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Next generation sequencing-based salivary biomarkers in oral squamous cell carcinoma

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Abstract (J Korean Assoc Oral Maxillofac Surg 2022;48:3-12)

Selection of potential disease-specific biomarkers from saliva or epithelial tissues through next generation sequencing (NGS)-based protein studies has recently become possible. The early diagnosis of oral squamous cell carcinoma (OSCC) has been difficult, if not impossible, until now due to the lack of an effective OSCC biomarker and efficient molecular validation method. The aim of this study was to summarize the advances in the application of NGS in cancer research and to propose potential proteomic and genomic saliva biomarkers for NGS-based study in OSCC screening and diagnosis programs. We have reviewed four categories including definitions and use of NGS, salivary biomarkers and OSCC, current biomarkers using the NGS-based technique, and potential salivary biomarker candidates in OSCC using NGS.

Key words: Next generation sequencing, Saliva, Biomarkers, Early diagnosis, Oral squamous cell carcinoma

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I. Introduction

Oral squamous cell carcinoma (OSCC) accounts for 90%-96% incidence of whole head and neck cancers, but there are no sensitive biomarkers for detection of OSCC. Definitive diagnosis has only been possible after examination of removed specimens. This diagnosis has been based on pathological findings such as angiogenesis, proliferation, and metastasis.

Even with radical resection combined with chemotherapy and concurrent radiation therapy, many OSCC cases demonstrate metastasis or recurrence without related or predictive symptoms. Complete eradication, early diagnosis, and metastatic and prognostic factor discovery have been research aims for OSCC management; until now, however, fulfillment of these aims has been elusive.

Like many cancers, early-stage OSCC is difficult to detect. A significant number of patients do not seek clinical care until the OSCC is in an advanced stage. The development and advancement of screening and early diagnosis approaches has been recommended as the most effective strategy for reducing the OSCC-related morbidity and mortality rate[1]. Biomarkers found in the saliva are an ideal non-invasive diagnostic tool for early diagnosis of cancer[2-4]. Compared to blood and tissue sampling, saliva sampling is a reliable, non-invasive, convenient, and economical alternative for disease diagnosis and prognosis determination[5]. Saliva sampling also provides an effective and easily-acquired liquid specimen for large-scale sampling, epidemiologic screening and long-term monitoring[6].

Several DNA or RNA sequencing techniques using salivary specimens have been used for karyotyping or submicroscopic chromosomal copy number changes such as microdeletions. These techniques include immunoprecipitation high-performance liquid chromatography, radioimmunoassay, electrophoretic immunoassay, mass spectrometry-based proteomics, microchips or microarrays, microfluidic devices, electrochemical biosensors[7,8], fluorescence *in situ* hybridization and comparative genomic hybridization microarrays. Maxam and Gilbert[9] DNA sequencing was first developed in 1976 and is based on nucleobase-specific partial chemical modification of DNA and subsequent cleavage of the DNA backbone at sites

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adjacent to the modified nucleotides. This sequencing method was advanced by Sanger et al.[10] in 1977. Sanger sequencing[10], also known as chain termination sequencing, was marketed commercially by Applied Biosystems in 1986[11] and was the most popular DNA sequencing method until now. These two methods are based on the dideoxy method, the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication[10,12]. These first generation sequencing methods have been improved upon in next-generation sequencing (NGS).(Fig. 1)

NGS is a massive and parallel DNA sequencing technology for large-scale, ultra-high throughput, and automated high-speed genome analyses. NGS is a less expensive method for determining the order of nucleotides in entire genomes or targeted regions of DNA or RNA and has revolutionized the biological sciences. NGS has a wide variety of applications for the study of biological systems at a new level. NGS can be used to sequence entire genomes or specific areas of interest[13]. Sanger sequencing continues to be useful for smaller-scale, short-read sequencing analysis and for the validation of NGS results[14].

The effectiveness of NGS in whole exome sequencing by targeted sequencing of cancer-related genes and in RNA sequencing has been demonstrated. NGS-applied OSCC research has also identified various genetic alterations and detected mutations with low variant allele frequency[15]. NGS is also becoming an essential method in characterization of salivary gland tumors[16]. However, NGS has only rarely been applied to the identification of salivary biomarkers of OSCC. We discuss four topics in this review article: definitions and use of NGS, salivary biomarkers and OSCC, current biomarkers using NGS-based technique, and potential candidates as NGS-based salivary biomarkers.

II. Definitions and Use of NGS

In the field of functional genomics, NGS is the most useful method for DNA and RNA analysis. This highly reproducible tool can reveal single nucleotide polymorphisms, related gene variants or spliced transcripts without input of direct DNA or RNA features as in microarray procedures. NGS can also be applied to cDNA molecules for RNA sequencing by reverse transcription from candidate RNA and sequencing-library construction by massive parallel deep sequencing. The most popular NGS methods are summarized in Table 1[17-31]. Illumina Solexa sequencing uses a fluorescence-based Illumina platform to identify 100-150 bp of DNA by emitting a particular fluorescent signal to each chain of nucleic acid. Adaptors can fragment, ligate and anneal longer sequences randomly. Reading is carried out by polymerase chain reaction (PCR) amplification and creation of a unique spot that repeats. The product can be separated into a single strand for final sequencing.(Fig. 1)

The sequencing-by-sequence methods using pyrosequenc-

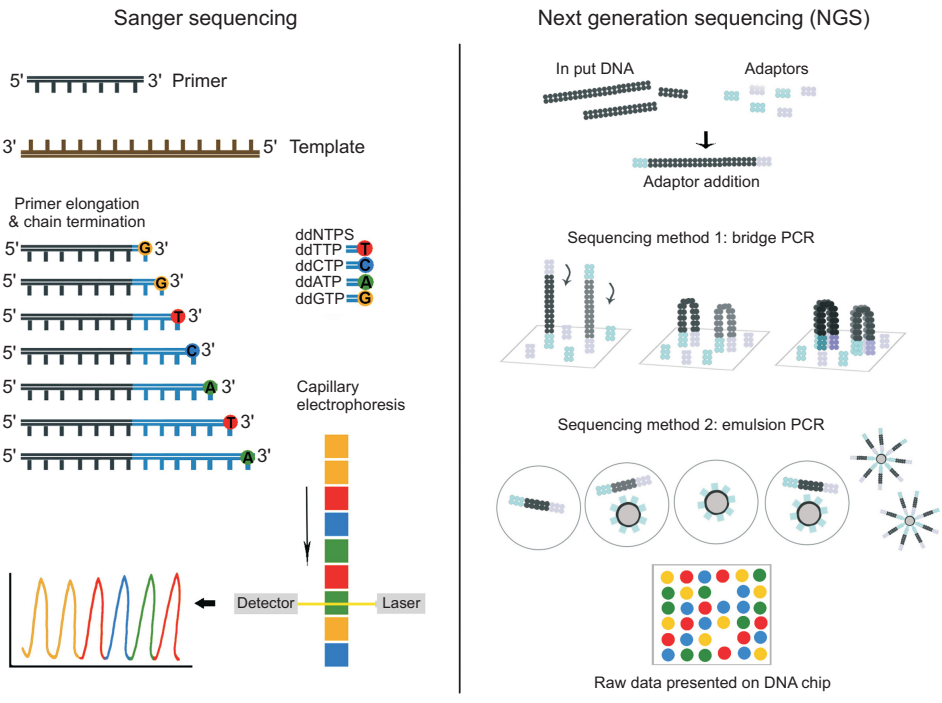

Fig. 1. Schematic drawing of the Maxam and Gilbert's chemical chain termination method for DNA sequencing developed in 1977 followed by Sanger's 'dideoxy method'[9,10]. (PCR: polymerase chain reaction)
Buyanbileg Sodnom-Ish et al: Next generation sequencing-based salivary biomarkers in oral squamous cell carcinoma. J Korean Assoc Oral Maxillofac Surg 2022

Table 1. Next generation sequencing platforms[17]

Platform	Template preparation	Detection method	NGS coverage (base)	Run time (day)	Gb per run	Essential descriptions
Roche 454 (Roche, Basel, Switzerland)[18]	Emulsion-based clonal amplification	Pyrosequencing	400*	0.42	0.40-0.60	First commercial platform for the NGS technology. The DNA amplification process is different from that of Illumina, which can sequence much longer reads[19].
GS FLX Titanium (Roche)[20]			400*	0.42	0.035	Able to sequence 400-600 million base pairs per run with 400-500 base pair read lengths[21].
Illumina MiSeq (Illumina, San Diego, CA, USA)[22]	Clonal bridge PCR	Reversible dye terminator	2×300	0.17-2.7	15	End-to-end sequencing solutions with reversible-terminator sequencing-by-synthesis. Smallest benchtop sequencer that can perform onboard cluster generation, amplification, genomic DNA sequencing, and data analysis in a single run. Performs both single- and paired-end runs with adjustable read lengths from 1×36 base pairs to 2×300 base pairs[22].
Illumina HiSeq (Illumina)[23]			2×150	0.3-11	1,000	Generate up to 1,000 Gb per run with the highest yield of data greater than Phred quality score of 30 (Q30). 1 hour's cycle time can be reduced to 10 minutes[23].
Illumina Genome Analyzer IIX (Illumina)[24]			2×150	2-14	95	Having a broad spectrum of genomic variation with short- and long-insert paired-end reads with insert sizes 200 bp to 5 kb. Used for studying the genome, epigenome, and transcriptome, and also yield greater than 85% of bases higher than Q30 at 2×50 bp[24].
Life Technologies SOLiD4 (Life Technologies, Waltham, MA, USA)[25]	Emulsion-based clonal amplification	Oligonucleotide ligation detection	35-50	4-7	35-50	Generates 10^8-10^9 small sequence reads at one time, and two-base base encoding to decode the raw data. This system utilizes four fluorescent dyes to interrogate all sixteen (4^2) possible two-base combinations, by a number of probes. Each probe is eight nucleotides long (8-mer)[26].
Life Technologies Ion Protons (Thermo Fisher Scientific, Waltham, MA, USA)[27]		Native deoxy-ribonucleotide triphosphates, proton detection	200	0.5	100	Does not use fluorescence or chemiluminescence. Instead, measures the H+ ions released during base incorporation. The lack of any optics allows rapid expansion of the output by approximately 10-fold every six months[27].
Complete Genomics (Complete Genomics, San Jose, CA, USA)[28]	Gridded DNA-nanoballs	Oligonucleotide ligation detection	7×10	11	3,000	A DNA nanoball sequencing, which assembles short DNA sequences into a full genome. The sequences are obtained by probe-ligation, but the clonal DNA amplification is performed by rolling circle amplification unlike the bead or emulsion amplification[28].
Helicos Biosciences Heliscope (Helicos Biosciences, Cambridge, MA, USA)[29]	Single molecule	Reversible dye terminator	35*	8	25	A highly sensitive fluorescence detection system for direct interrogation of single DNA molecules via sequencing by synthesis[30].
Pacific Biosciences SMRT (Pacific Biosciences, Menlo Park, CA, USA)[31]		Phospholinked fluorescent nucleotides or real-time sequencing	10,000 (N50); 30,000+ (max)	0.08	0.5	Long-read sequencing platforms with SMRT sequencing technology. Template preparation does not require any amplification steps, and the prepared library molecule is the sequencing template. The adapters have a hairpin structure (SMRT loop adapters) so that after ligation the double stranded DNA fragments will have become circular[31].

(PCR: polymerase chain reaction, NGS: next generation sequencing, SMRT: single molecule real-time)
*Average read lengths for the Roche 454 and Helicos Biosciences platforms.
Q30 is equivalent to the probability of incorrect base call 1 in 1,000 times with 99.9% base of accuracy. Run times and gigabase (Gb) output per run for single-end sequencing are noted. Run times and outputs approximately double when performing pair-end sequencing.

ing is the basis of Roche 454 platform sequencing. The release and incorporation of pyrophosphate into a new strand by polymerase is detected by fluorescence. Multiple reads over 1,000 bp long can be carried out by optical signal detection. One DNA fragment per bead can be annealed, and generic adaptors can add bases to the ends. The product is amplified by PCR as a routine adaptor-specific primers application.

The principle of measuring the direct hydrogen ion release from individual bases, not fluorescence, is the basic principle of Ion torrent and proton platform sequencing. Direct measurement of the emission of hydrogen ions by polymerase during the incorporation of deoxynucleoside triphosphate to a growing DNA strand. This is executed by uniform 200 to 400 bp fragmentation, and adapters are added to effect amplification by emulsion PCR.

III. Salivary Biomarkers and Oral Cancer

Saliva sampling is rapidly expanding; and arrays of analytes including proteins, messenger RNA (mRNA), and DNA in saliva have been studied for their potential use as biomarkers for OSCC screening programs[32,33]. OSCC is a malignancy in which salivary diagnosis has the greatest potential because the OSCC environment contains saliva. One of the useful aspects of saliva is its containment of exfoliated tissues or cells from the oral cavity. This aspect suggests the existence of potential salivary biomarkers for OSCC[33]. A number of candidate salivary biomarkers for oral cancer, including genomic and proteomic biomarkers for OSCC, have been reported and are summarized in Table 2.

Proteomic studies using human saliva have been targeting the biological activities of various peptides and proteins in normal individuals and in those with various pathologies. Proteomic targets such as CD59, catalase, M2BP, and MRP14 were suggested from a shotgun proteome analysis for oral cancer detection[34]. Li et al.[35] reported the presence of more than 3000 RNA species, mostly mRNAs; and reports of Park et al.[36] and Patel et al.[37] support the role of microRNAs (miRNAs) in oral cancer progression and various cancers.

Several salivary biomarkers such as DAPK, TIMP3, p16, and MGMT are potential screening markers for OSCC[38], and changes in DNA methylation patterns could be a useful screening tool for predicting the rate and the likelihood of malignant transformation. The methylation of DNA in saliva has been suggested as an effective biomarker for early detection of OSCC[39]. For example, hypermethylation of the DNA promoter of certain genes, such as p16, has been found in saliva as well as serum[40,41]. Promoter hypermethylation has been suggested to be an early event during OSCC genesis. Any change of transcription factor or receptor functions or other factors in tumorigenesis might be involved in loss of cell cycle control. This loss of control was reported by Liao et al.[42]; this group suggested the first salivary biomarker, genomic p53. Other cell cycle regulatory proteins such as mammary serine protease inhibitor and cyclin D1 were also increased in OSCC saliva[43].

Due to the basic precursor characteristics of mRNA for protein expression, other potential salivary biomarkers display increased mRNA production. These include IL-8, IL-1β, S100P, and spermidine/spermineN1-acetyltransferase[44]. The circulating levels of CD44 are reported to be related to head and neck cancer metastasis, and CD44 is a potential salivary protein biomarker[45]. Actin and myosin are also regarded as promising salivary biomarkers for premalignant differentiation and malignant oral lesions[33] IL-6 is another premalignant

Table 2. Candidate salivary biomarkers for oral cancer, based on the biomolecule markers[1]

Type of biomolecular markers	Biomarkers
Genomic	p53
	Promoter hypermethylation of DAPK, TIMP3, p16, and MGMT genes
	Cyclin D1 gene amplification
	Maspin
mRNA	IL-8
	IL-1β
	S100P
	SAT
	miR 31, miR 125, miR 200a
Protein	Elevated CD44
	IL-6
	Intermediate filament protein (Cyfra 21-1)
	8-OHdG
	Albumin
	Glutathione
	Actin and myosin
	L-phenylalanine
	EFNB2, ANGPT1, ANGPT2, CD31, VEGF
Others	HPV and EBV
	Inorganic compounds: Na, Ca, F, Mg
	Fucose

(DAPK: death-associated protein kinase, TIMP3: tissue inhibitor of metalloproteinase-3, MGMT: O6-methylguanine-DNA methyltransferase, Maspin: mammary serine protease inhibitor, IL-8: interleukin 8, IL-1β: interleukin 1 beta, S100P: S100 calcium binding protein P, SAT: spermidine/spermineN1-acetyltransferase, miR: microRNA, CD44: cluster of differentiation 44, 8-OHdG: 8-Oxo-2'-deoxyguanosine, ANGPT: angiopoietin, VEGF: vascular endothelial growth factor, HPV: human papilloma virus, EBV: Epstein–Barr virus)

Buyanbileg Sodnom-Ish et al: Next generation sequencing-based salivary biomarkers in oral squamous cell carcinoma. J Korean Assoc Oral Maxillofac Surg 2022

Table 3. NGS-based salivary genomic markers in oral cancer

Potential genomic markers[1]	Sample/ Collection method	NGS-based studies
p53 (TP53, P53; BCC7; LFS1; BMFS5; TRP53)[46,47]	Tumor tissue/ Formalin fixation and paraffin embedding	Approximately 30% of salivary gland cancers have mutations of p53 gene. A significant worse overall and disease-free survival was shown in cancers with p53 mutations.
Promoter hypermethylation of DAPK gene[48,49]	UWS/ Oragene DNA Self-Collection kit Salivary rinse/ 10-20 mL 0.9% NaCl, 15-60 s	DAPK methylation gene was reported with the development of cancers in women.
Promoter hypermethylation of TIMP3 gene (SFD; K222; K222TA2; HSMRK222)[50-52]	Salivary rinse/ rinsing and gargling with 20 mL 0.9% NaCl	Promoter hypermethylation of TIMP3 levels found in the circulating tumor DNA in saliva of disease-free survival HNSCC patients.
Promoter hypermethylation of p16 (CDKN2A, ARF; MLM; P14; P19; CMM2; INK4)[47,53]	Tumor tissue/ Formalin fixation and paraffin embedding Salivary rinse/ swishing with 15 mL of 0.9% normal saline, 15-30 s	The absolute frequencies of CDKN2A mutations are detected by NGS.
Promoter hypermethylation of MGMT gene[54]	Tumor tissue/ Formalin fixation and paraffin embedding Salivary rinse/ swishing with 15 mL of 0.9% normal saline, 15-30 s	MGMT methylation was detected in 29% of oral cancer/ dysplasia patient, showing MGMT gene is related to DNA repair and aberrant promoter hypermethylation.
Cyclin D1 gene amplification (CCND1, BCL1; PRAD1; U21B31; D11S287E)[55,56]	Tumor tissue/ Formalin fixation and paraffin embedding	14 of the 21 genes had copy number amplifications and losses, which included CCND1 (83.7%).
Maspin[57,58]	UWS/ samples were collected in the morning, without any oral stimulation for 90 minutes before collection	Downregulation of maspin; the functional importance of maspin includes the inhibition of tumor angiogenesis. The levels of maspin were reduced.

(NGS: next generation sequencing, DAPK: death-associated protein kinase, UWS: unstimulated whole saliva, TIMP3: tissue inhibitor of metalloproteinase-3, HNSCC: head and neck squamous cell carcinoma, MGMT: O-6-methylguanine-DNA methyltransferase, Maspin: mammary serine protease inhibitor)

Buyanbileg Sodnom-Ish et al: Next generation sequencing-based salivary biomarkers in oral squamous cell carcinoma. J Korean Assoc Oral Maxillofac Surg 2022

differentiation biomarker found especially in the saliva of oral leukoplakia patients. IL-6 inactivated the p53 tumor suppressor gene by hypermethylation of its promoter region resulting in uncontrolled cell proliferation and suppression of programmed cell death[33].

Researchers have also directed their research toward detection of human papilloma virus (HPV) and Epstein–Barr virus (EBV) in saliva; these viruses are etiological factors in cancer. The incidence for HPV positivity has been reported to be more than 45% in patients treated for oral cancer[33].

IV. Current Cancer Biomarkers Using NGS-based Technique

Currently, several authors have reported data from the search for biomarkers using a NGS-based technique[36,46-69]. (Tables 3, 4) Most of these studies started from Gene Expression Omnibus datasets (https://www.ncbi.nlm.nih.gov/gds) in combination with differentially expressed genes, the protein-protein interaction network, gene ontology and the Kyoto Encyclopedia of Genes and Genomes pathway. Shanmugam et al.[70] have reported a customized NGS analysis of unique coding regions of seven mutated genes from OSCC saliva, and similar data from whole-exome sequencing were also discovered in tumors from The Cancer Genome Atlas[71], the International Cancer Genome Consortium gingiva-buccal cohort[69], and MD Anderson Cancer Center OSCC cohort[72].

From this review, NGS-based salivary biomarkers in OSCC could be categorized primarily as genomic (Table 3) and transcriptome markers.(Table 4) Within these main categories, we could present promoter hypermethylation of p16 and three miRNAs as the main candidate salivary biomarkers. Especially, miRNAs, endogenous, non-coding, single-stranded RNA molecules 22 nucleotides long, have tumor-controlling characteristics including tumor-suppression.

Hypermethylation of the p16 promoter is a useful serum biomarker for the early detection of alimentary tract cancer, especially gastric cancer. Zammit et al.[73] reported on the etiology of OSCC using NGS while focusing on smoking and HPV factors in a prospective observational study. This group showed that the most frequent mutations were found in TP53 and CDKN2A in the salivary specimen.(Table 3) Fadhil et al.[74] reported five miRNAs using NGS data in saliva of 12 HNSCC patients and 12 healthy controls. Among these, miR-let-7a-5p and miR-3928 were suggested to be NGS-based salivary biomarkers for early diagnosis of HNSCC when compared with other miRNAs such as miR-7703, miR-345-5p, and miR-1470.(Table 4) A recent study also showed the

Table 4. NGS-based salivary mRNA markers in oral cancer

Potential mRNA markers[1]	Sample/ Collection method	NGS-based studies
IL-8 (IL8)[59,60]	UWS/ samples were collected between 9 am and 10 am, following the standard protocol.	In oral, esophageal, lung, pancreatic, ovarian and breast cancers, certain salivary mRNA biomarkers have been proposed as a possible cancer biomarker, including IL-8.
IL-1β (IL-1)[61,62]	UWS/ 3-5 mL salivary specimen was collected into a tube containing 10 mL of RNA*later* (Ambion, Austin, TX, USA), an aqueous tissue storage reagent that rapidly permeated tissue to stabilize and protect cellular RNA. The specimen was then placed on ice at 4°C; cell-free saliva supernatant was harvested.	IL-1β gene has been found to be an important biomarker for ovarian cancer.
S100P (S100 calcium binding protein P)[63,64]	UWS/ samples were collected between 6 am and 12 pm following standard protocol. A maximum of 8 mL of saliva were collected within 30 minutes.	Salivary S100 mRNA is a candidate biomarker for detecting OSCC development and in OLP patients determined by NGS.
SAT[69]	UWS/ Participants were asked to refrain from eating, drinking and any oral hygiene overnight and spit in 5-mL plastic vials used for biochemical examinations for 5 minutes. During the whole procedure and until centrifugation the vials were kept in ice.	The combination of SAT and IL-8 mRNA biomarkers are attractive candidates either for screening or for early diagnosis purposes. These exert a very good prediction ability together with a high sensitivity and specificity for screening oral squamous cell carcinoma.
miR 31 (MIRN31; miR-31; hsa-mir-31)[65,66]	UWS/ 3-5 mL saliva was collected from mouth floor after simple rinsing. Pre-treatment salivary sample was collected from 45 patients with OSCC and 10 patients with oral verrucous leukoplakia and 24 healthy participants.	A significantly high expression of miR-31 was found in the saliva sample of patients with OSCC at all clinical stages by RT-qPCR. More miR-31 levels were detected in the saliva than in plasma, suggesting salivary miR-31 to be a more sensitive.
miR 125 (MIRN125A; mir-125a; miRNA125A)[36,67]	UWS/ an aqueous tissue storage reagent was used to preserve UWS samples and SUPERase.In (Themo Fisher Scientific, Waltham, MA, USA) was used for supernatant saliva preservation.	Significantly lower levels of miR-125a was found in saliva sample of OSCC patient than that of healthy controls.
miR 200a[36,68]	UWS/ an aqueous tissue storage reagent was used for the UWS samples and SUPERase.In (Themo Fisher Scientific) was used for supernatant saliva preservation.	Significantly lower levels of miR 200a was found in saliva sample of OSCC patient than that of healthy controls.

(NGS: next generation sequencing, IL-8: interleukin 8, IL-1β: interleukin 1 beta, UWS: unstimulated whole saliva, OSCC: oral squamous cell carcinoma, OLP: oral lichen planus, miR: microRNA, RT-qPCR: quantitative real-time reverse transcriptase-polymerase chain reaction)
Standard protocol was summarized as 1) no drinking neither using any oral hygiene care on day of saliva collection, 2) mouth rinsing with water on 5 minutes prior, and 3) upright sitting position and spitting into a 50-mL Falcon tube kept on ice.
Buyanbileg Sodnom-Ish et al: Next generation sequencing-based salivary biomarkers in oral squamous cell carcinoma. J Korean Assoc Oral Maxillofac Surg 2022

Table 5. Known salivary biomarker and recommended NGS-based potential salivary biomarker

	Genomic	Transcriptome (mRNA)
Known salivary biomarkers	p53 FAT1 CASP8 PIK3CA HRAS NOTCH1 CDKN2A[70]	miRNA (saliva): miR-let-7a-5p and miR-3928[74] Oncogenic (up-regulated, tissue): miR-21, miR-22, miR-26a, miR34c, miR-34b, miR-117, miR-118, miR-130b, miR-135, miR-142, miR-143, miR-148a, miR-150, miR-221, miR-222, miR-423, miR-542, miR-1269a[81] Suppressive (down-regulated, tissue): miR-92b, miR-199, miR-214, miR-375, miR-486, miR-504, miR-499, miR-486[81]
Recommended NGS-based salivary biomarker	Mutation of p53 gene Promoter hypermethylation of DAPK, TIMP3, p16, and MGMT genes Cyclin D1 gene amplification Mammary serine protease inhibitor	miR 31[76], miR 125[77], miR 200[78,79]

(NGS: next generation sequencing, FAT1: AT atypical cadherin 1, CASPS: caspase 8, PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, HRAS: Harvey rat sarcoma viral oncogene homolog, NOTCH1: Notch homolog 1, translocation-associated, CDKN2A: cyclin-dependent kinase inhibitor 2A, DAPK: death-associated protein kinase, TIMP3: tissue inhibitor of metalloproteinase-3, MGMT: O6-methylguanine-DNA methyltransferase, miR: microRNA)
Buyanbileg Sodnom-Ish et al: Next generation sequencing-based salivary biomarkers in oral squamous cell carcinoma. J Korean Assoc Oral Maxillofac Surg 2022

potential benefits of NGS for mRNA expression profiling by using miR143-3p to detect chronic periodontitis[75].

Lu et al.[76] reported that miR-31-5p may be an independent OSCC biomarker by showing its tumor growth inhibition capacity in oral cancer patient-derived xenograft models. Wang et al.[77] showed that mature miR-125b could control metabolism and immunity of cancer cells by regulating NF-κB or p53 signaling pathways through translation inhibition of 3′ untranslated regions of target mRNAs. Korpal and Kang[78] also showed that miR-200 family members inhibit epithelial-mesenchymal transition (EMT) and metastasis. This occurs through a miRNA-mediated regulatory pathway by direct targeting of transcriptional repressors of E-cadherin, ZEB1, and ZEB2. The validity of this report was strengthened by Kabzinski et al.[79] who showed that DNA methylation of the miR-200c promoter in an epithelial originated tumor may occur during EMT. Majewska et al.[80] discovered a potentially targetable novel anaplastic lymphoma kinase fusion in an intraductal carcinoma of a minor salivary gland by NGS analysis.

Compared with known genomic salivary biomarkers, such as p53, FAT1, CASP8, PIK3CA, HRAS, NOTCH1, and CDKN2A, NGS-based genomics consider mutation of p53 and promoter hypermethylation of DAPK, TIMP3, p16, and MGMT, cyclone D1 and mammary serine protease inhibitors. Also, up-regulated oncogenic tissue miRNAs and down-regulated tissue suppressive marker miRNAs, including miR 31, miR 125, and miR 3928, are recommended as subjects for further research[70,74,76-79,81].(Table 5)

V. Discussion

This review article aimed to summarize current applications of NGS in cancer research and to propose potential genomic and proteomic saliva biomarkers for NGS-based study in OSCC screening and diagnosis programs. For the establishment of a standardized research and designed protocol for the detection of trace protein or nucleic acids biomarkers from saliva samples, efficient and stable collection, processing and preservation methods should first be confirmed. For the collection of saliva samples, confirmation of the patient's mouth cleanness is essential. This can be accomplished by rinsing the mouth with water to remove substances and by ensuring that the patient avoids any eating or drinking for at least 30 minutes prior to sampling. Approximately 2.5 mL of saliva in the buffered solution is recommended. Preparation should include addition of 2.5 mL of DNA stabilization buffer into a 10- or 15-mL conical tube. Excessive saliva might to be degraded due to insufficient DNA stabilization buffer, and insufficient saliva collection may not provide adequate results. The cap needs to be replaced, mixing needs to occur by inversion not shaking, and the specimen needs to be stored at room temperature for immediate use or at 4°C for future use.

NGS-based research has been used to identify factors in metastasis of lung, prostate, ovarian, and bile duct cancer. NGS-based evaluation of candidate biomarkers in saliva could also be routinely used as a simple, non-invasive test in patients with OSCC. NGS has also been used as a valuable tool for detecting novel biomarkers in periodontal disease[75]. More recently, NGS-based research has led the ability to differentiate patients with primary Sjögren's syndrome from those with non-Sjögren's sicca[82].

In various medical and non-medical institutions, prevention of unnecessary medical expenses may be possible by the quick and easy diagnosis of oral cancer in healthy or suspect patients. There would be no additional cost to the public health system, and provision of information on the potential of benefits to patients with OSCC by using NGS based salivary biomarkers can be effected. Since both proteins and genes can be applied as biomarkers, the accuracy of diagnosis can be increased. New biomarkers can be added quickly for expansion of a preventive platform for a variety of medical and dental diseases. Since both proteins and genes can be applied as biomarkers, the accuracy of diagnosis can be increased, and new biomarkers can be added quickly, so that it can be expanded to a preventive platform through early diagnosis of various diseases as well as various medical and dental treatments.

In conclusion, selection of potential OSCC biomarkers through NGS-based protein studies from epithelial tissue cells in collected saliva will be possible. These non-invasive methods could be a useful tool for the improvement of an immune biosensor for ensuring general public health and for diagnosis of OSCC before its metastasis or progressive infiltration to adjacent tissues. Based on NGS analysis, we will be able to verify the biomarkers of OSCC in saliva and to effect early diagnosis of oral cancer based on this.

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Authors' Contributions

B.S.I. and M.Y.E. participated in data collection and wrote the manuscript. H.M. and J.H.L. participated in the study design and helped to draft the manuscript. S.M.K. coordinated and approved the final manuscript.

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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