Original Article

Tumor angiogenesis: A potential marker of the ongoing process of malignant transformation in leukoplakia patients, removing the veil

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ABSTRACT

Context: The diagnosis of malignant and potentially malignant epithelial lesions of the oral mucosa is based on a careful histologic evaluation of a representative biopsy specimen. The site for the biopsy, however, is always a subjective choice that raises doubts about its representativeness. So far, no simple and reliable method is available for selecting the most appropriate area for biopsy. Colposcopy, a well-known gynecological diagnostic procedure, is helpful in the selection of these sites of epithelial dysplasia depending upon the vascular patterns. **Aims:** The present study aimed at assessing the vascular patterns by colposcopic findings and selecting the biopsy site in leukoplakia patients and compared the two methods, clinical criteria, and colposcopic examination, for selecting the biopsy site. **Settings and Design:** Sixty patients between the ages of 30-60 years clinically diagnosed with leukoplakia were included in the study. **Materials and Methods:** For each of the subject, a thorough clinical examination was carried out followed by colpospopic examination for the selection of biopsy site. The histopathological findings were then compared in the two cases. **Statistical Analysis Used:** The statistical analysis was done using paired *t*-test. **Results:** Based on clinical criteria, a sensitivity of 0.5714 (57%) and a specificity of 0.5000 (50%) and that guided by colposcopic examination to be 0.8571 (85%) and 0.6667 (66%), respectively, was found. **Conclusions:** From the study, it was concluded that colposcopic examination could prove to be a valuable diagnostic adjunct in the selection of biopsy site for leukoplakia patients.

Key words: Colposcopy, epithelial dysplasia, leukoplakia, vascular pattern

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common cancer in the oral cavity. It accounts for more than 90% of all oral cancers.[1] Each year, globally, there are 222,000 new cases of oral cancer diagnosed in men (5% of all cancer) and 90,000 new cases diagnosed in women (2% of all cancer).[2]

The incidence of premalignant lesions and oral cancers is steadily increasing globally. In spite of advancement in the early detection, there is seen increased mortality and morbidity related to oral cancers.[3] In India, the incidence of leukoplakia and carcinoma buccal mucosa is 46% as reported by Paymaster.[4] Carcinoma of buccal mucosa, in particular, deserves special mention with increased incidence because of numerous premalignant lesions and conditions seen predominantly in this part of oral mucosa, the most common being leukoplakia and pouch keratosis, attributed commonly to the quid habit in Indian population.

Clinical diagnosis of squamous cell carcinomas of the oral mucosa is not difficult when the lesion is obviously invasive or when the patient experiences pain, functional limitation, or regional lymphadenopathy. Conversely, it is more difficult to diagnose dysplasia and carcinomas, mainly in potentially malignant epithelial lesions (PMELs). With the aim of improving the efficiency of these diagnoses, techniques are being developed to complement clinical examination and to facilitate the identification of early dysplastic changes and initial carcinomas.[5]

Access this article online

Quick Response Code:

Website: www.ccij-online.org

DOI: 10.4103/2278-0513.102880

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Though biopsy with histopathological examination is still considered the gold standard in the diagnosis of oral cancer and precancerous lesions and conditions, the selection of the site for biopsy is the most important criteria to arrive at a correct diagnosis. But, as biopsy site is a subjective choice, it is possible that biopsy specimens are taken from unrepresentative sites of the lesion or before the morphologic changes could be detected in it. Even experienced clinicians cannot easily select a representative site for biopsy.

At present, though there are simple chair-side methods including staining with toluidine blue and exfoliative cytology to aid the diagnosis of such changes, there is a high risk of false positives, which can be as high as 30%.[6] Therefore, a technique for non-invasively detecting dysplastic changes or helping the clinician choose the appropriate site for biopsy can save patients from multiple biopsies and allow a broader range of diagnoses, which can aid early detection of oral cancers.[7]

Colposcopy (direct intra-oral microscopy) offers advantages in selecting the more representative sites for biopsy than routine clinical examination alone and is a simple, painless, chair-side diagnostic method.[8] Colposcopic criteria included vascular pattern, inter-capillary distance, surface pattern, color tone, and opacity, as well as clarity of demarcation of the mucosal lesions.[8] The accuracy of colposcopic examination for the detection of mucosal changes approximates between 70% and 98%.[9-11]

Various authors have tried to adapt gynecologic methods of examination to the oral cavity as there is similarity between the two types of mucosa. Colposcopy is one such method used to observe the mucosa of cervix for premalignant and malignant changes.[12] So far, a few studies have highlighted the value of colposcopy (direct intra-oral microscopy) in the diagnosis of oral mucosal lesions. Hence, the study was planned to assess the role of colposcopic examination in the biopsy site selection for leukoplakia, one of the most common pre-malignant lesions seen in relation to oral mucosa, preceding frank oral squamous cell carcinoma.

The present study aimed at assessing the vascular patterns by colposcopic findings and selecting the biopsy site in leukoplakia patients and compared the two methods, clinical criteria, and colposcopic examination, for selecting the biopsy site. The objectives of the study were to assess the feasibility of using colposcopic examination for leukoplakia patients; to compare the colposcopic examination findings with clinical criteria for selection of biopsy site in leukoplakia patients; to correlate the histopathological findings with colposcopic findings and clinical criteria; and to assess the respective sensitivity and specificity of colposcopic examination in the selection of the biopsy site in leukoplakia patients.

MATERIALS AND METHODS

Source of data: The study was conducted for a period of 1½ years from May 2010 to Nov 2012. The study group consisted of 60 cases of clinically diagnosed cases of leukoplakia between the age group of 30-60 years and control group consisting of 30 healthy individuals with matched age.

Method of Collection of Data: Clinically diagnosed cases of leukoplakia with extensive lesions of the oral mucosa with dimensions more than 2 x 2 cm were selected for the study. Patients with leukoplakia with secondary infection, those having other systemic diseases, and patients undergoing treatment for leukoplakia were excluded from the study. Before selecting the patients for study, details of the study were explained to the patients, and written informed consents were obtained for inclusion in the study. The ethical clearance for the study was obtained from the ethical committee appointed by the governing body of the institution.

Methodology

A total of 60 leukoplakia cases were selected for the study based on inclusion and exclusion criteria. The significance of the number of samples was analyzed statistically before their inclusion into the study. For each of the subjects, a detailed case history and thorough clinical examination was carried out, and under good illumination, intra-oral examination of the lesion was performed. Inspectory and palpatory findings were recorded in a prepared proforma. Following clinical examination of the lesion, the most representative site of the lesion was selected for biopsy based on set clinical criteria for dysplastic changes in leukoplakia.

Clinical criteria for selection of biopsy site for leukoplakia included erythema, granular consistency, and ulceration. The outline of the lesion was marked with a black color pen and the biopsy site with a red color pen with the help of a grid placed on the buccal mucosa. All the cases were then subjected to the colposcopic examination. Before taking up the patients for colposcopic evaluation, the normal colposcopic findings were standardized based on the colposcopic criteria. Colposcopic assessment was done, and the most representative site for biopsy was selected from the involved buccal mucosa depending upon the colposcopic criteria as mentioned by the author Gynther.[13]

All patients were subjected to routine blood investigations (Hb, BT, CT, RBS, TLC, DLC, and ESR) before the routine biopsy for histopathological examination, and punch biopsy after colposcopic evaluation with 6 mm diameter under local anesthesia was performed. Biopsy specimen was immediately

immersed in 10% neutral buffered formaldehyde solution and was coded. Later, it was embedded in paraffin by routine methods and subjected to histopathological examination.

Colposcopic examination: Following clinical examination, mucosa was wiped with saline. After the mucosa was wiped with saline, abnormal epithelium appeared much darker than the normal epithelium [Figure 1]. Using the blue (or green) filter and higher-power magnification, abnormal vascular patterns were evaluated. Then, 5% acetic acid was applied to the lesion for about 60 seconds. The grid was placed again on the buccal mucosa. Area that estimated to have the most extensive cell changes based on colposcopic criteria was selected for biopsy, and the area of the biopsy site was marked on the grid with a green pen.

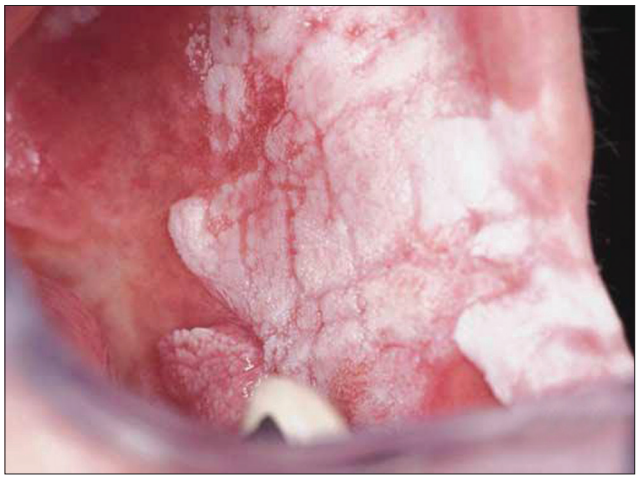

Figure 1: Proliferative verrucous leukoplakia on left buccal mucosa

Colposcopic criteria included vascular pattern, inter-capillary distance, surface pattern, color tone, and opacity, as well as clarity of demarcation of the mucosal lesions. In the normal mucosa of the uterine cervix, two basic types of capillary networks can be seen with direct microscopy (i.e. Colposcopy): Hairpin capillaries [Figure 2a] and network capillaries [Figure 2b]. In areas of dysplasia and carcinoma-*in-situ* of the uterine cervix, a specific vascular pattern, punctation (previously called ground), is seen commonly. Punctation [Figure 2c] is characterized by dilated, often twisted, irregular, hairpin-type vessels. Another pattern of the vessels in dysplasia is called mosaic [Figure 2d]. If the vessels do not reach the epithelial surface but extend only partially into the epithelium, they appear as red lines as surrounding blocks of epithelium. The colposcopic image resembles tiles of a floor. After application of acetic acid, this pattern is accentuated because of acetowhiteness of the atypical epithelium, forming a honeycomb pattern. True mosaic vessels are usually seen in sharply demarcated areas. When it is difficult to describe the pattern of the vessels, the term atypical vessels [Figure 2e] is used. Capillary, punctation, mosaic, or atypical patterns are encountered in malignant lesions. Therefore, if one of them is present, this is an indication for biopsy and histopathological examination.

When the area selected for biopsy by clinical criteria and colposcopy was superimposed (red and green area), then only one common biopsy sample was obtained. When two different areas were selected from the same lesion, two different areas, which were marked with red and green pen, were biopsied and subjected to histopathological

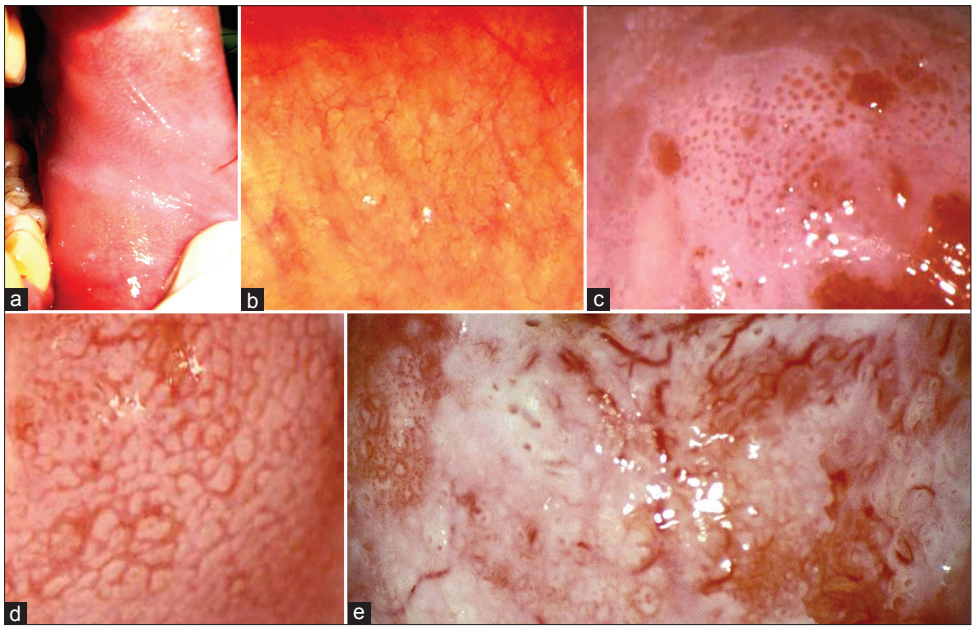

Figure 2: Vascular patterns seen in colposcopy. (a) Hair-pin capillary pattern in normal buccal mucosa. (b) Network capillary pattern in normal buccal mucosa. (c) Punctation vessel pattern in leukoplakia. (d) Mosaic pattern in leukoplakia, pointing towards high grades of epithelial dysplasia. (e) Atypical vessel pattern in leukoplakia, suggestive of epithelial dysplasia and ongoing process of malignant transformation

examination. Biopsy specimens were taken with 6 mm punch, biopsy wounds were sutured, and histopathological examination of the same was performed.

Histopathological examination: All the biopsied tissue specimens were sent for histopathological evaluation. The biopsied tissue was immediately transferred to the bottle containing 10% buffered formalin solution. Hematoxylin and Eosin staining was done for the microscopic examination of the sections.

Comparison of the histopathological diagnosis obtained with routine clinical examination and direct intra-oral microscopy was performed, and the data was subjected to statistical analysis.

Grid preparation: Printed graph on OHP sheet was used as a grid in marking the biopsy site. Each lesion was measured, and the grid was prepared to the approximate size of the lesion. The entire lesion was divided into 6 x 6 mm squares on a transparent grid. The outline of the lesion was marked with black color pen; the red color pen was used to mark the area of the biopsy site with clinical criteria, and green color pen was used to mark the area of the biopsy site performed with colposcopic criteria.

RESULTS

The study group consisted of 60 cases of clinically diagnosed leukoplakia and a control group consisting of 30 healthy individuals.

Age and gender distribution: The age [Table 1] and gender [Table 2] distribution of the cases and controls has been illustrated in the respective tables. In the patients, granular texture [Table 3] was observed in 36 while ulceration [Table 4] was the presenting complaint in 24 patients.

Vascular pattern observed in cases: Out of 60 leukoplakia patients, network capillary pattern was observed in 3 (5%) cases, hair-pin pattern in 3 (5%) cases, punctation vessel pattern in 21 (35%) cases, mosaic pattern in 9 (15%) cases, and atypical vessel pattern in 24 (40%) cases [Table 5].

In the control group, out of the 30 controls, network capillary pattern was observed in 15 (50%) cases and hair-pin pattern in 10 (33.3%) cases while in 5 (16.7%) cases, vascular pattern could not well be appreciated.

Histopathological findings observed in the biopsy specimen obtained from clinical presentation: Out of the 60 leukoplakia patients, dysplasia was absent in 24 (40%) cases, 12 (20%) cases were showing early dysplastic features, 9 (15%) cases were showing moderate dysplasia, 12 (20%) cases were showing severe dysplasia while 3 (5%) cases presented with carcinoma-*in-situ* [Table 6].

Histopathologic findings in colposcopically directed biopsy specimens: Out of the 60 leukoplakia patients, dysplasia was absent in 6 (10%) cases, 12 (20%) cases were showing early dysplasia, 18 (30%) cases were showing moderate dysplasia, 15 (25%) cases were showing severe dysplasia while 9 (15%) cases presented with carcinoma-*in-situ* [Table 7].

Table 1: Age distribution in the study sample

Age in years	Leukoplaia patients		Controls	
	No.	%	No.	%
31-40	12	20.0	6	20.0
41-50	33	55.0	12	40.0
51-60	15	25.0	12	40.0
Total	60	100.0	30	100.0
Mean ± SD	46.95 ± 5.96		49.72 ± 7.17	

Samples are age-matched between cases and controls with $P = 0.397$

Table 2: Gender distribution in the study sample

Gender	Leukoplakia patients		Controls	
	No.	%	No.	%
Male	36	60.0	18	60.0
Female	24	40.0	12	40.0
Total	60	100.0	30	100.0

Samples are gender-matched between cases and controls with $P = 0.191$

Table 3: Clinical presentation, granular texture in leukoplakia patients

Clinical presentation, (Granular texture) in leukoplakia patients	Leukoplakia patients, (n = 60)	
	No.	%
Absent	24	40.0
Present	36	60.0

Table 4: Clinical presentation, ulceration in leukoplakia patients

Clinical presentation, (Ulceration) in Leukoplakia patients	Leukoplakia patients, (n = 60)	
	No.	%
Absent	36	60.0
Present	24	40.0

Table 5: Vascular pattern seen in cases and controls

Vascular Pattern	Leukoplakia patients		Controls	
	No.	%	No.	%
Network Capillaries	3	5	15	50
Hairpin	3	5	10	33.3
Punctation	21	35	0	0
Mosaic	9	15	0	0
Atypical Vessels	24	40	0	0
Not detected	0	0	5	16.7
Total	60	100	30	100

Table 6: Histopathological findings in the biopsy specimens obtained from clinical examination

Histopathological findings Clinical examination	Leukoplakia patients, (n = 60)		*P* value
	No.	%	
No dysplasia	24	40.0	< 0.001
Early dysplasia	12	20.0	
Moderate dysplasia	9	15.0	
Severe dysplasia	12	20.0	
Poorly differentiated squamous cell carcinoma	0	0.0	
Moderately differentiated squamous cell carcinoma	0	0.0	
Well differentiated squamous cell carcinoma	0	0.0	
Carcinoma *in situ*	3	5.0	

Table 7: Histopathological findings in the biopsy specimens obtained from colposcopic examination

Histopathological findings in Colposcopically directed biopsy specimens	Leukoplakia patients, (n = 60)		*P* value
	No.	%	
No dysplasia	6	10.0	< 0.001
Early dysplasia	12	20.0	
Moderate dysplasia	18	30.0	
Severe dysplasia	15	25.0	
Poorly differentiated squamous cell carcinoma	0	0.0	
Moderately differentiated squamous cell carcinoma	0	0.0	
Well differentiated squamous cell carcinoma	0	0.0	
Carcinoma *in situ*	9	15.0	

Table 8: Sensitivity and specificity for biopsy specimens taken from clinical examination in leukoplakia patients

Clinical examination	Histopathology			Sensitivity	Specificity	PPV	NPV
	Positive	Negative	Total				
Positive	24	9	33	0.5714 (57%)	0.5000 (50%)	0.7273	0.3333
Negative	18	9	27				
Total	42	18	60				

Table 9: Sensitivity and specificity of colposcopically directed biopsy specimens in leukoplakia patients

Colposcopy	Histopathology			Sensitivity	Specificity	PPV	NPV
	Positive	Negative	Total				
Positive	36	6	42	0.8571 (85%)	0.6667 (66%)	0.8571	0.6667
Negative	6	12	18				
Total	42	18	60				

Sensitivity and specificity for biopsy samples taken from clinical presentation: Sensitivity and specificity for biopsy samples taken from clinical presentation came out to be 0.5714 (57%) and 0.5000 (50%), respectively [Tables 8 and 10].

Sensitivity and specificity of colposcopically directed biopsy specimens: Similar values in case of colposcopically directed biopsy specimens were 0.8571 (85%) and 0.6667 (66%), respectively [Tables 9 and 10].

DISCUSSION

Oral squamous cell carcinoma is a well-known malignancy, which accounts for more than 90% of all oral cancers. The overall 5-year survival rate of oral squamous cell carcinoma has not significantly increased in the last few years despite tremendous advancements made in the plethora of diagnostic and treatment modalities in the last 2-3 decades. Hence, the most important task is to establish an early diagnosis at the first stages of the disease.[14]

In our study, maximum patients with leukoplakia were seen to be in the age group of 41 to 50 years with a characteristic male predominance. This suggested the habit of smoking to be more common in males. The findings of our study were consistent with the age and gender of the oral cancer and pre-cancer patients reported by other studies by Silverman,[15] Neville,[16] and Swango.[17]

In our study, granular texture was present in 36 cases while ulceration was reported in 24 cases. These findings suggest that the patients report to the clinicians only when they develop symptoms of pain invariably associated with ulceration. The results were in confirmation with the various other reported studies. Bagan[18] reported in his study that the clinical presentation of these early malignant lesions was usually in the form of an erytholeukoplastic lesion. It consisted of a red or red and white area with a slight roughness and was seen to be well-demarcated. The elasticity of the soft tissue also changed to a harder sensation on palpation (''induration''). There was often no pain, but the patients reported with some discomfort.

A sensitivity of 0.5714 (57%) and a specificity of 0.5000 (50%)

Table 10: Correlation of sensitivity and specificity between biopsy directed with the help of colposcopy and clinical examination in leukoplakia patients

Method	Sensitivity (%)	Specificity (%)
Colposcopy	0.8571 (85)	0.6667 (66)
Clinical examination	0.5714 (57)	0.5000 (50)

[Tables 8 and 10] of the biopsy specimens taken with the help of clinical criteria for leukoplakia were in accordance with the studies reported by Lingen, Kalmar, Karrison, Speight[19] who reported similar findings in their study. Lingen, in his study, suggested the conventional oral examination (COE) using normal (incandescent) light as one of the standard method for oral cancer screening. Conventional visual cancer screenings for some anatomic locations can be highly successful. For example, visual inspection of skin lesions can be an effective screening method for melanoma, with sensitivity and specificity rates as high as 98%. However, while COE has traditionally been the mainstay of oral cancer screenings for decades, its utility remains controversial. A number of publications have suggested that COE may have limited value as a method for detecting pre-cancerous or early cancerous lesions. Conversely, some studies have also reported a relatively high degree of sensitivity, specificity, and positive predictive value of COE.

A study by Fedele[20] with 9 years randomized controlled trial revealed that screening via visual examination of the oral mucosa under white light is effective in reducing mortality in individuals exposed to risk factors. Simple visual examination, however, is well-known to be limited by subjective interpretation and by the potential, albeit rare, occurrence of dysplasia and early oral squamous cell carcinoma within areas of normal-looking oral mucosa. As a consequence, adjunctive techniques have been suggested to increase the ability to differentiate between benign changes of the mucosa from dysplastic/malignant changes as well as to identify areas of dysplasia/early oral squamous cell carcinoma that are not visible to the naked eye.

Hopman,[21] in his study, stated that colposcopy is an adequate tool for diagnosing cervical intra-epithelial neoplasia. It is part of the broadly accepted triage for women with abnormal cervical cytology. In his study, the predilection of the final histopathological diagnosis of cervical intra-epithelial neoplasia by the colposcopic impression was correct in 78.3% of the cases, when acolposcopically directed biopsy was taken, the correct prediction rate improved to 85.6%. The colposcopic impression of micro-invasive disease has been studied by many investigators since colposcopy was found to be more accurate in the identification of pre-malignant and invasive disease than in the identification of micro-invasive lesions. It has been suggested that micro-invasive carcinoma is suspected when mosaic, punctation, and acetowhite epithelium is seen covering the whole circumference around the external bone and when the thick white epithelium has a clear and elevated margin with an irregular surface contour and raised capillaries.

Shetty,[22] in his study, stated that the histopathological assessment of a biopsy specimen is regarded as the most reliable criterion for a correct diagnosis in cases of epithelial dysplasia; consequently the specimen must be taken from the most representative area of a suspicious looking lesion for increasing the diagnostic accuracy.

In our study, we have used the criteria for vascular changes described in colposcopic literature for the selection of biopsy site.[21,23,24] These include the vascular pattern, inter-capillary distance, surface pattern, color tone, and opacity as well as the clarity of demarcation of the mucosal lesions. Two basic types of capillary network can be seen with colposcopy: Network capillaries and hairpin capillaries. In dysplasia and carcinoma-*in-situ*, a specific vascular pattern, punctuation, is common. Punctation is characterized by dilated, often twisted, irregular, hairpin-type vessels. Another pattern of the vessels in dysplasia is called mosaic. Like punctation vessels, true mosaic vessels are usually seen in sharply demarcated areas. When it is difficult to describe the pattern of the vessels, the term atypical patterns are encountered in malignant lesions. Therefore, the presence of one of these indicates the need for biopsy and histopathological examination.

The results of our colposcopic examination regarding the selection of biopsy sites for leukoplakia reported a sensitivity of 0.8571 (85%) with a specificity of about 0.6667 (66%). [Tables 9 and 10] The results of our study were similar to the previously reported studies including the one conducted by Gynther[13] for assessing the value of colposcopy in diagnosing the mucosal lesions that found a total of 14 patients (40%) of the total, which were correctly diagnosed with the help of histopathological examination of the biopsy specimens taken with the colposcopic criteria concluding that the biopsy specimens selected with colposcopy appeared to be more representative of the histopathological findings than those selected with routine clinical examination ($0.01 < P \leq .05$). Seventeen (49%) patients showed no difference between the histopathological study of the specimens taken with COE and with the aid of colposcopic criteria.

Similar results were also reported by Shetty[22] who correlated the relevance of tumor angiogenesis pattern with the histopathological results in oral epithelial dysplasia of diagnostic value and found a total of 26 patients (52%) in whom the biopsy specimens selected with colposcopic

criteria appeared to be more representative than those selected with COE (0.01, $P < 0.05$).

In the present study, we found that the biopsy specimens selected with colposcopic criteria appeared to be more representative of the histopathological findings than those selected with routine clinical examination (COE) [Table 10]. The altered vascular patterns definitely helped with the correct selection of the biopsy site, which in turn helped us reach a more definitive diagnosis, thus avoiding false-negative results.

The results of colposcopic findings are based on vascular and tissue changes. The capillary changes preceding tumor growth with the pattern of tumor angiogenesis are different from the usual neo-vascularization taking place during repair and regeneration processes. At a cellular level, various molecules such as vascular endothelial growth factor, basic fibroblast growth factor, and transforming growth factor alpha are implicated, but the clinical perceptibility of these altered vascular patterns is poor. Direct optical visualization of these patterns would be helpful in the early determination of the underlying pathology and also aid in marking out the site of biopsy.[25] It is not possible to determine the progression of dysplasia to carcinoma on the basis of clinical findings. Regular follow-up examinations are, therefore, essential for pre-cancerous lesions such as non-homogenous leukoplakia.

This is a preliminary study that emphasized the selection of biopsy site using colposcopic examination as a method to select the most representative sites of epithelial dysplasia in leukoplakia patients. In the present study, we found that the biopsy specimens selected with colposcopy appeared to be more representative of the histopathological findings than those selected with set clinical criteria. The altered vascular patterns definitely helped with the correct selection of the site for biopsy, which, in turn, helped us reach a more definitive diagnosis, thus avoiding false-negative results. However, further studies are required with larger sample sizes to conclude the results and also studies on the use of various staining methods compared with colposcopic examination are recommended for selecting the biopsy site for such patients to diagnose the disease in the earliest of its stages for a better prognosis.

ACKNOWLEDGEMENT

We thank all the people who directly and indirectly contributed for the study as the study required intense efforts from the people outside our Department including Department of Oral Pathology, and Department of Gynecology and Department of General Pathology, Bangalore Medical College and Research Institute and Associated Hospitals.

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Cite this article as: Khan M, Nayyar AS, Gayitri HC, Bafna UD, Ahmed S. Tumor angiogenesis: A potential marker of the ongoing process of malignant transformation in leukoplakia patients, removing the veil. Clin Cancer Investig J 2012;1:127-34.

Source of Support: Nil, **Conflict of Interest:** None declared.