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Review

Improving accuracy of RNA-based diagnosis and prognosis of oral cancer by using noninvasive methods

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ABSTRACT

RNA-based diagnosis and prognosis of squamous cell carcinoma has been slow to come to the clinic. Improvements in RNA measurement, statistical evaluation, and sample preservation, along with increased sample numbers, have not made these methods reproducible enough to be used clinically. We propose that, in the case of squamous cell carcinoma of the oral cavity, a chief source of variability is sample dissection, which leads to variable amounts of stroma mixed in with tumor epithelium. This heterogeneity of the samples, which requires great care to avoid, makes it difficult to see changes in RNA levels specific to tumor cells. An evaluation of the data suggests that, paradoxically, brush biopsy samples of oral lesions may provide a more reproducible method than surgical acquisition of samples for miRNA measurement. The evidence also indicates that body fluid samples can show similar changes in miRNAs with oral squamous cell carcinoma (OSCC) as those seen in tumor brush biopsy samples – suggesting much of the miRNA in these samples is coming from the same source: tumor epithelium. We conclude that brush biopsy or body fluid samples may be superior to surgical samples in allowing miRNA-based diagnosis and prognosis of OSCC in that they feature a rapid method to obtain homogeneous tumor cells and/or RNA.

Few successes for RNA-based diagnosis or prognosis of cancer

Well over a decade ago the first studies were published that attempted to allow the identification of head and neck and, more narrowly, oral squamous cell carcinoma (OSCC) based on gene expression [1–3]. These studies relied on surgical biopsy to obtain tissue from which RNA was purified then analyzed. Unfortunately, for a number of reasons, this work did not lead to a method of tumor detection superior to that of histopathology of surgically obtained tissue by single biopsy, which is accurate 90% of the time [4]. While it has long been known that normal tissue expresses different RNAs than OSCC tissue [5], production of an RNA-based classifier to accurately differentiate tumor from normal has seen slow progress despite early advances [6,7]. Distinction between benign and malignant disease is even more difficult to achieve [8]. Work with miRNAs found the same: Classifiers designed to diagnose or provide a prognosis for oral lesions worked well when first developed, but less well when applied to external datasets [8–11]. RNA-based classifiers for other cancers have been approved for usage in the clinic [12,13]. Only one group of tests, which measure breast tumor RNA to predict treatment outcomes, represented by Oncotype DX and Mammaprint have met with wide, well-deserved acceptance as they clearly improve on the standard of care [14–16].

Possible explanation for poor results in diagnosis using OSCC tumor RNA

Over the years there have been many explanations for the poor function of RNA-based classifiers for OSCC detection when tested on external datasets (Table 1). Initially flawed statistical analysis was a major problem [17,18]. When this was largely eliminated as a source of error other causes were suggested to explain the persistent problem. This included usage of different platforms to

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Table 1
Sources of variability in identifying a miRNA signature for HNSCC and OSCC with surgically obtained tissue samples.

Type	Comments/solution
Statistical [17]	Use statistical analysis that avoids overfitting data
miRNA measuring platform [21]	Improvements in miRNA measurement with the usage of RT-PCR & NGS but still must be considered as an error source
Differences in etiology [23–25,27]	Tobacco vs. betel vs. HPV vs. unknown. It is not known if betel usage produces OSCC with a distinct RNA profile. Tobacco does not, HPV does.
Intertumor heterogeneity in RNA expression [23–25,27]	Not a major problem for OSCC
Tumor localization [8,26]	HNSCC can vary by site so focus on OSCC
Low sample number [25] Cancer Genome Atlas Network, 2015)	Small subject size may limit accuracy, large sample numbers available from the TCGA study
Tissue dissection [1]	a. Varying amounts of normal epithelium – as a result works better to detect miRNAs that increase in expression in tumors b. Varying amounts of stroma in surgical samples– best to remove with laser capture microdissection, Not necessary with brush biopsy

measure RNA, inter-tumor heterogeneity in RNA expression, variability in sample dissection, the limited size of most sample datasets, and cohort differences in head and neck cancer etiology etc. [1,10,19]. With time, platform-dependent differences in RNA measurement results have been greatly reduced, though those for miRNA still can occur [20–22]. Recent works suggest that inter-tumor heterogeneity and varied tumor etiology seem to contribute little to problems in RNA-based OSCC detection. Inter-tumor heterogeneity in RNA expression was originally reported for head and neck tumors in 2004 but was not corroborated till 2014 [23,24]. Four to six different classes of HNSCC tumors have been proposed based on mRNA and to some degree miRNA expression [23–26]. Most if not all of these subclasses are also found among oral tumors. The question arose that if these subclasses show substantially different RNA expression, then RNA-based detection of OSCC may be made difficult. Large studies like that of The Cancer Genome Atlas (TCGA), where hundreds of OSCC samples were measured, allowed construction of classifiers with a subset of samples for training the classifier, then usage of a second subset of samples from the same dataset for external validation. These, of course, would be obtained, dissected and prepared by the same group [25]. When this was done using RNA profiles from a subgroup of TCGA database, external validation with a miRNA-based classifier revealed near 100% accuracy in differentiating normal tissue from tumor tissue [27]. The accuracy of class prediction in two somewhat smaller studies was almost as good [8,28], suggesting that within a single study group it is possible to construct a highly accurate classifier to differentiate OSCC from normal. The issue of tumor etiology causing distinct patterns of RNA expression has also been explored. Human papilla virus (HPV)-expressing squamous cell carcinomas of the oral pharynx may indeed have distinct RNA profiles [24–26] so we mainly focus in this review on OSCC, which is almost always free of transforming HPV [24]. A further investigation of tumor etiology reveals little effect from tobacco exposure on miRNA or mRNA expression in OSCC [25,29]. However, it is not clear if the same is true for betel nut exposure, which may contribute to variability between studies.

Heterogeneous tissue in tumor samples leads to difficulty in RNA-Based diagnosis

The major source of the problems that have long been seen in RNA-based OSCC class prediction may be the variability in sample dissection within studies and between studies. Problems arise when RNA markers defined in one study are tested for classification of tumor samples from a different study, as has been observed [1]. Differences in platforms used to measure RNA can contribute to error, but another problem often overlooked may cause even more. While the tumor itself is epithelium, there can be variable amounts of stroma in samples. RNAs that are highly expressed in stroma but not in epithelium will obscure changes that occur in tumor epithelium. Descriptions of acceptable levels of stromal tissue in tumor samples are varied between studies, if present at all. The TCGA head and neck squamous cell carcinoma (HNSCC) study indicated that when sample acquisition is uniform, differentiation of normal versus malignant based on RNA is extremely accurate [25,27]. When we tested marker RNAs identified by Lager et al. for European OSCC, mainly tobacco users, versus those miRNAs measured in the TCGA study, over 30 percent were no longer differentially expressed [8,25]. In a study by Gombos et al. on 40 OSCC and 40 normal tissue controls, done in Europe, 4 miRNAs were shown to be differentially expressed at least 100% [28]. When we examined expression of these miRNAs in the TCGA dataset only one showed differential expression over 50% [25]. Perhaps because of this, only one microRNA, mir-21-5p, has been shown to be elevated in almost every study on OSCC or HNSCC [8,25,28,30,31]. Notably, mir-21-5p is expressed at high levels in epithelium and stroma, and is known to be further elevated in both tumor epithelium and the surrounding stroma. Thus, for mir-21-5p measurement, the presence of stroma may not be so important. Interestingly, in the study reported by Gombos et al. 4 miRNAs individually served as markers of OSCC with approximately 90% accuracy [28]. Remarkably when we tested them as a group for tumor classification of the TCGA sample set we found 92% accuracy [25]. However, accuracy of the classifier created using the recommended markers was largely dependent on mir-21-5p levels. When it was eliminated as a marker and the three remaining miRNAs, miR-221, miR-191, and miR-226, were used, accuracy slipped to 75%. We believe these results display the difficulty in applying results from one study to another. While other explanations are possible, we believe that the difficulty in achieving uniform tissue dissection between different research studies is a major factor. A second source of error is the variability in the amount of normal epithelium mixed in with the tumor epithelium, though this may be minimized by cancer field effects [32]. Nevertheless, this source of error makes it difficult to find RNAs that decrease in tumor since normal brush biopsy samples are 100% normal but malignant samples are a mix. Both of these sources of error can be limited by careful reproducible dissection of tumor samples as in the TCGA study where all tumor samples from which RNA was taken contained at least 60% tumor. In the TCGA study, laser capture microdissection of samples was done to insure this, a method at this time too cumbersome to do routinely [25].