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COMPARATIVE EVALUATION OF NATURAL KILLER CELL (CD57) EXPRESSION IN ORAL LEUCOPLAKIA AND ORAL SQUAMOUS CELL CARCINOMA: AN IMMUNOHISTOCHEMICAL STUDY

Oral Pathology

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ABSTRACT

Oral cancers are the sixth- to eighth- most common cancers worldwide, and account for 30-40% of all malignancies in India. About 80% of these are preceded by potentially malignant conditions like oral leucoplakia. Immune response against tumor cells is initially mediated by the innate immunity, mostly via Natural Killer (NK) cells; presence of CD57+ NK-cells in oral squamous cell carcinoma indicates good prognosis. The present study analyses the comparative expression of CD57+ NK cell in both these conditions. This hospital-based retrospective observational study included 90 paraffin-block samples of diagnosed cases of oral leucoplakia and squamous cell carcinoma, which were immunohistochemically evaluated for CD57 expression. Immunocompromised and immunosuppressed patients were excluded. Chi-square test and One-way ANOVA were used for analysis, where $p<0.05$ was considered significant, keeping α error at 5% and β error at 20%. The power of the study was 80%. 73.33% patients ranged from 31-60 years of age (51.01 ± 13.41 years). CD 57+ NK cell estimation (labelling index per high power field) was negative in normal mucosa, 58.33 ± 4.36 in leucoplakia, 30.80 ± 1.91 in well-differentiated, and 19.91 ± 3.62 in moderately-differentiated squamous cell carcinoma ($p<0.001$). Expression of CD57+ NK-cells decreases with increasing severity of the disease, while increased expression may point towards a better prognosis. Genetic modification of NK-cells by combining their two-pronged approach of tumor elimination is a prospect, while the possible toxicity may be eliminated by introducing a 'suicide gene' in the modified NK-cells.

KEYWORDS

Immunohistochemistry, CD57, Natural Killer Cell, Oral Leucoplakia, Oral Squamous Cell Carcinoma

INTRODUCTION

Cancer is one of the most prevalent illnesses in the world. On a global scale, oral cancers are ranked sixth to eighth as the most common cancers, and account for 30-40% of all malignancies in India.[1,2] More than 90% of oral cancers are squamous cell carcinomas (SCCs).[3] SCCs are heterogeneous cellular entities whose growth depends upon reciprocal interactions between genetically-altered cells and their micro-environment. This micro-environment is called the tumor stroma, and is made of connective tissue, blood vessels, and immune cells. It is required for nutritional maintenance and removal of discarded products.[4] Despite marked improvements in therapeutic modalities, the five-year-survival rate of oral cancer is still low.[5]

Oral squamous cell carcinoma (OSCC) is a hyper-proliferative disorder.[6] About 80% of oral cancers in India are preceded by potentially malignant conditions, of which Leucoplakia is the most common.[2] Leucoplakia is defined as a predominantly white lesion of the oral mucosa that cannot be clinically or histopathologically characterized as any other definable disease.[7,8] The prevalence of leucoplakia in India ranges from 0.2% to 5.2%, while its malignant transformation ranges from 0.13% to 10% in various Indian populations.[3,9,10]

The immune system plays a vital role in fighting cancers. Prolonged immunosuppression is a key contributor to malignant transformation of tumors and potentially malignant conditions.[11] Indeed, stromal infiltration by mononuclear inflammatory cells is a typical feature of the head, neck, and oral cancers.[12] The primary role of the immune system against cancers is to identify and eliminate cells undergoing carcinogenesis, which is a multifactorial process. The immune response against tumor cells is mediated initially by the innate immunity via Natural Killer (NK) cells, NK-T cells, cytokines and complement proteins, and later by the adaptive immune system (B- and T-cells).

NK-cells are bone-marrow-derived mononuclear cells, forming 10-20% of peripheral granulocytes. NK-cells routinely carry out the process of immune surveillance, which makes them the first line of defense against tumor cells, cells showing dysplastic changes and virally infected cells.[13,14] NK-cell-immune surveillance occurs via two mechanisms – release of the cytokine interferon (IFN)-γ, and perforin-dependent target cell elimination. While the presence of NK-cells in OSCC indicates good prognosis, its role in leucoplakia is still under research.[14]

The Fourth International Workshop of Human Leukocyte Antigens in 1989 assigned the cluster of differentiation (CD) designation, CD57 to those identified on NK-cells.15 CD57+ essentially represent "memory" NK-cells.13 Immature CD57− NK-cells may contribute to autoimmune inflammation and tissue damage whereas more differentiated, cytotoxic, CD57+ NK-cells perform an immunoregulatory role.[15,16]

The high prevalence of Oral Leucoplakia (OL) and OSCC, along with the fact that no study has yet been carried out to analyze the comparative expression of CD57+ NK cell in these conditions, formed the rationale behind this study.

MATERIAL AND METHODS

The study aimed at analyze the comparative evaluation of CD57+ NK-cells in OL and OSCC. The objectives were to evaluate expression of CD57+ NK-cells in OL and OSCC, and to compare the expression between them. Another objective was to compare the expression of CD57+ NK-cells in different grades of OSCC. This was a comparative, retrospective, analytical, observational study, carried out over a period of 18 months; study approved by the Institutional Ethics Committee. Paraffin embedded tissue sections clinico-histopathologically diagnosed as OL and OSCC from departmental archives. All the archived paraffin blocks of specimens that were clinico-histopathologically diagnosed as OL and OSCC from the department of Oral Pathology and Microbiology. Biopsy specimens of immuno-compromised patients having OL or OSCC, and those of patients on immunosuppressant drug therapy at the time of biopsy were excluded from the study. The samples were divided into three groups, each containing 30 samples:

- Control Group
- Oral Leucoplakia Group
- Oral Squamous Cell Carcinoma Group

For immunohistochemical staining of CD57+ NK-cells, slides were

dehydrated by increasing grades of alcohol and brought to distilled water, and then treated with hydrogen peroxide to eliminate endogenous peroxidase activity. Antigen retrieval with tri-sodium citrate for CD 57 was done with primary monoclonal antibody, and sections were rinsed with PBS for five minutes.

Blocking antibody was applied, incubated for 20 minutes at room temperature, and residue was drained off. Then, the primary antibody was applied for 60 minutes at room temperature and rinsed. After that, the array slide was incubated with a biotin-conjugated secondary antibody at 20-37ºC for 20 minutes, and then rinsed. Chromogen of final developmental DAB, or DAB Kit. Finally, the slides were stained and differentiated in hematoxylin, and positive and negative controls were examined for presence of staining.

Five high powered fields (HPF) were randomly selected in each slide, and cytoplasm of the cells stained with CD57 were counted as positive. The total number of positive cells in each HPF were labelled as "Total positive cells", and the Labelling index was calculated as (Total positive cells / 5) × 100

Per the manufacturer recommendations, known human liver samples and human tonsil samples, showing good CD57+ NK cell expression, were taken as positive control. Studies show that normal human oral mucosa does not show the presence of CD57+ NK-cells.[6] Therefore, the normal oral mucosa included in group I of the study acted as the negative control.

Data obtained were compiled on a Microsoft ® Excel ™. Statistical analysis was done using the IBM Statistical Package for Social Sciences (SPSS) software v 21.0. For all the statistical tests, $p<0.05$ was considered as statistically significant, keeping α error at 5% and β error at 20%, thus giving a power to the study as 80%.

Chi-square test was used to assess gender distribution across oral lesions. One-way ANOVA was used to assess age distribution across oral lesions. One-way ANOVA was used to assess Distribution of CD 57+ NK cell expression as per oral lesions. There were no ethical constraints to be considered in this study.

RESULTS

The present study included 90 samples distributed among three groups (Figure 1). Each study group was evaluated for expression of CD57+ NK-cells stained irrespective of the intensity of staining in the connective tissue stroma of the stained sections. Labelling index for the CD57+ NK-cells was calculated in all the three groups.

The age-wise distribution of the 90 samples is shown in Figure 2. Mean age of the overall population was 51.01 + 13.41 years. Mean age in Group 1 was 55.43 + 9.88 years. Group 2 had a mean age of 47.33 + 16.02 years, while the mean age in Group 3 was 50.27 + 12.76 years. ANOVA Test was applied to assess whether this age difference was statistically significant; the p-value turned out to be 0.059 (Not Significant). Similar tabulation and analysis were carried out for patient gender as well (Table 1).

Table 1

Disease	Group 1	Group 2	Group 3	P Value			
Gender	N	%	N	%	N	%	0.0016
Male	18	60.0	29	96.7	25	83.3	
Female	12	40.0	1	3.3	5	16.7	
Total	30	100	30	100	30	100	

Degree of freedom = 2, Chi-Square = 12.9167, $P < 0.05$, Significant.

Table 1: Shows the gender distribution across the study groups. The gender difference among groups is statistically significant.

The expression of CD 57, estimated as CD 57+ NK-cells, was measured in terms of the labelling index per HPF (Figure 3). The mean labelling index / HPF was 41.84 ± 17.46. Group-wise distribution, and statistical analysis of the same, is shown in Table 2.

Table 2

Gro--up	Oral Lesion	Mean Labelling Index (/HPF)	P Value
1	Normal Oral Mucosa	00	< 0.001
2	Oral Leucoplakia	58.33 ± 4.36	
3	Well-differentiated OSCC	30.80 ± 1.91	
	Moderately-differentiated OSCC	19.91 ± 3.62	
Total		41.84 ±17.46	

Degree of freedom = 2,57; ANOVA (F) = 624.73; $P < 0.05$, Significant.

Table 2: Shows the distribution of CD 57 expression according to oral lesions. The Mean Labelling Index/HPF steadily decreased with the differentiation of the lesion, which was statistically significant.

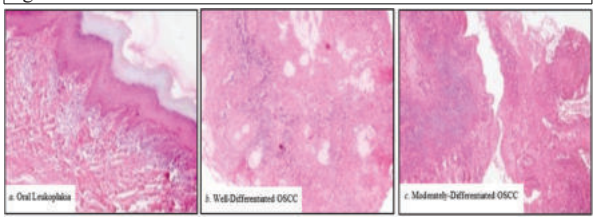

Figure 1 [Original Image; 100x]: Shows the distribution of samples included in the study. The included samples were distributed among three groups. Group 1 had normal oral mucosa (Used as Negative Control), Group 2 with Oral Leucoplakia (a), and Group 3 with OSCC. Group 3 was further divided into Well-Differentiated OSCC samples (b) and Moderately-Differentiated OSCC samples (c).

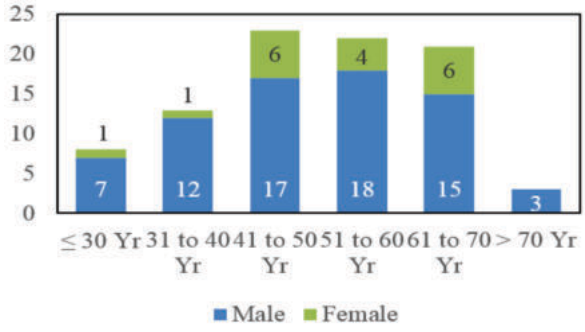

Figure 2: Shows the age distribution of samples included in the study.

DISCUSSION

With an annual incidence of over 300,000 cases, oral cancer is the sixth-most-common cancer reported globally. 62% of these arise in developing countries. In India, oral cancer ranks among the top three cancers, accounting for 30% of all cancers in the country. This variation in incidence and disease pattern is attributable to the regional differences in prevalence of disease-specific risk factors. The consistent risk factors are use of tobacco and alcohol.[17] OSSCs constitute over 95% of oral cancers, whose frequency in India (30-50%) remains the highest in the World.

This alarming trend can be attributed to sustained exposure to carcinogens like tobacco.18 As the morbidity and mortality this disease have not improved in decades, early recognition is essential to attempt improvement in survival rates, functional preservation, enhancement of aesthetic and psychological outcomes.[19]

OL has been found to be a frequent occurrence, of which, 15-20% have the potential to develop into OSCC.[20] Rapid progression of OL to OSCC has been observed after significant immunosuppression, leading to the hypothesis that immunosuppression and escape from immune recognition are major factors responsible for the establishment and progression of cancers.[21] The term 'Natural Killer Cell' was initially coined by Kiessling et al (1975), identified initially as large granular lymphocytes.

It was subsequently shown that NK-cells comprise a unique lineage of lymphocytes distinct from T-and B-cells, and have specific receptors for tumor cell recognition.[22] NK-cells have two significant functions – to support differentiation and to promote tissue regeneration.[21] Therefore, derangement of NK cell function may result in a chronic inflammatory process, causing continual tissue damage and release of pro-inflammatory cytokines. Indeed, NK-cells have been found to be downregulated by tumor cells, thus reinforcing their anti-tumor activity. As to cellular differentiation, acquisition of CD 57 by NK-cells represents a shift towards a higher cytotoxic capacity, and decreased responsiveness to cytokines. Whether the expression of CD 57 drives these changes, as opposed to being a marker for them is yet unclear.[15]

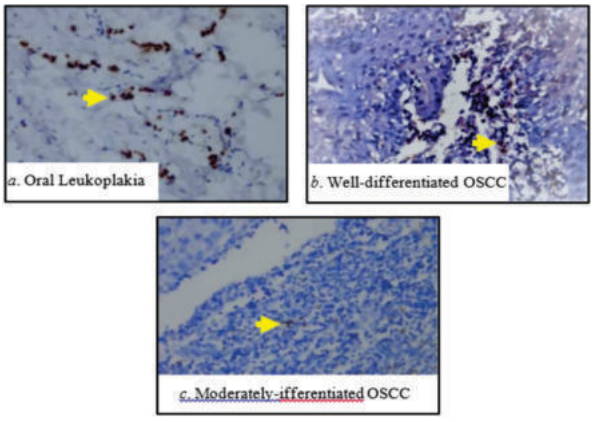

Figure 3 [Original Image; 400x]: Shows the expression of CD 57, estimated as CD 57+ NK cells, displayed as brown chromogen at the antigenic site. Expression of CD57+ NK cells was highest in Oral Leucoplakia (a), followed by well-differentiated OSCC (b), with least amount of CD57+ NK cell-expression in moderately-differentiated OSCC (c).

From a global standpoint, OL and OSCC are known to occur more frequently in middle aged and elderly males.[23] In the present study, the mean age of OL was 47.33 ± 16.02 years, and that for OSCC was 50.27 ± 12.76 years. At the same time, 96.7% of the OL and 83.3% of OSCC were found in males. These findings are consistent with the global scenario. Similar findings were obtained in a retrospective study in Spain (2010)[24], and on in India (2012)[25], which showed the highest occurrence of OL between 40-79 years, and OSCC between 50-60 years.[25] Retrospective studies in India carried out in 2010[26] and 2012[25] mention that OL and OSCC occur more commonly in males than in females.

The present study did not find CD 57+ NK cell expression in normal oral mucosa, whereas the mean labelling index of CD 57+ NK-cells in OL was 58.33 ± 4.36 / HPF. Thus, the expression of CD57+ NK cell expression was highest in OL, and decreased progressively as with the decrease in cellular differentiation. Although, a retrospective study carried out in Brazil (1991) found that the expression of CD 57+ NK-cells increased with increasing epithelial dysplasia.27 The present study, however, evaluated the expression in OL irrespective of epithelial dysplasia. .

Increased expression of CD57 on NK-cells is associated with increasing maturity and decreasing cellular activity.[16] As OL does not contain tumor cells, the NK cell seen have high maturity and low activity. Increased expression of NK-cells in OL can, in fact, be due to lack of NK-cell-downregulation in absence of tumor cells. Conversely, NK-cell-downregulation in the presence of tumor cells leads to NK-cell-exhaustion, preventing the cells from reaching maturity and expressing the CD57 antigen.[28] Therefore, as the disease progresses from OL to OSCC, there is progressive decline in the expression of CD57+ NK-cells in OSCC as compared to OL.

Upon the comparison of CD57+ NK cell expression in different grades of OSCC, a retrospective study in India (2016) stated that expression of CD57 decreased with the decrease in cellular differentiation, from well-differentiated to poorly differentiated OSCC.6 Findings of the present study afford a similar inference, as the mean labelling index of CD 57+ was 30.80 ± 1.91 / HPF in well-differentiated OSCC, and 19.91 ± 3.62 / HPF in moderately-differentiated OSCC. This signifies that decreasing cellular differentiation, i.e., increasing grade of OSCC leads to downregulation of NK-cells, as reported in 2017 by Bi and Tian.[29]

After extensive research, it has been observed that this is the first study to evaluate and compare CD57+ NK cell expression in OL and OSCC. It has however been established that CD57+ NK-cells determine the prognosis of the disease; more the number, better the prognosis.6 Recent advances regarding anti-tumor immune responses and cancer biology have shown a multi-faceted dynamic interaction between the immune effectors and the tumor cells. NK-cells have two distinct approaches to eliminate tumors – one targeting stem cells and the other targeting differentiated cells.[30] Therefore, a combination of NK-cell-directed therapy and chemotherapeutic agents could be considered for eliminating both, undifferentiated and differentiated tumors.

CONCLUSION

The present study, focusing on comparison of the expression of CD57+ NK-cells in OL and OSCC, concludes that the expression of CD57+ NK-cells decreases with increasing severity of the disease. Conversely, increased expression of CD57+ NK-cells may be associated with a better prognosis. This finding may be attributed to increased expression in OL: first, NK-cells are less active in OL due to lack of tumor cells which explains most of them being in their matured state (CD57+) signifying low activity and highest cytotoxicity; second, there is lack of downregulation of NK-cells in OL due to the lack of tumor cells. In OSCC as well, the CD57+ NK-cells decreased as the grade progressed from well-differentiated to moderately-differentiated OSCC. This demonstrates NK-cell-downregulation by tumors, and NK cell exhaustion.

Limitations

Although the present study was novel in its approach, it was limited by the relatively smaller sample size, and non-inclusion of poorly differentiated OSCC. Prognosis and outcome of the patients could not be assessed owing to loss to follow-up.

Recommendations

This study, however opens prospects for further research, in the form genetic modification of NK-cells by combining their two-pronged approach of tumor elimination. It may also be possible to incorporate a safety switch in the form of a 'suicide gene' in the modified NK-cells, to control any possible toxicity during immunotherapy by such genetically modified NK-cells.

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