RESEARCH ARTICLE

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An Automated Approach to Analyze *MMP1* Expression in Oral Squamous Cell Carcinoma Using Fluorescence in Situ Hybridization: An Image Based Analysis

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Abstract

Objectives: 1.To evaluate *matrix metalloproteinase 1 (MMP1)* expression in oral squamous cell carcinoma (OSCC) through fluorescence in situ hybridization (FISH). 2. To design an algorithm for the validation of FISH photomicrographs using a computer programming language. **Material And Methods:** The formalin fixed paraffin embedded (FFPE) tissue blocks from 20 histopathologically confirmed cases of OSCC and 24 normal oral mucosal (NOM) samples from healthy volunteers were considered. The inclusion and exclusion criteria were defined. After identifying the probe for *MMP1*, the protocol was optimized. 4 µm tissue sections were subjected to FISH for *MMP1* analysis. The expression was observed manually under fluorescence microscope by two pathologists independently. With computer programme, an algorithm was designed to quantify the data from the microscopic images of FISH-*MMP1*. **Results:** The *MMP1* expression was significantly more in OSCC when compared to NOM. The mean scores for staining intensity were significantly higher in OSCC [2.10 ± 0.72] as compared to NOM [1.54 ± 0.66] with p=0.01 and 95%CI [0.14, 0.98]. The ROC curve analysis showed a sensitivity of 80%, specificity of 54.2% and an accuracy of 65.9%. Further, the digital data quantification revealed the precision of the model used, to be 66.67 and recall 82.36, suggesting differential expression of *MMP1* between OSCC and normal oral samples. **Conclusion:** The present study has shown greater expression of *MMP1* in OSCC when compared to normal oral mucosal tissues. Further, a simple yet novel digital method to objectively quantify the expression of *MMP1* in OSCC has been shown.

Keywords: Biomarker- Cancer- Diagnosis- Digital algorithm- Python

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Introduction

Oral squamous cell carcinoma (OSCC) is the common type of head and neck cancer. OSCC shows well orchestrated complex molecular etiology attributed to genetic and epigenetic abnormalities. Extensive research is ongoing in the diagnostics and prognostication of this OSCC. Considering the prevailing higher mortality and morbidity of OSCC, there is constant evolution of various tools and techniques to understand the disease process, diagnose it at an early stage and to establish treatment plan. Despite these developments, the 5-year survival has not improved much; this has induced additional pressure on the researchers to understand the intricate molecular mechanisms involved in oral squamous cell carcinoma [1-10].

In view of complex molecular etiology, numerous biomarkers have been studied in OSCC [1, 11-20]; one such family represents matrix metalloproteinases (MMPs). The MMPs are the enzymes that break down extracellular matrix components, generate active peptides, and activate certain growth factors, fabricate a milieu that encourages tumour development, invasion, and metastasis. Altered expression of MMPs has been shown to have a vital role in the regulation of tumor microenvironment. MMPs break down ECM, produce active peptides, and activate certain growth factors, creating a milieu that encourages tumour development, invasion, and metastasis. Altered expression of matrix metalloproteinases (MMPs) may have a vital role in regulating the tumor microenvironment. MMPs have the potential to be used as cancer biomarkers in OSCC early detection, risk assessment, prognostic analysis, and therapy response evaluation. Additionally, a practical way to monitor OSCC non-invasively is through the detection of MMPs in blood and saliva. Among the 28 known MMPs with 6 subtypes, the *MMP1* is known to be overexpressed in OSCC. The greater expression in higher grades correlates with poorer prognosis. The *MMP1* is Collagenase type 1 that promotes breakdown of basement membrane and cleavage of extracellular matrix components, facilitating tumor invasion and metastasis [21-25].

Considering the prevailing higher mortality and morbidity of OSCC, there is continuous evolution of various tools and techniques for better understanding of the disease process, and to facilitate early diagnosis. One such established diagnostic tool is fluorescence in situ hybridization (FISH). The diagnostic applications of FISH exhibit advantages over other molecular techniques with respect to sensitivity, specificity and rapidity, making it a routinely used clinical laboratory tool for genomic diagnosis. Over polymerase chain reaction (PCR), immunohistochemistry (IHC) and southern blot hybridization (SBH), FISH shows distinct edge in using less tumor tissue, rapidity in diagnosis and refrainment from radioactivity [26-29]. Further, this FISH technique has been explored in tissue sections especially when morphology is to be assessed or to address challenge of sparse cellular material. While using formalin fixed paraffin embedded (FFPE) tissue sections in FISH, various technical challenges are encountered. The standard clinical manual scoring method used for FISH is laborious, requires good amount of time and also is subjective. Automation of FISH stained tissue sections is an interesting evolution to reduce inherent heterogeneity and subjectivity in interpretation.

In FISH technological field, the improvisation methods have been employed in probe labeling effectiveness and high resolution imaging systems for better visualization of chromosomal organization and RNA transcription profiling in single cells. Further, the advent of artificial intelligence and machine learning has revolutionalized decision making in the field of medicine. On the other hand, the complexities involved in designing the various algorithms and digital models to predict the disease development and progression seem to be gigantic. However, the collaborative work between health care professionals and data scientists has made this task more meaningful for health care applications [30-35].

Thus, the aim of this study was to design a digital algorithm for the interpretation and validation of *MMP1* FISH stained photomicrographs of oral squamous cell carcinoma and comparing the same with normal oral mucosa using a computer programming language (Python).

Materials and Methods

Sample size and recruitment

The 'G*Power software' was used to calculate the sample size. For current research, 20 histologically confirmed cases of OSCC were retrieved from the departmental archives and 24 samples of normal healthy oral tissues were obtained during surgical disimpaction of asymptomatic impacted teeth. Written informed consent from healthy volunteers for normal oral tissue samples and clearance from institutional ethical committee were procured. The tissue samples obtained during surgical disimpaction of asymptomatic impacted teeth were fixed in 10% neutral buffered formalin, routinely processed and prepared into paraffin embedded tissue blocks.

The demographic and clinical details were retrieved and documented for OSCC cases. The inclusion and exclusion criteria were defined and followed for enlisting the study subjects. Healthy individuals with history of tobacco / alcohol consumption or any systemic illness were excluded. For OSCC cases, those with secondary metastatic carcinoma / who have undergone prior surgery (recurrent case) or any other treatment for OSCC were excluded. The normal oral tissue samples were confirmed with microscopic evaluation and taken sections for FISH. Similarly, the FFPE tissue blocks of 20 oral squamous cell carcinoma cases were also sectioned for FISH staining. The extra number in control group (n=24) was suggested by the digital technical team for better validation of the designed algorithm in the current research.

Fluorescence In Situ Hybridization Protocol

Following soft tissue microtomy of the study blocks, 4 µm sections were taken on coated slides. L-polylysine was used for coating. The oligonucleotide probe for *MMP1* was identified to run FISH [37]. The sequence used was:

5'CTCAACTTCCGGGTAGAAGGGATTT GTGCGCGCATGTAGAA TCTGTC3 ' (Length 45); Scale: 50nm. This probe was procured from Bioserve Biotechnologies (India) Pvt. Ltd Hyderabad.

The FISH protocol was optimized at Central Research Laboratory (CRL). The tissue sections were deparaffinized in 2 changes of xylene and graded concentrations of ethanol. For FISH, the study slides were pretreated as: (i) PBS wash 3 times, 15 minutes each, followed by (ii) addition of 0.02 M HCl, once for 10 minutes. (iii) PBS wash 2 times, 3 minutes each (iv) 0.01% Triton X-100 (In PBS) 15 minutes (v) PBS wash 2 times, 3 minutes each (vi) Proteinase K (10µg/ml) 15 minutes at 37°C placed in incubator (vii) PBS wash 2 times, 3 minutes each (viii) 4% Paraformaldehyde in alcohol for 3 hours at 4°C followed by Phosphate Buffered Saline (PBS) wash once, 5 minutes. After pretreatment, the slides were taken for prehybridization by incubating with prehybridization buffer (20µl) at 37°C for 60 minutes followed by 2X Saline Sodium Citrate (SSC) Buffer wash, 2 times. After prehybridization, the study slides were loaded with denatured hybridization buffer mixed with probe (20µl) (Note:1ml buffer + 1 µl probe for 10 slides) and placed in moist chamber in an incubator at 37°C for hybridization for15 hours. Post hybridization, the slides were washed with 40% formamide in 2X SSC at 37°C, 1 hour ; Counterstained with DAPI; mounted with anti-fading agent and observed under fluorescent microscope.

The prehybridization buffer was prepared by adding 180 µl of 5M NaCl, 20 µl of 1 M Tris HCl, 400 µl of Formamide, 1 µl of 10% SDS with 399 µl of distilled water to make 1ml and placed in amber colored container. The hybridization buffer was prepared by adding 1ml of prehybridization buffer to 1 µl probe.

The FISH was carried out for *MMP1* on FFPE tissue sections from both OSCC and NOM study groups. The experiment was carried out in triplicates. The slides were observed under fluorescence microscope (Olympus BX 41) (Figure 1) and photomicrographs were captured using an image analyzer (Capture V2.3).

Tabulation of data

In the manual method, these photomicrographs were analyzed by 2 pathologists independently and were scored based on the intensity of FISH staining on a 4-point scale (0- negative, 1-light green, 2-dark green and 3- fluorescent green).

The results were tabulated. To overcome the subjectivity of scoring, the two pathologists had discussed and arrived at consensus. The obtained results were subjected to statistical analysis using non parametric tests.

Figure 1. Fluorescence Microscope (Olympus BX 41) Used to Take Photomicrographs with an Image Analyzer (Capture V2.3). The MMP1 expression from 44 study samples were observed under the microscope and photomicrographs were captured.

Application of Digital algorithm

In the digital method, using a computer language programming (Python: v-3.10), an algorithm was designed to quantify the data from the microscopic images. For automation, the image processing system comprised of several interconnected modules designed to accurately detect and analyze oral cancer cells (OSCC) from fluorescence microscopy (FM) images, providing crucial diagnostic insights. The modules were (i) Pre-Processing Module (ii) RBCs Extraction Module (iii) RBCs Elimination Module (iv) Cancer Cells Detection Module (v) Area Calculation of OSCC Module. The system begins with the Pre-Processing Module, where the uploaded FM image was initially converted into grayscale. This conversion eliminated color information, simplifying the image and reducing the intricacies for subsequent analysis. Additionally, the image was resized to specific dimensions, effectively minimizing noise while retaining essential details, thus producing a noise-reduced, resized grayscale image. The subsequent RBCs Extraction Module aimed to eliminate red blood cells (RBCs) present in blood vessels within the image. Operating on the pre-processed image, this module employed connected component (CC) analysis, leveraging the intensity characteristics of RBCs. By selectively extracting RBCs based on their intensity and employing boundary-based methods, the module generated an image wherein RBCs were efficiently isolated and retained. The RBCs elimination module further refined the image to facilitate accurate cancer cell identification. Taking both the RBCs-extracted image and the pre-processed image as inputs, this module effectively removed the RBCs which were previously extracted. This step enhanced the precision of detecting oral cancer cells, as it refined the image to focus solely on the target cells of interest. The core of the system was within the cancer cells detection module, which took the refined image from the RBCs elimination module. Here, the module identified and marked cancerous cells within the image using a combination of intensity and shape-based criteria. By leveraging advanced image analysis techniques, this module accurately identified and highlighted the presence of OSCC, providing critical information for diagnosis and treatment planning. Lastly, the area calculation of OSCC module performed an essential quantitative analysis. Operating on the result image generated by the OSCC cells detection module and this module calculated the percentage of the area affected by oral cancer cells. This quantification aided in assessing the extent of the disease and predicting the presence of oral cancer in the patient. By providing a numerical representation of the affected area, healthcare professionals could make informed decisions regarding treatment strategies.

The images captured with 4X &10X resolutions were validated (Figure 2).

Results

Study sample characteristics

Out of 20 cases of OSCC, 14 were males and 6 were females with mean age of 53.55 years (Range - 39 years). Among 20 cases of OSCC, all 14 males and 4 females showed association with mixed tobacco habits for about 15-20 years. Out of 20 OSCC cases, 16 lesions were in the buccal mucosa and 4 in buccal mucosa along with gingivo-buccal sulcus. Among 24 healthy volunteers, 11 were males and 13 were females with mean age of 24.91 years (Range -19 years). These control group subjects were free from gingivitis, periodontitis or any other oral diseases and deleterious habits.

Expression analysis of MMP1

The *MMP1* expression in OSCC and normal oral mucosa samples was analyzed on FISH photomicrographs by manual and digital methods. The tabulated observations by manual scoring were statistically analyzed using Mann Whitney and Chi Square Tests followed by Receiver Operator Characteristic (ROC) curve.

The mean scores for intensity of staining were seen to be significantly higher in OSCC group [2.10 ± 0.72] as compared to normal oral mucosa [1.54 ± 0.66]. This mean difference in the scores between 2 groups was statistically significant at p=0.01 (Table 1). The Chi-Square test results demonstrated that OSCC samples were predominately stained dark green and fluorescent green [50.0% & 30.0%] as compared to normal oral mucosal samples, that had stained majorly with light green & dark green [54.2% & 37.5%]. This proportional difference in the staining intensity scores between the 2 study groups was statistically significant at p=0.04 (Table 2). The ROC curve was plotted to analyze the diagnostic accuracy of FISH (Tables 3 & 4 and Figure 1). The ROC curve analysis showed a sensitivity of 80%, specificity of 54.2% and an accuracy of 65.9% (Figure 3, 4). Thus, the expression of *MMP1* was found to be significantly more in oral squamous cell carcinoma when compared to normal oral mucosa. Further, the quantification of data using python programming had confirmed this observation with precision of the model used, as 66.67, recall of 82.36 and

Table 1. Comparison of Mean Scores for Intensity of Matrix Metalloproteinase 1 (MMP1) Staining by Fluorescent in Situ Hybridization (FISH) between 2 Groups Using Mann Whitney Test

Comparison of mean scores for Intensity of Staining between 2 groups using Mann Whitney Test								
Groups	N	Mean	SD	Median	Mean Diff	95% CI for the Diff		p-value
						Lower	Upper	
OSCC	20	2.10	0.72	2	0.56	0.14	0.98	0.01*
Normal Mucosa	24	1.54	0.66	1				

OSCC, Oral Squamous Cell Carcinoma; *, Statistically Significant; The mean scores for Intensity of Staining was significantly higher in OSCC group [2.10 ± 0.72] as compared to Normal Mucosa [1.54 ± 0.66]. This mean difference in the scores between 2 groups was statistically significant at p=0.01.

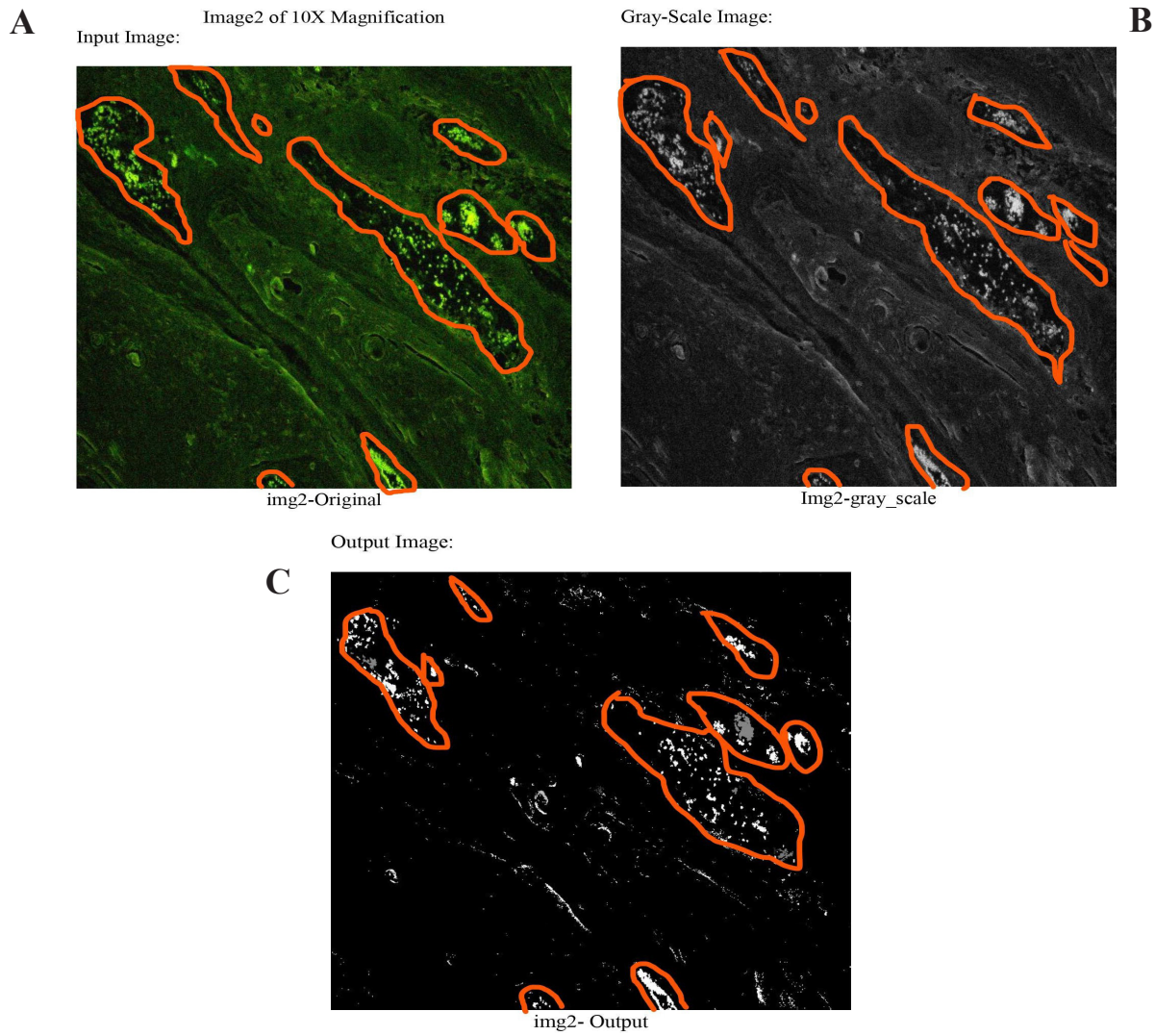

Figure 2. A: Input image, 10X Magnification in the validation of FISH photomicrograph by digital algorithm. The blood cells have been outlined by subject expert as a step in digital algorithm to quantify the expression of *MMP1* in OSCC sample. B: Grey scale image, 10X Magnification in the validation of FISH photomicrograph by digital algorithm. In the quantification of *MMP1* expression in OSCC sample, the outlined blood cells were excluded later. C: Output image, 10X Magnification in the validation of FISH photomicrograph by digital algorithm. Final output image for quantification of *MMP1* expression in OSCC sample after excluding the blood cells.

accuracy of 48.79% (Table 5).

The digital method was found to be more objective in analyzing the expression of *MMP1* in OSCC when compared to subjective manual method of scoring.

Discussion

Oral squamous cell carcinoma is the common malignancy of the oral cavity exhibiting complex etiopathogenesis with genetic and epigenetic vulnerabilities. OSCC research has witnessed advent of various molecular methods for better understanding of the

Table 2. Comparison of Intensity of Matrix Metalloproteinase 1 (*MMP1*) Staining scores between 2 Groups Using Chi Square Test

Comparison of Intensity of Staining scores between 2 groups using Chi Square Test						
Variable	Scores	OSCC		Normal Mucosa		p-value
		N	%	n	%	
Intensity of Staining	Light Green	4	20.0%	13	54.2%	0.04*
	Dark Green	10	50.0%	9	37.5%	
	Fluorescent Green	6	30.0%	2	8.3%	

*, Statistically Significant; The test results demonstrated that Oral Squamous Cell Carcinoma (OSCC) samples were predominately stained Dark Green and Fluorescent Green [50.0% & 30.0%] as compared to Normal Mucosa samples, which were stained majority with Light Green & Dark Green [54.2% & 37.5%]. This proportional difference in the staining intensity scores between 2 groups was statistically significant at p=0.04.

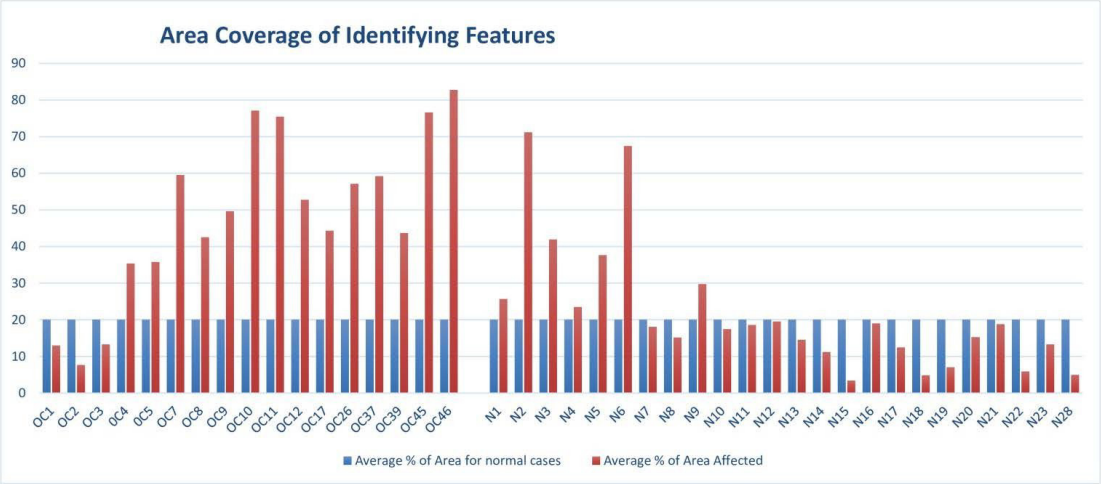

Figure 3. Area Coverage of Identifying Features in Digital Algorithm. The graph shows area covered by *MMP1* expression in Oral Squamous Cell Carcinoma (OSCC) and Normal oral mucosa (NOM) study groups. There was significantly higher expression of *MMP1* in OSCC when compared to NOM

Table 3. ROC Curve Analysis for Intensity of *Matrix Metalloproteinase 1* (*MMP1*) Staining Scores between 2 Groups

ROC Curve analysis for Intensity of Staining scores between 2 groups								
Parameter	AUC	Std. Error	95% Conf. Interval		p- value	Cut off	Sn (%)	Sp (%)
			Lower	Upper				
Intensity Scores	0.71	0.07	0.55	0.83	0.005*	> 1	80.00	54.17

*, Statistically Significant; ROC is a plot of the true positive rate against the false positive rate for the different possible cut- off points of a diagnostic test; Accuracy is measured by the area under the ROC curve. An area of 1 represents a perfect test; an area of .5 represents a worthless test. A rough guide for classifying the accuracy of a diagnostic test is the traditional academic point system:0.90-1 = excellent (A); .80-.90 = good (B).70-.80 = fair (C) .60-.70 = poor (D) .50-.60 = fail (F)

pathogenesis and its early diagnosis. One such diagnostic tool is FISH. Among the various biomarkers being studied in OSCC, the MMPs represent an important known family. While using FFPE tissue sections in FISH based diagnosis of a biomarker in OSCC, various technical challenges have been encountered related to paucity of given tissue and inherent subjectivity in manual scoring. Thus, the current study aimed at developing and validating a simple digital

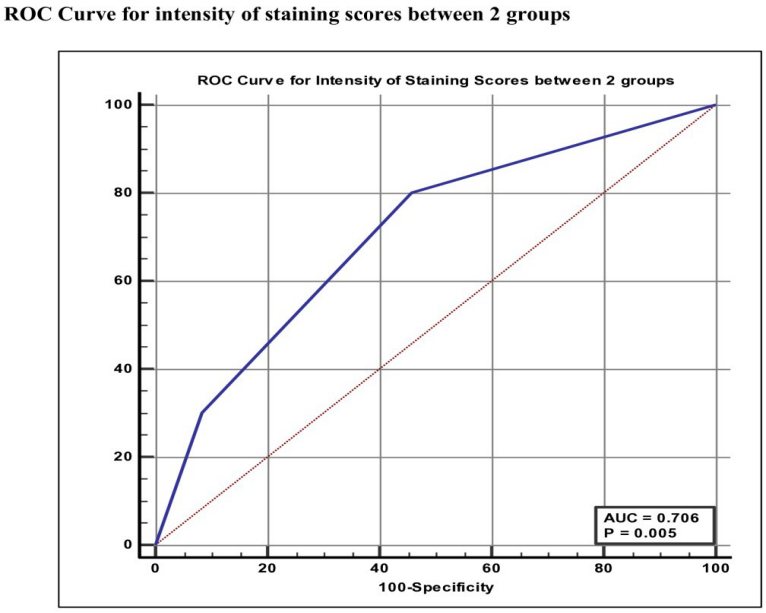

Figure 4. Receiver Operator Characteristic (ROC) Curve. The ROC curve was plotted to analyze the diagnostic accuracy of FISH. The ROC curve analysis showed a sensitivity of 80%, specificity of 54.2% and an accuracy of 65.9%. Thus, the expression of MMP1 was found to be significantly more in oral squamous cell carcinoma when compared to normal oral mucosa.

Table 4. Sensitivity & Specificity Analysis for estimating the accuracy of Fluorescent in Situ Hybridization (FISH) between OSCC & Normal Oral Mucosa

Sensitivity & Specificity Analysis for estimating the accuracy of Fluorescent in Situ Hybridization between OSCC & Normal Mucosa			
	Actual		
FISH	OSCC	Normal	Total
OSCC	16 [True +ve]	11 [False +ve]	27
Normal	4 [False –ve]	13 [True –ve]	17
Total	20	24	44
Diagnostic accuracy of FISH	%	95% CI	
		Lower	Upper
Sensitivity	80.0%	56.3%	94.3%
Specificity	54.2%	32.8%	74.5%
PPV	59.3%	47.2%	70.3%
NPV	76.5%	55.7%	89.4%
Accuracy	65.9%	50.1%	79.5%

Table 5. Confusion Matrix for Assessing Diagnostic Accuracy of Digital Algorithm

Confusion Matrix		Predicted Classification	
		Negative	Positive
Actual Classification	Negative	17	7
	Positive	3	14
Result Analysis			
	Precision	0.66667	66.67
	Recall	0.82353	82.36
	Accuracy	0.4878	48.79
	F1	25	25

algorithm to quantify the expression of a known cancer biomarker, *MMP1* in OSCC in an objective manner. For this, 44 study samples (n=44; 20 OSCC and 24 NOM) were identified. After procuring oligonucleotide probe for *MMP1* and optimizing the FISH protocol at CRL, all 44 tissue sections were analyzed for *MMP1* expression under fluorescence photomicrographs by two pathologists independently by manual scoring method using 4 point scale. In addition, a digital algorithm was developed using Python for quantification of *MMP1* expression in OSCC and NOM study groups. The developed digital algorithm was also validated. The tabulated observations from manual scoring and digital evaluation were subjected to statistical analyses. The biostatistical interpretation with ROC confirmed higher expression of *MMP1* in OSCC when compared to normal oral tissues, as analyzed by both manual and digital methods. Thus, the present study advocates the use of a simple yet novel digital algorithm for objective quantification of a cancer biomarker in oral tissue sections.

Looking into the reported English literature on *MMP1* expression in OSCC and FISH automation techniques, few observations have been made as follows: The analysis of *MMP1* expression in OSCC by IHC has been reported where the greater expression was observed in higher grades of OSCC. Moreover, MMP-1 expression was correlated with the histopathological grading of OSCC. The expression of MMP-1 protein in epithelium was significantly elevated with the increase in histopathological grade [23]. In another study, higher *MMP1* expression has been reported in those precursor lesions undergoing malignant transformation (Nishizawa 2007) to OSCC [24]. Recently, a set of MMPs and angiogenic factors have been studied in tissue and saliva samples of OSCC by IHC, protein chip array and RT-qPCR (Real Time quantitative Polymerase Chain Reaction), proposing these molecules as less invasive diagnostic and prognostic biomarkers [37]. In contrast to these techniques, the present study has shown analysis of *MMP1* expression in OSCC tissue sections using FISH with a greater expression in OSCC when compared to NOM.

The reported automation techniques for FISH include: Automated 3D scoring by confocal whole slide imaging [43], segmentation, pattern analysis, Modified Radial Basis Function Network and such others. The Cas9-mediated FISH (CASFISH) has facilitated in situ labeling of repetitive sequences and single-copy sequences in fixed or living cells without the disruption of nuclear genomic organization. The chromosome haplotypes could be distinguished from differentially specified single-nucleotide polymorphism loci using oligopaint-FISH and super-resolution imaging.

Now we can measure mRNA expression of multiple genes within single cells by using single molecule RNA FISH (smRNA-FISH) using combinatorial labeling or sequential barcoding with multiple rounds of hybridization. Further, these single molecule single cell DNA and RNA FISH techniques have enabled visualization of genomic structure within cell nuclei and transcriptional dynamics of multiple genes; revealing their functions in various biological processes.

Multi-gene FISH analysis studies are being reported with 3D imaging and tissue reconstruction for volumetric spatial analysis. Further, digital interfaces and deep learning algorithms enable precise detection of abnormal signal variations in nuclear patterns, facilitating automated detection of tumor area in lymphomas and solid tumors [38- 43]. In contrast to all these, the present study is one of its kinds to adopt a simple digital algorithm for quantification of data from FISH stained photomicrographs of a known biomarker (*MMP1*) in oral carcinoma (OSCC).

Although, it was observed that the accuracy of the model with respect to statistical figures was more with the manual scoring method, the digital algorithm has the potential to give more objective quantification of *MMP1* in OSCC making it a superior alternative to overcome inherent subjectivity in manual scoring of FISH photomicrographs. However, the digital model does very well with greater sample size as recommended for future research.

Python is a high-level programming language, widely used and famous for its simplicity and clarity. It was established by Guido van Rossum (1991) and has since acquired enormous popularity among developers. Python's elegant, easy-to-understand syntax, which depends

on indentation for code sections, makes it a favorite among novices and professionals alike. It supports many programming paradigms, such as procedural, object-oriented, and functional programming, and provides a big standard library for a broad variety of activities. Additionally, Python's wide ecosystem contains various third-party libraries and frameworks that extend its capabilities in fields like data analysis, machine learning, web development, and more. Its flexibility, along with its active community, has made Python a go-to language for different applications [44-48].

In conclusion, our study has shown that the *MMP1* expression was found to be significantly more in oral squamous cell carcinoma than in normal oral tissues. The current research has shown the application of a simple yet novel digital method to quantify the expression of a known biomarker (*MMP1*) in oral squamous cell carcinoma using fluorescence in situ hybridization in oral tissue sections, helping in evading the subjective error involved with manual method. With a larger study sample size, the validated diagnostic algorithm may be used in the clinical setting in a swift manner.

The current research with adoption of Python in the quantification of a cancer biomarker (*MMP1*) may assist in precision and tailored treatment of oral carcinoma in future. Moreover, MMPs have also been implicated in the response of OSCC to the treatment. As adjunct therapies have their own inherent side effects, effective biomarkers are needed to predict the patient's response to therapy.

Author Contribution Statement

Murittige Gopalakrishna Madhura, Kotrashetti S Vijayalakshmi, and Kishore Bhat S had conceptualized the study. Murittige Gopalakrishna Madhura, Preethi Patil N, R Rakshith, and Kumar J Ranjith, had developed and validated digital algorithm. Preethi Patil N, R Rakshith, and Kumar J Ranjith had performed computations. Kugaji S Manohar had done Experimentation and provided technical support. Kumar B Veerendra, Vasudev Sunil, Aghanashini Suchetha, Kumar Kiran, Krishnamoorthy Naveen, Muttagi S Sidramesh, Nayak S Ramakant, BR Patil and Kodaganur Srinivaschar Gopinath had encouraged to investigate. Murittige Gopalakrishna Madhura, Kotrashetti S Vijayalakshmi had written the mansuscript and revised with inputs from all authors. All authors have discussed and contributed to the final manuscript.

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If it was approved by any scientific Body/ if it is part of an approved student thesis

This study was approved by institutional review board of Maratha Mandal's NGH Institute of Dental Sciences and Research Centre, Belagavi, Karnataka, India and is part of PhD thesis of Dr. Murittige Gopalakrishna Madhura approved under State Government University-Rajiv Gandhi University of Health Sciences, Karnataka, India

How the ethical issue was handled (name the ethical committee that approved the research)

The study has been approved by the institutional review board of Maratha Mandal's NGH Institute of Dental Sciences and Research Centre, Belagavi, Karnataka, India. The written informed consent was obtained by study subjects. The patient details were kept confidential and used judiciously.

Any conflict of interest

None.

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