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Review Article

Preliminary Evaluation Salivary Biomarkers in Patients with Oral Cancer

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Abstract: *Any cancer that develops in the lips, throat, or oral cavity is referred to as oral cancer; oral squamous cell carcinoma accounts for 90% of all oral cancer cases. Even with the most recent advancements in treatment, oral cancer is still known to have one of the highest fatality ratios of all cancers, largely due to delayed detection. Saliva has long been used as a useful diagnostic tool for systemic disorders, including oral cancer, and drug monitoring. The present review discusses the new molecular markers (DNA, RNA, and protein markers) for oral cancer diagnosis and surveillance that have been made possible by newly developed molecular biology technology.*

Keywords: *OSCC, DNA, mRNA, miRNA.*

Introduction

More than 481,000 new individuals worldwide are affected by oral cancer, which is defined as any cancer originating from the lips, oral cavity, or pharynx [1]. Oral squamous cell carcinoma accounts for ninety percent of all mouth malignancies. The survival rate for this cancer is 80–90% if detected early. The World Health Organization reports that oral cancer has one of the highest fatality ratios among other malignancies, with a death rate of 45% at five years following diagnosis, despite this fact and the considerable advancements in therapy [3]. The late detection of the disease is certainly the cause of this high morbidity rate [4]. There are currently insufficient countrywide screening programs and appropriate and conclusive biological indicators [5-7] for early oral cancer detection has re- sulted in late stage diagnosis of oral cancer [8]

Attempts on Early Oral Cancer Detection

The most reliable method for the diagnosis of oral can- cer is a tissue biopsy followed by a histopathological evalua- tion of the tissue specimen [9, 10]. This however takes as granted that a usually asymptomatic lesion will be detected by the patient who will be alerted and will then soon visit a dentist's or other practitioner's office [11]. Because oral can- cers usually lack early signs, there have been in the past sev- eral attempts towards the direction of early oral cancer detec- tion and attention has been drawn to cancer screening pro- grams [12, 13]. Most oral cancer screening programs include the simple visual inspection [9, 14], whereas

others attempt the use of toluidine blue [15, 16], brush biopsy (exfoliative cytology) [17, 18], chemiluminesce [19, 20] and fluores-cence imaging [21]. The last three screening methods in fact deal with the diagnosis of lesions that have already been de- tected by the patient, dentist or other clinician but a defini- tive diagnosis can only be made by a tissue biopsy.

However, according to Kujan et al. [22, 23], "there is not enough evidence to decide whether screening by visual in- spection reduces the death rate for oral cancer and also no robust evidence exists to suggest that other methods of screening, toluidine, fluorescence imaging or brush biopsy are either beneficial or harmful".

Cancer Related Genetic Alterations Identified in Bodily Fluids

The model of multiple stem tumorigenesis is established by the progressive genotypic and phenotypic changes that occur in the affected cells during the development of neoplastic disease. These changes include the activation of protoncogenes and ongogenes and the inactivation of tumor suppressor genes, which are linked to tumorigenesis [24]. Similar mutations have been demonstrated to exist in physiological fluids that drain a tumor [7], but more recently, they have also been found in bodily fluids released at the initial site where a solid tumor is growing [25, 26]. Plasma/serum [27–29], urine [30, 31], saliva [32, 33], bronchoalveolar lavage fluid [27], cerebrospinal fluid [34], and other body fluids have all been found to contain nucleic acids and proteins associated with cancer cells. These proteins and nucleic acids have been employed as molecular indicators for the early diagnosis of the disease [33, 35, 36], recurrence markers [37] survival and metastasis predictors [38, 39] and decide the therapeutic approach [40, 41].

Saliva as a Perfect Diagnostic Medium

The substance known as whole saliva is a mixture of secretions from the parotid, submandibular, and sublingual salivary glands as well as numerous minor salivary glands, along with bronchial and nasal secretions, blood components from cuts or bleeding gums, bacteria, viruses, fungi, exfoliate epithelial cells, and food particles [42, 43]. Saliva has been suggested and used as a diagnostic medium for a long time [44–46] because it is readily available, non-invasive, quick to collect, low-cost, low-training required, and suitable for mass screening of sizable population samples [46, 47]. Saliva can be collected in its whole either stimulated or not. Masticatory movements or gustatory stimulation (citric acid) can be used for stimulation [48]. Saliva as a Perfect Diagnostic Medium Whole saliva is the product of the secretions of the 3 ma- jor

salivary glands (parotid, submandibular, sublingual) and the numerous minor salivary glands mixed with crevicular fluid, bronchial and nasal secretions, blood constituents from wounds or bleeding gum, bacteria, viruses, fungi, exfoliate epithelial cells and food debris [42, 43]. Saliva has been long proposed and used as a diagnostic medium [44-46] because it is easily accessible and its collection is non-invasive, not time-consuming, inexpensive, requires minimal training and can be used for the mass screening of large population sam- ples [46, 47]. Whole saliva can be collected with or without stimula- tion.Stimulation can be performed with masticatory move- ments or by gustatory stimulation (citric acid) [48]. Stimu- lated saliva however, it can be collected in larger quantities, is a little bit altered in content [49]. Unstimulated saliva can be collected by merely spitting in a test tube or by leaving saliva drool from the lower lip [50] and it is more often used for the diagnosis or follow up of systemic diseases. Saliva has long been used for the monitoring of drug abuse (drugs and addictive substances) such as cocaine, her- oin, amphetamine, barbiturates [51] etc. Moreover salivary testing has largely performed for the diagnosis of HIV- infection [52, 53]. Analysis of salivary parameters such as salivary flow rate, pH, buffer capacity, lactobacillus, and yeast content, presence of IgG, IgM and anti-La autoantibod- ies and raised protein levels such as that of lactoferrin and cystatin C as has been proposed for the diagnosis of Sjogren's syndrome [54, 55]. Concerning cancer diagnostics and follow up altered levels of certain mRNA molecules [33, 56] have been detected in saliva in oral cancer patients and of certain proteins in several cancers [25, 26, 57].

Speculations about Possible Mechanisms that Lead to the Presence of Genotypic and Phenotypic Markers in the Saliva

Saliva contains proteins and cell-free nucleic acids that can be created locally or derived from serum [58]. Nucleic acids and proteins derived from serum that are present in saliva may be a natural byproduct of salivary secretion (by the acinar cells) [59] or they may arrive via extracellular channels like tight junction ultrafiltration [61] or active transport or passive diffusion [60] from serum to saliva across cell membranes. However, trauma, lysis, apoptosis, and necrosis of cells can all produce cell free nucleic acids and proteins locally. Normal epithelial or malignant cells can even actively release these substances into the saliva. One potential process that results in the release of of cell free nucleic acids and proteins in the saliva and this idea is also supported by the large amount of DNA in the plasma of patients with cancers in an advanced stage. Moreover, mounting evidence exists con- cerning the presence of cell-free nucleic acids and proteins in apoptotic bodies [62] which also protect these molecules from degradation [63]. The active release of these

molecules in exosomes or microvesicles is another strong possibility [64]. Exosomes or microvesicles are released by living cells. They are membrane vesicles, 40–100-nm in diameter [65], originating from the endoplasmic reticulum and are released when fused with the cell membrane. They contain mRNA [66], miRNA [67] and proteins [68, 69] and are thought to play a role in the cell-free intercellular communication [69- 71], Table 1.

Table 1. Mechanisms that Lead to the Presence of Genotypic and Phenotypic Markers in the Saliva

Cell-free nucleic acids & proteins in saliva	*Serum derived*	*Normal salivary secretion*
		Passive diffusion
		Active transport
		Ultrafiltration through tight junctions
		Outflow of crevicular fluid
	Locally produced	*Cell necrosis, lysis*
		Apoptosis
		Trauma
		Active release

Table 2. Molecular Markers for the Diagnosis of Oral Squamous Cell Carcinoma

Changes in the cellular DNA	Altered mRNA transcripts	Altered protein markers
Allelic loss on chromosomes 9p	Presence of IL8	Elevated levels of defensin-1
Mitochondrial DNA mutations	Presence of IL1B	Elevated CD44
p53 gene mutations	DUSP1 (dual specificity phosphatase 1)	Elevated IL-6 andIL-8
Promoter hypermethylation of genes (p16, MGMT, or DAP-K)	H3F3A (H3 histone, family 3A)	Inhibitors of apoptosis (IAP)
Cyclin D1 gene amplification	OAZ1 (ornithine decarboxylase antizyme 1)	Squamous cell carcinoma associated anti- gen (SCC-Ag)
Increase of Ki67 markers	S100P (S100 calcium binding protein P)	Carcino- embryonic antigen (CEA)
Microsatellite alterations of DNA	SAT (spermidine/spermine N1-acetyltransferase)	Carcino-antigen (CA19-9)
Presence of HPV		CA128
		Serum tumor marker (CA125)
		Intermediate filament protein (Cyfra 21-1)

		Tissue polypeptide specific antigen (TPS)
		Reactive nitrogen species (RNS)
		8-OHdG DNA damage marker
		Lactate dehydrogenase (LDH)
		Immunoglobulin (IgG)
		s-IgA
		Insulin growth factor (IGF)
		Metalloproteinases MMP-2 and MMP-11

Salivary Markers for Oral Cancer Detection

Molecular markers for the diagnosis of OSCC can be quested in 3 levels; (I) changes in the cellular DNA, which result in (II) altered mRNA transcripts, leading to (III) altered protein levels (intracellularly, on the cell surface or extracellularly). All these markers are summarized in Table 2.

Changes in the Cellular DNA

Typical changes in the host DNA of dysplastic or cancer cells include point mutations, deletions, translocations, am- plifications and methylations, cyclin D1, epidermal growth factor receptor (EGFR), microsatellite instability and HPV presence. Allelic loss on chromosomes 9p has been observed in OSCC [72]. Mitochondrial DNA mutations have also been useful targets to detect exfoliated OSCC cells in saliva. They have been identified in 46% of head and neck cancers. The same mitochondrial DNA mutations were detected in 67% of saliva samples from OSCC patients by direct sequencing alone [73]. p53 gene mutations are also present in approxi- mately one-half of head and neck cancers [74, 75]. Using plaque hybridization, Boyle et al. [75] identified tumor spe- cific p53 mutations in 71% saliva samples from patients with head and neck cancer.

Promoter hypermethylation of several genes has been re- ported in head and neck cancer. Rosas et al. identified aber- rant methylation of at least one of three genes (p16, MGMT, or DAP-K) in OSCC. Abnormal promoter hypermethylation was also detected in the matched saliva sample in 65% of OSCC patients [76].Cyclin D1 gene amplification has been found to be asso- ciated with poor prognosis in OSCC [77]. In

another study Ki67 markers were increased, while 8-oxoguanine DNA glycosylase, phosphorylated-Src and mammary serine prote- ase inhibitor (Maspin) were found decreased in the saliva of patients with OSCC [78].Microsatellite alterations of DNA were also observed in the saliva of patients with small cell lung cancer [79]. In the same study it was further demonstrated that 93% of the pa- tients with microsatellite instability in tumor DNA also had similar microsatellite alterations in the corresponding plasma DNA.

The presence of HPV (human papilloma virus) and Ep- stein Barr virus genomic sequences have been identified as possible DNA molecular markers in detecting OSCC and tumor progression [80, 81].

Altered mRNA Transcripts

RNA for years was thought to quickly degrade in saliva due to the various RNAses that saliva contains [82]. Despite the opposite reports [83], cell-free RNA molecules however, seem to exist in saliva both intact but also fragmented [84]. An intriguing question that remains to be answered is the mechanism by which mRNA in saliva is protected by degra- dation. A speculation is that salivary mRNA is contained in apoptotic bodies [63, 64] or actively released in exosomes or microvesicles [66, 68,70]. Lately microRNAs, small RNA molecules, 18-24 molecules in length, that seem to regulate transcription were also discovered existing in saliva [85-87].

mRNA detection in saliva has been extensively reported enabling body fluid identification in Forensic Medicine [88, 89]. Moreover mRNA markers in the saliva have been pro- posed for the diagnosis of primary Sjögren's syndrome [90] and for the identification of sleep drive both in flies but also in humans [91].

Various mRNA molecules were found up-regulated in the saliva of patients suffering from OSCC by the team of Li et al. [33]. Seven mRNA molecules: transcripts of: 1. IL8 (interleukin 8) playing a role in angiogenesis; replication; calcium-mediated signaling pathway; cell adhesion; chemotaxis; cell cycle arrest; immune response, 2. IL1B (interleu- kin 1B) which takes part in signal transduction; proliferation; inflammation and apoptosis 3. DUSP1 (dual specificity phosphatase 1) with a role in protein modification; signal transduction and oxidative stress, 4. H3F3A (H3 histone, family 3A) having a DNA binding activity, 5. OAZ1 (or- nithine decarboxylase antizyme 1) taking part in polyamine biosynthesis 6. S100P (S100 calcium binding protein P) with a role in protein binding and calcium ion binding, and 7. SAT (spermidine/spermine N1-acetyltransferase) which takes part in enzyme and transferase activity- were found significantly elevated in OSCC patients rather than in healthy controls [56].

Altered Protein Markers

Several salivary protein markers for OSCC have been in- vestigated in various studies and have shown relatively mod- erate sensitivity and specificity values relative to prognosis prediction.

For example, defensins are peptides which possess an- timicrobial and cytotoxic properties. They are found in the azurophil granules of polymorphonuclear leukocytes [92, 93]. Elevated levels of salivary defensin-1 were found to be indicative for the presence of OSCC, since higher concentra- tions of salivary defensin-1 were detected in patients with OSCC compared with healthy controls [94].

In another study soluble CD44 [95] was found to be ele- vated in the majority of patients with OSCC and distin- guished cancer from benign disease with high specificity. Whereas the solCD44 test lacks sensitivity by itself, methy- lation status of the CD44 gene seems to complement the solCD44 test and provides very high sensitivity and specific- ity for the detection of OSCC.

St John et al. [32] investigated whether IL-6 and/or IL-8 could serve as informative biomarkers for OSCC in saliva. Interleukin 8 was detected at higher concentrations in saliva, while IL-6 was detected at higher concentrations in serum of patients with OSCC. Thus, they concluded that IL-8 in saliva and IL-6 in serum hold promise as biomarkers for OSCC.

A group of leading researchers [33, 56, 96-99] using new and sophisticated approaches, such as, Luminex Multianalyte Profiling (xMAP) technology, shotgun proteomics, capillary reversed-phase liquid chromatography with quadruple time- offlight mass spectrometry and matrix-assisted laser desorp- tion/ionization–mass spectrometry (MALDI–MS), has con- tributed significantly in recent years to the research in saliva for cancer diagnosis. Their studies have shown that saliva contains proteins that may serve as biomarkers for OSCC, since 46 peptides/proteins were found at significantly differ- ent levels between the OSCC and control groups. For exam- ple Arellano-Garcia et al. [100] using Luminex xMAP technology showed that both IL-8 and IL-1þ were expressed at significantly higher levels in OSCC subjects.

Other salivary biomarkers which have been shown to be significantly altered in OSCC patients as compared with healthy controls are inhibitors of apoptosis (IAP) [101], squamous cell carcinoma associated antigen (SCC-Ag) [102- 104], carcino- embryonic antigen (CEA) [102, 103], carcino-antigen (CA19-9) [102, 104], CA128 [102, 104], serum tumor marker (CA125) [105], intermediate filament protein (Cyfra 21-1) [106-108], tissue polypeptide specific antigen (TPS) [108, 109], reactive nitrogen species (RNS) and 8- OHdG

DNA damage marker [106], lactate dehydrogenase (LDH) and immunoglobulin (IgG) [107], s-IgA [110], insu- lin growth factor (IGF) [110], metalloproteinases MMP-2 and MMP-11 [110].

Conclusions

For years saliva is tested as a diagnostic fluid and com- pared to blood (serum or plasma) in parameters such as sen- sitivity, specificity and applicability of the method, cost and duration of the procedure, patient compliance [111] etc. Due to the recent advances and emerging technologies in molecu- lar biology new molecular markers (DNA, RNA and protein markers) have been discovered existing in the saliva in meas- urable quantities [112]. OSCC can be diagnosed with high sensitivity and specificity by merely testing saliva samples from the subjects. This does not of course undermine the value of screening tests by visual examination neither the importance of the tissue biopsy.

Despite the scepticism in the scientific community and the conservatism of the patients, saliva seems to emerge as a valuable tool in cancer diagnostics and mass population screening. In our opinion much attention must be given to the saliva collecting method. An attempt to integrate the si- multaneous testing of different salivary molecular markers in order to raise the possibility of an accurate diagnosis by sim- ply using micro- and nano-electrical-mechanical systems biosensors is on the way raising much hope in its future ap- plications [113].

Finally, since the present methods are not ready for immediate clinical use as diagnostic tools, much work is necessary and it can be envisaged that simple, fast, portable and cost-effective clinical diagnostic systems could be available in the near future.

References

[1]The international statistical classification of diseases and related health problems. Geneva: World Health Organization, 1992; 1(10).

[2]Ferlay J, Bray F, Pisani P, Parkin DM. GLOBOCAN 2000, cancer incidence, mortality and prevalence worldwide, Version 1.0, Lyon: IARC Press 2001.

[3]Peacock S, Pogrel A, Schmidt BL. Exploring the reasons for delay in treatment of oral cancer. Am Dent Assoc 2008; 139: 1346-52.

[4]Schantz SP. Biologic markers, cellular differentiation, and metas- tatic head and neck cancer. Eur Arch Otorhinolaryngol 1993; 250: 424-8.

[5]Schantz SP. Carcinogenesis, markers, staging, and prognosis of head and neck cancer. Curr Opin Oncol 1993; 5: 483-90.

[6]Sidransky D. Emerging molecular markers of cancer. Nat Rev Cancer 2002; 3: 210-9.

[7]Ellison MD, Campbell BH. Screening for cancer of the head and neck: addressing the problem. Surg Oncol Clin N Am 1999; 8: 725- 34.

[8]Fedele S. Diagnostic aids in the screening of oral cancer. Head Neck Oncol 2009; 30; 1-5.

[9]Trullenque-Eriksson A, Munoz-Corcuera M, Campo-Trapero J, Cano-Sánchez J, Bascones-Martínez A. Analysis of new diagnostic methods in suspicious lesions of the oral mucosa. Med Oral Patol Oral Cir Buccal 2009; 14: E210.

[10]Dolan RW, Vaughan CW, Fuleihan N. Symptoms in early head and neck cancer: an inadequate indicator. Otolaryngol Head Neck Surg 1998; 118: 463-7.

[11]Sankila R, Coll EC. Evaluation and monitoring of screening pro- gram. Luxembourg: Office for the Official Publication of the Euro- pean Communities 2001; pp. 243-254.

[12]Warnakulasuriya S, Nanayakkara BG. Reproducibility of an oral cancer and precancer detection program using a primary health care model in Sri Lanka. Cancer Detect Prev 1991; 15: 331-4. http://www.jdentaled.org/cgi/external_ref?access_num=1751941&l ink_type=MED

[13]Zakzerwska JM, Hindle I, Speight PM. Practical considerations for the establishment of an oral cancer screening programme. Commun Dent Health 1993; 10(Suppl 1): 79-85.

[14]Onofre MA, Sposto MR, Navarro CM. Reliability of toluidine application in the detection of oral epithelial dysplasia and in situ and invasive squamous cell carcinomas. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001; 91: 535-40.

[15]Zhang L, Williams M, Poh CF, et al. Toluidine blue staining identi- fies high-risk primary oral premalignant lesions with poor outcome. Cancer Res 2005; 65: 8017-21.

[16]Christian DC. Computer-assisted analysis of oral brush biopsies at an oral cancer screening program. J Am Dent Assoc 2002; 133: 357-62.

[17]Mehrotra R, Hullmann M, Smeets R, Reichert TE, Driemel O. Oral cytology revisited. J Oral Pathol Med 2009; 38: 161-6.

[18]Kerr AR, Sirois DA, Epstein JB. Clinical evaluation of chemilumi- nescent lighting:an adjunct for oral mucosal examinations. J Clin Dent 2006; 17: 59-63.

[19]Epstein JB, Silverman S, Jr, Epstein JD, Lonky SA, Bride MA. Analysis of oral lesion biopsies identified and evaluated by visual examination, chemiluminescence and tolouidine blue. Oral Oncol 2008; 44: 538-44.

[20]Onizawa K, Saginoya H, Furuya Y, Yoshida H. Fluorescence pho- tography as a diagnostic method for oral cancer. Cancer Lett 1996; 108: 61-6.

[21]Kujan O, Glenny AM, Oliver R, Thakker N, Sloan P. Screening programmes for the early detection and prevention of oral cancer. Aust Dent J 2009; 54: 170-2.

[22]Omar K, Glenny A, Duxbury J, Thakker N, Sloan P. Evaluation of screening strategies for improving oral cancer mortality: a cohrane systematic review. J Dent Educ 2005; 69: 255-65.

[23]Farber E. The multistep nature of cancer development. Cancer Res 1984; 44: 4217-23.

[24]Bigler LR, Streckfus CF, Dubinsky WP. Salivary biomarkers for the detection of malignant tumors that are remote from the oral cavity. Clin Lab Med 2009; 29: 71-85.

[25]Streckfus C, Bigler L, Dellinger T, Dai X, Kingman A, Thigpen JT. The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. Clin Cancer Res 2000; 6: 2363-70.

[26]Schmidt B, Engel E, Carstensen T, et al. Quantification of free RNA in serum and bronchial lavage: a new diagnostic tool in lung cancer detection? Lung Cancer 2005; 48: 145-7.

[27]Kopreski MS, Benko FA, Gocke CD. Circulating RNA as a tumor marker: detection of 5T4 mRNA in breast and lung cancer patient serum. Ann NY Acad Sci 2001; 945: 172-8.

[28]Hasselmann DO, Rappl G, Rössler M, Ugurel S, Tilgen W, Rein- hold U. Detection of tumor-associated circulating mRNA in serum, plasma and blood cells from patients with disseminated malignant melanoma. Oncol Rep 2001; 8: 115-8.

[29]Bryzgunova OE, Skvortsova TE, Kolesnikova EV, et al. Isolation and comparative study of cell-free nucleic acids from human urine. Ann NY Acad Sci 2006; 1075: 334-4.

[30]Yoneda K, Iida H, Endo H, et al. Identification of cystatin SN as a novel tumor marker for colorectal cancer. Int J Oncol 2009; 35: 33- 40.

[31]St John M, Li Y, Zhou X, et al. Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2004; 130: 929- 35.

[32]Li Y, St John MA, Zhou X, et al. Salivary transcriptome diagnos- tics for oral cancer detection. Clin Cancer Res 2004; 10: 8442-50.

[33]de Bont JM, van Doorn J, Reddingius RE, et al. Various compo- nents of the insulin-like growth factor system in tumour tissue, cerebrospinal fluid and peripheral blood of pediatric medulloblas- toma and ependymoma patients. Int J Cancer 2008; 123: 594-600.

[34]Johnson PJ, Lo YM. Plasma nucleic acids in the diagnosis and management of malignant disease. Clin Chem 2002; 48: 1186-93.

[35]Neves AF, Araújo TG, Biase WK, et al. Combined analysis of multiple mRNA markers by RT-PCR assay for prostate cancer di- agnosis. Clin Biochem 2008; 41: 1191-8.

[36]Honma H, Kanda T, Ito H, et al. Squamous cell carcinoma-antigen messenger RNA level in peripheral blood predicts recurrence after resection in patients with esophageal squamous cell carcinoma. Surgery 2006; 139: 678-85.

[37]El-Abd E, El-Tahan R, Fahmy L, et al. Serum metastasin mRNA is an important survival predictor in breast cancer. Br J Biomed Sci 2008; 65: 90-4.

[38]Voorzanger-Rousselot N, Goehrig D, Journe F, et al. Increased Dickkopf-1 expression in breast cancer bone metastases. Br J Can- cer 2007; 97: 964-70.

[39]Siddiqua A, Chendil D, Rowland R, et al. Increased expression of PSA mRNA during brachytherapy in peripheral blood of patients with prostate cancer. Urology 2002; 60: 270-5.

[40]Ogawa O, Iinuma M, Sato K, et al. Circulating prostate-specific antigen mRNA during radical prostatectomy in patients with local- ized prostate cancer: with special reference to neoadjuvant hormo- nal therapy. Urol Res 1999; 27: 291-6.

[41]Mandel ID. The functions of saliva. J Dent Res 1987; 66: 623-7.

[42]Sreebny LM. Salivary flow in health and disease. Compend Suppl 1989; 13: S461-9.

[43]Kaufman E, Lamster I. The diagnostic applications of saliva: a review. Crit Rev Oral Biol Med 2002; 13: 197-212.

[44]Streckfus CF, Bigler L. Saliva as a diagnostic fluid. Oral Dis 2002; 8: 69-76.

[45]Malamud D. Saliva as a diagnostic fluid. Br Med J 1992; 8: 207-8.

[46]Samaranayake L. Saliva as a diagnostic fluid. Int Dent J 2007; 57: 295-9.

[47]Fox PC. Salivary enhancement therapies. Caries Res 2004; 38: 241-6.

[48]da Mata AD, da Silva Marques DN, Silveira JM, et al. Effects of gustatory stimulants of salivary secretion on salivary pH and flow: a randomized controlled trial. Oral Dis 2009; 15: 220-8.

[49]Navazesh M. Methods for collecting saliva. Ann NY Acad Sci 1993; 8: 72-7.

[50]Bosker WM, Huestis MA. Oral fluid testing for drugs of abuse. Clin Chem 2009; 55: 1910-31.

[51]Pink R, Simek J, Vondrakova J, et al. Saliva as a diagnostic me- dium. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2009; 153: 103-10.

[52]Roberts KJ, Grusky O, Swanson AN. Outcomes of blood and oral fluid rapid HIV testing: a literature review, 2000-2006. AIDS Pa- tient Care STDS 2007; 21: 621-37.

[53]Giusti L, Baldini C, Bazzichi L, Bombardieri S, Lucacchini A. Proteomic diagnosis of Sjögren's syndrome. Expert Rev Proteomics 2007; 4: 757-67.

[54]Sreebny LM, Zhu WX. The use of whole saliva in the differential diagnosis of Sjögren's syndrome. Adv Dent Res 1996; 10: 17-24.

[55]Zimmermann BG, Wong DT. Salivary mRNA targets for cancer diagnostics. Oral Oncol 2008; 44: 425-9.

[56]Di-Xia C, Schwartz P, Fan-Qin L. Salivary and serum CA 125 assays for detecting malignant ovarian tumors. Obstet Gynecol 1990; 8: 701-4.

[57]Kaufman E, Lamster IB. The diagnostic applications of saliva: a review. Crit Rev Oral Biol Med 2002; 13: 197-212.

[58]Baum BJ. Principles of saliva secretion. Ann NY Acad Sci 1993; 694: 17-23.

[59]Haeckel R, Hanecke P. Application of saliva for drug monitoring: an in vivo model for transmembrane

transport. Eur J Clin Chem Clin Biochem 1996; 34: 171-91.

[60]Aps JK, Martens LC. Review: the physiology of saliva and transfer of drugs into saliva. Forensic Sci Int 2005; 150: 119-31.

[61]Halicka HD, Bedner E, Darzynkiewicz Z. Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. Exp Cell Res 2000; 260: 248-56.

[62]Hasselmann D, Rappl G, Tilgen W, Reinhold U. Extracellular tyrosinase mRNA within apoptotic bodies is protected from degra- dation in human serum. Clin Chem 2001; 47: 1488-9.

[63]Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leuke- mia 2006; 20: 1487-95.

[64]Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: pro- teomic insights and diagnostic potential. Expert Rev Proteomics 2009; 6: 267-83.

[65]García JM, García V, Peña C, et al. Extracellular plasma RNA from colon cancer patients is confined in a vesicle-like structure and is mRNA-enriched. RNA 2008; 14: 1424-32.

[66]Yuan A, Farber E, Rapoport A, et al. Transfer of microRNAs by embryonic stem cell microvesicles. PLoS One 2009; 4: e4722.

[67]Skog J, Würdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and pro- vide diagnostic biomarkers. Nat Cell Biol 2008; 10: 1470-6.

[68]Simpson RJ, Jensen SS, Lim JW. Proteomic profiling of exosomes: current perspectives. Proteomics 2008; 8: 4083-99.

[69]Al-Nedawi K, Meehan B, Rak J. Microvesicles: messengers and mediators of tumor progression. Cell Cycle 2009; 8: 2014-8.

[70]Aharon A, Brenner B. Microparticles, thrombosis and cancer. Best Pract Res Clin Haematol 2009; 22: 61-9.

[71]Nawroz H, van der Riet P, Hruban RH, Koch W, Ruppert JM, Sidransky D. Allelotype of head and neck squamous cell carci- noma. Cancer Res 1994; 54: 1152-5.

[72]Fliss MS, Usadel H, Caballero OL, et al. Facile detection of mito- chondrial DNA mutations in tumors

and bodily fluids. Science 2000; 287: 2017-9.

[73]Liao| PH, Chang YC, Huang MF, Tai KW, Chou MY. Mutation of p53 gene codon 63 in saliva as a molecular marker for oral squamous cell carcinomas. Oral Oncol 2000; 36: 272-6.

[74]Boyle JO, Hakim J, Koch W, et al. The incidence of p53 mutations increases with progression of head and neck cancer. Cancer Res 1993; 53: 4477-80.

[75]Rosas SL, Koch W, Carvalho MGC, et al. Promoter hypermethyla- tion patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer Res 2001; 61: 939-42.

[76]Vielba R, Bilbao J, Ispizua A, et al. p53 and cyclin D1 as prognos- tic factors in squamous cell carcinoma of the larynx. Laryngoscope 2003; 113: 167-72.

[77]Shpitzer T, Hamzany Y, Bahar G, et al. Salivary analysis of oral cancer biomarkers. Br J Cancer 2009; 101: 1194-8.

[78]Chen XQ, Stroun M, Magnenat JL, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med 1996; 2: 1033-5.

[79]Paz IB, Cook N, Odom-Maryon T, Xie Y, Wilczynski SP. Human papillomavirus (HPV) in head and neck cancer: an association of HPV 16 with squamous cell carcinoma of Waldeyer's tonsillar ring. Cancer 1997; 79: 595-604.

[80]Shimakage M, Horii K, Tempaku A, Kakudo K, Shirasaka T, Sasa- gawa T. Association of Epstein-Barr virus with oral cancers. Hum Pathol 2002; 33: 608-14.

[81]Eichel HJ, Conger N, Chernick WS. Acid and alkaline ribonucle- ases of human parotid, submaxillary, and whole saliva. Arch Bio- chem Biophys 1964; 107: 197-208.

[82]Kumar SV, Hurteau GJ, Spivack SD. Validity of messenger RNA expression analyses of human saliva. Clin Cancer Res 2006; 12: 5033-9.

[83]Hu Z, Zimmermann BG, Zhou H, et al. Exon-level expression profiling: a comprehensive transcriptome analysis of oral fluids. Clin Chem 2008; 54: 824-32.

[84]Park NJ, Zhou H, Elashoff D, et al. Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. Clin Cancer Res 2009; 15: 5473-7.

[85]Michael A, Bajracharya SD, Yuen PS, et al. Exosomes from human saliva as a source of microRNA biomarkers. Oral Dis 2010; 16: 34- 8.

[86]Hanson EK, Lubenow H, Ballantyne J. Identification of forensi- cally relevant body fluids using a panel of differentially expressed microRNAs. Anal Biochem 2009; 387: 303-14.

[87]Juusola J, Ballantyne J. Multiplex mRNA profiling for the identifi- cation of body fluids. Forensic Sci Int 2005; 152: 1-12.

[88]Juusola J, Ballantyne J. Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identifica- tion. Forensic Sci Int 2003; 135: 85-96.

[89]Hu S, Wang J, Meijer J, et al. Salivary proteomic and genomic biomarkers for primary Sjögren's syndrome. Arthritis Rheum 2007; 56: 3588-600.

[90]Seugnet L, Boero J, Gottschalk L, Duntley S, Shaw P. Identifica- tion of a biomarker for sleep drive in flies and humans. Proc Natl Acad Sci USA 2006; 103: 19913-8.

[91]Lichtenstein A, Ganz T, Selsted ME, Lehrer RI. In vitro tumor cell cytolysis mediated by peptide defensins of human and rabbit granu- locytes. Blood 1986; 68: 1407-10.

[92]Lehrer RI, Ganz T, Selsted ME. Defensins: endogenous antibiotic peptides of animal cells. Cell 1991; 64: 229-30.

[93]Mizukawa N, Sugiyama K, Fukunaga J, et al. Defensin-1, a peptide detected in the saliva of oral squamous cell carcinoma patients. Anticancer Res 1998; 18: 4645-9.

[94]Franzmann EJ, Reategui EP, Pedroso F, et al. Soluble CD44 is a potential marker for the early detection of head and neck cancer. Cancer Epidemiol Biomarkers Prev 2007; 16: 1348-55.

[95]Hu S, Arellano M, Boontheung P, et al. Salivary proteomics for oral cancer biomarker discovery. Clin Cancer Re 2008; 14: 6246- 52.

[96]Tan W, Sabet L, Li Y, et al. Optical protein sensor for detecting cancer markers in saliva. Biosens Bioelectron 2008; 24: 266-71.

[97]Hu S, Yen Y, Ann D, Wong DT. Implications of salivary pro- teomics in drug discovery and development: a focus on cancer drug discovery. Drug Discov Today 2007; 12: 911-6.

[98]Yang CY, Brooks E, Li Y, et al. Detection of picomolar levels of interleukin-8 in human saliva by SPR.

Lab Chip 2005; 5: 1017-23.

[99]Arellano-Garcia ME, Hu S, Wang J, et al. Multiplexed immuno- bead-based assay for detection of oral cancer protein biomarkers in saliva. Oral Dis 2008; 14: 705-12.

[100]Kurokawa H, Tsuru S, Okada M, Nakamura T, Kajiyama M. Evaluation of tumor markers in patients with squamous cell carci- noma in the oral cavity. Int J Oral Maxillofac Surg 1993; 22: 35-8.

[101]Hoffmann J, Munz A, Krimmel M, Alfter G. Intraoperative and postoperative kinetics of serum tumor markers in patients with oral carcinoma. J Oral Maxillofac Surg 1998; 56: 1390-3.

[102]Krimmel M, Hoffmann J, Krimmel C, Cornelius CP, Schwenzer N. Relevance of SCC-Ag, CEA, CA 19.9 and CA 125 for diagnosis and follow-up in oral cancer. J Craniomaxillofac Surg 1998; 26: 243-8.

[103]Nagler RM, Braun J, Daitzman M, Laufer D. Spiral CT angiogra- phy – analternative vascular evaluation technique for head and neck microvascular reconstruction: a preliminary experience. Plast Reconstr Surg 1997; 100: 1697-703.

[104]Kurokawa H, Yamashita Y, Tokudome S, Kajiyama M. Combina- tion assay for tumor markers in oral squamous cell carcinoma. J Oral Maxillofac Surg 1997; 55: 964-6.

[105]Nagler RM, Barak M, Ben-Aryeh H, Peled M, Filatov M, Laufer

D. Early diagnostic and treatment monitoring role of Cyfra 21-1 and TPS in oral squamous cell carcinoma. Cancer 1999; 35: 1018- 25.

[106]Yen TC, Lin WY, Kao CH, Cheng KY, Wang SJ. A study of a new tumour marker, CYFRA 21-1, in squamous cell carcinoma of the head and neck, and comparison with squamous cell carcinoma an- tigen. Clin Otolaryngol 1998; 23: 82-6.

[107]Nagler R, Bahar G, Shpitzer T, Feinmesser R. Concomitant analy- sis of salivary tumor markers: a new diagnostic tool for oral cancer. Clin Cancer Res 2006; 12: 3979-84.

[108]Bahar G, Feinmesser R, Shpitzer T, Popovtzer A, Nagler RM. Salivary analysis in oral cancer patients DNA and protein oxidation, reactive nitrogen species and antioxidant profile. Cancer 2007; 109: 54-9.

[109]Shpitzer T, Bahar G, Feinmesser R, Nagler RM. A comprehensive salivary analysis for oral cancer diagnosis. Cancer Res Clin Oncol 2007; 133: 613-7.

[110]Pesce MA, Spitalnik SL. Saliva and the clinical pathology labora- tory. Ann N Y Acad Sci 2007; 1098:

192-9.

[111]Segal A, Wong DT. Salivary diagnostics: enhancing disease detec- tion and making medicine better. Eur J Dent Educ 2008; 12: 22-9.

[112]Wong DT. Towards a simple, saliva-based test for the detection of oral cancer. 'Oral fluid (saliva), which is the mirror of the body, is a perfect medium to be explored for health and disease surveil- lance.' Expert Rev Mol Diagn 2006; 6: 267-72.