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NARRATIVE STUDENT REVIEW

Molecular Pathology Signatures in Predicting Malignant Potentiality of Dysplastic Oral Pre-cancers

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Abstract The role of dysplastic oral pre-cancers in oral squamous cell carcinoma development is well recognized, but the notion is not exclusive. Diagnostic gold standards in predicting malignant potentiality of such pre-cancers suffer from ambiguity due to inter- and intra-observer variability. In addressing such diagnostic challenges, combinatorial appraisement of molecular pathology attributes encompassing cancer hallmarks is thought to provide a wider analytical sense. Two major premalignant disorders, viz. oral leukoplakia and oral submucous fibrosis have been considered as candidate precursors of cancer here. This review highlights the molecular pathology signatures expressed in oral epithelial dysplasia and revisits the usefulness of combinatorial analysis of expressional pattern of existing molecular biomarkers in the context of proper selection of cardinal attributes from each cancer hallmark for better malignant potentiality assessment.

Keywords Oral epithelial dysplasia · Molecular pathology · Malignant potentiality

Introduction

Oral squamous cell carcinoma (OSCC), the sixth largest cause of death due to malignancy in the world [95], is a complex multistep phenomenon. Its progression from benign hyperplasia to dysplasia [68], to carcinoma in situ, and then into OSCC is reported [3]. Although oral epithelial dysplasia (OED) is considered to be an intermediate step for transformation of varied pre-cancerous lesions in oral cancer development, interestingly OSCC can develop from non-dysplastic lesions too [3]. Among many oral premalignant disorders (PMDs), premalignant lesion like oral leukoplakia (OLK) and premalignant conditions like oral submucous fibrosis (OSF) are commonly prevalent and histopathological evaluation of their biopsy is the diagnostic gold standard. However, this diagnostic approach suffers from non-specificity as well as intra- and inter-observer variability [96]. In spite of significant development in molecular pathology, the lack of specific molecular markers in predicting the malignant potentiality of PMDs is remarkable. Current research focuses on more selective and specific marker expression which can provide a better insight. As OED is also considered as a histopathologic marker of the malignant potentiality of PMDs [89], the molecular mechanism of dysplasia in different PMDs to assess malignant potentiality of PMDs will be reviewed in this article. The importance of a combinatorial approach will also be revisited to evaluate the expression profile of prime molecules in the context of cancer hallmarks.

Definition and Classification of OED

For OED, the initial definition provided by Pindborg (1977) and Lumermann et al. (1995) has not been accepted due to a lack of objectivity [3]. Architectural and cytological changes were considered as the major attributes of OED [54]. Grading of oral epithelial dysplasia is currently performed by interpretation of the epithelial features, like

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loss of maturation and stratification of intact epithelia as well as cytological changes, consisting of cellular atypia in the epithelial tissue of the oral mucosa. Loss of cell adhesion, cellular apicobasal polarity, hyperplastic basal cells, drop-shaped rete ridges consisting of keratin pearls, increased number of mitotic figures, atypical mitotic figures, abnormally superficial mitoses, anisonucleosis, anisocytosis, pleomorphism, increased nuclear size and nuclear-cytoplasmic ratio, increased number and size of nucleoli and hyperchromasia, and dyskeratosis are a few other epithelial features [96]. Current research suggests that, inclusion of DNA ploidy status analysis when combined with conventional histopathological OED grading can potentiate the malignant potentiality prediction [86]. There are still few other criteria which are considered during grading of OED which are not included in the cluster of features currently accepted to evaluate degree of dysplasia, but can potentiate the classification [90].

OED can be divided into three types according to severity; mild, moderate, and severe dysplasia [97]. Mild dysplasia is associated with minor architectural disturbance where only the lower third of the epithelium is affected with cytological atypia. In moderate dysplasia, architectural disturbance invades up to the middle third of the epithelium, as well as cytological atypia being increased, whereas severe dysplasia is characterized by architectural disturbance that extends to more than two-thirds of the epithelium with many other changes of oral mucosa [97]. Gradations of OED, based on histopathological observations, have been augmented nowadays with clinicopathological features, immunohistochemical examinations by useful molecular markers, as well as newer clinical diagnostic and treatment modalities [33, 66]. Even the malignant potentiality of a dysplastic condition can be predicted through characterized molecular pathological features. Nuclear translocation of β -catenin in upper layer, as well as simultaneous loss of E-Cadherin in basaloid cells in the lower layer of a dysplastic keratinized tissue, indicates increased recurring and malignant potentiality [4].Along with immunohistochemistry-based studies, the expression profile of protein biomarkers using RNA-based or proteomics studies is also now often considered for better assessment [79]. The basis of molecular expression studies for dysplastic PMDs will be emphasized with OLK and OSF. The definition of OLK, as amended in the workshop of WHO Collaborating Centre for Oral Cancer and Precancer in 2005 by the working group, is that: 'The term leukoplakia should be used to recognize white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer' [68], whereas OSF is a chronic, premalignant condition, characterized by progressive submucosal fibrosis inside oral cavity [57].

Current concepts to understand the molecular pathway of dysplasia in PMD and its correlation with malignant potentiality through expression studies of different molecular markers of PMDs are intriguing and rapidly evolving. In this review, cardinal molecules of cancer hallmarks will be highlighted to understand OED and associated carcinogenesis through mentioned dysplastic PMDs. An extensive literature search has described the expression features of genes in OED that could be indicative for malignant potentiality of the PMDs, but they suffer from non-specificity. Hence, combinatorial assessment of cardinal molecules from cancer hallmarks could be contributory and may be important in developing robust integrated biomarkers to assess the malignant potentiality of dysplastic PMDs. Table 1 shows the association between the genes involved in OED and cancer hallmarks. Figure 1 depicts the route of carcinogenesis through different grades of OED.

Molecular Pathology Features of Dysplastic Oral PMDs

Impairment of Epithelial Progressive Maturation and Epithelial Mesenchymal Transitions (EMT) Features

Deregulation in Epithelial Master Regulator and EMT Markers

If molecular expressions are taken into account in respect to development, proliferation, maintenance, and maturation of stratified epithelia, p63 is considered as a master regulator [21]. It has a major impact on stemness preservation as well [13]. Increased expression of p63 protein and mRNA, as well as of its isoform ΔNp63 is noted not only in dysplasia, but also in impairment of epithelial maturation with the severity of oral dysplasia [17, 21, 55]. It was also found to be associated with EMT, a fundamental biological process embedded in development, wound healing, and metastasis [20]. Further simultaneous elevation of ΔNp63, podoplanin, and intraepithelial inflammatory cells in OED is reported to indicate its high malignant potentiality [76]. Loss of E- Cadherin, another fundamental feature of EMT is also seen in OED caused by OSF (16). It is used to grade OED to avoid confusion arising from intra-observer and inter-observer variability [99]. The association between E-Cadherin–catenin complex, especially β- and γ-catenin expression are deregulated with the grade of dysplasia leading to cytoplasmic dislocation of E-Cadherin [21, 51]. In addition, P-cadherin expression was found to be upregulated in moderate and severe dysplasia but in dysplasia adjacent to infiltrating carcinomas it is lost [98].

When adhesion molecules are taken into account, reduced but extended expression of CD44s throughout the

Table 1 Genes involved in OED having association with Cancer Hallmarks

Carcinogenic feature/cancer hall mark	Genes involved
Epithelial mesenchymal transition and impairment of progressive maturation	CK1, CK2, CK4, CK5, CK6, CK13, CK14, CK15, CK17, CK19, CK17 and CK10, MCM2, Geminin[a], KI 67, P63, ΔNp63, PCNA, Cyclin D1[a] CD 44, CD44v5, CD44v6, CD44v7-8, E-Cadherin, P-Cadherin, β-catenin, γ-catenin, Laminin 5, collagen IV, collagen XVII (BP180), Perlecan, MMP-1, MMP-2, MMP-7, MMP-9 Moesin, α9 integrin, α6β4, α3β1, Claudin, TWIST[b], α-smooth muscle actin (SMA), Syndecan-1, Vimentin, Maspin[b]
Deregulation of apoptosis	P53[a,b,c], BCL-2, Survivin, Bax, TRAIL, DcR2, c-myc[d], p73[c], p14 ARF, c-jun[a,d]
Insensitivity to anti-growth signals and growth-stimulatory signaling proteins	p21, p16INK4a, PTEN, P57, p27(Kip1), FGF-2[b], FGFR-2[b], EGFR, FGF-1, TGF-α^2
Neo-angiogenesis	Furin, VEGF-C, VEGF-A, CD 105, NOS, VEGF 1

[a] Cell cycle control

[b] Angiogenesis

[c] Cell differentiation and proliferation

[d] Oncogenes, (References for the mentioned genes are endorsed in the relevant portion of the text)

dysplastic layers are noted which is indicative of malignant potentiality [10, 11]. Downregulation of CD44v7-8 is also known to be associated with increased malignant potentiality [8, 44]. Further CD44v6 is also reported to be downregulated in OED of PMDs [28]. In the context of impairment in the basement membrane, the focal breaks of laminin and collagen IV could be noted [78]. Discontinuous laminin 5 expressions along with increased expression of laminin 5 γ^2 chain, with slightly altered thickness of mucosa are observed in OED [37]. Increasing expression of collagen XVII (BP180), a component responsible for keratinocyte adhesion and motility, can also be correlated according to the grade of dysplasia and may be linked to carcinogenesis, as it is induced by a tumor promoter, PMA [65]. Perlecan, another basement membrane molecule present in the cell border of parabasal cells in normal mucosa, migrates to the surface layer and deposits in the cytoplasm as well as in intercellular spaces with increased degree of dysplasia [32]. The intercellular deposit of perlecan is known to induce MMP-7, a component having E-Cadherin lysis potential. The coexpression of increased MMP-7 and perlecan has a crucial impact on the progression of dysplasia toward carcinoma [67]. Further, it has been reported that the expressions of MMP-1 and -9 in oral dysplastic lesions are associated with carcinogenesis [36]. MMP-2 along with MMP-9 overexpression and the loss of basal lamina collagen IV α chain indicate progression toward malignancy [20, 88].

Abberation in Cytokeratin Expressions

During assessment of deregulation in epithelial progressive maturation in dysplastic PMDs, the expression profiles of different cytokeratins are important. Studies showed the upregulation of CK1, CK2, CK6, and CK10 in suprabasal layers along with the same trend of CK5, CK 14, and CK17 in both basal and suprabasal layers in OED. CK15 and CK19 show downregulation only in the basal layer [12]. Extension of CK19 expression from basal layer to suprabasal layer is also an indication of OED which is known to be a marker of premalignant changes of oral epithelium [47] and its use is suggested in grading of OED [75]. Further, there are reports of downregulation of CK4 and CK13 in the suprabasal layer, where the former was found to be more specific, while marked loss of the latter is an indicator for dysplastic transformation, and its progression [44]. Another study showed increased CK 4 and CK 13 overexpression in OED [63], and interestingly, despite being a biomarker of dysplasia, downregulation of these two cytokeratins and cornulin shows ambiguity in the prediction of malignant transformation [79]. However, their aberrant expressions were found to be linked with impaired epithelium maturation too, with increased cell motility along with altered CK6, CK16, and CK17 expressions [77].

Cell Growth and Adhesion Markers

Integrins, the cell surface receptors responsible for cell–cell and cell–extracellular matrix adhesion as well as cell growth are important in assessing OED. The α9 integrin was found to be upregulated [30] and suprabasal expressions of α6β4 [27] and α3β1 were found to be altered [67]. In OED, cytoplasmic localization of claudin, one of the main tight junction forming proteins indicates loss of activity. It can be correlated with moderate to severe [15]. Basal and suprabasal TWIST expression, a downstream protein of FGF, in dysplastic OLK indicates malignant

transformation [83]. Interestingly, while one reported no expression of alpha-smooth muscle actin (SMA) and vimentin in OED, another study finds SMA expression in high-risk OED [16, 23].

Cell Proliferation Markers

Cell proliferation is a fundamental criterion in maintaining epithelial functional homeostasis. The important epithelial proliferation markers, viz. MCM2, geminin, and KI 67 were found to be elevated in OED. MCM2 possesses efficiency for indicating malignant potentiality [91]. In dysplastic OLK, KI 67 is overexpressed, and expression was reported up to the superficial layer [48]. Frequency of upregulation of suprabasal KI67 expression in dysplasia adjacent to OSCC indicates severity of the lesion [29], whereas coexpression of KI 67 and p53 with increased dysplasia points toward increased chances of malignancy [31, 43]. Centromere-associated protein CENP-F, and another two proliferation markers, KI 67 and cyclin D1 are also upregulated in OED, and mainly in the superficial layer of dysplastic OLK [49]. Cyclins and cyclin-dependant kinases (CDK) are the important factors for cellular proliferation. Cyclin D, is found to be overexpressed in OSCC, but not in dysplastic epithelia, whereas Cyclin E is overexpressed in severe dysplasia and its progression. Again overexpression of cyclin D1 was also reported by many [49, 74]. Interestingly, CDK2 is not expressed in normal or dysplastic epithelium, but overexpressed in OSCC, whereas CDK4 expression is parallel with the degree of dysplasia in cases of OED [82]. Expression of HER2/neu was not found to have any contributory effect on OED-associated carcinogenesis [80] and its expression was found to be very low in OED too [25].

Neo-Angiogenic Attributes

Neo-angiogenesis is one of the major hallmarks of cancer. VEGF expression study and vessel counting are the major methods still followed for angiogenesis determination in PMDs [56]. Most of the studies suggest a positive correlation between increasing grade of dysplasia with increasing VEGF expression from basal to suprabasal layer and increased microvessel density (MVD). Conflicting reports are also noted about such correlation [18]. Interestingly, another study indicated that though expression of VEGF-A did not correlate with grade of dysplasia in PMDs [34], the mean MVD was greater in dysplastic conditions than non-dysplastic ones [62]. VEGF-A is also found to be more upregulated in dysplastic OLK than non-dysplastic OLK [26, 62]. Further an increase in furin and VEGF-C expression during normal to dysplastic change of the oral mucosa is considered important as increase in new blood vessel formation was detected. It also indicates the presence of neo-angiogenic switch in OED [69]. Study of tumorigenic angiogenesis through molecular expression of CD105 or endoglin is the current method to determine the malignant potentiality. In dysplastic OSF, its expression was found to be upregulated [7, 21]. Other important modulators of neo-angiogenesis, like, fibroblast growth factor (FGF), transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, etc. [93], also manifest their role in growth stimulation. FGF-binding protein- 1, (FGFBP-1) may be considered as one of the important angiogenic factors which can induce FGF-2 and VEGF-1, which are found to be upregulated in angiogenesis [67]. FGF-2 and FGFR-2 mainly express in the basal and parabasal layers, but in OED, it extends to the entire level with increased severity and there is subsequent loss of FGF-1 [94]. TGF- α level also increases with increasing dysplasia [87]. Enzyme nitric oxide synthase (NOS), a component responsible for synthesis of NO and induced NOS, may have role in angiogenesis and is related to dysplasia; the expression of the latter is found to be increased with severity of OED [14, 73]. Decorin, a leucine-rich proteoglycan has a role in both proliferation and angiogenesis, is also overexpressed in OED [9] nuclear localization of which may indicate increased malignant potentiality [22].

Deregulation of Apoptosis

Apoptosis is a very complex phenomenon, which occurs by two major pathways, intrinsic and extrinsic. Apoptotic proteins, consisting of anti-apoptotic and pro-apoptotic factors regulate apoptosis in cells. Bcl-2-associated x proteins (Bax) like Bax, Bad, Bak, and Bcl-xS are pro-apoptotic and Bcl-2 and Bcl-xL are anti-apoptotic proteins. p53, an important regulator of genomic integrity initiates the molecular cascade of apoptosis, or activates DNA repair when required. c-myc, a proto-oncogene can also induce apoptosis with p53 [38]. In PMDs, suprabasal expression of p53 along with an increase in dysplasia is an indicator of malignancy [5, 19]. Reddy et al. suggested moderate and severe dysplastic lesions showing intracellular localization of p53 indicating increased malignant potentiality [71]. Kerdpon et al. also found increasing expression of p53 in normal to dysplasia to OSCC, and thus suggested its association with carcinogenesis [40]. Abraho et al. found p53 expression in the entire dysplastic area of oral lesions, but did not find any correlation in expression and the degree of dysplasia [1]. p73, a functional homolog of p53 also induces apoptosis and overexpression from the basal to suprabasal layer during normal to dysplastic to OSCC [17].

The expression profile of BCL-2 is controversial in OED. Loro et al. found decreased expression of BCL-2 in the basal layer of OED, but in severe dysplasia the loss is

less pronounced [53], whereas Singh et al. suggested that an increase in the expression of BCL-2 is directly proportional to the degree of epithelial dysplasia, but is downregulated in differentiating carcinomas [84]. Upregulation of BCL-2 protein in dysplastic epithelium adjacent to an invasive tumor may also indicate its role in malignant transformation [35]. BCL-2 is also expressed in OLK with apparent dysplasia [70]. The expression profile of Bax in dysplastic lesions is similar to the normal ones, but with an increased degree of dysplasia, an inverse relationship is observed in Bcl-2/Bax ratio [52]. Survivin, an inhibitor of apoptosis was also found to be overexpressed in OED [64]. One study suggested increased expression of the component, while another study suggested p34cdc2-cyclin B1-mediated phosphorylation of survivin on Thr34 as the underlying cause of carcinogenesis [100, 101]. A proto-oncogene c-jun, important in apoptosis as well as cell cycle regulation is increased in OED, according to the degree of dysplasia [92]. The main executioner caspase of apoptosis, caspase 3 expression is not affected in dysplastic tissues [46].

Molecules Related to Growth-Stimulatory and Anti-growth Signaling Proteins

Self-sufficiency in growth signals and insensitivity to anti-growth signals are considered as two major hallmarks of cancer. Epidermal growth factor receptor (EGFR) and its ligand, Transforming Growth Factor alpha (TGF-α), and their receptors are few which are upregulated in carcinomas, but their expression is also dysregulated in OED. Two- to fourfold of EGFR gene amplification is found in OED [58]. In OLK, one study suggested linear correlation between EGFR overexpression in stratum spinosum with increasing degree of dysplasia [87]. Another stated that its expression is not associated with dysplasia. It did not also vary between smokers and non-smokers, and there was a positive correlation with malignant potential [72].

A number of events, namely loss of heterozygosity, hypermethylation, deletion, and mutation are found to be associated with OED. Heterogeneity in p21 expression in PMDs, along with overexpression in proliferating dysplasia, suggests its role in oral oncogenesis [2]. Both p16INK4a and p14ARF are inactivated in OED, but to a greater extent in the former which is found to be inactivated in dysplastic oral epithelial lesions by the deletion of exon 1α of the CDKN2A gene. This gene encodes p16 and p14ARF, and is supposed to be more evident in conversion from dysplasia to malignancy [81]. In proliferative verrucous OLK, loss of p16INK4a and p14ARF is more than other dysplastic OLK lesions with elevated p14ARF exon 1β deletions [41]. A cell cycle inhibitory protein p57 expression is downregulated in OED and leads to carcinogenesis [24]. Downregulation of p27 (Kip1) along with p53 indicates an increase in the malignant potentiality of OED [42].

Role of Combinatorial Analysis of Molecular Expression in OED

In the context of value addition to analytical approaches for high-precision pathological grading of malignant potentiality of OED, association to biomarkers needs evaluation from different dimensions. Recent studies focus on the development of risk assessment tool using a combinatorial grading through computational analysis of clinico-epidemiological, demographic, and molecular perspectives [45]. When only clinicopathological aspects are considered, malignant potentiality could be assessed only after follow-up studies using the Kaplan–Meier method and log-rank test [39, 50]. Oral cancer-free survival (OCFS) studies in the OED are another approach. The study is determined using time-to-event analysis. Previously concurrent salivary marker detection was proposed as cancer diagnostic tool [59]. Analysis of gene profile data through multivariate predictive models generated for assessment of malignant potentiality has shown better prediction than existing models generated by clinicopathological risk factors [39]. However, it is a costly alternative. Hazard ratio analysis—when considered alone as a tool for the selection of biomarkers can assess increment in malignant potentiality, whereas Cox proportional hazard model was proposed to utilize clinical, demographic, and molecular factors to predict malignant potentiality [39]. The backward variable selection method was also used to generate a tool for assessing malignant potentiality of OED through selecting optimal combinations of biomarkers from biomarkers from the EGFR pathway in a recent study [60]. This method has limitations as it is confined within a particular hallmark. Utilization of tissue microarrays (TMA) for biomarker discovery is a novel, but a costly approach [60]. A recent study utilized computer-based stain separation techniques for intensity quantification in immunohistochemistry studies in search for molecular selection to predict malignant potentiality too [6]. So, when follow-up studies are not possible, or multilevel data required for such above-mentioned models are scarce, the proposition intends to hypothesize a molecular pathology-based computational tool for selection of cardinal biomarkers from different cancer hallmarks for better malignant potentiality prediction of oral PMDs, considering the role of each biomarker expression pattern noted in different studies associated with cancer hallmarks. The concept has been depicted in Fig. 2.

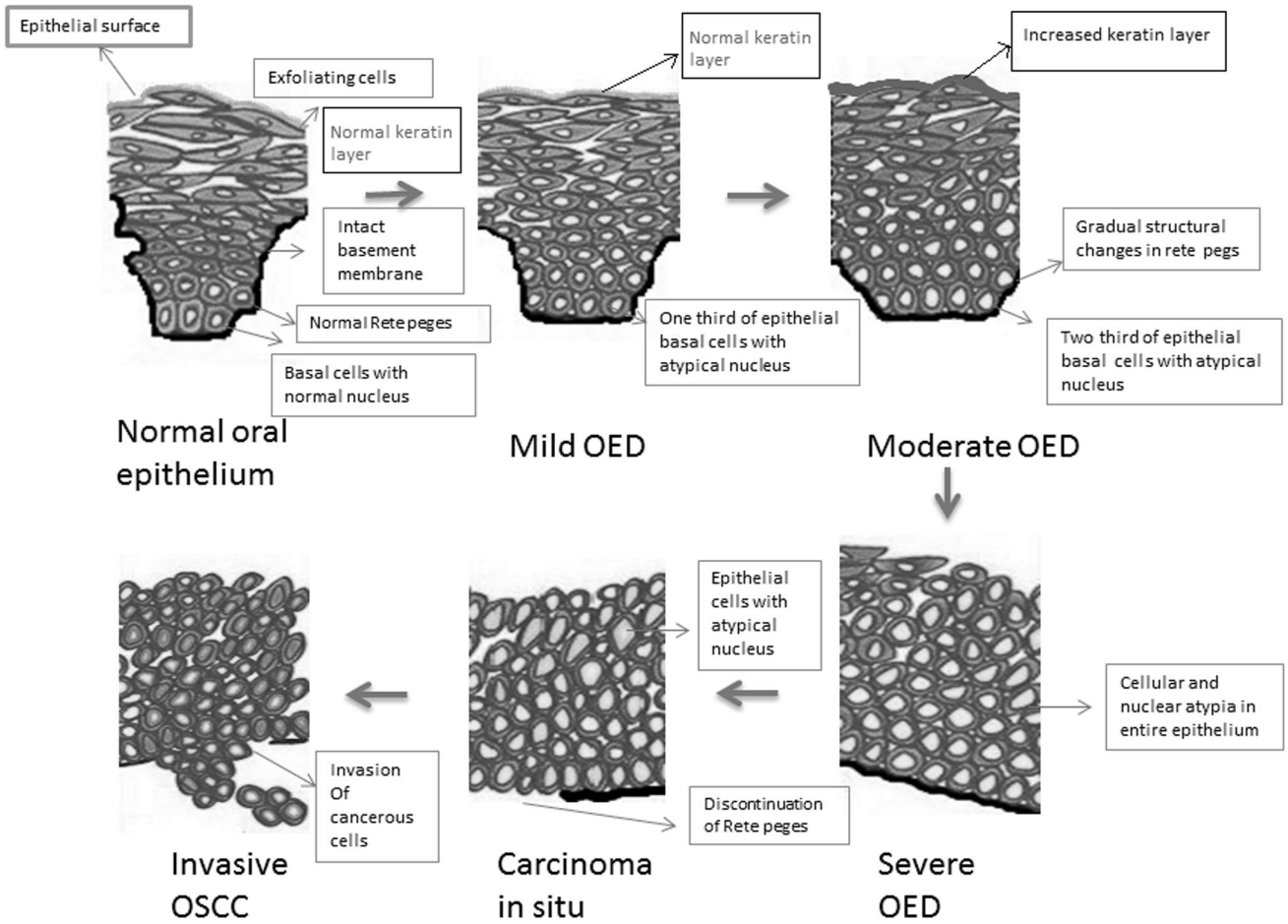

Fig. 1 Stages of carcionogenesis through different grades of OED

Conclusion

Proposing a specific biological threshold for predicting the transformation of PMDs with OED into carcinoma still suffers from ambiguity and is an open diagnostic challenge. Molecular expression studies provide information about the important roles of the genes and proteins expressed in the progression of dysplastic condition to carcinoma. Indeed, biomarker studies can help to find pathobiological thresholds to predict the irreversible changes towards malignancy. Differences in the reagents and protocol used, the clinical condition of the study subjects, site of biopsy taken etc., are of immense value in the context of addressing controversies by using the information on expression of biomarkers in OED and their significance. Each of the individual cancer hallmarks is often connected by multiple biological pathways and is interrelated. Even many molecules have a role in multiple biological mechanisms. Utilization of more than one of those molecules, especially cardinal molecular attribute to the cancer hallmarks, may provide an overall picture of onset of dysplasia and its progression toward malignancy. For example, abnormality in the onset of dysplasia was diagnosed by simultaneous expression study of p16/pRb/cyclin D1 [85], similarly many other pathways can be utilized by this way. Studies have also encountered the simultaneous expression of more than one biomarker. Another study showed that dual p53/p16INK4a and triple p53/p16INK4a/Ki-67 aberrations in dysplastic OLK have higher malignant potentiality [61]. Inclusion of mathematical modeling studies like principle component analysis also helps in the selection of two to three specific markers for accurate diseases grading. Therefore, implementation of conventional histopathological study along with multiple molecular expression features may help the pathologists to design combinatorial diagnostic markers for early diagnosis of malignant potentiality of OED in PMDs. The high-precision oral dysplasia grading embedded with a combinatorial approach will thus enable medical practitioners to predict the malignant potentiality with irreversible changes toward carcinoma (Fig. 2).

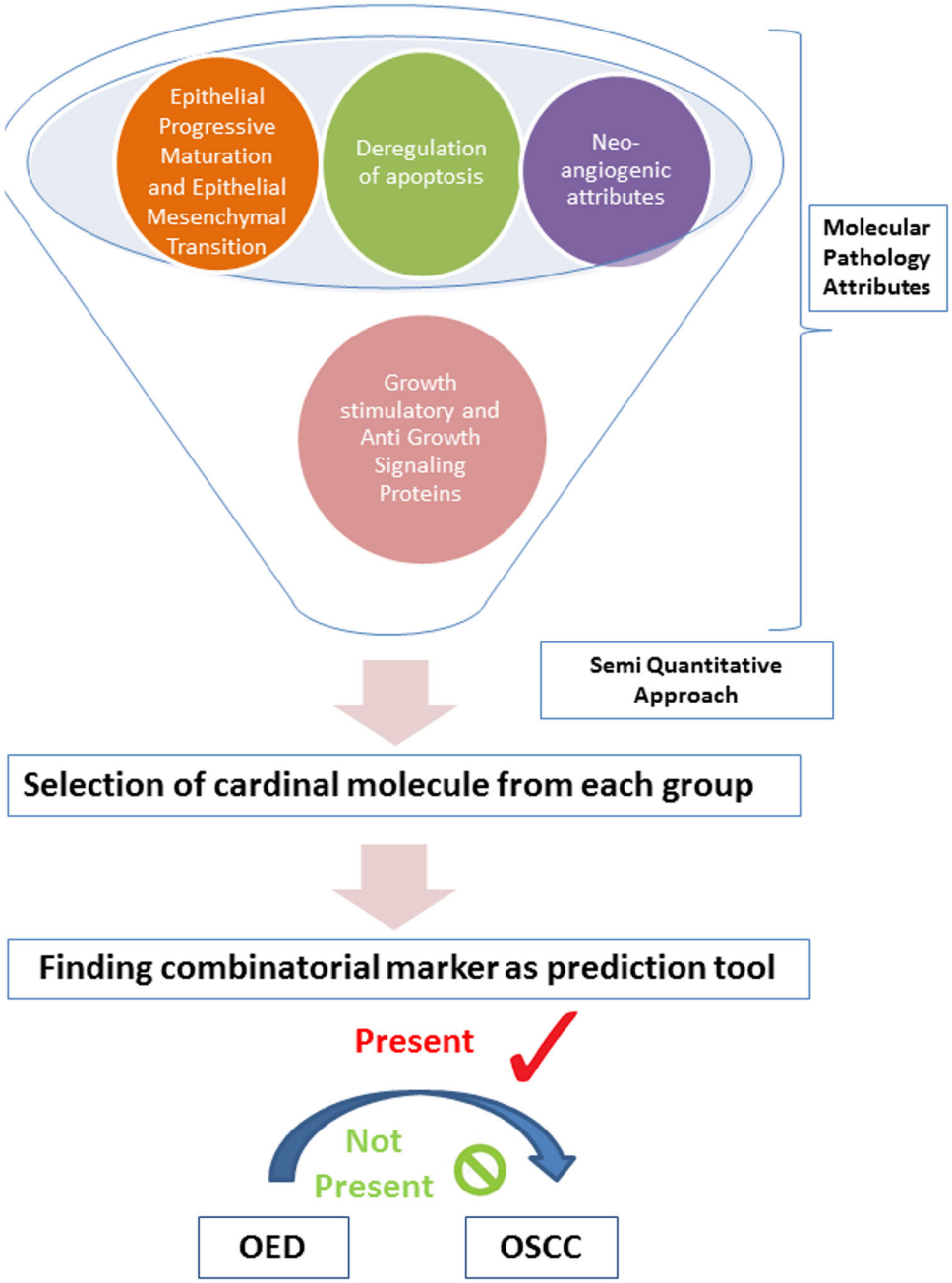

Fig. 2 Proposed model to predict malignant potentiality of OED using molecular pathology attributes

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