

Estimation of Salivary Levels of an Inhibitory Apoptotic Protein Survivin in Oral Leukoplakia and Squamous Cell Carcinoma

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ABSTRACT

The process of carcinogenesis is initiated by an excessive proliferation of tumor cells, accompanied by a reduction in the rate of apoptosis. A number of molecules are involved in this process, with Survivin being one such molecule that is known to control it. The expression of Survivin has been observed in a range of embryonic and adult tissues with a high rate of turnover, as well as in tissue sections of oral precancerous and cancerous lesions. However, data regarding the detection of Survivin in saliva are scarce. The aim of this study is to estimate and compare salivary Survivin levels (SSL) in normal oral mucosa (NOM), oral leukoplakia (OL), and squamous cell carcinoma (OSCC) in order to demonstrate its potential as a predictive biomarker. Whole saliva samples were collected from all subjects and assessed for salivary Survivin levels using the ELISA technique. A Kruskal-Wallis test was employed to ascertain any significant differences in SSL. The mean SSL in the control group was found to be 301.9 ± 180.35 ng/dl, while the study groups of OL and OSCC exhibited mean values of 285 ± 140.4 and 316.5 ± 176.72 ng/dl, respectively. In the OL group, the highest value was observed in cases of severe dysplasia (407.4 ng/dl), while in the OSCC group, the highest value was noted in WDSCC (311.5 ng/dl). Due to the inconsistency in the distribution of the sample, our findings were found to be non-significant ($p = 0.796$). This study is the first to detect Survivin in normal oral mucosa (NOM). The results suggest that Survivin plays a definitive role in maintaining the normal homeostasis of oral mucosa. The gradual upregulation and downregulation of SSL in OL and OSCC can be used to predict the aggressive nature of the malignancy. Therefore, the detection of Survivin in saliva can be utilized as a predictive marker for the progression of oral precancerous and cancerous lesions.

Keywords: Survivin, Salivary Protein, Oral Precancer, Oral Cancer.

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1. Introduction

Oral squamous cell carcinoma (OSCC) represents a significant global health burden, with an estimated 500,000 new cases reported annually (1). Its etiology is complex and multifactorial, involving various risk factors such as tobacco and alcohol consumption, dietary habits, immunodeficiency, and viral infections (2). The correlation between tobacco use and the development of OSCC has been firmly established in regions where tobacco consumption is highly prevalent (3). The prognosis of OSCC is considered to be poor due to a number of factors, including delays in diagnosis, lack of follow-up, and incomplete treatment. In addition to these factors, the disease progression is also considered to be a complex phenomenon, as it is involved with undiscovered multiple functions of biomarkers that are protein in nature. Therefore, an understanding of the molecular role of these individual biomarkers may facilitate improvements in prognosis and the development of new therapeutic strategies (4, 5). The majority of biomarkers are identified in tissue samples and also in body fluids such as serum and saliva (6). Collection of serum is an invasive procedure and the technique is highly sensitive, whereas saliva collection is non-invasive and does not require expertise in the procedure. Furthermore, biomarkers for both systemic conditions and localized pathology have been identified in saliva. The process of carcinogenesis is characterized by an excessive proliferation of tumor cells accompanied by a reduction in the rate of apoptosis. One molecular family with established regulatory functions with respect to both cell division and apoptosis is the Inhibitor of Apoptotic Protein (IAP) family. The proteins in this family are distinguished by the presence of a domain of approximately 70 amino acids, which has been designated the baculoviral IAP repeat (BIR). The IAP family comprises six members that are known to be expressed in humans. These include NAIP, c-IAP1/HAIP-2, c-IAP2/HAIP-1, XIAP/hILP, Survivin, and BRUCE (7). Among these, Survivin has found its way into human medicine after its discovery in 1997 by hybridization screening of a human genomic library with cDNA of the effector cell protease receptor-1 (EPR-1) (7). Structurally, survivin is a 16.3 kDa molecule that contains a baculoviral IAP repeat (BIR) domain at the amino-terminal end and a long alpha-helical region at the carboxy-terminal end. The two principal functions of Survivin are the inhibition of apoptosis, which is achieved either directly or indirectly by interfering with the function of caspases. Secondly, Survivin is a chromosomal passenger protein that is necessary for the process of cell division (7). The protein Survivin is expressed in embryonic tissues and the majority of human tumors, but remains undetectable in terminally differentiated normal cells and tissues. However, survivin is secreted in various body fluids (e.g., urine, milk, serum, saliva), rendering it a valuable biomarker for the early diagnosis of numerous systemic cancers (8). Additionally, survivin expression has been demonstrated in tissues of oral premalignancy and

malignancy. It has been demonstrated that Survivin exhibits nuclear expression in cases of well-differentiated squamous cell carcinoma and cytoplasmic expression in poorly differentiated carcinoma. The authors are able to determine the prognosis based on this nuclear/cytoplasmic expression of Survivin (9). The detection of Survivin in the saliva of patients with oral squamous cell carcinoma (OSCC) has been attempted (10), but thus far, no studies have been conducted to determine the salivary levels of Survivin in cases of leukoplakia with evidence of dysplasia and between the grades of OSCC. Furthermore, the normal salivary levels of Survivin have yet to be established in order to ascertain its normal physiological role. Therefore, the objective of the present study is to evaluate the salivary Survivin levels (SSL) in order to demonstrate the predictive behavior of this protein molecule in normal healthy individuals and in the most common pre-malignant lesion, oral leukoplakia (OL) with dysplasia and oral squamous cell carcinoma (OSCC).

2. Materials and Methods

2.1. Data Collection

Following the acquisition of institutional ethical clearance for the study, informed consent was obtained from all participants. The control group (n=30) was defined as normal healthy individuals with no evidence of any oral pathology or underlying systemic conditions. The study group comprised 30 cases each of clinically diagnosed and histologically confirmed cases of OL with dysplasia and OSCC. Informed consent was obtained from all participants before the collection of saliva and also for tissue biopsy.

2.2. Inclusion & Exclusion Criteria

In accordance with the research protocol approved by the ethical committee, only those cases exhibiting a white patch concomitant with tobacco use were classified as OL. Conversely, cases displaying clinical characteristics of frank ulceroproliferative growth with an indurated margin and a raised border were designated as OSCC. Cases of OL lacking histological evidence of dysplasia were excluded. Patients presenting with scrapable white lesions, recurrent cases, ongoing treatment of OL and OSCC, or any other pathology in the oral cavity were excluded from the study.

2.3. Protocol of Saliva Collection

Following the acquisition of consent to participate in the study, saliva was collected from all cases within the study groups prior to treatment in order to circumvent any potential bias associated with molecular alteration. Participants were instructed to refrain from consuming any food or beverages, or from engaging in any oral hygiene procedures, for a minimum of one hour prior to saliva collection. Subsequently, the participants were requested to rinse their oral cavity with distilled water. Thereafter, five millilitres of unstimulated whole saliva was collected into sterilized Euricol bottles. Subsequently, the saliva samples were subjected to centrifugation at 2,000 rpm, after which the supernatant was collected and stored at -20°C.

2.4. Estimation of Salivary Survivin Levels (SSL) by ELISA

The ELISA technique was performed using the Human anti-Survivin antibody (Surv) ELISA kit (Cat No. E1612 Hu) produced by Bioassay Technology. The kit's principle is based on a double-antibody sandwich type of ELISA, and the protocol was followed in accordance with the instructions recommended by the manufacturing company. The wells of the ELISA plate were marked with codes, and subsequently, the saliva samples were thawed and centrifuged at 2000 rpm for 20 minutes. The supernatant was then utilized as the sample. Approximately 40 μ L of saliva sample, 10 μ L of SURVIVIN antibody, and 50 μ L of streptavidin HRP were added to the wells of the ELISA plate. Subsequently, the plate was incubated at 37°C for 60 minutes. Subsequently, the excess reagents were removed through the use of the provided washing concentrate (30X). Subsequently, 50 μ L of Chromogen A and 50 μ L of Chromogen B were added to the wells and incubated for 10 minutes at 37°C. After a 10-minute incubation period, a blue coloration developed. Subsequently, 50 μ L of stop solution was added to the wells, which promptly transformed the color from blue to yellow. The density of this color was then quantified using an ELISA reader with a wavelength of 450nm. The Survivin marker in saliva was validated by repeating the same saliva sample three times, and the mean of the readings was considered for all samples.

2.5. Histological Grading Criteria

A total of 60 biopsy tissues from the study groups were obtained, formalin-fixed, processed, embedded in paraffin wax, sectioned at a thickness of 4 microns, and stained with hematoxylin and eosin for histological assessment. Oral leukoplakia cases were evaluated for dysplasia and graded in accordance with the three-tier system proposed by the WHO 2005 Oral Epithelial Grading System (11). The histological grading of OSCC tissue biopsies was conducted in accordance with the degree of differentiation as delineated by the Broder's grading system (12) (Figure 1).

2.6. Statistical Analysis

All data were entered into an Excel spreadsheet, and statistical analysis was conducted using SPSS software, version 1.5. A probability value of less than 0.05 with a 95% confidence interval was deemed statistically significant. A Kruskal-Wallis test was employed to ascertain whether there were any statistically significant differences in salivary survivin levels (SSL) between the two study groups.

3. Results

Upon examination of the SSL, it was determined that the mean SSL in the control group was 301.9 \pm 180.35 ng/dl, while the study groups of OED and OSCC exhibited mean values of 285 \pm 140.4 and 316.5 \pm 176.72 ng/dl, respectively (Table 1). A slight decrease in mean Survivin value was observed in the OED group, yet overall, no significant

difference in salivary Survivin levels was found across all groups. This lack of statistical significance was confirmed by the Kruskal-Wallis test ($p=0.771$) (Table 1). A histological assessment of the OL cohort ($n=30$) revealed that 14 cases were classified as mild and moderate dysplasia, while only two cases were categorized as severe dysplasia. A comparison of salivary Survivin levels across dysplasia grades revealed that the highest mean value was observed in severe dysplasia (407.4 ng/dL), which was significantly higher than the mean values observed in mild and moderate dysplasia. The histological grades of OSCC revealed 19 cases of WDSCC, 7 cases of MDSCC, and only 4 cases of PDSC (Figure 1). A comparison of salivary Survivin levels between grades of OSCC revealed that the highest value, with a mean of 311.5 ng/dl, was observed in WDSCC cases. This was followed by MDSCC and PDSC cases. However, due to an inconsistency in the equal distribution of samples across all groups, the above findings were found to be non-significant ($p = 0.796$) upon application of the Kruskal-Wallis test (Table 2).

4. Discussion

The development of oral carcinogenesis is a gradual process whereby the disease progresses through a series of hyperplastic alterations in the epithelium, culminating in dysplastic changes and ultimately invasive carcinoma (13). During the transition from dysplasia to OSCC, several biomarkers are instrumental in predicting the disease state and prognosis. The majority of these markers can be identified at the tissue level and also quantified in bodily fluids such as serum, saliva, plasma, urine, and so forth. In tissue, established biomarkers in OED and OSCC include epidermal growth factor receptor (EGFR), p53, Ki67, vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), B-Cell Lymphoma 2 (BCL-2), and MIB-1 (5, 14). The biomarkers that can be detected in body fluids, and most commonly in serum, of patients with oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC) are interleukin-1 (IL-1), IL-1 α , IL-6, IL-8, tumor necrosis factor- α (TNF- α), tissue polypeptide antigen, endothelin-1, and carcinoembryonic antigen (CEA) measured in terms of serum cytokeratin fragment (CYFRA) (14, 15). Among the various biomarkers studied in OED and OSCC, one belonging to the inhibitor of apoptosis protein (IAP) family is survivin. This molecule is distinctive due to its dual function in cell division and apoptosis. Consequently, Survivin has been the subject of investigation in a number of systemic malignancies, including gastric, breast, and urinary bladder cancers, as well as in cardiac disease (16,17,18,19,20). Furthermore, some studies have demonstrated the expression of Survivin in OSCC and OED at the tissue level (20,21,22,23). A significant number of authors have concentrated their research efforts on the study of survivin in the context of a normal physiological state, with the objective of gaining insight into its role in cancer. In light of the existing literature, we sought to ascertain the presence of survivin in

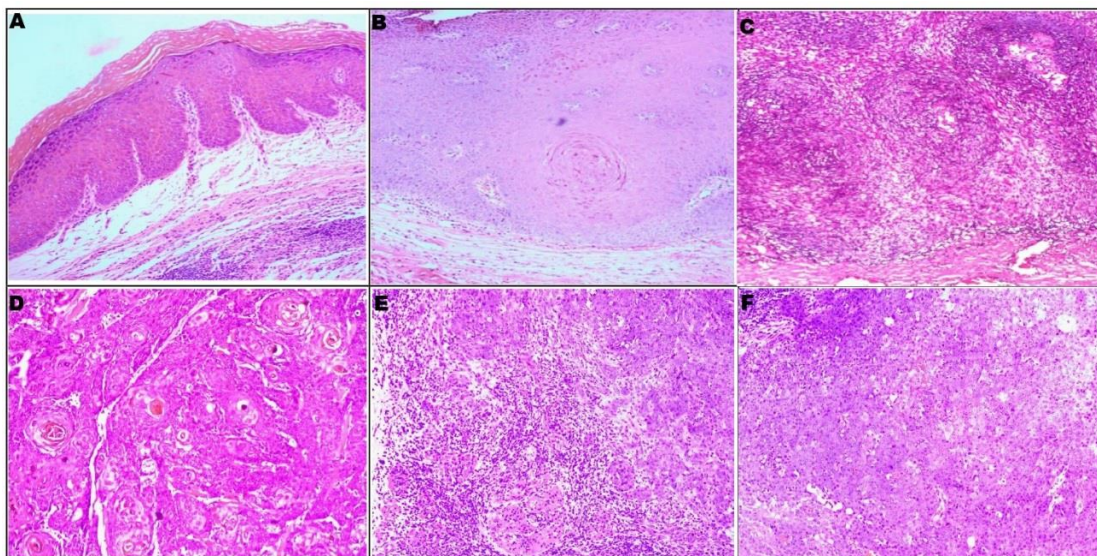


Figure 1: The photomicrographs of hematoxylin and eosin-stained section images at x10 magnification illustrate the histological grades of oral leukoplakia (A: mild dysplasia, B: moderate dysplasia, C: severe dysplasia) and oral squamous cell carcinoma (D: Well-differentiated squamous cell carcinoma (E), moderately differentiated squamous cell carcinoma (F), and poorly differentiated squamous cell carcinoma (F).

Table 1: Evaluation & Comparison of Salivary Survivin Levels in Control & Study Groups

Groups	Salivary Survivin (ng/dl)		Mean of Salivary Survivin (ng/dl)
	Minimum	Maximum	
Control Groups (Normal Healthy Individuals)	87.28	1063.32	301.9 ± 180.35
Oral Epithelial Dysplasia	-2.18	529.27	285 ± 140.4
Oral Squamous Cell Carcinoma	116.73	1113.72	316.5 ± 176.72

Kruskal-Wallis Test: p=0.771.

Table 2: Comparison of levels of Salivary Survivin and Histopathological Grade of OED & OSCC

Study Groups	Histopathological Grades	Number of Cases(n)	Mean of Salivary Survivin	Standard Deviation
OED	Mild Dysplasia	14	271.3	144.88
	Moderate Dysplasia	14	281.2	138.94
	Severe Dysplasia	2	407.4	124.75
OSCC	Well differentiated squamous cell carcinoma (WDSCC)	19	311.5	228.91
	Moderately Differentiated Squamous cell carcinoma (MDSCC)	7	280.4	105.63
	Poorly Differentiated Squamous cell carcinoma (PDSCC)	4	264.7	94.37

Kruskal Wallis Test: 0.796

one of the most readily accessible body fluids, namely saliva. The presence of SSL was detected in both normal healthy individuals and in individuals with varying grades of OED and OSCC. Although no statistically significant correlation was identified, a gradual increase in levels was observed with an increase in grades of OED. The highest levels were found in severe dysplasia, with a mean of 407.4 ng/dl. This gradual increase can be explained by the fact that as dysplastic changes progress, there is an increase in the number of abnormal mitosis. Given that the molecule Survivin is linked to its role during cell division, an increase in SSL is to be expected in line with an increase in the demand for cell division. Our findings align with those of other researchers who have also observed an increase in Survivin expression with the severity of dysplasia (20,21,22). Furthermore, an examination of SSL within the histopathological grades of OSCC revealed a decline in SSL with an increase in the grade of OSCC. Consequently, the lowest levels of SSL were observed in PDSCC, with an average of 264.7 ng/dl. Additionally, it was observed that the level of Survivin in mild dysplasia was nearly identical to that observed in PDSCC. Therefore, it can be postulated that Survivin plays a role in the initiation and progression of the carcinogenic process. The reason behind the gradual rise and fall of Survivin may be attributed to a negative feedback mechanism that is activated once the protein has reached a certain limit. In contrast with these findings, some studies have demonstrated an increase in Survivin expression with an elevated grade of malignancy (23,27,28). As previously discussed, the development of OSCC is dependent on multiple factors, including the protein Survivin. In the course of our investigation, we observed that SSL was present in both the control and study groups. However, the highest levels were observed in the OSCC group, with a mean of 316.5 ± 176.72 ng/dl. Given the longer duration of tobacco use in the OSCC group than in the OED group, it was found that there were higher

levels of SSL, which suggests a potential link between tobacco-induced secretion of Survivin in saliva. Similarly, Santarelli et al. observed an enhanced expression of Survivin in OSCC and proposed that during the early stages of OSCC development, the levels of Survivin are altered, which could be used as a potential marker (10). The observed variation in SSL may be attributed to inherent limitations of the ELISA technique, particularly its low sensitivity in detecting low concentrations of targeted molecules, especially in saliva. Additionally, the presence of autoantibodies may potentially interfere with the antigen-antibody reaction. Furthermore, the ELISA technique provides an endpoint measurement at a single point in time. Although the levels of SSL were observed to be higher in OSCC, a decline was noted from WDSCC, MDSCC to PDSCC. Therefore, we postulate that a regulatory system exists for the secretion of Survivin in saliva (see Proposed Hypothetical Diagram Showing the Role of Survivin in Normal Tissues and Oral Carcinogenesis, Figure 1). In a typical cell cycle, the expression of Survivin is at its highest during the G2/M phase, where it plays a crucial role in the separation of mitotic spindles. In human adults, along with cytokines such as IL-2 and IL-11, Survivin plays a role in the proliferation of hematopoietic cells, including neutrophils, lymphocytes, and certain adult stem cells. In order to inhibit apoptosis, Survivin, in conjunction with other IAP families, impedes the conversion of procaspase 9 to its active form. In the context of carcinogenesis in OED, the highest levels were observed in cases of severe dysplasia. With further progression to PDSCC, a decline in levels was noted (Figure 2). The variability of Survivin in saliva represents a promising target for cancer therapies, as evidenced by previous studies (29,30,31). Furthermore, our study is the inaugural investigation to detect salivary levels of Survivin in normal oral mucosa. Consequently, we propose that Survivin does possess a normal physiological function and plays a definitive role in maintaining the

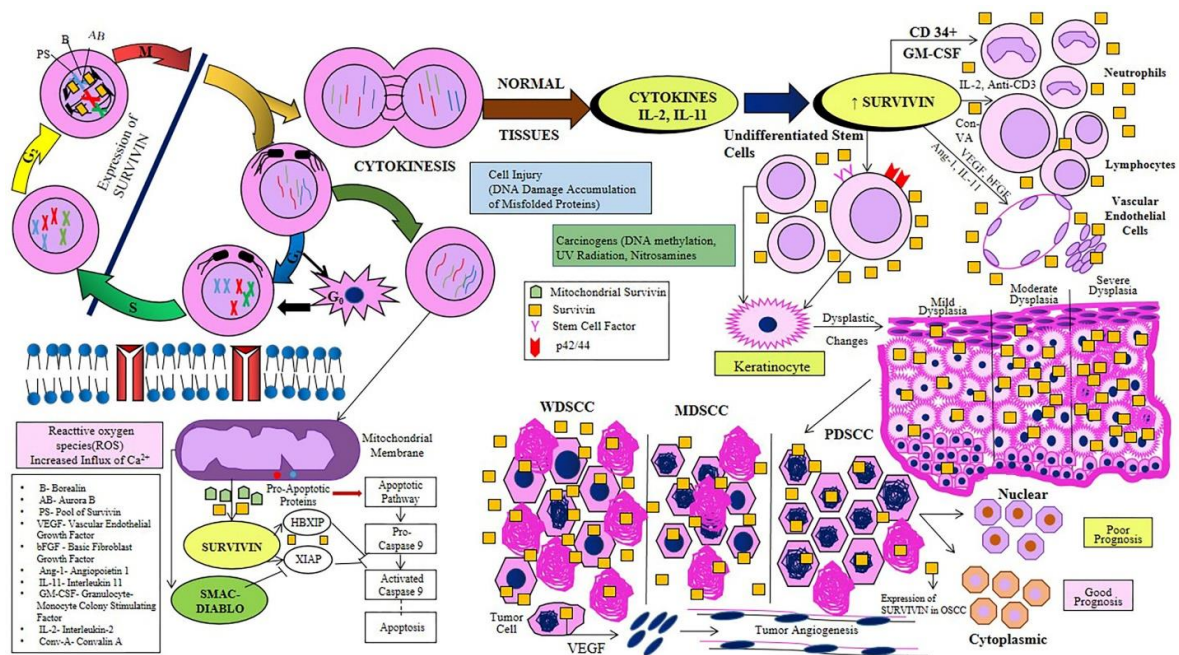


Figure 2. Hypothetical Diagram: Digital Diagram created by author PS

normal homeostasis of oral mucosa. To further validate the present findings, additional studies should be conducted with a larger sample size and an equal distribution of samples based on histological grades. Furthermore, the time of saliva collection should be taken into account to ascertain whether there are any alterations in the diurnal variation in the secretion of Survivin in saliva. Subsequent studies should be conducted to ascertain whether there are any alterations in SSL in the apparently normal mucosa of individuals who smoke tobacco and in oral cancer cases, both before and after treatment. Our findings indicate a gradual increase in Survivin-like protein (SSL) in the progression of dysplasia, suggesting a probable role for Survivin in the high mitotic index and altered apoptotic function observed in oral pre-cancer. The OSCC group demonstrated the highest SSL in WDSCC cases, which was significantly higher than that observed in PDSCC cases. Furthermore, Survivin was identified in the saliva of NOM, suggesting that the aggressiveness of malignancy can be predicted by the downregulation of salivary Survivin levels.

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Authors' Contribution

PS: Performed the experiments, collected the data, analyzed, and approved the manuscript, DM: Conceptualized the study design, analyzed the data, Wrote, edited, and approved the manuscript.

Ethics

Ethical clearance was obtained from the Research and Ethical Committee of KLE University V. K. Institute of Dental Science, Belagavi, India (IRB no. 890). The principles and practices of research were rigorously adhered to.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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