Original Article / Özgün Araştırma

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Fractal Analysis of Nuclear Architecture in Oral Squamous Cell Carcinoma by Using Transmission Electron Microscopy: An Original Research

Oral Skuamöz Hücreli Karsinomda Nükleer Mimarinin Transmisyon Elektron Mikroskobu Kullanılarak Fraktal Analizi: Orijinal Bir Araştırma

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Abstract

Objective: A tumor's histological traits can be digitally assessed and quantified using fractal dimension (FD), a mathematical measure of a shape's irregularity and complexity. The goal of this study was to examine and contrast the nuclear FD between normal and oral squamous cell carcinoma (OSCC).

Materials and Methods: The present study comprised 15 OSCC patients and 15 patients in an age- and sex-matched control group. Fresh biopsy was taken from both groups reported over a period of one year at St. Joseph Dental College, Duggirala, Italy. Half of the tissue sample was used for transmission electron microscopy and the remaining half for H&E staining for grading. Fractal box counting on the ImageJ program serves for the analysis of images in the assessment of nuclear architecture. The FD of the nucleus was calculated in accordance with the Sarkar fractal box counting method.

Results: Results showed a statistically significant increase in the mean nuclear FD value of OSCC compared with normal mucosa. The minimum FD value of the nucleus obtained for normal mucosa is 0.7516 and the maximum value is 1.7982, whereas for OSCC, the minimum FD value of the nucleus is 1.8230 and maximum value of 1.9587. Mean ± standard deviation value of FD of the nucleus in normal mucosa is 1.5806±0.2928 and in OSCC is 1.9244±0.0414.

Conclusion: Significant differences in FD values were obtained compared with normal oral mucosa, thus crediting this as a novel and interesting tool in the diagnosis of cancer. Because the fractal analysis technique is non-invasive and cost-effective, it can be used in developing countries.

Keywords: Fractal dimension, nuclear architecture, oral squamous cell carcinoma, transmission electron microscopy

Öz

Amaç: Fraktal boyut (FD), bir şeklin düzensizliği ve karmaşıklığının matematiksel bir ölçüsüdür ve tümördeki histolojik özelliklerin dijital değerlendirmesi ve miktarının belirlenmesi için kullanılabilir. Çalışmanın amacı OSCC'deki ve normaldeki nükleer FD'yi analiz etmek ve karşılaştırmaktır.

Gereç ve Yöntemler: Bu çalışma 15 oral skuamöz hücreli karsinom (OSCC) hastası ve yaş ve cinsiyet açısından eşleştirilmiş 15 kontrol grubundan oluşmaktadır. Duggirala'daki St. Joseph Dental College'da bir yıllık bir süre boyunca bildirilen her iki gruptan da taze biyopsi alındı. Doku örneğinin yarısı TEM için, geri kalan yarısı ise sınıflandırma amacıyla H&E boyama için kullanıldı. Nükleer mimarinin değerlendirilmesi, ImageJ yazılımında fraktal kutu sayımı kullanılarak görüntü analizi yoluyla yapılır. Çekirdeğin FD'si sarkar fraktal kutu sayma yöntemine göre hesaplanır.

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Bulgular: Sonuçlar normal mukoza ile karşılaştırıldığında OSCC'nin ortalama nükleer fraktal boyut değerinde istatistiksel olarak anlamlı artış olduğunu gösterdi. Normal mukoza için elde edilen çekirdeğin minimum FD değeri 0,7516 ve maksimum değeri 1,7982 iken, OSCC için çekirdeğin minimum FD değeri 1,8230 ve maksimum değeri 1,9587'dir. Normal mukozadaki çekirdeğin FD'sinin ortalama ± standart sapma değeri; 1,5806±0,2928 ve OSCC'de 1,9244±0,0414'tür.

Sonuç: Normal ağız mukozası ile karşılaştırıldığında FD değerlerinde önemli farklılıklar elde edildi, bu da bunun kanser tanısında yeni ve ilginç bir araç olduğunu gösteriyor. Fraktal analiz tekniği non-invaziv ve uygun maliyetli olduğundan gelişmekte olan ülkelerde kullanılabilir.

Anahtar Kelimeler: Fraktal boyut, nükleer mimari, oral skuamöz hücreli karsinom, transmisyon elektron mikroskobu

Introduction

With an incidence of over 3 lakh cases annually, 62% of which occur in poor nations, oral cancer is the sixth most frequent type of cancer. With an incidence rate of 12.8 and 7.5 for men and women, respectively, per 100,000 persons, it is the second most frequent cancer in India among men and the third most common cancer among women. The primary risk factors are drinking alcohol and using tobacco products, such as chewing and smoking. Up to 40% of all malignancies in India are oral cancers, making them a serious health concern (1).

The combined incidence and mortality of oral squamous cell carcinoma (OSCC) are increasing despite a number of diagnostic and treatment advancements; age-standardized incidence and death estimates for men and women respectively are 1.6/100,000 and 3.1/100,000. Hence, the use of screening and detection aids and the development of molecular markers may improve the early diagnosis of this dreadful disease and may help in reducing mortality rate. Chutta smoking is widespread in coastal areas of Andhra Pradesh. Chuttas are coarsely prepared cheroots (2).

The "reverse chutta smoking" is a special variety of cheroot which is smoked with the burning end inside the mouth due to which local effects of heat and smoke cause chronic stomatitis, leukoplakia and OSCC. In cancer cell undergoes many changes in shape of nucleus, nuclear membrane or margin, chromatin pattern, nucleoli and organization of nuclear chromatin (3). An important factor in determining the malignancy and alterations of tumors is the nuclei of neoplastic cells. Fractal geometry has recently been applied to histomorphometrical techniques, quantifying the morphological traits that pathologists often utilize to characterize and describe cancers. Fractal dimensions (FD) are a perfect tool to describe a naturally occurring item with an irregular shape (1,3).

Recent researches on OSCC have highlighted various diagnostic techniques to detect cancer at initial its stages. This study is the first of its kind to assess FD in OSCC. While clinical and histological variables have been helpful in predicting OSCC, more specialized diagnostic techniques are needed to detect cancer. So, in the present study, interest has been cultivated to use FD as a diagnostic tool. By calculating FD of OSCC and normal mucosa in transmission electron microscopy (TEM) images having higher resolving power to visualize even small changes occurring at ultrastructural level in great detail can felicitate early diagnosis (4).

Materials and Methods

The present study comprises of age and sex matched 30 patients which were divided into two study groups. Group 1: Comprises of 15 histologically diagnosed cases of well differentiated squamous cell carcinoma according to Broder's classification. Group 2: Comprises of 15 normal oral mucosa tissues from patients undergoing minor dental surgery. Explanation of study design was done to both the study groups, written consent and detailed case history was recorded from each individual and Institutional ethical clearance was requested and granted. Fresh biopsy was taken from the clinically diagnosed cases of OSCC and half of the tissue sample was used for TEM and remaining for H&E staining for grading.

The St. Joseph Dental College's Institutional Ethics Committee in Duggirala, Eluru, granted ethics approval for this study (approval no: CEC/10/2015-16, date: 22.12.2015). We got informed consent from each and every study participant.

Specimen Preparation Protocol Followed

Fixation: Fixation was the first and most important step in any electron microscope study. Tissues can be fixed by immersion or perfusion. In present study 2.5% glutaraldehyde was diluted in 100 mM phosphate buffer at pH 7.0 for 4 hours. Fixation was done at room temperature and after 15-30 minutes fixed at 4 *°C*. Fixation at 4 °C slows down autolytic processes and reduces tissue shrinkage.

Post fixation: After post-fixing the tissue for two hours with 1% aqueous osmium tetroxide, the tissue was cleaned four times every 45 minutes with deionized distilled water.

Dehydration: Dehydrated by means of a progression of acetones.

Resin embedding (epon mix): Embedding was done using Araldite 6005 resin or spur resin (Spurr 1969) next Propylene oxide used for 2 changes, each of 15 minutes.

Sectioning: Sections of one micron were taken, and copper grids were used to hold these incredibly thin sections. Glass knife and ultra microtome (Leica Ultra cut UCT-GA-D/E-1/00) were used to cut ultra thin (60 nm) sections,

which were then examined under a TEM (Model: Hitachi, H-7500 from JAPAN) at the necessary magnifications.

Fractal Analysis

Image preprocessing was the initial step in the analysis of nFD, which was then followed by segmentation and feature extraction. For fractal analysis, color images were simply thresholded to create binary versions. By utilizing the sarkar fractal box counting method in ImageJ software 1.43u with the FracLac plugin Java 1.6 (Wayne Rasband, National Institutes of Health, Bethesda, USA), nFD of each image was calculated (Figure 1, 2).

In ImageJ, choosing the nucleus required manual intervention. The box count approach was straightforward. This technique superimposes a grid of several small boxes, each with a specific pixel length, over the digital image. The software then automatically "boxed" irregular image profiles into the proper number of boxes based on the size of the image in pixels. As an outcome, a regression line graph was generated. The object's FD was equivalent to the slope of the regression line. The box counting technique that implements the formula is used by the software to automatically estimate the fractal dimension: DF=log N/log ε (where DF=Fractal dimension, N=number of pieces which the line can be broken into when using pieces of scale ε).

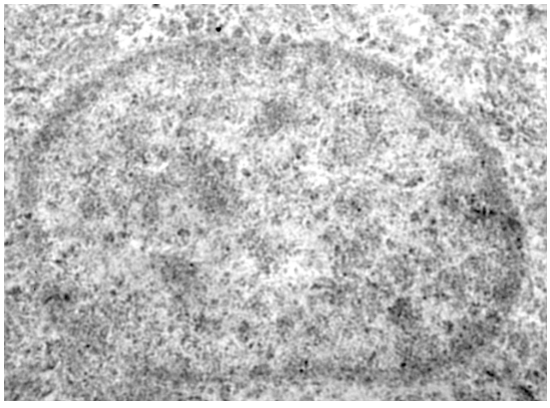


Figure 1. Nucleus of normal mucosa in magnification of 15440X19 bar in TEM
TEM: Transmission electron microscopy

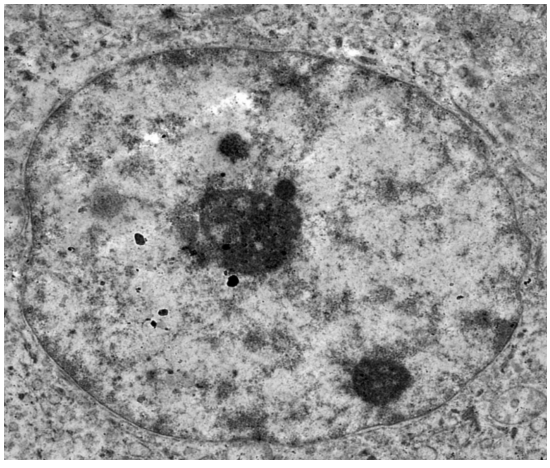


Figure 2. Nucleus of OSCC in magnification of 15440X19 bar in TEM
OSCC: Oral squamous cell carcinoma, TEM: Transmission electron microscopy

Statistical Analysis

The obtained FD values were compared with those of OSCC and normal mucosa. The independent simple t-test was used to compare the mean FD values for the normal mucosa and OSCC. A significance level of $p<0.05$ was set, and SPSS statistical software version 22 was utilized for the statistical analysis.

Results

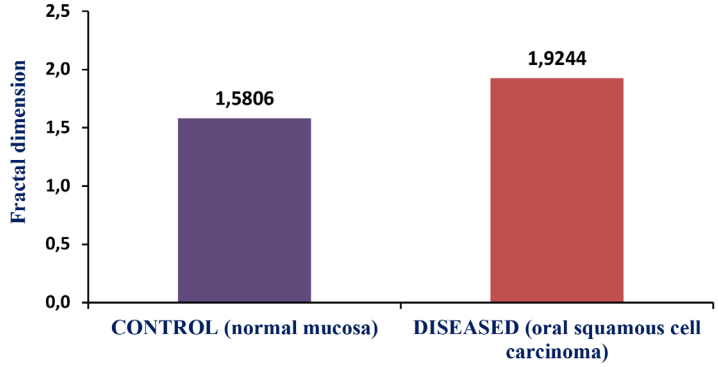
The present study comprised of 15 cases of OSCC and 15 cases of normal mucosa. FDs were calculated for all the cases using box counting fractal analysis method. The most typical clinical characteristics of an OSCC were elevated rolled borders surrounding an ulcerated lesion with a necrotic central area. Also associated with symptoms of pain, difficult in swallowing and tooth mobility. The minimum FD value of nucleus obtained for normal mucosa is 0.7516 and maximum value is 1.7982, whereas for OSCC minimum FD value of nucleus is 1.8230 and maximum value of 1.9587 (Table 1). Mean ± standard deviation value of FD of nucleus in normal mucosa is 1.5806±0.2928 and in OSCC is 1.9244±0.0414. Table 1 summarizes the fracture dimension values in relation to various clinic-pathological features of patients with oral squamous carcinoma.

Results showed increased mean nFD value of OSCC when compared with normal mucosa Graphic 1-3. Fractal values were determined using the independent simple t-test, and FD values between the two groups were found to be significantly different ($p<0.05$).

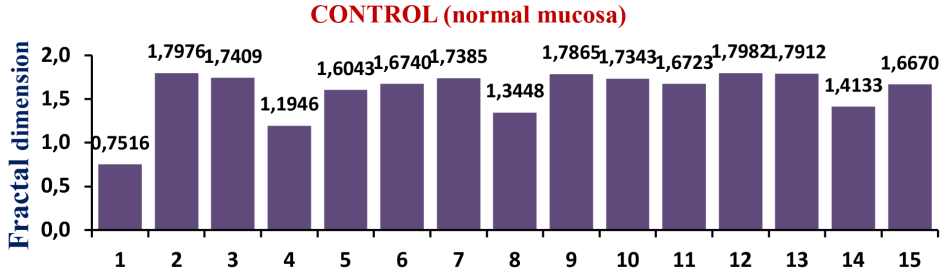
Table 1. Fractal dimension values respect to different clinicopathological characteristics of patients with oral squamous carcinoma

Groups	Mean ± SD	Std. error mean	p-value
Gender			
Male	1.9271±0.04725	0.01494	0.736
Female	1.9190±0.03054	0.01366	
Topography			
Tongue	1.9500±0.00756	0.00252	0.001
Mucosal tissue	1.8861±0.04221	0.01723	
Grade			
I	1.8773±0.04063	0.01817	0.000
II	1.9480±0.00954	0.00302	

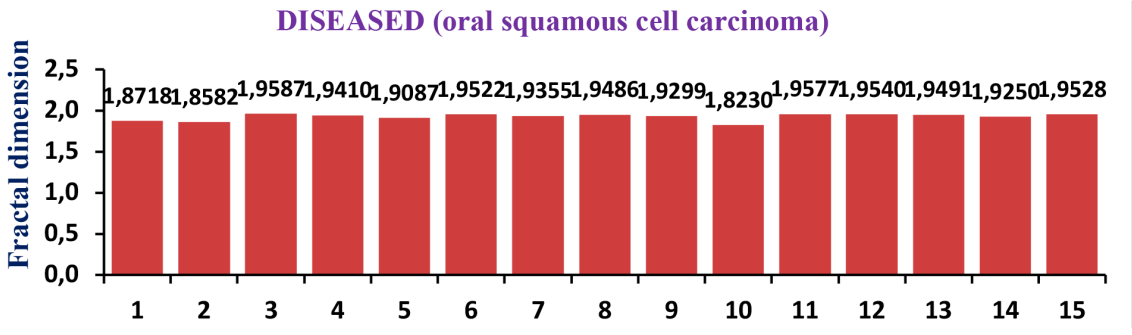
SD: Standard deviation, Std.: Standard



Graphic 1. Graph showing comparison of FD values between normal mucosa and OSCC. The mean ± SD value in normal mucosa was 1.5806±0.2928 and mean ± SD value of OSCC was 1.9244±0.0414. The graph showing significant statistical difference between both the groups
FD: Fractal dimension, OSCC: Oral squamous cell carcinoma, SD: Standard deviation



Graphic 2. Graph illustrating the FD values of normal mucosa. The highest FD value obtained for normal mucosa is 1.7982 and lowest is 0.7516
FD: Fractal dimension



Graphic 3. Graph illustrating the FD values of OSCC. The highest FD value obtained for OSCC is 1.9587 and lowest is 1.8718, but when comparison of FD values done it suggested that there was an increase in FD value of OSCC
FD: Fractal dimension, OSCC: Oral squamous cell carcinoma

Discussion

A cell's biological potential and activity are reflected in its nucleus. A normal cell typically has a single, round or oval-shaped nucleus with uniform chromatin distribution, a regular nuclear membrane or border, one or two small nucleoli, and normal mitotic figures. During carcinogenesis, changes occur in the size, shape, number, nuclear membrane, margin, nucleoli, chromatin pattern, and organization. These changes result in modifications to nuclear architecture, which in turn affect FD in OSCC (5,6).

Variations in the degree of chromatin condensation as well as chromatin condensation or de condensation are likely the causes of chromatin texture changes, which are commonly seen in cancer cells. Because of increased protein synthesis, OSCC higher grades display coarse clumped chromatin and heterogeneous chromatin pattern, while lower grades display delicate chromatin strands and homogenous chromatin pattern (7,8).

Tumor cell chromatin causes chromatin configurations such as open chromatin and chromatin coarsening, which correlate to an increase or decrease in heterochromatin

aggregates and are highly diagnostically significant. As a result, the nuclear architectures of cancer cells differ characteristically from those of normal cells. It's also critical to recognize that certain tumor types are linked to distinctive changes in the course of cancer. Newer diagnostic techniques thus serve as the foundation for cancer treatments (9,10).

The pathologist typically uses the qualitative and empirical characteristics of the cells in biopsy sections or cytological preparations to make the final diagnosis of neoplasia (11). In order to improve the examination of the internal components, such as the nucleus, number of nucleoli, amount of chromatin, and abnormalities in the nuclear membrane, these approaches have been aided by morphometric methods, such as surface area determination, volume, axes ratios, and population density estimation (12,13).

The need for efficient treatment protocols and the recognition of oral cancer as a serious public health issue have led to the development of new diagnostic systems like FD, which use clinical and histopathological criteria to help with early diagnosis. Over the past 20 years, medicine has used fractal geometry-first presented by Mandelbrot in 1982-to describe unique patterns found in a variety of organs and normal tissues (14,15).

It is unclear if nuclear fractals will eventually play a significant role in oncologists' toolkits as society shifts away from traditional methods and toward a reliance on technology in all areas. Further comparisons between fractal-related studies and traditional pathological procedures are necessary to achieve this radical shift (16,17).

The nuclear FD (nFD) in OSCC was assessed in a study by Yinti et al. (17) in 2015 utilizing computer-aided image analysis. According to the study's findings, when compared to buccal mucosa that was normal, nuclear FD gradually rose towards the worst tumour stage. These findings are consistent with the current research, which suggests that nuclear FD can be used to quantify nuclear architectural changes as a prognostic indication in OSCC (17).

Normal mucosa had a mean nFD of 1.5806, while well-differentiated squamous cell carcinoma had a mean nFD of 1.9244. This suggests a significant rise in the mean, indicating a continued use of fractal geometry to histological investigation. However, because only the well-differentiated OSCC carcinoma/Grade I tumour according to the Broders classification was included in the study, the maximum and lowest FD values for normal mucosa and OSCC were near. These findings were in line with those reported by Phulari et al. (18), who found that normal mucosa had a mean nFD of 1.7578, epithelial dysplasia had a mean nFD of 1.8363, and squamous cell carcinoma had a mean nFD of 1.9621.

A study by Goutzanis et al. (19) investigated the potential prognostic value of the nFD in tissue samples from patients with oral cavity carcinomas. The present inquiry and the research conducted by Goutzanis et al. (19) examined the connections between FD and additional variables, including clinicopathologic characteristics. Both studies showed that in OSCCs, there are several clinically and statistically significant associations between FD and other morphometric or clinicopathologic variables (19).

Mincione et al. (20) conducted a study in 2015 to evaluate the FD in tissue samples from patients with oral squamous cell cancer (OSCC). Studies looked into relationships between patient survival and clinicopathological factors, as well as FD values at different stages of OSCC. The study's conclusions, which agreed with the results of the current investigation, suggested that fractal geometry might shed light on tumor morphology and be a useful tool for looking at abnormal tumor growth patterns. Furthermore, the current study found statistically significant differences in survival between patients with lower FD values and patients with higher FD values. Thus, one could consider FD to be a predictor of OSCC (20).

The present study adopted the use of TEM images to evaluate the nFD of OSCC and normal mucosa. Significant differences in FD values were obtained when compared with normal oral mucosa thus crediting FDs as a novel, interesting tool, independent diagnostic factor with promising significance in detection of cancer. nFD may be able to assist in classifying a tumor as a low- or high-grade lesion by determining an appropriate cutoff, which would allow us to determine the necessary course of treatment (21).

Despite having a small sample size, the current study has produced encouraging findings that pave the way for more investigation. The techniques described here are quantitative, repeatable, and low in subjectivity and error. They could be modified for use in automated diagnostic systems and expedited screening of numerous histopathological sections (22).

Present study has emphasized the diagnostic importance thereby carrying out many studies in this sector by improving sample size may help in determining more reliable, accurate methods to diagnose OSCC. Further research should be carried out to use fractal analysis in the field of diagnostic pathology.

Conclusion

Scientists around the world are taking part in oral and oro-pharyngeal cancer research in many universities, institutes and medical centers. This research focuses more on what causes the disease, newer strategies for its prevention and how to improve treatment aspects. As of right now, the best approach to manage oral cancer is to combine prompt, appropriate treatment with an early diagnosis. To get a conclusive diagnosis, patients with complaints lasting longer than two to four weeks should be referred to the appropriate specialist.

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Ethics

Ethics Committee Approval: The St. Joseph Dental College's Institutional Ethics Committee in Duggirala, Eluru, granted ethics approval for this study (approval no: CEC/10/2015-16, date: 22.12.2015).

Informed Consent: Informed consent was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.A., P.H.K., B.B., M.K.P., V.S.G., Concept: S.S., A.A., V.S.G., Design: S.S., A.A., P.H.K., B.B., V.S.G., Data Collection or Processing: S.S., M.K.P., Analysis or Interpretation: A.A., V.S.G., Literature Search: P.H.K., B.B., M.K.P., Writing: S.S., M.K.P., V.S.G.

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