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Role of salivary transcriptomics as potential biomarkers in oral cancer: a systematic review

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ABSTRACT

Introduction: Transcriptomes in saliva can be used as potential biomarkers for both diagnostic and response to treatment in oral squamous cell carcinoma (OSCC). In this review, we explored their application in this increasingly common disease

Materials and methods: PubMed, EMBASE, Scopus, Web of Science and grey literature from January 1990 to May 2017 were searched. Two independent reviewers performed the study selection according to eligibility criteria.

Results: A total of nine studies were included. Three studies showed increased expression of DUSP1, IL8, IL1B, OAZ1, SAT1, S100P and two showed increased expression of miRNA-31 amongst study groups compared to normal healthy controls. The sensitivity ranged from 14% - 100%, while the specificity ranged from 38% - 100%. miRNA-27b had the highest AUC (write in full) of 0.9643 and DUSP1 had the minimum AUC of 0.41.

Conclusion: Salivary transcriptomics may play an effective role as a robust and non-invasive biomarker sighting tool for the diagnosis and management of OSCC.

Key words: Biomarker; Oral squamous cell carcinoma; RNA; Saliva; Transcriptomics, Systematic review

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer with a global incidence of approximately 275,000 new cases.[1,2] Developing countries in South East Asia have the highest incidence of OSCC, with oral cancer being the most common cancer among men and accounting for nearly 25% of all new cases annually.[3] In addition, many countries in the European Union and parts of the United States have reported a rising incidence in oral and oropharyngeal cancer and mortality rates in young adults.[4-6] In addition, the five-year survival rate of cancers of the oral cavity and oropharynx is approximately 50%.[2,7] In an attempt to reduce the high mortality and morbidity rates of oral cancer, it is critical to develop tools that can accurately identify and distinguish OSCC in its early stages.

Saliva may provide pertinent information regarding the disease status of oral mucosa due to its direct contact with oral lesions. An increasing number of studies are utilizing saliva as a potentially diagnostic tool for diseases including oral cancers,[8] pancreatic cancer,[9] Sjögren's syndrome,[10] HIV ,[11,12] Hepatitis A, B, and C,[13-15] diabetes mellitus,[16] Alzheimer disease.[17] Saliva contains high levels of miRNAs that are non-coding single-stranded RNAs comprised of approximately 20 nucleotides, that can be used as potential biomarkers for the diagnosis of OSCC as well as evaluating response to different treatment modalities and predicting disease prognosis.[18]

In recent years, transcriptomics has emerged as a robust and cost-effective biomarker sighting tool capable of simultaneously quantifying a large set of transcriptomes in a miniaturized, automated format. A transcriptome is defined as mRNA in a cell which is the template for protein synthesis through translation and gene expression. Transcriptomics have proven benefit in oncology, mainly due to the relative ease of collecting samples.[19,20] In addition, the technique has also shown promise in identifying genetic fingerprints that are predictive of

disease outcome using gene expression profiling .[20] The aim of this study was to systematically quantify the existing literature and assess the role of salivary transcriptomics in the diagnostic and prognostic prediction of OSCC.

MATERIAL AND METHODS

Protocol and registration

International Prospective Register of Systematic Reviews (PROSPERO) database was searched for any registered protocols on similar topic. In addition, the current systematic review was registered as a protocol in the PROSPERO platform (ID: 121630). The systematic review was reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement .[21]

Eligibility Criteria

Inclusion criteria

The PECOS framework (Population, Exposure, Comparison, Outcomes, Studies) was used to formulate the focused question of the review, of which: P) Patients with diagnosis of OSCC; I) Transcriptomics analysis; C) Patients with no history of HNC; O) Diagnostic and prognostic prediction of OSCC; S) Observational studies and/or Clinical trials.

Observational studies (case-control, cross-sectional or population-based) and/or clinical trials (randomized controlled trial) that recruited patients with clinically and/or histologically confirmed diagnosis of OSCC and evaluated the diagnostic and prognostic prediction by utilizing analytics technology of transcriptomics were included. Only articles published in English language were included.

Exclusion criteria

The following exclusion criteria were applied: (1) Studies that did not evaluate the analytics technology of transcriptomics; (2) case-reports, reviews, experimental studies, short communications and personal opinions, letters to the editor, and conference abstracts.

Focused question

The focus question was: *"*Does salivary transcriptomics play a role in diagnostic and prognostic prediction of oral cancer*?"*

Search strategy

Detailed automated literature searches were performed in PubMed, EMBASE, Scopus, and Web of Science from January 1990 up to and including May 2017. An additional search of the grey literature was carried out on Google Scholar, ProQuest, and OpenGrey. Reference lists of all included articles were manually searched to identify any potentially relevant articles. EndNote software (EndNote X7®, Thomson Reuters, Philadelphia, USA) was used to manage the references and remove any duplicate articles.

Various combinations of descriptors extracted from Medical Subject Headings (MeSH) and free terms were used; "omics" AND "oral cancer" or "omics" AND "oral submucous fibrosis" or "omics" AND "oral leukoplakia" or "omics" AND "oral erythroplakia" or "omics" AND "oral lichen planus" or "omics" AND "oral squamous cell carcinoma."

Study selection and data extraction

The study selection process was completed in two stages. First, titles and abstracts of all identified articles were screened by two independent reviewers (KHA and SP) using a standardized guide. This was followed by retrieval of full texts of studies that met the eligibility criteria and reviewed independently by the same two reviewers using a standardized and pilot tested form. Any disagreements on study selection were mutually discussed and a consensus was made before inclusion of the study.

Two reviewers (KHA and SP) independently collected the data on study characteristics (author, year of study and country), study design, sample population, OSCC sub-site, methods used for transcriptomics analysis, statistical findings, and conclusions.

Risk of bias assessment

The risk of bias of included studies was assessed using the Newcastle Ottawa scale (NOS).[22] Two reviewers (KHA and SP) independently evaluated the quality of studies based on the following parameters: Selection, Comparability, and Outcome/Exposure. A maximum of 4 stars in selection domain, 2 stars in comparability domain and 4 stars in outcome/exposure domain were given. The included studies were qualified as "Good", "Fair" and "Poor" quality based on the total NOS score they achieved. Studies with a NOS score ≥ 7 and were considered good-quality studies.

Statistical analysis

Cohen's kappa statistic was used to calculate the agreement between the two reviewers (KHA and SP). Descriptive statistics for all included studies were populated and reviewed. NOS scores based on the assessment of quality of each study were also reported.

RESULTS

Study selection

Of 23 full texts assessed, 14 articles were excluded, giving, nine included articles that met the eligibility criteria (Fig. 1). The inter-examiner agreement (Kappa) was 0.98 in the first stage (title and abstract screening stage) and 1.00 in the second stage (full-text reading stage).

Studies characteristics

Of the 9 included studies,[23-31] five were from United States,[23-25, 27, 28] two from Taiwan,[26, 30] and one each from Saudi Arabia,[29] and Turkey[31]. All included studies had a case-controlled design and all utilized quantitative polymerase chain reaction (qPCR) to quantify salivary miRNA; three studies also utilized ELISA,[25] In-situ hybridization [30] and micro-array based miRNA analysis. [31] Five studies reported sites of the oral cancer among the study group,[25, 26, 29-31] while the remaining studies did not report. The studies were carried out among Caucasian, Asian, African, Taiwanese, and Arabic populations. Table 1 provides the detailed characteristics of the included studies.

Risk of bias assessment

The NOS score for the quality of the included studies ranged from 5 to 7 (Figure 2). Only three studies [23, 26, 27, 31] had required NOS score of '7' to be considered good-quality studies, while three studies[24, 28-30] scored '6' and only one study[25] had a NOS score of '5'. The majority of studies scored high in the selection domain and outcome/exposure domain. Studies also scored high in the comparability domain with the exception of one study[25] that scored '0'.

Analyzed salivary transcriptomes

All nine included studies provided significant data for the analyzed salivary transcriptomes. Three studies showed increased expression of DUSP1, IL8, IL1B, OAZ1, SAT1 and S100P in OSCC patients compared to healthy controls. [23, 25, 27] Two studies showed that expression of miRNA-31 was upregulated among OSCC and oral pre-malignant disease (OPMD) patients compared to healthy controls.[26,30] One study reported 11 miRNAs were downregulated (miRNA-136, miRNA-147, miRNA-1250, miRNA-148a, miRNA-632, miRNA-646, miRNA668, miRNA-877, miRNA-503, miRNA-220a, miRNA-323-5p), and 2 miRNAs were upregulated (miRNA-24, miRNA-27b) in OSCC patients.[28]

Sensitivity, specificity and receiver operator characteristic (ROC) curve analysis

Majority of the studies reported sensitivity, specificity and AUC (Area under ROC curve) analysis for the salivary transcriptomes (Table 2). The sensitivity ranged from 14% - 100%, while the specificity ranged from 38% - 100%. OAZ1, miRNA-21 and miRNA-31 were reported to have the highest sensitivities, and miRNA-31 and miRNA-27b had the highest specificities. DUSP1 was reported to have the lowest sensitivity of 14% and OAZ1 had the lowest specificity of 38%. AUC reported was in the range of 0.41 – 0.9643; miRNA-27b had the highest AUC of 0.9643 and DUSP1 had the minimum AUC of 0.41.

DISCUSSION

Transcriptomics is a cost-efficient technology that can help in the quantification of many defined mRNA species in a miniaturized automated manner.[32] Identifying altered transcriptomes along with RNA sequencing can facilitate in classification and progression of diseases.

While assessing quality of the included studies using NOS score, only four studies were classified as good-quality.[23,25,26,31.] These reported OSCC patients that were confirmed either

through histological analysis or hospital records and adjusted their risk estimates for other confounding factors, including smoking. In addition, controls were age, sex, smoking and alcohol history matched, and had the same method of exposure assessment as the cases. In contrast, studies that scored low on NOS did not provide adjustment for other confounding factors and did not report any histological and/or hospital records.

A wide range of salivary transcriptomes were analyzed in the included studies. mRNA transcripts of IL8, IL1B, DUSP1, H3F3A, OAZ1, S100P, and SAT were evaluated using the saliva samples of OSCC patients and healthy controls.[23] All of these potential salivary RNA biomarkers had higher sensitivity and specificity in identifying and differentiating OSCC. In a similar study, Brinkmann et al.[25] (2011) evaluated salivary transcriptomes IL8, IL1B, DUSP1, OAZ1, S100P, SAT1 and reported significant expression IL8, IL1B, S100P, SAT1 in OSCC patients when compared to healthy controls.[25] Another found significantly elevated levels of all these salivary transcriptomes amongst OSCC patients; expression of IL-8 and SAT was increased in all 5 cohorts, expression of IL1B, DUSP1, OAZ1 and H3F3A was increased in only 3 cohorts, Expression of S100P was increased in 2 cohorts.[27]

Park et al[24] (2009) found two salivary miRNAs, miR-200a and miR-125a, that were present in significantly lower levels in OSCC patient than in healthy controls (24). Studies have reported differential expression of miR-200a in head and neck and other cancer cell lines.[33-36] In addition, miR-125a along with its homolog miR-125b have been associated with reduced ERBB2 and ERBB3 oncogenic protein levels in a human breast cancer cell line SKBR3.[37] Although this study reported reduced levels of miR-200a in OSCC patients, higher levels of miR-200a have been presented in various oral squamous cell lines.[33-36] These contrasting results may be due to the difference in examining cell free state of miRNAs when compared to those in living cells. The supernatant saliva used for salivary transcriptomic analysis in the study is cell free phase of saliva, hence, the supernatant saliva contained some miRNA that

were byproducts of cell death. Similar to regulatory mRNAs, cancer-specific miRNAs may also have a more rapid degradation and/or a shorter half-life during cell death.[38]

Two studies evaluated the role of miR-31 as a biomarker for early detection and prognostic indicator of OPMD and OSCC.[26, 30] Both reported increased expression levels of miR-31 in OPMD and OSCC patients when compared to healthy controls. Although there is accumulated evidence that show a strong association of miR-31 in the pathogenesis of various cancers including oral cancer, other studies that utilized salivary microarray analysis did not have similar findings, [28, 39] perhaps due to discrepancies in the predisposing factors of the OPMD patients and/or histological features and lesion location. Mucosal lesions with distinct histological features may demonstrate varied expression of miRNAs. In addition, predisposing factors such as tobacco have shown to affect the expression of miR-31 in lung cancer specimens compared to adjacent normal lung tissues.[40] An experimental study on mice treated with 4NQO as carcinogen also showed increased expression of salivary miR-31.[41]

There are a few limitations to this review. Firstly, there was a lack of population variation in majority of the included studies. The studies were carried out mostly among Caucasian, Asian, African, Taiwanese, and Arabic populations. There is a need for exclusive studies in other ethnic population such as Indian population, especially keeping in mind the fact that these regions have some of the highest prevalence rates for oral cancer. Furthermore, the included studies were case-control biomarker development studies and did not follow the essential aspects of eliminating bias for biomarker research such as biomarker performance criteria, the biomarker test, study size and vigorous follow-up.

CONCLUSION

Salivary transcriptomes may be potentially useful biomarkers in the diagnostic and prognostic prediction of OSCC. However, further well-designed large-scale studies with detailed investigations and vigorous follow-up are needed to validate the sensitivity and specificity of these biomarkers before their more widespread use can be recommended.

CONFLICT OF INTEREST

None declared.

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legends

Fig. 1. Flow diagram of literature search and selection criteria.

Figure 2. Assessment of the quality of studies included using Newcastle-Ottawa (NOS) scale [22]

Table 1. Characteristics of the included studies

Table 2. Sensitivity, specificity and receiver operator characteristic (ROC) curve analysis of OSCC-associated salivary biomarkers

Author et al. (year), Country	Study design	Sample population	Oral cancer site	Platform	Molecules analyzed	Outcome	Conclusion
Li et al. (2004), USA[23]	• Case-control • Medical Centers at University of California, Los Angeles (UCLA) and University of Southern California (USC), Los Angeles, CA; and University of California San Francisco, San Francisco, CA	• Cases: 32 OSCC patients; Mean age 49.8±7.6 y • Controls: 32 healthy individuals; age-sex- and smoking history matched; Mean age 49.1±5.9 y	• NS	• qPCR	• IL8 • IL1B • DUSP1 • H3F3A • OAZ1 • S100P • SAT	• Expression of DUSP1, H3F3A, IL1B, IL8, OAZ1, S100P, and SAT was increased in the saliva of OSCC patients compared to controls	• Salivary transcriptomics is a useful tool for oral cancer detection
Park et al. (2009), USA[24]	• Case-control • UCLA School of Dentistry Dental Research Institute, Los Angeles, CA	• Cases: 50 OSCC patients; Average age 56 y; 32M, 18F • Controls: 50 healthy individuals; age-gender-ethnicity and smoking history matched; Average age 52 y; 29M, 21F	• NS	• RT-preamp-qPCR	• miR-142-3p • miR-200a • miR-125a • miR-93	• miR-125a and miR-200a were present in significantly lower levels ($p<0.05$) in the saliva of OSCC patients than in control samples	• Saliva miRNAs can be used for oral cancer detection
Brinkmann et al. (2011), USA[25]	• Case-control • Clinical Center of Serbia and Stomatology Faculty University of Belgrade, Belgrade, Serbia	• Cases: 35 OSCC patients; Mean age 60.94±12.30 y; 30M, 5F • Controls: 51 healthy individuals; Mean age 38.24±12.50 y; 28M, 23F	• Buccal mucosa • Gingiva • Others	• qPCR • ELISA	• DUSP1 • IL8 • IL1B • OAZ1 • SAT1 • S100P	• Expression of IL8, IL1B, SAT1, S100P was increased in OSCC patients compared to controls	• Salivary transcriptomic biomarkers are discriminatory & reproducible in OSCC
Liu et al. (2012), Taiwan[26]	• Case-control • Department of Stomatology, Taipei Veterans General Hospital, Taipei, Taiwan	• Cases: 45 OSCC patients; 53.7±1.4 y; 43M, 2F • Cases: 10 OVL patients; 49.5±2.5 y; 9M, 1F • Controls: 24 healthy individuals; age, sex, and oral habits matched; Mean age 51.1±1.7 y; 23M, 1F	• Buccal mucosa • Gingiva • Tongue • Others	• qRT-PCR	• miR-31	• Expression of salivary miR-31 was increased in OSCC patients • Expression of salivary miR-31 was not increased in OVL patients compared to controls	• Salivary miR-31 can be a potential biomarker for early detection and postoperative follow-up of OSCC

Elashoff et al. (2012), USA[27]	• Case-control • Medical Centers at the University of California, Los Angeles (UCLA) and University of Southern California (USC) and Veteran Hospital in greater Los Angeles (VAGLA); 2004 to 2007	• Cohort 1: Cases - 48 OSCC patients; Mean age 62.7±12.1 y; 34M, 16F. Controls – 48 healthy; Mean age 31.4±12.7 y; 33M, 15F • Cohort 2: Cases - 24 OSCC patients; Mean age 64.9±15.2 y; 14M, 10F. Controls - 24 healthy; Mean age 41.1±13.4 y; 14M, 10F • Cohort 3: Cases - 30 OSCC patients; Mean age 54.5±8 y; 21M, 9F. Controls - 30 healthy; Mean age 51.5±11.4 y; 20M, 10F • Cohort 4: Cases - 36 OSCC patients; Mean age 58.8±13.5 y; 30M, 6F. Controls - 54 healthy; Mean age 59.9±9.1 y; 50M, 4F • Cohort 5: Cases – 31 OSCC patients; Mean age 63.3±11.0; 26M, 4F. Controls - 70 healthy; Mean age 60.7±10.0; 61M, 8F	• NS	• qPCR	• IL8 • SAT • IL1B • DUSP1 • OAZ1 • H3F3A • S100P	• Expression of IL-8 and SAT was increased in all 5 cohorts • Expression of IL1B, DUSP1, OAZ1 and H3F3A was increased in only 3 cohorts • Expression of S100P was increased in 2 cohorts	• Biomarkers showed their feasibility in discrimination of OSCC from healthy controls
Momen-Heravi et al. (2014), USA[28]	• Case-control • Stomatology Center, Texas A&M University–Baylor College of Dentistry, Texas, USA; 2010 to 2011	• Cases: 35 patients • 9 OSCC patients before treatment; Mean age 60.6±11.8 y; 8M, 1F • 9 patients with OSCC-R; Mean age 69.71±16.8 y; • 9 patients with OLP; Mean age 66.25±13.67 y • Controls: 8 healthy individuals; 60.19±9.6 y	• NS	• RT-qPCR	• miR-136 • miR-147 • miR-1250 • miR-148a • miR-632 • miR-646 • miR-668	• miR- 191, miR- 136, miR- 147, miR- 1250, miR- 632, miR- 646, miR- 668, miR- 877, miR- 503, miR- 200a and miR- 323- 5p were downregulated in OSCC • miR- 24 and miR- 27b were upregulated in OSCC • miR-136 was underexpressed in	• miR-27b can be a potential OSCC salivary biomarker

					• miR-877 • miR-503 • miR-220a • miR-323-5p • miR-24 • miR27b	OSCC vs. HC and OSCC vs. OSCC-R • miR-27b levels were significantly higher in OSCC patients compared to HC, patients with OSCC-R, and patients OLP	
Zahran et al. (2015), Saudi Arabia[29]	• Case-control • Outpatient clinic of Oral Medicine and Periodontology Department, Faculty of Oral and Dental Medicine, Cairo University and National Cancer Institute in Cairo, Egypt	• Cases: • 40 OPMD patients; Mean age 54.2±9.7 y; 22M, 18F • 20 OSCC patients; Mean age 58±9.2 y; 8M, 12F • Controls: • 20 healthy individuals; Mean age 51.1±9.3 y; 9M, 11F • 20 RAS patients; Mean age 28±7.3 y; 7M, 13 F	• Buccal mucosa • Tongue • Floor of mouth • Retro-molar • Lower alveolar	• qRT-PCR	• miR-21 • miR-184 • miR-145	• Expression of miR-21 and miR-184 was increased in OSCC and OPMD patients compared to healthy and disease controls • Expression of miR-145 was reduced in OSCC and OPMD patients	• miR-184 may be a potential diagnostic biomarkers for oral malignant transformation
Hung et al. (2016), Taiwan[30]	• Case-control • Department of Dentistry, School of Dentistry, National Yang-Ming University, Taipei, Taiwan	• Cases: 46 patients newly diagnosed as OPMD; Mean age 53.3±3.7 y; 42M, 4F. • Controls: 24 healthy individuals; Mean age 52.9±3.2 y; 20M, 4F.	• Buccal mucosa • Gingiva • Lip • Palate • Tongue	• qRT-PCR • In situ hybridization (ISH)	• miR-21 • miR-31	• Expression of salivary miR-21 and miR-31 was increased in OPMD patients compared to control individuals • Patients with recurrent OPMD and/or malignant transformation exhibited a further augmented expression of miR-31	• Salivary miR-21 and miR-31 are useful OPMD screening tools

Duz et al. (2016), Turkey[31]	• Case-control • Department of Otorhinolaryngology, Cerrahpasa Medical School, Istanbul University	• Cases: 25 TSCC patients; Mean age 54.08±2.4 y; 19M, 6F. • Controls: 25 healthy individuals; age, gender matched; similar smoking and alcohol habits as cases; Mean age 46.88±3.6 y; 21M, 4F.	• Tongue	• qRT-PCR • Microarray-based miRNA	• miR-139-5p	• Expression of miR-139-5p was reduced in TSCC saliva samples compared to control saliva samples • In post-operative saliva samples of TSCC patients the miR-139-5p expression levels returned to normal	• miR-139-5p may serve as a potential biomarker for early TSCC detection

OSCC – Oral squamous cell carcinoma; NS – Not stated; M – Male; F – Female; HC – Healthy controls; OSCC-R – Oral squamous cell carcinoma in remission; OPMD – Oral potentially malignant disorders; TSCC – Tongue squamous cell carcinoma; qRT-PCR - Quantitative reverse transcription polymerase chain reaction; RT-qPCR - Real-time quantitative polymerase chain reaction; qPCR - Quantitative polymerase chain reaction; RT-preamp-qPCR - reverse transcriptasepreamplification-quantitative PCR ()

Table 1. Characteristics of the included studies

Author et al. (year)	Molecules analyzed	Sensitivity	Specificity	AUC
Li et al. (2004)[23]	IL8 IL1B DUSP1 H3F3A OAZ1 S100P SAT	88% 63% 59% 53% 100% 72% 81%	81% 72% 75% 81% 38% 63% 56%	0.85 0.70 0.65 0.68 0.69 0.71 0.70
Park et al. (2009)[24]	miR-200a miR-125a miR-142-3p miR-93	NS	NS	0.65 0.62 0.58 0.57
Brinkmann et al. (2011)[25]	IL8 S100P SAT1 OAZ1 IL1B DUSP1	60% 54% 54% 40% 23% 14%	78% 88% 82% 92% 94% 98%	0.75 0.71 0.70 0.60 0.42 0.41
Liu et al. (2012)[26]	miR-31	NS	100%	0.82
Elashoff et al. (2012)[27]	IL8 SAT IL1B DUSP1 OAZ1 H3F3A S100P	68% 66% 65% 60% 62% 61% 60%	64% 63% 60% 65% 58% 56% 56%	0.74 to 0.86 across the cohorts
Momen-Heravi et al. (2014)[28]	miR-27b	85.71%	100%	0.9643
Zahran et al. (2015)[29]	miR-184 miR-21 miR-145	80% 65% 60%	75% 65% 70%	0.86 0.73 0.68
Hung et al. (2016)[30]	miR-21 miR-31	100 % 100 %	NS	0.74 0.76
Duz et al. (2016)[31]	miR-139-5p	NS	NS	0.805

AUC - Area under ROC curve; NS – Not stated

Table 2. Sensitivity, specificity and receiver operator characteristic (ROC) curve analysis of OSCC-associated salivary biomarkers

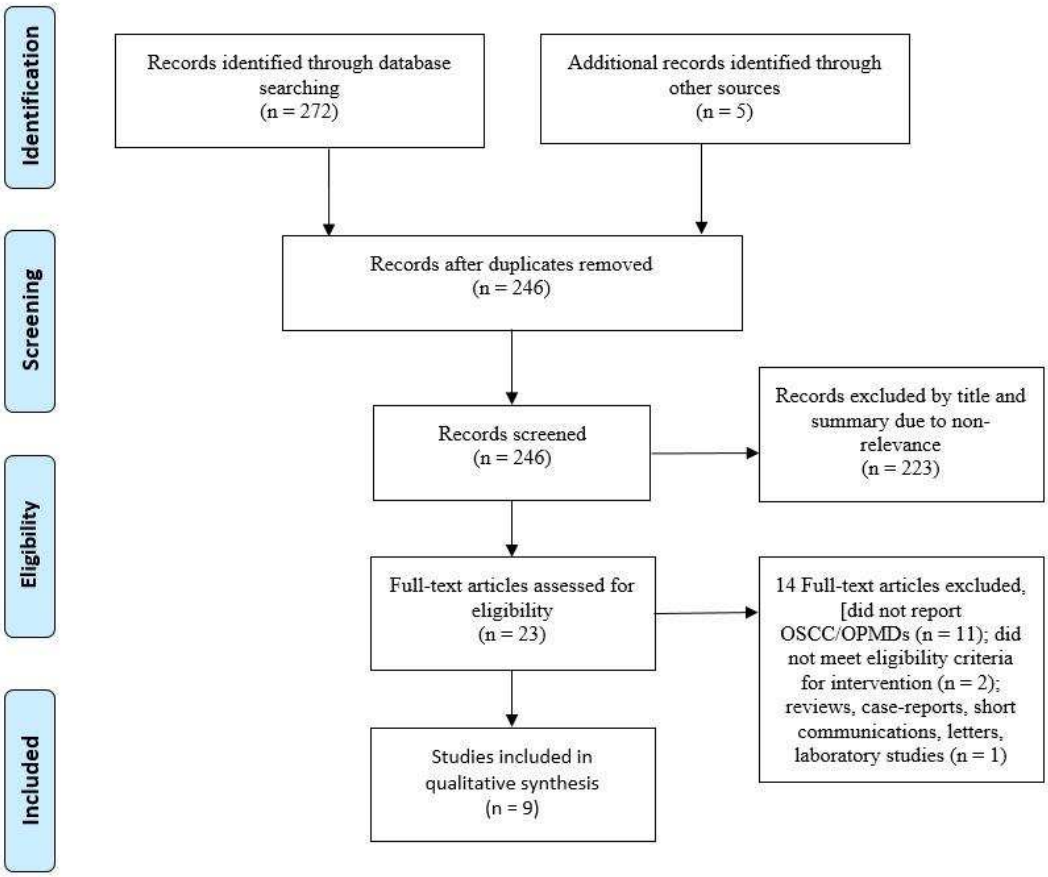

Fig. 1. Flow diagram of literature search and selection criteria.

Figure 2. Assessment of the quality of studies included using Newcastle-Ottawa (NOS) scale (22)

Study	Selection	Comparability	Outcome/exposure	NOS
Li et al. (2004)[23]	●●○●	●●	○○●●	7
Park et al. (2009)[24]	●●○●	○●	○○●●	6
Brinkmann et al. (2011)[25]	●●○●	○○	○○●●	5
Liu et al. (2012)[26]	●●○●	●●	○○●●	7
Elashoff et al. (2012)[27]	●●○●	●●	○○●●	7
Momen-Heravi et al. (2014)[28]	●●○●	●○	○○●●	6
Zahran et al. (2015)[29]	●●○●	●○	○○●●	6
Hung et al. (2016)[30]	●●○●	●○	○○●●	6
Duz et al. (2016)[31]	●●○●	●●	○○●●	7