A Study on Diagnostic Accuracy of Modified Cajal's Trichrome Stain in Suspected Cases of Microinvasion

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Abstract: *Microinvasive oral squamous cell carcinoma (OSCC) is an early stage malignancy which exhibits invasion of superficial lamina propria without involving invasion of deeper structures. Even though Hematoxylin and eosin staining remains the cornerstone of contemporary cancer diagnosis, histopathological diagnosis of initial epithelial pathology may be challenging. Masking of small and obscure invasive components and the basement membrane by the inflammatory reaction poses a hindrance for detection of microinvasion. The present study endeavours to use Modified Cajal's trichrome stain for cost effective, easy diagnosis of microinvasive squamous cell carcinoma.*

Keywords: Microinvasion, Hematoxylin and eosin, Modified Cajal's trichrome stain

1. Introduction

Ramon Y Cajal initially described Cajal's trichrome in 1897. It is considered as a good stain for tissues composed of different structural components and as such is applicable in cases with connective tissue framework associated with epithelial cells. In SCC, it gives different tonalities of colours for varying degrees of keratinization and cellular differentiation[1]. Immunohistochemical markers commonly used for the diagnosis of microinvasive SCC include Pancytokeratins, E-cadherin, Laminin and collagen IV. Pancytokeratins help in the identification of keratinocytes in the stroma[2]. The nuclei show varying cell-type and cancer-type specific patterns of heterochromatin condensation, which is well appreciated under hematoxylin stain that is of diagnostic significance[3]. Histopathological diagnosis of initial epithelial pathology may be challenging even with H and E, especially in cases of early, micro-invasive squamous cell carcinoma (SCC), carcinoma *in situ*, and atypical epithelial malignancies[4].

2. Objectives

To evaluate efficacy of Modified Cajal's Trichrome Stain (CTS) in relation to Haematoxylin and Eosin in detection of microinvasion.

3. Materials and Methods

All suspected and confirmed cases of microinvasion from 2015 to 2019 collected from archives of Department of Oral Pathology and Microbiology, Government Dental College, Kottayam, comprised the study sample. Cases with inadequate or no tissue samples were excluded from the study. A total of 66 cases which included epithelial dysplasia (43 cases), carcinoma in-situ (2 cases) and early invasive squamous cell carcinoma (21 cases) wherein the report suggested presence/suspicion of microinvasion was selected. Cases of epithelial hyperplasia were selected as negative control and those of well differentiated squamous cell carcinoma as positive control. All cases were stained with pan cytokeratin immunohistochemical marker for confirmation of presence or absence of microinvasion which is considered as gold standard with respect to the other stains used. In this study, microinvasion was considered as "invasion of neoplastic/dysplastic epithelial cell(s) into the superficial lamina propria without invasion of deeper structures". Three sections were taken from each selected paraffin embedded tissue block of the respective case, which was processed for IHC, H&E and CTS staining.

Protocol for trichrome stain of Cajal modified by Gallego[5]
1) Four-micron thick sections have to be obtained from the formalin-fixed, paraffin-embedded tissue blocks. The sections are then to be deparaffinized and hydrated
2) The sections are to be stained with Ziehl's acetic fuchsin for 2 min (Ziehl's fuchsin - 10 drops, acetic acid - 1 drop, distilled water 10 cc)
3) The sections have to be subsequently washed in water
4) After the wash, the sections are to be differentiated in formalin-acetic acid solution for 5 min (formalin -2 drops, glacial acetic acid - 2 drops, distilled water 10cc)
5) The sections have to be washed in water again

6) As the final staining step, stain the sections with picroindigocarmine stain for 3–5 min (aqueous solution of indigocarmine for 1% one part, aqueous saturated solution of picric acid two parts)
7) The sections are then to be dehydrated, cleared, and mounted.

Slides stained with H and E and CTSwereobserved by two separate observers for presence or absence of microinvasion without any exchange of information regarding study specimen details.Data was entered in excel spreadsheet and statistical analysis was done using SPSS software.Sensitivity and specificity of both stains were computed and compared. For interobserver variability kappa analysis was done.

Figure 1: Early invasive OSCC stained by H&E(left) and Modified CTS(right)

Figure 2: Well differentiated OSCC stained by H&E (left) and Modified CTS (right)

4. Results

Cases of severe dysplasia were easily diagnosed with modified CTS. In SCC cases stained with Cajal's trichrome stain, invasion of tumor cells into connective tissue and the presence of keratin pearls were strikingly evident [Figure 4]. Kappa value indicated moderate agreement between both observers. Both stains showed moderate agreement in detection of microinvasion with statistically significant p value <0.05 within a confidence interval of 95%.(Table 2)
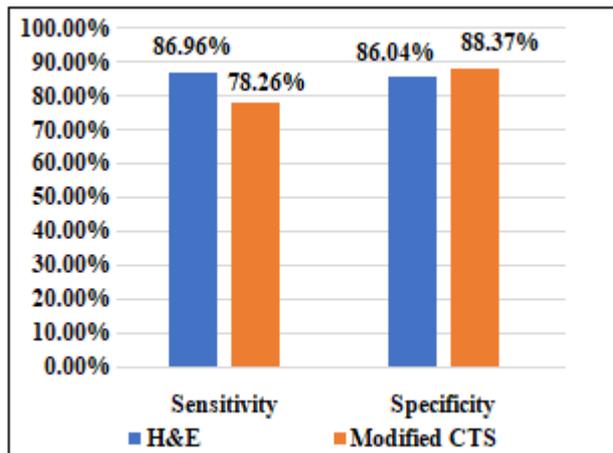
Table 2

Stain		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
1	Measure of Agreement — Kappa	.738	.086	6.011	.000
	N of Valid Cases	66			
2	Measure of Agreement — Kappa	.591	.106	4.801	.000
	N of Valid Cases	66			

a. Not assuming the null hypothesis.
b. Using the asymptotic standard error assuming the null hypothesis

Table 1

Type of lesion (no of cases)	No of cases with microinvasion present (+) or absent (-)					
	IHC (pan CK)		H&E		Modified CTS	
	+	_	+	-	+	_
Epithelial dysplasia (43)	0	43	6	37	5	38
Carcinoma Insitu (2)	2	0	1	1	1	1
Early invasive squamous cell carcinoma (21)	21	0	19	2	17	4

Figure 3: Column plot showing sensitivity and specificity of H&E and Modified CTS in detection of microinvasion

5. Discussion

Cajaltrichrome stain initially described by Cajal and later modified by Calleja and Gallego is rarely used in the histopathology laboratory. The various staining techniques and principles of Cajal trichrome stains was extensively described by Castroveijo in 1932 in his review. According to his studies, this stain is one of the best for the study of corneal pathology and for diagnosis of tumors such as neurofibroma, myofibroma, and fibrosarcoma. He elucidated that this stain shows variation in hues with degree of keratinization and is particularly useful in tumors where connective tissue framework is associated with epithelial cells. This was the basis for the hypothesis that this stain will be particularly useful in the diagnosis of early micro-invasive SCC.

As mentioned by Castroviejo, the triple stain developed by Cajal is hardly known and practiced despite being one of the best differential stains[5].

The major predicament a pathologist faces when the question of "early SCC" arises is deciding whether or not frank invasion is present[6]. The cases ofmicro-invasive SCC needsintense deliberation in this regard. Micro-invasive SCC is considered as a comparatively "thin" tumor in the early stages of tumor progression and is devoid of deeper invasion into the connective tissue[7].

For classifying an early invasive lesion as a micro-invasive epithelial tumor, it should be only restricted to papillary lamina propria as defined by the depth of rete ridges. In such cases where diagnosisof micro-invasivetumorsis difficult, modified Cajal's trichrome provides remarkable differentiation between epithelial and connective tissue elements[8]. In this study it was noted that, withCajal's trichrome stain, cytoplasm stained predominantly green in dysplastic epithelium while normal epithelium showed primarily pale pink cytoplasm. Also the nuclei stained red, epithelial cytoplasm pink-green, collagen fibers blue, muscle olive green, and keratin and red blood cells grass green, which was in accordance to previous studies in the literature. However, nuclear features like vesiculation, atypia and heterochromatin condensation were better discernable with conventional H and E stain. Sensitivity for detection of

microinvasion and invasion was better for H and E while modified CTS showed more specificity in cases of invasion and microinvasion than H and E (Table 2, Fig 5 and 6).Hence grading of dysplasia can be done more accurately with H and E whereas Cajal's trichrome stain will be helpful in screening of large samples.

While molecular and immunohistochemical markers such as cytokeratins are being increasingly used in the investigation of OSCC, these are expensive, time-consuming, and labor intensive[9]. Since Cajal's trichrome stain is easy to use and comparatively inexpensive, it may well prove to be a time-saving aid to the pathologist[10].

6. Conclusion

Histopathological evaluation of biopsy tissue still remains as the foundation of cancer diagnosis and pathological grading and staging. Even though the standard protocols for reporting head and neck cancer have been described and are being widely used world over, there are probable difficulties and pitfalls in the assessment of incisional biopsy specimens, surgical resection specimens, and neck dissections[11]. Modified Cajal's Trichrome stain is a potential, economic and easy-to-use stain which can be used as an adjunct in detection of microinvasion. It can also be used in scenarios requiring screening of large samples of potentially malignant or malignant lesions and in special cases where the invasive nature of the tumor is not readily discernable. Further studies with larger sample size are required to elicit the use of this differential stain in solving the dilemma of microinvasion.

7. Ethical Consideration

Ethical clearance from Institutional Ethics Committee was obtained.

8. Financial Support

Nil

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