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REVIEW ARTICLE

Potential biomarkers for the cytologic diagnosis of oral squamous cell carcinoma

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Unlike other deep cancers, oral squamous cell carcinoma (OSCC), which occurs in the oral cavity, is more easily monitored, and specimens for diagnosis are easier to collect. However, identifying reliable and sensitive markers for the early diagnosis and markers for identification of the recurrence of OSCC remains a challenge. This article mainly focused on reviewing recently identified potential biomarkers for a cytologic diagnosis during OSCC development. In addition, markers used in modern diagnostic technologies, including a sensitive labeling method and a photodynamic approach for cytologic diagnosis, are also described. Cytologic examinations using sensitive and specific biomarkers can improve the accuracy of the diagnosis. Hence, they can lead to appropriate treatment without delay and to the reduced recurrence of OSCC.

Introduction

Unlike other deep cancers, oral squamous cell carcinoma (OSCC), which occurs in the oral cavity, is more easily monitored, and specimens are easier to collect for diagnosis. However, the 5-year mortality rate with oral cancer, at about 50%,[1] has not significantly changed in the past 50 years despite advances in surgery, radiotherapy, and chemotherapy. This may be because most OSCC cases are diagnosed at a late stage, and no reliable early diagnostic markers are available. In addition, OSCC has a very high recurrence rate, and the early identification of recurrence or second primary tumors remains a challenge.[2] OSCC detection is currently based on an expert clinical examination and histologic analysis of suspicious areas, but the disease may be undetectable in hidden sites, and most oral lesions are often neglected by clinical health care providers. Therefore, cytologic sampling combined with sensitive and specific biomarkers for molecular detection may be the most convenient, low invasive and low-cost method for an OSCC diagnosis.

Cytologic diagnoses of malignancies originated with Papanicolaou and Traut, who introduced new methods for collecting and staining cells in gynecologic diagnoses.[3] During the 1980s, a brush was introduced for a smear biopsy for better cell spreading on objective slides, which was more convenient than a wooden spatula.[4] The cytologic observation and description may include the following features: enlarged nuclei, variations in nuclear

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Table 1. Modern methods of oral cytology for oral squamous cell carcinoma

Classification	Analytic method	Markers	References
Cytomorphometry	Conventional cytologic diagnosis	Cell morphology	3, 6, 7
	Computer-assisted image analysis (OralCDx)	Cell morphology	5, 8
DNA analysis	DNA image cytometry	DNA ploidy, DNA methylation	9, 10
Optical diagnosis	Autofluorescence	Flavin, tryptophan, elastin, collagen (endogenous fluorophores)	11
	Photodynamic diagnosis	5-Aminolevulinic acid	12, 13
		Photofrin	14
	Immunophotodiagnosis antibody targeting	EGFR	15

size and shape (pleomorphism), nuclear borders, a decreased nuclear to cytoplasmic ratio, increased number of nuclei (multinucleation), binucleation, keratinization, tadpole forms, and hyperchromatism. According to those criteria, a cytologic diagnosis can be staged as inadequate, negative, atypical, dysplastic, and tumor cell-positive. However, sometimes this may fail using a conventional cytologic diagnosis for OSCC because of the oral topology or a hidden lesion. In addition, it is difficult to access the deeper cell layer.

Adequate cell sample collection is essential for histomorphologic evaluations. Unlike other deep cancers, OSCC develops in the oral cavity and is much easier to monitor, and shed cancer cells in the saliva are easier to collect for diagnosis. In addition, the low invasive brush sampling to observe cancerous transformation is also convenient and easily applied. Furthermore, compared with other common diagnostic methods for cancer such as detecting biomarkers in body fluids, a cytologic diagnosis is more sensitive. Therefore, a cytologic diagnosis for OSCC is a noninvasive, convenient and ideal approach for screening high-risk patients with OSCC. In fact, application of liquid-based cytology on oral smears collected using a cytobrush showed a significant improvement in cell distribution and smear thickness, leading to easier identification of abnormal cells. The combined use of a cytobrush and liquid-based cytology that allows evaluation of the epithelial structure of oral epithelial carcinomas showed improved accuracy of diagnosis with a sensitivity of 95.1% and a specificity of 99.0%.[5]

Genomic and proteomic approaches have facilitated the identification of altered gene or protein expressions associated with OSCC. However, few such approaches have been integrated into clinical practice. In this report, we review recently identified cytologic markers that were histopathologically confirmed in patients with OSCC. These biomarkers might have higher potential to be developed as liquid-based cytologic diagnostic markers for OSCC and biomarkers in body fluids, such as the blood, urine or saliva, for detecting the recurrence of OSCC in deep areas. In addition, the sensitive labeling method and photodynamic approaches that might improve the accuracy of cytologic diagnosis of OSCC are also described. In addition, potential biomarkers that were correlated with typical behaviors such as betel quid chewing, which causes OSCC in Taiwan, are also included. Modern cytologic diagnostic methods and potential biomarkers for cytologic diagnoses of OSCC are summarized in Tables 1 and 2.

Modern methods of oral cytology for OSCC

Cytomorphometry

The OralCDx BrushTest is a computer-assisted method for the analysis of cellular samples collected using the brush biopsy. Using a neural network-based image processing system, this method can analyze digitalized microscopic images of collected cells to detect oral precancerous and cancerous cells. OralCDx was shown to have a sensitivity and specificity of $>90\%$.[48,49]

DNA image cytometry

DNA cytometry is a method to measure DNA ploidy. By comparing Feulgen dye-stained cytologic samples with normal epithelial cells, the malignancy of oral mucosal cells can be determined. Remmerbach et al.[50] reported that DNA aneuploidy might detect oral cancer 1–15 months prior to histologic confirmation in a clinical setting. In addition, it was also suggested that DNA aneuploidy could be an early marker to confirm the cytologic diagnosis of cancer in patients at high risk of dysplasia.[50,51] Sudbo et al.[52] analyzed archival material and reported that the nuclear DNA content of oral leukoplakia cells can be used to predict the risk of oral epithelial

Table 2. Biomarkers of oral cytology for oral squamous cell carcinoma

Classification	Analytic method	Markers	References
Extracellular matrix proteins	Immunochemical stain	laminin-5 γ2 chain	16
	Immunochemical stain	High-molecule-weight tenascin-C	17
	Immunochemical stain	Mini chromosome maintenance	18
Cell surface markers	Immunochemical stain	Syndecan-1	19
	Proteomics analysis	Mac-2-binding protein	20
	Immunochemical stain	Epithelial adhesion molecule	21, 22
	Immunochemical stain	γ2 chain of laminin-5 (lam-γ2)	23
	Immunochemical stain	Urokinase receptor	23, 24
	Immunochemical stain	Plasminogen activator inhibitor-1	23
	Immunochemical stain and proteomic analysis	EMMPRIN (CD147)	25, 26
	Immunochemical stain	CD44s and CD44v6	27
	Immunochemical stain	CD109	28
	Immunochemical stain	B7-H1	29
	Immunochemical stain	E-, P- and N-cadherin	30, 31
	Immunochemical stain	Endoglin (CD105)	32
	Immunochemical stain	P185 (HER-2/neu)	33
	Immunochemical stain	CD46, CD55, CD59	34
	Immunochemical stain	Podoplanin, human PA2.26 antigen (T1α-2, podoplanin)	34–36
	Immunochemical stain	LOXL4	34, 37
Cytoplasmic markers	LC-MS/MS, Western blotting	SERPINB1	38
	RT-PCR	PIN1	39
	Immunohistochemistry	HIF1α	40–42
	Immunohistochemistry	ANXA1	43
	Immunohistochemistry	RANK/RANKL	44
Macrophage	Immunohistochemistry and infiltrated cell count	CD68	40, 45
Cancer stem cells	Cell sorting	CD44, CD133, CD34	46
	Immunohistochemistry	Oct-4, Nanog, CD117, nestin, CD133, and ABCG2	47

LC = liquid chromatography; MS/MS = tandem mass spectrometry; RT-PCR = reverse transcriptase-polymerase chain reaction.

dysplasia up to 5 years before a histologic diagnosis is possible. In addition, a current study reported that promoter hypermethylation was associated with head and neck squamous cell carcinoma.[9] Using a DNA cytometric method, an increase in sensitivity and specificity of the oral brush biopsy to 100% for the early diagnosis of oral cancer was reported.[53]

In oral cancerization, patients with one oral lesion may be at increased risk of developing a further malignant tumor. But the detection of an early oral lesion is difficult owing to the innocuous appearance. The accuracy of diagnosing high-risk oral neoplasia may sometimes be poor. Several modern technologies, including the sensitive labeling method or photodynamic approach, were developed as an adjunctive method to oral cytologic diagnosis.

Optical diagnosis

An optical diagnosis was proven to be a reliable and noninvasive technique for detecting fluorescence in tissues that arises from a photosensitizer that is taken or endogenously occurs.

Autofluorescence

The use of autofluorescence to detect malignant lesions emanated from photodynamic therapy, a technique for cancer treatment. Autofluorescence describes the biologic characteristic of tissues possessing endogenous fluorophores such as flavin, tryptophan, elastin and collagen, which become fluorescent when exposed to certain wavelengths of light. The presence of disease can change the concentrations of these fluorophores as well as the light-scattering and absorption properties of the tissue, owing to changes in blood concentration, nuclear size distribution, collagen content, and epithelial thickness. The tissue is usually illuminated with a light source, mostly in the near-ultraviolet to green range of the spectrum. In the human oral cavity, the first investigation of *in vivo* autofluorescence

imaging was performed by Harris and Werkhaven[54] and Harris et al.,[55] who noted endogenous autofluorescence at around 630 nm in tumors of the oral mucosa. However, autofluorescence of normal tissues was also observed. The sensitivity of this fluorescence detection method was improved by an associated porphyrin injection for detection. Porphyrin, which may rapidly accumulate in tumors compared with that in the surrounding normal tissue, is the most commonly used target in autofluorescence-detecting oncology. However, porphyrin-based tumor detection might only be useful for diagnosing advanced tumors because of its high false-positive potential.

Photodynamic diagnosis

Photodynamic diagnosis of an oral carcinoma and precursor lesions mainly uses 5-aminolevulinic acid (5-ALA), which is a precursor of the fluorescent photosensitizer, protoporphyrin IX (PpIX), which can be administered systemically or applied topically to the oral mucosa and facial skin. Excessive accumulation of 5-ALA-induced PpIX results in the accumulation of intracellular porphyrins, especially PpIX, which increases the tissue fluorescence. When Sharwani et al.[12] used 5-ALA in the form of a mouth rinse to test 71 patient with clinical suspicion of leukoplakia to identify dysplasia or carcinoma *in situ*, a sensitivity of 83–90% and a specificity of 79–89% were obtained. Chang and Wilder-Smith[14] reported that a photodynamic diagnosis, Photofrin, was used topically to detect early oral cancer with a sensitivity of 92.45% in a macroscopic study and 93.75% in a microscopic study. The epidermal growth factor receptor (EGFR) is overexpressed in oral cancer. Soukos et al.[15] used an anti-EGFR monoclonal antibody coupled to the fluorescent dye, *N,N′*-di-carboxypentyl-indodicarbocyanine-5,5′-disulfonic acid, to detect a tumor-associated antigen of the EGFR. This method using a specific antibody coupled with a fluorescent dye improved the detection accuracy to specificities of 95.65% in the macroscopic study and 97.50% in the microscopic study. In addition, 5-ALA-induced PpIX accumulation might lead to selective killing of macrophages at the site of photodynamic treatment in OSCC[56] and downregulate the invasion.[57] Recent studies demonstrated improved sensitivity and specificity, and the potential for the immunophotodiagnosis of OSCC.

Molecular analysis using biomarkers for a cytologic diagnosis of OSCC

An immunocytochemical analysis of oral brush biopsies can recognize atypical cells by detecting altered protein expressions by tumor cells. In brush biopsies of OSCC, the following immunocytochemical patterns may be observed: (1) staining of cytoplasm, (2) banded markings between clumped carcinoma cells, and (3) positive hazes surrounding atypical cells. The immunocytochemical marking of atypical cells in brush biopsies makes it easier to locate suspicious cells, which facilitates the diagnosis and raises the sensitivity of the biopsy. Many proteins that were successfully detected by immunochemical staining and correlated with OSCC were reported and described as follows. These proteins may be potential biomarkers for a molecular analysis of oral cytology of OSCC.

During the malignant transformation of cells, the structure or expression level of some proteins in the extracellular matrix (ECM) may be altered. Transformation of squamous cell carcinomas with invasiveness and metastatic potential is associated with a poor survival rate. The cell surface molecules involved in cell migration and invasion might be potential markers for monitoring malignant phenomena. In addition, there is considerable interest in exploiting improved understanding of the biology of cancer cells to develop biomarkers capable of distinguishing malignant and dysplastic cells[18]. Using the minimally invasive brush biopsy, sufficient numbers of suspicious oral mucosal cells can be collected to search for potential biomarkers using genomic, proteomic or immunochemical staining approaches. Potential cell surface markers of OSCC were surveyed by clinical histopathologic immunochemical staining methods listed in Table 2.

ECM proteins and cell surface markers

Both the laminin-5 γ2 chain and tenascin-C proteins have key functions in the cascade of invasion and metastasis of OSCC.[58] Their expressions at both the messenger RNA and protein levels are significantly high.[16,17,59] Laminin-5 is a key protein of the epithelial adhesion complex that is involved in the process of invasion and metastasis. Significantly higher expression levels of those ECM molecules were demonstrated in cells of oral lesions in OSCC using the brush biopsy.[60]

In contrast to the intact laminin-5 molecule, the isolated laminin γ2 chain acts as a potent factor in cell migration and invasion. The laminin γ2 chain was found to be resynthesized in the squamous cell carcinoma invasion zone and eventually deposited in the stroma of the invasion zone.[61] Increased expression of the laminin γ2 chain was associated with poor survival in patients with OSCC.[62] The combination of conventional cytologic and immunochemical analyses using an anti-laminin γ2 chain antibody achieved a sensitivity of 93% and a specificity for brush biopsy diagnostics of up to 98%.[16] Formation of ECM with

a high tenascin-C content is a characteristic feature of a variety of carcinoma types.[17] The tenascin-C matrix is synthesized by the carcinoma cells themselves, especially by those in the invasion zone and in precursor lesions. In inflammatory oral mucosal lesions, excessive tenascin-C of the ECM is also seen. Unlike normal tenascin-C, it is a high-molecular-weight variant. By including high-molecular-weight tenascin-C detection, the cytologic analysis increased the sensitivity and specificity of the conventional biopsy diagnosis from 78% to 96% and 95% to 99%.[17] Chuang et al.[63] reported that high podoplanin expression was statistically significantly correlated with clinical nodal metastasis.

Cytoplasmic markers

Some cytoplasmic proteins which regulate oncogenic mechanisms may be potential targets for early tumor detection or tumor recurrence. The expression of SERPINB1 (serine protease inhibitor, clade B, member 1) was significantly higher in oral cancer cells with high motility, and the overexpression of SERPINB1 in invasive OSCC was clinicopathologically confirmed.[38] Overexpression of PIN1, a prolyl isomerase which regulates phosphorylation of Ser/Thr-Pro motifs, was associated with progression of OSCC.[39] Overexpression of hypoxia-inducible factor-1α (HIF-1α), which reflects the presence of hypoxia, was correlated with poor survival and tumor progression in patients with OSCC.[40–42,64–66] In addition, messenger RNA expression of annexin A1 (ANXA1) was found in the peripheral blood of patients with OSCC.[67] A proteomics approach demonstrated downregulation of ANXA1 in OSCC-derived cell lines.[68] More interestingly, the evidence of nuclear localization of the ANXA1 protein from the cytoplasm to nuclei was associated with poor survival of patients with OSCC.[43] Marked downregulation of plasma membranous ANXA1 was correlated with the poorly differentiated status of OSCC cells.[69,70] Monitoring of the nuclear translocation of ANXA1 can be a potential marker for a diagnosis and prognosis of OSCC. The receptor activator of nuclear factor-κB (RANK) and its ligand (RANKL), which are involved in osteoclastogenesis leading to bony destruction, were potentially associated with bony invasion in patients with OSCC.[44]

Marker cells

The presence of typical cells may be correlated with tumor progression. The infiltrating macrophage count was correlated with the progression of OSCC and is a prognostic marker.[40,45]

Cancer stem cells (CSCs) are tumor cells, which have stem features such as self-renewal, a high migration capacity, drug resistance, and high proliferation abilities. There is increasing evidence that the growth and spread of cancers is driven by a small subpopulation of CSCs, which are the only cells that are capable of long-term self-renewal and generation of a phenotypically diverse tumor cell population. Failure of cancer therapies may be due to quiescent CSCs that remain vital and retain their full capacity for tumor repopulation.[71] In addition, the development of new CSC-targeted therapy is hindered by the lack of reliable markers for identifying CSCs and a poor understanding of their behavioral determinates. A cytofluorimetric analysis targeting stem antigens may be an efficient method to identify and recognize CSCs. CSC-specific surface markers such as CD44, CD133 and CD34 have been used in CSC isolation.[46] Chiou et al.[47] found that enriched oral cancer stem-like cells highly expressed stem/progenitor cell markers such as Oct-4, Nanog, CD117, nestin, CD133 and ABCG2, and positive correlations of Oct-4, Nanog and CD133 expressions with the tumor stage were detected.

Biomarkers correlated with environmental carcinogens, areca (betel) chewing, and smoking in patients with OSCC

The prevalence of OSCC in Taiwan as well as in the other Asian countries is highly associated with betel quid chewing and smoking.[72–75] Chewing areca quid generates reactive oxygen species that might cause oxidative DNA damage to surrounding tissues in the oral cavity.[76] In addition, the areca nut extract can impair actin organization that causes fibroblastoid morphologic changes of oral keratinocytes.[77] The genetic susceptibility to such environmental carcinogens and the resulting altered molecular expressions might be potential markers for a diagnosis and prognosis of OSCC.[75] Studies in patients with OSCC associated with the use of betel nut and/or tobacco showed significantly altered expressions of genes and proteins (Table 3) such as the fragile histidine triad (FHIT),[80] p53,[81] loss of nuclear retinoic acid receptor-β,[88] EGFR, Her-2,[82,83] vascular endothelial growth factor (VEGF),[84] high-molecular-weight microtubule-associated protein 2 (hmw-MAP2),[98] and soluble receptor II for tumor necrosis factor-α (sTNF-RII),[85] downregulation of S100A2,[89] increased expressions of matrix metalloproteinase (MMP)-1[78] and MMP-8,[79] and increased activities of Src family kinases in invasive tumor fonts[86] and in smoking/betel-using patients associated with somatic mitochondrial DNA mutations.[87] In addition, chromosomal mutations, the polymorphism including CYP1A1 at exon 7 containing the

Table 3. Potential biomarkers for a cytologic diagnosis of environmental carcinogen-induced oral squamous cell carcinoma

Classification	Analytic method	Markers	References
Extracellular matrix molecule expression	RT-PCR, antibody neutralization and antisense oligodeoxynucleotides to reverse cell mobility	MMP-1	78
	Immunoblot analysis	MMP-8	79
Cytoplasmic markers	RT-PCR and immunochemistry	FHIT	80
	Immunohistochemistry	p53	81
	Enzyme immunoassay, ELISA, and immunohistochemistry	EGFR, Her-2	82, 83
	Immunohistochemistry	VEGF	84
	Immunohistochemistry	hmw-MAP2	84
	ELISA	sTNF-RII	85
	Immunohistochemistry	Src family kinase	86
	Temporal temperature gradient gel electrophoresis	Mitochondrial DNA mutation	87
Nuclear markers	*In situ* hybridization	Nuclear retinoic acid receptor-β	88
	RT-PCR, Southern blotting, immunochemical stain	S100A2	89
Chromosomal mutations	Polymorphism	CYP1A1	90
	Polymorphism	HO-1	91
	Polymorphism	Nuclear factor-κB	92
	Genomic hybridization	Chromosome 4p and 9q	93
	Q-PCR	CCND1 and CTTN	94
	PCR-restriction fragment length polymorphism	XRCC1, XRCC2, XRCC3, XRCC4	95, 96
	PCR-restriction fragment length polymorphism	Cyclooxygenase 2	97

RT-PCR=reverse transcriptase-polymerase chain reaction; ELISA=enzyme-linked immunosorbent assay; Q-PCR=quantitative polymerase chain reaction; PCR=polymerase chain reaction.

A/G (Ile/Val) genotype,[90] longer $(GT)_n$ repeat alleles in the heme oxygenase (HO)-1 promoter[91] and nuclear factor-κB promoter,[92] deletion of chromosomes 4p and 9q associated with poor outcomes of betel-using patients with OSCC,[93] and aberrant copy numbers of cyclin D1 (CCND1) and/or cortactin (CTTN) on chromosome 11q13 correlated to areca-associated OSCC[94] were also reported. Combinational polymorphisms of DNA repair genes of XRCC1, XRCC2, XRCC3, and XRCC4,[95] and the XRCC4 intron 3 delete genotype[96] were highly associated with people who have the betel nut chewing habit and are susceptible to OSCC; in addition, the polymorphism in the promoter of cyclooxygenase-2-1195A/A contributed to the development of betel-related OSCC.[97]

Biomarkers for oral cancers in Taiwan

In Taiwan, oral cancer has been one of the top ten causes of death from cancer since 1991.[99] The prevalence showed a 5.3-fold increase for men and a twofold increase for women over two decades.[100] In addition, the annual death toll for oral cancer in males has rapidly increased.[101] As described in the previous section, betel quid chewing and smoking are the two major risk factors associated with the prevalence of OSCC in Taiwan.[102–104] The expression of more than 20 biomarkers in betel quid chewing-related OSCCs from Taiwan was studied by immunohistochemistry in the past two decades.[21,41,43,105–121] In comparisons of protein expression levels in specimens of normal oral mucosa, oral epithelial hyperplasia and oral epithelial dysplasia, the majority of these biomarkers are overexpressed in OSCC specimens,[21,41,43,105–119] with very few biomarkers including $p27^{KIP1}$, metastasis-associated protein 1 and epithelial cell adhesion molecule (Ep-CAM) being downregulated in OSCC samples.[21,118,119] Overexpression of cyclin D1, p53, $p21^{WAF1}$, cyclin A, survivin, human telomerase reverse transcriptase (hTERT), HIF-1α, receptor-binding cancer antigen expressed on SiSo cells (RCAS1), ANXA1, histone deacetylase 2, and B-cell lymphoma 10 (BCL10) in OSCC samples were correlated with poorer overall survival of OSCC patients.[21,41,43,105–120] Downregulation of $p27^{KIP1}$ and Ep-CAM in OSCC

samples was also associated with poorer overall survival of OSCC patients.[21,118,119] In addition, overexpression of cyclin A, hTERT, HIF-1α, RCAS1, histone deacetylase 2, BCL10, ras p21, hepatocyte growth factor, c-met, erythropoietin, erythropoietin receptor, p34^{cdc2}, and cellular inhibitor of apoptosis protein 1 in OSCC samples was correlated with larger tumor sizes, more regional lymph node metastasis, and more-advanced clinical stages of OSCCs.[41,107,109–116,121] Furthermore, downregulation of metastasis-associated protein 1 and Ep-CAM was also related to larger tumor sizes, more regional lymph node metastasis, and more-advanced clinical stages of OSCCs.[21,119] Overexpression of cyclin A and BCL10 and downregulation of Ep-CAM in OSCC samples were associated with the recurrence of OSCCs.[21,107,121] Moreover, the overexpressions of hTERT and ras p21 were correlated with betel quid chewing and smoking habits of OSCC patients.[109,112]

Conclusion

In summary, cytologic observations are convenient and can be a noninvasive method for the diagnosis and prognosis of OSCC, since shed cancer cells can be collected from saliva in the oral cavity. Advanced oral cytology associated with a sensitive detection method using specific biomarkers as described in this review may improve the accuracy of the preliminary clinical inspection of oral cancer and the prognosis after treatment. Therefore, cytologic diagnostic methods may be valuable for screening high-risk cases of OSCC before further histologic confirmation. Larger-sized clinical studies are required to confirm the specificity and sensitivity of some of the markers listed in this review. This article provides a view of future work to search for useful biomarkers and put them into clinical use.

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