Original Research Article

Assessment of immunohistochemical expression of minichromosome maintenance protein (MCM-5) in oral squamous cell carcinoma and its clinicopathological correlation

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A R T I C L E   I N F O

A B S T R A C T

Introduction: Oral and oropharyngeal carcinomas are common cancers in the world. Therefore, for early diagnosis, prognosis, disease progression monitoring and treatment response, cancer biomarkers are identified as signature molecules.
Aim and Objectives: To evaluate and compare the immunohistochemical expression of MCM-5 in oral squamous cell carcinoma (OSCC) between clinical stages and its histopathological grades.
Study Design: Descriptive cross sectional – in vitro study.
Materials and Methods : This study used immunohistochemistry to examine expression of MCM5 protein in 40 specimens of OSCC and evaluated for localization and staining intensity of MCM-5.
Results: Among 40 cases 39 showed positivity of MCM-5 regarding localization, with highly significant (p < 0.001) correlation among all grades (I, II and III) of OSCC. Findings showed, as histopathological grade increased, nuclear expression and immunostaining intensity of MCM-5 also increased.
Conc lusion: MCM-5 can be promising diagnostic and prognostic markers, as well as potential targets for anticancer therapy.

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1. Introduction

Oral and oropharyngeal carcinomas are the sixth most common cancers in world. Overall survival (OS) rate of patients with OSCC has not significantly improved, with a year survival rate of 29 of 45%.[1] The most important risk factors for oral SCC are use of tobacco or betel quid and the regular drinking of alcoholic beverages.[2] Oral potentially malignant disorders (OPMDs) characterized by an increased risk for malignant transformation (MT) to OSCC.[3] Hence, there is a need for the knowledge about prevalence rate,

early detection of these OPMDs to decrease the burden of cancer incidence.[4]

Replication licensing system, coordinates DNA replication and initiation events at chromosomal origins with cell cycle progression. Proliferating cells are characterized by high expression levels of the MCM proteins throughout the cell division cycle, with cyclical binding to origins occurring in late M/early G1 phase and displacement from chromatin during S phase. Consequently, MCM 2-7 complex have emerged as novel biomarkers of proliferation.[5]

This study aimed to evaluate the immunohistochemical expression of MCM-5 in OSCC and to compare the

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immunohistochemical expression of MCM-5 between clinical stages and histopathological grades of OSCC.

2. Materials and Methods

2.1. Patients and specimens

After approval by Institutional Review Board, we obtained formalin-fixed, paraffin-embedded specimens from 40 patients with OSCC, which was diagnosed by histological examination of hematoxylin and eosin-stained tissue sections.

2.2. Inclusion criterion

Patients with oral squamous cell carcinoma, diagnosed with clinical stage and histopathological grade.

2.3. Exclusion criteria

1. Patients who had received or were receiving any treatment for carcinoma.
2. Patients with history of any other neoplastic or non-neoplastic diseases.
3. Patients with any other oral disease.

Relevant history of each patient was recorded and thorough clinical examination. The diagnosis of OSCC was confirmed in each case by histopathological examination. Study commenced after obtaining informed consent.

Immunohistochemistry for MCM5 protein:

A) Specimens for immunohistochemical staining were fixed in 10% neutral formalin, embedded in paraffin, and cut in serial sections of 5 um (Rotary microtome – Semi automatic (Leica RM 2245). Immunohistochemical staining was performed using a super-sensitive polymer-horseradish peroxidase (HRP) technique (Biogenetics). Briefly, tissues sections were deparaffinized and rehydrated. Then, sections were heated in an antigen retrieval jar containing antigen retrieval solution and then kept in pressure cooker filled with 500ml -1 liter distilled water.

B) After which.

Set watt at 1000 W – wait till 1st whistle, Set watt at 800 W – 5-6 min. After washing with PBS buffer for 3 times 1 minute each, sections were covered with peroxide for 10 minutes. Following this, sections were gently washed with PBS buffer and after tapping of excess buffer from slide, sections were covered with power block (casein and proprietary additives in PBS with 15mM sodium azide) for 10 minutes which was later tapped off. Then sections were covered completely with primary antibody for MCM-5. For negative control PBS was used instead of primary antibody. Slides were incubated for 45 minutes at room temperature in humidifying chamber. Then slides were washed gently with PBS for 3 times 1 minute each. Excess buffer was tapped off and sections were covered with super-enhancer for 20 minutes following which it was gently washed with PBS

for 3 times 1 minute each. Then, sections were completely covered with freshly prepared substrate chromogen solution using disposable dropper for 10 minutes. The slides were then washed with distilled water and counterstained with haematoxylin for 2 minutes.

Expression Presence of brown colored end product at the site of target antigen was indicative of positive immunoreactivity. Negative control tissue demonstrated absence of staining. Immunoreactivity was observed at membrane, cytoplasmic and nuclear level. For scoring, semiquantitative method, considering proportion of positive cells and staining intensity. Under high power, 10 random fields were selected, and 500 cells were assessed. Number of immunoreactive cells was recorded.[6]

2.4. Statistical analysis

More than two categorical variables were compared by Chi-square ($\chi 2$) test. Statistical analyses were performed using statistical software SPSS. p-values of <0.05 were considered statistically significant.

3. Results

In present study age of OSCC cases ranged from 24-65 years, with mean age values of 50.82 ± 11.93 years with male predominance, male: female ratio of 2.63:1.

Distribution and comparison of MCM-5 (Localization and Immunostaining intensity) among various clinical stages of OSCC was done according to TNM system (AJCC 2009). The OSCC cases were maximum in stage III 24(60%) followed by stage IV 9(22.5%), stage I 05(12.5%) and stage II 02(5%) respectively. (Graph 1) In all cases of stage I, MCM-5 was localized only at cytoplasm 5(100%) with 3(33.3%) showed moderate and 2(66.7%) showed strong immunostaining intensity. In stage II 01(50%) case each showed in cytoplasm and nuclear localization of MCM-5 with 02(100%) cases showed strong immunostaining intensity. In stage III MCM-5 was localized in 13(54.2%) in cytoplasm and 10(41.7%) in nucleus. Strong staining intensity was seen in 10(41.7%) cases, moderate in 12(50%) and weak in 01(4.2%) cases and negative expression in 1(4.2%) case While in stage IV only nuclear expression of MCM5 was 8(88.9%) seen and strong and moderate immunostaining intensity of MCM-5 expression in 06(66.7%) and 03(33.3%) cases respectively. (Graph 2)

Expression of MCM5 in OSCC samples represents immunohistochemical staining microphotographs for OSCC are shown in Graph 1 . In OSCC samples, nuclear and/or cytoplasmic MCM5 staining was either focal or diffuse, and number of nuclear MCM5-positive staining cells gradually increased from well-, through moderately-, to poorly differentiated OSCCs (Graph 1 E–G). In tumor nests of well differentiated OSCC, nuclear MCM5-positive
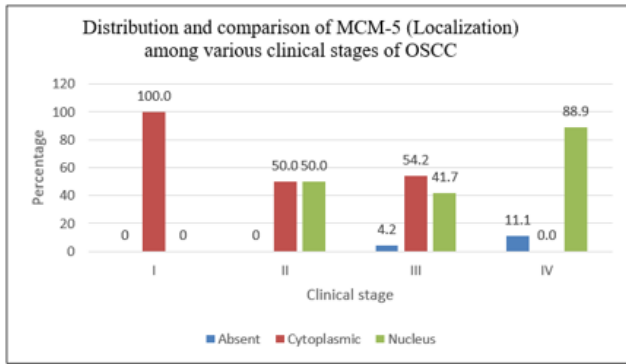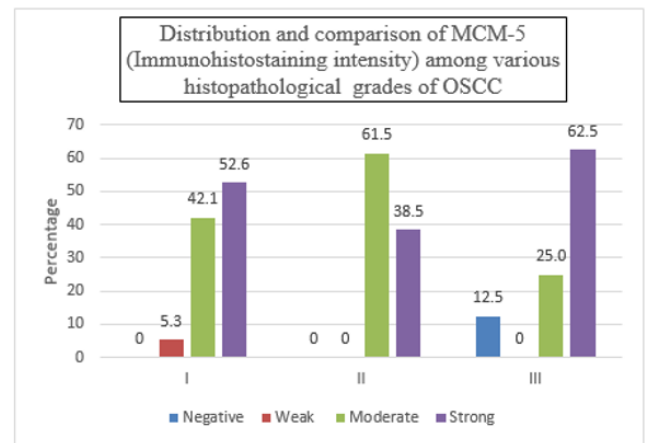
Graph 1: Distribution and comparison of MCM-5 (Localization) among various clinical stages of OSCC
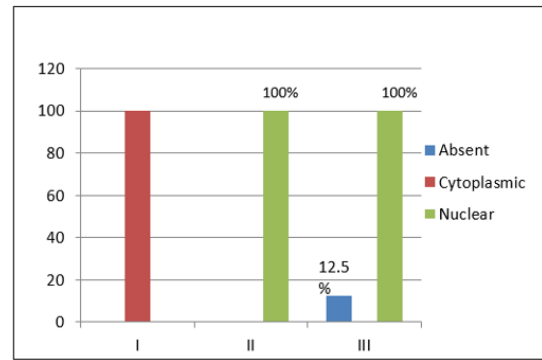
Graph 3: Distribution and comparison of MCM-5 (Localization) among various histopathological grades of OSCC
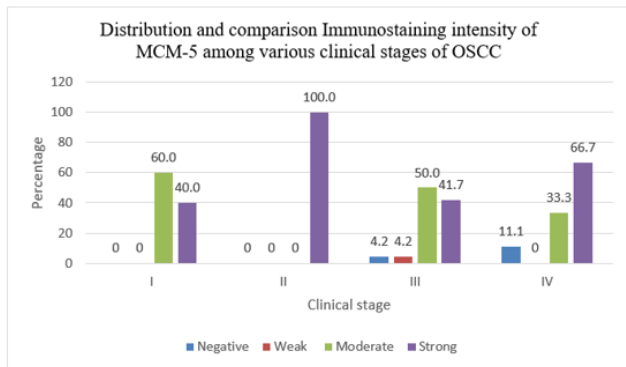
Graph 2: Distribution and comparison Immunostaining intensity of MCM-5 among various clinical stages of OSCC

Graph 4: Distribution and comparison of MCM-5 (Immunostaining intensity) among various histopathological grades of OSCC.

staining was noted predominantly in peripheral cells and cytoplasmic MCM5 staining mainly in central keratinized cells of cancer nests.

Distribution and comparison of MCM-5 (Localization) among various histopathological grades of OSCC showed that in grade I cases, localization of MCM-5 expression was cytoplasmic in all 19(100%) cases. In grade II all 13(100%) cases showed nuclear expression. In grade III, 07(100%) showed nuclear MCM-5 expression with remaining cases showed negative result. As grade advanced, expression and immunostaining intensities of MCM-5 changed from cytoplasmic to nuclear in all grades (I, II, III) of OSCC (Graphs 3 and 4)

4. Discussion

Cancers are most common cause of death in adults.[7] Worldwide, oral cancer accounts, 2%–4% of all cancer cases.[8] In India, 90 -95% of oral cancers is SCC.[9] Among other cancers, head and neck cancer is 14th in terms of incidence but 13th in terms of mortality.[10] In South Asian countries, consumption of smokeless tobacco and areca
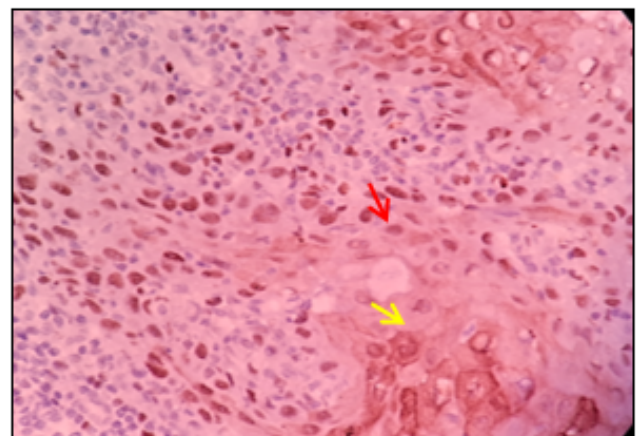

Figure 1: IHC expression MCM-5 in Grade I Oral squamous cell carcinoma (400x) predominantly in cytoplasm (yellow arrows), nuclear expression in cells at invading front (red arrow)
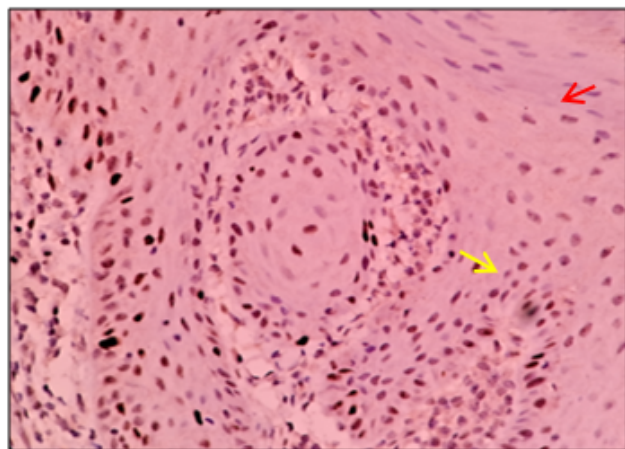
Figure 2: IHC expression MCM-5 in Grade II, Oral squamous cell carcinoma (400x) predominantly in nuclear (yellow arrows) weak expression in cytoplasm (red arrow)
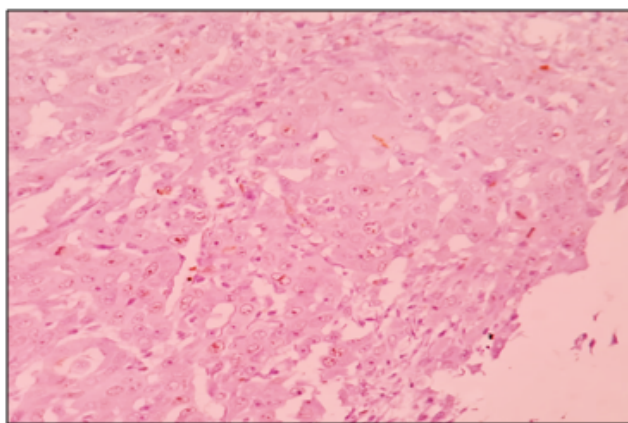

Figure 3: IHC expression MCM-5 in Grade III Oral squamous cell carcinoma (400x)

nut products are main etiological factors associated with OSCC.[9] Tobacco may contain more than 60 potential carcinogens that can increase relative risk of cancer through different mechanisms, including oxidative stress on tissues, persistent reactive oxygen species, lipids, carbohydrates and DNA (Deoxyribonucleic acid) to disrupt cell cycle-regulated mutations or through effects on immune system.[11]

Cell cycle is governed by CDKs, activities of which are regulated positively by cyclins and negatively by CKIs. Cyclins, CDKs, and CKIs are frequently altered in cancer or disrupted secondarily by oncogenic events. Several reports demonstrated overexpression of cyclin D1 gene and alterations of specific CKIs, such as p21CIP1, p15INK4B, p16INK4A in head and neck cancers.[12]

Therefore, for early diagnosis, prognosis, disease progression monitoring and treatment response, cancer biomarkers are identified as signature molecules. DNA, mRNA, metabolites, nucleic acids, peptides,

enzymes, antibodies, genomics, mi-RNAs act as a biomarker.[13] Though several molecular based assays have increased in recent years, but histopathology remains the gold standard for most diagnostic and therapeutic decisions.[14]

MCM2-7 complex is part of pre-replicative complex, plays an essential role in DNA replication and it is a proliferative biomarker. MCM5 is highly expressed in OSCC, which promotes proliferation of OSCC cells and regulates cell cycle. Cyclin E and cell cycle-related gene expression levels were decreased, while p21 was significantly upregulated. Therefore, MCM5 modulate OSCC cell proliferation by regulating cell cycle. Studies have confirmed that MCM5 is highly expressed in numerous human malignancies, such as renal cell carcinoma, pancreatic ductal adenocarcinoma, cervical cancer and skin cancer.[15]

OSCC is common in middle-aged men, which may be due to changing social habits in high socioeconomic groups or cultural habits of some rural areas of India.[16] Risk of oral cancer increases with age. Maximum incidence of OSCCs was seen in 4th and 5th decades.[17] The present study, age of OSCC cases ranged from 24-65 years with mean value of 50.82 ± 11.93 years. OSCC cases showed peak incidence in 4th - 5th decade comprising of about 28% of study population. This finding was in accordance with the observations of Sandeep Sharma et al (2019).[17] Shah S et al (2019)[18] in which larger number of cases were seen developing in the 4th to 6th decade. In this present study, highest incidence was present among male (72.5%) followed by female (27.5%) with male to female ratio of 2.63:1. The demographic data of by, Kanyilmaz G et al (2014)[9] showed significantly more incidence in males than females with OSCC (male: female (M: F) =2.92:1).

Based on tumor size, lymph node metastasis and distant lymph node involvement, oral cancers are subjected to staging as per AJCC (8th edition) protocol. In present study, 12.5% (n=05) cases belonged to Stage I, 5% (n=2) cases belonged to stage II and bulk was seen in stage III and stage IV with 60% (n=24) and 22.5% (n=9) cases respectively. Histopathologically, 47.5% (n=19) cases belonged to grade I, 32.5% (n=13) belonged to grade II and remaining 20% (n=8) belonged to grade III according to Anneroth's grading criteria. Our study findings are in agreement with Yu SY et al (2014)[21] where a significant correlation was found between the higher MCM5 and OSCCs with site larger tumor size (T3 and T4), positive lymph node metastasis (N1, N2, and N3), more advanced clinical stages (stages 3 and 4).

4.1. Immunohistochemical expression of MCM-5 among study group:

In present study, MCM-5 expression in OSCC cases was positive in 97.5% cases, located primarily on nucleus of epithelial cells. This finding is in accordance with the

findings of Freeman A et al (1999),[19] Hao M (2020),[20] Yu SY (2014),[21] Giaginis C et al (2009),[22] Korkolopoulou P et al (2005)[23] in which MCM-5 was detected in many malignancies, most notably skin, kidney, prostate, pancreas, breast, cervix, uterus, lung and oral cavity. In present study, expression of MCM-5 in OSCC cells was observed for its localization (cytoplasm, nucleus) and immunostaining intensity (weak, moderate, strong) and analyzed among clinical stages (I-IV) and histopathological grades (I-III) of OSCC.[24]

4.2. Immunohistochemical expression of MCM-5 in OSCC:

About 97.5% OSCC cases were positive for MCM-5 in present study, which is in accordance with high percentage of positivity reported by Yu SY (2014)8 in 61% cases. MCM-5 positive cases were further analysed for its localization and immunostaining intensity. Among MCM-5 positive cases of OSCC, cytoplasmic staining was evident in 47.5 % cases and nuclear staining in 50% cases. Amongst all cases, 01(2.5%) did not show MCM-5 expression. Among these cases, there was variability in intensity of expression of MCM-5, which was described as weak, moderate and strong expression. 20(50%) cases showed strong expression, 18(45%) showed moderate expression and 01(2.5%) showed weak expression of MCM-5.

Similar results were observed in study done in OSCC samples. Yu SY (2014).[21] Well-differentiated OSCC demonstrated nuclear MCM5 staining in peripheral cells and cytoplasmic MCM5 staining in central keratotic cells of cancer nests, whereas moderately differentiated OSCC showed nuclear MCM5 staining in nearly all cells of the tumor nest while poorly differentiated OSCC showed nuclear MCM5 staining in nearly all hyperchromatic and pleomorphic cancer cells.

Hao M (2020)[20] suggested that MCM5, mRNA was significantly overexpressed in OSCC tissues compared with that in adjacent normal tissues. Moreover, silencing of MCM5 expression in OSCC cell line (SCC-15) significantly impaired proliferation and colony formation. Furthermore, negative regulation of the mRNA and protein expression of MCM5, demonstrated that MCM5 served as a cancer-promoting gene modulating OSCC cell proliferation through induced G2/M phase arrest.

4.3. MCM-5 expression among various histopathological grades of OSCC

With regard to localization of MCM-5, in grade I OSCC, cytoplasmic expression was seen in 100%. This finding of predominant cytoplasmic expression in grade I was comparable with observation of Yu SY (2014).[21] Reported 59% with cytoplasmic staining in grade I cases of OSCC. In present study, all (100%) positive cases of grade II &

grade III showed nuclear expression which was comparable with results of Yu SY (2014)[21] who found 69 % nuclear staining in grade III cases of OSCC. There was a highly significant (p<0.001) correlation of MCM-5 localization between all grades of OSCC. Above findings showed that as histopathological grade increased, nuclear localization of MCM-5 increased. Present study showed highly significant (p<0.001) difference between all grades (I, II and III) of OSCC regarding localization for MCM-5. This finding was in line with Yu SY (2014)[21] who found significant correlation between higher nuclear expression and advanced histopathological grade. Similar findings appeared to be in accord with Kearsey (1999)[25] who founded that, in invasive TCCs (Transitional cell carcinoma) of the bladder, there was staining of 40% of cells in grade 1 tumors and staining of > 80% of cells in grade 2 and 3 tumors. Freeman A et al (1999)[18] also showed similar results in adenocarcinomas (AC) of colon, endometrial adenocarcinomas and prostatic adenocarcinoma in which there was a correlation between increased frequency of nuclear staining and tumor grade.

In present study, among positive cases of OSCC, immunostaining intensity for MCM-5 varied widely between different histopathological grades. In grade I, intensity of MCM-5 expression varied from strong (52.6%) to moderate 42.1%) and weak (5.3%). In grade II, immunostaining intensity was moderate in 61.5% case and strong expression in 38.5%. In present study, 62.5% cases of grade III showed strong immunostaining intensity, 25% with moderate while remaining 12.5% cases showed negative staining intensity. The present study showed statistically significant (p=0.049) positive correlation between all grades (I, II and III) of OSCC regarding immunostaining intensity for MCM-5.

In the present study, significant correlation was observed between histopathological grade of tumor with localization and immunostaining intensity of MCM-5. Nuclear accumulation of MCM-5 associated with increased cellular proliferation supports the role of MCM-5 as a prognostic marker. However, there was a direct correlation among degree of differentiation and MCM-5 expression based on histopathologic grades.

Molecular characterization of localization of MCM-5 clarifies role of MCM-5 in cancer cell survival and proliferation. This provides insight into its utility as a diagnostic and prognostic marker and its further exploitation as a target for cancer therapies in grades of OSCC. Thus, the present study showed a significant association between degree of tumor differentiation with immunohistochemical expression of MCM-5.Toluidine blue staining is considered a highly sensitive adjunctive method for the detection of early-stage OSCC and high-grade dysplasia. As it is a catirole in visualizing proteoglycans within tissues because of its strong affinity for sulfate groups present in these molecules.[26]

5. Conclusion

Incidence of OSCC has shown a sharp acceleration since last two decades, and difficulties in monitoring and managing these conditions require more intense efforts. Many investigators have been searching for a specific, reliable and easily identifiable biomarker to identify patients with OSCC for early diagnosis and prognosis. [13]

In present study, MCM5 was found to be overexpressed in OSCC and correlated with increased histopathological grade and stage. Therefore, the results suggested that MCM5 might be one of the important pathogenic factors of OSCC and is expected to be used as a potential tumor biomarker for OSCC target drugs. Also, further study can be planned to assess MCM-5 expression in OPMD patients which will help us to screen the subjects with or without risk of malignant transformation, as increase in MCM-5 expression signifies increased risk of malignant changes. Hence this could be used as a screening as well as prognostic marker which may help further in diagnostic information and disease monitoring.

6. Source of Funding

None.

7. Conflict of Interest

None.

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