducted by Qiaozhen Yang et al. in 2017 they evaluated the role of differentially expressed genes and IncRNAs in the malignant transformation of oral lichen planus. The mapping of the IncRNAs were conducted using RNA sequencing. From the study it was concluded that keratininsation and major histocompatibility complex class I antigen processing and also the antigen presentation was activated during malignant transformation of oral lichen planus and found that numerous genes were expressed as well.

Junjun Chen et al. (2017) in their study on evaluation of the role of differentially expressed miRNAs and differentially expressed genes using next generation sequencing with DESeq. The gene expression profiling suggested a possible role in the development and progression of lichen planus [19].

In a study conducted by Keumjin Baek (2020), used high throughput sequencing of 16S rRNA gene to identify the bacterial communities present in lesions of oral lichen planus to recognize the role of these organisms in the pathogenesis of oral lichen planus. Both high throughput sequencing of 16S rRNA gene and whole genome sequencing revealed that there was an elevation in E.coli in biopsy tissues obtained from patients with oral lichen planus which is suggestive of potential role in triggering or developing the disease [20].

Studies in lichen planus done with RNA sequencing revealed newer clues as to possible role of oral microbiota in the development as well as progression of oral lichen planus. There are numerous gene expression studies which showed that there are variations in the expression profile. Like with the other two PMODs, RNA sequencing may play an important role in diagnostic and prognostic evaluation of lichen planus (**Figure 4**).

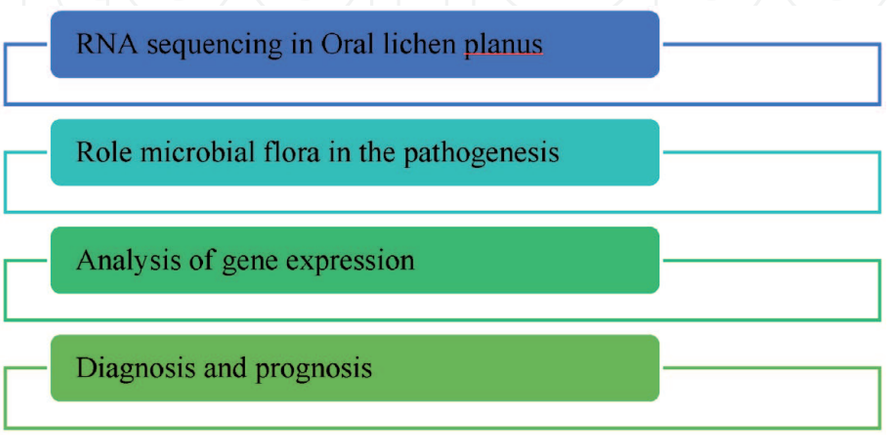

Figure 4.
Applications of RNA sequencing in oral lichen planus.

9. Scope of RNA sequencing in potentially malignant oral disorders

Potentially Malignant oral disorders have eluded the medical community for long due to lack of the right means to assess its molecular signature. Ground breaking research focusing on new molecular techniques to assess the molecular signature set by the Potentially malignant oral disorders is the need of the hour. RNA profiling serves to be a valuable tool in deciphering the molecular signature and serves as a guiding light on which the therapeutics working on similar principles can be based.

10. RNA sequencing aiding in generation of molecular signature

A significant advantage of RNA sequencing over the other diagnostic techniques is that it is based on Next generation sequencing where in the complete set of altered genome can be assessed through transcriptome analysis, thereby providing a comprehensive outlook on the genetic profile of a disease model. This elaborate guide of genetic set up of the disease serves to provide a valuable insight into the unique molecular signature of a particular disease, thereby enabling prompt and accurate diagnosis of the disease [21].

10.1 Sensitivity and specificity

RNA profiling is characteristically known for its high degree of sensitivity and specificity as it encodes for genetic alterations at the nuclear level and hence can be used as a confirmatory tool in the diagnosis of PMODs and elimination of Oral cancer in cases where clinical and histological appearance can be elusive and misleading.

10.2 RNA therapeutics

RNA therapeutics is a branch of therapeutics dealing with treatment strategies targeting the RNA profile of the disease which is unique to the individual. RNA profiling provides details about the genomic alterations unique to a particular individual. Targeted therapy towards the altered components of the genome helps eliminating the disease and offers better prognosis and avoids recurrence, thereby improving the overall survival and disease free survival rates of the individual [22].

10.3 Monitoring the prognosis and prediction of recurrence

The treatment protocol for most disorders is standard and has been in practice for decades, however, a proper protocol to assess the prognosis and the propensity for recurrence has not been established for any disease model. Obtaining the RNA profile of the individual suffering from PMODs can not only aid in diagnosis and treatment planning, but also serve as a tool in predicting the prognosis of the disease. Several genes, she upregulated serves as a poor prognostic marker where as several unregulated genes serve as markers of good prognosis and decreased recurrence rates. Hence obtaining the genomic profile through RNA sequencing can serve to be a valuable tool in predicting the same, thereby improving the overall quality of life of the individual post treatment.

10.4 RNA sequencing-a tool for research continuum

Apart from offering patient specific and disease specific outcomes, obtaining the RNA profile of a particular disease will serve as a valuable tool for furthering

the cause of research pertaining to the particular disease. Most of the data obtained through RNA profiling is specific and permanent. It is not subject to change over a period of time. Thus RNA sequencing profile obtained can be used in further research, aimed at diagnosis and development of targeted therapeutics, thereby enabling continuous research up gradation.

11. Conclusion

RNA sequencing is an advanced diagnostic aid that serves to be a valuable tool in diagnosis, targeted therapy and prognostic marker for Potentially Malignant Oral Disorders. The technique is highly sensitive and specific and provides valuable information regarding the genomic set up of a particular disease. We have summarized the potential genetic alterations in PMODs and highlighted the research efforts undertaken in order to obtain the RNA profile of the particular disease. The knowledge of RNA profile added with the clinical data can serve to be a diagnostic tool par excellence in detecting Potentially Malignant Oral Disorders.

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