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Original Article

Expression of Sirtuin 4 in Oral Squamous Cell Carcinoma and it's Correlation with Clinicopathological Parameters

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Abstract

Introduction: SIRTs (Sirtuins) are class III histone deacetylase enzymes that use NAD+ as a co-substrate for their enzymatic activities. In mammals, there are seven sirtuin proteins (SIRT1–SIRT7) among which SIRT4, SIRT3 and SIRT5 are mitochondrial sirtuins that regulate enzymes and other mitochondrial proteins to coordinate oxidative production of ATP with the availability of energy in the diet. SIRT4 is known to have tumor suppression activity in many human cancers. However, the role of SIRT4 in oral squamous cell carcinoma is not known. It is present at higher levels under nutrient-rich conditions, and inhibits glutamine catabolism through ADP-ribosylation and hence repression of glutamate dehydrogenase (GDH) activity, a rate-limiting enzyme in glutamine catabolism. Due to higher requirement of energy and bio-molecules for proliferation, cancer cell often resort to various metabolic pathways that are otherwise uncommon in normal cells. One of such mechanism is switching to Glutamine metabolism. SIRT4 acts as a tumor suppressor by repressing glutamine utilisation by cells.

Purpose: Study the role of SIRT4 in oral squamous cell carcinoma and evaluate its tumor suppressor role.

Method: Here we studied expression of SIRT4 in oral cancer tissues by immuohistochemistry and compared it with that of normal tissue.

Results: SIRT 4 was seen to significantly down regulated in oral squamous carcinoma.

Conclusion: The present study suggests SIRT4 as a marker of tumor aggressiveness and as a therapeutic target for OSCC.

Key words: Cancer cell metabolism, Sirtuin 4, Tumor suppressor

INTRODUCTION

The global burden of cancer has kept on increasing with each passing year. According to the latest data released by the WHO, an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared to 12.7 million and 7.6 million, respectively, in 2008. It has also been predicted that by 2025, new cancer cases per year will increase substantially to 19.3 million, due to growth and aging of the global population.[1]

The incidence of oral cancer along with lip and pharyngeal cancer in 2012 was 529,500 that correspond to 3.8% of all cancer cases and is predicted to rise by 62% to 856,000 by 2035 because of demographic change.[1] It is the sixth leading cancer by incidence worldwide.[2] The figures are alarming and there is a need for local tailored approaches for prevention, screening, and therapeutic interventions that will optimally reduce the burden of the above anatomical areas in future decades. One of the major hindrances in formulation of these tailored approaches of interventions is lesser understanding of metabolic reprogramming that drives oncogenesis. Although numerous studies are done on cancer genomics, the literature is in dearth of studies

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that focus on altered cellular metabolism in cancer cells. The present study highlights one of such genes that are integrally associated with altered metabolism of cancer cells.

Sirtuins (SIRTs) are Class III histone deacetylase enzymes that use NAD+ as a cosubstrate for their enzymatic activities.[3] These proteins have been shown to counter aging in a broad range of organisms, from yeast to mammals.[4] The effect of SIRT activation in mammals is to forestall the progression of diseases of aging, including neurodegeneration, diabetes, cardiovascular diseases, and many types of cancer.[5] SIRT4, SIRT3, and SIRT5 are mitochondrial SIRTs that regulate enzymes and other mitochondrial proteins to coordinate oxidative production of ATP with the availability of energy in the diet.[6] In humans, SIRT4 expression is reduced in several types of cancers, including small-cell lung carcinoma,[7] leukemia,[8] gastric cancer,[9] bladder cancer,[10] and breast cancer.[11] Unlike other SIRTs that are activated under energy-limiting conditions, SIRT4 is present at higher levels under nutrient-rich conditions, and this SIRT inhibits glutamine catabolism through ADP-ribosylation and hence repression of glutamate dehydrogenase (GDH, also known as GLUD) activity, a rate-limiting enzyme in glutamine catabolism.[11-13]

Although the expression of SIRT4 has been studied in few human cancers, no study on oral cancer has yet been reported in English literature. The present study aims to evaluate and compare the expression of SIRT4 in oral squamous cell carcinoma (OSCC) tissue with adjacent non-tumor tissue using immunohistochemistry (IHC). We also intend to correlate SIRT4 expression in tumor tissue with various clinicopathological parameters of the tumor.

MATERIALS AND METHODS

The study was conducted with prior approval of the Institutional Ethical Committee, SCB Medical College and Hospital, Cuttack. After obtaining necessary consent, 45 formalin-fixed paraffin-embedded tissue samples of diagnosed cases of OSCC and their corresponding microscopically healthy tissue margin were obtained from the tissue archive of the Department of Oral Pathology and Microbiology, SCB Dental College and Hospital, Cuttack. The samples included 31 males and 14 females in the age range of 28 years–72 years. All the patients had undergone radical neck dissection surgery for OSCC between January 2016 and August 2017 at the Department of Oral and Maxillofacial Surgery, SCB Dental College, Cuttack. None of the patients received chemotherapy or radiotherapy before surgery. Recurrence cases were excluded from the study. The OSCC tissue samples were divided into three groups according to the histological differentiation. Group 1 contained 30 tissue samples of well-differentiated OSCC (T1-T30), whereas Group 2 and Group 3 had 10 (T31-T40) and 5 samples (T41-T45) of moderately differentiated and poorly differentiates OSCC, respectively. Tumor margins which were microscopically free of tumor cells were taken as control and were numbered C1-C45.

All the samples were stained with hematoxylin and eosin and viewed under a light microscope to confirm the rendered diagnosis. The samples were subjected to immunohistochemical study using primary antibody anti-SIRT4 polyclonal antibody produced in rabbit (Product code – HPA029692, Sigma-Aldrich Corporation, USA)

The IHC stained slides were scored as per the standard protocol of scoring, 10 random high-power fields were selected for each sample (×400; Leica, Germany). The fields were scored for staining area (0 = <5%; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; and 4 = More than 75%) and staining intensity (0 = No staining; 1 = Weak staining appearing as light yellow; 2 = Moderate staining appearing as yellowish-brown; and 3 = Strong staining appearing as brown). The overall staining score was calculated by multiplying staining area score with staining intensity score. Average scores of 10 fields were considered as the final staining score of the sample. Staining score <4 was considered as low expression and score ≥4 was considered as high expression of SIRT4.

Statistical analysis was performed using the SPSS software package version 22.0 (SPSS, Inc. IBM, USA). Fisher's exact test was used to analyze the final score of the tumor and non-tumor tissues. Paired *t*-test and ANOVA were employed to evaluate the correlation between expression of SIRT4 and various clinicopathological parameters such as age, gender, site of lesion, size of lesion, differentiation, Union for International Cancer Control staging, and involvement of lymph nodes.

RESULTS

SIRT4 was expressed in cell cytoplasm. It was found to be significantly downregulated in tumor tissues in

Table 1: Sirtuin 4 protein expression in oral squamous cell carcinoma and adjacent normal oral mucosa tissues

Tissue type	Sample No.	Sirtuin 4 expression		X^2	*P* value*
		Low (IHC score <4)	High (IHC score ≥4)		
Normal	45	31 (68.88%)	14 (31.12%)	16.58	<0.001
Tumor	45	45 (100%)	-		

*Fisher's exact test applied. IHC: Immunohistochemistry

comparison to adjacent microscopically healthy appearing tissue [Figure 1]. The difference in the staining score was found to be highly significant ($P < 0.001$). About 31.12% of the normal tissues showed high expression of SIRT4 compared to none of the tumor cases [Tables 1 and 2, Graph 1].

Further on evaluating the expression of SIRT4 protein in various stages of the tumor, patients with higher stage (Stage III and Stage IV) showed significantly lower expression of SIRT4 than early-stage (Stages I and II) disease [Table 3]. The mean value of SIRT4 protein expression was found to be 1.39 ± 0.60 in Stages I and II, 0.51 ± 0.20 in Stage III, and 0.26 ± 0.10 in Stage IV cancers. The difference in the expression was found to be statistically significant ($P < 0.001$). When the scores of Stages I and II cancers were compared with that of Stage III cancer, a significant difference in expression was noted ($P = 0.002$). The difference was even more prominent when compared with Stage IV cancer ($P < 0.001$). However, no significant difference was seen between Stage III and Stage IV cancers ($P = 0.331$) [Table 4].

To further evaluate the role of SIRT4 in tumor spread, we compared the expression of the protein in tumor tissues from patient who had at least one lymph node metastasis with non-metastatic cases. The mean of SIRT4 expression

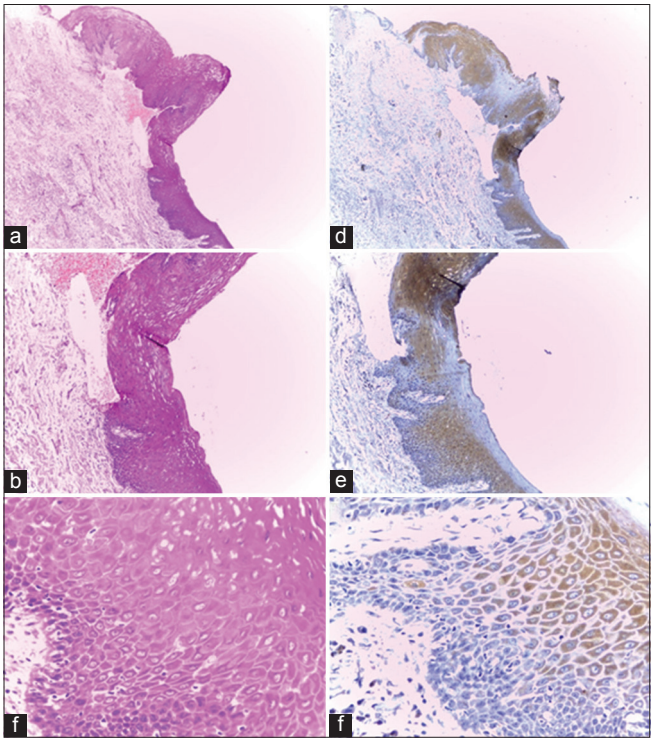
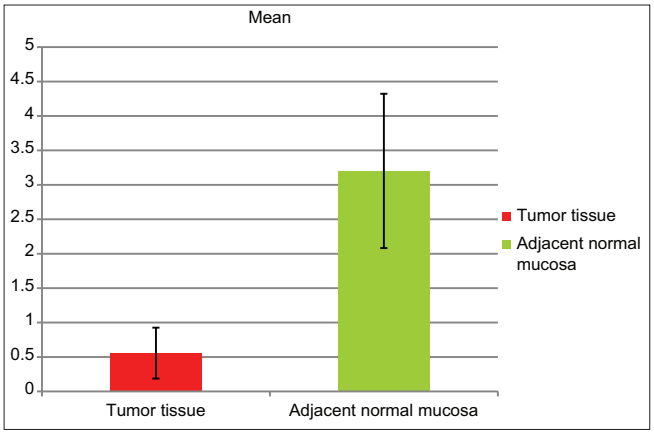


Figure 1: Expression of sirtuin 4 (SIRT4) in microscopically healthy mucosa taken from tumor margins; (a-c) HE stained samples shown at different magnifications ([a] – ×40, [b] –×100, [c] – ×400); (d-f) Samples stained with anti-SIRT4 antibody



Graph 1: Sirtuin 4 protein expression in oral squamous cell carcinomas and adjacent normal oral mucosa tissues

Table 2: Sirtuin 4 protein expression in oral squamous cell carcinoma and adjacent normal oral mucosa tissues

Samples	Mean	Std. deviation	Std. error mean	*t*	Mean difference	*P* value
Tumor tissue	0.55	0.37	0.1000	−13.586	−2.6467	<0.001
Adjacent normal mucosa	3.20	1.12	0.1672			

*Student's *t*-test applied

Table 3: Correlation of sirtuin 4 protein expression in oral squamous cell carcinoma and pathological stage of tumor

Stage	Mean	Std. deviation	Std. error	F	*P* value
1 and 2	1.39	0.60	0.19	11.05	<0.001
3	0.51	0.20	0.22		
4	0.26	0.10	0.09		
Total	0.72	0.53	0.13		

Table 4: Difference in sirtuin 4 protein expression in oral squamous cell carcinoma between pathological stages of tumor

(I) Stage	(J) Stage	Mean difference (I-J)	Std. error	*P* value
1 and 2	3	0.88	0.25	0.002
1 and 2	4	1.13	0.25	0.000
3	4	0.25	0.15	0.331

Table 5: Evaluation of sirtuin 4 protein expression in oral squamous cell carcinoma with respect to lymph node metastasis

Lymph node metastasis	Mean	Std. deviation	Std. error mean	*t*	*P* value
Present	0.19	0.06	0.0526	−3.212	0.003
Absent	0.98	0.46	0.1716		

was found to be 0.98 ± 0.46 in non-metastatic cases and 0.19 ± 0.06 in metastatic lymph node cases. The difference in the expression was statistically significant ($P = 0.003$) [Table 5].

There was no significant difference in expression of SIRT4 across various grades of tumor. The size, site of tumor, gender, and age of patient had no significant effect on expression of SIRT4.

DISCUSSION

Morphological changes in transforming cells are late to appear as they are secondary to the genomic changes that trigger carcinogenesis in the cell. This makes it difficult to diagnose a cell as non-tumorous solely by relying on morphological parameters. Several immunohistochemical markers have been employed to see the changes in a transforming cell at molecular level which is otherwise not appreciated by morphological study under microscope. SIRT4 is one such marker that plays a significant role in deciphering the molecular changes occurring in a cell in the process of carcinogenesis.

Different family members of SIRTs were found to play various roles in carcinogenesis, in a tumor-type dependent manner.[14] For instance, SIRT1 was found to be upregulated in gastric carcinoma,[15] colon cancer,[16] prostate cancer,[17] and skin cancer,[18] whereas the same is downregulated in breast cancer[19] and induced intestine cancer in mouse model.[20] In a similar fashion, an SIRT2 expression is decreased in breast cancer,[21] glioma,[22] and skin cancer[23] but upregulated in acute myeloid leukemia[24] and prostate cancer.[25] Among the mitochondrial SIRTs, the role of SIRT3 in cancer progression has been extensively studied. SIRT3 has been seen to suppress a wide number of caners;[26-34] on the contrary, the same has also been seen to be upregulated in some cancers.[35-39] Unlike SIRT3, limited information are available about SIRT4. The expression of SIRT4 has been evaluated in few cancers, but none of the study was performed on OSCCs.

In the present study, we evaluated the expression of SIRT4 in OSCC and correlated it with various clinical and pathological parameters of OSCC. Expression of SIRT4 was found to be decreased in cancer cells which can be attributed to the activation of mammalian target of rapamycin complex 1 (mTORC1) pathway during carcinogenesis.[13] The tumor cells live in a dynamic environment where the nutrient availability keeps on changing. To survive the nutrient level variation, the anabolism and catabolism need to be regulated. The decision of process between anabolism and catabolism is highly conserved. Atypical serine/threonine kinase mTORC1 which drives nutrient uptake and subsequent proliferation[40] has been seen to be dysregulated in many cancers.[41] mTORC1 get activated by various pathways such as downstream effect of PI3K pathway that is one of the most frequently activated pathways in human malignancies,[42-44] mutation of tumor suppressor gene PTEN which is the second most mutated gene to be involved in carcinogenesis after p53, tumor suppressor LKB1 mutation in the upstream,[45] and Wnt and tumor necrosis factor-alpha pathway.[46,47] PI3K pathway is seen to be frequently mutated in head-and-neck squamous cell carcinoma[48] with a downstream effect on mTORC1 activation. Analyzing the result of the present study, we assume that decreased expression of SIRT4 protein in OSCC is an effect of upstream mutation of PI3K pathway acting through mTORC1.

Further, we found the decreasing expression of SIRT4 with increasing pathological stage of tumor further. As the stage of the tumor is related with its spread, SIRT4 can be assumed to play a significant role in keeping a rein on the spread of tumor by decreasing cellular proliferation. Glutamine is known to be an essential metabolite for proliferation[49-53] and also required for transition from G1 to S phase[54] during cell division. SIRT4 is known to repress mitochondrial glutamine metabolism in response to DNA damage.[11] This phenomenon by SIRT4 might be affecting the cellular proliferation negatively contributing to the tumor suppressor nature.

One more important aspect of tumor spread is lymph node metastasis. Lymph node metastasis cases showed significantly decreased expression than non-metastatic cases. Loss of cell to cell adhesion is an important step associated with tumor invasion and metastases that are frequently accompanied by downregulation of the epithelial molecule E-cadherin.[55] Loss of E-cadherin has been related to cancer development, progression, and poor prognosis.[56] High glucose was found to suppress the mRNA expression of E-cadherin compared to low glucose in pancreatic cancer.[57] The role of glutamine and SIRT4 in cell migration and invasion has started getting attention recently. Wang *et al.*[58] reported that in cell invasion assays, the migratory activity of transformed fibroblasts and cancer cells is highly compromised by glutaminase inhibitor 968, suggesting a role of glutamine metabolism in cancer cell migration. Fu *et al.*[59] indicated that glutamine restriction inhibited attachment, spreading, and migration of melanoma cell lines through the inhibition of specific integrin expression and modulation of actin cytoskeleton remodeling. Miyo *et al.*[60] demonstrated that suppression of glutamine metabolism by SIRT4 resulted in positive regulation of E-cadherin expression. Further, it was

suggested that SIRT4 inhibits EMT through reducing levels of intracellular α-ketoglutarate through the inactivation of GDH. In the present study, decreased expression of SIRT4 in lymph node metastatic cases supports the role of that SIRT4 in suppression of tumor invasion and metastasis.

CONCLUSION

In the present study, the role of SIRT4 as a tumor suppressor in OSCC was established by comparing expression of SIRT4 in various tumor tissues with normal healthy appearing adjacent mucosa. It was observed that tumors with higher stages and with lymph node metastasis showed marked decrease in the expression of the protein; hence, SIRT4 could be considered as a novel prognostic marker of tumor aggressiveness. As SIRT4 plays an important role in suppressing glutamine anaplerosis, it is downregulation in OSCC tissues indicated the dependence of the tumor cells on glutamine for cellular proliferation. This fact can be advantageous and contribute to the development of effective therapeutics for oral cancer. SIRT4 in conjunction with metabolic and cytotoxic chemotherapeutic agents can serve as a promising strategy in the treatment of OSCC. The present study suggests SIRT4 as a marker of tumor aggressiveness and as a therapeutic target for OSCC.

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