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Original Article

Morphometric approach to decipher the Verrucous Carcinoma - oral squamous cell carcinoma enigma using fractal geometry

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Abstract

Introduction: *The entire continuum of Oral potentially malignant- malignant disorders is dogged with numerous uncertainties during their diagnosis. Particularly affected by these indecisions and predicaments are verrucous hyperplasia, verrucous carcinoma and squamous cell carcinoma.*
Substantial and objective studies are required in order to better define the biologic behavior and prognosis of these lesions and to distinguish closely resembling lesions from one another.

Aim: *To evaluate and compare nuclear fractal dimensions (nFD) & fractal dimensions of epithelial connective tissue interface (eFD) for -Leukoplakia histopathologically diagnosed as Oral Epithelial Dysplasia (OED), Oral Verrucous Carcinoma (VC) and Oral Squamous Cell Carcinoma (OSCC).*

Materials & Method: *60 archived paraffin embedded tissue sections stained with H&E which were segregated into 3 groups – OED (20), VC (20), OSCC (20). Five fields each at low power (10x) and high power (40x) were captured for each section using Magnus Image-Pro System. Histo-morphometric analysis of nucleus and ECTI was carried out with the help of Computer aided image analysis software - Image ProPremier 9.1.*

Results: *The nuclear fractal dimensions for OED= 1.1151, VC=1.2012, OSCC= 1.2493 were detected. Analogous trend of increasing fractal dimensions was observed with respect to the ECTI of OED=1.0962, VC=1.1266, OSCC= 1.1357. The statistical analysis was done using one-way ANOVA and the p value in both the groups was found to be <0.005 (statistically significant).*

Conclusion: *Fractal dimensions may be used as a single digital marker to asses nuclear abnormalities and the abnormalities of the ECTI in order to solve ambiguities in diagnosis of OED, VC and OSCC and also to predict their biologic behavior.*

Keywords: *Fractal diamensions, digital imaging, epithelial-connective tissue interface, nuclear morphometry*

Key Messages: *The fractal dimensions of the nucleus of the neoplastic cells and the changes occurring at the Epithelial- connective tissue interface of OED, VC and OSCC were computed and correlated to their pathobiology. The purpose of this study was to use morphometry in order to point towards a substantial diagnosis whenever histopathology is insufficient to rule out ambiguous presentation. A similar study with a larger sample size should be undertaken to reinforce the findings obtained in the current study.*

Introduction

Discrepancies of the oral epithelium may present as a continuous spectrum of clinical and histopathological manifestation, ranging from simple hyperkeratosis, oral epithelial dysplasia (OED), verrucous hyperplasia (VH), verrucous carcinoma(VC) and conclude with invasive squamous cell carcinoma. Often these entities present with overlapping histopathological findings which may result in diagnostic uncertainties, holding the pathologist back from making an accurate diagnosis[1].

The entire spectrum of Oral premalignant-malignant disorders is plagued with innumerable ambiguities. Particularly affected by these uncertainties and predicaments are verrucous hyperplasia and verrucous carcinoma. Among the many squabbles, the ones which have garnered the most attention include-

1) Presence of histological inconsistencies in the literature regarding the presence or absence of dysplasia; diagnosing it as VH or VC?
2) Labelingverrucous carcinoma as "Carcinoma" in presence of intact basement membrane and lack of cellular atypia.
3) Prognosis of verrucous carcinoma- is it truly good or should we be looking at it as a precursor of OSCC? [2]

Upon referring to the available literature, it was perceived that some authors consider VH as a morphologic variant of VC owing to a close histologic similarity between the two entities.

Others often regarded it as an irreversible precursor of the carcinoma, albeit with many of the biologic consequences of verrucous carcinoma. However, Batsakis JG in 1999 put forth a compelling opinion stating that the term "hyperplasia" may fall short to succinctly convey the aggressiveness of the lesion. And hence, when in dilemma, VH should be denoted as VC.[3]

Similar vague clarifications were encountered for the other two questions as well.

Rationale

The malignant transformation of a premalignant lesion is a multi-step alteration wherein numerous cellular and molecular processes are deranged. This instability paves way for altered cellular metabolism, replication, signaling etc. resulting in various structural and sub-structural modifications.

Nucleus of a cell is a primary site for these alterations. The nuclear changes may range from irregularities of nuclear lamina and nucleoli to presence of coarse chromatin texture, presence of heterochromatin etc[4,5]. By the same token, the cell invasion mechanisms viz. individual cell migration and collective cell migration, results in irregularities of the epithelial connective tissue interface (ECTI)[6]. These fluctuations become increasingly apparent as the lesion progresses along the premalignant – malignant spectrum. The tumor invasion front is the hub for all these mutational activities[7]

Thus, newer pertinent studies employing the advances in routine pathology should be explored in order to better comprehend and predict these changes.

Aim

To evaluate and compare nuclear fractal dimensions (nFD) & fractal dimensions of epithelial connective tissue interface (eFD) for - Leukoplakia histopathologically diagnosed as Oral Epithelial Dysplasia (OED), Oral Verrucous Carcinoma (VC) and Oral Squamous Cell Carcinoma (OSCC).

Materials & Method

Study sample comprised of 60 archived paraffin embedded tissue sections stained with H&E which were segregated into 3 groups – OED (20), VC (20), OSCC (20). Five fields each at low power (10x) and high power (40x) were captured for each section using *Magnus Image-Pro System*.

Histo-morphometric analysis of nucleus and ECTI was carried out with the help of

Computer aided image analysis software - *Image ProPremier 9.1*

Algorithm for computing the Nuclear Fractal dimensions (nFD) and the Fractal dimensions of ECTI (eFD)–

1. Image selection &
2. Selecting Region Of Interest
3. Apply red filter
4. Subtract bright background
5. Adjust threshold histogram
6. Select object & background
7. Give count command.

Results

The nuclear fractal dimensions for OED= 1.1151, VC=1.2012, OSCC= 1.2493 were detected. Analogous trend of increasing fractal dimensions was observed with respect to the ECTI of OED=1.0962, VC=1.1266, OSCC= 1.1357. The statistical analysis was done using one-way ANOVA and the p value in both the groups was found to be <0.005 (statistically significant).

Discussion

Oral squamous cell carcinoma is one of the very few malignancies that are preceded by a clinically and histopathologically detectable phase- "the potentially pre-malignant lesions". Although these lesions may be predictive, they do not suggest invariability and there can be considerable inter- and intra-examiner variation in their diagnosis as well [8]. Thus, utmost significance has been given in predicting the malignant transformation as well as the biologic behavior of these entities.

In the last couple of decades, morphometry has emerged as a steadfast tool in studying different parameters of pathology. It has enabled conversion of subjective data into quantifiable data. Various morphometric parameters such as cell size, cell area, nuclear size, nuclear area, aspect ratio, cell circumference have been extensively studied in the past[9]. Amongst these, fractal geometry is a relatively newer division that is being explored by pathologists for its efficacy.

In an attempt to comprehend Fractal dimensions, it is obligatory to understand the concept of "fractals". Derived from the Latin word fractus ("fragmented," or "broken"), the term denotes the reiteration of details or patterns that occur at progressively smaller scales and can continue indefinitely, so that each part of each part, when magnified, will look like a fixed part of the whole object[10]. To simplify, fractal dimensions measure the degree of complexity or irregularity of self-similar objects.

Cross and Cotton in 1992 hypothesized that fractal dimension will be beneficial in discerning between different diagnostic categories and may also allow more precise prognostic stratification within single diagnostic groups[11].Fractal geometry has found applications in quantifying quite considerable histopathological parameters such as assessing the tumor microenvironment through nuclear alterations, lymphatic and vascular networking, ECTI of the malignant lesions etc.[7, 12,13]

One of the earlier studies appraising the effectiveness of FDs in malignancies was conducted by Sedivyand Windischbergerin 1998. They concludedthat use of fractal analysis might provide a specific determinate value of the growth pattern of a tumor[14]. In the subsequent year, the same authors also published their findings on the applicability of FDs as an objective method for detecting atypical nuclei in dysplastic lesions of the cervix[15]. Later, in *2015Waliszewski Pet al* published a study wherein they analyzed the relationship between tumor structure and complexity of the spatial distribution of cancer cell nuclei of prostate carcinoma with the help of fractal geometry[16].

Application of fractal geometry in OSCC was assessed much later. Various tumor parameters such as nuclear morphology, vascularity, and epithelial-connective tissue interface etc. were analyzed by different researchers. *Khandekar S et al in 2013* conducted a pilot study to assess the fractal dimension of ECTI of normal mucosa and OSCC and found an extremely statistically

significant difference between the diseased and healthy tissue[13]. On similar lines, *Cristopher V (2015)* studied histomorphometric parameters such as nuclear diameter, nuclear area, cell area, and nuclear cytoplasmic ratio and observed them to increase significantly in OED, VC, and OSCC patients[17]. FD in different stages of OSCC, and their correlations with clinic-pathological factors and patient survival were examined by *Mincione et al in 2014* [18].

In agreement to the previously performed studies, *Phulari RG et al (2016)* also concluded that nFD can offer reliable data to differentiate between normal mucosa, dysplasia and carcinoma objectively without subjective discrimination[19].

The perplexity that belies the VH, VC and OSCC has also been approached through few different trails such as immunohistochemistry using panel of several biomarkers such as p53, matrix metalloproteinase-1, E-cadherin, Ki67 (*Klieb HB;2007*)[20.] A more pragmatic line of immunohistochemical investigation was attempted by *Paral KM et al* in 2014. They evaluated a panel of stromal markers such as CD34 and α Smooth muscle actin (sma) to delineate VH from VC and OSCC. A strong positivity for α-sma was exhibited by OSCC and VC as opposed to the weak expression by VH and conversely, CD34 was positive for VH and an insignificant expression was shown by VC & OSCC. Loss of CD34+ dendritic cells, together with a gain of α-SMA+ myofibroblasts, supports a diagnosis of OSCC and VC, while the reverse reaction supported VH[21].

Our study is a forerunner of its kind in commissioning fractal dimensions to demystify the conundrums which have plagued VC in terms of its nomenclature, clinical course and prognostic considerations. As was seen in our results, the difference between the fractal dimensions of the entities was significant. However, the numerals of FD for VC and OSCC were fairly akin, further hinting towards the malignant nature of VC.

The irregularities of the nucleus that resulted in increased FD as the lesion progressed from OED to VC to OSCC were attributed to a plethora of factors. *Pianese G in 1896* reported that hypertrophic and irregularly shaped nucleoli were characteristic of malignant cells[22]. So was the conversion of normal, centrally placed, inactive euchromatin to active, peripherally placed heterochromatin which lead to the irregularities of nuclear lamina. Along with these increased number of micronuclei, aneuploidy, chromatin clumping and chromosomal aberrations were some of the cytogenetic checkpoints contributing to increased nuclear anomalies etc[5, 23, 24].

On the other hand, the alterations disturbing the stability of the ECTI vary for all the three entities. FD of OED is a reflection of focal epithelial proliferation and expansion and proteolytic remodeling of the lamina propria[25]. According to the review of *Salo T et al 2014*, tumor spread critically relies on extracellular matrix proteolysis, mediated by tumor microenvironment (TME) cells. Different TME key components such as carcinoma-associated fibroblasts (CAFs) and inflammation (CAI) cells, angiogenesis, stromal matrix molecules and proteases are the proposed perpetrators behind this remodeling[26].

In VC, the cells remain coupled by cell-cell junctions at the leading edge inside the moving cell group wherein a relatively small proportion of ‘‘trail-blazing’’ cells often act to guide others. Also of interest is the fact that a very small population of actively ‘‘invasive’’ cells is needed to cause a transition to collective motion of a large body of cells[6,26]. The translocation of cells occurs through physical coupling. The activity of actin-rich lamellae in multiple cells along or underneath the collective cell mass also aid in their movement. The secondary remodeling of the extracellular matrix along the migration track mutually leads to the formation of a basement membrane or the widening of a 3D track to encompass an increasing volume of the cell mass[27, 28]. Thus, offering a plausible explanation for the patent integrity of the basement membrane which is pathognomic to VC.

And lastly OSCC, in which extensive studies have been conducted to elaborate the tumor invasion and infiltration. These modifications have been attributed to several factors such as change in the cytoskeleton assembly, formation of lamellopodia or Invadopodia, epithelial-mesenchymal transition, mesenchymal-ameboid transition etc[29, 30]. Thus, the interplay between two dominant mechanisms i.e. collective cell migration and single cell migration are responsible for the variable phenotype of cancer invasion [26, 31, 32.]

Our study aimed at assessing and relating both the components of the tumor invasion front i.e. the nuclei of the active tumor proliferating cells as well as the ECTI at TIF. This correlation was attempted in order to solidify our perspective to the OSCC-VC dilemma.

Conclusion

Our study was an attempt to compare the nuclear alterations and ECTI of OED, VC and OSCC using fractal geometry which, as demonstrated by *Bose P et al, 2015* was a reliable single digital integrate to assess the tumor[7]. Thus comparing the nFD and the eFD of VC with those of OED and OSCC was a rational way of ascertaining its pathobiology. The analogous yet different value of VC & OSCC backs up the malignant nature of VC at the same time implies a better prognosis than OSCC.

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