Original Article

Immunohistochemical expression of stathmin in oral dysplasia: An original study with an insight of its action on microtubules

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Abstract

Background and Objectives: Stathmin is a phosphoprotein, which in its phosphorylated/unphosphorylated states plays a major role in polymerization/depolymerization of microtubules, respectively. Assembly of microtubules is an important aspect of cell division called mitosis. Hinderance in the function of stathmin could lead to damage in the mitotic process resulting in aneuploidy which is common manifestation of malignancies. Hence, stathmin could be used as a tumor marker for oral dysplasias and cancers. The purpose of the study is to compare the expression of stathmin in normal subjects to the patients with oral leukoplakia and to correlate its expression with different histopathological grades of oral leukoplakia This is the first ever study conducted to examine the expression of stathmin in oral dysplasia.

Methodology: Thirty histopathologically confirmed cases of oral dysplasia were selected for the study. These tissues were evaluated immunohistochemically for stathmin. To enumerate the stathmin stained cells, 300 cells were examined manually in at least 5 areas and a mean percentage of positive–stained slides were determined. Then, each sample was assigned to one of the following staining scores: (0) – (<10% of stained cells); (1) – (11%–25% of stained cells); (2) – (26%–50% of stained cells); (3) – (51%–75% of stained cells); (4) – (76%–90% of stained cells) and (5) – (91%–100% of stained cells). The results were analyzed statistically using ANNOVA test.

Results: When comparison was made with respect to staining scores of stathmin between normal and dysplasia groups, the results were found to be statistically significant with a $P = 0.0001$. A statistically significant difference was observed between various histopathological grades of dysplasia with respect to stathmin immunohistochemistry scores with a $P = 0.0001$.

Conclusion: These results suggest stathmin as a tumor marker and prognostic indicator.

Keywords: Leukoplakia, microtubules, stathmin

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INTRODUCTION

In cell division, there is segregation of the duplicated chromosomes by bipolar spindle formation that is made up of spindle microtubules which are attached to sister chromatids by kinetochores.[1]

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Kinetochores are the protein complexes located at the centromeres that activate the mitotic checkpoint. Kinetochores lacking spindle microtubules do not allow the complete formation of mitotic spindle thus delaying mitotic process and causing defects. Most malignant tumors show chromosomal instability due to mitotic checkpoint defects, aberrant number of spindle poles and spindle attachment defects, thus resulting in aneuploidy.[2]

In mitosis, microtubules polymerize to form the mitotic spindle, allowing chromosome segregation and cell division. Stathmin also known as Op18 is a cytosolic phosphoprotein that plays an important role in the regulation of microtubule cytoskeleton.[3] Dephosphorylated stathmin which is its general representation, regulates microtubule checkpoint by either depolymerizing microtubules or by preventing polymerization of microtubules which do not allow mitotic spindle formation. Phosphorylation of stathmin switches off its ability to depolymerize microtubules thus allowing microtubule polymerization and mitotic spindle assembly. For the cells to exit mitosis and enter a new interphase, phosphorylated stathmin has to be again dephosphorylated, thus losing the ability of mitotic spindle formation.[4]

Forced expression of stathmin leads to a total lack of mitotic spindle assembly and further lack of cell division hence, arresting the cells in the early stages of mitosis. On the other hand, absence of stathmin expression leads to accumulation of cells in the G2/M phases and is associated with severe mitotic spindle abnormalities and difficulty in the exit from mitosis.

Thus, stathmin is critically important for the formation and regulation of a normal mitotic spindle upon entry into mitosis and also for the timely exit in the later stages of mitosis. These data suggest stathmin could be used as a tumor marker for predicting more aggressive nature of oral dysplasias and cancers.[5]

The purpose of the study is as follows;

1. To compare the expression of stathmin in normal subjects, patients with oral leukoplakia
2. To correlate the expression of stathmin with respect to the different histopathological grades of Oral Leukoplakia.

This is the first ever study conducted to examine the expression of stathmin in oral dysplasia.

METHODOLOGY

Thirty neutral buffered formalin fixed, paraffin embedded tissues of oral leukoplakia were retrieved from the department of Oral Pathology and Microbiology, Mamata Dental College, Khammam for the purpose of this study and compared with that of normal tissues. Ethical clearance was obtained from the ethical committee of the institution. The histological grading of oral leukoplakia was done based on the World Health Organization criteria.[6]

Immunohistochemical staining

Two to three serial sections of 3 μm thickness were made and taken onto silanized slides. The sections were deparaffinized by keeping the slides on the slide warmer at 60°C for 15–20 min. Rehydration was done by taking the tissue sections through 2 changes of xylene, absolute alcohol, 95% alcohol and 70% alcohol for 5 min, respectively. Then, the slides were immersed in distilled water for 30 s.

Antigen retrieval was done by placing the slides in a plastic container containing a metal slide rack which in turn was kept in a microwave oven containing boiling tris buffered saline. The slides were heated four times at 100°C for 5 min. All the slides were allowed to cool to room temperature. All the reagents stored in the refrigerator were brought to room temperature (24°C–28°C) prior to immunostaining. All the incubations were performed at room temperature using a humidifying chamber. At no time, the tissue sections were allowed to dry during the IHC staining procedure. They were washed gently with phosphate buffer saline (PBS) three times for 2 min each. After tapping off the excess buffer from the slide, the sections were covered with 3% hydrogen peroxide for 15–20 min. They were then washed gently with PBS three times for 2 min each. After tapping off the excess buffer from the slide, the sections were covered with Power block for 15–20 min. After power block was tapped off; the sections were covered completely with prediluted stathmin primary antibody except the negative control. The slides were incubated for 1 h at 21°C in a humidifying chamber then washed gently with PBS three times for 2 min each. Then Super Enhancer was applied and left for 30 min and washed gently with PBS three times for 2 min each. After tapping off the excess buffer, the sections were then incubated with secondary antibody for 30 min then washed gently with PBS three times for 2 min each. Excess buffer was tapped off and tissue sections were completely covered with freshly prepared substrate chromogen solution using Pasteur pipette for 10 min. Then the sections were washed gently with distilled water for 2 min. The sections were then counterstained by immersing them in Mayer's hematoxylin for 2 min, washed gently under running tap water for bluing. Dehydration procedure was done by taking the tissue sections through absolute

alcohol, 95% alcohol, 70% alcohol for 5 min respectively. The sections were kept immersed in xylene bath and later were mounted using dibutyl phthalate in xylene.

Interpretation of staining

Presence of brown colored end product at the site of target antigen was indicative of positive immunoreactivity. The negative control tissue (normal mucosal tissue omitting the primary antibody) demonstrated absence of staining [Figure 1]. Brain tissue was taken as positive control with each batch of staining and normal oral mucosal tissue was taken as negative control [Figure 2]. The evaluation of study cases was done subsequently in a similar way and was graded as positive or negative.

To enumerate the stathmin stained slides, 300 cells were examined manually in at least five areas and a mean percentage of positive-stained slides were determined. Then, each sample was assigned to one of the following staining scores: 0– <10%; 1%–11%–25%; 2%–26%–50%; 3%–51%–75%; 4%–76%–90% and 5%–91%–100%. All these observations were carried out by two observers to eliminate inter observer bias. The results were analysed statistically using ANNOVA test.

RESULTS

Thirty tissues of oral leukoplakia (n = 30) were evaluated for the immuohistochemical expression of stathmin and compared with normal tissues (n = 30). Stathmin staining was evaluated on the basis of the presence or absence of staining in the cytoplasm. The mean number of positive tumor cells was further graded. Two observers independently evaluated the staining scores and the average of the observations was taken.

The staining score was graded in all the cases from 0 to 5 (i.e.,), with "0" indicating <10% of positive stained cells, "1" indicating 11%–25% of positive stained cells, "2" indicating 26%–50% of positive stained cells, "3" indicating 51%–75% of positive stained cells, "4" indicating 76%–90% of positive stained cells and "5" indicating 91%–100% of positive stained cells.

Out of 30 cases in dysplasia group, the staining score was found to be 0 in three cases (10%), 1 in fifteen cases (50%), 2 in six cases (20%), 3 in two cases (6.66%) and 4 in four cases (13.33%). Out of 30 cases in normal group, the staining score was found to 0 in thirty cases (100%). When comparison was made with respect to staining scores between normal and dysplasia groups, the results were found to be statistically significant with a P = 0.0001 [Tables 1 and 2].

Out of 16 mild dysplasia cases, the staining score was found to be 0 in three cases (18.75%), 1 in thirteen cases (81.25%) [Figure 3]. In 10 moderate dysplasia cases, the staining score was found to be 1 in two cases (20%), 2 in six cases (60%) and 3 in two cases (20%) [Figure 4]. In 4 severe dysplasia cases, the staining score was found to be 4 in four cases (100%) [Figure 5]. A statistically significant difference was observed between various histopathological grades of dysplasia with respect to immunohistochemistry scores with a P = 0.0001 [Table 3].

DISCUSSION

Stathmin, also called oncoprotein 18, is a microtubule destabilizing phosphorprotein wherein microtubules are essential for many cellular processes, including intracellular transport, mitosis, sustain of cell shape and cell motility.[7]

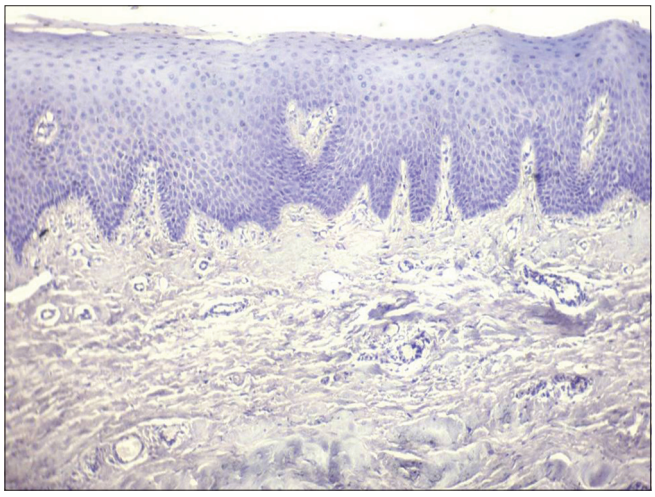

Figure 1: Photomicrograph of normal oral mucosa as a negative control for stathmin immunoexpression (×10)

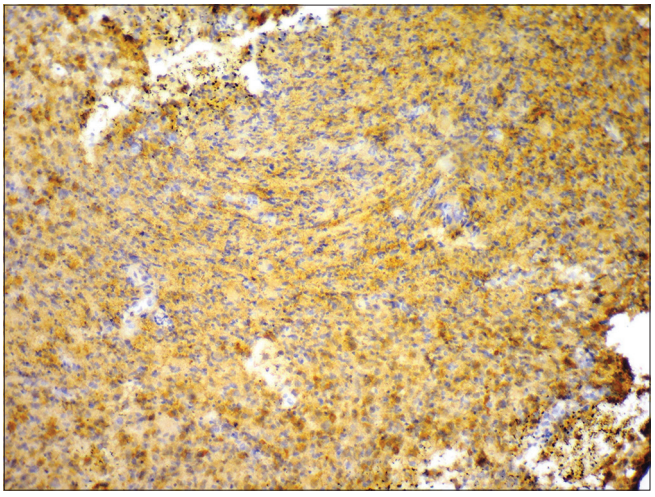

Figure 2: Photomicrograph of brain as a positive control for stathmin immunoexpression (×10)

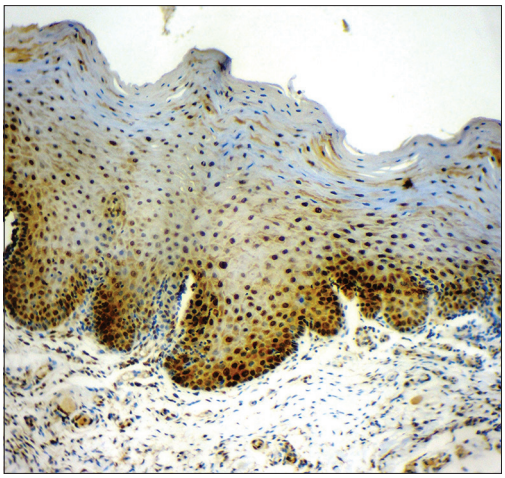

Figure 3: Photomicrograph of stathmin immunoexpression in Mild dysplasia (×10)

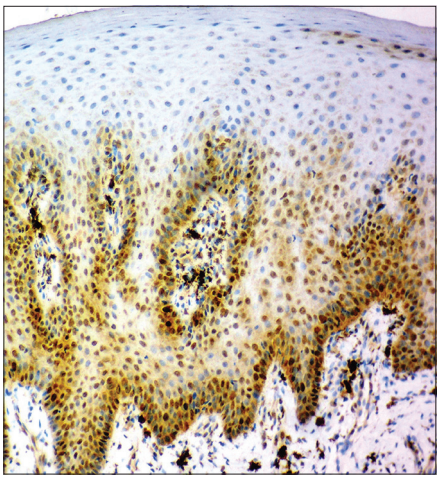

Figure 4: Photomicrograph showing stathmin immunoexpression in Moderate dysplasia (×10)

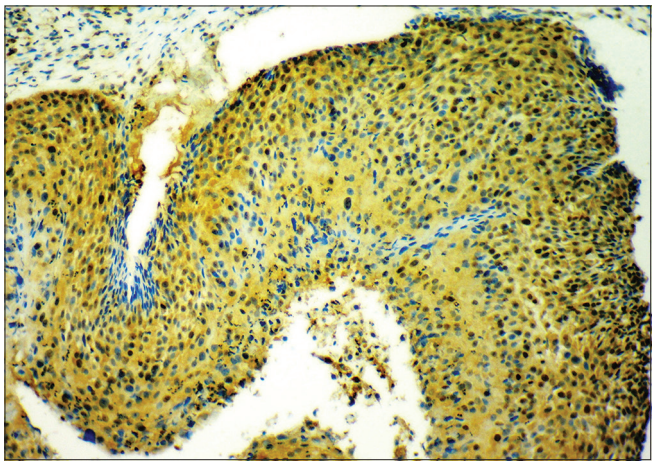

Figure 5: Photomicrograph showing stathmin immunoexpression in severe dysplasia (×10)

Stathmin is a protein that plays critically an important role in the regulation of the microtubule dynamics. At molecular level, stathmin depolymerizes microtubules by either seizing free tubulin dimers or by inducing microtubule catastrophe. Studies have showed that stathmin, which is frequently overexpressed in a variety of human cancers and has a close correlation with cancer cell differentiation, tumor node metastasis (TNM) classification and lymph node metastases.[8]

Overexpression of stathmin maintains proliferation of cancer cells suggesting the role of stathmin in tumorigenesis making it an oncobiological marker and a molecular target for cancer therapy.[9]

Stathmin also plays an important role in the cell cycle progression which is merely dependent on its phosphorylated/unphosphorylated states. This hypothesis came from the observation made that the level of phosphorylation of stathmin increases markedly when K562 erythroleukemia cells enter the mitotic phase of the cell cycle.[10]

Luo *et al.* suggested that the antisense RNA inhibition of the stathmin expression in K562 leukemic cells results in decreased cellular proliferation and cell cycle arrest thus contributing to the accumulation of cells in the G2/M phases of the cell cycle.[10]

With this background it is noted that, over expression of stathmin has been observed in various human cancer cell lines and that stathmin gene plays an important role in mitosis and other cellular processes which attracted many investigators to evaluate its role in cancer growth and progression.[5]

Curmi *et al.* observed stathmin overexpression in breast carcinomas which was correlated with higher histological grade, advance pathological state, tumor recurrence and disease progression. The results of the present study were in accordance with Curmi *et al.*[11]

Kang *et al.* reported that high stathmin expression in the gastric carcinoma cell lines and predicted poor prognosis of the same as the rate of expression increases and also subsequently decreases the overall survival rate which was in accordance with the present study.[12]

Cheng *et al.* identified increased expression of stathmin in primary nasopharyngeal carcinoma and its expression was associated with the advanced stages of the disease. This was also associated with higher grade of the tumor and was a predictor of the worst prognosis of the disease which was in accordance with the present study.[13]

Table 1: Number of cases in each grade of dysplasia

Histological grades	Number of cases
Mild dysplasia	16
Moderate dysplasia	10
Severe dysplasia	4

Table 2: Comparison of normal and dysplasia groups for stathmin expression with respect to the staining intensity scores

Groups	Mean	SD	Median	Sum of ranks
Dysplasia group	2.11	1.54	2.00	1689.5
Normal group	0.00	0.00	0.00	510
H			48.7312	
P			0.0001*	

*Significant, *P*=0.0001, Kruskal-Wallis ANOVA test. SD: Standard deviation

Table 3: Comparison of various histological grades of dysplasia (mild, moderate, severe) for stathmin expression with respect to staining intensity scores

Histopathology grading	Mean	SD	Sum of ranks
Mild dysplasia	0.55	0.33	91.00
Moderate dysplasia	1.97	0.45	160.00
Severe dysplasia	3.89	0.52	184.00
H			25.6171
P			0.0001*
Mild dysplasia versus moderate dysplasia			*P*=0.0001*
Mild dysplasia versus severe dysplasia			*P*=0.0002*
Moderate dysplasia versus severe dysplasia			*P*=0.0001*

*Significant, *P*=0.0001, Kruskal-Wallis ANOVA test. SD: Standard deviation

In the present study, an attempt was made to evaluate the expression of stathmin immunohistochemically with respect to different histopathological grades of oral dysplasia. The results from the present study showed an increased expression of stathmin with respect to varying histological grades of oral epithelial dysplasia.

Kouzu *et al*. examined stathmin expression in oral squamous cell carcinoma (OSCC) and found significant correlation to clinical staging. Moreover the state of expression differed significantly between Stage I/II and Stage III/IV, suggesting its role in tumor progression. 65% of the OSCCs were positive for stathmin in immunohistochemistry with no immunoreaction normal tissues. Real-time quantitative reverse transcriptase polymerase chain reaction data were consistent with the protein expression status. Moreover, stathmin expression status was correlated with the TNM stage grading. Furthermore, they found a statistical correlation between the protein expression status and disease-free survival suggesting that the expression of stathmin could contribute to cancer progression and that stathmin may have potential as a biomarker. Thus, based on the abovementioned study, the current study will be further extended to study carcinoma groups and comparison will be made between carcinoma and dysplasia groups for the expression of stathmin.[14]

In the present study, the results were found to be statistically significant with a $P = 0.001$ with respect to the expression between normal and dysplasia groups. The present study is the first of its kind to compare the two groups.

In the present study, the results were found to be statistically significant with a $P = 0.001$ when the various histological grades of dysplasia were compared stathmin expression was found to increase with increasing grades of dysplasia that is from mild dysplasia to severe dysplasia.

CONCLUSION

The results suggest a statistical significance in the expression of stathmin, suggesting its role as a prognostic indicator for tumorigenesis thus making it a biomarker. Since the current study was the first study done in oral dysplasias, further studies must be done in oral carcinomas such as OSCCs and stathmin expression must be evaluated.

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Conflicts of interest
There are no conflicts of interest.

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