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TESI DI DOTTORATO

Oral Cancer: from the research of biomarkers for diagnosis and prognosis to the treatment with nutraceuticals

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Abstract

Oral Squamous Cell Carcinoma (OSCC) accounts for more than 500.000 new cancer cases every year globally, and in high-risk countries is one of the most common cancer in men. When diagnosed at a late stage, treatment of OSCC is highly debilitating and the disease if often fatal. Patients diagnosed in early stage OSCC has a favorable prognosis and requires less aggressive treatment. The overall 5-year survival rate for OSCC is below 50% and has only improved modestly over the past two decades despite improvements in treatment modalities.

The diagnostic delay seems to be one of the main obstacles to overcome in order to diagnose tumors in early stage. The research of clinical biomarker aims to identify molecules capable of predicting the risk of the disease' presence and help clinicians in the early diagnosis. Furthermore, another field of research is related to the research of prognostic biomarker, in order to address patients towards personalized treatment modalities on the basis of the molecular aggressiveness of the neoplasia. Moreover, there is the need to find new therapeutic drugs aiming to improve the survival rate of OSCC patients. In this field, nutraceuticals, any substance considered to be a food or a food ingredient that provides medical and health benefits, showed promising effects for the treatment of some types of cancer. However, further studies are useful before to include them in the current therapeutic protocols.

The aim of this thesis was twofold, first the research of possible clinical biomarkers of OSCC diagnosis and prognosis; and secondly addressed to assess the effects of treatment with Curcumin and Genistein on OSCC cell lines.

Results of several studies included in this thesis, performed for the research of clinical biomarker, showed the identification of promising proteins for the diagnosis of OSCC. Among these, neurotrophin-3 (NT-3) revealed the better diagnostic prediction values when quantified in plasma of patients with OSCC.

Related to the prognostic prediction we performed meta-analysis of the literature for the long non-coding RNA (lncRNA) HOTAIR and demonstrated as this marker seems to be able to predict words prognosis when expressed at higher levels in the tumor bulk.

Finally, we assessed the treatment effects with curcumin and genistein on OSCC cancer cell lines. Results showed a reduction in: viability, migration, proliferation of cancer cells and increasement in the rate of apoptosis after treatment with different dosages of this nutraceuticals.

In conclusions, we identified promising biomarker for the prediction of diagnosis and prognosis of OSCC patients. In addition, results of our studies showed that treatment with curcumin and genistein lead to inhibition of: viability, migration, proliferation, and increase in the rate of apoptosis of OSCC cell lines.

SUMMARY

Dedicated to the memory of my mother

Introduction

1.1. Introduction and thesis motivation

The most recent statistics from the US estimated the incidence for cancer of the oral cavity at 2.7 per 100,000 (3.7 in men and 1.8 in women) in 2012, with substantial differences by sex, age, and region (1). Furthermore, highest incident for oral cavity cancer was reported in south-central Asia (40.9% of all incident cases) (2). The incidence of oral cavity cancer was consistently greater among men than women, with an M/F rate ratio of 2.1, ranging from 5.2 for Central and Eastern Europe to 1.4 for Northern Africa, Western Asia and Oceania (2). Risk factors involved in the development of oral squamous cell carcinoma (OSCC) are considered: tobacco smoke, alcohol consumption, reverse smoke and betel chewing. Reaching an early diagnosis of symptomatic OSCC is crucial for a better prognosis in terms of disease stage, survival and quality of life (3). In fact, patients diagnosed in early stages have a longer life expectancy compared to patients who get the diagnosis in advanced stages (4). The overall 5-year survival rate for OSCC is below 50% and has only improved modestly over the past two decades despite considerable improvement of treatment modalities (5).

Recently the 8th edition of the American Joint Committee on Cancer (AJCC) introduced in the staging system of the oral cavity squamous cell carcinoma (OSCC) the depth of invasion (DOI) as a variable for the T category (6). The new staging manual highlights the importance to distinguish between tumors with different DOI and the need to use the latter for staging purposes. However, although tumor-node-metastasis (TNM) staging is routinely used to predict tumor behavior and, hence, to inform the choice of treatment strategies for OSCC, patients with same TNM stages may result in dramatically different survival time (7).

Due to these reasons, the scientific community put its attention to the research of biomarkers for the early diagnosis and prognosis prediction. The prognosis prediction aims to develop personalized treatment planned on the basis of the molecular profiling of the tumor. In this way, clinicians could

modulate the aggressiveness of the treatment on the basis of a molecular risk profile. From the point of view of diagnosis, a biomarker of early diagnosis could decrease the rate of patients treated in advanced stages and strongly increase the overall survival rate of patients with OSCC.

In fact, as previously mentioned, the advent of new treatment modalities who demonstrated benefits for other types of malignancies, failed to improve survival for OSCC patients. With regards to the advanced OSCC, guidelines from the National Comprehensive Cancer Network still today recommend surgical excision followed by concurrent single– agent cisplatin chemo-radiotherapy in T3 or T4 lesions demonstrating adverse features of extracapsular spread and/or positive margins (8). For such reasons, there is the need to figure out the molecular basis of this pathology in order to provide clinicians new treatment options they can use in the clinical practice.

'Nutraceutical'' term (a combination of the words ''nutrition'' and ''pharmaceutical'') refers to any substance considered to be a food or a food ingredient that provides medical and health benefits (9). Recently, some nutraceutical agents showed anti-inflammatory, antioxidant and anticancer properties in several studies (10, 11). Among these, curcumin and genistein are among the nutraceuticals the most promising (12-14).

Therefore, the aim of this thesis was twofold, first, the research of possible clinical biomarkers of OSCC diagnosis and prognosis; and secondly to assess the effects of treatment with Curcumin and Genistein on OSCC cell lines.

Research of diagnostic and prognostic biomarkers in OSCC

- 2.1. Background

The World Health Organization (WHO) defines a biomarker as any substance, structure or process that can be measured in the body or its products and influences to predict the incidence of outcome or disease (Biomarkers in Risk Assessment: Validity and Validation, Environmental Health Criteria Series, No222, WHO). Biomarkers can be used for patient assessment in multiple clinical settings, including estimating the risk of disease and distinguishing benign from malignant tissues.

Cancer biomarkers can be classified according to the disease state, such as predictive, diagnosis and prognosis biomarkers (15). A diagnostic biomarker aims to detect or confirm the presence of a disease or condition of interest or to identify individuals with a subtype of the disease. Instead, a prognostic biomarker informs about a likely cancer outcome (e.g., overall survival, disease-free survival, and cause-specific survival) independent of the received treatment. The identification of prognosis biomarkers could guide clinicians in modulating the best therapeutic approach based on the molecular aggressiveness of the pathology. Predictive biomarkers, sometimes referred to biomarkers used to identify individuals who were more likely than similar individuals without the biomarker to experience a favorable or unfavorable effect from exposure to a medical product or an environmental agent (16).

These biomarkers allow clinicians to select a set of chemotherapeutic agents which will work best for an individual patient. For example, Herceptin is useful in breast cancer lesions showing only Her2/Neu overexpression, whereas tamoxifen is the preferred treatment for other breast cancer lesions. Thus Her-2/Neu is a predictive cancer biomarker for a subset of breast cancer therapies (17). Likewise, drugs such as erlotinib or gefitinib work only in lung cancer patients with specific mutations in the epidermal growth factor receptor (EGFR) gene (18). Although, several studies have been performed aiming to find out biomarkers of diagnosis and prognosis for OSCC, no molecule

has still been introduced in the clinical practice. For such reason, there is the need to go deep in the study related to biomarkers of diagnosis and prognosis in OSCC.

- 2.2. Literature Review of circulating miRNA for the detection of OSCC

Although, proteins still represent the final effectors of the cell processes, more recently the discovery of non-coding RNA added a new layer to our understanding of biological processes. The term non-coding RNA (ncRNAs) encompasses microRNA (miRNAs), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), and others (19). Although ncRNAs do not encode proteins, they are master regulators of gene expression through various mechanisms (20, 21). In the field of cancer research, it has become apparent that aberrations within the noncoding genome drive fundamental cancer phenotypes in addition to the best-known protein coding mutations (22). MiRNAs are the most studied group of ncRNAs. They are long in between 19 and 23 nucleotides. They are able to negatively regulate target messenger RNAs (mRNAs) by partially binding to their 3' untranslated region (23). In addition, miRNAs seem to be generated from intergenic genomic sequences or intronic regions of protein-coding genes (23).

The majority of mammalian miRNAs are transcribed by the RNA polymerase II into a long precursor containing a typical stem-loop structure (pri-miRNAs) (Fig. 1) [11].

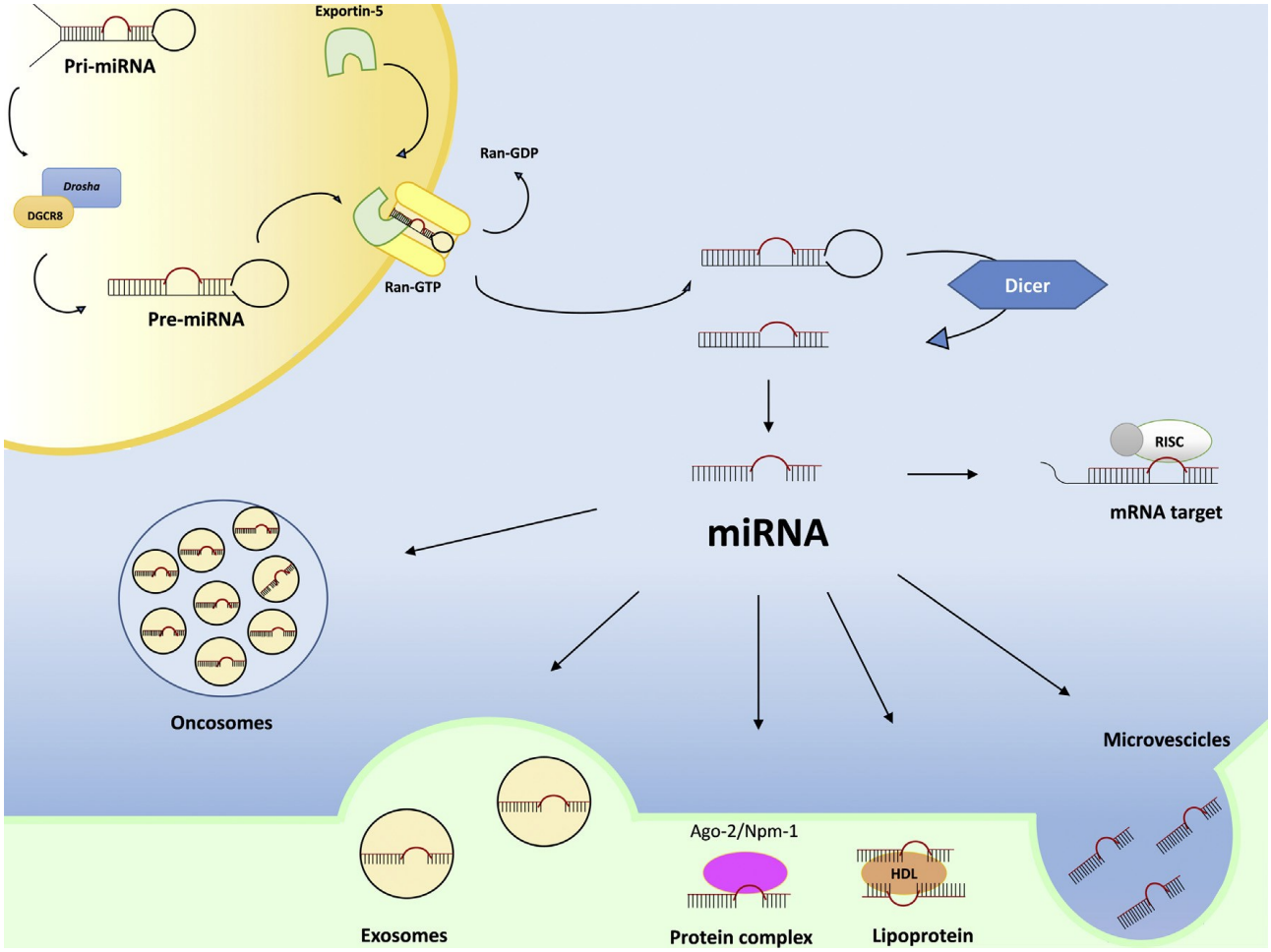


Figure 1: Mechanisms involved in synthesis of miRNAs. It starts in the nucleus and ends with secretion once the miRNa has reached its final stage.

These initial transcripts undergo cleavage by the ribonuclease enzyme III Drosha to produce the pre-miRNAs of around 70 nucleotides and with hairpin structure. Pre-miRNAs are then transported into the cytoplasm by a nuclear export factor (Exportin-5), where they are cleaved by an enzyme called Dicer into duplexes of 21–23 nucleotides (24). Afterwards, one strand of the duplex, the passenger strand (miRNA*), is degraded and the other incorporated together with Argonaute proteins or lipoproteins to form the RNA-induced silencing complexes (RISC) leading to the formation of mature miRNA (25). The presence of miRNAs was also discovered in blood and several other body fluids including: urine, tears, pleural effusions and saliva, exhibiting different expression patterns in cancer patients compared to healthy individuals. In order to clarify the role of miRNAs as possible biomarkers of diagnosis in OSCC we performed a systematic review of the literature including studies

performed on blood, serum or plasma who investigated the expression of specific miRNAs. We reported their potential use as cancer biomarkers. This systematic review was performed according to the Preferred Reporting Items for Systematic reviews and meta- Analysis (PRISMA). The criteria for inclusion in this systematic review were: studies on human blood, plasma or serum evaluating miRNA expression in patients with oral cancer compared to healthy controls. Presence of at least one miRNA dysregulated in the test group confirmed by either qRT-PCR or ddPCR. At the end of the selection process 16 studies [56–71] were assessed as adequate for inclusion in the study. Of the studies included, only 11 reported Receiver Operating Characteristics (ROCs) of circulating miRNAs in order to discriminate between cancer and non-cancer patients (Table 1).

Study	Year	miRNA Investigated	AUC	Specificity	Sensitivity
Liu et al.	**2016**	**miR-187***	0,73 95%CI: 0,62-0,83	Not Reported	Not Reported
Xu et al.	**2016**	**mir-483-5p**	0.85 95%CI: 0,73-0,93	0,746	0,853
Lu et al.	**2015**	**miR-196a + miR196b**	0,963	0,878	0,925
Ren et al.	**2014**	**miR-21**	0,788 95%CI: 0,692-0,883	0,906	0,621
Ries et al.	**2014**	**miR-185-5p** **miR-3651** **miR494-5p**	0,69 0,824 0,715	Not Reported	Not Reported
Hung et al.	**2013**	**miR-146a**	0,79	0,92	0,86
Liu et al. [63]	**2013**	**miR-196a**	0,75	Not Reported	Not Reported
Lu et al.	**2012**	**miR-10b**	0,932	0,8	0,944
Yang et al.	**2011**	**miR-181a + miR181b**	0,89	Not Reported	Not Reported
Lin et al.	**2010**	**miR-24**	0,82	0,92	0,7
Liu et al.	**2010**	**miR-31**	0,82	Not Reported	Not Reported

Table 1: Data for Receiver Operating Characteristic (ROC) as: Area Under the Curve (AUC), specificity and sensitivity from 11 studies.

These studies revealed very promising results of Area Under Curve (AUC), showing promising diagnostic ability in the detection of OSCC through the quantification of specific miRNAs in blood and derivatives. However, most of the included studies suffer from the presence of different source of bias bound to the heterogeneous sampling and to the use of not fully reliable housekeeping genes. Such shortcomings should be overcome in future studies focusing on single subsites of tumors arising in the oral cavity and using a panel of reliable reference genes.

- 2.3 Analysis of circulating miRNAs and proteins for the detection of patients with tongue squamous cell carcinoma

- 2.3.1 *Introduction*

As above mentioned, in the last years the research of biomarkers has been enriched by the possibility of studying a multitude of molecules besides the well-known proteins. Among these cell-free miRNAs have a great potential because they are stable, even when subjected to extreme conditions such as high temperature, wide variation in pH or multiple freeze-thaw cycles (26). The use of amplification reactions, such as qRT-PCR, for the study of circulating miRNAs allows the detection of molecules also at a low concentration. Unlike detection of circulating nucleic acids, two potential problems with serum protein biomarkers were their low concentrations due to the dilution in the circulation and the limited ability to assay multiple proteins from a small sample volume. Recently, the proximity extension assay (PEA) has been developed. It is a highly sensitive method based on pairs of antibodies linked to oligonucleotides with partial complementariness for each other (PEA probes) (27). When the two different antibodies bind to their target protein, probes are brought into proximity and the two oligonucleotides accordingly bind (28).

The new sequence achieved can be extended, amplified and measured by quantitative real-time PCR, where the number of PCR templates is proportional to the initial concentration of antigen in the sample (29). In addition for a high sensitivity, specificity is also improved due to the requirement for simultaneous binding of two different antibodies to epitopes on the same antigen (30). The purpose

of this study was to analyze the expression of both circulating protein and miRNAs in the plasma of patients with squamous cell carcinoma of the tongue.

2.3.2 Materials and methods

- Patients Material

Whole blood was collected from a total of 19 patients and 23 healthy volunteers, mean age 58 and 50 years respectively. Tumor biopsies (T) and biopsies from corresponding clinically normal tumor free tissue (TF) adjacent to the tumor was collected from 15 patients, with a mean age of 59 years.

Three milliliters of peripheral blood were collected into vacutainers containing EDTA using standardized venipuncture procedures. Handling and processing was the same for all samples. Tubes were left standing for at least 30 minutes at room temperature after collection, centrifuged at 1300 g for 10 min at room temperature and the top layer, the plasma, was immediately aliquoted.

- Extraction of miRNAs from plasma and tissues

Total RNA including miRNA was extracted from frozen plasma samples using miRCURY RNA Isolation kit –Biofluids (Exiqon, Vedbaek, Denmark). A standard protocol for RNA isolation was used according to the manufacturer, with 250 µl of plasma as starting material and stored at -80 °C until further use. Total RNA including miRNA was extracted from frozen plasma samples using miRCURY RNA Isolation kit –Biofluids (Exiqon, Vedbaek, Denmark). A standard protocol for RNA isolation was used according to the manufacturer, with 250 µl of plasma as starting material.

After centrifugation to remove debris, 200 µl supernatant was extracted and RNA eluted in 50 µl RNase free water. AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) was used for miRNA extraction from tumor and tumor free tissue. The fresh frozen biopsies (less than 20 mg) were homogenized in 600 µl Buffer RLT Plus containing β-mercaptoethanol using a Precellys Tissue homogenizer (Bertin Technologies, Artigus Pres Boreaux, France). After DNase treatment and washing, total RNA including miRNA was eluted twice in 30 µl and the eluates pooled. Purified RNA

was stored at -80 °C until cDNA preparation. Quantity and purity of RNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

- *Discovery and validation of miRNAs in plasma*

Twenty microliters of RNA extracted from plasma from 13 patients and 13 controls was sent on dry ice to Exiqon Services (Vedbæk Denmark) for miRNA analysis with real-time PCR panel. The panel used was miRCURY LNA Universal RT microRNA PCR Human Panel I. Briefly 10 µl RNA was reversed transcribed in 50 µl using the miRCURY LNA Universal RT microRNA PCR, polyadenylation and cDNA synthesis kit (Exiqon, Vedbæk, Denmark). cDNA was diluted 50x and assayed in 10 µl PCR reactions according to the kit protocol. Amplification was performed in a LightCycler® 480 Real-time PCR system (Roche) in 382 well plates, and amplification curves analyzed using Roche LC software, both for determination of Cq (by the 2nd derivative method) and for melting curve analysis. All samples went through a quality control of RNA isolation and cDNA synthesis using Exiqon´s RNA spike-in kit. All data were therefore normalized by the formula; normalized Cq = global mean Cq – assay Cq (sample). For validation of findings from the panel in plasma, miR-150 and three miRNAs used for normalization, miR-30e, miR-93 and miR-222, were quantified by individual qRT-PCR assays. RNU6 and SNORD 44 were used for normalization of miR-150 tissue data, obtained from 8 of the 13 SCCT samples included in the plasma analysis plus an additional 7 samples. Real time RT-PCR was performed using an IQ5 multicolor real-time PCR detection system with IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). For each assay, 4 µl cDNA, diluted 40X, was used in 10 µl reactions. Cycling conditions were enzyme activation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 60 sec.

- *Proximity Extension Assay*

Plasma from 19 controls (9 of the samples included in the miRNA analysis and an additional 10) and 19 SCCT patients (12 of the 13 analyzed for miRNA in plasma and an additional 7 patients with SCCT) were sent to Clinical biomarkers facility, Science for Life Laboratory (Uppsala, Sweden) for analysis with proximity extension assay. One µl EDTA-plasma was used in each of two different panels comprising 92 and 71 proteins respectively, Proseek multiplex Oncology I, v2 and Proseek multiplex Inflammation I (Olink Bioscience, Uppsala, Sweden).

2.3.3 Results

- *miRNA expression in TSCC patients*

A panel of circulating miRNAs was analyzed in plasma from healthy individuals (13 controls) and patients with SCCT (13 patients). Of the 372 miRNAs analyzed, 152 were detected in all samples. None of the samples showed signs of hemolysis, evaluated by analyzing the ratio between miR-451 and miR-23a-3p. All samples had values below 7.0, where a ratio above 7.0 is considered as risk of being affected by hemolysis. Principal component analysis (PCA) showed that the levels of these miRNAs did not discriminate healthy individuals from SCCT patients (Figure 2).

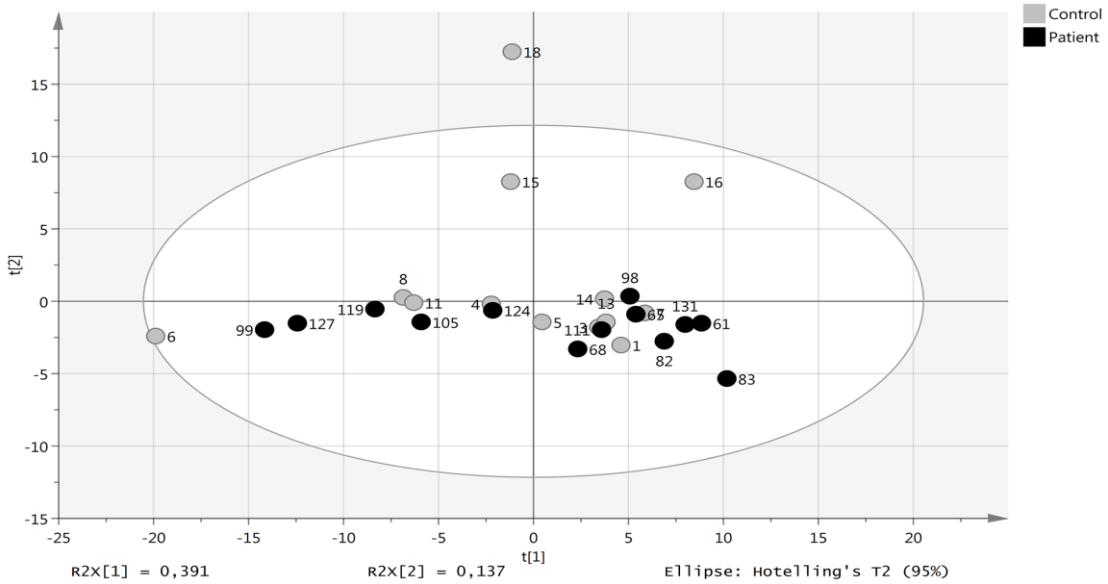
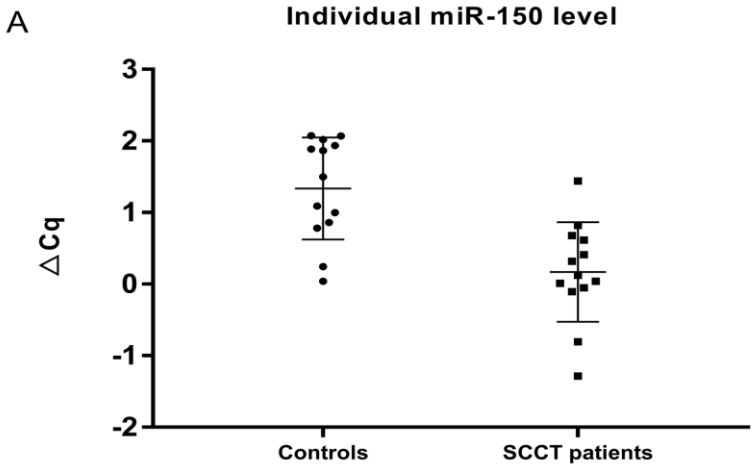


Figure 2: miRNA levels in patients with tongue tumor compared with healthy controls. Score plot (t1/t2) from PCA modelling based on 152 miRNAs detected in all samples. Each dot represents one patient indicated by the patient ID.

Statistical evaluation of individual miRNA levels showed miR- 150 to be significantly decreased in patients with SCCT compared to controls, Benjamini-Hochberg FDR adjusted p-value 0.007 (Figure 3A). Single qRT-PCR assay was then applied to validate miR-150 results using a combination of three miRNAs for normalization (miR-93, miR-222 and miR-30e). The normalized Cq value from single qRT-PCR assays of plasma samples showed high consensus with the normalized Cq value from the panel ($p < 0.001$, Rho = 0.913).

miR-150 was further analyzed in tissue samples using paired tumor and tumor free samples from 15 SCCT patients; 8 of the patients included in the plasma analysis and an additional 7 patients. In contrast to the decreased plasma levels in tumor patients, there was significant up-regulation (p-value 0.02) of miR-150 in tumor tissue compared to tumor free tissue adjacent to the tumor (Figure 3B).



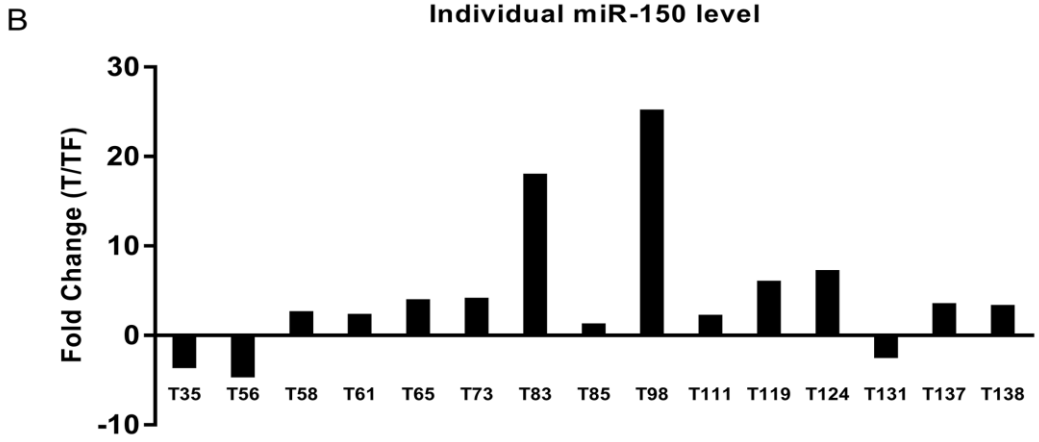


Figure 3: miR-150 levels in plasma and tissue. (A) Individual miR-150 levels in plasma from controls and tumors based on Cq values from the miRNA-panel. Normalized Cq = global mean Cq – assay Cq (sample). A higher value thus indicate that miR-150 is more abundant in that particular sample. (B) Fold change in miR-150 level in tongue tumor compared to tumor free tongue tissue from the same patient.

- *Protein expression in TSCC patients*

A total of 146 proteins involved in oncogenesis and inflammation were analyzed by proximity extension assay of plasma from 19 SCCT patients (the same 13 as included in the miRNA analysis and an additional 6 patients) and 19 healthy controls (9 that were included in the miRNA analysis and 10 additional controls). Using a cut-off of $p < 0.05$, 23 markers were differentially expressed between SCCT patients and healthy individuals. Principal component analysis of these 23 proteins separated SCCT patients and healthy individuals from each other, although not completely (Figure 4).

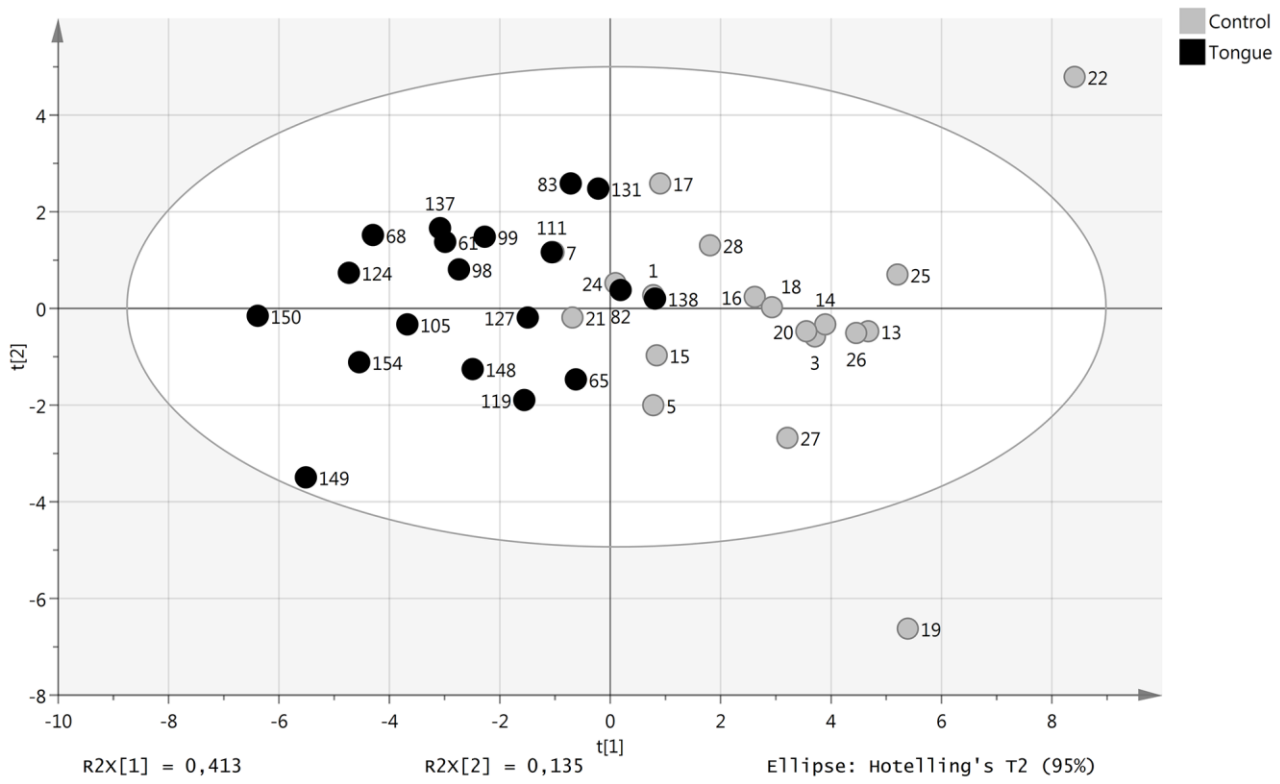


Figure 4: Levels of plasma proteins with tongue tumor compared with healthy controls. Score plot (t1/t2) from PCA modelling based on 23 plasma proteins with significantly different levels in patient and control samples.

All but one of these proteins showed down-regulation in SCCT patients compared to healthy controls, the exception being IL1-ra, and there was a large overlap in protein levels between the groups for all proteins (Figure 5).

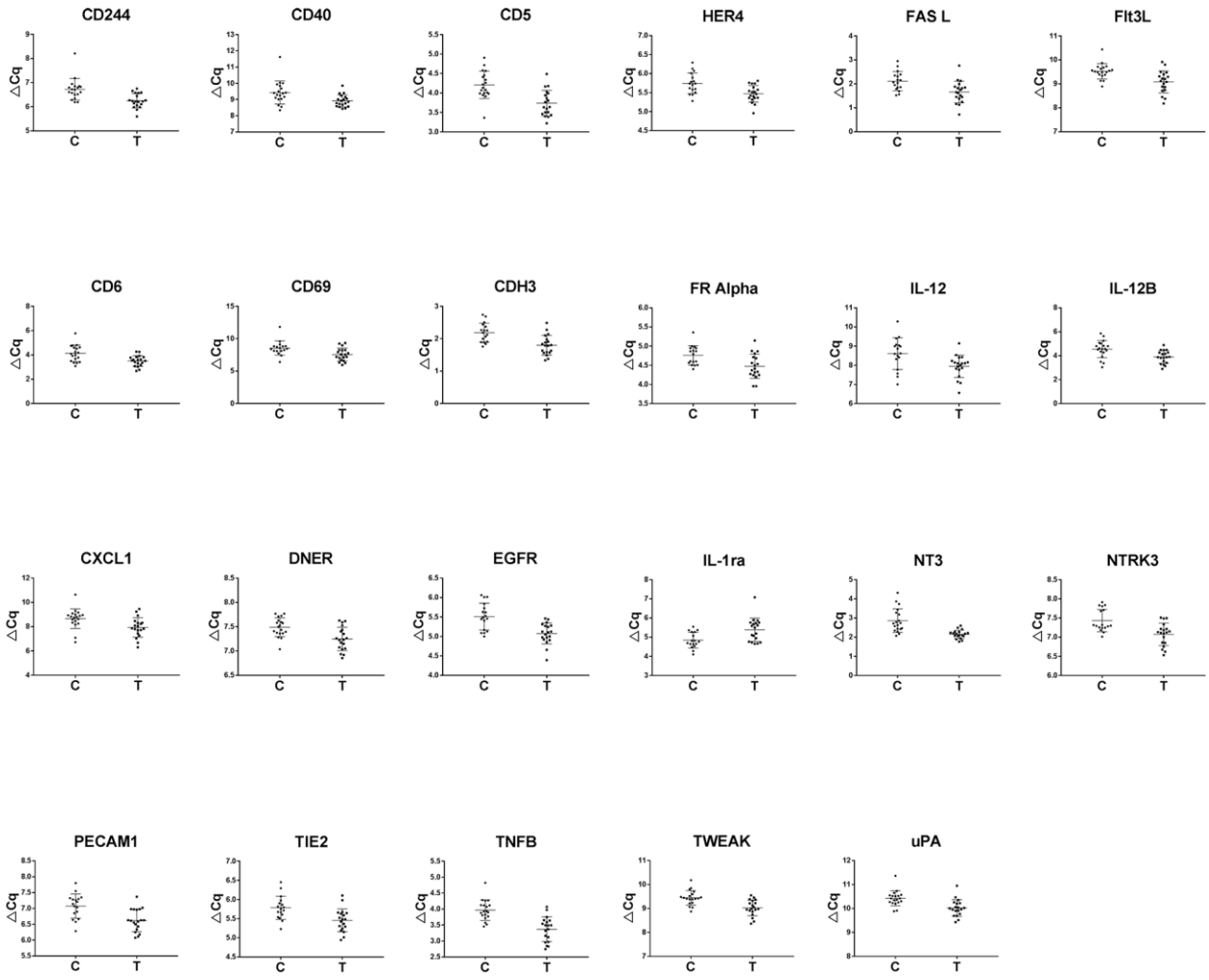


Figure 5: Differentially expressed proteins. Scatter plots of significantly differentially expressed proteins in plasma from patients with squamous cell carcinoma of the tongue compared to healthy controls

Calculating a receiver operating curve (ROC) showed the AUC for individual proteins to range from 0.676 to 0.903, indicating fair to good discrimination between those with and without disease (Table 2).

	Protein levels in circulation		mRNA expression in tissue [1]		AUC Circulating proteins		
						Confidence interval	
	p-value	SCCT vs C	P-value	SCCT vs C	AUC	Lower Bound	Upper Bound
NT-3	0,002	Down*	1,11E-02	Down*	0,903	0,805	1,000
TNFB	0,001	Down*	6,64E-05	Up*	0,889	0,786	0,993
CD5	0,008	Down*	4,04E-04	Up*	0,831	0,694	0,968
uPA	0,014	Down*	1,21E-10	Up*	0,825	0,686	0,965
IL-1ra	0,031	Up*	3,77E-05	Down*	0,806	0,670	0,942
Flt3L	0,018	Down*	1,04E-02	Up*	0,803	0,656	0,950
DNER	0,031	Down*	1,08E-05	Down*	0,781	0,631	0,931
CXCL1	0,047	Down*	2,31E-06	Up*	0,770	0,607	0,933
CD6	0,018	Down*	2,02E-07	Up*	0,767	0,615	0,919
CD40	0,047	Down*	4,69E-07	Up*	0,765	0,605	0,924
CDH3	0,014	Down*	1,80E-11	Up*	0,737	0,570	0,904
PECAM-1	0,014	Down*	1,21E-02	Up*	0,731	0,560	0,902
TIE2	0,031	Down*	2,22E-02	Down*	0,712	0,538	0,886
FasL	0,031	Down*	7,72E-03	Up*	0,709	0,538	0,881
FR-alpha	0,047	Down*	3,55E-02	Up*	0,693	0,519	0,867
CD69	0,044	Down*	4,69E-07	Up*	0,676	0,495	0,856
CD244	0,012	Down*	5,33E-01	Down	0,845	0,723	0,967
TWEAK	0,010	Down*	6,71E-01	Down	0,834	0,705	0,962
IL-12B	0,026	Down*	4,23E-01	Down	0,795	0,644	0,946
EGFR	0,008	Down*	2,58E-01	Up	0,756	0,593	0,920
NTRK3	0,016	Down*	2,58E-01	Down	0,731	0,562	0,900
ErbB4/HER4	0,031	Down*	6,71E-01	Down	0,690	0,515	0,864
IL-12	0,047	Down*	6,47E-01	Up	0,676	0,487	0,865

* Significant differential expression
[1]Data from Boldrup et al. 2017 ([26])

Table 2: Protein levels in circulation and mRNA expression in tissue

The protein with highest AUC value was NT-3 (neurotrophin-3; NTF3), which is also the only candidate with both high sensitivity and high specificity. For the 23 differentially expressed proteins, corresponding gene expression was available in a recently published study of RNA profiling in nine of the patients. Sixteen mRNAs showed significantly different expression when comparing tumor tissue to control, whilst mRNA levels for 7 of the proteins was not significantly different between control and tumor tissues. Consistency in the direction of change for circulating protein and tissue mRNA levels were identified in only three of the 16 differentially expressed genes (*DNER*, *NT3* and *TIE2*), where protein levels were lower in plasma from patients than controls and mRNA levels were lower in tumor tissue compared to tumor-free tissue. Consistency in the direction of change for circulating protein and tissue mRNA levels were identified in only three of the 16 differentially expressed genes (DNER, NT3 and TIE2), where protein levels were lower in plasma from patients than controls and mRNA levels were lower in tumor tissue compared to tumor-free tissue.

- 2.4 Meta-analysis of HOTAIR as prognostic factor in patients with squamous cells carcinoma of the head and neck

Squamous cell carcinoma of the head and neck (SCCHN) encompasses a wide and frequent range of neoplasms arising from the epithelium of different sites such as: oral cavity, nasal cavity, oropharynx, hypopharynx and larynx. It has been reported that around 90% of all tumors in this region are squamous cell carcinomas. SCCHNs are among the ten most frequent malignant neoplasms in humans; and due to the high rate of early metastasis to regional cervical lymph-nodes treatment of these patients is a challenge for clinicians. OSCC belongs to the wide group of SCCHN, being the most present subtype with about 24% of all the SCCHN cases. Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides previously defined as “junk DNA” (31). They are usually transcribed by RNA polymerase II and can undergo splicing and polyadenylation processes. Although they are not translated into proteins, many of them are linked to some complexes involved in chromatin modification causing an overexpression or overexpression or silencing of target genes of

which most are in cancer pathogenesis silencing of target genes of which most are in cancer pathogenesis. The HOX transcript antisense intergenic RNA (HOTAIR) is one of the most studied lncRNAs (32). Different studies have focused on the capacity of HOTAIR to cooperate with different chromatin modifying complexes, above all the Polycomb Repressive Complex 2 (PRC2), through its 5′-terminal binding domain (33).

HOTAIR lets PRC2 recognize the target gene, leading to Histone H3 lysine-27 trimethylation, causing a silencing effect. HOTAIR is also able to cooperate with the LSD1 complex and can be regulated by different factors such as miR141, Ago2, c-Myc, TGF-β and small interfering RNAs (siRNAs). The goal of this systematic review and meta-analysis was to assess the link between HOTAIR expression and patient prognosis in SCCHN in order to highlight its potential role as prognostic biomarker. Inclusions criteria were: (i) studies focusing on the expression of HOTAIR in SCCHN including more than 50 patients in total, (ii) studies showing analysis of correlation between different levels of HOTAIR expression and overall survival, (iii) studies reporting a Hazard Ratio (HR) and 95% confidence interval (CI) or Kaplan-Meier curve for HR estimation and (iv) studies including quantitative analysis performed with qRT-PCR, in situ hybridization (ISH), fluorescence in situ hybridization (FISH), droplet digital PCR (ddPCR) or RNA sequencing data. Accordingly, studies performed on cell lines or animal models, reviews, case reports, overlapping publications and all studies reporting insufficient data for estimation of HR and 95% CI, were excluded. No restriction was applied concerning year of publication. The bibliographic research was performed on the databases: PUBMED, SCOPUS, Web of Knowledge and EMBASE. In addition, a direct search on the bibliographies of previously published systematic reviews on the topic was also performed. Overall effects were investigated with a fixed effect model where $I2$ was less than 50%, if higher, a random effects model was used. Data for overall survival were synthetized as HR and standard error (SE), while for lymph-node metastasis, tumor stage and histological grade the odds ratio (OR) was evaluated. In cases in which HR and its 95% CI was not reported in the article, it was extracted from Kaplan- Meier curves using the method of Tierney et al (34).

At the end of the selection process, 4 studies met the inclusion criteria and were included in the meta-analysis. All studies were performed in China and published between 2012–2016. Results of meta-analysis, on the basis of two studies, revealed that higher levels of HOTAIR expression were not associated with higher degree of differentiation *(OR, 2.31; 95% CI: [0.89, 6.02]; p = 0,09)* (Figure 6A), whereas a correlation to advanced tumor stages was seen *(OR, 3.44; 95% CI: [1.84, 6.43]; p < 0,001)* (Figure 6B). As heterogeneity was not detected *(I2 = 0%)* a fixed effects model was used. Analysis of correlations performed with a random effect model *(I2 = 58%)* revealed a correlation between HOTAIR expression and the rate of LNM *(OR, 3.31; 95% CI: [1.24, 8.79]; p = 0,02)* (Figure 6C).

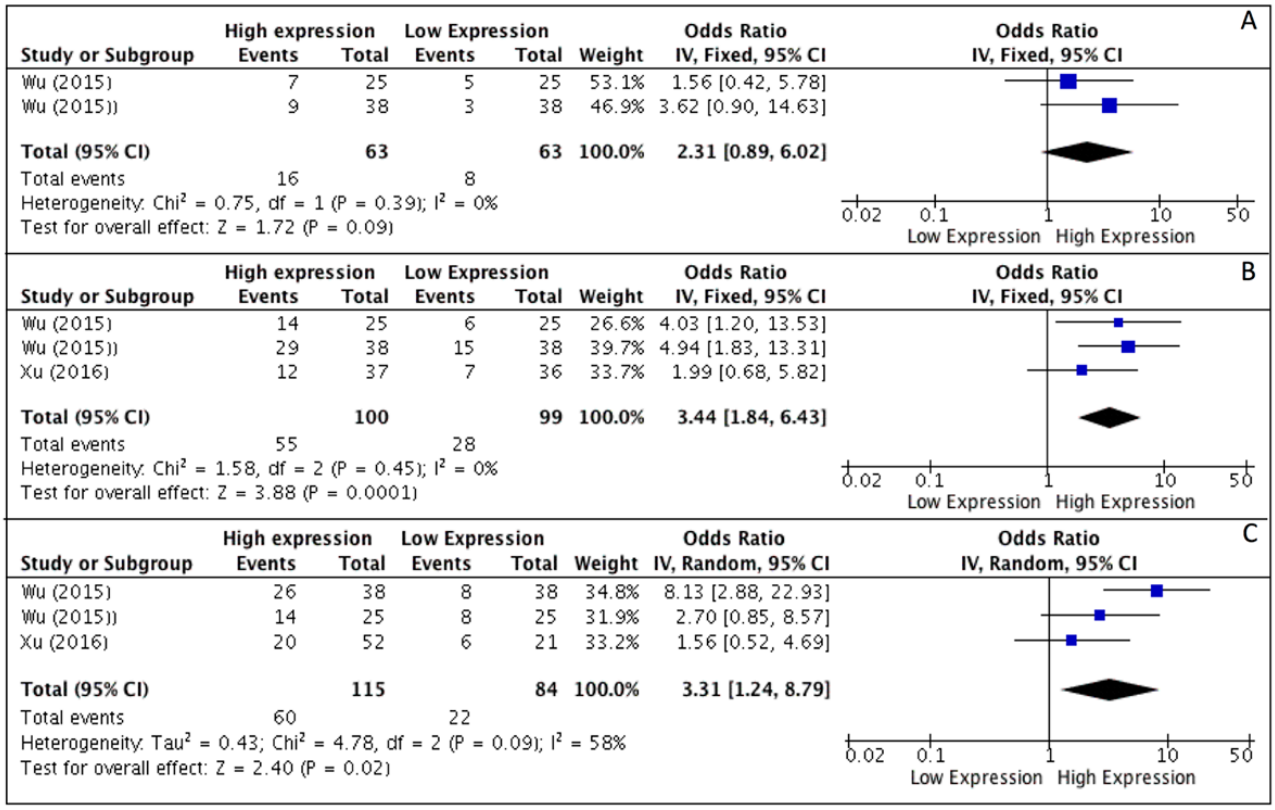


Figure 6: (A–C) Forest plot showing different expression of lncRNA HOTAIR and histological grade (A), tumor stage (B) and lymph-node metastasis (C); the frequency of HOTAIR expression was considered in patients with: Advance Stage (III–IV), positive LNM and Poor/Low Grade of differentiation

Analysis of overall survival pooling HRs showed high expression of HOTAIR to be associated with poor OS in patients with SCCHN *(HR, 1.90; 95% CI: [1.42, 2.53]; p < 0,0001)*, (Figure 7).

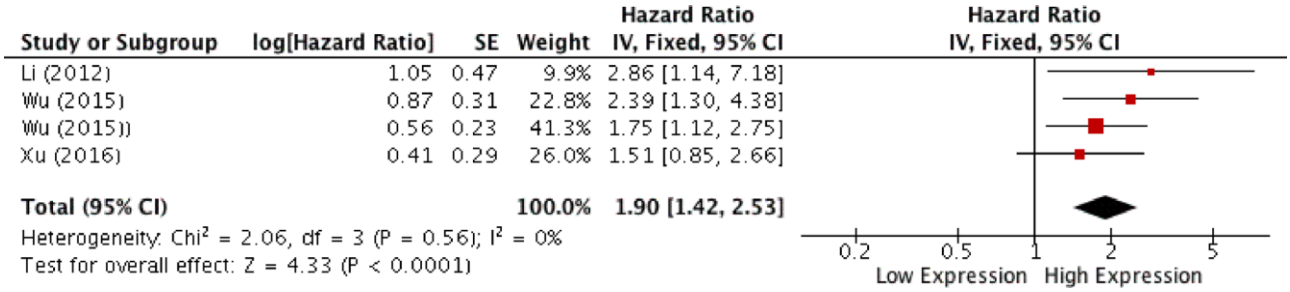


Figure 7: Forest plot for the association between HOTAIR expression and overall survival.

Summary of data extracted from the included studies used for the pooled analysis are shown in Table 3.

	HOTAIR expression									
Study	**High with LNM**	**Low with LNM**	**High with high grade**	**Low with high grade**	**High with high staging**	**Low with high staging**	**High expression**	**Low expression**	**HR statistic**	**Hazard ratios (95% CI)**
Li	NA	NA	NA	NA	NA	NA	33	39	Survival curve and data in paper	2,41–2,856
Wu	14	8	7	5	14	6	25	25	Survival curve	2.3
Wu	26	8	9	3	29	15	38	38	Survival curve	2.39
Xu	20	6	NA	NA	12	7	52	21	Survival curve	1.51
Total	**60**	**22**	**16**	**8**	**55**	**28**	**148**	**123**		

Table 3: Synthesis of data extracted from the included studies related to outcomes pooled in the meta-analysis

Effects of treatment with Curcumin and Genistein on OSCC cell lines

- 3.1. Background

‘‘Nutraceutical’’ (a combination of the words ‘‘nutrition’’ and ‘‘pharmaceutical’’) refers to any substance considered to be a food or a food ingredient that provides medical and health benefits. The use of natural agents is promising because not only they have minimal toxicity to humans compared to conventional chemotherapies, but also, they could target numerous signaling pathways. This is beneficial as malignant transformation and progression are multistage processes caused by gene alterations in more than one signaling pathway. This is one of the most plausible explanations why monomodal therapy typically fails in cancer treatments as the specific inhibitors often target only a single gene in a signaling pathway. Therefore, the impact of natural agents on cancer treatment could be more efficacious, as they can be used alone or as adjuvant in combination chemotherapy to improve therapeutic efficacy by overcoming drug resistance and/or reducing drug-induced toxicities. Hence, many of the anticancer agents currently used in cancer therapies have been developed from natural products such as plants (vincristine, vinblastine, etoposide, paclitaxel, camptothecin, topotecan, and irinotecan), marine organisms (cytarabine), and microorganisms (dactinomycin, bleomycin, and doxorubicin). Besides these, there are also plant-derived dietary polyphenols such as curcumin, genistein, resveratrol, epigallocatechin-3-gallate, indole-3-carbinole and others. A number of studies involving cultured cancer cells and animal models have illustrated the protective role of these dietary polyphenols, and mechanistic studies have demonstrated that they exert their antiproliferative and/or proapoptotic effects to prevent the occurrence and/or spread of various cancers by targeting numerous key elements in intracellular signaling network involved in carcinogenesis.

Curcumin is one of the components of curry and a popular dietary spice worldwide. It is the primary active constituent of turmeric, a botanical agent derived from the rhizome (root) of the Curcuma longa, a perennial herb belonging to the ginger family that is broadly cultivated in south and south-

east Asia. Turmeric is comprised of a group of three curcuminoids, i.e., curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin, as well as volatile oils, sugars, proteins and resins. Curcumin is a hydrophobic polyphenol that is nearly insoluble in water. Importantly, it has limited systemic bioavailability due to its rapid metabolism, largely through conjugation to sulfates and glucuronides. In humans, this metabolism presumably occurs in the gastrointestinal tract rather than in the liver. Curcumin has been considered pharmacologically safe, based on the fact that it has been consumed for centuries as a dietary spice at doses up to 100 mg/day. Moreover, its safety and tolerability became evident in phase I studies when it was administered at doses as high as 8 g per day.

Genistein (4′,5,7-trihydroxyisoflavone) is an isoflavone, found in soybeans and in all its derivatives such as flour, sauces, oil, milk and cheese. It is also found in other legumes such as lentils, beans, peas, chickpeas and whole grains such as wheat, rice, barley, rye and oats. It has highanti-cancer properties, including the inhibition of tyrosine kinase proteins and inhibition of cell cycle at G2/M phase; it is also able to promote apoptosis by activation of caspase-9 and -3.

The aim of this study was to assess the effects of treatment with different dosages of curcumin and genistein on oral cancer cell lines.

- 3.2. Materials and Methods

-Cell cultures and treatments

PE/CA-PJ15, PE/CA-PJ49, HSC-3 are cell lines of tongue squamous cells carcinoma (European Collection of Cell Cultures, ECACC). Cells were maintained at standard conditions of temperature and atmosphere (37°C and 5% CO2, respectively) for all tests used. DMEM culture medium with 4,500 mg/L glucose was used for PE/CA-PJ15 and PE/CA-PJ49 cells and Roswell Park Memorial Institute 1640 medium (RPMI 1640) for HSC-3 cells (Life Technologies, Gibco, Grand Island, NY, USA). Both culture media were supplemented with 10% fetal bovine serum, L-glutamine (2 mM) and penicillin–streptomycin (100 U/mL) (Sigma Aldrich, Saint Louis, MO, USA). Genistein (Abcam,

Cambridge, UK) and curcumin were dissolved in dimethylsulfoxide (DMSO) at a stock concentration of 5 mM. Serial dilutions of Genistein at 20, 50 and 100 μM and of Curcumin at 1, 5, 10, 20 and 50μM were prepared. The cell lines were treated for 24, 48 and 72 hours at the concentrations previously mentioned.

- *xCELLigence System*

The xCELLigence DP Real-Time Cell Analyzer (RTCA) is used for real-time monitoring of cell proliferation, cytotoxicity, and migration. This system is based on recording the electronic impedance with a unitless parameter called Cell Index (CI). The CI calculation is based on the following formula: CI = (Zi - Z0)/15çç where Zi is the impedance at the start of the experiment. Hence, CI is a self-calibrated value derived from the ratio of measured impedances. When many cells are attached on the surface of a particular E-Plate the CI gets high. For this reason, we have used it to determine the variation of cell adhesion after curcumin treatments. The RTCA System was used accordingly to the manufacturer instructions.

- *MTT assay and Trypan blue exclusion test*

Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate cell viability of PE/CA-PJ15, PE/CA-PJ49, and HSC-3 cells after treatments with genistein. We seeded 5×104 cells in a total volume of 250 μL/well in a 96-well plate with 20, 50 and 100 μM of genistein for 24, 48 and 72 hours. Then, 100 μL of fresh culture medium and 10 μL of 12 mM MTT stock solution were added to each well after the cells were incubated for 4 hours at 37°C. To each well, SDS-HCl solution (10 mL of 0.01 M HCl to 1 gm of SDS) was added for an incubation period of 12 hours in a humidified chamber at 37°C. The absorbance was read at 570 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific). While PE/CA-PJ15, PE/CA-PJ49, HSC-3 cells were treated with genistein and curcumin at the same concentrations and time points, Trypan blue exclusion method was used to validate the cell viability results obtained with

MTT assay. A suspension of each cell line was mixed with 0.4% Trypan blue solution and, after 10 minutes, cells were counted automatically with JuLI™ FL (NanoEntek, Pleasanton, CA, USA).

- *Scratch assay*

A monolayer of each cell line was scraped with a p200 pipette tip and then washed twice with Dulbecco's phosphate buffered saline 1X (Life Technologies, Gibco) in order to remove debris. Then, cells were treated with the IC50 dose of genistein at 24 hours. Untreated cells were used as control. Initially, we acquired the first image (T0) and then the subsequent ones after 1 hour (T1), 2 hours (T2), 3 hours (T3), 5 hours (T4), 6 hours (T5) and 24 hours (T6) after treatment. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to calculate the size of wound and analyze all the acquired images. GraphPad Prism 7 (GraphPad Software, Inc., CA, USA) software was used for statistical evaluation.

- *Western blotting analysis*

After treatments cells were lysated to obtain proteins. They were measured and, then, separated by 15% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Bovine serum albumin (BSA) 5% was used for 1 hour as blocking solution. Membranes were incubated with vitronectin (1:150; BD Biosciences), OCT4 (1:700; Novus Biologicals, Littleton, CO, USA), survivin (1 : 1,000; Cell Signaling Technology Inc., Danvers, MA, USA), and β-actin (1:5,000; Sigma Aldrich), overnight at 4°C. Then, peroxidase-conjugated secondary antibody was used (1 : 2,500; Santa Cruz Biotechnology, Dallas, TX, USA). Signals were acquired with enhanced chemiluminescence kit (ClarityTM Western ECL Substrate, Bio-Rad). UVP ChemiDoc-It®TS2 Imaging System (Analytik Jena AG, Jena, Germany) was used.

- *Statistical analysis*

GraphPad Prism 7 software was used for Student's t-test and one-way analysis of variance (ANOVA) and all data were expressed as the M ± SEM;17 a P-values ,0.05, P,0.01, P,0.001 were accepted as statistically significant.

- 3.3. Results

- Effects on cell adhesion

Through xCELLigence system, we monitored adhesion kinetics in real time of the treated cells, using untreated cells as control. After 24, 48 and 72 hours, the CI values were taken and adhesion curves were analyzed. Data obtained show clear effects of genistein and curcumin on all cells used even at 24 hours after the treatment. The resulting curves for Curcumin showed a difference of adhesion between control and treated cells. The reduction of adhesion was proportionally increased respect to the concentration of curcumin used for all cell lines. Even after long times of treatment, the effects of curcumin were significant.

CI values to 72 hours were considerably reduced in all conditions analyzed, even considering the limitations of xCELLigence system, such as the normal reduction in cell growth space for long analysis times. However, the results obtained confirmed that the increase in curcumin concentrations leaded to a proportional reduction of cell adhesion, even for very long treatments. In the evaluation of adherence rate, we converted the CI values in percent values in order to obtain a better indication of the reduction of cell adhesion, considering the control value of CI, at each time point, as the maximum value of adhesion at that period. In order to better evaluate the effect of curcumin on cell adhesion, we evaluated the expression of integrin β1, an integral membrane glycoprotein that binds the extracellular matrix proteins playing a key role in cell adhesion. Figure 8 a, b showed the reduction of integrin expression by 50% in cells treated with 10 μM of curcumin after 24 hours compared to control.

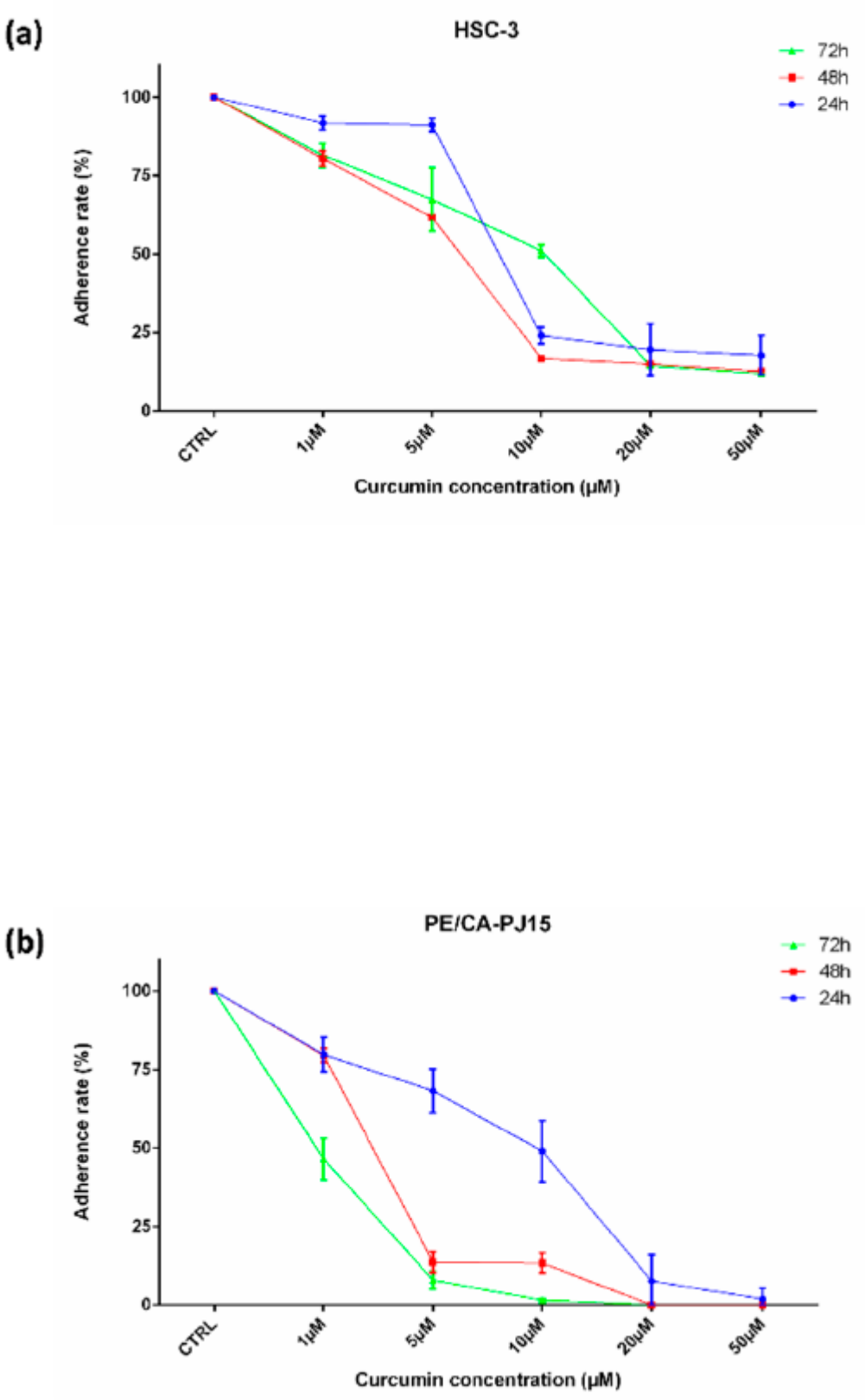


Figure 8 (A/B): Expression of integrin β1. Downregulation of integrin β1 expression by 50% in cells treated with 10 μM of curcumin after 24 hours compared to control (a), (b).

While, after 48 hours the same reduction was present for cells drawn with about 5μM of curcumin. Instead, at 72 hours after treatment the values were significantly reduced compared to the treatments after 24 and 48 hours. In particular, a reduction of 50% adhesion relative to the control was evident in cells treated with 5μM of nutraceutical. In addition, we found a great reduction of integrin expression in all the cells treated with 20 and 50μM of curcumin to 48 hours and 72 hours after treatment.

- *Effects on cell viability*

Through the MTT assay, we verified a decrease in cell viability proportional to increasing concentrations of curcumin and genistein for all treated cell lines. We observed a 50% reduction of viability in HSC3 cells treated with 5-10 μM of curcumin at 24 hours after treatment and 1-5 μM at 48 and 72 hours. PE/CA-PJ15 cells showed the same reduction for the concentrations of 10 μM of curcumin at 24 and 48 hours and about 10-20 μM at 72 hours. A reduction of about 50% was observed in PE/CA-PJ49 cells treated with 5-10 μM of curcumin at all time points. Through trypan blue test, the cell viability was reduced for HSC-3 and PE/CA-PJ49 cells in the same way as MTT assay. While for the PE/CA-PJ15 cells a 50% reduction in cell viability was observed for cells treated with 10-20 μM of curcumin at all time points unlike MTT test. For genistein, we detected a reduction of about 50% of cell viability in each treated cell line with concentrations between 20 and 50 μM at 24, 48 and 72 hours. Trypan blue assay confirmed almost all the data obtained with the MTT assay.

- *Effects on cell migration*

A monolayer of each cell line (PE/CA-PJ15, PE/CA-PJ49, HSC-3) was scraped with a p200 pipette tip and then washed twice with Dulbecco's phosphate buffered saline 1X (Life Technologies, Gibco) in order to remove debris. Then, cells were treated with the IC50 dose of genistein at 24 hours. Untreated cells were used as control. Initially, we acquired the first image (T0) and then the subsequent ones after 1 hour (T1), 2 hours (T2), 3 hours (T3), 5 hours (T4), 6 hours (T5) and 24 hours (T6) after treatment. The treated cells migrated very little in the cut area. The most significant effects were evident starting from T4 and, specifically, the wound of treated cells does not show any healing

after 24 hours. The untreated control showed an increasing level of healing of the wound during the considered times total healing after 24 hours.

Evaluations of the timing at T4, T5 and T6 were most significant with P-value ,0.05, P,0.01, P,0.001 (Figure 9 a,b).

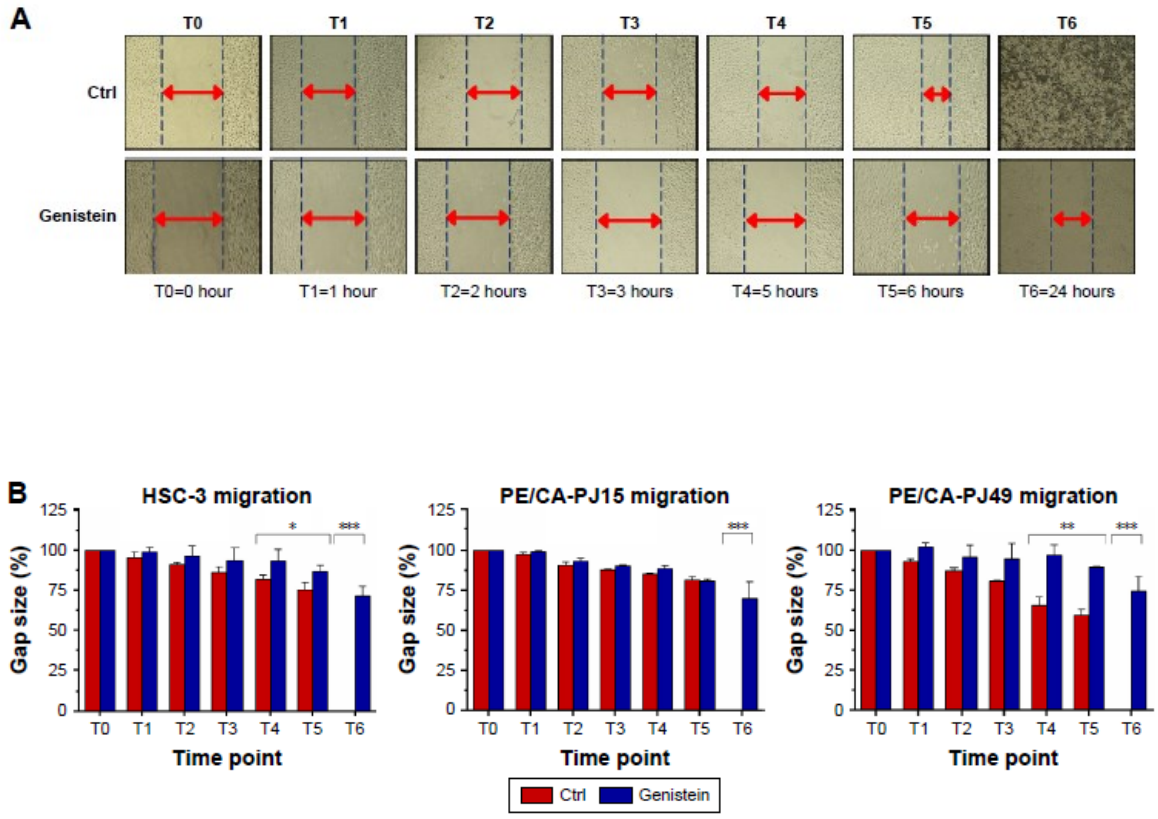


Figure 9: Effects of genistein on cell migration(A) and Significant increase of the gap size is more evident from T4 in tongue carcinoma cells compared to untreated control (Ctrl) (A). All experiments were performed at least 3 times and results are presented as the mean ± SD. *P,0.05, **P,0.01, ***P,0.001 (B).

ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to calculate the size of wound and analyze all the acquired images. GraphPad Prism 7 (GraphPad Software, Inc., CA, USA) software was used for statistical evaluation. The same protocol was adopted using curcumin standing for the genistein. To evaluate cell migration, we captured images at the beginning of the treatment with 10 µM of curcumin (T0) and at intervals (T1, T2, T3, T4, T5 and T6) during the migration of the cells to close the scratch, and we compared the images to determine the rate of cell migration (Figure 10a).

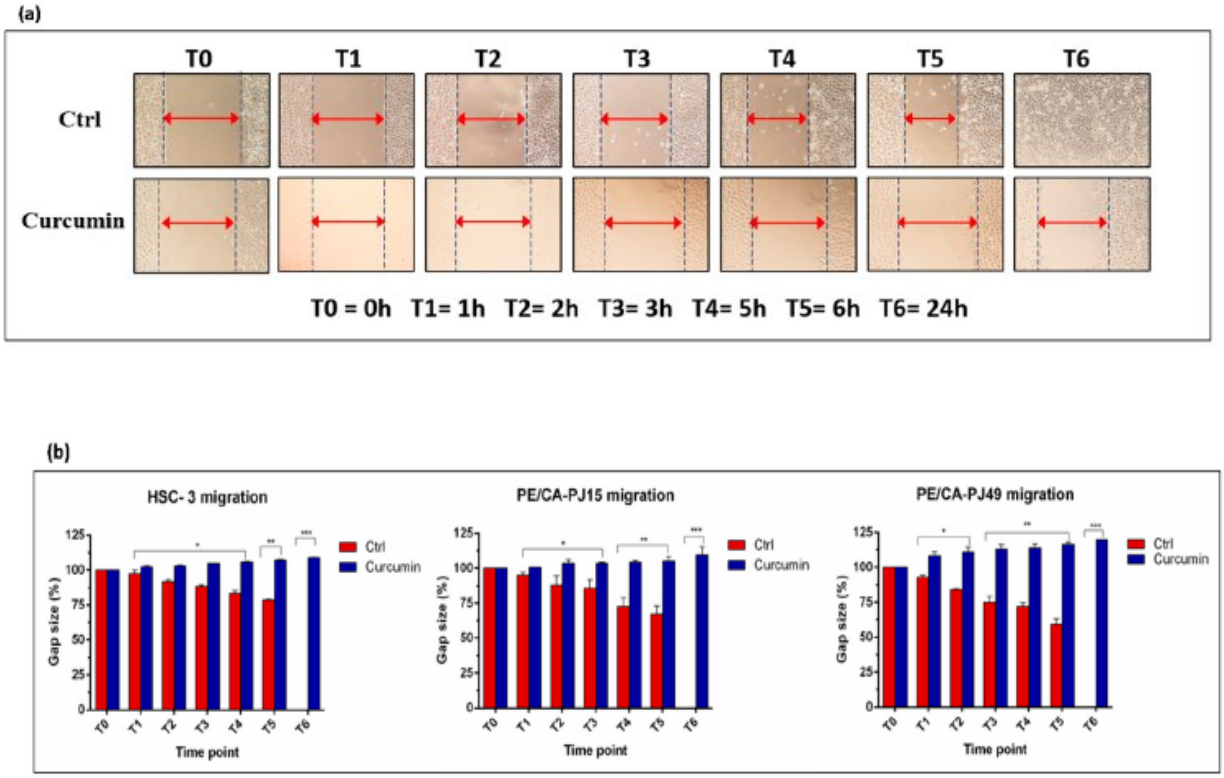


Figure 10: Effects of curcumin on cell migration(a); All experiments were performed at least three times and results are presented as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 (b).

The concentration of curcumin used was the average IC50 value at 24 hours, previously estimated. All experiments were performed at least three times and the results were presented as means ± SD. We noticed a significant increase of the gap size directly proportional to the increase of the treatment time with curcumin, while it decreased in the untreated control. The increase was most evident from T3-T4 onwards and it was present in all of TSCC cell lines. For T4 and T5 times we had a very high significance compared to control ($p < 0.05$ and $p < 0.01$) for all cell lines. Instead, the wound treatment with curcumin after 24 hours (T6) prevented the complete healing of the wound, while it disappeared completely in the untreated control ($p < 0.001$).

- *Effects on cell apoptosis*

After treating tongue cancer cells with different curcumin concentrations, we evaluated the expression of PAR4. PAR4 is a pro-apoptotic and tumor suppressor protein that selectively induces apoptosis in cancer cells by activating the extrinsic mechanisms. We noticed an increased expression of PAR4, pro-apoptotic protein, for concentration higher than 10 μM at 24 and 48 hours and for all treatments at 72 hours. This demonstrated, probably, that curcumin induces apoptosis in cells treated with 10 μM already after 24 hours and that possible apoptosis is more evident after 72 hours of treatment (Figure 11).

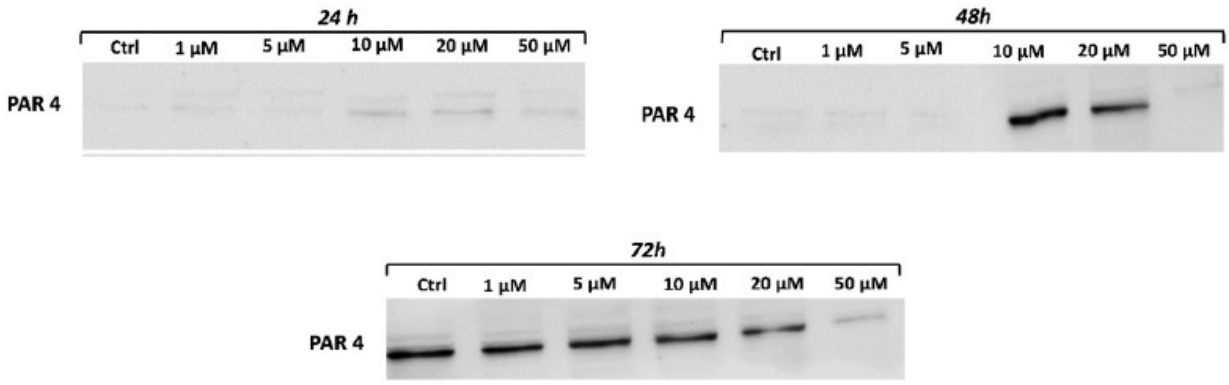


Figure 11: Curcumin reduces promotes apoptosis. Variation in level expression of PAR4 showed at 24 hours (a), 48 hours (b) and 72 hours (c). Curcumin upregulates PAR4. The results shown in Figure b belong to two gels.

Discussion and Conclusions

The tumor markers are playing an increasingly important role in cancer detection and management. (35). A cancer biomarker may be a molecule secreted by a tumor cell or a specific response of the body to the presence of cancer (36). Cancer biomarkers can be classified based on the disease state, including predictive, diagnosis and prognosis biomarkers (16). In our research activities, we focused mainly on the research of diagnostic and prognostic biomarkers. The first ones are used to detect or confirm presence of a disease or condition of interest or to identify individuals with a subtype of the disease. In OSCC such kind of molecules have been studied mainly in blood and saliva, aiming to find a marker that can help clinicians in the early diagnosis of the disease, decrease the diagnostic delay and improve the survival rate of OSCC patients (37-39). In the last years, the research of circulating biomarkers has been enriched by the study of new molecules in addition to the "classic" proteins. Among these, great attention has been paid on the study of miRNAs. Such molecules are a class of small non-coding RNAs long 19–23 nucleotides, able to negatively regulate target messenger RNAs (mRNAs) by partially binding to their 30 untranslated region (40). In cancer miRNAs are often dysregulated and implicated in different cellular process such as differentiation, proliferation, apoptosis and metastasis (41, 42). The presence of miRNAs was also discovered in blood and several other body fluids including: urine, tears, pleural effusions and saliva, exhibiting different expression patterns in cancer patients compared to healthy individuals (43). Although, the exact mechanism with which miRNAs are released into the body fluids is still unclear a lot of studies have been performed in order to assess whether circulating miRNAs could be used as diagnostic biomarker in OSCC.

In order to synthesize results of literature, we performed a systematic review about the evaluation of circulating miRNAs in blood and derivatives as diagnostic biomarkers in OSCC (44). Results of the review revealed that 11 studies reported very good values of Area Under the Curve (AUC), sensitivity and specificity. However, issues related to methods and sampling have to be overcome before to use circulating miRNAs in the clinical practice.

Unlike detection of circulating nucleic acids that can be amplified prior to detection using array technologies, two potential problems with serum protein biomarkers are their low concentrations due to dilution in the circulation and the limited ability to assay multiple proteins from a small sample volume by means of immunoenzymatic assays (45). Recently, the development of proximity extension assay (PEA) has overcome such limitations allowing the simultaneous quantification of proteins in body fluids (46).

On the basis of such evidences, we performed a study aiming to assess both circulating proteins (with PEA) and miRNA (by qPCR-panel) in patients with squamous cells carcinoma of the tongue (47). We identified 23 proteins that seems to be able to detect the disease, among these neurotrophin-3 (NT-3) showed the better predictive values. While, no one of the 372 circulating miRNAs analyzed showed good values for use as diagnostic biomarker. To note, miR-150 was downregulated in blood but upregulated in tissues of patients, suggesting a possible role in the pathogenesis of OSCC.

Focusing on the prognosis, the research aims to find a biomarker used to identify likelihood of a clinical event, disease recurrence or progression in patients who have the disease or medical condition of interest (16). On the prognostic side, focused on the study of a new molecules called long non-coding RNAs. tools for treatment of the disease. Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides that do not code for protein production (31).

Their action seems to be linked to chromatin modification causing an overexpression or silencing of target genes of which most are in cancer pathogenesis (48). In particular, we performed a meta-analysis of studies that quantified the expression of the lncRNA HOTAIR in patients with squamous cells carcinoma of the head and neck (SCCHN) (49). HOTAIR is a well-known oncogene, 2.2 kb long and transcribed from the antisense strand of the HOXC gene located on chromosome 2, and consisting of 6 exons (50). Although the exact mechanism of action is not well known, HOTAIR seems to be able to regulate the expression of very important genes, such as: p53, PTEN, E-cadherin and HER-2 (32, 33, 51). Results of our meta-analysis based on the inclusion of 4 studies revealed that HOTAIR is a promising prognostic biomarker in SCCHN patients. HOTAIR expression correlated

with: advanced tumor stages (OR, 3.44; 95% CI: [1.84, 6.43]; p < 0,001), development of lymphnode metastasis (OR, 3.31; 95% CI: [1.24, 8.79]; p = 0,02) and poor overall survival (HR, 1.90; 95% CI: [1.42, 2.53]; p < 0,0001). However, such preliminary results need to be further expanded increasing the power of evidences and focusing on the single subsite of SCCHN.

Although treatment options for OSCC patients have improved in the last decades, the overall survival rate is still low, underlining the importance of expanding research in new therapeutic options. We addressed our attention to the study of the effects of two nutraceuticals (Curcumin and Genistein) on OSCC cell lines. Results of our studies revealed that both molecules exert dose dependent inhibition of viability, adhesion proliferation, migration of OSCC cell lines. In addition, curcumin seems to be able to induce apoptosis through downregulation of survivin, that is a well-known inhibitor of apoptosis (52).

Although such effects are very promising, it is to underline that one of the major barriers to the use of nutraceuticals in clinical practice is related to their low systemic bioavailability (53). In order, to increase the amount of the products absorbed systemically further studies on new pharmaceutical formulations of nutraceuticals should be addressed, aiming to overcome such limitations and allow the use of nutraceuticals in the clinical practice.

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Appendix 1: Journal publications

1) Linda Boldrup, **Giuseppe Troiano**, Xiaolian Gu, Philip Coates, Robin Fåhraeus, Torben Wilms, Lena Norberg-Spaak, Lixiao Wang, Karin Nylander. Evidence that circulating proteins are more promising than miRNAs for identification of patients with squamous cell carcinoma of the tongue. Oncotarget. 2017 Sep 30; doi: /10.18632/oncotarget.21402.

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28) Mario Dioguardi, Giorgia Apollonia Caloro, **Giuseppe Troiano**, Giovanni Giannatempo, Luigi Laino, Massimo Petruzzi, Lorenzo Lo Muzio: Oral manifestations ***in chronic uremia patients.*** Renal Failure 10/2015; DOI:10.3109/0886022X.2015.1103639

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Appendix 2: Abstracts and Conference Proceedings

1) **G. Troiano**, A. Santarelli, R. Mauceri, O. di Fede, L. Lo Muzio. Prognostic predictive value of microRNA expression in oral squamous cell carcinoma: a systematic review, meta-analysis and trial sequential analysis. XIV CONGRESSO NAZIONALE SIPMO, ROMA, 26-28 OTTOBRE 2017

2) C. Lajolo, **G. Troiano**, A. D'Addona, L. Lo Muzio, M. Giuliani. Oral Lichen Planus and Malignant Transformation: a Systematic Review. Poster #: 0795. IADR general session, San Francisco, USA, 22-25 March 2017

3) **G. Troiano**, K. Zhurakivska, V.C.A. Caponio, L. Boldrup, K. Nylander, A. Santarelli, G. Campisi, L. Lo Muzio. I long non-coding RNAs (LNCRNAS) sono espressi in modo anomalo nel carcinoma della lingua e rappresentano promettenti biomarcatori clinici. Collegio dei docenti di odontoiatria, XXIV CONGRESSO NAZIONALE, Milano, 6-8 aprile 2017. Journal of Osseointegration January-April 2017; 9(1):196

4) V. Caponio, **G. Troiano**, K. Zhurakivska, A. Santarelli, L. Guida, L. Lo Muzio. Espressione di LNCRNA, un promettente fattore prognostico nel carcinoma a cellule squamose testa-collo: meta-analisi. Collegio dei docenti di odontoiatria, XXIV CONGRESSO NAZIONALE, Milano, 6-8 aprile 2017. Journal of Osseointegration January-April 2017; 9(1):150

5) K. Zhurakivska, **G. Troiano,** A. Albanese, G. Pizzo, L. Lo Muzio, L. Lo Russo. Clinical effectiveness of platelet concentrates for periodontal regeneration. Collegio dei docenti di odontoiatria, XXIII CONGRESSO NAZIONALE, Roma, 6-8 Aprile 2016.MINERVA STOMATOLOGICA 2016; 65 (Suppl. 1 to No. 3):189.

6) L. Coppola, A. Cocco, M. Dioguardi, G. Giannatempo, L. Laino, **G. Troiano,** Third molar surgery: two methods of dexamethasone's administration to reduce postoperative discomforts. Vol. 65 - Suppl. 1 to No. 3 MINERVA STOMATOLOGICA 2016; 21-22

7) C. Arena, L. Laino, G. Capocasale, V. Panzarella, **G. Troiano,** L. Lo Muzio, What is the best surgical approach for the treatment of solid/multicystic ameloblastoma? Vol. 65 - Suppl. 1 to No. 3 MINERVA STOMATOLOGICA 2016; 192

8) C. Mangione, M. Dioguardi, A. De Lillo, D. Ciavarella, **G. Troiano,** G. La Torretta. Clinic and prejudict: case report of partial glossectomy in 92 years old woman. Vol. 65 - Suppl. 1 to No. 3 MINERVA STOMATOLOGICA 2016; 205-206

9) K. Zhurakivsa, **G. Troiano**, A. Albanese, G. Pizzo, L. Lo Muzio, L. Lo Russo, Clinical effectiveness of platelet concentrates for periodontal regeneration. Vol. 65 - Suppl. 1 to No. 3 MINERVA STOMATOLOGICA 2016; 189

10) **G. Troiano**, A. De Lillo, L. Lo Russo, D. Ciavarella, G. Giannatempo, R. Prisco, Use of T-Scan device for the occlusal balancingof Michigan splint. Vol. 65 - Suppl. 1 to No. 3 MINERVA STOMATOLOGICA 2016; 70

11) **G. Troiano**, F. Ardito, D. Perrone, N. Termine, L. Lo Muzio. Synergic effects of curcumin and polydatin on MG63-osteosarcoma cells proliferation are mediated through down-regulation of Survivin. Annali di Stomatologia 2015; Suppl. 1 to n. 2: 1-71

12) G. Capocasale, R. Mauceri, **G. Troiano,** L. Lo Muzio, G. Campisi. Optical coherence tomography as a simple and not invasive tool for the diagnosis of oral disease: a case report. Annali di Stomatologia 2015; Suppl. 1 to n. 2: 1-71

13) L. Laino, M. Giuliani, R. Mauceri, F. Giancola, **G. Troiano**. Use of porcine collagen matrix (Mucograft®) to promote the wound healing in the oral cavity. Annali di Stomatologia 2015; Suppl. 1 to n. 2: 1-71

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15) V. Panzarella, R. Mauceri, M. Dioguardi, **G. Troiano**, O. Di Fede, Evidence of field cancerization of oral squamous cell carcinoma: a case report. Annali di Stomatologia 2014; Suppl. 3 to n. 2: 1-41

16) G. Troiano, L. Lo Russo, V. Panzarella, N. Termine, L. Laino, Synchronous tumors arising in the hard palate. Annali di Stomatologia 2014; Suppl. 3 to n. 2: 1-41

17) G. Troiano, D. Ciavarella, M.E. Licata, O. Di Fede, L. Laino. Use of a new collagen matrix (mucograft) in soft tissue recostruction after bioptic excision. Annali di Stomatologia 2014; Suppl. 3 to n. 2: 1-41

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20) B. Polizzi, P. Tozzo, A. De Lillo, **G. Troiano,** F. Frezza, G. Campisi, Oral lesions due to ACINETOBACTER BAUMANNII infection in a patient affected by autoimmune hemolytic anemia(AIHA): first clinical report. Minerva Stomatologica – Vol. 63 Suppl. 1 al N. 4 (Aprile 2014)

21) F. Giancola, O. Di Fede, A. De Lillo, **G. Troiano**, L. Lo Russo, G. Campisi, Restitutio ad integrum in a case of ONJ related to bevacizumab. Minerva Stomatologica – Vol. 63 Suppl. 1 al N. 4 (Aprile 2014)

22) Troiano G, Staibano S, Licata ME, Termine N, Lo Muzio L, Sebaceous carcinoma of the lip. Ann Stomatol (Roma). 2013 Oct 24;4(Suppl 2):46-7.

Appendix 3: Oral Presentations

- Title: "Update on circulating microRNAs as potential clinical biomarkers in oral carcinoma." SIMPOSIO SIPMO SESSION, XXIV NATIONAL CONGRESS, College of teachers, Milan, 6-8 April 2017.
- Title: "Molecular biology in the early diagnosis of oral carcinoma". Refresher course: "New diagnostic techniques of oral carcinoma." At the Master's Degree in Dentistry and Dental Prosthetics Catholic University of the Sacred Heart - A. Gemelli University Hospital, Largo Agostino Gemelli, 8 - Rome

Appendix 4: Honors and Awards

Date	Honor/Prize	Awarding Body	Subject/Title
2017	College of teachers in dentistry National Congress, Milan 6-8 April 2017. (Best Poster)	Italian College of Teachers in Dentistry	Long non-coding RNAs (lncRNAs) are abnormally expressed in tongue carcinoma and represent promising clinical biomarkers
2017	College of teachers in dentistry National Congress, Milan 6-8 April 2017. (Honorable Mention)	Italian College of Teachers in Dentistry	Expression of a lncRNAs as promising prognostic factors in head and neck squamous cell carcinoma: meta-analysis
2017	XIV SIPMO (Italian Society of Oral Pathology and Medicine) National Congress (Honorable mention)	SIPMO (Italian Society of Oral Pathology and Medicine)	Prognostic predictive value of microRNA expression in oral squamous cell carcinoma: a systematic review, meta-analysis and trial sequential analysis
2015	XIII SIPMO (Italian Society of Oral Pathology and Medicine) National Congress (Honorable mention)	SIPMO (Italian Society of Oral Pathology and Medicine)	Optical coherence tomography as a simple and non-invasive tool for the diagnosis of oral disease: a case report

Appendix 5: Original PDFs of Journal publications

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Contents lists available at ScienceDirect

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journal homepage: www.elsevier.com/locate/oraloncology

Review

Circulating miRNAs from blood, plasma or serum as promising clinical biomarkers in oral squamous cell carcinoma: A systematic review of current findings

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ABSTRACT

The purpose of this systematic review was to summarize current findings on the use of circulating miRNAs from blood, serum and plasma as cancer biomarkers in patients with oral squamous cell carcinoma. Studies were gathered after searching four different electronic databases: PUBMED, SCOPUS, Cochrane Library and Web of Science. Additional search was carried out through cross check on bibliography of selected articles. After the selection process made by two of the authors, 16 articles met the inclusion criteria and were included in the review. Results showed that circulating miRNAs from blood, serum or plasma represent promising candidates as cancer biomarkers in patients suffering from oral cancer. The possibility to predict recurrences and metastases through follow-up quantification of candidate miRNAs represents another potential feature to be addressed in future studies. However, methodological standardization and uniform sampling is needed to increase the power and accuracy of results.

Introduction

Background

Oral squamous cell carcinoma (OSCC) is the most common cancer type arising in the oral cavity and belongs to the more wide group of head and neck cancers (HNCs), the sixth most common cancer type in humans [1]. An incidence of 300,000 OSCC cases worldwide has been estimated, accounting for about 24% of all HNCs [2]. The five-year survival for OSCC patients remains less than 50 percent, and late diagnosis may be responsible for the poor prognosis in these patients [3,4]. In fact the five-year survival rate for patients with stage I disease is about 80%, but strongly decreases to approximately 40% in higher stages [5]. No reliable biomarkers are, however, available to detect OSCC aggressiveness and predict disease outcome. In the future new diagnostic and prognostic systems must be developed, in order to allow personalized therapies for individual tumors [6].

microRNAs (miRNAs) are a class of small non-coding RNAs 19–23 nt long, able to negatively regulate target messenger RNAs (mRNAs) by partially binding to their 3′ untranslated region [7]. Until today, about 2500 miRNAs have been recognized, targeting about two thirds of all human genes [8,9]. miRNAs seem to be generated from intergenic genomic sequences or intronic regions of protein-coding genes [10]. The majority of mammalian miRNAs are transcribed by the RNA polymerase II into a long precursor containing a typical stem-loop structure (pri-miRNAs) (Fig. 1) [11]. These initial transcripts undergo cleavage by the ribonuclease enzyme III Drosha to produce the pre-miRNAs comprising around 70 nucleotides and a hairpin structure [12]. Pre-miRNAs are then transported into the cytoplasm by a nuclear export factor (Exportin-5), where they are cleaved by an enzyme called Dicer into duplexes of 21–23 nucleotides [13]. Afterwards, one strand of the duplex, the passenger strand (miRNA*), is degraded and the other incorporated together with Argonaute proteins or lipoproteins to form the RNA-induced silencing complexes (RISC) leading to the formation of mature miRNA [14]. It has, however, been reported that in some cases the miRNA* strands escape the degradation and are loaded on carrier proteins and act as mature

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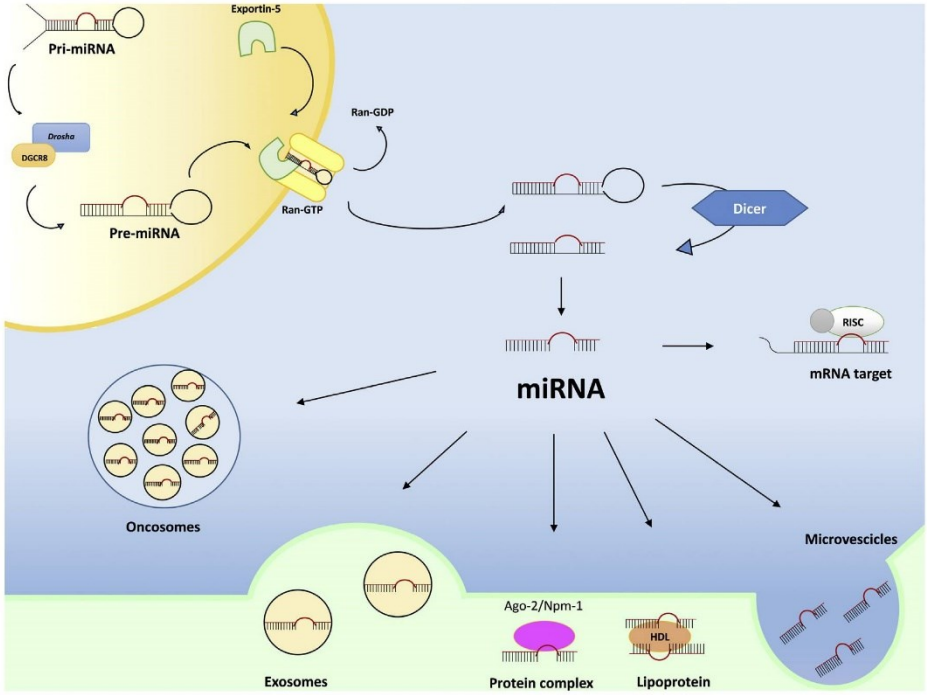

Fig. 1. Scheme of processes involved in synthesis of miRNAs. The process starts in the nucleus and ends with secretion once the miRNa has reached maturity.

miRNAs [15]. Due to their short structure, a single miRNA is able to bind to a lot of target genes and regulate their expression. But a single 3′ untranslated region can also be targeted by a great number of different miRNAs [16]. In cancer miRNAs are often dysregulated and implicated in different cellular process such as differentiation, proliferation, apoptosis and metastasis [17–20]. The presence of miRNAs was also discovered in blood and several other body fluids including: urine, tears, pleural effusions and saliva, exhibiting different expression patterns in cancer patients compared to healthy individuals [21,22]. The exact mechanism with which miRNAs are released into the body fluids is, however, still unclear but various mechanisms seem to be involved [23]. Passive release into body fluids as a result of cell death is supported by the fact that extracellular miRNA levels in conditioned media increased with higher rate of cell death [24]. This does, however, not seem to be the only way for miRNA release as they also can be loaded into microvesicles (also called oncosomes) through membrane budding, or incorporated in exosomes and then released in the extracellular space [25–28] (Fig. 1).

Exosomes are microvesicles of 30–120 nm, initially considered cell waste products, but more recently having gained a lot of interest as they seem to play an important role in intercellular communication [29,30]. Exosomes can be released into the extracellular environment through inward budding of the cell membrane into the cytoplasm, or invagination into multivesicular bodies as intraluminal vesicles (ILVs) and then released after the fusion of ILVs with the cell membrane [31–33]. Exosome-miRNAs have been reported to represent a subset of about the 3% of the entire amount of cell-free miRNAs [24]. A large amount of miRNAs are released through an RNA-binding protein dependent pathway thanks to the association of miRNAs with Argonaute-2 (Ago-2), high density lipoprotein (HDL) and nucleophosmin 1 (Npm1) [23,34–36]. Due to the exosome-carriage and binding to RNA-binding proteins miRNAs have demonstrated to be highly stable in body fluids leading to rising hopes for their future use as cancer biomarkers [37]. In fact it has been seen that circulating miRNAs are not digested by RNase and stable at high pH, boiling and multiple freeze-thaw cycles [27,34,38].

Challenges in the research of circulating-miRNAs

Despite the stable features of miRNAs and the encouraging initial results, researchers were faced with different challenges for the routine use of circulating miRNAs as cancer biomarkers. In fact, before the routine utilization of circulating miRNAs for clinical purposes, a number of issues must be solved for better standardization and safer use in the clinical practice. Firstly inconsistent levels of miRNAs have been reported among different investigated samples [39]. For example serum has been reported to show higher miRNA concentration compared to corresponding plasma, suggesting that the coagulation process may affect the total amount of miRNA [40]. Other issues related to differences in processing and handling of the sample may also lead to significant variation in results [41]. The variation in speed and duration of centrifugation may influence number of platelets and microvesicles remaining in the supernatant, and platelets may in turn unleash their amount of miRNA leading to a variation in total amount of cell free-miRNAs [42]. In addition, when studies are carried out on blood/plasma/serum samples each procedure should aim at reducing the risk of hemolysis as rupture of erythrocytes strongly can increase the amount of miRNA [37,38]. To reduce this risk, the time from blood collection to centrifugation should not exceed 2 h [43]. A

prolonged incubation (more than 5 h at room temperature) should also be avoided since that has been demonstrated to decrease the concentration of non-exosomal miRNA [44]. Furthermore, the type of anticoagulant in the collection tube can also affect results of the quantification [45]. The amount of sample used was also seen to influence results, as presence of inhibitors can affect synthesis of complementary DNA (cDNA) [37]. Although using a large amount of plasma leads to high activation of inhibitors, a too small amount may be inefficient in detection of circulating miRNAs due to lack of material [46]. In addition, a very important issue is normalization of miRNA expression data. Since many studies are performed with quantitative real time polymerase chain reaction (qRT-PCR) there is a need to find reliable "housekeeping" miRNAs for normalization. Some of the most common housekeeping genes in miRNA studies (eg. RNU6B and SNORD) have demonstrated unstable expression in serum and plasma and are considered unreliable for normalization [40,47]. It has been reported that the use of droplet digital PCR (ddPCR) systems may overcome this problem since it provides absolute quantification of the expression using a spike-in approach to control for RNA extraction and cDNA synthesis [48,49]. This technique has proved a comparable sensitivity, but greater precision and reproducibility for absolute quantification compared to qRT-PCR [50]. ddPCR is also more tolerant to presence of inhibitors in the reaction [51] and comparable results have been seen using either TaqMan or EvaGreen chemistries [52]. Another, promising technique is next-generation deep sequencing (miRNA-seq) which, however, needs more normalized protocols for wider and easier use [27,53].

Objectives

The aim of this paper was to review the literature on altered expression of circulating miRNAs from blood, serum or plasma in patients with oral cancer to report about their potential use as cancer biomarkers.

Methods

This systematic review was performed according to the Preferred Reporting Items for Systematic reviews and meta-Analysis (PRISMA) [54]. The systematic review has also been registered on the PROSPERO database (registration number: CRD42016041740). Two of the authors (GT, FA) independently performed search on the following electronic databases: PUBMED, SCOPUS, Cochrane Library and Web Of Science in the period between June 1 and 25 2016. When searching these databases a combination of 'MESH terms' and 'free text words' were pooled through boolean operators ('AND', 'OR'). The following protocol was uploaded: "((((mirRNA OR microRNA OR microRNAs OR miRNAs))) AND ((OSCC OR 'oral squamous cell carcinoma' OR 'oral cancer' OR 'Tongue Cancer' OR TSCC OR OTSCC OR Gingiva OR 'Head Neck'))) AND (Plasma OR Blood OR 'Whole Blood' OR Serum)". The criteria for inclusion in this systematic review were:

Studies on human blood, plasma or serum evaluating miRNA expression in patients with oral cancer compared to healthy controls.
Presence of at least one miRNA dysregulated in the test group confirmed by either qRT-PCR or ddPCR.

Exclusion criteria were:

Studies including heterogeneous sampling with other head and neck cancers (oropharynx, nose, maxillary sinus, larynx and tonsil).
Studies with less than 10 samples in each group.
Studies written in other language than English.
Studies performed on saliva or other body fluids.

No restrictions were imposed about date of publication. Additionally, a direct online research was carried out on the official websites of: Oral Oncology, Journal of Dental Research, British Journal of Cancer, Journal of Pathology, Biomarkers, Cancer Research, Clinical Cancer Research, Clinical Oral Investigation, Oral Diseases and Journal of Oral Pathology and Medicine. A further search was made on bibliography of articles that were read in full-text, in order to find other studies to include in the review. The first round of evaluation was performed reading only title and abstract of the studies. At the end of this first round all studies considered eligible were included for full-text evaluation. A direct research on bibliographies of articles read in full-text was also carried out, in order to find further articles to include. After full-text reading, only studies considered eligible by both authors were included in the systematic review and if disagreement between reviewers was present, it was solved through discussion. The value of Kappa Statistic was calculated to show the level of agreement between the two reviewers [55].

Results

A total of 419 studies were screened by title and abstract and of these 23 exceeded the first round of selection and were read in full-text. At the end of the selection process 16 studies [56–71] were assessed as adequate for inclusion in the study, while 7 studies [72–78] were excluded. A flow chart showing the processes for identification, inclusion and exclusion is seen in Fig. 2. The value of K-statistic was 0.72 showing a good level of agreement between reviewers [55]. For each study data for the following factors were extracted: sample size for each group, tumor stage, oral subsite of the tumors, type of sample, method performed in the discovery phase [79], number of miRNAs found to have different expression in the discovery phase, method performed in the validation phase [80], miRNAs discovered to be differentially expressed after the validation phase and correlation with clinicopathological behaviour. Additionally, statistical information from Receiver Operating Characteristic (ROC) such as: Area Under the Curve (AUC), specificity and sensitivity were extracted if reported. Data were extracted and collected through an ad hoc extraction sheet. Of the 16 studies included all reported differential expression of at least one circulating miRNA between patients with oral cancer and healthy control (Tables 1 and 2), while only 11 studies reported statistical evaluation of circulating miRNA as predictor biomarkers (Table 3). The stage of the tumor was reported in 8 studies [61,63,65–67,69–71], 4 studies [59,60,64,68] showed tumor size and neck status only, while 4 [56–58,62] did not report TNM features of the tumors. Subsite of the tumor was reported in 6 studies [59,63,64,69,70], while the others did not mention it. Looking at the discovery phase from which the search screening started, it is seen that 9 studies [56,60,61,63,65–68,71] performed a preliminary high throughput screening, while in the remaining the discovery phase was based on literature search. Most of the studies were conducted on plasma samples, while whole blood and serum samples were less investigated. All studies included performed a qRT-PCR to confirm results of the preliminary evaluation, none had used ddPCR. All results from data extraction are shown in Tables 1–3. Correlations with clinicopathological parameters have been reported in 9 studies [56,59,63–65,68,70,71], while 2 studies [60,61] failed to find correlations and 5 studies [57,58,62,66,69] did not investigate these features.

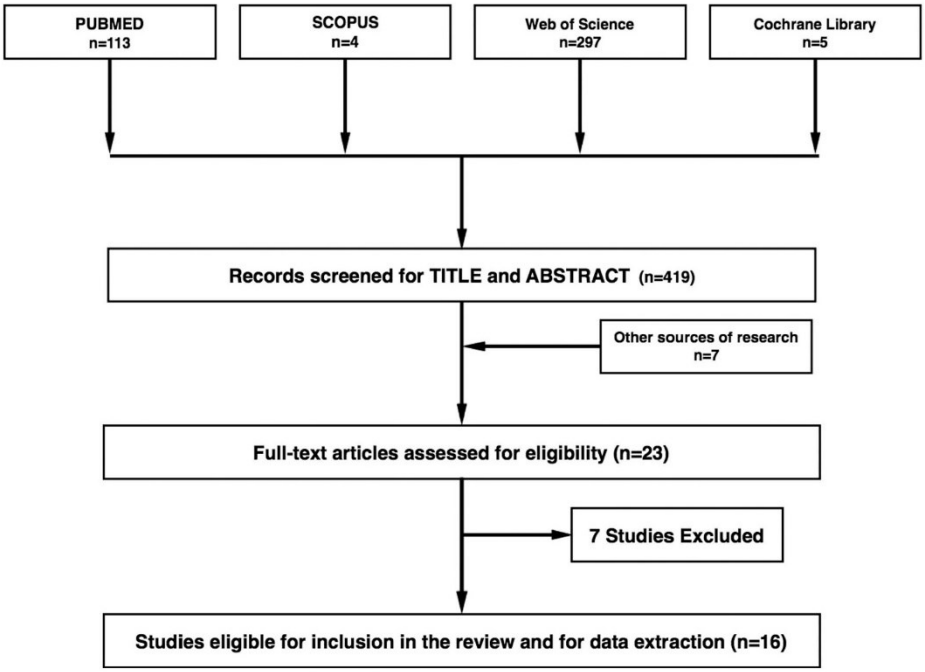

Fig. 2. Flow-chart of the selection process for studies included in the systematic review.

Table 1
Data for: name of the study, year of publication, number and stage of the tumors, type of sample and oral subsite involved.

Study	Year	Samples in the validation phase	OSCC tumors stage	Type of sample	Tumor from oral subsites
Liu et al. [69]	2016	OSCC: 63 Control: 26	Stage 1–3: 35 Stage 4: 20	PLASMA	Buccal Mucosa: 35 Tongue: 15 Others: 13
Xu et al. [71]	2016	OSCC: 101 Control: 103	Stage 1/2: 51 Stage 3/4: 50	SERUM	Not reported
Li et al. [68]	2016	OSCC: 108 Control: 108	T(1–2) = 39 T(3–4) = 69 N0 = 33 N(1–3) = 75	SERUM	Not reported
Sun et al. [70]	2016	OSCC: 104 Control: 40	Stage 1/2 = 66 Stage 3/4 = 38	SERUM	Tongue: 34 Others: 70
Lu et al. [67]	2015	OSCC: 90 Control: 53	Stage 1/2 = 20 Stage 3/4 = 60	PLASMA	Not reported
Ren et al. [64]	2014	OSCC: 58 Control: 32	T(1–2) = 19 T(3–4) = 39 N^- = 33 N^+ = 25	BLOOD	Gingiva: 16 Buccal Mucosa: 9 Tongue: 18 Others: 15
Ries et al. [65]	2014	OSCC: 57 Control: 33	Stage 1/2 = 26 Stage 3/4 = 31	BLOOD	Not reported
Ries et al. [66]	2014	OSCC: 50 Control: 35	Stage 1/2 = 27 Stage 3/4 = 23	BLOOD	Not reported
Hung et al. [62]	2013	OSCC: 51 Control: 12	Not reported	PLASMA	Not reported
Liu et al. [63]	2013	OSCC: 65 Control: 24	Stage (1–3) = 26 Stage 4 = 69	PLASMA	Buccal Mucosa: 34 Tongue: 25 Others: 36
Lo et al. [60]	2012	OSCC: 37 Control: 20	T1 = 10 T2 = 10 T3 = 10 T4 = 07	PLASMA	Not reported

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Table 1 (*continued*)

Study	Year	Samples in the validation phase	OSCC tumors stage	Type of sample	Tumor from oral subsites
Lu et al. [61]	2012	OSCC: 54 Control: 36	Stage 1/2 = 20 Stage 3/4 = 34	PLASMA	Not reported
Yang et al. [59]	2011	OSCC: 39 Control: 12	T(1–3) = 13 T4 = 26 N0 = 20 N1 = 7 N2 = 12	PLASMA	Gingiva: 10 Buccal Mucosa: 11 Tongue: 9 Others: 9
Lin et al. [57]	2010	OSCC: 33 Control: 10	Not reported	BLOOD	Not reported
Liu et al. [58]	2010	OSCC: 43 Control: 21	Not reported	PLASMA	Not reported
Wong et al. [56]	2008	OSCC: 30 Control: 38	Not reported	PLASMA	All OSCC samples from tongue cancer

Table 2
Data for: methods performed discovery phase, number of candidate miRNAs, methods performed in the validation phase, name of miRNAs dysregulated and correlation with clinico-pathological parameters.

Study	Method performed in the discovery phase	Number of dysregulated miRNAs after discovery phase	Method performed in the validation phase	Housekeeping control	miRNAs differentially expressed after validation phase	Correlation with clinicopathological features
Liu et al. [69]	Based on literature research	2 deregulations to be investigated	qRT-PCR	miR-16	miR-187* Upregulation	Not investigated
Xu et al. [71]	miRNAs microarray analysis on serum through qRT-PCR panels (miRCURY™ LNA Array, Exiqon, Denmark)	26 miRNAs deregulated: 16 up-regulated 10 down-regulated	qRT-PCR	RNU6	miR-483-5p Upregulation	Correlation with lymph-node metastasis, high TNM stages and overall survival
Li et al. [68]	miRNA expression profiles of exosomes derived from normoxic and hypoxic OSCC cells using high-throughput sequencing for miRNA-seq (Illumina HiSeq 2500)	108 miRNAs deregulated: 105 up-regulated 3 down-regulated	qRT-PCR	cel-miR-39	Exosomal miR-21 Up-regulation	Correlation with Tumor size and lymph-node metastasis
Sun et al. [70]	Based on literature research	1 deregulation to be investigated	qRT-PCR	RNU6	miR-9 Downregulation	Correlation with Tumor size, lymph-node metastasis and tumor Stage
Lu et al. [67]	miRNA microarray global profile (Agilent Technology) on oral cancer lines and normal oral keratinocytes lines	23 miRNAs deregulated: 19 up-regulated 4 down-regulated	qRT-PCR	Not Reported	miR-196a miR-196b Up-regulation	Correlation for both miRNAs with tumor Stage
Ren et al. [64]	Based on literature research	1 deregulation to be investigated	qRT-PCR	RNU6	miR-21 Up-regulation	Correlation with differentiation grade and nodal status
Ries et al. [65]	miRNA microarrays profile on whole blood (GeniomH real-time analyzer)	3 miRNAs deregulated	qRT-PCR	RNU6 and SNORD44	miRNA-3651 miRNA-494 Upregulation miRNA-186 Downregulation	miRNA-3651 correlates with tumor Stage, Grade and lymph-node status
Ries et al. [66]	miRNA microarrays profile on whole blood (GeniomH real-time analyzer)	21 miRNAs deregulated	qRT-PCR	RNU6 and SNORD44	miR-3651 miR-494 Upregulation miR-186 Downregulation	Not investigated
Hung et al. [62]	Based on literature research	1 deregulation to be investigated	qRT-PCR	RNU6 and miR-16	miR-146a Upregulation	Not investigated
Liu et al. [63]	qRT-PCR panel analysis on tissues sample	2 deregulations to investigate	qRT-PCR	RNU6B	miR-196a Upregulation	Correlation with loco regional recurrence
Lo et al. [60]	Evaluation of 3′UTRs of target proteins found with iTRAQ proteomics and then the mirSVR predicted target site scoring and an online database	7 overlapping miRNAs	qRT-PCR	RNU6	miR-27b Downregulation	No correlations found
Lu et al. [61]	miRNA microarray global profile (Agilent Technology) comparing miRNAs from oral cancer lines and normal oral keratinocytes lines	23 miRNAs deregulated: 19 up-regulated 4 down-regulated	qRT-PCR	RNU6	miR-10b Upregulation	No correlations found

Table 2 (*continued*)

Study	Method performed in the discovery phase	Number of dysregulated miRNAs after discovery phase	Method performed in the validation phase	Housekeeping control	miRNAs differentially expressed after validation phase	Correlation with clinicopathological features
Yang et al. [59]	Based on literature research	2 deregulations to be investigated	qRT-PCR	RNU6 and Let-7a	miR-181a miR-181b Upregulation	Correlation with lymph-node metastasis
Lin et al. [57]	Based on literature research	1 deregulation to be investigated	qRT-PCR	RNU6 and Let-7a	miR-24 Upregulation	Not investigated
Liu et al. [58]	Based on literature research	1 deregulation to be investigated	qRT-PCR	miR-16	miR-31 Upregulation	Not investigated
Wong et al. [56]	Taq Man microRNA Assay on laser microdissected cells (Applied Biosystems)	37 miRNAs deregulated: 24 up-regulated 13 down-regulated	qRT-PCR	miR-16	miR-184 Upregulation	Correlation with sex, age, tumor Size and lymph-node metastasis

Table 3
Data for Receiver Operating Characteristic (ROC) as: Area Under the Curve (AUC), specificity and sensitivity from 11 studies.

Study	Year	miRNA Investigated	AUC	Specificity	Sensitivity
Liu et al. [69]	2016	miR-187*	0.73 95%CI: 0.62–0.83	Not Reported	Not Reported
Xu et al. [71]	2016	mir-483-5p	0.85 95%CI: 0.73–0.93	0.746	0.853
Lu et al. [67]	2015	miR-196a + miR196b	0.963	0.878	0.925
Ren et al. [64]	2014	miR-21	0.788 95%CI: 0.692–0.883	0.906	0.621
Ries et al. [65]	2014	miR-185-5p miR-3651 miR494-5p	0.69 0.824 0.715	Not Reported	Not Reported
Hung et al. [62]	2013	miR-146a	0.79	0.92	0.86
Liu et al. [63]	2013	miR-196a	0.75	Not Reported	Not Reported
Lu et al. [61]	2012	miR-10b	0.932	0.8	0.944
Yang et al. [59]	2011	miR-181a + miR181b	0.89	Not Reported	Not Reported
Lin et al. [57]	2010	miR-24	0.82	0.92	0.7
Liu et al. [58]	2010	miR-31	0.82	Not Reported	Not Reported

Discussion

Identification of clinical biomarkers from liquid may help clinicians to obtain diagnostic and prognostic information in a less invasive manner. Due to their stability in blood increasing hopes have been posed on circulating miRNAs as non-invasive biomarkers. The aim of this study was to systematically review the current findings on miRNAs from blood (and its derived samples) in patients with oral cancer. The search was carried out in four of the most common databases with focus on studies with statistically differential expression of one miRNA in blood in patients suffering from oral cancer compared to healthy control. Studies from the whole head and neck region were excluded due to the heterogeneity of tumors and differences in prevalence of HPV infection between different sites of head and neck cancer tumors [81–83]. After the evaluation performed by two of the authors only 16 studies fulfilled the inclusion criteria. Data were extracted by one of the authors and summarized in Tables 1–3. The year of publication ranging from 2008 to 2016, a total of 983 samples from cancer patients and 565 samples from healthy controls have been investigated. All studies presented heterogeneous sampling regarding stage of the tumor. Of note the subgroup analysis performed by Liu et al. [58] demonstrated overexpression of miR-31 for the early stages (T:1–2 with N0) compared to healthy control. The possibility to discover a biomarker differentially expressed in the early stages represents a major goal for predicting the possibility to discriminate what tumors that will metastasize during follow-up. In all studies the selection process for miRNA candidates started with a discovery phase and ended with a validation phase to confirm the preliminary results [79]. In 6 studies [57–59,62,64,69,70] the discovery phase was based on literature search, while in the remaining 10 a lot of different methods had been performed. The methods included miRNA microarray profile, wide qRT-PCR panels, proteomic analysis and further analysis of miRNAs in order to obtain promising candidate miRNAs as cancer biomarkers. These potential biomarkers have subsequently been investigated in the validation phase with qRT-PCR. A possible shortcoming of these studies is represented by the heterogeneous sampling of tumors from different subsites in the oral cavity used in the validation phase. In fact, a differential expression of miR-424 was seen in tissue samples from different subsites of the oral cavity, suggesting that this could cause differential release of miRNAs also in the blood [84]. Of the included studies only the study of Wong et al. [56] focused on tumors from a single subsite in the oral cavity (tongue) while the others did not report about subsite of the tumor or did not perform subgroup analysis to investigate possible differences. To increase

the power of the findings, a subgroup analysis for each different subsite should be carried out in the validation phase. Another problem is the housekeeping control used for data normalization in the validation phase. In fact as mentioned in the introduction section it has been demonstrated that RNU6 is not a reliable housekeeping control [47], however, it has been used in many studies included in this review. Focusing on the correlation with clinicopathological parameters, it is possible to see that promising findings have been reported (Table 2). Very interesting is the correlation with overall survival found in the study of Xu et al. [71] regarding expression of miR-483 in serum. In addition authors reported overexpression of miR-483 also in tumor tissue, suggesting a possible role of this miRNA as target for future therapies. Furthermore, two recent studies focusing on the exosomal fraction of the miR-21 [68] and of the recovered passenger strand of miR-187 [69], show that this small fraction of circulating miRNAs represent challenging and promising fields for research on candidate biomarkers in the future. Of the studies included, only 11 reported Receiver Operating Characteristics (ROCs) of circulating miRNAs in order to discriminate between cancer and non-cancer patients (Table 3). Findings from the studies are very promising, in fact according to the classification of Swets [85] an Area Under The Curve (AUC) between 0.7 and 0.9 reveals a moderate accuracy of the test and an AUC over 0.9 high accuracy of the biomarker. However, for a correct validation of results, further prospective wide cohort studies are needed paying attention to the possible shortcomings mentioned above. Another important issue is addressed by the possibility to predict recurrence or metastasis monitoring the expression levels of candidate miRNAs during follow-up. Four studies [56,58,62,69] included in this review reported overexpression of miRNAs after surgical resection of the tumor, suggesting a possible direct production of the candidate miRNA by the tumor bulk. Higher survival rate for patients in which expression of miRNAs had decreased compared to those in which expression remained stable was also seen. Although, the power of the findings is low due to low power of the sampling and short follow-up, this behaviour represents another important finding in the research of cancer biomarkers for oral squamous cell carcinoma.

Conclusions

The current findings present in the literature reveal that circulating miRNAs from plasma, serum or whole blood represent promising candidates for future use as cancer biomarkers for patients suffering from oral cancer. In the future issues related to methods and sampling have to be overcome through standardized large cohort studies. The possibility to predict recurrence and metastases through follow-up quantification of candidate miRNAs is a factor to be addressed in future studies.

Conflict of interest statement

All authors declare no conflict of interest related to this study.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2016.11.001.

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Expression of the long non-coding RNA HOTAIR as a prognostic factor in squamous cell carcinoma of the head and neck: a systematic review and meta-analysis

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ABSTRACT

Introduction: Long noncoding RNAs (lncRNAs) are often dysregulated in cancer tissue and seem to play an important role in neoplastic processes. Recent studies have shown that the HOX transcript antisense intergenic RNA (HOTAIR) may play a role as a marker of prognosis in squamous cell carcinoma of the head and neck (SCCHN). The aim of this study was to perform a meta-analysis of studies focused on the prognostic role of HOTAIR in SCCHN.

Results: At the end of the selection process, four studies were considered eligible for inclusion in the meta-analysis, comprising a total of 271 patients. Meta-analysis revealed that high expression of HOTAIR was associated with poor overall survival (HR, 1.90; 95% CI: [1.42, 2.53]; $p < 0,0001$), advanced tumor stage (OR, 3.44; 95% CI: [1.84, 6.43]; $p < 0,001$) and lymph-node metastasis (OR, 3.31; 95% CI: [1.24, 8.79]; $p = 0,02$).

Materials and Methods: The literature search was performed in the following databases: PUBMED, SCOPUS, EMBASE and Web of Science, in order to find studies that met the inclusion criteria.

Conclusions: Findings from this systematic review and meta-analysis revealed that HOTAIR represents a potential biomarker of prognosis in patients with squamous cell carcinoma of the head and neck.

INTRODUCTION

Squamous cell carcinoma of the head and neck (SCCHN) encompasses a wide and frequent range of neoplasms arising from the epithelium of different sites such as: oral cavity, nasal cavity, oropharynx, hypopharynx and larynx [1, 2]. It has been reported that around 90% of all tumours in this region are squamous cell carcinomas [3]. SCCHNs are among the ten most frequent malignant neoplasms in humans; and due to the high rate of early metastasis to regional cervical lymph-nodes treatment of these patients is a challenge for the clinicians, [4]. It has been estimated that there will be > 300.000 new cases of lip and oral cavity SCC, > 140.000 of oropharyngeal SCC, > 85.000 of nasopharyngeal and > 155.000 of laryngeal SCC annually worldwide, with an increasing incidence and mortality rate [5]. Despite improvement in both surgical and adjuvant therapies, the 5-year survival rate for SCCHN has shown very little improvement over the last decades [6]. For such reasons, searching for biomarkers for diagnosis and prognosis represents a rising hope in order to provide clinicians with new tools for treatment of the disease.

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides previously defined as "junk DNA" [7]. They are usually transcribed by RNA polymerase II and can undergo splicing and polyadenylation processes. Although they are not translated into proteins, many of them are linked to some complexes involved in chromatin modification causing an

overexpression or silencing of target genes of which most are in cancer pathogenesis [8].

The HOX transcript antisense intergenic RNA (HOTAIR) is one of the most studied lncRNAs [9, 10]. Different studies have focused on the capacity of HOTAIR to cooperate with different chromatin modifying complexes, above all the Polycomb Repressive Complex 2 (PRC2), through its 5′-terminal binding domain [11]. HOTAIR lets PRC2 recognize the target gene, leading to Histone H3 lysine-27 trimethylation, causing a silencing effect. HOTAIR is also able to cooperate with the LSD1 complex and can be regulated by different factors such as miR141, Ago2, c-Myc, TGF-β and small interfering RNAs (siRNAs) [12]. It is also involved in a regulatory control of p53 expression [13]. Different studies suggest that HOTAIR plays an important role in the metastatic process and may be a predictor of poor patient prognosis when highly expressed [14–17].

The aim of this systematic review and meta-analysis was to investigate the link between HOTAIR expression and patient prognosis in SCCHN in order to highlight its potential role as prognostic biomarker.

MATERIALS AND METHODS

Protocol and eligibility criteria

This systematic review and meta-analysis was performed according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines [18] and the Cochrane Handbook [19]. This review has also been registered on the PROSPERO database (registration number: CRD42017057317), before the holding. Studies published in English and fulfilling the following criteria were considered eligible for inclusion in the study: (1) studies focusing on the expression of HOTAIR in SCCHN including more than 50 patients in total, (2) studies showing analysis of correlation between different levels of HOTAIR expression and overall survival, (3) studies reporting a Hazard Ratio (HR) and 95% confidence interval (CI) or Kaplan-Meier curve for HR estimation and (4) studies including quantitative analysis performed through quantitative PCR (qPCR), *in situ* hybridization (ISH), fluorescence *in situ* hybridization (FISH), droplet digital PCR (ddPCR) or RNA sequencing data. Accordingly, studies performed on cell lines or animal models, reviews, case reports, overlapping publications and all studies reporting insufficient data for estimation of HR and 95% CI, were excluded. No restriction was applied concerning year of publication.

Information sources and search strategies

The literature search was performed in the following databases: PUBMED, SCOPUS, EMBASE and Web of Science, and independently performed by two of the authors (GT and CC). Mesh terms and free text words were combined through Boolean operators for research on databases. The following terms were used: ("long non-coding RNA" [Mesh] OR "lncRNA" [Mesh] OR "lincRNAs" [Mesh] OR "HOTAIR" [free word] OR "HOX transcript antisense RNA" [Mesh]) AND ("prognosis" [free word] OR "prognostic factor" [Mesh] OR "survival" [Mesh] OR "relapse" [Mesh]) AND (("head neck" [free word] OR "oral cancer" [Mesh] OR "tongue" [Mesh] OR "pharynx" [Mesh] OR "HNSCC" [free word] OR "OSCC" [free word] OR "tonsil" [Mesh] OR "larynx" [Mesh])). In addition, a direct search on the bibliographies of previously published systematic reviews on the topic was also performed.

Study selection, data collection process and data items

Eligibility assessment of the studies was performed independently in an unblinded standardized manner by two of the authors (GT and CC). In the first round title and abstract was read from the articles found, and studies meeting the inclusion criteria as well as those presenting insufficient data to make a clear decision were then read in full-text. Any disagreement was solved by discussion in a joint session. In order to investigate the level of agreement between the two reviewers, a value of K statistics was calculated. At the end of the selection process papers fulfilling all inclusion criteria were included in the quantitative synthesis. Data extraction was performed through an ad hoc extraction sheet and checked by two authors. In the quantitative synthesis a difference in overall survival was seen for patients with SCCHN showing different levels of HOTAIR expression. Furthermore, differences in: lymph-node metastasis, tumor stage and histological grade were seen between patients with Low or High HOTAIR expression.

Risk of bias assessment, summary measures and planned methods for analyses

Analysis of risk of biases of the included studies was conducted using the Newcastle-Ottawa Scale (NOS) for case control studies. Risk of biases across studies was quantified evaluating the presence of heterogeneity among studies and also investigated through Q and I^2 tests. A p-value of Q statistic < 0.05 was considered significant for presence of heterogeneity. Risk of biases across studies was quantified evaluating presence of heterogeneity among studies through Q and I^2 tests. The Higgins index was also assessed and classified as follow: $< 30\%$ low heterogeneity, 30% to 60% medium heterogeneity, and $> 60\%$ high heterogeneity [20]. Overall effects were investigated with a fixed effect model where I^2 was less than 50%, if higher, a random effects model was used. Data for overall survival were synthetized as HR and

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standard error (SE), while for lymph-node metastasis, tumor stage and histological grade the odds ratio (OR) was evaluated. In cases in which HR and its 95% CI was not reported in the article, it was extracted from Kaplan-Meier curves using the method of Tierney et al. [21]. All calculations were performed using Review Manager version 5.2.8 (Cochrane Collaboration, Copenhagen, 153 Denmark; 2014). Results of the meta-analysis were summarized in forest plots, and overall effects were compared using the inverse of variance test. A p-value lower than 0.05 was considered significant for all tests performed in this meta-analysis.

RESULTS

Study selection

In order to evaluate inclusion in the review, a total of 135 records were screened by title and abstract. Among these only 7 were read full-text and at the end of the selection process 4 articles [22–25] were assessed as eligible for inclusion in the study. Three articles [26–28] were excluded. The reasons for exclusion and the flowchart of the selection process are reported in Figure 1. The value of k-statistic was 0.78 revealing an excellent level of agreement between reviewers.

Study features and risk of bias within studies

In the 4 studies included in the meta-analysis [22–25], a total of 271 samples were analyzed, from tumors in different subsites of the head and neck area (Table 1). All studies were performed in China and published between 2012–2016. Two studies reported data for univariate analysis only [23, 24], while in the other two [22, 25] also multivariate analysis had been performed. In all studies quantification of HOTAIR expression was performed by the use of qPCR. In two studies GADPH was used as

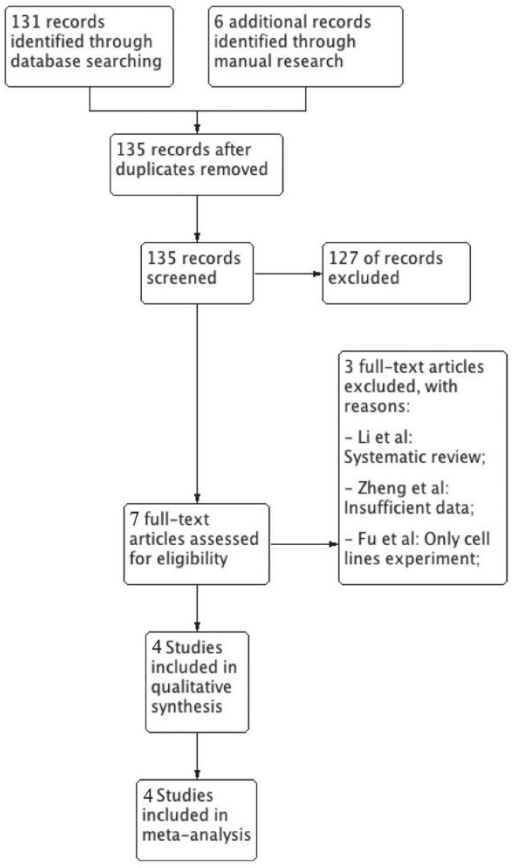

Figure 1: Flowchart for inclusion of studies in the meta-analysis and reasons for exclusion of articles read in full text.

Table 1: Features of the four studies included in the meta-analysis

Study	Year	Country	Involved Site	Total number	Detection method	Cut-off	Survival analysis	Multivariate analysis	Follow-up, months
Li	2012	China	Larynx	72	RT-qPCR	NA	OS	Yes	60
Wu	2015	China	Oral Cavity	50	RT-qPCR	Median	OS	No	60
Wu	2015	China	Tongue	76	RT-qPCR	NA	OS	No	100
Xu	2016	China	Head and Neck	73	RT-qPCR	NA	OS	Yes	150

Table 2: Summary of the risk of bias assessment using the Newcastle-Ottawa scale for case-control studies

Author/year	Country	Adequacy of case definition	Representativeness of cases	Controls Selection	Definition of controls	Comparability cases/controls	Ascertainment of exposure	Same method of ascertainment	Nonreponse rate
Li/2012	China	★	★	★	★	★★	★	★	NA
Wu/2015	China	★	★	★	★	★★	★	★	NA
Wu/2015	China	★	★	★	★	★★	★	★	NA
Xu/2016	China	★	★	★	★	★★	★	★	NA

a single reference gene [23, 24], another study used 18 rRNA as internal control [25], while in the remaining study authors didn't specify the name of the reference gene used [22]. In addition, all studies reported a follow-up period of at least five years. Risk of bias assessment, performed with the Newcastle-Ottawa scale, showed that all included studies were at low risk of bias (Table 2).

Synthesis of results and risk of bias across studies

Results of meta-analysis, on the basis of two studies, revealed that higher levels of HOTAIR expression were not associated with higher degree of differentiation *(OR, 2.31; 95% CI: [0.89, 6.02]; p = 0,09)* (Figure 2A), whereas a correlation to advanced tumor stages was seen *(OR, 3.44; 95% CI: [1.84, 6.43]; p < 0,001)* (Figure 2B). As heterogeneity was not detected *(I^2 = 0%)* a fixed effects model was used. Analysis of correlations performed with a random effect model *(I^2 = 58%)* revealed a correlation between HOTAIR expression and the rate of LNM *(OR, 3.31; 95% CI: [1.24, 8.79]; p = 0,02)* (Figure 2C). Analysis of overall survival pooling HRs showed high expression of HOTAIR to be associated with poor OS in patients with SCCHN *(HR, 1.90; 95% CI: [1.42, 2.53]; p < 0,0001)*, (Figure 3). Summary of data extracted from the included studies used for the pooled analysis are shown in Table 3.

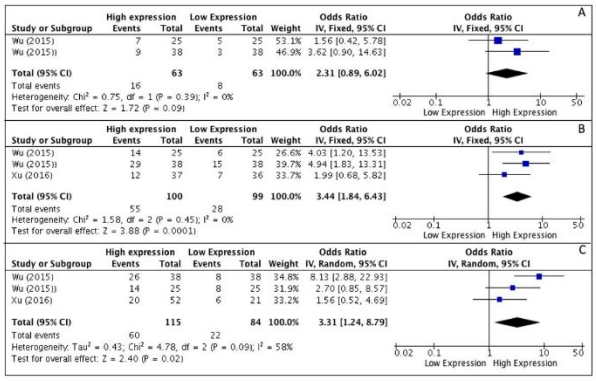

Figure 2: (**A–C**) Forest plot showing different expression of lncRNA HOTAIR and histological grade (A), tumor stage (B) and lymph-node metastasis (C); the frequency of HOTAIR expression was considered in patients with: Advance Stage (III–IV), positive LNM and Poor/Low Grade of differentiation.

DISCUSSION

Non-coding RNAs (ncRNAs) are a class of transcripts involved in various cellular processes [29], and classified into three major groups: short ncRNAs (20–50 nt long), medium ncRNAs (50–200 nt long) and long ncRNAs (> 200 nt long). Long non-coding RNAs (lncRNAs) are widely transcribed in the genome and their expression seems to be dysregulated in many diseases [30–32]. Dysregulation of the expression of lncRNAs has been seen in different cancer types, suggesting that these molecules could be involved in tumorigenesis [33–35]. Due to results indicating an important role in cancer, HOTAIR is one of the most studied lncRNAs [11]. HOTAIR is 2.2 kb long and transcribed from the antisense strand of the *HOXC* gene located on chromosome 2, and consisting of 6 exons [36, 37]. After interaction with the polycomb repressive complex 2 (PRC2) and lysine-specific histone demethylase 1 (LSD1) It is able to regulate gene expression through chromatin dynamics [38, 39]. A recently published study, however, showed that HOTAIR can lead to transcriptional changes independent of PRC2 activity [40]. Furthermore, HOTAIR can act as a competitive endogenous RNA (ceRNA) and regulate levels of some microRNAs [41], for example it can decrease expression of miR-331–3p leading to upregulation of the human epithelial growth factor 2 (HER2), a well-known oncogene [42]. In SCCHN, HOTAIR is able to regulate the activity of the well-known oncogene PTEN, by influencing promoter methylation [25]. In addition, it plays an important role in the epithelial-mesenchymal transition (EMT) repressing expression of E-cadherin through interaction with EZH2 [23, 27]. Overexpression of HOTAIR can also inhibit apoptosis through modification of the mitochondrial membrane and activation of the mitochondrial calcium uptake dependent cell death [43]. It also plays a role in progression and metastasis through a regulatory loop with HuR. In addition, it can work as ceRNA for miR-7 thus avoiding its inhibitory effect on HuR expression [22].

Previous meta-analyses have demonstrated higher expression of HOTAIR to correlate with poor prognosis in patients with cervical [44], ovarian [45], esophageal [44] and gastric [46] cancer. However, to our knowledge no meta-analysis has evaluated its role as a prognostic predictor in SCCHN. In this meta-analysis four studies evaluating correlations between clinical, pathological and survival parameters were included, and a total of 271 patients analyzed. Pooled analysis showed reliable evidence for higher levels of HOTAIR expression to correlate with poor prognosis in SCCHN (HR, 1.90; 95% CI: [1.42, 2.53]; $p < 0{,}0001$). In addition, meta-analysis demonstrated a correlation between higher levels of HOTAIR and higher rates of LNM (OR, 3.31; 95% CI: [1.24, 8.79]; $p = 0{,}02$). However,, presence of heterogeneity *($I^2 = 58\%$)* limits the reliability of these findings, it could be speculated that increasing the number and power of the included studies could render more reliable results. Very interestingly, increased levels of HOTAIR were also associated with advanced tumor stage (OR, 3.44; 95% CI: [1.84, 6.43]; $p < 0{,}001$) in the absence of heterogeneity *($I^2 = 0\%$)*. Looking at results for histological degree of differentiation, meta-analysis failed to demonstrate a correlation with HOTAIR expression (OR, 2.31; 95% CI: [0.89, 6.02] $p = 0.09$). However, only two studies were pooled in the evaluation of this outcome, resulting in very low power of evidences.

Taken together, the present results suggest a potentially important role for HOTAIR as a biomarker of aggressiveness in SCCHN. Results of this meta-analysis should, however, be read with caution due to some obvious limitations. First of all, the number of studies included was low. Secondly a geographical bias may be present as all studies were performed in China, rendering data only from one ethnic group, which could influence the validity of the results. As demonstrated in previous studies, people of different race/ethnicity vary in their risk of developing SCCHN [47, 48]. In addition, differences in prognosis have been seen for patients of different ethnicities, linked both to the genetic and the lifestyle variabilities [49]. As it is known that the frequency of smoking or chewing tobacco, alcohol consumption and HPV infection differs among populations, there can be a "geographical bias" between studies [48, 50]. In addition, the studies included in the meta-analysis have evaluated samples from different subsites within the head and neck area, sites that can differ in both clinical and molecular behavior [51–53]. Furthermore, not all studies reported time-to-event

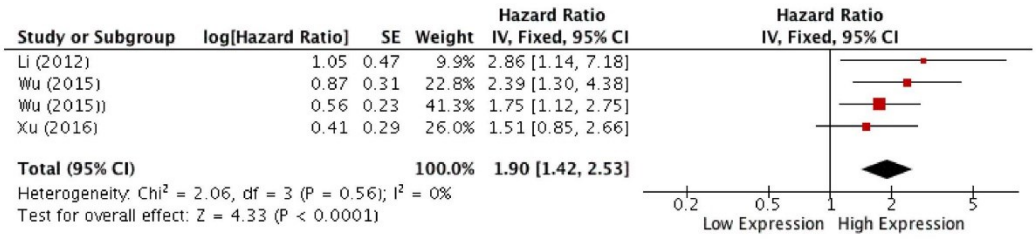

Figure 3: Forest plot for the association between HOTAIR expression and overall survival.

Table 3: Synthesis of data extracted from the included studies related to outcomes pooled in the meta-analysis

	HOTAIR expression									
Study	**High with LNM**	**Low with LNM**	**High with high grade**	**Low with high grade**	**High with high staging**	**Low with high staging**	**High expression**	**Low expression**	**HR statistic**	**Hazard ratios (95% CI)**
Li	NA	NA	NA	NA	NA	NA	33	39	Survival curve and data in paper	2,41–2,856
Wu	14	8	7	5	14	6	25	25	Survival curve	2.3
Wu	26	8	9	3	29	15	38	38	Survival curve	2.39
Xu	20	6	NA	NA	12	7	52	21	Survival curve	1.51
Total	**60**	**22**	**16**	**8**	**55**	**28**	**148**	**123**		

outcome with a multivariate approach, and cut-off values differed and were, in some of the included articles, not even reported . Multivariate analysis allows adjustment for patient-related factors, known as covariates or confounders, which could potentially affect the survival time of the patients [54, 55]. Whether for example a higher number of patients with advanced stage is present in a group, the difference in prognosis between groups could be influenced by the Staging distribution and not HOTAIR expression. On the other hand, the low rate of heterogeneity recorded in three of the studies, combined with a follow-up time of at least 60 months enhances the reliability of findings.

In conclusion, findings from this meta-analysis indicate that higher expression of lncRNA HOTAIR is associated with poor prognosis, advanced tumor stage and higher rate of lymph-node metastasis.

Based on the present results, quantification of HOTAIR could be recommended as a complement associated with evaluation of other prognostical, clinical and molecular biomarkers, in order to categorize patients with high risk of death and thus to help clinicians in the choice of best personalized treatment option. Furthermore, this study encourages the execution of large and well-standardized multicenter studies on human samples of different ethnicity in order to confirm these preliminary findings.

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CONFLICTS OF INTEREST

Authors declare to not have any type conflicts of interest related to this study.

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Evidence that circulating proteins are more promising than miRNAs for identification of patients with squamous cell carcinoma of the tongue

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ABSTRACT

Despite intense research, squamous cell carcinoma of the tongue remains a devastating disease with a five-year survival of around 60%. Late detection and recurrence are the main causes for poor survival. The identification of circulating factors for early diagnosis and/or prognosis of cancer is a rapidly evolving field of interest, with the hope of finding stable and reliable markers of clinical significance. The aim of this study was to evaluate circulating miRNAs and proteins as potential factors for distinguishing patients with tongue squamous cell carcinoma from healthy controls. Array-based profiling of 372 miRNAs in plasma samples showed broad variations between different patients and did not show any evidence for their use in diagnosis of tongue cancer. Although one miRNA, miR-150, was significantly down-regulated in plasma from patients compared to controls. Surprisingly, the corresponding tumor tissue showed an up-regulation of miR-150. Among circulating proteins, 23 were identified as potential markers of squamous cell carcinoma of the tongue. These findings imply that circulating proteins are a more promising source of biomarkers for tongue squamous cell carcinomas than circulating miRNAs. The data also highlight that circulating markers are not always directly associated with tumor cell properties.

INTRODUCTION

Squamous cell carcinoma of the tongue (SCCT) is a devastating disease with a 5-year survival of around 60% [1] which has not improved over the last decades [2]. One major problem is that around 60% of patients develop a local relapse [3], the main cause (24%) of death for SCCT [4]. It is therefore of great importance to find markers for prediction and early detection of relapses. However, it has been challenging to identify biomarkers of clinical significance for SCCT due to the heterogeneous nature of this tumor type [5].

Circulating miRNAs were discovered in 2008 [6], 15 years after the discovery of miRNA in tissue [7]. While the majority of miRNAs exist intracellularly, some enter the circulation. miRNAs have been isolated from blood, urine, saliva, pancreatic juice and breast milk [8]. How miRNAs enter these body fluids is poorly understood [9, 10], but is suggested to be via passive leakage from cells due to necrosis, apoptosis or inflammation, through active

release by complex formation with lipoproteins (HDL) or RNA binding proteins (AGO2), or by packaging in exosomes or microvesicles before being released [10–12], or a combination of these events.

Regardless of how they are released, cell-free miRNAs are stable, even when subjected to extreme conditions such as high temperature, wide variation in pH or multiple freeze-thaw cycles [13, 14]. Although there are inconsistencies between studies, some have suggested that miRNA profiles in plasma can differentiate patients with oral squamous cell carcinoma (OSCC) from healthy individuals (reviewed in [15]).

Circulating factors are potentially ideal biomarkers for simple, rapid and inexpensive screening of patients for cancer diagnosis and monitoring, such as the use of prostate-specific antigen for prostate [16] or CA125 for ovarian cancer [17]. In both cases, these proteins can be measured easily to provide diagnostic information and to monitor therapeutic response and tumor relapse, but suffer from problems with specificity and/or sensitivity. Circulating proteins have also been evaluated in patients with squamous cell carcinoma of the head and neck (SCCHN) [18–20], although not as extensively as miRNAs. Studies using panels of cytokines and growth factors have found promising markers for early detection of SCCHN [18, 20]. For example, macrophage inflammatory protein 1b (MIP 1b), interleukin 13 (IL13), metalloproteinase 3 (MMP3), epidermal growth factor (EGF) and vascular cell adhesion molecule (VCAM) are down-regulated in plasma from SCCHN patients compared to controls. Furthermore, both squamous cell carcinoma antigen (SCC-Ag) and neurite growth-promoting factor 2 (Midkine) have been suggested as independent prognostic factors for patients with SCCHN [21, 22].

Unlike detection of circulating nucleic acids that can be amplified prior to detection using array technologies, two potential problems with serum protein biomarkers are their low concentrations due to dilution in the circulation and the limited ability to assay multiple proteins from a small sample volume. Proximity extension assay (PEA) is a highly sensitive method based on pairs of antibodies linked to oligonucleotides with partial complementariness for each other (PEA probes). When the two different antibodies bind to their target protein, probes are brought into proximity and the two oligonucleotides accordingly bind. The new sequence thus achieved can be extended, amplified and measured by quantitative real-time PCR [23, 24], where the number of PCR templates is proportional to the initial concentration of antigen in the sample. In addition to high sensitivity, specificity is also improved due to the requirement for simultaneous binding of two different antibodies to epitopes on the same antigen. However, the technique is dependent on cross reactivity and sensitivity of the antibodies used. Recent technological advances have enabled simultaneous measurement of multiple proteins, and a 96-plex PEA-based immunoassay has made it possible to analyze 92 known or putative biomarkers simultaneously [25].

In this study we have compared two different sensitive technologies for the detection of miRNAs or proteins in plasma as potential diagnostic markers for squamous cell carcinoma of the tongue.

RESULTS

miR-150 down-regulation in plasma and up-regulation in tissue from patients with SCCT compared to healthy controls

A panel of circulating miRNAs was analyzed in plasma from healthy individuals (13 controls) and patients with SCCT (13 patients). Of the 372 miRNAs analyzed, 152 were detected in all samples. None of the samples showed signs of hemolysis, evaluated by analyzing the ratio between miR-451 and miR-23a-3p. All samples had values below 7.0, where a ratio above 7.0 is considered as risk of being affected by hemolysis.

Principal component analysis (PCA) showed that the levels of these miRNAs did not discriminate healthy individuals from SCCT patients (Figure 1). Statistical evaluation of individual miRNA levels showed miR-150 to be significantly decreased in patients with SCCT compared to controls, Benjamini-Hochberg FDR adjusted p-value 0.007 (Figure 2A). Single qRT-PCR assay was then applied to validate miR-150 results using a combination of three miRNAs for normalization (miR-93, miR-222 and miR-30e). The normalized Cq value from single qRT-PCR assays of plasma samples showed high consensus with the normalized Cq value from the panel ($p < 0.001$, Rho = 0.913).

miR-150 was further analyzed in tissue samples using paired tumor and tumor free samples from 15 SCCT patients; 8 of the patients included in the plasma analysis and an additional 7 patients. In contrast to the decreased plasma levels in tumor patients, there was significant up-regulation (p-value 0.02) of miR-150 in tumor tissue compared to tumor free tissue adjacent to the tumor (Figure 2B). There was no correlation between plasma and tissue miR-150 in the 8 patients for whom both sets of data were available. One well known target for miR-150 is the *MYB* proto-oncogene. RNA expression of *MYB* was down-regulated in all SCCT samples compared to tumor free controls adjacent to tumor [26]. However, there was no direct correlation between miR-150 and *MYB* levels.

Using proximity extension assay 23 proteins separated SCCT patients from healthy individuals

A total of 146 proteins involved in oncogenesis and inflammation were analyzed by proximity extension assay

of plasma from 19 SCCT patients (the same 13 as included in the miRNA analysis and an additional 6 patients) and 19 healthy controls (9 that were included in the miRNA analysis and 10 additional controls). Using a cut-off of $p < 0.05$, 23 markers were differentially expressed between SCCT patients and healthy individuals. Principal component analysis of these 23 proteins separated SCCT patients and healthy individuals from each other, although not completely (Figure 3). All but one of these proteins showed down-regulation in SCCT patients compared to healthy controls, the exception being IL1-ra, and there was a large overlap in protein levels between the groups for all proteins (Figure 4). Calculating a receiver operating curve (ROC) showed the AUC for individual proteins to range from 0.676 to 0.903, indicating fair to good discrimination between those with and without disease (Table 1). The protein with highest AUC value was NT-3 (neurotrophin-3; NTF3), which is also the only candidate with both high sensitivity and high specificity.

DNER, NT3 and *TIE2* mRNA are also down-regulated in tumor tissue

For the 23 differentially expressed proteins, corresponding gene expression was available in a recently published study of RNA profiling in nine of the patients [26]. Sixteen mRNAs showed significantly different expression when comparing tumor tissue to control (Table 1; upper part), whilst mRNA levels for 7 of the proteins was not significantly different between control and tumor tissues (Table 1, lower part). Consistency in the direction of change for circulating protein and tissue mRNA levels were identified in only three of the 16 differentially expressed genes (*DNER*, *NT3* and *TIE2*), where protein levels were lower in plasma from patients than controls and mRNA levels were lower in tumor tissue compared to tumor-free tissue. The remaining thirteen genes that were significantly dysregulated in tumor tissue showed the opposite direction when comparing mRNA to corresponding circulating protein.

DISCUSSION

The use of circulating markers for diagnosis, prognosis and disease monitoring has been employed for many years for specific cancer types. However, such markers suffer from a lack of specificity and/or sensitivity, and are only applicable to a limited number of cancer types. With the advent of new technologies, many reports are appearing for the identification of new disease markers. Here, we evaluated the potential value of circulating miRNAs and proteins in distinguishing patients with squamous cell carcinoma of the mobile tongue (SCCT) from healthy controls. In contrast to

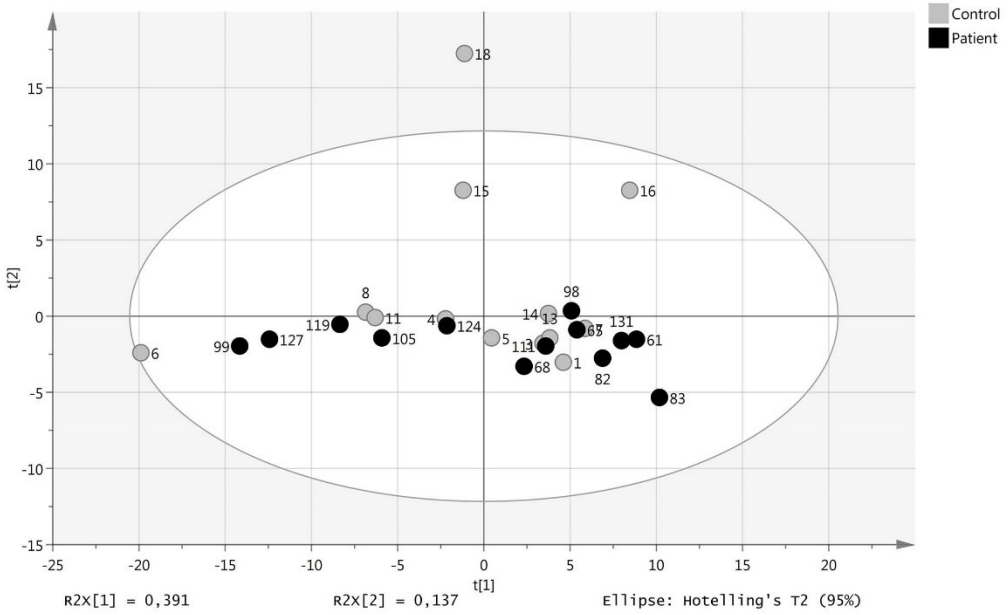

Figure 1: miRNA levels in patients with tongue tumor compared with healthy controls. Score plot (t1/t2) from PCA modelling based on 152 miRNAs detected in all samples. Each dot represent one patient indicated by the patient ID.

previous studies on plasma suggesting that, for example, miR-21, miR-31, miR-184 and miR-186-5p are altered in patients with SCCHN compared to controls [27–30], we found only miR-150 to be significantly different in plasma from SCCT patients, a specific subgroup of SCCHN.

The discrepancies seen between studies of miRNA is one of many challenges when evaluating circulating miRNAs as potential biomarkers (reviewed in [31]). Factors known to affect results are choice of method for normalization and the use of different patient cohorts, making comparisons between studies difficult. In contrast

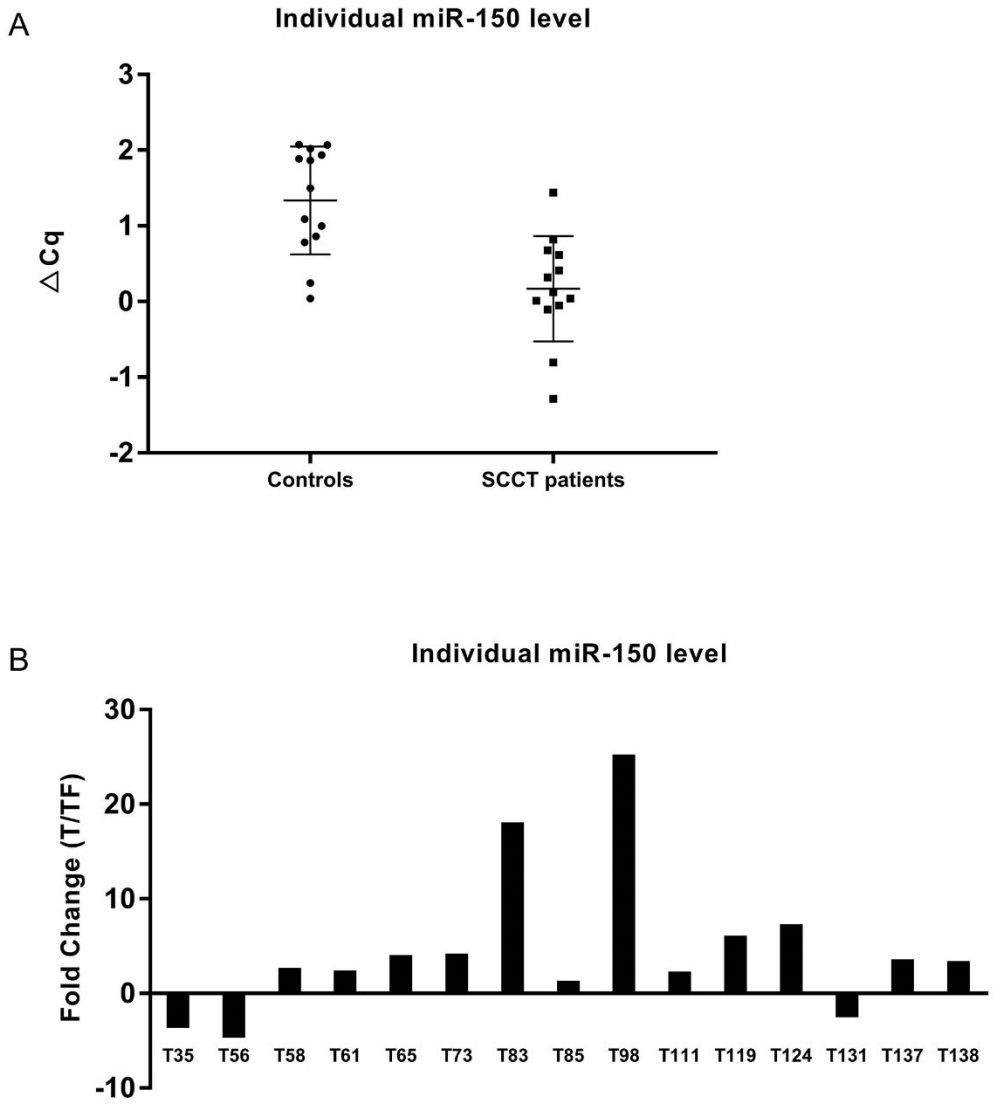

Figure 2: miR-150 levels in plasma and tissue. **(A)** Individual miR-150 levels in plasma from controls and tumors based on Cq values from the miRNA-panel. Normalized Cq = global mean Cq – assay Cq (sample). A higher value thus indicate that miR-150 is more abundant in that particular sample. **(B)** Fold change in miR-150 level in tongue tumor compared to tumor free tongue tissue from the same patient.

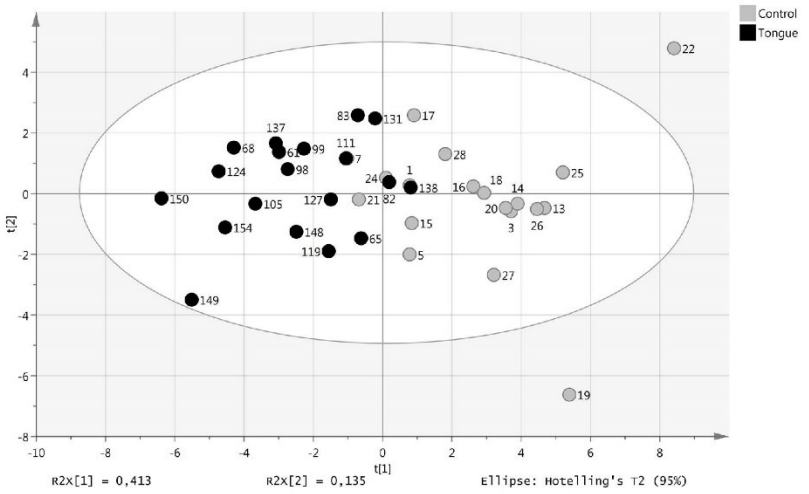

Figure 3: Levels of plasma proteins with tongue tumor compared with healthy controls. Score plot (t1/t2) from PCA modelling based on 23 plasma proteins with significantly different levels in patient and control samples.

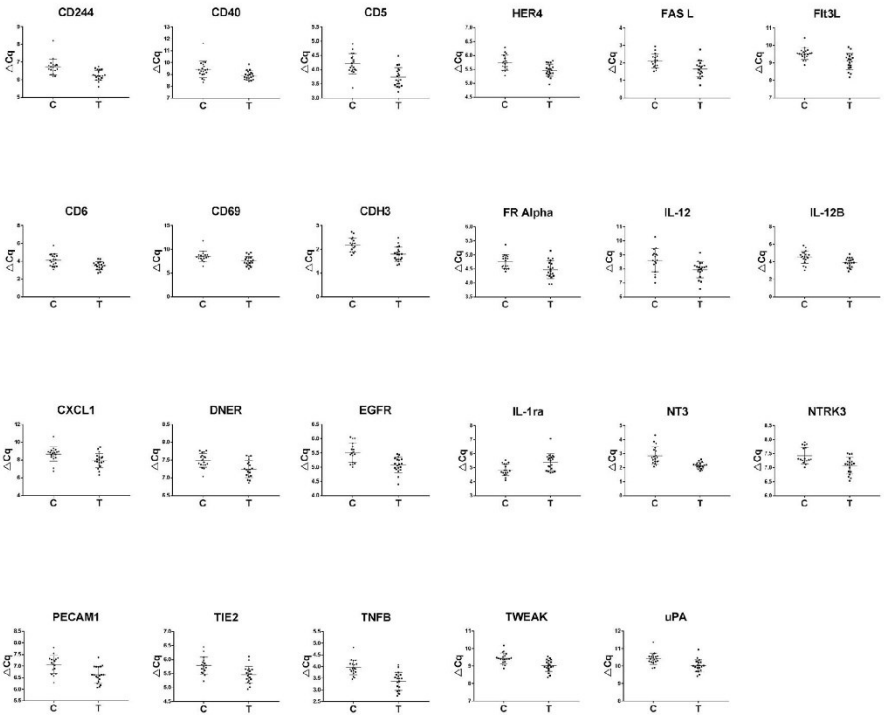

Figure 4: Differentially expressed proteins. Scatter plots of significantly differentially expressed proteins in plasma from patients with squamous cell carcinoma of the tongue compared to healthy controls.

Table 1: Protein levels in circulation and mRNA expression in tissue

	Protein levels in circulation		mRNA expression in tissue [1]		AUC Circulating proteins		
						Confidence interval	
	p-value	SCCT vs C	P-value	SCCT vs C	AUC	Lower Bound	Upper Bound
NT-3	0,002	Down*	1,11E-02	Down*	0,903	0,805	1,000
TNFB	0,001	Down*	6,64E-05	Up*	0,889	0,786	0,993
CD5	0,008	Down*	4,04E-04	Up*	0,831	0,694	0,968
uPA	0,014	Down*	1,21E-10	Up*	0,825	0,686	0,965
IL-1ra	0,031	Up*	3,77E-05	Down*	0,806	0,670	0,942
Flt3L	0,018	Down*	1,04E-02	Up*	0,803	0,656	0,950
DNER	0,031	Down*	1,08E-05	Down*	0,781	0,631	0,931
CXCL1	0,047	Down*	2,31E-06	Up*	0,770	0,607	0,933
CD6	0,018	Down*	2,02E-07	Up*	0,767	0,615	0,919
CD40	0,047	Down*	4,69E-07	Up*	0,765	0,605	0,924
CDH3	0,014	Down*	1,80E-11	Up*	0,737	0,570	0,904
PECAM-1	0,014	Down*	1,21E-02	Up*	0,731	0,560	0,902
TIE2	0,031	Down*	2,22E-02	Down*	0,712	0,538	0,886
FasL	0,031	Down*	7,72E-03	Up*	0,709	0,538	0,881
FR-alpha	0,047	Down*	3,55E-02	Up*	0,693	0,519	0,867
CD69	0,044	Down*	4,69E-07	Up*	0,676	0,495	0,856
CD244	0,012	Down*	5,33E-01	Down	0,845	0,723	0,967
TWEAK	0,010	Down*	6,71E-01	Down	0,834	0,705	0,962
IL-12B	0,026	Down*	4,23E-01	Down	0,795	0,644	0,946
EGFR	0,008	Down*	2,58E-01	Up	0,756	0,593	0,920
NTRK3	0,016	Down*	2,58E-01	Down	0,731	0,562	0,900
ErbB4/HER4	0,031	Down*	6,71E-01	Down	0,690	0,515	0,864
IL-12	0,047	Down*	6,47E-01	Up	0,676	0,487	0,865

* Significant differential expression
[1]Data from Boldrup et al. 2017 ([26])

to many of the previous studies that included tumors originating from the whole head and neck area and the majority being N+ [27–29], we only included patients with SCCT, with the majority (11/13) being N0. The importance of taking sub-site into consideration in analysis of SCCHN has previously been shown by us and others [32–34].

Results showed high variation in miRNA levels within controls, even though samples had been collected in the same way and analyzed using the same method on the same day and by the same person. This again could reflect the dynamic expression of miRNA and the importance of global protocols [35]. Even though there was a significant down-regulation of miR-150 in SCCT patients in group wise comparison, only two patients had a lower value than the mean minus two S.D. of the controls. In a previous study of colorectal cancer, lower plasma levels of miR-150 were seen in patients with advanced cancer compared to patients with polyps and adenomas. Also here the variance within the groups was large, particularly within the control group [36].

Various mechanisms of how tumors release miRNAs into the circulation have been suggested [37] making it difficult to judge if miRNA levels in plasma really reflect miRNA expression in tumor tissue. It has been proposed that part of the circulating miRNAs originate from blood cells and that the miRNA content therefore reflects the blood count. miR-150 is strongly correlated with lymphocyte count [38] and specifically expressed by

Table 2: Characteristics and clinical parameters of tongue squamous cell carcinoma patients and controls

						Circulating miRNA				
Patient ID	**Gender**	**Age**	**TNM**	**Survival**	**Recurrence**	**Panel**	**Verification**	**miRNA tissue**	**Protein plasma**	**RNA tissue**
35	F	24	T2N0M0	Dead	Yes			X		X*
56	F	41	T2N2bM0	Dead	Yes			X		X*
58	M	62	T1N0M0	Alive	No			X		X*
61	M	70	T4aN0M0	Alive	No	X	X	X	X	X*
65	F	81	T2N0M0	Alive	No	X	X	X	X	X*
68	M	62	T2N0M0	Dead	Yes	X	X		X	X*
73	M	81	T4aN0M0	Dead	No			X		X*
82	F	19	T4aN0M0	Dead	Yes	X	X		X	X*
83	F	64	T1N0M0	Alive	No	X	X	X	X	
85	F	88	T2N0M0	Dead	Yes			X		X*
98	M	31	T2N0M0	Alive	No	X	X	X	X	
99	M	65	T4aN2cM0	Alive	No	X	X		X	
105	M	64	T1N0M0	Alive	No	X			X	
111	F	31	T1N0M0	Alive	No	X	X	X	X	
119	M	67	T2N0M0	Alive	No	X	X	X	X	
124	M	54	T4N2bM0	Dead	Never TF	X	X	X	X	
127	M	28	T1N0M0	Alive	No	X	X		X	
131	F	74	T2N0M0	Alive	No	X	X	X	X	
137 ¤	F	71	T2N0M0	Alive	No			X	X	
138 ¤	M	50	T2N1M0	Alive	No			X	X	
148 ¤	M	81	T1N0M0	Alive	No				X	
149 ¤	F	69	T1N0M0	Alive	No				X	
150 ¤	M	79	T3N0M0	Alive	No				X	
154 ¤	F	42	T1N1M0	Alive	No				X	

*Data published in Boldrup et al, 2017 ([26])
¤ Follow up time less than 2 years

Controls			
	n	**Mean age**	**Age range**
Circulating miRNAs			
Female	7	46,4	21- 82
Male	6	44,7	23- 59
miRNA verification plasma			
Female	7	46,4	21- 82
Male	5	43,6	23- 59
Circulating proteins			
Female	11	55,2	39- 82
Male	8	56,8	38- 83

(Continued)

Controls			
	n	Mean age	Age range
RNA tissue*			
Female	9	40,3	25- 59
Male	5	39,2	27- 57

*Data published in Boldrup et al, 2017 ([26])

mature lymphocytes [39], but can also be expressed at a lower level by keratinocytes [40]. One of the top targets for miR-150 is *MYB*, a transcription factor essential during lymphocyte development, especially B cell development [41–43]. In accordance with previous findings, *MYB* mRNA was down-regulated in all our SCCT samples compared to tumor free controls adjacent to tumor. However, no direct correlation between miR-150 and *MYB* was seen, indicating that other factors are also important in regulation of *MYB*.

In our analysis of circulating proteins involved in tumor development and inflammatory processes, 23 were differentially expressed and could aid in distinguishing SCCT patients from controls. A previous report including patients with tumors in the oral cavity, pharynx and larynx using an equivalent method measuring 60 cytokines, growth factors and tumor antigens from SCCHN patients [20] presented a panel of 25 proteins as a promising new approach for early detection of head and neck cancer. Seven of the proteins included in that panel were also included in our panel (CEA, ErbB2, EGFR, IL-1Ra, IL-8, HGF and IL-6) but only two showed significant alteration (EGFR and IL-1Ra) in patients with SCCT.

In our material, 16 of the 23 proteins analyzed showed significant dysregulation both at RNA level in tissue and protein level in plasma. However, only three genes/proteins, DNER, NT-3 and TIE2 showed the same direction of down-regulation in both plasma and tumor tissue. Using area under the curve (AUC) analysis, neurotrophin-3 (NT-3, or NTF3) showed the best discrimination between controls and SCCT patients. Apart from being connected to somatosensory innervation in the mouse tongue [44], NT-3 also plays a role in breast cancer [45], lung cancer [46] and adenoid cystic cancer [47]. Based on its role in metastatic growth, tumor behavior and invasion in these tumor types, its role in SCCT will be worth further elucidation.

As circulating proteins show promising results as potential markers for SCCT, future studies will aim at clarifying their role in a bigger group of stage I tumors only where such markers could be of value in early diagnosis.

In summary we have shown the potential of circulating proteins as markers of squamous cell carcinoma of the mobile tongue. In particular, NT-3 with known roles in other cancer types requires further investigation in SCCT. For circulating miRNAs, our results were less promising, showing wide inter-individual variations and even for the best performing miRNA, miR-150, only two patients had levels outside the area of the controls. With the detection tools available today, circulating miRNAs are unlikely to be valuable in the diagnosis/monitoring of SCCT. However an extended panel of circulating proteins and the PEA assay is a promising approach for detection of clinically useful markers in the future.

MATERIALS AND METHODS

Patient material

The study was approved by the local Ethical committee (Dnr 08-003M). After informed consent from all participants diagnosed with primary SCCT, blood or/ and tissue biopsies were collected before treatment of the SCCT. Whole blood was collected from a total of 19 patients and 23 healthy volunteers, mean age 58 and 50 years respectively. Tumor biopsies (T) and biopsies from corresponding clinically normal tumor free tissue (TF) adjacent to the tumor was collected from 15 patients, with a mean age of 59 years. Detailed patients information is shown in Table 2. All biopsies were collected from mobile tongue. Samples were snap-frozen in liquid nitrogen and stored at -80°C until miRNA was extracted.

Collection and storage of blood plasma

Three milliliters of peripheral blood was collected into vacutainers containing EDTA using standardized venipuncture procedures. Handling and processing was the same for all samples. Tubes were left standing for at least 30 minutes at room temperature after collection, centrifuged at 1300 g for 10 min at room temperature and the top layer, the plasma, was immediately aliquoted and stored at -80 °C until further use.

miRNA extraction from plasma and tissue

Total RNA including miRNA was extracted from frozen plasma samples using miRCURY RNA Isolation kit –Biofluids (Exiqon, Vedbaek, Denmark). A standard protocol for RNA isolation was used according to the manufacturer, with 250 µl of plasma as starting material.

After centrifugation to remove debris, 200 µl supernatant was extracted and RNA eluted in 50 µl RNase free water. AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) was used for miRNA extraction from tumor and tumor free tissue. The fresh frozen biopsies (less than 20 mg) were homogenized in 600 µl Buffer RLT Plus containing β-mercaptoethanol using a Precellys Tissue homogenizer (Bertin Technologies, Artigus Pres Boreaux, France). After DNase treatment and washing, total RNA including miRNA was eluted twice in 30 µl and the eluates pooled. Purified RNA was stored at -80 °C until cDNA preparation. Quantity and purity of RNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

miRNA profiling in plasma

Twenty microliters of RNA extracted from plasma from 13 patients and 13 controls was sent on dry ice to Exiqon Services (Vedbæk Denmark) for miRNA analysis with real-time PCR panel. The panel used was miRCURY LNA Universal RT microRNA PCR Human panel I. Briefly 10 µl RNA was reversed transcribed in 50 µl using the miRCURY LNA Universal RT microRNA PCR, polyadenylation and cDNA synthesis kit (Exiqon, Vedbæk, Denmark). cDNA was diluted 50x and assayed in 10 µl PCR reactions according to the kit protocol. Amplification was performed in a LightCycler® 480 Real-time PCR system (Roche) in 382 well plates, and amplification curves analyzed using Roche LC software, both for determination of Cq (by the 2nd derivative method) and for melting curve analysis. All samples went through a quality control of RNA isolation and cDNA synthesis using Exiqon´s RNA spike-in kit.

Data analysis: the amplification efficiency was calculated using algorithms to the LinReg software. Using NormFinder (http://moma.dk/normfinder-software), the best normalizer was the average of assays detected in all samples (average-assay Cq). All data were therefore normalized by the formula; normalized Cq = global mean Cq – assay Cq (sample).

miRNA validation with qRT-PCR in plasma and tissue samples

For validation of findings from the panel in plasma, miR-150 and three miRNAs used for normalization, miR-30e, miR-93 and miR-222, were quantified by individual qRT-PCR assays. miR-30e, miR-93 and miR-222 were selected by Normfinder and recommendations from Exiqon (Biofluids Guidelines, http://www.exiqon.com/ls/Documents/Scientific/microRNA-serum-plasma-guidelines.pdf). All samples but one control and one SCCT were subjected to the validation.

RNU6 and SNORD 44 were used for normalization of miR-150 tissue data, obtained from 8 of the 13 SCCT samples included in the plasma analysis plus an additional 7 samples. All primers were from Exiqon. cDNA preparation and qRT-PCR analyses used the protocol for individual assays in miRCURT LNA Universal RT microRNA PCR kit from Exiqon. Two microliters of extracted RNA from plasma were used for each 10 µl cDNA reaction as recommended by miRCURY RNA Isolation kit – Biofluids. For tissue samples, 10 ng of total RNA was used in each cDNA reaction.

Real time RT-PCR was performed using an IQ5 multicolor real-time PCR detection system with IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). For each assay, 4 µl cDNA, diluted 40X, was used in 10 µl reactions. Cycling conditions were enzyme activation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 60 sec.

Proximity extension assay

Plasma from 19 controls (9 of the samples included in the miRNA analysis and an additional 10) and 19 SCCT patients (12 of the 13 analyzed for miRNA in plasma and an additional 7 patients with SCCT) were sent to Clinical biomarkers facility, Science for Life Laboratory (Uppsala, Sweden) for analysis with proximity extension assay. One µl EDTA-plasma was used in each of two different panels comprising 92 and 71 proteins respectively, Proseek multiplex Oncology I, v2 and Proseek multiplex Inflammation I (Olink Bioscience, Uppsala, Sweden).

Statistical analysis

For multivariate data analysis we used Simca –P 14 (MKS Data analytics Solutions, Umea, Sweden). Multiple Experiment Viewer (MeV) (http://www.tm4.org/mev.html) with the LIMMA package was used to identify significantly differentially expressed miRNAs from the panel and also circulating proteins from the protein panel. Spearman correlation was used for statistical correlations between miRNA panel results and results from single assays and for correlations between plasma and tissue miRNA. Wilcoxon test was used for evaluation of tissue expression of miR-150 in paired tissue samples. The predictive analytical software SPSS was used for the above statistical analysis and receiver operating characteristics (ROC) and area under the curve (AUC) analysis to test how well individual factors separate the groups. All graphs were prepared in Graphpad Prism 7 (Graphpad prism software).

Abbreviations

AUC; Area under the curve
C; Control
DNER; Delta/Notch like EGF repeat containing
EGF; epidermal growth factor

IL13; interleukin 13
miRNA; microRNA
Midkine; neurite growth-promoting factor 2
MIP 1b; macrophage inflammatory protein 1b
MMP3; metalloproteinase 3
NT-3; Neurotrophin-3
OSCC; Oral squamous cell carcinoma
PCA; Principal component analysis
PEA;Proximity extension assay
ROC; receiver operating curve
SCC-Ag; squamous cell carcinoma antigen
SCCHN; Squamous cell carcinoma of the head and neck
T; Tumor
TF; Tumor free
TIE2; TEK Receptor tyrosine kinase
SCCT; Squamous cell carcinoma of the tongue
VCAM; vascular cell adhesion molecule

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CONFLICTS OF INTEREST

The authors of the manuscript have no conflicts of interest to declare.

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ORIGINAL RESEARCH

In vitro study on anti-cancer properties of genistein in tongue cancer

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Purpose: Tongue cancer is an extremely aggressive disease and is characterized by a poor prognosis. It is a complex disease to treat and current therapies have produced mediocre results with many side effects. Some facts suggest that natural essences can support traditional cancer therapy by carrying out a synergistic function with chemotherapy. Therefore, we evaluated the antitumor effects of genistein on tongue carcinoma cells.

Methods: Genistein 20, 50 and 100 μM were used for 24, 48 and 72 hours on 3 tongue carcinoma cell lines. xCELLigence system was used to evaluate the effects on cell adhesion, proliferation and to calculate IC_{50} values. Both MTT assay and Trypan blue assay were used to evaluate alterations in cell viability, scratch assay for cell migration and Western blot analysis for expression of some proteins.

Results: Cell adhesion was inhibited especially between 20 and 50 μM of genistein treatment. Proliferation was reduced by 50% for treatments with 20 μM at 24 hours, with 20 or 50 μM at 48 and 50 μM at 72 hours ($P<0.0001$). Viability tests confirmed a proportional reduction in concentration of genistein and duration of treatments. Even cell migration was reduced significantly ($P<0.001$). Genistein down-regulates vitronectin, OCT4 and survivin.

Conclusion: This in vitro study clarifies the anti-tumor effect of genistein on tongue carcinoma. In vivo studies are needed to confirm these data and develop a suitable delivery system that is capable of acting directly on tumor.

Keywords: genistein, tongue cancer, xCELLgence system, cell adhesion, cell proliferation

Introduction

Tongue cancer is the most common tumor of oral squamous cell carcinoma (OSCC). Survival rates at 5 years since diagnosis appears stable, despite new treatments.[1–3] In addition, the side effects have not been completely eliminated.

This has led to a growing interest in researching new molecules with selective effects against cancer cells without causing any damage to healthy cells and tissues.

Genistein (4′,5,7-trihydroxyisoflavone) is an isoflavone, found in soybeans and all its derivatives such as flour, sauces, oil, milk and cheese.[4] It is also found in other legumes such as lentils, beans, peas, chickpeas and whole grains such as wheat, rice, barley, rye and oats.

It has potent anti-cancer properties, including the inhibition of tyrosine kinase proteins and inhibition of cell cycle at G2/M phase; it is also able to promote apoptosis by activation of caspase-9 and -3.[5–7]

Recently, clinical trials have confirmed the chemopreventive effects of genistein on many types of cancers.[8–17]

Many studies have been conducted on breast cancer demonstrating that genistein modulates oxidative stress, acting on estrogen receptor (ER)α/ERβ ratio,[18]

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down-regulates the activity of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor (HER)-2/neu modulating the expression levels of nuclear factor (NF)-kB[19] and it inhibits protein phosphatase (PP2A), inducing growth reduction and apoptosis.[20]

However, genistein has a poor bioavailability in nature. It can be found as β-glucoside, which is hydrolized, in the small intestine, in the form of aglycones,[21–23] its active pharmacological part.

During digestion, due to the action of microflora, the genistein is transformed in various metabolites. In this phase, the absorption efficiency is often reduced because the intestinal flora can degrade aglycones and produce aromatic acids.

It is known, however, that the glycosidic form can also be transported intact into the cell through a carrier and then hydrolyzed by cytosolic β-glucosidases.[24–26]

Shehata et al have fabricated self-emulsifying phospholipid pre-concentrates using some bioactive surfactants and they have demonstrated that they can be good nanocarriers for genistein, thereby overcoming its poor oral bioavailability.[27]

Meanwhile, Gavin et al studied a nanomocoadhesive system using lipid-based nanocarriers containing genistein, named nanoemulsions, and achieved excellent results in patients with oral cancer.[28]

This study suggests that in situ application of genistein in tumors could help in overcoming obstacles associated with its poor bioavailability. This may be simpler for oral cancer since they are present in an anatomical site of easier access.

There are not many studies in literature on the effects of genistein on oral cancer, except for some that studied its capacity to inhibit tyrosine kinases,[29] in combination with other isoflavones, such as quercetin[30,31] and biochanin A.[32] Moreover, there are no studies that specifically investigate the effects of genistein on tongue squamous cell carcinoma.

For this reason, we evaluated in vitro anti-tumor action of genistein using 3 cell lines of OSCC; PE/CA-PJ15, PE/CA-PJ49 and HSC-3 cell lines.

Three concentrations of genistein have been used to assess adhesion, proliferation, migration, cell viability at 3 time points, and we calculated the IC_{50} values.

In addition, we studied the expression of OCT4 and survivin, 2 important proteins responsible for promoting tumorigenesis, after treatment with 20, 50 and 100 μM of genistein at 24, 48 and 72 hours of treatment.

Materials and methods

Cell culture and treatment

PE/CA-PJ15, PE/CA-PJ49, HSC-3 are cell lines of tongue carcinoma (European Collection of Cell Cultures, ECACC) maintained at standard conditions of temperature and atmosphere (37°C and 5% CO_2, respectively) for all tests used. We have used DMEM culture medium with 4,500 mg/L glucose for PE/CA-PJ15 and PE/CA-PJ49 cells and Roswell Park Memorial Institute 1640 medium (RPMI 1640) for HSC-3 cells (Life Technologies, Gibco, Grand Island, NY, USA). Both culture media were supplemented with 10% fetal bovine serum, L-glutamine (2 mM) and penicillin–streptomycin (100 U/mL) (Sigma Aldrich, Saint Louis, MO, USA). Genistein (Abcam, Cambridge, UK) was dissolved in dimethylsulfoxide (DMSO) at a stock concentration of 5 mM and serial dilutions of 20, 50 and 100 μM of genistein were prepared. The cell lines were treated for 24, 48 and 72 hours.

xCELLigence real-time cell analyzers

PE/CA-PJ15, PE/CA-PJ49, HSC-3 cells were resuspended in culture medium and then seeded, in triplicate, inside an E-plate of xCELLigence RTCA DP system (ACEA Biosciences Inc., San Diego, CA, USA). Genistein at concentrations of 20, 50 and 100 μM were added to cells in adhesion phase and the cells were monitored for 24, 48 and 72 hours. To evaluate its effects on cell adhesion, genistein was added to cells after their adhesion phase. Control cells were treated with equivalent volume of the vehicle of genistein; DMSO. The concentrations used were the same as indicated previously. Moreover, the same concentrations of genistein were added in the proliferation phase to evaluate the effects of this nutraceutical on cellular proliferation. xCELLigence system measured the impedances parameter called "Cell Index (CI)" and hence we calculated also IC_{50} values at 24, 48 and 72 hours. The measurement of the IC_{50} values was performed by the instrument as described in the literature, with a high reliability.[33,34] They were expressed as the mean (M) ± standard error of the mean (SEM) (n=3).

MTT assay and Trypan blue exclusion test

Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate cell viability of PE/CA-PJ15, PE/CA-PJ49, and HSC-3 cells after treatments with genistein. We seeded 5×10^4 cells in a total volume of 250 μL/well in a 96-well plate with 20, 50 and 100 μM of genistein for 24, 48 and 72 hours. Then, 100 μL of fresh culture medium and 10 μL of 12 mM MTT stock solution were added to each well after we had incubated the cells for 4 hours at 37°C. To each well, SDS-HCl solution (10 mL of 0.01 M HCl to 1 gm of SDS) was added for an incubation period of 12 hours in a humidified chamber at 37°C. The absorbance was read at 570 nm using the

Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific).

While PE/CA-PJ15, PE/CA-PJ49, HSC-3 cells were treated with genistein at the same concentrations and time points, Trypan blue exclusion method was used to validate the cell viability results obtained with MTT assay. A suspension of each cell line was mixed with 0.4% Trypan blue solution and, after 10 minutes, cells were counted automatically with JuLI™ FL (NanoEntek, Pleasanton, CA, USA).

Scratch assay

A monolayer of each cell line was scraped with a p200 pipette tip and then washed twice with Dulbecco's phosphate buffered saline 1X (Life Technologies, Gibco) in order to remove debris. Then, cells were treated with the IC_{50} dose of genistein at 24 hours. Untreated cells were used as control. Initially, we acquired the first image (T0) and then the subsequent ones after 1 hour (T1), 2 hours (T2), 3 hours (T3), 5 hours (T4), 6 hours (T5) and 24 hours (T6) after treatment. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to calculate the size of wound and analyze all the acquired images. GraphPad Prism 7 (GraphPad Software, Inc., CA, USA) software was used for statistical evaluation.

Western blotting analysis

After treatments with genistein at the same concentrations and time points previously used, cells were lysated to obtain proteins. They were measured and, then, separated by 15% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Bovine serum albumin (BSA) 5% was used for 1 hour as blocking solution.

Membranes were incubated with vitronectin (1:150; BD Biosciences), OCT4 (1:700; Novus Biologicals, Littleton, CO, USA), survivin (1:1,000; Cell Signaling Technology Inc., Danvers, MA, USA), and β-actin (1:5,000; Sigma Aldrich), overnight at 4°C.

Then, peroxidase-conjugated secondary antibody was used (1:2,500; Santa Cruz Biotechnology, Dallas, TX, USA). Signals were acquired with enhanced chemiluminescence kit (ClarityTM Western ECL Substrate, Bio-Rad). UVP ChemiDoc-It®TS2 Imaging System (Analytik Jena AG, Jena, Germany) was used.

Statistical analysis

GraphPad Prism 7 software was used for Student's *t*-test and one-way analysis of variance (ANOVA) and all data were expressed as the M ± SEM;[17] a *P*-values <0.05, $P<0.01$, $P<0.001$ were accepted as statistically significant.

Results

Effects of genistein on cell adhesion

PE/CA-PJ15, PE/CA-PJ49 and HSC-3 cell lines were treated with 20, 50 and 100 μM of genistein during cell adhesion phase. Through xCELLigence system, we monitored adhesion kinetics in real time of the treated cells, using untreated cells as control. After 24, 48 and 72 hours, the CI values were taken and adhesion curves were analyzed. Data obtained show clear effects of genistein on all cells used even at 24 hours after the treatment.

The CI values showed that the adhesion of HSC-3 treated with 20 μM of genistein appears to have reduced by 50% compared with the same untreated cells (Figure 1A and B). This reduction is evident at all time points considered, while, the values of CI of HSC-3 treated with 100 μM of genistein after 48 and 72 hours are negative (Figure 1A and B).

PE/CA-PJ15 cell adhesion was reduced by ~50%, following treatment with concentrations of genistein ranging between 50 and 100 μM, 24 and 48 hours after the treatment. However, for longer treatments, such as 72 hours, even 50 μM of genistein caused a halving of cell adhesion, compared with the untreated control (Figure 1C and D).

Cell adhesion of the PE/CA-PJ49 showed a reduction of about 50% when treated with an intermediate concentration between 20 and 50 μM of genistein, at all time points (Figure 1E and F). Furthermore, the values of CI of PE/CA-PJ49 treated with 100 μM of genistein were negative (Figure 1E and F).

To evaluate the effects of genistein on cell adhesion of tongue carcinoma cells, we also studied the expression levels of the vitronectin protein. It is a glycoprotein found mainly in the extracellular matrix and promotes cell adhesion and spreading. From the Western blotting performed on protein lysates of the 3 cell types used, we noticed that the expression of vitronectin seems to have decreased as a result of treatments with increasing concentrations of genistein, especially those long-lasting. In fact, at 48 hours after treatment with 100 μM of genistein, vitronectin seems to be little expressed and almost unexpressed for treatments with 50 and 100 μM of genistein at 72 hours (Figure 2A and B).

Variation on cell proliferation and IC_{50} determination

Genistein was added to all cancer cell lines in the cell proliferation phase. All cells were monitored in real time and all cell index values were taken at 24, 48 and 72 hours. In addition, they were converted into percentage values and we

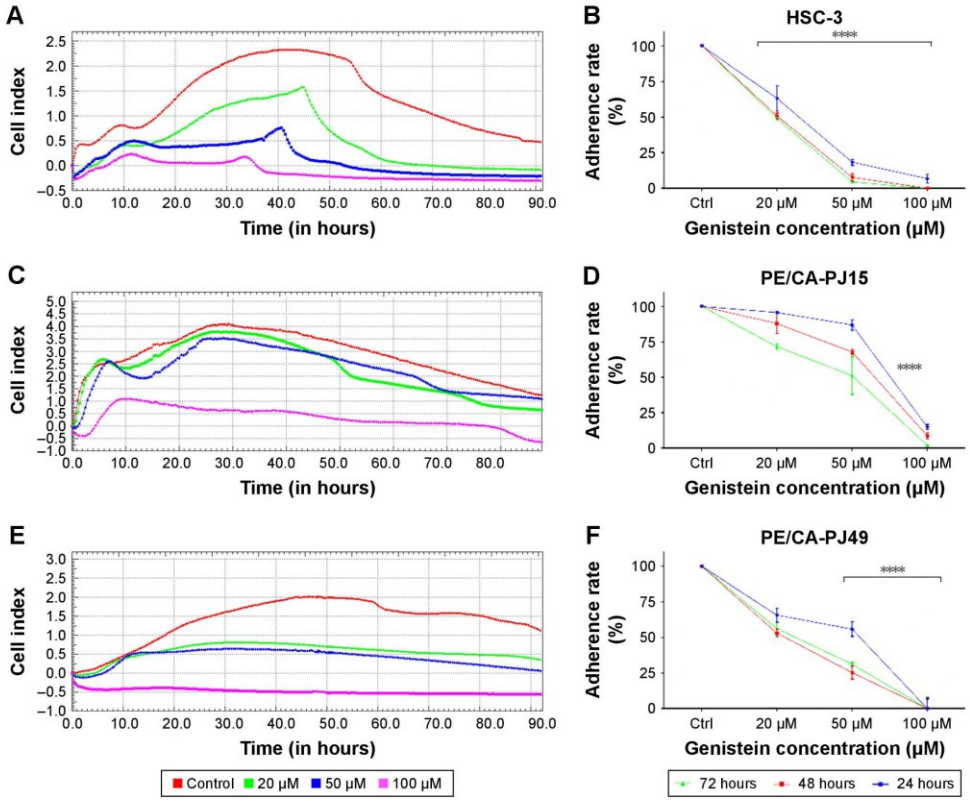


Figure 1 Genistein inhibited adhesion of tongue cancer cells.
Notes: All cell lines were treated with 20, 50 and 100 μM of genistein. In the graphs, there is an initial phase of cell adhesion, followed by a plateau phase prior to a gradual period of proliferation. The adhesion of (**A**) HSC-3 cells, (**C**) PE/CA-PJ15 cells and (**E**) PE/CA-PJ49 cells is shown. Untreated cells were used as control (red curves). We monitored in real time the adhesion for 24, 48 and 72 hours after treatment and all cell index values of HSC-3 cells (**B**), PE/CA-PJ15 cells (**D**) and PE/CA-PJ49 cells (**F**). The results are the mean CI for 3 replicates ± SD. ****$P<0.0001$.

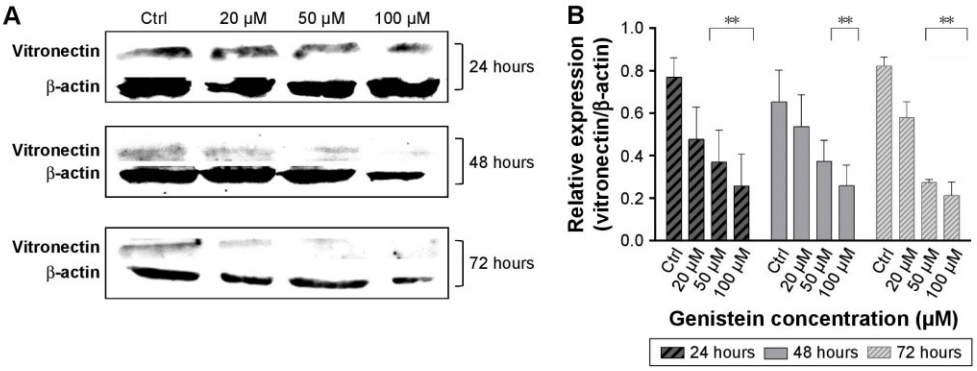


Figure 2 Inhibition of vitronectin expression.
Notes: Western blotting shows the inhibition of vitronectin expression after genistein treatment. Vitronectin is especially down-regulated at 48 hours with 100 μM of genistein and at 72 hours with 50 and 100 μM of genistein (**A**). Also shown is significance degree of tests used **$P<0.005$ (**B**).
Abbreviation: Ctrl, control.

used the ANOVA as the statistical test to evaluate the cell proliferation percentage.

We found a 50% significant reduction of cell proliferation for all cells treated with 20 µM of genistein after 24 hours of treatment (Figure 3A); while, the same reduction was found at 48 hours in a concentration range between 20 and 50 µM of genistein (Figure 3B). Instead, cell proliferation seems to be reduced by 50% compared to the control at 72 hours in all cells treated with 50 µM of genistein (Figure 3C). In all cell lines, 100 µM of genistein resulted in a reduction of about 25% of proliferation at all time points considered. All the data were considered statistically significant for $P<0.005$, $P<0.001$, $P<0.0001$ (Figure 3A–C).

From the growth curves obtained by treating cells with 20, 50 and 100 µM of genistein, CI values were used to calculate the value of IC_{50} at 24, 48 and 72 hours. In particular, it was found that at 24 hours, the IC_{50} value was approximately 46 µM for all cell lines used (Figure 4A–F); while for treatments at 48 hours, the average IC_{50} value of the 3 cell lines used was approximately 40 µM (Figure 4A–F). However, there was a slight discrepancy between the lines for 72-hour treatments. In fact, for HSC-3 and PE/CA-PJ15 the IC_{50} value seemed to be around 22 µM (Figure 4A–D). Only for PE/CA-PJ49, this value was around 46 µM. This was probably because this line showed a higher resistance to treatment with genistein than the other (Figure 4D and E).

Effects of genistein on cell viability

For a better evaluation of genistein on cell viability, we used 2 simple tests: the MTT assay and the Trypan blue assay. In both assays, we noticed that genistein changed cell viability even 24 hours after treatment.

In fact, we noticed a reduction of about 50% of cell viability in each treated cell line with concentrations between 20 and 50 µM at 24, 48 and 72 hours (Figure 5A). Trypan blue assay confirmed almost all the data obtained with the MTT

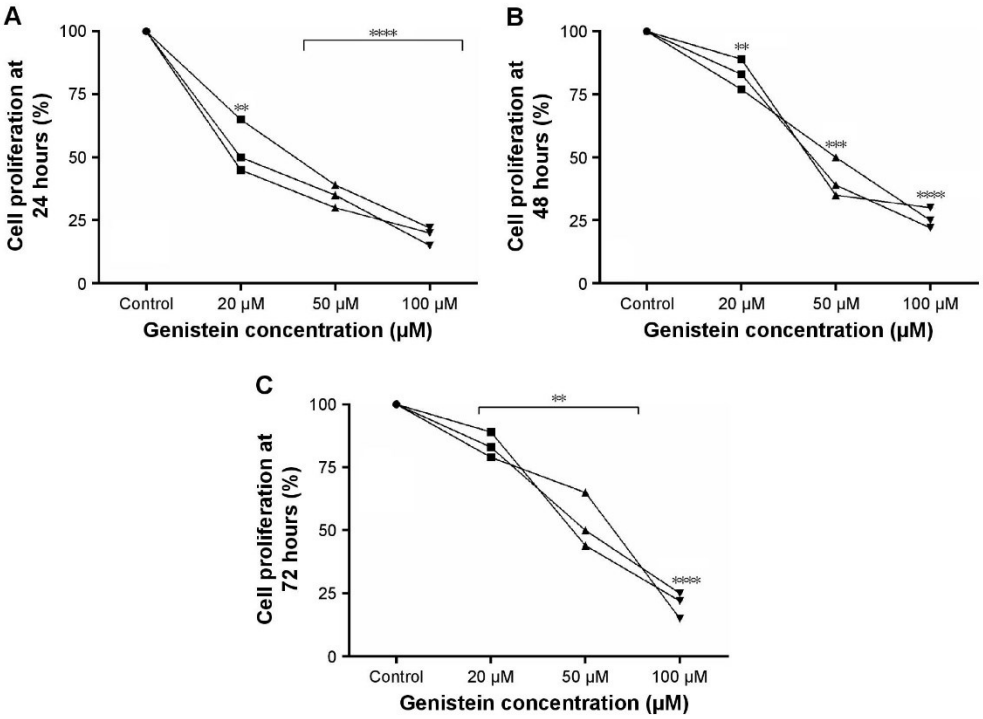
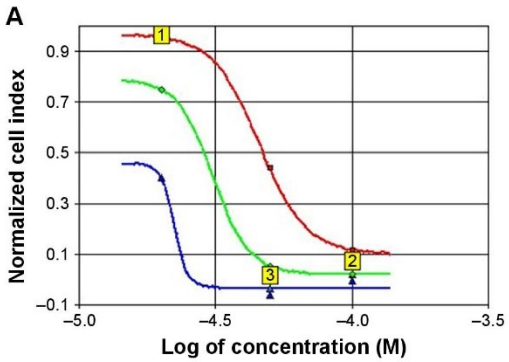
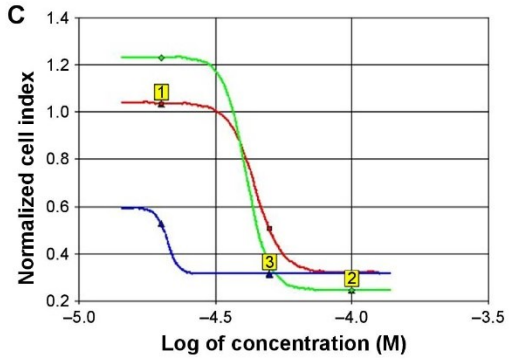


Figure 3 Variation in cell proliferation rate (%).
Notes: Cell index (CI) values were measured and converted to percentage rate using untreated cells as control (100% adhesion). (**A**) This shows a 50% reduction of adhesion posttreatment with 20 µM genistein at 24 hours. (**B**) The same reduction is present for treatment between 20 and 50 µM of genistein at 48 hours, and for 50 µM of genistein at 72 hours (**C**). Also shown is significance degree of tests used (**$P<0.005$, ***$P<0.001$, ****$P<0.0001$).



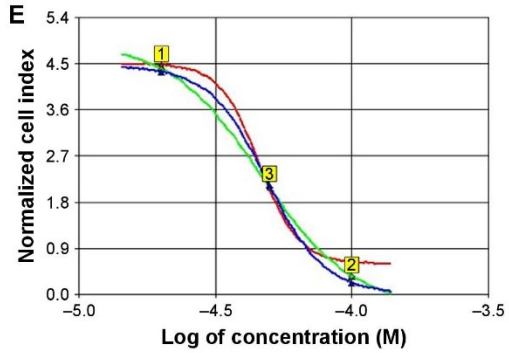
B

	24 hours	48 hours	72 hours
IC_{50}	~46 μM	~32 μM	~23 μM



D

	24 hours	48 hours	72 hours
IC_{50}	~45 μM	~42 μM	~22 μM



F

	24 hours	48 hours	72 hours
IC_{50}	~46 μM	~46 μM	~46 μM

Figure 4 IC_{50} values.
Notes: Dose-response curves are shown for HSC-3 cells (**A**), PE/CA-PJ15 cells (**C**) and PE/CA-PJ49 cells (**E**). In each graph, the yellow square with the numbers 1, 2 and 3 indicates, respectively, IC_{50} values at 24, 48 and 72 hours. IC_{50} values are expressed as the mean (M) ± standard error of the mean (n=3) and they are shown as the average of all IC_{50} values of HSC-3 cells (**B**), PE/CA-PJ15 cells (**D**), PE/CA-PJ49 cells (**F**) at each time point. R^2 of IC_{50} was 0.99.

assay (Figure 5B). All the data were considered statistically significant for $P<0.001$ and $P<0.0001$.

Determination of cell migration rate

To evaluate if genistein is able to reduce the migration of the tongue cancer cells, we used the Scratch assay. Tongue cells were treated with IC_{50} at 24 hours, previously calculated. The control had not undergone any treatment.

The treated cells migrated very little in the cut area (Figure 6A). The most significant effects were evident starting from T4 and, specifically, the wound of treated cells does not show any healing after 24 hours (Figure 6A).

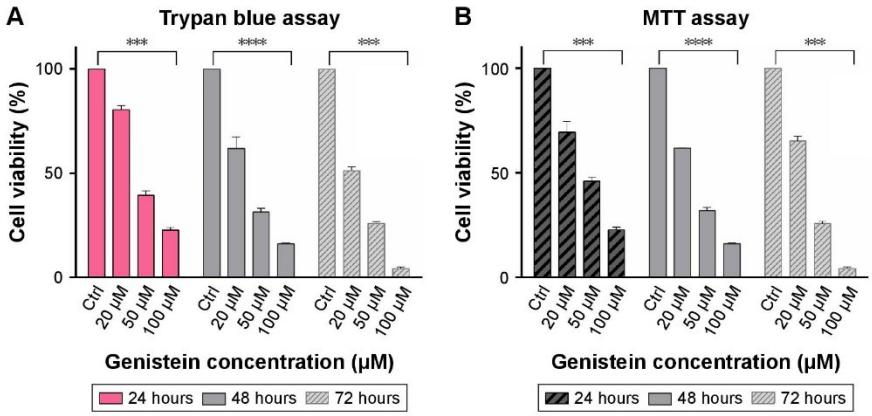


Figure 5 Genistein reduces the viability of tongue cancer cells.
Notes: Trypan blue test (**A**) and MTT assay (**B**) show a proportional reduction in cell viability with increasing concentrations of genistein. Experiments were performed at least 3 times and results are presented as the mean ± SD, and are statistically significant for ***$P<0.001$ and ****$P<0.0001$.
Abbreviation: Ctrl, control.

The untreated control showed an increasing level of healing of the wound during the considered times total healing after 24 hours (Figure 6A).

Evaluations of the timing at T4, T5 and T6 were most significant with P-value <0.05, $P<0.01$, $P<0.001$ (Figure 6B).

Effects of genistein on tumorigenesis

After treating cancer cells of the tongue with different concentrations of genistein, we evaluated the expression of OCT4 and survivin. OCT4 is a known protein because its overexpression promotes tumorigenesis in different types of cells. In tongue

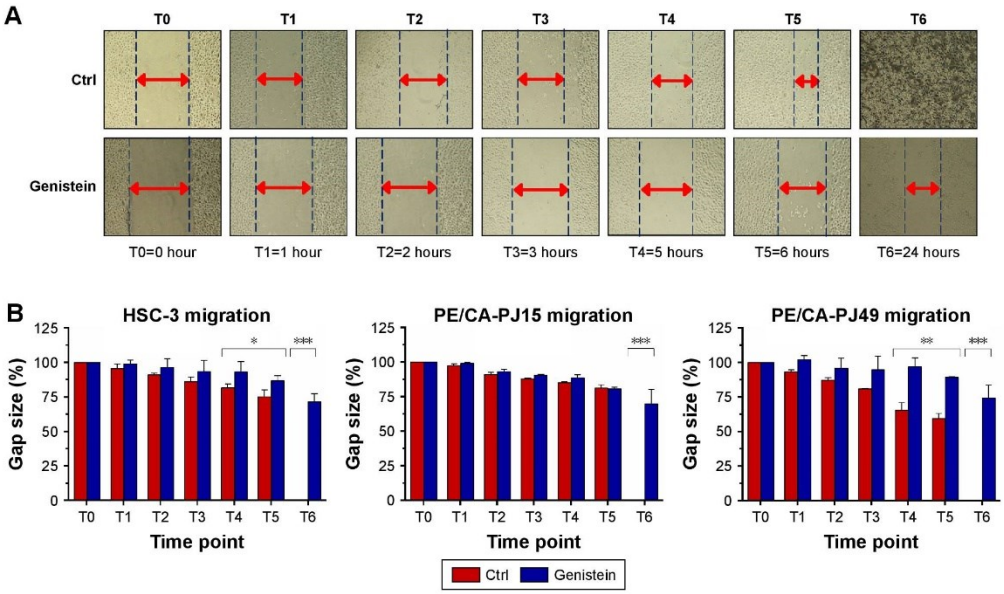


Figure 6 Effects of genistein on cell migration.
Notes: Significant increase of the gap size is more evident from T4 in tongue carcinoma cells compared to untreated control (Ctrl) (**A**). All experiments were performed at least 3 times and results are presented as the mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (**B**).

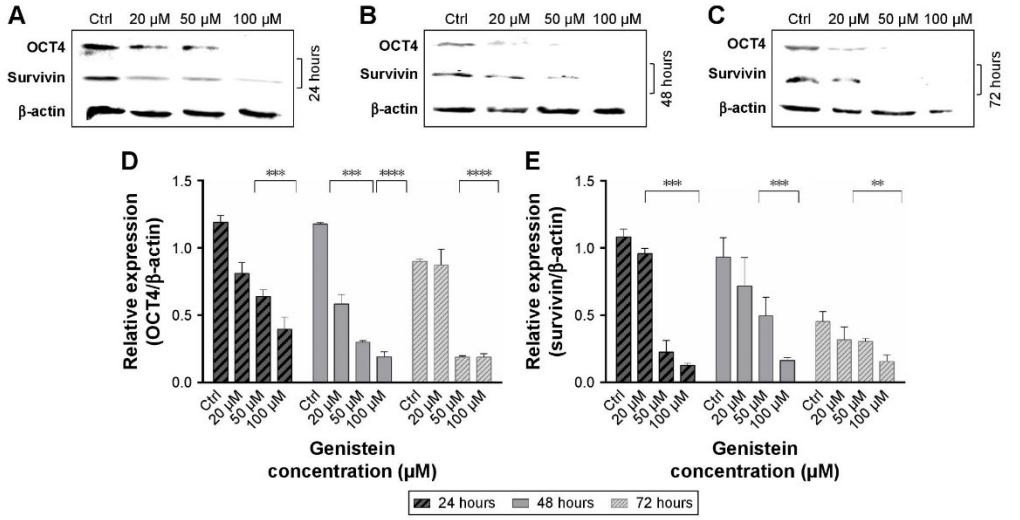


Figure 7 Genistein reduces tumorigenesis and promotes apoptosis.
Notes: Variation in level of expression of OCT4 and survivin. Genistein inhibits survivin and OCT4 at all time points considered (**A–C**). The significance degree of tests used is $^{**}P<0.005$, $^{***}P<0.001$, $^{****}P<0.0001$ (**D–E**).
Abbreviation: Ctrl, control.

cancer cells, genistein appears to have an action on the expression of OCT4 only after high concentration treatments.

In fact, Figure 7 shows a reduction in those cells that were treated with 50 and 100 μM of genistein for each time point considered.

Moreover, expression of survivin appears to be extremely reduced as a result of treatments with concentrations of 50 and 100 μM at 24 hours (Figure 7A and E). This seems to be confirmed at all time points considered, and especially at a concentration of 100 μM, there is almost a complete inhibition (Figure 7A–E).

Discussion

Modern treatment protocols of OSCC did not lead to great results despite adoption of new surgical techniques and innovative chemotherapy formulations.

In recent years, the scientific community has been fascinated by the remarkable properties that natural substances possess, especially by many features that make them anticancer substances.

Genistein is an isoflavone isolated in 1899 from dyeing of genistra, characterized in 1926 and synthesized for the first time in 1928. It is well known to be present mainly in soybeans. It has a structure similar to estrogen and this characteristic seems to play an important role to help improve menopause symptoms.[35]

Notable are also its anti-tumor effects, demonstrated especially in breast cancer[20,36–38] and prostate cancer.[39–41]

Even for OSCC, there are studies on genistein action, but they investigate the inhibitory activity of the protein tyrosine kinase,[29] its action on tumor angiogenesis[42] and its chemopreventive effects when used with quercetin[30,31] and biochanin A,[32] which are other isoflavones.

Park et al have carried out an in vitro study on oral cancer cells, HSC-3 and KB, validating their results on nude mice. They demonstrated that genistein and cetuximab have a synergistic action in the inhibition of EGFR signaling pathway.[43]

In literature, there are no papers that demonstrate the effects of genistein, as the only treatment against tongue carcinoma, which is considered the most common oral cancer and the most aggressive of all oral cancers.

Therefore, we studied the effects of this isoflavone on 3 lines of squamous cell carcinoma of the tongue; HSC 3, PE/CA-PJ15 and PE/CA-PJ49 cell lines.

We treated the cells with 3 concentrations of genistein; 20, 50 and 100 μM. The choice of concentrations was made by studying the literature data and, in particular, inspired by the work of Johnson et al. Even when the genistein was used with the biochanin A, the results on oral carcinoma cells were good. All in vitro assessments were made considering 3 time points; 24, 48 and 72 hours.

In this paper, our goal was to show how genistein inhibits adhesion, proliferation, viability and migration of cancer cells of the tongue.

It is known that alterations in cell adhesion affect the ability of cells to move. In particular, cancer cells need to be able to move and migrate in order to spread, and cell adhesion plays an important role in regulating cell movements and the spread of metastases.[44]

Our data show that cell adhesion is decreased by almost 50% at concentrations between 20 and 50 μM, at 24 hours, in almost all cell types used compared with untreated cells. This decrease was confirmed at other time points within the range of the concentrations used.

This showed that the adhesion of the tongue cancer cells is greatly compromised by treatment with genistein.

Kingsley et al have shown that the adhesion of cells of OSCC (CAL 27 and SCC25 cell lines) was clearly decreased when cells were treated with soy protein extract[45] belonging to different isoflavones, including genistein.[46]

In addition, to confirm the strong action of genistein on adhesion, we also evaluated the expression of vitronectin, a well known adhesion protein. Western blotting analysis demonstrates that the inhibition of the vitronectin after treatment with genistein is dose-dependent and is also down-regulated by treatment at 48 and 72 hours with high concentrations (50–100 μM).

Inhibitory effects of genistein are also confirmed by Skogseth et al. They demonstrate that genistein is able to reduce the adhesion of prostate cancer cells because it inhibits many adhesion proteins. This is probably because it is a strong inhibitor of tyrosine kinases.[47]

Even for Haier et al, genistein has strong cell adhesion inhibition capacity in colon carcinoma cells. Indeed, it also seems to inhibit the expression of vitronectin in HT-29 cell lines.[48]

In our study, we also evaluated the effects of genistein on cell proliferation. Also, in this case, we obtained a strong reduction of proliferation with high significance ($P<0.0001$) for all tongue cancer cells treated with concentrations between 20 and 50 μM at all time points.

Davis et al showed that genistein also has anti-proliferative action on prostate tumor cells.[49] Alhasan et al confirmed that genistein has a strong anti-proliferative effect in OSCC HN4 cell line treated with 50 μM of genistein, even favoring an arrest of the cell cycle and promotion of apoptosis.[50]

In addition, we used the CI values obtained with the xCELLigence system to calculate the IC_{50} values of genistein at 24, 48 and 72 hours.

In all cell lines, on average, we found IC_{50} values of approximately 46 μM at 24 hours and approximately 40 μM at 48 hours. However, we obtained discordant IC_{50} values at 72 hours in each cell line used. This was probably because PE/CA-PJ49 cells are more aggressive than the other 2 cell lines used.

Johnson et al affirm that the IC_{50} value for the SCC15 and SCC25 cell lines, OSCC cells, is ~50 μM.[32] The same IC_{50} value was attributed to the breast cancer cells.[51]

In addition, we showed that tongue carcinoma cells' viability decreased by 50% when treated with concentrations between 20 and 50 μM of genistein, almost at all time points.

Alhasan et al have reported a significant reduction of the vitality and growth of OSCC cells after treatment with genistein.[50] Ye et al also argued that genistein inhibits the viability and proliferation of cells of head and neck cancer by also inducing apoptosis.[52]

In our opinion, one of the most significant results, among those obtained, relates to cell migration. In fact, the treatment of all cells with the IC_{50} value of genistein at 24 hours showed a very significant reduction of cell migration after 24 hours; $P<0.001$. This shows that genistein can inhibit tongue carcinoma cells migration and so it can have a big effect on the inhibition of the metastasis processes.

This power of genistein seems to be confirmed for ovarian cancer[53] and OSCC.[42]

Finally, we have shown that genistein is able to strongly inhibit, with treatment >50 μM, the expression of OCT4 and survivin in tongue cancer cells, thus reducing the tumorigenesis enough to be considered a good agent against this kind of tumor.

This inhibitory action of OCT4 expression seems to be also confirmed in embryonal carcinoma,[54] while Tian et al have shown that genistein down-regulates the expression of survivin in H446 small-cell lung cancer cells.[55]

Conclusion

Genistein is a natural isoflavone whose effects have been studied fully in many neoplastic diseases.

However, its anti-tumor properties were evaluated, especially in combination with other isoflavones, against OSCC and there are no data in literature that make clear its effect, in particular on tongue carcinoma. There are several studies on the use of genistein for other cancers, very few on oral cancer and almost no specific study on tongue squamous cell carcinoma.

With this in vitro study, we can confirm that genistein acts on the tongue cancer cells by inhibiting efficaciously cell viability, proliferation, adhesion and migration.

This shows that genistein has excellent anti-cancer properties that can be explored for this type of tumor.

However, in vivo studies are needed to further validate these results and develop a suitable delivery system that acts directly in situ to overcome the problems concerning the bioavailability.

Author contributions

Dr FA designed the paper, collected data, drafted the manuscript, drew the figures and approved the final manuscript as submitted. Dr GT, Dr AC and Dr DP did statistical analysis with the data collected. Dr MRP drafted the initial manuscript with Dr FA and approved the final manuscript as submitted. Prof LLM coordinated and supervised data collection, critically reviewed the manuscript, and approved the final manuscript as submitted. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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UNDER REVIEW

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Effects of curcumin on squamous cell carcinoma of tongue: an in vitro study

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Abstract: **Background**: The squamous cell carcinoma of tongue (TSCC) is the most frequent cancer of oral mucosa often characterized by poor prognosis. Conventional therapies used are not very efficient and often cause several side effects. Use of natural substances as possible adjuvant in the treatment and prevention of cancer is very much studied. In particular, curcumin is used for decades in Chinese medicine for its beneficial effects. Curcumin has anti-cancer properties in many tumors but its action on TSCC is not entirely evident and many other investigations are necessary. **Methods**: In this study, we evaluated the effects of curcumin on TSCC cells using different concentrations (1, 5, 10, 20 and 50 μM) and 3 different treatment times (24, 48 and 72 hours). The inhibition of adhesion, proliferation, viability, migration and apoptosis was studied by *xCELLigence System*. **Results**: IC50 value of curcumin is about 10 μM and there have been inhibitory effects even for treatments at low concentrations. Curcumin reduces migration and progression of TSCC cells and promotes apoptosis inhibiting tumorigenesis. **Conclusions**: These results suggest the possible use of curcumin as an anti-cancer agent in TSCC. However, in vivo studies are needed to confirm these results and overcome its low bioavailability.

Keywords: curcumin; TSCC; tumor adhesion; IC50 value; xCELLigence system

1. Introduction

Oral squamous cell carcinoma is the most frequent cancer of head and neck region and it represents 90% of all malignant tumor of oral cavity (1). The most common site of this tumor is tongue (2, 3) and five-year survival rate for this cancer is about 44%. These data has remained almost unchanged over the past decades, despite advances in oncology (1).

Moreover, the treatment is essentially surgical, with a consequent poor quality of life. Depending on the stage of cancer, it can be very invasive, disfiguring, debilitating and in almost every case it results in a radical dissection of the neck (4).

Cisplatin (cis-diamminedichloroplatinum) is a widely used chemotherapic to treat Oral squamous cell carcinoma and its efficacy is increased when combined with other chemotherapeutic agents, such as 5-fluorouracil (5, 6).

On the other hand, chemotherapy and radiotherapy can produce unpleasant and often permanent side effects such as xerostomia, mucositis, oral candidiasis, osteoradionecrosis (4, 7, 8).

Recent studies showed that Cetuximab (anti-EGFR monoclonal antibody) is a good adjuvant in radiotherapy increasing patient survival (9, 10) even if causing many side effects.

New literature data suggested that many dietary compounds can have an anti-cancer effect, used alone or together with other chemotherapic agents (11-15).

Thus, a great effort is dedicated to study dietary polyphenols such as Curcumin (diferuloylmethane) (16-18), extracted by rhizome of Curcuma longa plant and commonly used in Ayurvedic medicine as a drug to treat numerous disease (19).

This substance is well known for its anti-inflammatory, antioxidant, antiplatelet and anticancer properties (20-23). Curcumin binds many proteins, including cyclooxygenase-2 (COX2) and lipoxigenase (LOX) (24), NF-κB (25), protein kinase (PTK) (26), inflammatory cytokine (27). Curcumin modulates multiple pathways in breast cancer, including PI3K/Akt, MAPK, NF-κB, cyclin D, c-Myc, etc. (28) and can inhibit the proliferation and survival of almost all types of tumor cells since it has a selective role on cancer cells rather than normal cells (24).

In this study, we have evaluated the in vitro effects of curcumin on the tongue carcinoma cells (TSCC) using the same concentrations of this nutraceutical previously tested in other tumors [16, 24].

For the determination of its action on cell adhesion, proliferation and cytotoxicity we used xCELLigence® RTCA system, which monitors real-time biological cellular events (29-31). This system has gold microelectrodes placed on the bottom of plates that measure the electronic impedance through an untitled parameter, termed Cell Index (CI), in order to represent cell status. Furthermore, we have evaluated the effects of curcumin on vitality, migration, apoptosis and tumorigenesis of TSCC.

2. Results

2.1. Evaluation of cell adhesion

The PE/CA-PJ15, PE/CA-PJ49 and HSC-3 cell lines were seeded in E-Plate of xCELLigence system with 1, 5, 10, 20 and 50 μM of curcumin to verify some variation in adherence of treated cells compared to untreated ones (*Fig.1a, 1b, 1c*). We monitored in real time the adhesion for 24, 48 and 72 hours after treatment and all CI values analyzed.

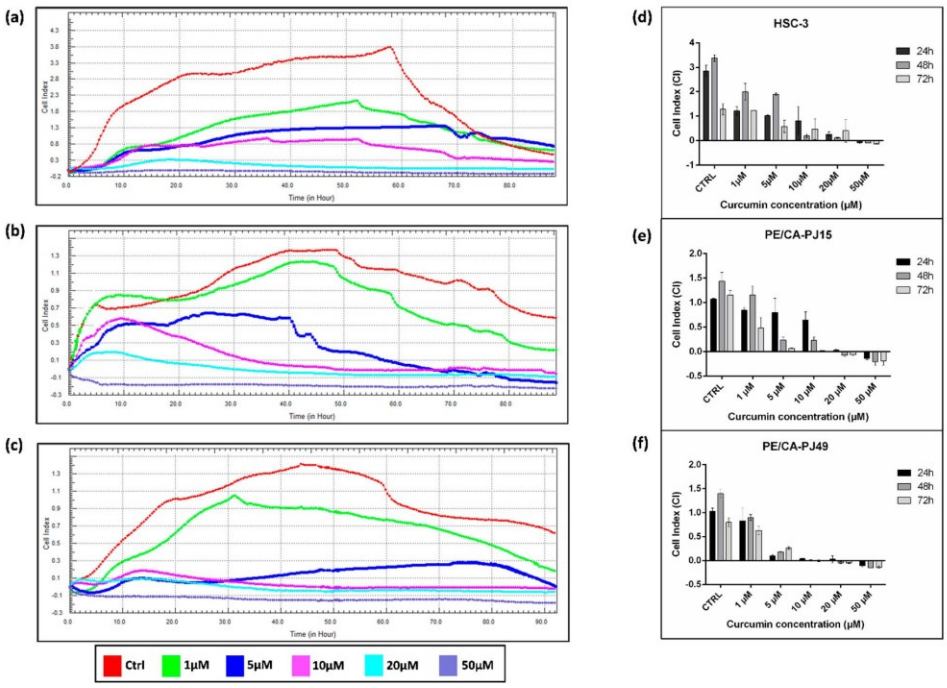


Figure 1. Curcumin inhibited adhesion of tongue cancer cells. Different Cell Index (CI) adhesion curves are obtained from 3 tongue carcinoma cells types. All cells treated with different concentration of curcumin 1, 5, 10, 20 and 50 μM. The adhesion of (**a**) HSC-3 cells, (**b**) PE/CA-PJ15 cells and (**c**) PE/CA-PJ49 cells is shown. Cells untreated used as control (red curves). The results are the mean CI for three replicates ± SD. CI values was measured and it was converted in % rate using untreated cells as control (100% adhesion). (**d**) HSC-3 cells shows a 50% reduction of adhesion post treatment with 1

μM at 24 hours, 5 μM at 48 hours, 5-10 μM at 72 hours. (**e**) PE/CA-PJ15 cells treated with 10 μM of curcumin for 24, and those treated with 1-5 μM for 48 and 72 hours showed the same reduction. (**f**) PE/CA-PJ49 cells treated with 1-5 μM of curcumin was reduced by 50% compared to control at 24 and 48 hours, while with 5 μM of curcumin. $^{**}p < 0.01$, $^{***}p < 0.001$.

The resulting curves (*Fig.1a, 1b, 1c*) showed a difference of adhesion between control and treated cells. The reduction of adhesion was proportionally increased respect to the concentration of curcumin used for all TSCC lines. Even after long times of treatment, the effects of curcumin were significant.

The CI values to 72 hours were considerably reduced in all conditions analyzed, even considering the limitations of xCELLigence system, such as the normal reduction in cell growth space for long analysis times. However, the results obtained confirmed that the increase in curcumin concentrations leaded to a proportional reduction of cell adhesion, even for very long treatments.

In the evaluation of adherence rate, we converted the CI values in percent values in order to obtain a better indication of the reduction of cell adhesion, considering the control value of CI, at each time point, as the maximum value of adhesion at that period (*Fig.1d, 1e, 1f*).

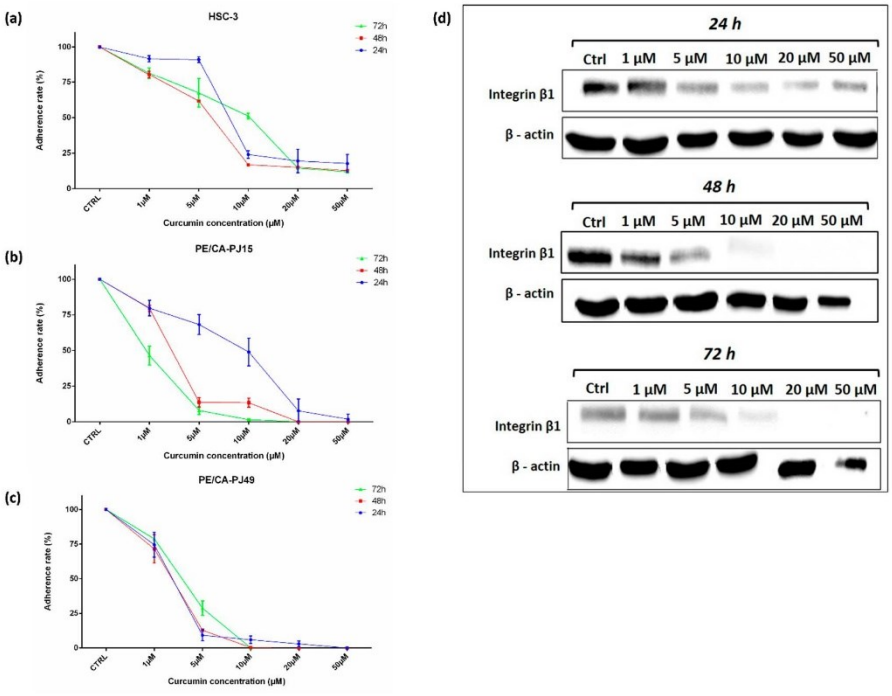
In particular, CI values showed a 50% reduction of cell adhesion in HSC-3 cells treated with 1 μM of curcumin at 24 hours (*Fig.1d*). We had a very high significance compared to control ($p < 0.001$). The same reduction was also proved at 48 hours after treatment with 5 μM of curcumin (*Fig.2a*). While, HSC-3 cells treated with curcumin concentration between 5-10 μM showed a 50% reduction of adhesion at 72 hours compared to control (*Fig.1d*).

Also, PE/CA-PJ15 treated with about 10 μM of curcumin for 24 hours showed the same reduction ($p < 0.001$) (*Fig.1e*). However, this reduction was evaluated for this cell line treated for 48 and 72 hours at low concentration (between 1 and 5μM) (*Fig.1e*).

Furthermore, adhesion of PE/CA-PJ49 cells treated with 1-5 μM of curcumin was reduced by 50% compared to control at 24 and 48 hours (*Fig.1f*). While, the same reduction is observed after 72 hours of treatment with 5 μM of curcumin. This data showed a high significance ($P < 0.001$). In addition, the adhesion is decreased by 75% compared to control after 24 and 48 hours with 5 μM, while at 72 hours with concentrations of around 10 μM (values of $P < 0.01$ and $P < 0.001$)

All TSCC cells showed negative CI values after being treated with 20 and 50 μM of curcumin at all time points considered (*Fig.1d, 1e, 1f*).

In order to better evaluate the effect of curcumin on cell adhesion, we evaluated the expression of integrin β1, an integral membrane glycoprotein that binds the extracellular matrix proteins playing a key role in cell adhesion. *Figure 2a, b* showed the reduction of integrin expression by 50% in cells treated with 10 μM of curcumin after 24 hours compared to control. While, after 48 hours the same reduction was present for cells drawn with about 5μM of curcumin. Instead, at 72 hours after treatment the values were significantly reduced compared to the treatments after 24 and 48 hours. In particular, a reduction of 50% adhesion relative to the control was evident in cells treated with 5μM of nutraceutical. In addition, we found a great reduction of integrin expression in all the cells treated with 20 and 50μM of curcumin to 48 hours and 72 hours after treatment (*Figure 2a, b*).



111

112 **Figure 2. Expression of integrin β1.** Downregulation of integrin β1 expression by 50% in cells treated
113 with 10 µM of curcumin after 24 hours compared to control (**a**), (**b**). A great reduction of integrin
114 expression is at 20 and 50µM concentration of curcumin for 48 hours and 72 hours to treatments (**a**),
115 (**b**). WB showing expression of integrin β1 in HSC3 (**e**), PE/CA-PJ15 (**f**) and PE/CA-PJ49 cells (**g**).

116 *2.2. Cell Proliferation and half maximal inhibitory concentration (IC50)*

117 xCELLigence system was also used to evaluate the effect of the nutraceutical on tongue
118 carcinoma cells. Proliferation rate (%) showed a decrease by 50% compared to control at 24 hours for
119 cells treated with concentration of curcumin about 5-10 µM with p< 0,001 (*Fig. 3a*). There was only
120 about 25% of tumor cell proliferation compared to the control after treatment with 20 µM of curcumin
121 with p < 0,0001 (*Fig. 3a*). Even with 50 µM of curcumin (p < 0,001), the proliferation was less than 25
122 (p < 0,001) (*Fig. 3a*).

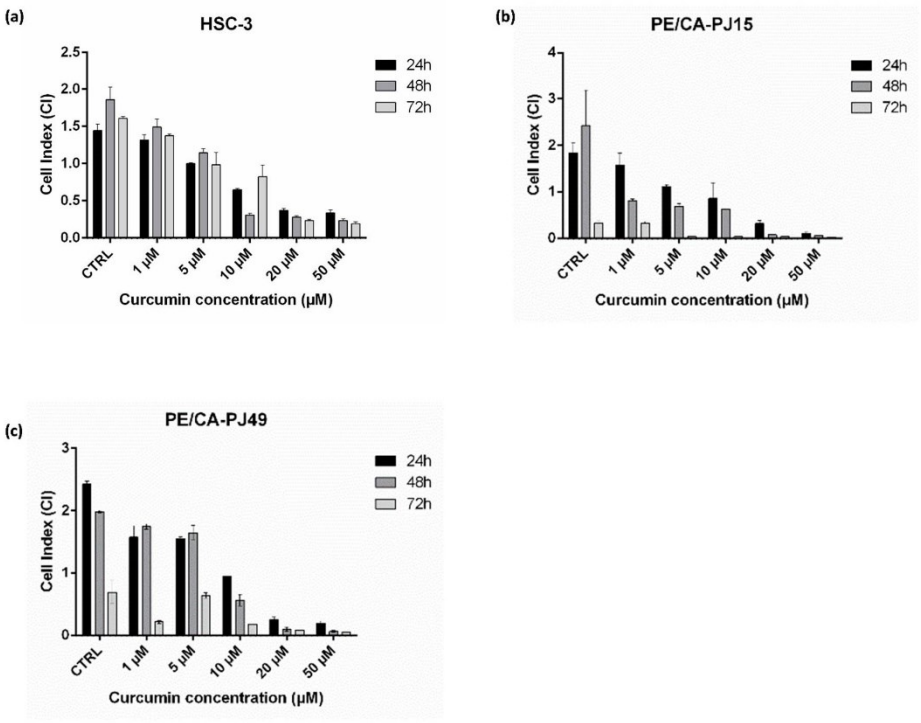


Figure 3. Curcumin dose-dependent effects on tongue carcinoma. We added curcumin to all cells in proliferation phase. Curcumin inhibitory effects at 24 hours (**a**), 48 hours (**b**) and 72 hours (**c**) in all cell lines with $^{**}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Furthermore, treatments at 48 hours, with 1-10 μM of curcumin, showed a reduction of the proliferation of 50% compared to control (*Fig. 3b*). The reduction of cell proliferation observed between 10 μM and 50 μM was highly significant compared to the control ($p < 0,0001$) (*Fig. 3b*).

While, the proliferation of cancer cells was halved compared to the control after treatment with 5-10μM at 72 hours with $p < 0,05$ (*Fig. 3c*). The proliferation was significantly reduced by treatment with 10 μM of curcumin $p < 0,0001$ (*Fig. 3c*).

All CI values were used for the calculation of half maximal inhibitory concentration (IC50) by RTCA Software 1.2 (*Fig. 4 a, b, c*). IC50 value was expressed as the mean (M) ± S.E.M. (n = 3).

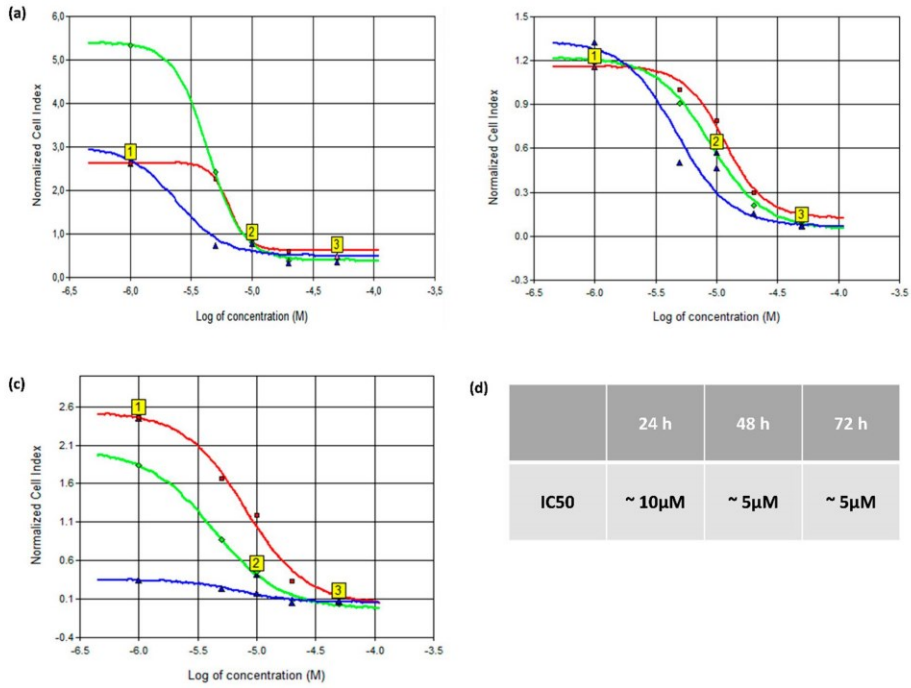


Figure 4. Determination of half maximal inhibitory concentration (IC50). Curcumin cytotoxicity was quantified by calculating the IC50 24, 48 and 72 hours post curcumin treatments. Dose-response curves are shows for HSC-3 cells (**a**), PE/CA-PJ15 cells (**b**) and PE/CA-PJ49 cells (**c**). In each graph, the yellow square with the number 1, 2 and 3 indicates respectively IC50 values at 24, 48 and 72 hours. IC50 values are expressed as the mean (M) ± S.E.M. (n=3) and they are show as the average of all IC50 values of the cell lines used at each time point (**c**). Square R of IC50 was 0.99. Figure 4 d is reporting IC50 values obtained from the averages of the IC 50 values of the individual cell lines for each time point.

In *Figure 4 d,* we reported the IC50 values obtained from the averages of the IC 50 values of the individual cell lines for each time point.

IC50 at 24 hours post treatment was around 10 μM, while IC50 at 48 and 72 hours was around 5 μM (*Fig. 4d*). For the calculation of all IC50, the square R was 0.99.

2.3. Effects of curcumin on cell viability

Through the MTT assay, we verified a decrease in cell viability proportional to increasing concentrations of curcumin for all treated cell lines (*Fig.5a, b, c*). We observed a 50% reduction of viability in HSC3 cells treated with 5-10 μM of curcumin at 24 hours after treatment and 1-5 μM at 48 and 72 hours (*Fig.5a*). PE/CA-PJ15 cells showed the same reduction for the concentrations of 10 μM of curcumin at 24 and 48 hours and about 10-20 μM at 72 hours (*Fig.5b*).

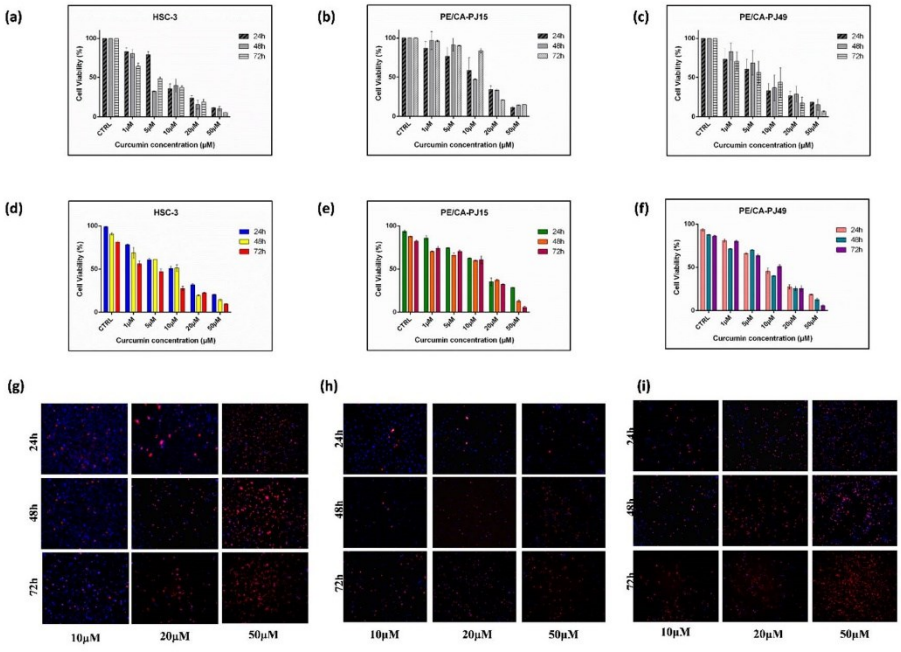


Figure 5. Curcumin reduces the viability of tongue cancer cells. MTT assay (**a**) (**b**) (**c**), Trypan blue test (**d**) (**e**) (**f**) and Fluorescence assay (**g**) (**h**) (**i**) show a proportional reduction in cell viability with increasing concentrations of curcumin. (**g**) (**h**) (**i**) show the effects of turmeric respectively for HSC-3, PE/CA-PJ15, PE/CA-PJ49 cells at the indicated concentrations. Results were performed at least three times and they are presented as the means ± SD.

A reduction of about 50% was observed in PE/CA-PJ49 cells treated with 5-10 μM of curcumin at all time points (*Fig.5c*). Through trypan blue test, the cell viability was reduced for HSC-3 and PE/CA-PJ49 cells in the same way as MTT assay (*Fig.5d, e, f*). While for the PE/CA-PJ15 cells a 50% reduction in cell viability was observed for cells treated with 10-20 μM of curcumin at all time points unlike MTT test (*Fig.5e*). We assessed cell viability fluorescence with ReadyProbes® Cell Viability Imaging Kit.

Figure 5g, h, i showed the important effects of curcumin at concentrations of 10, 20 and 50 μM for all cells used. Data showed the same effects evaluated in the previous tests and, in particular, at 50 μM of curcumin we noticed huge reductions in cell viability for all cells and at all time points.

2.4. Determination of cell migration rate

To evaluate cell migration, we captured images at the beginning of the treatment with 10 μM of curcumin (T0) and at intervals (T1, T2, T3, T4, T5 and T6) during the migration of the cells to close the scratch, and we compared the images to determine the rate of cell migration (*Fig.6a*). The concentration of curcumin used was the average IC50 value at 24 hours, previously estimated.

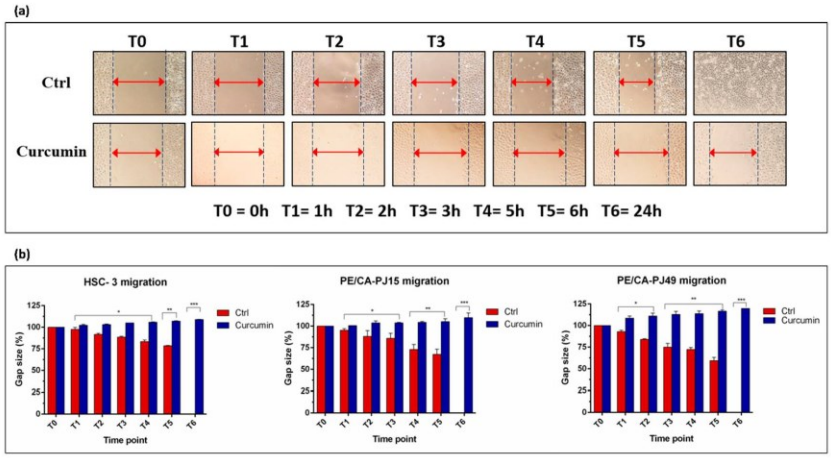


Figure 6. Effects on cell migration. Significant increase of the gap size is most evident from T3-T4 in tongue carcinoma cells compared to control untreated (Ctrl) (**a**). All experiments were performed at least three times and results are presented as the means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (**b**).

All experiments were performed at least three times and the results were presented as means ± SD. We noticed a significant increase of the gap size directly proportional to the increase of the treatment time with curcumin, while it decreased in the untreated control (*Fig.6a, b*).

The increase was most evident from T3-T4 onwards and it was present in all of TSCC cell lines. For T4 and T5 times we had a very high significance compared to control ($p < 0.05$ and $p < 0.01$) for all cell lines (*Fig.6b*). Instead, the wound treatment with curcumin after 24 hours (T6) prevented the complete healing of the wound, while it disappeared completely in the untreated control ($p < 0.001$) (*Fig.6 a, b*).

2.5. Effects on apoptosis and tumorigenesis

After treating tongue cancer cells with different curcumin concentrations, we evaluated the expression of PAR 4, OCT4 and survivin (*Fig. 7a, b, c*).

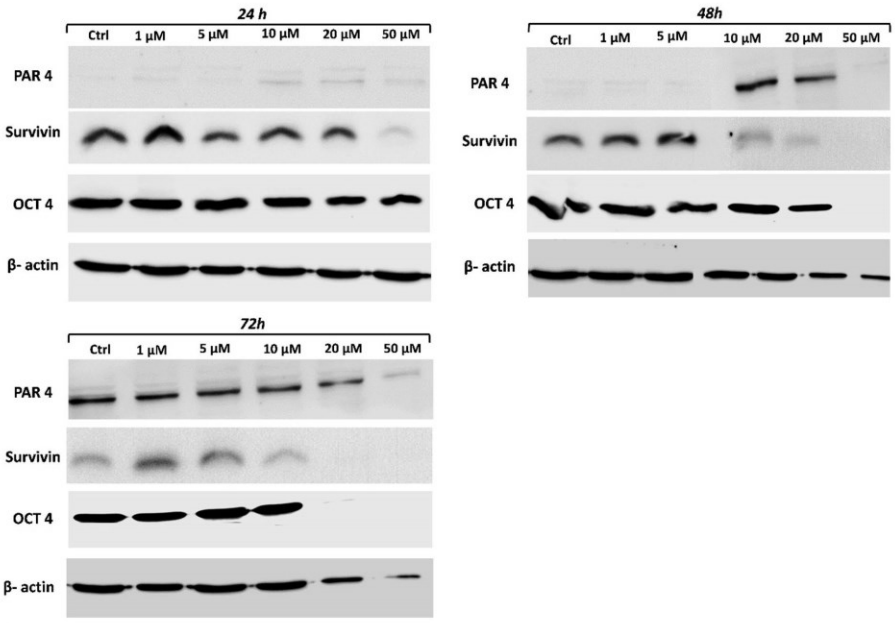


Figure 7. Curcumin reduces tumorigenesis and promotes apoptosis. Variation in level expression of PAR4, survivin and OCT4 show at 24 hours (**a**), 48 hours (**b**) and 72 hours (**c**). Curcumin inhibits survivin and OCT4 but up regulates PAR4. The results shown in Figure **b** belong to two gels.

We noticed an increased expression of PAR4, pro-apoptotic protein, for concentration higher than 10 μM at 24 and 48 hours and for all treatments at 72 hours (*Fig. 7a, b, c, d*). This demonstrated, probably, that curcumin induces apoptosis in cells treated with 10 μM already after 24 hours and that possible apoptosis is more evident after 72 hours of treatment (*Fig. 7a, c, d*).

In addition, there was an inhibition of OCT4 with 50 μM of curcumin at 24 hours and with 20 and 50 μM at 72 hours showing that curcumin can inhibits tumorigenesis (*Fig. 7c, f*).

Furthermore, curcumin downregulated expression of survivin with concentrations of 50 μM at 24 hours, greater than 10 μM after 48 hours and at all concentrations at 72 hours. There was a total inhibition for concentrations greater than 20 μM for 72 hours (*Fig. 7a, b, c, e*).

3. Discussion

Surgery, radiation and chemotherapy are the most widely used treatments in head and neck tumors. Despite the use of modern techniques and treatments, the survival rate of patients at 5 years after diagnosis has not changed significantly (32).

For these reason, scientific attention has been focused in recent decades on possible use of natural substances, well known as nutraceuticals, how adjuvant therapy.

Curcumin, also known as tumeric, is a vegetal extract for long used in Asian countries to treat inflammation, liver disorders and rheumatic diseases and, in recent years, it has been confirmed as an antiseptic, analgesic, anti-inflammatory and anti-malarial agent.

To date, literature showed that curcumin has an antiproliferative effect on tumor cells of breast, lung, prostate (33-35).

The molecular basis of its antitumor effect are attributed to inhibition of transcription factors, growth regulators, adhesion molecules and genes involved in apoptosis (36).

So, curcumin appears to have a strong inhibitory power against adhesion, proliferation, viability, migration, invasion and apoptosis(37) .

In recent decades, many studies showed the powerful action of curcumin in head and neck tumors. *Borges et al* summarize all studies conducted to date on the action of curcumin on head and neck cancer (38). They reported that curcumin inhibits cell proliferation and promotes apoptosis (38).

However, in literature there are not many studies to clarify the effect of curcumin on TSCC, the most frequent cancer of head and neck with worst prognosis.

In this study, we treated 3 TSCC cell lines with curcumin concentrations of 1, 5, 10, 20 and 50μM for 24, 48 and 72 hours and in vitro effects showed a dose-dependent correlation.

We found a halving of cell adhesion after treatment with 1 μM of curcumin for 24 hours, 5 μM of curcumin for 48 and 10 μM of curcumin 72 hours.

The same inhibition was found in PE/CA-PJ49 cells, at increasing concentrations of curcumin, over time. For PE/CA-PJ15 cells, however, we had the same level of inhibition with concentrations in a range from 1 to 5 μM, for all time points considered.

These data showed that curcumin has a strong inhibitory power on adhesion in vitro on TSCC at concentrations even lower than 10μM.

Even, the integrin expression supports the theory that curcumin inhibits adhesion of TSCC cells.

While, its inhibition on the proliferation was showed by 50% reduced after treatment with 5-10μM curcumin at all time points considered and IC50 value was to these same concentrations.

Xi et al identified the same IC50 for all head and neck cancers (39), even if *Sivanantham et al* noted a dose-depend growth inhibition on cells (40).

However, IC50 of the other tumors are much higher. In fact, 50% inhibiting concentration (IC50) of curcumin in human liver cancer HepG2 cells was 23.15 ± 0.37 μmol/l (41).

All tests done to evaluate the viability have showed that it acts with large inhibitory power and it caused a halved of viability compared to the control for 5-10 μM curcumin concentrations at 24 hours and for lower concentrations or equal to 5 μM for longer treatments.

Scratch assay allowed us to evaluate the effects on cell migration considering various treatment times.

After treating the cells with 10 μM of curcumin (the mean value of IC50 at 24 hours), the scratch size grew proportionally to the increase of incubation time and, especially after 24 hours, did not undergo any healing contrary to what happens with the control. In this way, we showed that curcumin has an interesting effect on tumor cells migration of TSCC cells, also for very long treatment times (as 24 hours).

In addition, treatment with curcumin showed ability to inhibit tumorigenesis and promote cell apoptosis.

PAR4 is a pro-apoptotic and tumor suppressor protein that selectively induces apoptosis in cancer cells by activating the extrinsic mechanisms (42). For this reason, PAR 4 is an attractive therapeutic option. We showed that level expression of PAR 4 increased after curcumin treatment in TSCC cells and then curcumin promoted apoptosis.

Survivin is a member of the inhibitor of apoptosis family (43) considered an oncogene for its overexpression in cancer cells, making them able to escape to apoptotic stimuli and conventional therapies (44). Its overexpression is especially known in oral cancer (45).

This study reported that curcumin action leads to survivin reduction after 24 hours treatment, showing that curcumin induces apoptosis in TSCC cells.

OCT4 (octamer-binding transcription factor 4), also known as **POU5F1** (POU domain, class 5, transcription factor 1) is a protein that in humans is encoded by the *POU5F1* gene(46). Oct-4 is a homeodomain transcription factor of the POU family. This protein is critically involved in the self-renewal of undifferentiated embryonic stem cells. As such, it is frequently used as a marker for undifferentiated cells. Oct-4 expression must be closely regulated; too much or too little will cause differentiation of the cells(47). The overexpression of this protein promotes tumorigenesis in different cell types (48, 49), including the cervical cancer. In TSCC cells, curcumin can have an action on OCT 4 expression only after long treatments at high concentrations. This shows that it acts on tumorigenesis but it does not act very effectively on OCT 4 pathway. Therefore, we should study how it acts on tumorigenesis more vigorously.

From these data, curcumin acts in vitro as a potent adjuvant agent in tongue cancer. However, its action should be further investigated in vivo. Moreover, curcumin is known for its poor bioavailability, problem that could be overcome through the local use of the drug or by nanoparticles that can carry turmeric directly into the tumor site (50).

4. Materials and Methods

4.1. Cell culture and treatment

The cell lines PE/CA-PJ15, PE/CA-PJ49, HSC-3 have been isolated from human Oral Squamous Cell Carcinoma of the tongue (TSCC). All lines were obtained from the European Collection of Cell Cultures (ECACC). Dulbecco's modified Eagle's medium with 4 500 mg/L glucose was used for PE/CA-PJ15 and PE/CA-PJ49 cells, while RPMI 1640 was used for HSC-3 cells. Both culture media were supplemented with 10% fetal bovine serum, L-glutamine (2 mM) and penicillin–streptomycin (100 U/ml). All cell lines were maintained at 37 ºC in a 5% CO2 humidified atmosphere. All these reagents were purchased from Sigma-Aldrich (Sigma Aldrich, MO, USA). Curcumin (Santa Cruz Biotechnology Inc, CA, USA) was prepared by dissolving it in dimethylsulfoxide (DMSO) at a stock concentration of 5 mM and serial dilutions 1, 5, 10, 20 and 50μM of curcumin were prepared in culture medium. The cell lines have been for 24, 48 and 72 hours at the concentrations indicated above.

4.2. xCELLigence System

The xCELLigence DP Real-Time Cell Analyzer (RTCA) is used for real-time monitoring of cell proliferation, cytotoxicity, and migration. This system is based on recording the electronic impedance with a unitless parameter called Cell Index (CI). The CI calculation is based on the following formula: CI = (Zi - Z0)/15çç where Zi is the impedance at the start of the experiment. Thus CI is a self-calibrated value derived from the ratio of measured impedances(29). When many cells are attached on the surface of a particular E-Plate the CI gets high. For this reason, we have used it to determine the variation of cell adhesion after curcumin treatments. The RTCA System was used accordingly to the manufacturer instructions.

4.2.1 Cell adhesion

The cell lines PE/CA-PJ15, PE/CA-PJ49, HSC-3 were washed with PBS and treated with 0.05% trypsin/EDTA (Sigma Aldrich, MO, USA). Cells were resuspended in media after 2 min and they were counted by using a hemocytometer. Then, $5X10^4$ cells per well were seeded in triplicate inside E-plate with 1, 5, 10, 20 and 50μM of curcumin. Cells without curcumin treatments were considered as negative control and they were treated with only DMSO as vehicle. Cells were monitored for 24, 48 and 72 h.

4.2.2 Cell Proliferation and IC50 values

Curcumin was added to cells after their adhesion phases previously estimated. The concentrations of curcumin are the same used to evaluate cell adhesion. IC50 values of curcumin treatments have been obtained using the software of xCELLigence RTCA DP system. It was expressed as the mean (M) ± S.E.M. (n=3).

4.3. MTT Assay

The cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (51) of Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). PE/CA-PJ15, PE/CA-PJ49, HSC-3 cells were treated in a 96-well plate with 1, 5, 10, 20 and 50μM of curcumin for 24, 48 and 72 h at a concentration of $5X\ 10^4$ cells in a total volume of 250 μl/well. At the end of each incubation period, the culture medium was removed and 10 μl of 12 mM MTT stock solution was added to 100 μL of fresh medium for each well and the cells were allowed to incubate for 4 hours at 37°C. Subsequently, 100 μL of the SDS-HCl solution (10 mL of 0.01

M HCl to 1 gr of SDS) were added to each well and then the plate was incubated at 37°C for 12 hours in a humidified chamber. Absorbance was read at 570 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

4.4. Trypan blue exclusion test of cell viability

In the trypan blue exclusion method 0.1 ml of the PE/CA-PJ15, PE/CA-PJ49, HSC-3 cell suspensions and 0.1 ml of 0.4% trypan blue solution were mixed (52). After 10 minutes, the cells were counted automatically with JuLI™ FL (NanoEntek, CA, USA), and all blue and damaged cells were counted as dead. The cell count was performed on all cells treated at different concentrations of curcumin (1, 5, 10, 20 and 50μM) and at three different time points (24, 48 and 72 h).

4.5. Fluorescence Cell Viability

ReadyProbes® Cell Viability Imaging Kit (Invitrogen, CA, USA) was used to determine the viability of cells. NucBlue® Live reagent stains the nuclei of all cells in blue while Propidium iodide stains in red the nuclei of only dead cells with compromised plasma membrane. The images were acquired with fluorescent microscope EVOS™ FL Cell Imaging System (Thermo Fisher Scientific, Massachusetts, USA). Fluorescence test was performed on all the cells treated at three time points and with the same concentrations of curcumin (1, 5, 10, 20 and 50μM).

4.6. Scratch assay

To study cell migration in vitro we used scratch assay. We seeded on 24-well culture plate a number of cells to obtain a confluent monolayer after 24 hours of incubation. With a p200 pipet tip we scraped the cell monolayer with a straight line to create a scratch. After, the cells have been washed twice with PBS to remove the debris. We have added to the cells used as control only their respective culture medium, while the other cells treated with IC50 dose of curcumin at 24 hours, previously calculated. We acquired the first image of the scratch as soon as it was made (T0) and the subsequent after 1 hour (T1), 2 hours (T2), 3 hours (T3), 5 hours (T4), 6 hours (T5) and 24 hours (T6) later. All scanned images were analyzed with software ImageJ to calculate the gap size of scratch. Statistical evaluation was done with software GraphPad Prism 7.

4.7. Western blotting analysis

Both control cells (not treated) and cells treated with 1, 5, 10, 20 and 50μM of curcumin concentrations were lysed after 24, 48 and 72 hours. The protein concentration was measured using the Bradford assay (Bio-Rad, Hercules, California, USA). The proteins were separated by 15% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, California, USA).

After blocking with 5% Bovine Serum Albumin (BSA) for 1 hour, the membranes were incubated with integrin β1 (1:5000; BD Biosciences), PAR 4 (1:200; Santa Cruz Biotechnology), OCT4 (1:700; Novus Biologicals), survivin (1:1000; Cell Signaling Technology), and β-actin (1:5000; Sigma Aldrich), overnight at 4°C. After incubation with corresponding suited 1:2500 horseradish peroxidase-conjugated secondary antibody (1:2500; Santa Cruz Biotechnology).

Signals were developed using the enhanced chemiluminescence kit (ClarityTM Western ECL Substrate, Bio-Rad) and the UVP ChemiDoc-It®TS2 Imaging System.

4.8. Statistical analysis

The unpaired Student's *t*-test and one-way ANOVA followed by Newman–Keuls tests were used to compare continuous variables. All the data are expressed as the mean ± standard error mean (SEM); a p value < 0.05, $p < 0.01$, $p < 0.001$, $p < 0.0001$ were accepted as statistically significant.

5. Conclusions

Curcumin is a nutraceutical agent with beneficial effects for human cells and many studies showed its effects against various tumors.

Our data showed that curcumin has potent effects on TSCC cells. Therefore, future in vivo studies are needed to validate these results.

In addition, we believe that curcumin can be easily applied in situ, since TSCC is localized in an easy access area, and this could increase the bioavailability of curcumin.

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Author Contributions: A.F. designed the paper, collected data, drafted the manuscript, drew the figures and approved the final manuscript as submitted. P.D. and T.G. done statistical analysis with the data collected. G.M. drafted the initial manuscript with T.N.F. and approved the final manuscript as submitted. L.M.L. coordinated and supervised data collection, critically reviewed the manuscript, and approved the final manuscript as submitted. All authors have read and approved the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CI Cell index
COX2 Cyclooxygenase-2
DMEM Dulbecco's modified Eagle's medium
DMSO Dimethylsulfoxide
ECACC European Collection of Cell Cultures
IC 50 The half maximal inhibitory concentration
LOX Lipoxigenase
MAPK Mitogen-activated protein kinases
NF-Kb Nuclear Factor Kappa-light-chain-enhancer of activated B cells
OSCC Oral Squamous Cell Carcinoma
PI3/Akt Fosfatidilinositolo 3 chinasi/Akt
PTK Protein Kinase
RTCA Real Time Cell Analyzer
TSCC Squamous cell carcinoma of the tongue

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Biological and therapeutic activities, and anticancer properties of curcumin (Review)

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Abstract. Curcumin (diferuloylmethane) is a polyphenol derived from the *Curcuma longa* plant. Curcumin has been used extensively in Ayurvedic medicine, as it is nontoxic and exhibits a variety of therapeutic properties, including antioxidant, analgesic, anti-inflammatory and antiseptic activities. Recently, certain studies have indicated that curcumin may exert anticancer effects in a variety of biological pathways involved in mutagenesis, apoptosis, tumorigenesis, cell cycle regulation and metastasis. The present study reviewed previous studies in the literature, which support the therapeutic activity of curcumin in cancer. In addition, the present study elucidated a number of the challenges concerning the use of curcumin as an adjuvant chemotherapeutic agent. All the studies reviewed herein suggest that curcumin is able to exert anti-inflammatory, antiplatelet, antioxidative, hepatoprotective and antitumor activities, particularly against cancers of the liver, skin, pancreas, prostate, ovary, lung and head neck, as well as having a positive effect in the treatment of arthritis.

Contents

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Key words: curcumin, nutraceutical, cancer, anti-inflammatory activity, anticancer agents

1. Introduction

Curcumin [1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene 3,5-dione] is among the most extensively studied naturally-derived therapeutic products in recent decades, due to its various properties. It is the primary biologically active curcuminoid of *Curcuma longa*, a herbaceous perennial plant belonging to the ginger family (Zingiberaceae) (1).

Curcuma longa, commonly known as turmeric, is native to South Asia, India and Indonesia and is predominantly grown in South India (2,3). The root and rhizome (underground stem) of *Curcuma longa* is crushed and powdered into ground turmeric. Ground turmeric is used worldwide as a seasoning and as a key ingredient in curry. Curry contains ~2% curcumin, which was first identified in 1910 by Miłobędzka *et al* (4). Furthermore, curcumin is responsible for the yellow color of the spice, in addition to the majority of the therapeutic effects attributed to turmeric (3,5). The other two curcumoids obtained from *Curcuma longa* are desmethoxycurcumin (DMC) and bis-desmethoxycurcumin (BDMC; Fig. 1). In addition, turmeric contains a number of volatile oils (e.g. zingiberone, atlantone and tumerone), sugars, resins and proteins. However, other than curcumin, turmeric contains no known agents with anti-inflammatory and anti-proliferative activity (6).

Following its extraction and purification, curcumin is used for its attributed medicinal properties as a natural treatment for numerous diseases. In Ayurvedic medicine, turmeric has been used for centuries for its medicinal properties (7) and has been administered through various routes, including topically, orally and by inhalation. It is well known that the curcumin exerts certain antioxidant, analgesic, anti-inflammatory and antimalarial properties (7-15).

Furthermore, curcumin is considered to be pharmacologically safe (9), and is classed as safe for human consumption by the US Food and Drug Administration (16). It is widely consumed as a condiment without any known side effects.

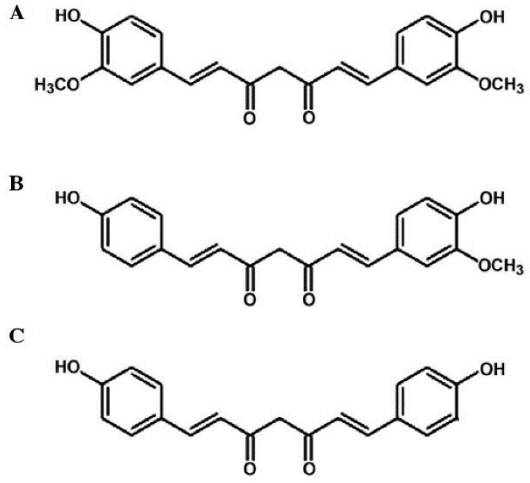

Figure 1. Primary extracts of the *Curcuma longa* plant. (A) Curcumin (diferuloloymethane), (B) demethoxycurcumin and (C) bisdemethyoxycurcumin.

2. Chemical composition

Curcumoids consist of two methoxylated phenols connected through two α,β-unsaturated carbonyl groups. Curcumin is rich in terpene derivates and contains predominantly monocyclic sesquiterpenes and oxygenated derivatives, such as turmerone and zingibrene (17). The rhizome contains 3-5% curcuminoids and 2-7% essential oil (18,19). Curcumin does not readily dissolve in water, whereas it is soluble in organic solvents, such as dimethyl sulfoxide, ethanol, methanol or acetone, and has a melting point of 183˚C. Curcumin presents a maximum spectrophotometric absorption of 430 nm in methanol and 415-420 nm in acetone, while a 1% solution of curcumin has 1,650 absorbance units (20).

3. Anti-inflammatory activity

Molecular studies have indicated that curcumin blocks the activation of factors or enzymes present in human cells able to trigger the inflammatory response. For instance, Surh *et al* revealed that curcumin is able to inhibit the activity and induced expression of cyclooxygenase-2 (COX-2) in various cell lines and animal models (21,22).

Topical application of curcumin inhibits the lipopolysaccharide (LPS)-mediated induction of COX-2 expression. This effect, rather than the catalytic inhibition of COX, may contribute towards the reduced formation of prostaglandin E_2 (PGE_2), while in macrophages not stimulated by LPS, curcumin increases the levels of COX-2 (23). Zhang *et al* observed that curcumin suppresses the expression of COX-2 protein and mRNA, in addition to TPA- or chenodeoxycholate-induced PGE_2 production (24). Furthermore, curcumin reduces the expression levels of COX-2 and PGE_2 synthase 1, which act on the PGE_2 formation, and prostaglandin, which serves a key function in inflammation and tumor development. Curcumin was also demonstrated to reversibly inhibit the conversion of prostaglandin H_2 (PGH_2) to PGE_2 by microsomal PGE_2 synthase 1 in A549 lung cancer cells stimulated with interleukin (IL)-1β, with a half maximal inhibitory concentration between 0.2 and 0.3 $\mu mol \cdot L^{-1}$ (25) In human whole blood stimulated with LPS, curcumin inhibits the formation of PGE_2 by COX-2 from arachidonic acid (AA), while the formation of 6-keto $PGF_{2\alpha}$ and 12 (1)-hydroxy-5-cis-8,10-transeptadecatrienoico by COX-1 is suppressed at markedly higher concentrations (26). A previous study indicated that the deletion of microsomal PGE_2 synthase 1 by curcumin is crucial to its anti-inflammatory and anticancer activities (26).

However, curcumoids exert a significant inhibitory effect on the peroxidase activity of COX-1, but not that of COX-2. In addition, curcumin and the curcumoids markedly inhibit the activity of 5-lipoxygenase (5-LOX), as curcumin interferes with the metabolism of AA by blocking cytosolic phospholipase A_2 phosphorylation, and thus reducing the expression of COX-2 and inhibiting the catalytic activities of 5-LOX. These activities may to explain the anti-inflammatory action of curcumin and the curcumoids in general (23).

The inhibitory effects of curcumin on proinflammatory gene expression may be associated with its inactivation of activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (27). *NF-κB* and *AP-1* are two transcription factor genes, which are overexpressed in cancer cells, and are crucial to the LPS-induced proinflammatory response. They control numerous cellular activities: NF-κB mediates immune activity, inflammation, collagenase and cell proliferation, while AP-1 mediates cell proliferation (3,28). By inhibiting NF-κB, curcumin results in the death of malignant cells and inhibits inflammation (29).

Furthermore, AP-1 is involved in the activation of the stress-activated c-Jun N-terminal kinase (JNK), which is markedly expressed in various types of tumors, including head and neck, cervical and breast cancers. Curcumin is able to inhibit AP-1 signaling that is induced by tumor promoters by interacting with the AP-1 DNA-binding motif, in addition to inhibiting JNK activation by carcinogens (30).

In addition, NF-κB and AP-1 mediate the expression of inducible nitric oxide synthase (iNOS), and thus the production of nitric oxide (NO), COX, prostaglandins and tumor necrosis factor (TNF)-α. These proinflammatory factors, in addition to the action of hypoxia through heterochromatin protein 1 (HF-1), AP-1 and various other co-factors, induce the expression of vascular endothelial growth factor (VEGF) and increase inflammation (31). VEGF may subsequently cause a metabolic cascade that leads to a degradation of the extracellular matrix (ECM), endothelial proliferation and ultimately angiogenesis (32,33). Activation of NF-κB is elevated in numerous types of cancer, and is associated with various steps in the development of malignancy. Curcumin may exert a scavenger action directly against free radicals, and modulate the signaling pathways controlled by NF-κB and mitogen-activated protein kinase (MAP). In addition, curcumin reduces the expression of matrix metalloproteinases (MMPs), adhesion molecules and growth factors that stimulate the expression of histone deacetylase oxidized abnormally in the lung. Biswas and Rahman (34) indicated that curcumin may be a potential antioxidant and anti-inflammatory agent against chronic inflammatory diseases of the lung. In addition, Yadav *et al* (35) demonstrated that curcumin significantly inhibited the production of the proinflammatory cytokine, IL-18, which induces severe inflammatory reactions.

Curcumin significantly inhibited the production of IL-18 in *Escherichia coli* LPS-stimulated murine macrophage-like RAW264.7 cells in a concentration-dependent manner, without exerting a cytotoxic effect (35).

4. Effect on arthritis

In Ayurvedic medicine, curcumin is used to treat inflammatory disorders, including arthritis, and is administered as a nutritional supplement. Funk *et al* (36) documented the effectiveness of an extract devoid of essential oils in preventing joint inflammation, while a hydroalcoholic extract of turmeric was demonstrated to inhibit joint inflammation and periarticular tissue destruction in a dose-dependent manner (37).

A recent study indicated that oral administration of curcumin was able to attenuate the neutrophil inflammatory response against zymosan-induced arthritis in rats (38). Furthermore, Panahi *et al* argued that curcuminoid treatment represents an effective and safe alternative treatment for osteoarthritis (39).

5. Antiplatelet activity

Curcumin inhibits the production of thromboxane (TX) by platelets *in vitro* and *ex vivo* and increases fibrinolysis. In addition, it inhibits platelet aggregation *in vitro* and *ex vivo* induced by ADP, collagen or norepinephrine (effective as aspirin) without decreasing the synthesis of prostacyclin in the epithelium aortic arch (the opposite of aspirin). With this differential action on prostacyclin, a progressive increase in doses of curcumin protects against collagen or norepinephrine-induced thrombosis (40).

The hydroalcoholic extract of turmeric inhibits platelet aggregation *in vitro* induced by AA instead of ADP or collagen, increases fibrinolysis, inhibits the production of TX by exogenous AA, and inhibits the release of AA. The cause of these actions may involve the inhibition of TX synthase. By contrast, the essential oil of turmeric does not exhibit any evident anti-platelet activity (41). In addition, Liu *et al* observed that the combination of curcumin and clopidogrel (antiplatelet family of thienopyridines) had no significant effect on the maximum platelet aggregation rate of rats compared with the use of clopidogrel alone (42).

6. Antioxidant activity

The antioxidant activities of curcuminoids are a result of their chemical composition. Curcumin inhibits lipid peroxidation using linoleate, a polyunsaturated fatty acid that is able to oxidize and form fatty acid radicals. Curcuminoids markedly reduce the hemolysis and lipid peroxidation of erythrocytes (lower vitamin E levels), and function as a scavenger of NO by blocking the enzyme that produces it, thus exerting a promoter activity (43). NO is a lipophilic molecule with a short half-life that is generated from L-arginine by various NADPH-dependent enzymes, known as NO synthases (44,45). NO is involved physiologically in vasorelaxation, neurotransmission, inhibition of platelet aggregation, immunity and intracellular signaling (44,45).

Additional studies have evaluated the potential use of curcumin in the prevention of neurodegenerative diseases, such as Alzheimer's disease. Oxidative damage and inflammatory processes are known to be particularly high in the brains of Alzheimer patients, and as the aqueous extract of turmeric reduces and prevents oxidation, it may be used concurrently in long-term therapy (46-49).

Motterlini *et al* confirmed that curcumin promotes the increased expression of heme oxygenase-1 (HO-1), an enzyme that catalyzes the degradation of heme and produces biliverdin, iron and carbon monoxide and exhibits cytoprotective activity against oxidative stress (50). Beneficial effects were also obtained using a mixture of curcuminoids that are commonly used as a nutritional supplement, particularly curcumin-95. The expression of HO-1 is increased in astrocytes treated with curcumin at 5-15 μM concentrations (51).

Recently, Miao *et al* described the beneficial effects of curcumin as an antioxidant and investigated the underlying mechanisms in diabetic rat model (52). In addition, Liu *et al* demonstrated that curcumin and resveratrol were able to regulate drug-metabolizing enzymes in addition to antioxidative enzymes during lung carcinogenesis in mice (53).

7. Hepatoprotective action

An *in vivo* study examined the protective effect of curcumin on the hepatotoxicity induced by carbon tetrachloride (CCl_4) (54). When administered at a dose of 3 ml/kg/day for three months, CCl_4 caused a marked increase in transaminases, alkaline phosphatase and plasma levels of γ-glutamyl transpeptidase thiobarbituric acid and lipoperoxide, and a reduction in plasma levels of glutathione, vitamins C and E. In addition, the liver and kidneys exhibited marked increases in thiobarbituric acid and lipoperoxide levels and an evident reduction in the levels of glutathione, vitamin C and vitamin E. The administration of curcumin with CCl_4 significantly reduced these phenomena in the plasma, kidneys and liver (54). In addition, the authors observed histological damage in these organs, with thickening of the portal vessels and the deposition of fat droplets in the vessel walls (54). An additional study indicated that curcumin prevents CCl_4-induced liver damage by inhibiting the activation of NF-κB and exerting an antioxidative effect (55).

A previous study demonstrated that curcumin does not alter the levels and activity of cytochrome P450 in the liver, except at high doses of ≥5 g/kg/day (56). Instead, CCl_4 caused a marked reduction in the activity of these isoenzymes, particularly of cytochrome P450 2E1 (CYP2E1), with the formation of high levels of free radicals. Pretreatment with curcumin at a dose of 0.5 g/kg/day significantly reduced the effects of CCl_4 on these isoenzymes, but not on CYP2E1, possibly due to its marked antioxidative activity. Furthermore, the study indicated that the protection of the liver guaranteed by curcumin against CCl_4-induced damage may depend, at least in part, on its contrasting effects against damage caused by CCl_4 to cytochrome P450 (56).

8. Anticancer activity

A number of activities of curcumin, which are exerted in a chemopreventive and a directly therapeutic manner, indicate that it may be a potential anticancer remedy. Although the results have been obtained in animal models, curcumin has been

demonstrated to be active in various other *in vitro* models, and the dosages are comparable to those used in humans. *In vitro* and *in vivo* studies have indicated that curcumin prevents carcinogenesis by affecting two primary processes: Angiogenesis and tumor growth (57).

Turmeric and curcuminoids influence tumor angiogenesis through multiple, interdependent processes (58): i) Action at the level of transcription factors NF-κB, AP-1 (associated with inflammatory processes) and early growth response protein 1, which attenuates the expression of IL-8 in pancreatic and head and neck cancer cell lines and prevents the induction of VEGF synthesis; ii) inhibition of angiogenesis mediated by NO and iNOS; iii) inhibition of COX-2 and 5-LOX; iv) action at the level of angiogenic factors: VEGF, the primary factor for migration, sprouting, survival and proliferation during angiogenesis, and basic fibroblast growth factor; and v) action at the level of stability and coherence of the ECM, including the downregulation of MMP-2 and MMP-9, and upregulation of tissue inhibitor of metalloproteinase-1. Turmeric also interferes with the release of angiogenic factors stored in the ECM (58).

Curcumin induces cell death in numerous animal and human cell lines, including leukemia, melanoma, and carcinomas of the breast, lung, colon, kidney, ovaries and liver (59). It appears to function by caspase-dependent and independent (mitochondrial) mechanisms, which are associated with the presence and absence of p53. Certain data have demonstrated that curcumin exhibits a biphasic action, which acts on the proteasome, with an activation at lower doses and with inhibition at higher doses. As the inhibition of the proteasome leads to apoptosis, and its stimulation leads to cell survival, it is possible that curcumin results in apoptosis or survival depending on the dosage used. In addition, turmeric at different doses may also affect the type of cell death: Low doses lead to oxidative stress and apoptosis, while higher doses lead to reduced production of reactive oxygen species, reduction of ATP and necrotic cell death (60).

Curcumin also appears to be able to cause cell death in various cell lines resistant to apoptosis, possibly by activating cell death mechanisms other than apoptosis, such as mitotic catastrophe that is characterized by aberrant mitosis, and the formation of multinucleated and giant cells. The mitotic catastrophe caused by curcumin is linked to the reduction of gene expression of various apoptotic inhibitor proteins, in particular survivin (61).

Furthermore, a previous study demonstrated that curcumin administration was able to significantly reduce the levels of the cell cycle regulators CDK4 and cylin D1, and inhibit the expression of p53, which is an upstream regulator of the CDK4-cylin D1 complex (62). Recently, Vallianou *et al* (63) discussed the ability of curcumin to cause apoptosis in tumor cells by inducing severe endoplasmic reticulum stress, which serves a crucial function in the apoptotic process. The study suggested that curcumin may act by suppressing specific protein 1 activation and consequently was unable to prevent cancer formation, migration and invasion (63).

9. Liver cancer

Curcumin has been demonstrated to impede the formation of hepatic hyperplastic nodules, hypoproteinemia and body weight loss in Wistar rats (64). In an animal experiment, N-nitrosodimethylamine (DENA), a powerful hepatocarcinogen, was injected intraperitoneally in five-week-old C3H/HeN mice (64). One group of mice received a diet containing 0.2% curcumin, from 4 days prior to DENA injection until the end of the study. At the age of 42 weeks, the curcumin group exhibited an 81% reduction in the multiplicity and a 62% reduction in the incidence of hepatocarcinoma compared with the non-treated group (64). Busquets *et al* studied the chemopreventive potential of curcumin in rats that were inoculated with Yoshida AH-130 ascites hepatoma, a fast-growing tumor that results in fatality in ~10 days after inoculation (65). Curcumin significantly decreased tumor growth by 31% (21).

10. Skin carcinogenesis

Topical application of curcumin combined with the tumor promoter TPA, twice per week for 20 weeks, to female CD-1 mice markedly inhibited papilloma formation (66). In an additional study, topical application of relatively low doses of curcumin (20 or 100 nmol) markedly abrogated TPA-induced tumor promotion. Topical application of commercial-grade curcumin (containing ~77% curcumin, 17% demethoxycurcumin and 3% bis-demethoxycurcumin), pure curcumin or demethoxycurcumin exhibited almost equipotent inhibitory effects on TPA-induced tumor promotion in DMBA-initiated mouse skin carcinogenesis. Furthermore, in female Swiss mice dietary administration of 2% turmeric significantly inhibited DMBA and TPA-induced skin tumor formation. In a benzo[a]pyrene-initiated and TPA-promoted two-stage skin tumorigenesis model, curcumin reduced the number of tumors per mouse and decreased the number of tumor-bearing mice. In further studies, Huang *et al* demonstrated that curcumin inhibited UV-induced dermatitis in mouse skin (67-69).

Jiang *et al* demonstrated that curcumin is able to induce apoptosis and inhibit the proliferation of melanoma cells (70). In addition, curcumin treatment altered the expression levels of the apoptosis-associated proteins, NF-κB, p38 and p53 (70).

11. Pancreatic cancer

In a xenograft model study, pancreatic cancer cells were injected subcutaneously into the side of the abdomen of female nude mice (71). Subsequently, liposomal curcumin was injected into these animals. This treatment reduced tumor size and decreased the expression of CD31 in addition to that of VEGF and IL-8, indicating that curcumin suppressed pancreatic carcinoma growth in murine xenograft models and inhibited tumor angiogenesis (71).

Bao *et al* demonstrated that the administration of difluorinated-curcumin (CDF) inhibited tumor growth in a manner associated with the reduced expression levels of EZH2, Notch-1, CD44, EpCAM and NANOG and increased expression levels of let-7, miR-26a and miR-101, which are typically not expressed in pancreatic cancer (72).

Furthermore, Ali *et al* demonstrated that the administration of CDF induced the re-expression of let-7, resulting in decreased tumor growth and Ras gene expression in pancreatic cancer cells (73).

12. Prostate cancer

Androgen-dependent LNCaP prostate cancer cells were injected subcutaneously into mice fed with a 2% curcumin containing diet for up to 6 weeks (74). Curcumin significantly increased the extent of apoptosis, as measured by an *in situ* cell death assay, and caused a reduction in cell proliferation, as measured by a BrdU incorporation assay (74). In addition, curcumin has been observed to induce a marked reduction in MMP-2 and MMP-9 activity in tumor-bearing sites. A previous study demonstrated significantly fewer metastatic nodules in a curcumin-treated group compared with the untreated group (75).

Another study employed a xenograft prostate cancer model to evaluate the anti-tumor, radiosensitizing and chemosensitizing effect of curcumin (76). Prostate cancer cells were injected into the left inguinal area of nude mice, and curcumin was administered by gavage, while gemcitabine was introduced by intraperitoneal injection. Reduced expression of the *Mdm2* oncogene was detected in xenografts treated with curcumin alone, in addition to those treated with combinations of curcumin and gemcitabine or irradiation (76). Furthermore, researchers have demonstrated that GO-Y030, a curcumin analogue, reduced the expression of Bcl-XL in prostate cancer (77).

13. Ovarian cancer

In order to evaluate the effect of curcumin against ovarian cancer, a group of animals were treated with curcumin alone or in combination with docetaxel (78). Curcumin alone induced a 49-55% reduction in mean tumor growth compared with control animals, while the combination of curcumin with docetaxel resulted in a 77% reduction in mean tumor growth compared with the controls. In both cases, curcumin induced a decrease of proliferation and microvessel density and a significant increase in tumor cell apoptosis (78). In a recent *in vitro* study, the authors showed that the combination of curcumin and triptolide was able to synergistically inhibit ovarian cancer cell growth (79).

14. Lung cancer

In an animal study the administration of curcumin decreased the number of lung tumor nodules and inhibited lung metastasis of melanoma (78). Therefore, it is possible to use curcumin in order to arrest the metastatic growth of tumor cells. In addition, exposure of lung cells to curcumin was demonstrated to inhibit cigarette smoke-induced NF-κB activation, which correlated with the suppression of CS-induced cyclin D1, COX-2 and MMP-9 expression (78). Yang *et al* (80) observed that curcumin is able to inhibit cell proliferation, modifying the expression of proliferative and anti-proliferative proteins (survivin, Bcl-XL and cyclin B1), cell cycle, migration and invasion, downregulating the invasive proteins VEGF, MMP-2, MMP-7 and intercellular adhesion molecule-1. Furthermore, curcumin appeared to reduce angiogenesis through suppression of the STAT3 signaling pathway in small cell lung cancer (80).

15. Head and neck cancer

Curcumin administration (0.5 g/kg) in male F344 rats caused a 91% reduction in the frequency of 4-nitroquinoline 1-oxide-induced tongue carcinoma, with a marked reduction in the incidence of oral preneoplastic lesions (81). Azuine *et al* (82) used a Syrian golden hamster model to demonstrate that curcumin, alone or in combination with catechin, inhibited methyl(acetoxymethyl)nitrosamine-induced oral mucosal tumors. In addition, treatment with 10 mmol curcumin caused reductions in the visible oral papillomas and papilloma volume of 39.6 and 61.3%, respectively (82). Furthermore, treatment with curcumin caused a reduction in the incidence of oral squamous cell carcinoma (SCC), and the number of oral SCC lesions decreased by 51.3% (30). Following curcumin treatment, a reduction of the tumor proliferation index in hyperplasia, dysplasia and papilloma was observed (83).

Chakravarti *et al* indicated that curcumin is able to suppress the growth of immortalized oral mucosal epithelial cells and squamous cell carcinoma cells, while exerting minimal effects on normal oral epithelial cells (84). In SAS oral cancer cells, curcumin induced the promoter activity of insulin-like growth factor binding protein-5 and CCAAT/enhancer-binding protein α, which are involved in the suppression of head and neck cancer. Curcumin exerted an inhibitory effect on these factors through the activation of p38, and resulted in decreased *in vivo* tumorigenesis in a mouse xenograft model (85). The activity of curcumin has been investigated in a number of head and neck SCC (HNSCC) cell lines, including CAL27, CCL23 (laryngeal), UM-SCC1 and UMSCC14A (oral) (86). The growth suppression effect was represented primarily by the effect of curcumin on the NF-κB signaling pathway. Curcumin caused a reduction in the expression of NF-κB and, in addition, inhibited its nuclear localization. The activity of curcumin on the NF-κB in this type of tumor is due to inhibition of IκB kinase (IKK), thus blocking the phosphorylation of IκB-α and resulting in NF-κB sequestration in the cytoplasm (87). It has been demonstrated that the inhibition of IKK occurs via an AKT-independent mechanism (87). AKT, also known as protein kinase B, is involved in signal transduction from oncogenes and growth factors. The effects of curcumin on the AKT signaling pathway are various: In certain tumors, including malignant gliomas and pancreatic cancer, curcumin suppresses the AKT signaling pathway, whereas in HNSCCs and melanoma, it has been demonstrated that curcumin functions independent of AKT (88). The AKT signaling cascade is stimulated by epidermal growth factor receptor (EGFR) and represents one pathway by which NF-κB may be activated (89). EGFR is overexpressed in numerous types of head and neck cancer, and molecular therapies targeting the EGFR/AKT signaling cascade the therapeutic efficacy of standard platinum-based chemotherapy (90). In addition, the expression levels of multiple NF-κB-regulated gene products, including IL-6, IL-8, MMP-9, COX-2, CCL2 and Bcl-XL, were reduced (86,91-95).

Additionally, in a study using a mouse model of SCC-1 tumors, curcumin was shown to decrease COX-2 expression and inhibit EGFR phosphorylation (96). In other types of tumors, including prostate, colorectal and ovarian, the use of curcumin as a radiosensitizer has been supported in prostate, colorectal and ovarian cancers, in addition to HNSCC (97,98).

Several studies have demonstrated the potential use of curcumin as an adjuvant compound in combination with standard platinum-based chemotherapy for the treatment of head and neck tumors (96). In particular, a basic component of

curcumin, known as FLL32, is able to increase the effectiveness of this type of chemotherapy-regressing tumor cells by inhibiting STAT3 phosphorylation, reducing survival signaling, and increasing susceptibility to apoptosis and sensitization to cisplatin (99).

The potential anti-cancer properties of curcumin have been widely investigated; however, the spice it contained is not able to be absorbed by the body. By contrast, FLL32 is easily assimilated and able to sensitize cancer cell lines that are resistant to platinum-based chemotherapy. In order to eliminate cancer cells that are resistant to cisplatin, it may be necessary to increase the dose of chemotherapy, which entails increased risk. The use of FLL32 to sensitize such cells may reduce the required dose of cisplatin, and therapy may therefore be conducted with reduced toxicity and potential damage to the body (99).

Numerous studies have demonstrated that, *in vivo*, curcumin exerts growth suppressive effects, using nude mouse xenograft models (81,82,100,101). Clark *et al* observed an inhibited tumor growth in mice, via the inhibition of the AKT/MTOR pathway, following treatment with an oral curcumin solution prior to inoculation of SCC40 tongue SCC cells (102). In addition, Chang *et al* demonstrated the suppression of oral carcinogenesis in mice xenografts (85). Furthermore, Kumar *et al* (103) developed a novel class of curcumin analogs (H-4073), based on diarylidenylpiperidones (DAP), incorporating a piperidone link to the β-diketone structure and fluoro-substitutions on the phenyl groups. These authors demonstrated the potent anti-tumor effects of H-4073, a parafluorinated variant of DAP, using *in vitro* and *in vivo* head and neck cancer models (103).

Curcumin may also have a potential application as an enhancer of radiation therapy. Rao *et al* (104) compared the effects of curcumin and single-dose radiation alone and in combination in the HNSCC cell lines SCC-1, SCC-9, A431 and KB. The results demonstrated that curcumin inhibited HNSCC cell growth and augmented the effect of radiation *in vitro* and *in vivo*. The underlying mechanism may have involved the inhibition of COX-2 expression and EGFR phosphorylation.

In a recent study *in vitro*, the authors confirmed that curcumin used in combination with AG490, a JAK-2 inhibitor, reduced the expression of JAK-2/STAT-3 in laryngeal squamous cell carcinoma. In particular, the expression of JAK-2, p-STAT3, MMP-2 and VEGF at the protein levels were decreased ($P<0.01$) (105). Another study hypothesized that H-4073, an analog of curcumin, may be useful as an anticancer agent for mitigating resistance to chemotherapy in patients with HNSCCs (103).

16. Conclusion

Curcumin (diferuloylmethane) is a polyphenol derived from the *Curcuma longa* plant that has numerous therapeutic properties, including antioxidative, analgesic, anti-inflammatory and antiseptic activities. Recently, a number of studies have indicated the anticancer activities of curcumin by investigating its effect on a variety of biological pathways involved in mutagenesis, apoptosis, tumorigenesis, cell cycle regulation and metastasis.

The results reviewed in the current study indicated that curcumin may exert positive effects against various types of tumor. Notably, combination of curcumin with other nutraceuticals, such as resveratrol, have been used to combat the mechanism underlying tumorigenesis, and the prevalence of studies have employed curcumin analogues as effective potential treatments.

Therefore, further *in vivo* studies elucidating the mechanisms underlying the effects of this nutraceutical may be useful in the treatment of tumors and elimination of the use of cancer treatments that have known side effects.

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Conservative vs Radical Approach for the Treatment of Solid/Multicystic Ameloblastoma: A Systematic Review and Meta-analysis of the Last Decade

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Purpose: To examine whether a difference exists in the relapse rate between the conservative and radical approaches after the treatment of solid/multicystic ameloblastoma (SMA), a systematic review of the literature based on evidence of the last decade was performed.

Materials and Methods: The search strategy incorporated examinations of electronic databases, supplemented by hand searches. A search of four electronic databases, including Ovid MEDLINE, PubMed, EMBASE and Web of Science, was carried out for relevant studies published in the English language from January 2005 to September 2015. Cross referencing and hand research was used to identify further articles. Relative Risk (RR) as effect estimates was calculated in both fixed and random effects models.

Results: Of 4234 abstracts screened, only 26 articles met the inclusion criteria and were screened in full text. Of these, only 4 were included in the final meta-analysis.

Conclusion: The inverse of variance test revealed a statistical difference in the relapse rate for SMA treatment with the conservative vs radical approach. The higher recurrence rate after a conservative approach compared to the surgical approach is significant. However, this review cannot give any recommendation due to the lack of clinical evidence.

Key words: ameloblastoma, ameloblastoma surgical treatment, conservative approach, odontogenic tumour, radical approach, solid multicystic ameloblastoma

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Ameloblastoma is the second most common odontogenic tumour arising in the maxillary bones; it accounts for 1% of all oral neoplasms, and about 15% of all odontogenic tumours.[20] The World Health Organization (WHO) classified benign ameloblastoma into four different variants: 1. solid/multicystic (SMA); 2. unicystic (UCA); 3. peripheral; 4. desmoplastic. The rare malignant forms are classified as: 1. metastasising ameloblastoma; 2. ameloblastic carcinoma.[2] Ameloblastoma seems to originate in the degeneration of the tooth-forming apparatus, such as: the enamel organ, reduced enamel epithelium odontogenic remains, and the epithelial lining of odontogenic cysts.[11] This pathology is more frequent in the mandible (ratio mandible:maxilla 5:1) and in the third decade of life. The average age of the patients at the time of diagnosis of solid/multicystic ameloblastomas is nearly 39 years. However, a peak can also be seen at the ages of 10–19 years.[16] The WHO describes the age span between 30 and 60 years as the one with most diagnoses. To date, no difference in gender predilection has been demonstrated.[15] SMA is the most common subtype and accounts for about 80% of benign cases, and it is often diagnosed following a routine radiographic examination.[6] In fact, in the majority of cases it is totally asymptomatic. Local symptoms such as pain, swelling, malocclusion and pares-

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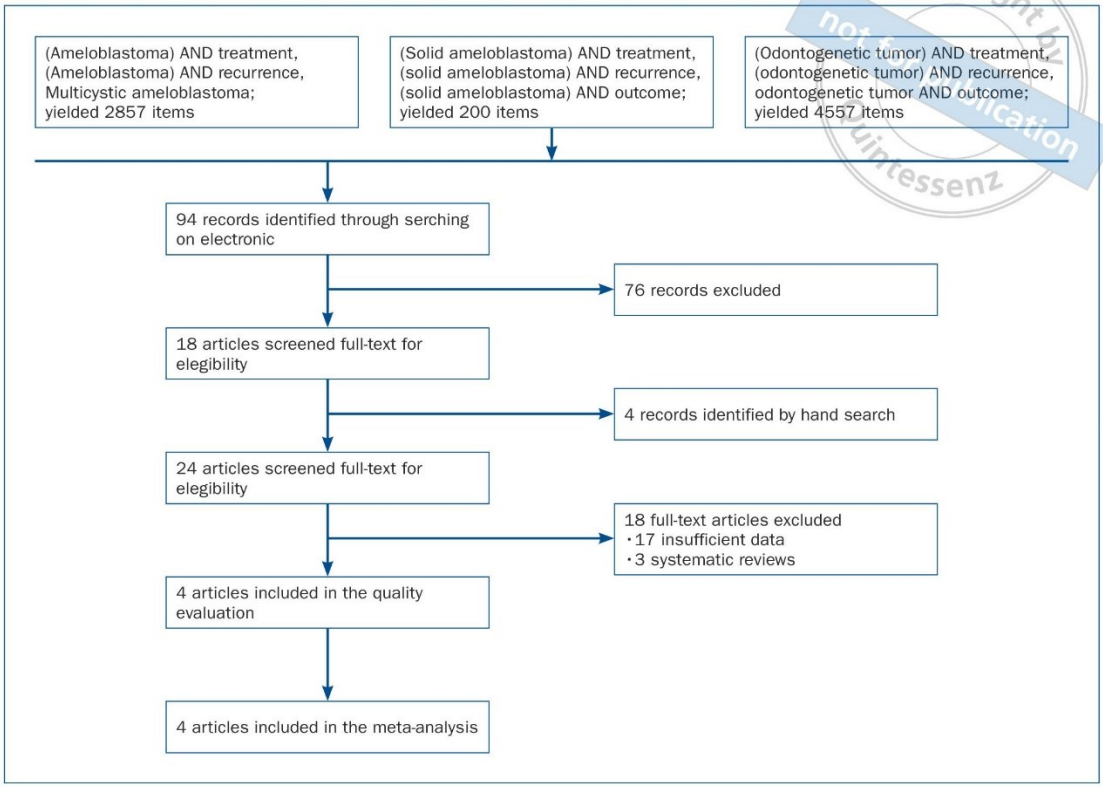

Fig 1 Flow chart of the different phases of the systematic review.

thesia may be associated with large lesions.[4] Radiographically, SMA appears as a uni- or multilocular lesion often found in contact with the roots of adjacent teeth.[17] Because of its great infiltrative potential, SMA is characterised by a higher rate of recurrence.[18] Currently, surgery represents the best therapeutic option for this odontogenic lesion.[21] A conservative or radical approach may be performed for SMA treatment to achieve total excision of the lesion.[19]

The aim of the present study was to highlight the recurrence rate after SMA treatment performed with different surgical modalities. To examine whether there is a different relapse rate after treatment with a conservative or radical approach, a systematic review of randomised and observational studies of articles published in the last decade was performed.

MATERIALS AND METHODS

Protocol and Inclusion Criteria

This systematic review was carried out according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (the PRISMA Statement)[12] and the Cochrane Handbook.[10] Although these schedules are indicated for the revision of prospective randomised clinical studies (PRCs), this review also included retrospective non-randomised studies because of the absence of PRCs for ameloblastoma. This paper followed the recommendations of the MOOSE group (Meta-analysis of Observational Studies in Epidemiology) for the execution of this study.[22] The review question was formulated by following the PICOS (patient population, intervention, comparison, outcome and study design) framework:[13] patient/ population, patients suffering for SMA; intervention, surgical excision of SMA; comparison, conservative and radical approach; outcome: recurrence rate after the different approaches, and study design: both randomised and non-randomised studies. The inclusion criteria were:

- Studies published from January 2005 to September 2015
- Only English-language articles
- Diagnosis of SMA obtained after histological examination
- Recurrence rate reported for SMA subtype
- At least 10 cases of SMA treated with the conservative or radical approach present in the study
- No case reports or letters to the editor.

Table 1 Reason for the exclusion of studies read in full text for eligibility

Study	Reason for rejection
Krishnapillai et al (2010)	No data about treament modality
Hertog et al (2010)	No data for solid/multycystic subtype
Siar et al (2012)	Data available only for treatment of relapses
Filizzola et al (2014)	No data about treatment modality
Adeline et al (2008)	No data about treatment modality
Sammartino et al (2007)	No data for solid/multycystic subtype
Franca et al (2012)	No data for solid/multycystic subtype
Carneiro et al (2014)	No surgical modality
Hertog et al (2011)	No data about treatment modality
Bianchi et al (2013)	No data about relapse for the solid/multicystic subtype
Ooi et al (2014)	No data about recurrence rate
Antonoglou et al (2015)	Systematic review including papers published before 2006
Eckardt et al (2009)	Review read to search further data
Yongsa et al (2013)	No data for solid/multicystic subtype
Huang et al (2007)	Less than 10 SMA treated
Hammarfsord et al (2013)	No data for solid/multycystic subtype
Bansal et al (2015)	Systematic review including papers published before 2006
Butt et al (2012)	No data for solid/multycystic subtype
Hertog et al (2012)	Insufficient data
Hasegawa et al (2013)	Insufficient data

All the included studies included were retrospective. For this reason, studies that did not clearly document a follow-up period were not considered. A focused question was then formulated: 'Is there a difference in the recurrence rate after surgical treatment of SMA using the conservative vs radical approach?'

Information Sources, Search and Study Selection

Searching electronic databases about recurrence rate after SMA treatment identified the studies. The electronic databases screened for the present research were MEDLINE, Web of Science Core Collection, EBSCO library and Cochrane Database of Systematic Reviews, using the combination of the following key words: ameloblastoma, solid ameloblastoma, multicystic ameloblastoma, treatment, relapse and outcomes. In addition, hand searches from the reference list of other systematic reviews were performed to find missed articles eligible for inclusion in our study.

Study Selection, Data Collection Process and Data Items

Two unblinded reviewers carried out the study selection process in an independent manner. Disagreements between reviewers about the studies included were resolved by discussion. In the first round, the authors excluded studies that did not focus on treatment modality and relapse for SMA by screening the titles and abstracts of the search results.[3] Only 26 articles met the inclusion criteria (23 from electronic search and 3 from hand search) and were read in full text. In the second screening, the authors excluded studies which did not meet the criteria regarding participants, intervention characteristics, comparisons, outcome measures and study design (PICOS). From this search, only 6 studies were included in the quantitative analysis for systematic review (Fig 1). The reasons for the exclusion of the other articles are given in Table 1. Some studies were excluded because data were not clear or because the full text was not available. The main reason for exclusion was the inability to extract data regarding the recurrence rate for the solid/multicystic subtype. Because this review focuses on the recurrence rate after surgical treatment, data regarding cryotherapy or Carnoy solutions added to a conservative approach were excluded. For comparison, treatment modalities considered conservative include: curettage, enucleation, marsupialisation/decompression alone or followed by

Table 2 Assessment of the risk of bias within the studies

Study	Adequate sequence generation	Allocation concealment	Blinding of personnel and partipants	Blinding of examiners	Incomplete outcome data	Selective reporting	Other bias
Fregnani et al (2008)	Yes	Unclear	No blinding	No blinding	No missing data	No selective reporting	No
Darshani et al (2010)	Yes	Unclear	No blinding	No blinding	No missing data	No selective reporting	No
Singh et al (2015)	Yes	Unclear	No blinding	No blinding	No missing data	No selective reporting	No
Zhang et al (2010)	Yes	Unclear	No blinding	No blinding	No missing data	No selective reporting	No

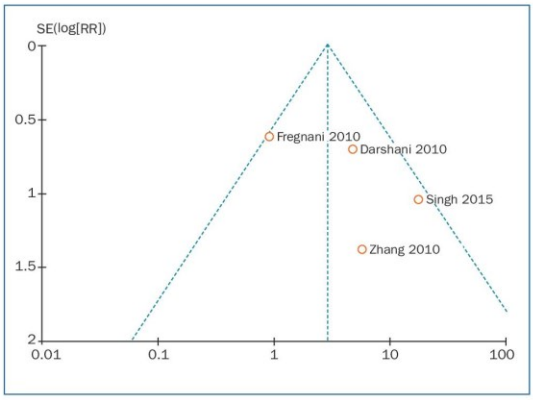

Fig 2 Funnel plot of the studies reporting the relative risk of ameloblastoma treatment.

enucleation, or enucleation with peripheral ostectomy. Radical surgery was defined as the procedure in which the ameloblastoma was resected, with a safety margin of at least 1 cm of normal bone, with or without a continuity defect.[14] The radical approach group includes: enucleation with peripheral ostectomy and/or resection, segmental or marginal resection, hemimandibulectomy and segmental resection of the mandible. Data extraction was carried out independently by two reviewers (LL and AC) regarding PICOS. They collected year of publication, number of patients, treatment modality, number of relapses and study design.

Risk of Bias in the Individual Studies and Across Studies

The analysis of the risk of bias of the included studies was conducted using the Cochrane Collaboration tool. The analysis of each study was based on six criteria: appropriate sequence generation, concealment of the allocation sequence, blinding of participants, incomplete outcome data, selective outcome reporting, and other sources of bias (Table 2).

The risk of bias across studies was evaluated graphically. The I^2 statistic was used to express the percentage of heterogeneity across the studies, and confirmed by a chi-squared test. A funnel plot of the Relative Risk (RR) among the studies was evaluated to assess the publication bias.

Summary and Synthesis of Results

Only four studies reported a direct comparison between conservative and radical approaches and were included in the meta-analysis. Data were collected as RR with a 95% confidence interval. The Higgins Index[9] was used to assess the heterogeneity and classified as follow: <30% low heterogeneity, 30%–60% medium heterogeneity, > 60% high heterogeneity. The inverse of variance test was used both for fixed and random effects models. Data were analysed and summarised in a forest plot. All the statistical analyses have been carried out using the software Review Manager version 5.2.8 (2014, Cochrane Collaboration; Copenhagen, Denmark).

RESULTS

Study Selection and Characteristics

A total of 6 studies were identified for inclusion in this review. Of these, 2 studies[4,8] presented data only for the conservative group and were not included in the meta-analysis, while the remaining 4 studies[5,7,19,23] exhibited a direct comparison of the two methods and were included in meta-analysis. The search of electronic databases provided 4234 abstracts, which were read by two independent reviewers. Only 26 articles met the inclusion criteria (23 from electronic database and 3 from the hand search) and were read in full text. Twenty articles did not meet the inclusion criteria for data extraction and were not included in the subsequent evaluation. The reasons for exclusion are summarised in Table 1. All the included studies were non-randomised ob-

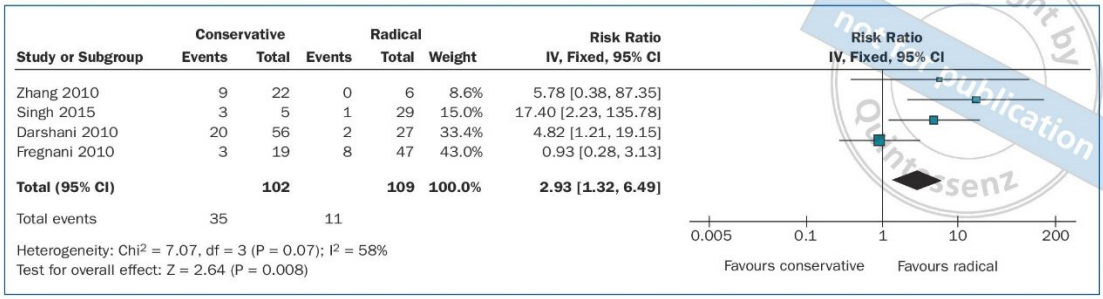

Fig 3 Forest plot of fixed effects models of the meta-analysis.

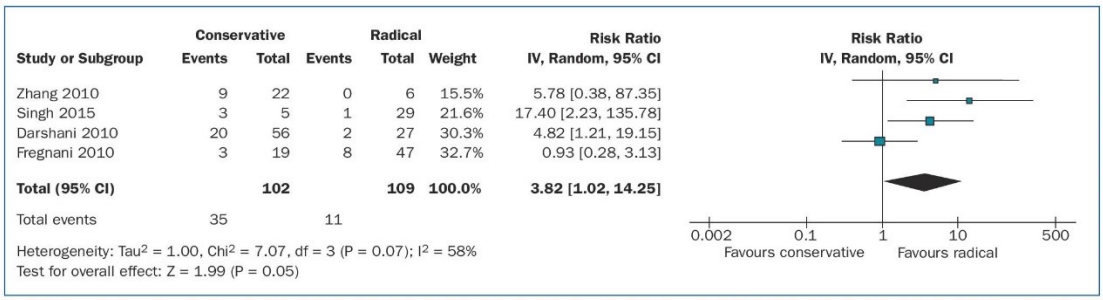

Fig 4 Forest plot of random effects models of the meta-analysis.

servational retrospective cohorts published in English. For each study, a blinded examiner (LL) extracted data about treatment modality and recurrence rate. The included studies involved 256 participants, of which 147 were treated with a conservative approach and 109 with a radical approach. The recurrence rate was 40% (59/147) for the conservative and 10% (11/109) for the radical treatment.

Risk of Bias Within and Across Studies

Because the studies included in this review are retrospective non-randomised clinical studies, the risk of bias within studies was considered high. In fact, it is difficult in the review of a single-centre retrospective study to gain information about concealment and randomisation. However, it is likely that more surgeons perform interventions in a single institution, but it is difficult to recognise this in the studies. Risk of bias across studies was graphically evaluated with a funnel plot (Fig 2). It was estimated as medium because, although there is asymmetry in the graph, all the included studies fall within the area of the triangle.

Synthesis of Results

Four studies were included in the meta-analysis because they reported about recurrence after either conservative or radical treatments. The heterogeneity within the studies was not significant ($\chi^2 = 7.07$, df = 3 (p = 0.07); $I^2 = 58\%$), although the Higgins Index showed intermediate results. The inverse of variance test revealed a statistically significant difference in the possibility of relapse (p = 0.008). Because the Higgins Index was 58%, both fixed (Fig 3) and random effects models were performed. Both tests revealed a favourable result with the use of a radical approach for the treatment of solid/multicystic ameloblastoma. Among the included studies, three argue strongly in favour of the radical approach, while only one did not detect differences between the two groups.

DISCUSSION

Six studies that included treatment modality and relapse rate after a surgical treatment for SMA were included in this systematic review. Of these, only four reported about a direct comparison between conservative and radical approaches and were included in the subsequent meta-analysis. The Relative Risk (RR) was evaluated for each study and a direct comparison with the inverse of variance test was performed. All the included studies were retrospective and nothing was reported about randomisation. For these reasons, the risk of bias was considered to be medium/high. Because the Higgins Index ($I^2 = 58\%$) was evaluated as medium, it was decided to perform the evaluation both for fixed and random effects models. Both models demonstrated a statistically significant difference between the

conservative and radical approach ($p < 0.05$). Thus, this systematic review, based on the literature of the last ten years, revealed a lower possibility of recurrence when the radical approach was performed. Due to the level of risk of bias and characteristics of included studies, the results of this meta-analysis should be interpreted with caution.

However, these data are in accordance with other systematic reviews from the same field. Antonoglou and Sandor[1] performed a systematic review on the treatment of both unicystic and multicystic forms, and the results were also in favour of the radical approach. Although the studies included in their systematic review were all prior to 2006, the results are very similar with regard to the SMA subtype. They also noted that the follow-up period was not reported in many studies, and the same problems in the most recent literature analysis were found. In addition, numerous studies reported outcomes not classified for histologic subtype, and were thus unavailable for this systematic research.

CONCLUSIONS

In this systematic research of observational studies, a lower possibility of recurrence after the treatment of solid/multicystic ameloblastoma was found when a radical approach was performed. However, studies published in the recent literature are not well organised and the strength of evidence is very poor. Randomised clinical trials should be conducted based on treatment modality and histological subtypes to determine the best surgical approach for the ameloblastoma treatment.

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ORIGINAL ARTICLE

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Patients with high c-MYC-expressing squamous cell carcinomas of the tongue show better survival than those with low- and medium-expressing tumours

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Background: c-MYC is a potent oncoprotein with roles in a wide range of cellular processes such as differentiation, apoptosis and growth control. Deregulation of the *MYC* gene is commonly seen in human tumours resulting in overexpression of the protein. Here we studied expression of c-MYC in correlation to clinical outcome in patients with primary squamous cell carcinoma of the mobile tongue.

Methods: Immunohistochemistry was used to identify c-MYC in a group of 104 tongue squamous cell carcinomas with an antibody directed against the N-terminal part of the protein. Staining was evaluated by multiplying the percentage of c-MYC-expressing cells with staining intensity, giving a quick score for each tumour.

Results: All 104 tumours expressed c-MYC at varying levels. Quantitation according to per cent of positive cells and staining intensity revealed that most (15/21; 71%) high-expressing tumours were seen in males. Within the group of high c-MYC-expressing tumours, the majority were alive 2 and 5 years after treatment.

Conclusions: The present findings show that expression of c-MYC has prognostic value in squamous cell carcinoma of the tongue, and could be useful in choice of therapy.

KEYWORDS
c-MYC, radiotherapy, squamous cell carcinoma, tongue

1 | INTRODUCTION

One of the most studied oncoproteins in human tumours is c-MYC which belongs to a family of nuclear DNA-binding proteins.[1] There are five members in the family, of which three have neoplastic potential (reviewed in ref. 2). c-MYC is encoded by the *MYC* gene and may regulate up to 10-15% of all genes, but at a weak level making it difficult to understand its role in cancer development.[3] c-MYC acts as a transcription factor with roles in differentiation, apoptosis and growth control[4] and its expression is high in rapidly

proliferating cells whereas it is low or absent at quiescence.[5] *MYC* mRNA and c-MYC protein are both short lived, and the half-life of the protein after post-translational modification, ubiquitination and degradation is 15-20 minutes (reviewed in ref. 6). In transcription and repression of its target genes c-MYC dimerises with its partner, MYC-associated protein X (MAX), through the helix loop helix/leucine zipper (HLH/LZ) region in the C-terminal part of c-MYC. Via the c-MYC N-terminus, the complex binds to specific E-boxes in the promoters of target genes,[6] including the cell cycle related genes *CCND2*, *CCNE1* and *CDK4*.[7] c-MYC is important also in apoptosis, as it has the ability to induce the apoptotic process or sensitise cells to apoptosis.[4] *MYC* is deregulated in many human tumours by chromosomal translocation, amplification, activation of upstream growth stimulatory pathways, or there may be an increase in c-MYC protein stability.[4,8] *MYC* is also deregulated in oral squamous cell carcinoma (OSCC) and higher expression has been reported with advanced stage and advanced degree of dysplasia.[1]

Within the group of OSCC there are several subgroups of tumours based on location, and we and others have shown tongue SCC (TSCC) to be a specific group with different behaviour from other OSCCs.[9-11] When comparing TSCC to buccal SCC, c-MYC was found to be one of the four most significant independent prognostic factors in TSCC but not in buccal SCC.[11] As the tongue has two parts, tongue base and mobile tongue, where the former has more in common with the neighbouring tonsils, another study divided TSCCs into anterior and posterior tongue, showing slightly higher expression of c-MYC in the posterior tongue.[12] Looking at the percentage of c-MYC-expressing tongue tumours, studies show good concordance with a positivity of 67%[11] to 73-76%.[12] In accordance with previous results,[1] these studies also showed worse prognosis connected to the expression of c-MYC.

Here we studied the levels of c-MYC in 104 cases of primary SCC in the mobile tongue, to our knowledge the largest material of this subsite studied so far. Results showed more high c-MYC-expressing tumours in males, as well as more patients with high c-MYC-expressing tumours being alive 2 and 5 years after treatment compared to patients with low/medium-expressing tumours.

2 | MATERIALS AND METHODS

2.1 | Materials

One hundred and four primary squamous cell carcinomas of the mobile tongue (TSCC) were included. Formalin-fixed and paraffin-embedded tissues were retrieved from the archives of Clinical Pathology, Umeå University Hospital, Sweden, and Second University of Naples, Multidisciplinary Department of Medical, Surgical and Dental Specialties, Naples, Italy. The age at diagnosis was 19-93 (mean 63.3) years, 54 patients were female and 50 male. Patients were grouped based on age into three groups: ≤40 years, 41-65 years or >65 years. Follow-up time ranged between 1 and 179 (mean 45.7) months, with only four patients having a follow-up of less than two years. Seventy tumours were located on the border of the tongue, 20 on the ventral side, two on the dorsal side and 12 showed overgrowth from the mobile tongue into the surroundings. Clinical data were obtained from the clinical files, or when necessary from the Swedish Death Registry. Sixty eight of the patients (65%) had been treated primarily with radiotherapy and 48 of these (71%) had also received surgery after radiotherapy. Of the remaining 36 patients, 19 had been treated with surgery only, 14 had surgery followed by radiotherapy, and three patients had not received any treatment at all. The project was approved by the local Ethical Committee (dnr 01-057, 03-201).

Clinical data taking age and gender into account are summarised in Tables 1 and 2 including data on status at the end of the study where ADF, alive disease free; AWD, alive with disease; DOD, dead of disease; DAD, dead of other disease and DWD, dead with disease but not with oral cancer as first cause of death.

2.2 | Immunohistochemistry

Sections with a thickness of 5 μm were cut and after pre-treatment in EDTA-buffer, pH 8.4 for 64 minutes, slides were incubated with a rabbit monoclonal antibody directed against residues in the N-terminus of c-MYC (EP121; Cell Marque, Rocklin CA, USA). Detection was performed with OptiView detection chemistry (760-700; Ventana Medical Systems, Inc, Tucson, AZ, USA). Pre-treatment and

TABLE 1 Patient data in correlation to age group, where T, tumour size; N, nodal metastasis; M, distant metastasis; ADF, alive disease free; DAD, dead of other disease; AWD, alive with disease; DOD, dead of disease and DWD, dead with disease but not with oral cancer as first cause of death

Age group	Male/Female ratio	2-year survival	5-year survival	T1/T2	T3/T4	N0	N+	M0	M+	ADF DAD	AWD DOD DWD	No	Total number
≤40	7/9 1:1.3	8 50%	6 38%	12 75%	4 25%	12 75%	4 25%	16 100%	0 0%	6 38%	10 62%	16	104
41-65	25/12 2.1:1	24 65%	17 55%	25 68%	12 32%	27 73%	10 27%	36 97%	1 3%	22 59%	15 41%	37	
>65	18/33 1:1.8	23 49%	13 33%	32 63%	19 37%	38 75%	13 25%	50 98%	1 2%	21 41%	30 59%	51	
No	50/54	55	36	69	35	77	27	102	2	49	55	104	

TABLE 2 Patient data in correlation to gender, where T, tumour size; N, nodal metastasis; M, distant metastasis; ADF, alive disease free; DAD, dead of other disease; AWD, alive with disease; DOD, dead of disease and DWD, dead with disease but not with oral cancer as first cause of death

Gender	Male/Female ratio	2-year survival	5-year survival	T1/T2	T3/T4	N0	N+	M0	M+	ADF DAD	AWD DOD DWD	No	Total number
Female	54/104	26/51	14/42	36	18	41	13	54	0	18	36	54	104
	52%	51%	33%	67%	33%	76%	24%	100%	0%	33%	67%		
Male	50/104	29/49	22/44	33	17	36	14	48	2	31	19	50	
	48%	59%	50%	66%	34%	72%	28%	96%	4%	62%	38%		
No	50/54	55	36	69	35	77	27	102	2	49	55	104	

staining was performed under standardised conditions using a Bench Mark Ultra (Ventana Medical Systems, Inc, Tucson, AZ, USA) according to a protocol from the supplier. Positive controls were included with each batch.

2.3 | Scoring

The quick score system developed by Detre and collaborators was used to quantify c-MYC immunohistochemistry.[13] Percentage of c-MYC-expressing tumour cells was divided into six classes with class 1 representing 0-4% c-MYC-expressing cells, class 2 5-19%, class 3 20-39%, class 4 40-59%, class 5 60-79% and class 6 80-100%. Staining intensity was further classified as negative=0, weak=1, intermediate=2 or strong=3. The quick score (QS) was achieved by multiplying class-number for percentage of c-MYC-expressing cells with class-number representative of staining intensity, giving a QS in the range of 0-18 for each case. Scoring of the 104 cases was performed individually by KS, GT, FA and KN. In cases of disagreement, slides were re-evaluated and then discussed in a joint session.

2.4 | Statistics

Version 24 of SPSS was used to correlate clinical data to QS values. The Pearson chi2-test was used to calculate *P*-values, where a limit at $P<.05$ was set to be considered statistically significant. Survival analysis was estimated using Kaplan-Meier.

3 | RESULTS

All cases of TSCC showed nuclear staining of tumour cells with the c-MYC antibody and the calculated QS for the 104 cases varied between 2 and 18, meaning that no tumour was negative. Based on QS values, patients were divided into two groups, where group 1 included tumours with a QS of 2-10 (83 patients) and group 2 tumours with a QS of 12-18 (21 patients). Examples of staining are shown in Figure 1.

No correlation to c-MYC staining was seen for age, specific localisation of tumours on the tongue, T-, N- or M-stage. Of the 21 high-expressing tumours, 15 (71%) were seen in males, compared to only six in female patients (29%) ($P=.017$) (Table 3). No difference in c-MYC expression was seen based on ethnicity. The majority of patients with high c-MYC-expressing TSCC were alive after 2 and 5 years (67% and 62%, respectively) ($P=.278$ and $P=.005$, respectively). Within the group of low- and medium-expressing TSCC, 65% were dead after 5 years, although no difference in survival was seen within this group after 2 years (Table 4). Kaplan-Meier analysis did not show any statistical significance of c-MYC ($P=.773$).

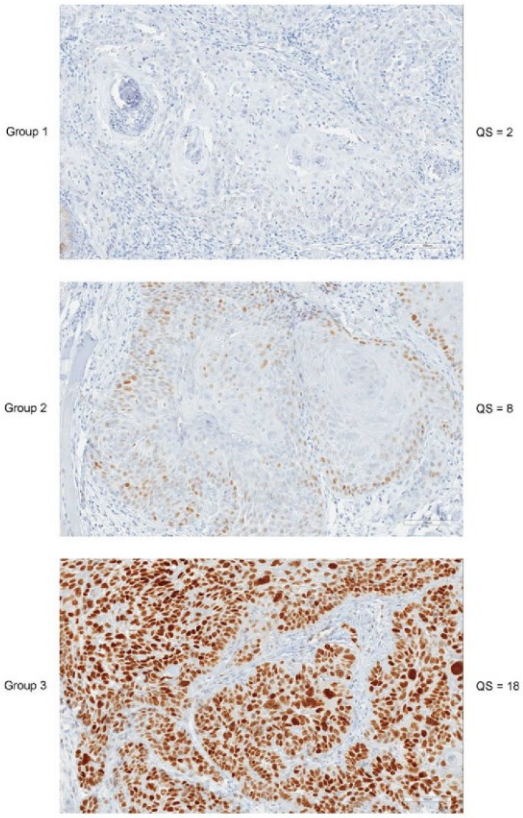

FIGURE 1 Examples of c-MYC expression in three different TSCC with quick score (QS) values of 2, 8 and 18, respectively

Among the 63 patients that were treated primarily with radiotherapy and had been followed for 5 years or more, 16 had high

TABLE 3 Quick score (QS) groups in correlation to gender, showing more low- and medium-expressing tumours, defined as a QS of 2-10, in females and more high-expressing TSCC, defined as a QS of 12-18, in males (*P*=.017). Ethnicity did not show any significant difference, even if no Italian patients showed high-expressing tumours (*P*=.064)

QS	2-10	12-18	Total	*P*-value
Female	48 58%	6 29%	54	.017
Male	35 42%	15 71%	50	
Swedish patients	71 77%	21 23%	92	.064
Italian patients	12 100%	0 0%	12	
	83	21	104	

TABLE 4 Looking at 2- and 5-year survival, the majority of patients, 42 of 65 (65%), with low- or medium-expressing TSCC, defined as a Quick score (QS) of 2-10, were dead after 5 years whereas the majority, 13 of 21 (62%), of patients with high-expressing TSCC, defined as a QS of 12-18, were alive 5 years after treatment. Already after 2 years the majority of patients with high-expressing TSCC, 14/21 (67%), were alive, whereas no difference in survival was seen for low- and medium-expressing TSCC

	2-year survival			5-year survival			
	Yes	No	Not	Yes	No	Not	Total
QS 2-10	41 49%	38 46%	4	23 28%	42 65%	18	83
QS 12-18	14 67%	7 33%	0	13 62%	8 38%	0	21
No	55	45	4	36	50	18	104

TABLE 5 Quick score (QS) groups in correlation to 5-year survival for the 63 patients that were primarily treated with radiotherapy, either alone or followed by surgery. Results showed that the majority of patients with low or medium QS were dead after 5 years, whereas the majority of patients with a QS of 12, 15 or 18 were alive

	5-year survival		
QS	Yes	No	Total
QS 2-10	18 38%	29 62%	47
QS 12-18	9 56%	7 44%	16
Total	27	36	63

expression of c-MYC, of which nine (56%) were alive 5 years after treatment. In contrast, only 18 of the remaining 47 patients (38%) with low or medium expression were alive after 5 years (Table 5; *P*=.191).

4 | DISCUSSION

Squamous cell carcinoma of the tongue (TSCC) is a distinct subtype of intraoral SCC[9,10] with a worldwide increase seen in young patients (≤40 years).[14] So far there is no explanation for this increase. Apart from showing higher recurrence rate in young patients,[15] we have previously shown specific characteristics for young patients with TSCC such as higher levels of the podoplanin protein and worse prognosis when lacking expression of the p16 tumour suppressor protein.[16] The former finding has implications for therapeutic use as there are antibodies available against podoplanin.[17]

When looking at the oncoprotein c-MYC, age did not seem to influence its levels in TSCC, whereas, in accordance with previous findings,[12] gender did, with females showing more low-expressing and males more high-expressing tumours.

Compared to other studies of TSCC showing c-MYC in 67-76% of tumours,[11,12] we had 100% positivity using a scoring system that includes individual positive tumour cells.[13] As c-MYC is known to act rather weakly at many of its targets[3] we thought it important to include all positive tumours in the analysis. Previous studies have shown high expression of c-MYC to correlate to worse prognosis in TSCC,[1,11,12] whereas our results showed the opposite, with the majority of patients with high-expressing tumours being alive 5 years after treatment. An explanation for these contrasting results can be the antibody used; other studies used an antibody recognising amino acids 408-439 in the C-terminus,[11,12,18] whereas our antibody recognises residues in the N-terminus of the c-MYC protein. The N-terminus of c-MYC contains the transcriptional regulatory domain and the C-terminus contains the domain for dimerisation with MAX. The c-MYC/Max complex binds to E-boxes in the promoter of target genes and thus stimulates transcription.[3] Whether the c-MYC protein detected by our antibody is free or bound to E-boxes cannot be judged by the present results. Alternatively, the rabbit monoclonal antibody we used may have an improved affinity and/or may perform better in clinical material than the mouse monoclonal used previously, as is generally seen when comparing mouse and rabbit monoclonal antibodies.[19,20] Higher affinity with concurrent improved sensitivity and discriminatory power may also explain why we did not find patients that lack c-MYC, unlike other studies. Whether the differences reflect epitope masking due to complex formation/DNA binding, or reflect different antibody affinities and performance, it is clear from our data that high levels of c-MYC as detected with an N-terminal rabbit monoclonal antibody is prognostically advantageous in TSCC.

Overexpression of c-MYC can be due to many different reasons, such as gene amplification, chromosomal translocation and insertions. It has also been shown that activation of hormones or growth factors, their receptors, second messengers, *etc.* can be involved in deregulation of c-MYC.[19] Tumours overexpressing c-MYC often show mutations disabling apoptosis,[4] a process in which c-MYC plays a role by either inducing or sensitising cells to apoptosis.[4] Based on cell type and type of apoptotic trigger, c-MYC-induced

apoptosis can be either dependent or independent of p53.[4] Treating tumours with high levels of c-MYC primarily with radiotherapy, which leads to radiation-induced apoptosis,[20] could thus restore the apoptotic activity and be a factor of therapeutic advantage. This is supported by our findings that the majority of TSCC with high c-MYC scores and primarily treated with radiation were alive at 5-year follow-up.

Taken together, we have used an N-terminal-specific c-MYC rabbit monoclonal antibody and found that high levels of this oncoprotein indicate higher survival rates 2 and 5 years after treatment. We also show indications that treating TSCC patients with high c-MYC tumours with radiotherapy is prognostically advantageous, a finding with potential clinical impact.

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CONFLICT OF INTEREST

None declared.

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REVIEW ARTICLE

Can Inspection of the Mouth Help Clinicians Diagnose Crohn's Disease? A Review

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Purpose: Crohn's disease (CD) is a chronic inflammatory bowel disease characterised by localised areas of non-specific, noncaseating granulomas. Approximately 1/3 of CD patients develop extraintestinal manifestations in the course of their disease. This study focuses on oral manifestations of CD to understand if oral lesions could help clinicians in the diagnosis of systemic CD.

Materials and Methods: Literature for the review was retrieved using PubMed Medline, Ebsco Library and Web of Science.

Results: After a careful preliminary evaluation, only 43 articles were eligible for inclusion in the qualitative evaluation, whereas only seven mentioned oral CD as the first sign of a systemic disease and were included in the quantitative evaluation.

Conclusions: Oral manifestations of CD can be classified as specific and non-specific. The aetiology of oral CD seems to be linked to particular bacterial infections. Although the evidence from the literature is weak, it seems that in some cases the inspection of the mouth could assist in the diagnosis of a systemic Crohn's disease.

Key words: extraintestinal manifestations of Crohn's disease, inflammatory bowel disease (IBD), oral manifestions of Crohn's disease (CD), orofacial granulomatosis

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Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterised by granulomatous inflammation that may affect any site along the gastrointestinal tract from the mouth to the anus.[2] The reported incidence of CD in Europe ranges from 3.9 to 7.0 cases per 100,000 persons/year.[24] Increasing evidence suggests that IBD results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host.[1] Risk factors include smoking, age, ethnicity (predominantly Eastern European) and family history of CD.[7] The diagnosis of CD is based on the discovery of typical clinical, radiological, or histopathological findings.[37] The prevalence rate of oral involvement in Crohn's disease ranges from 0.5% to 50%.[25,28] In 1969, Dyes et al[10] were the first to describe an oral involvement of CD; on the other hand in 1972, Varley[40] reported the first case of oral Crohn's disease in the absence of intestinal findings.[40] Due to its anatomic location, the oral cavity is easily examined directly; this could help clinicians in performing an early and correct diagnosis.[11]

In clinical practice, is not uncommon to see patients showing swelling of the lips and gums (especially upper) that appear red, also involving the cheeks, chin and periorbital region. Clinically, this appears as erythematous hypertrophy, with oedema of the gums and lips. One of the first diagnostic hypotheses is that it may be orofacial granulomatosis (OFG), which should be confirmed by an oral biopsy.[19] This should be characterised by lymphedema and non-caseating granulomas. The aetiology of oral lesions with non-caseating granulomas includes CD (some patients with oral lesions develop typical bowel symptoms of CD after months or years), dental infections, sarcoidosis and severe contact or food allergies.[38] Very often, however, the result of the biopsy is negative, at least for the presence of non-caseating granulomas, and this greatly complicates the diagnosis and the therapy of this clinical condition.[34]

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Table 1 Incidence of oral Crohn's disease (CD) as early symptoms of a systemic condition reported in the literature

Author	Year	Oral CD manifestation/systemic CD cases	Percentage
Gale et al[16]	2015	12/29	41%
Lourenco et al[25]	2010	4/6	66%
Harty et al[20]	2005	0/20	0%
Pittock et al[28]	2001	12/25	48%
Dupuy et al[11]	1999	2/9	22%
Plauth et al[29]	1991	43/79	54%
Scully et al[35]	1982	7/19	36%
Total		80/187	42%

This study focuses on the incidence and clinical aspects of oral CD lesions through a literature review of the last decade. In addition, a quantitative evaluation of studies present in literature was performed to determine in how many cases the oral manifestations of CD may be the first sign of a systemic condition.

MATERIALS AND METHODS

The literature reviewed was retrieved using the computerised databases PubMed Medline, Ebsco Library and Web of Science, without specifying the year of publication. The electronic research was carried out independently by two blinded reviewers (TG and DM); disagreement on the inclusion of studies was resolved by discussion. In each database, citations were searched by key word or title using the following combination of words: ('oral manifestations' OR 'oral signs' OR 'orofacial granulomatosis') AND ('Crohn's disease' OR 'inflammatory bowel disease' OR 'ulcerative colitis' OR 'early Crohn's disease'). Moreover, we performed a direct online search of relevant articles in three impact journals in the sector of interest (Inflammatory Bowel Disease, World Journal of Gastroenterology and Journal of Crohns and Colitis). Abstracts from papers were retrieved in order to exclude irrelevant studies and clinical opinion. A full-text reading of those papers cited in the reference list of articles previously selected was also performed. After the preliminary investigation, only articles written in English were included. The search was carried out in the months of March and April 2015. Neither authors nor journals were blinded to reviewers.

RESULTS

Using a series of combined search terms, the first step of the search yielded 366 abstracts. Of these, only 43 met the initial inclusion criteria and were thus read in the full-text version. Only 7 of these reported cases of oral CD as the first sign of a systemic condition and were included in the subsequent quantitative evaluation. Consensus for inclusion of articles in this study was obtained after discussion between the two reviewers, while data were extracted in a separate step by a third reviewer (LL). The studies included in the review were published between 1969 and 2014. All 43 articles extracted were evaluated as full-text and the reading focused on the aetiology, clinical aspects and treatment of oral CD. In addition, particular attention was placed on the first manifestation of systemic CD in the oral cavity, to determine whether oral manifestation could assist in the diagnosis of systemic CD. All studies included in the quantitative evaluation were observational clinical studies and reported the experience of a single institution; two of these were prospective and the others were retrospective. Because none were prospective randomised clinical trials, a possible risk of bias existed. Aggregated data revealed that, for the included studies, oral CD could be an early sign of a systemic condition in about 42% (80/187) of cases (Table 1).

DISCUSSION

Crohn's disease (CD) is a chronic granulomatous inflammatory disorder that can affect any part of the gastrointestinal tract, including the oral cavity. Evidence that CD aetiology is based on autoimmune mechanism is given in the literature.[6,22] It is also known that smoking increases the risk of developing CD. Moreover, smoking cessation represents an important strategy for CD management.[8] Diagnosis is not simple to perform, as a precise correlation of clinical, endoscopic, and histopathological findings is required.[30] Approximately 1/3 of CD patients develop extraintestinal manifestations (EIM) over the course of disease, which could help clinicians in the diagnosis of a systemic form. A susceptibility to develop EIM seems to be linked with particular features in the major histocompatibility complex (MHC) gene region on chromosome 6.[31] The most common EIM are located in joints, skin, eyes, the oral cavity and biliary tract.

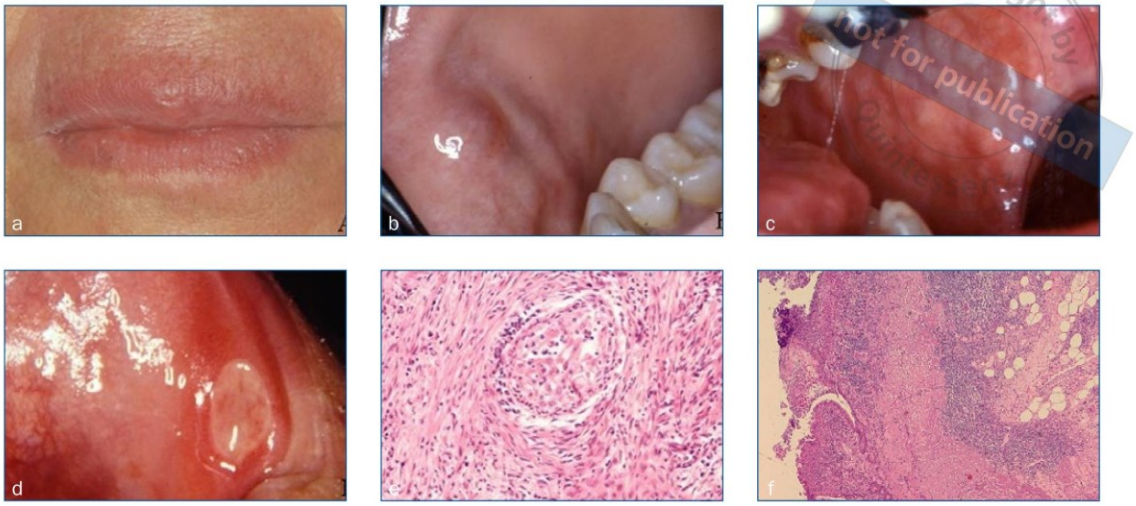

Fig 1 Signs of oral Crohn' disease found in different subjects. A: Indurated tag-like lesions of the lip with vertical fissures; B and C: Cobblestoning-like lesion located on the cheek. Fissured swollen buccal mucosa with corrugation is evident. D: Non-specific aphthous stomatitis of the oral mucosa; E: Histological sample of oral tissues in Crohn's disease showing the presence of a noncaseating granuloma; F: At gastrointestinal level the granulomas are not always visible; in the case reported above, it is possible to note fissures with lymphoid aggregates scattered in the thickness of the intestinal wall.

With regard to the oral manifestations, it is difficult to distinguish between oral CD and other types of orofacial granulomatosis (OFG). The latter is a generalised term used to describe a group of chronic disorders of unknown aetiology, characterised by facial and lip swelling, gingival enlargements, oral ulceration, and in some cases a history of facial nerve paralysis. Recently, evidence has been found of a different composition in the inflammatory infiltrate in patients with OFG compared to patients with OFG+CD, suggesting a different immune mechanism in these two conditions.[15] To date, however, it is not clear whether OFG and CD must be considered as two different entities, or if some OFG lesions represent early lesions of undiagnosed CD.[43] Some studies have found a higher prevalence of periodontitis in CD patients than in people without CD.[3] Oral lesions may manifest contiguous with intestinal involvement, or could represent metastatic CD.[18] The aetiology of oral lesions could be linked to an infection with *Mycobacterium avium* subspecies *paratuberculosis*.[27] Additionally, changes in oral microbiome have been found in patients with systemic CD; particular differences concern *Firmicutes* and *Fusobacterium* species.[9] More recently data reported increased levels of lysozime and IL-1β in CD patients, suggesting a possible link between oral and intestinal dysbiosis.[33]

Oral CD manifestations can be divided into specific and non-specific lesions, based on the presence or absence of granuloma during the histopathology examination. Specific lesions are less common than non-specific lesions, and upon histopathological examination, they present typical granuloma features.[29] These signs are strongly associated with oral CD involvement and can be recognised by clinical analysis. Indurated tag-like lesions are white reticular tags referred to as mucosal tags, epithelial tags, or folds (Fig 1A). Another characteristic feature is a cobblestoning aspect (Fig1B), i.e. fissured, swollen buccal mucosa with corrugation and hyperplastic appearance of the mucosa. Lesions often appear as coloured papules that produce firm plaques on the buccal mucosa and palate (Fig 1C). These lesions are pathognomonic for CD, but are not necessarily associated with intestinal CD activity.[42] Another oral sign of CD is a particular form of mucogingivitis: the whole gingiva up to the mucogingival line can be involved. The gingiva may become oedematous, granular, and hyperplastic with or without ulceration.[21] Other specific lesions are deep swelling with vertical fissures and deep linear ulceration; because they are very painful, these lesions may be invalidating for patients. Oral Crohn's involvement may also appear as non-specific lesions. This group includes aphthous stomatitis (Fig 1D), pyostomatitis vegetans, angular cheilitis, glossitis and persistent submandibular lymphadenopathy.[14]

Although clinical examination is very important, oral Crohn's disease may be diagnosed only through the histopathological identification of noncaseating granulomas in the oral tissues (Fig 1E), although this sign is not always present in the gastrointestinal tract (Fig 1F). Oral involvement seems to be more frequent in children than in adults. The higher prevalence of oral manifestation in the paediatric group could be due to the indirect effect of Crohn's disease via malabsorption.[17] Lesions seem to be more severe during active disease, but up to 30% of patients might continue to manifest oral lesions despite disease control.[23] In

addition, in children, a direct correlation between oral and perianal lesions has been reported in the literature.[20] In some cases, oral manifestations of CD may be the first and/or exclusive presentation and precede other gastrointestinal symptoms by days, months, or even years.[16,35] This association has been found by many authors and is summarised in Table 1. However, the data are not homogeneous and significantly differ between oral pathologists and gastroenterologists. It seems, especially in children, that examination of the mouth for signs of oral CD is of value only for the initial diagnosis; it has no predictive value for the progression of the disease. Nevertheless, the oral cavity provides an easily accessible source of diagnostic material when CD is suspected.[12] The results of this literature review indicate that the oral cavity could be the first anatomic site involved in systemic CD in about 40% of cases. However, the data are very heterogeneous, making future prospective randomised clinical trials with high scientific standardisation necessary.

The treatment goal of oral CD is the negative modulation of the immune response. Systemic corticosteroids currently represent the most effective treatment; however, they should be used sparingly in cases of oral manifestation without systemic disorders.[12] In these cases, the topical or intralesional application of triamcinolone acetonide may be considered a valid therapeutic option.[26] The use of many other medications has also been reported in literature: methotrexate, azathioprine, thalidomide, tacrolimus and infliximab are among the most common.[4,32,36,39,41] The use of these compounds was partly abandoned because of the possibility of severe adverse reactions.[5] In addition, patients treated with infliximab have failed to show a long-term response: in these cases, treatment with adalimumab may be a valid alternative.[13] At present, however, the poor quality of data in the literature data makes it impossible to confirm this.

In our opinion based on several scientific papers, where OFG and CD occur together and are mainly characterised by the presence of non-caseating granulomas, the two diseases can represent two aspects of the same condition. Conversely, if the disease appears as 'autonomous' OFG or at any length of time before the intestinal CD, without the pathognomonic histopathological features, it is possible that the two entities are not the expression of the same disease.

CONCLUSIONS

Within the limitation of this narrative review, it can be concluded that oral manifestation of Crohn's disease could help clinicians in the diagnosis of the systemic condition. Because the data currently available in the literature are poor, prospective randomised clinical trials should be performed in order to discern in which cases a collaboration between oral pathologists, endoscopists, gastroenterologists and general pathologists may help in the diagnosis of systemic Crohn's disease.

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