



Influence of NK Cells during Epithelial to Mesenchymal Transition in Oral cancer

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Abstract

Oral carcinogenesis is a major health problem in the world. Over 90% of all Oral Cancer is classified as Oral Squamous Cell Carcinoma with loco regional relapse and cervical lymph node metastasis. Considering epithelial malignancies, the role of immune system in governing Epithelial to Mesenchymal Transition (EMT) has not been much investigated in oral cancer. The present investigation suggests a possible role of NK cells during EMT and its correlation to invasive properties of tumour. Although a direct correlation couldn't be established but the influence of NK cells on EMT could be seen which open up new horizon for immunotherapeutic strategies in the coming years.

Keywords: NK cells, invasion, Oral squamous cell carcinoma (OSCC), EMT, E-cadherin, Vimentin.

Introduction

NK cells are cells of the innate immune system that display cytotoxic activity against an extensive range of tumor cells and tend to mark a response against process of carcinogenesis⁽¹⁾. In humans, there is a correlation between low NK cell cytotoxicity in peripheral blood and increased risk of cancer⁽²⁾. Conversely, NK cell infiltration into tumor tissue is associated with better patient prognosis in multiple malignancies⁽³⁾. Epithelial to Mesenchymal Transition (EMT) a hallmark of invasion, is a biological phenomenon that allows a polarized epithelial cell to undergo biochemical changes to attain a mesenchymal cell phenotype⁽⁴⁾ that is often characterised by the loss of E-cadherin and gain of Vimentin expression⁽⁵⁻⁸⁾. Given the extensive molecular and cellular changes that occur during

EMT, it is likely that tumor cell interactions with both innate and adaptive immune cells in the tumor microenvironment will be altered⁽⁹⁾. Work by Lehenbre et al. in 2008⁽¹⁰⁾ reveals that the Neural cell adhesion molecule (NCAM) has a role in initiating E-cadherin loss during EMT.

However, studies investigating the role of NK cells in tumor progression and metastasis are very limited. Therefore, it becomes important to understand NK cell responses in circulation as well as in tumor microenvironment, for better understanding of its role in initiating EMT in Oral carcinogenesis.

Aim of the study was to investigate the role of Peripheral blood NK Cells using Flow cytometry and Tumor infiltrating NK cells along with E-cadherin (an epithelial marker) and Vimentin (a mesenchymal marker) using Immunohistochemistry in OSCC.

Materials and Methods

In this prospective study, NK cells were evaluated in circulation as well as in tumour tissue front of 30 Male Oral cancer patients (Buccal mucosa). Peripheral blood NK cells were also compared with those of 10 age-matched healthy controls. These patients were diagnosed and treated at The Gujarat Cancer and Research Institute within the period of November 2017-June 2018. None of the patients received any form of neo-adjuvant therapy and no patient suffered recurrence upto the follow-up period of 8 months.

Immunophenotyping of peripheral blood NK cells, was performed by Flow cytometer BD FACS Canto TM II, (7 colour set up beads system) obtained from BD Biosciences (San Jose, USA) using a combination of monoclonal antibodies CD45 (PerCP, clone 2D1) to identify the expression of lymphocytes, CD3 (APC H-7, clone SK7) for T cells, CD56 (PE, clone MY31) for NK cells. For NK cells detection, antibodies were added to the peripheral blood (100 µl) sample and incubated for 15 minutes. After incubation, 2 ml of erythrocyte lysing solution (1:10 dilution with double distilled water) was added and incubated for 15 minutes at room temperature. Then cells were centrifuged at 400g for 5 minutes and supernatant was discarded. Remaining pellet was washed twice with 2ml PBS and then resuspended in 500 µl of PBS. Total 30,000 Lymphocytes were acquired (Figure 1).

Immunohistochemical localization of Tumor infiltrating NK cells and expression of E-cadherin and Vimentin were evaluated on FFPE blocks using Ventana Benchmark XT Autoimmunostainer (Ventana, USA). The commercially available antibodies used were anti-CD56 (clone 123C3, Dako), anti-E-cadherin (clone NCS38, Dako) and anti-Vimentin (cloneV9, Biogenex). 3-4µm thin sections were cut on microtome (Leica, Germany) and taken on to 3-Aminopropyltriethoxysilane (APES) coated slides. Briefly, the protocol includes following steps of deparaffinization using EZ solution, Antigen retrieval for 60 minutes using retrieval solution CC1, and incubation with ultra-view DAB Inhibitor for 4 minutes, Addition of 100

µl of anti-E-cadherin, anti-Vimentin antibody at 370°C for 32 minutes, anti-CD56 antibody at 370°C for 48 minutes respectively. Followed by Ultra-view HRP Multimer for 8 minutes, counterstained with Hematoxylin for 8 minutes and mounted with DPX. For NK cells, the numbers of tumor infiltrating NK cells were counted in three different fields and mean value for each field was calculated and based on the its median value patients were grouped into Low expression group and High expression group. For E-cadherin and Vimentin expression, scoring was done on the basis of intensity and percentage of cell population (%) in to Negative (<10% cells), and positive (>10% cells). They were further grouped in to grade I (10-30%), II (30-50% and III (>50%). Statistical analysis was carried out using SPSS statistical software version 20 (SPSS Inc, USA).

Results

In comparison with Healthy Controls, OSCC patients had a significant increased Mean ± SD value of WBC and a trend of decreased Mean ± SD value of Lymphocytes. While an incidence of higher Mean ± SD values were obtained for NK cells populations. NK cells were further categorised into CD56^{dim} and CD56^{bright} population (Table 1).

Table 1: WBC and NK cells comparison in blood of OSCC patients with healthy controls

	Healthy controls (N=10)	OSCC (N=30)	P Value
WBC	59.8×10 ³ ±10.8	89.5×10 ³ ±28.1	0.002*
Lymphocytes	27.5±3.60	23.5±6.20	0.061
ALC	1.6±0.36	2.0±0.75	1.07
NK CD56 ^{Dim}	8.1±2.70	10.0±1.8	0.461
NK CD56 ^{Bright}	0.8±2.70	1.0±0.08	0.660

*p values ≤ 0.05 was considered significant

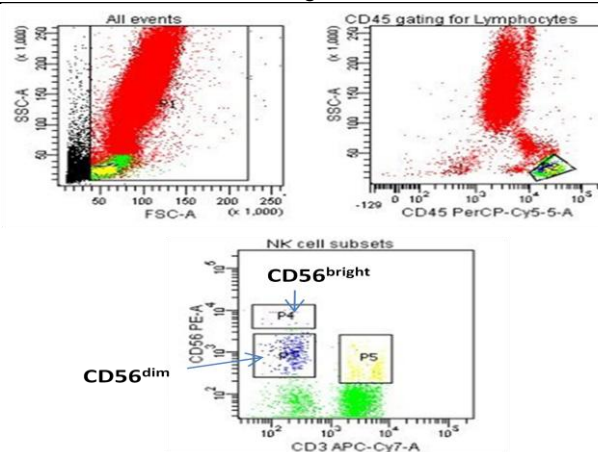


Figure: 1 Representative dot plot showing NK cells population of Oral cancer patients

Based on Immunohistochemistry analysis of OSCC patients (N=30), Tumor infiltrating NK cells expression was found lower in 60% (18/30) and higher in 40% (12/30) patients. E-cadherin expression was found to be lower in 40% (12/30) and higher in 60% (22/30) of patients. While Vimentin expression was found to be lower in 73% majority of patients (22/30) and Higher in 24% (8/30). It was further correlated with the clinicopathological parameters such as age, gender, habit, tumor site, tumor size, disease stage, histological grade, lymphatic permeation and Perineural invasion.

In accordance with clinicopathological characteristics, a higher incidence of Tumor infiltrating NK cells was observed in patients with perineural invasion (80%, (4/5). In case of EMT markers, a significant higher E-cadherin expression was observed in patients who were habituated to tobacco (71%, $p=0.050$) and as insignificant increase of Vimentin expression (40%, p value= 0.020) is observed with highly invasive type of tumor. Characteristic trend of higher Vimentin expression was observed in patients who had poorly differentiated Grade III tumors (100%, 4/4, p value=0.060) (Table 2, supplementary document).

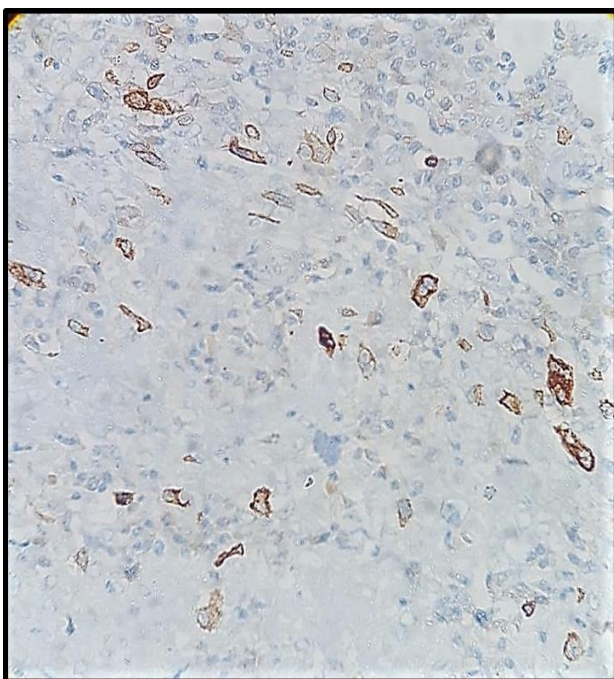


Figure 2 Membranous expression of Tumor infiltrating NK cells

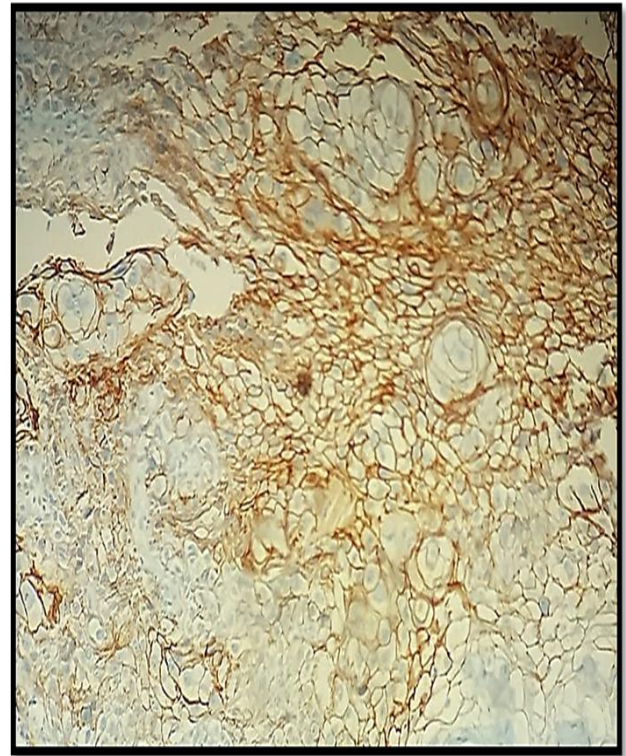


Figure: 3 Membranous expression of E-cadherin

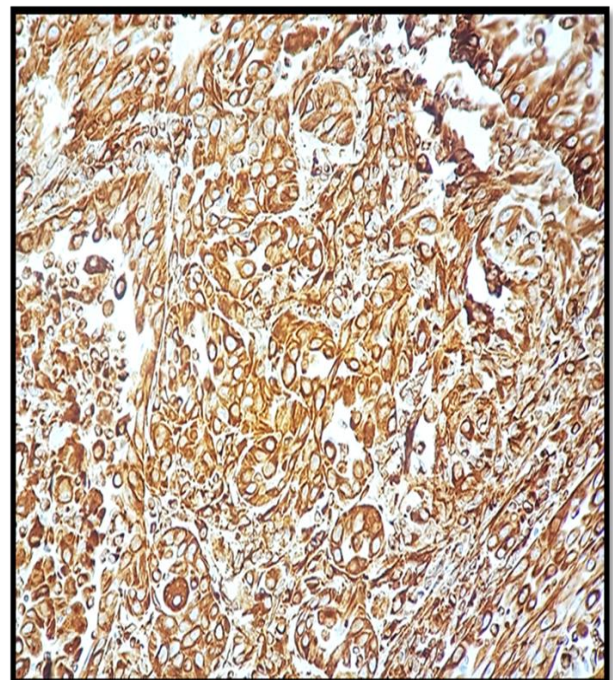


Figure: 4 Cytoplasmic expression of Vimentin

Table:2 Correlation of Tumor infiltrating NK cells and EMT markers with Clinicopathological parameters

Characteristics		N	Tumor infiltrating NK cells		N	E-cadherin		N	Vimentin	
			Negative	Positive		Negative	Positive		Negative	Positive
Age	<45	14	9 (64%)	5 (36%)	14	6 (43%)	8 (57%)	14	12 (86%)	2 (14%)
	≥45	16	9 (56%)	7 (44%)	16	6 (38%)	10 (62%)	16	10 (63%)	6 (37%)
Habit	Without Habit	9	5 (56%)	4 (44%)	9	6 (67%)	3 (33%)	9	6 (67%)	3 (33%)
	With Habit	21	13 (62%)	8 (38%)	21	6 (29%)	15 (71%)	21	16 (76%)	5 (24%)
Tumor Size	T1	13	8 (62%)	5 (38%)	13	6 (67%)	7 (54%)	13	10 (77%)	3 (23%)
	T2	11	8 (73%)	3 (27%)	11	2 (18%)	9 (82%)	11	8 (73%)	3 (27%)
	T4	6	2 (33%)	4 (67%)	6	4 (67%)	2 (33%)	6	4 (67%)	2 (33%)
Nodal Status	N0	21	11 (52%)	10 (48%)	21	8 (38%)	13 (62%)	21	15(71%)	6 (29%)
	N1	5	5 (100%)	0 (0%)	5	1 (20%)	4 (80%)	5	4(80%)	1 (20%)
	N2	3	1 (33%)	2 (67%)	3	3 (100%)	0 (00%)	3	2 (67%)	1 (33%)
	N3	1	1 (100%)	0 (0%)	1	0 (0%)	1 (100%)	1	1 (100%)	0 (0%)
Stage	Stage I	12	7 (58%)	5 (42%)	12	6 (50%)	6 (50%)	12	9 (75%)	3 (25%)
	Stage II	11	8 (73%)	3 (27%)	11	2 (19%)	9 (81%)	11	8 (73%)	3 (27%)
	Stage IV	7	3 (43%)	4 (57%)	7	4 (57%)	3 (43%)	7	5 (71%)	2 (29%)
Histological Grade	G1	13	8 (62%)	5 (38%)	13	4 (30%)	9 (70%)	13	4 (30%)	9 (70%)
	G2	13	6 (46%)	7 (54%)	13	8 (62%)	5(38%)	13	8 (62%)	5 (38%)
	G3	4	4 (100%)	0 (0%)	4	0 (0%)	4 (100%)	4	0 (0%)	4 (100%)
Lymphatic Permeation	Absent	26	14 (54%)	12 (46%)	26	12 (46%)	14 (54%)	26	19 (73%)	7 (27%)
	Present	4	4 (100%)	0 (0%)	4	0 (0%)	4 (100%)	4	3 (75%)	1 (25%)
Perineural Invasion	Absent	25	17 (68%)	8 (32%)	25	10 (40%)	15 (60%)	25	18 (72%)	7 (28%)
	Present	5	1 (20%)	4 (80%)	5	6 (43%)	8 (57%)	14	12 (86%)	2 (14%)

p value ≤ 0.05 was considered significant, X² = Chi-square value, r= Correlation coefficient
 *p value=0.050, X²=3.810, r= 0.356

Further, we correlated, circulating NK cells with NK cells infiltrated at tumor front and EMT markers along with disease stage, a significant positive correlation of circulating CD56^{Dim} NK cells was observed with tumor infiltrating NK cells (P=0.02) and a negative correlation was observed with E-cadherin though a value was not statistically significant. With disease stage, a positive correlation of CD56^{Dim} and negative correlation CD56^{Bright} was observed (P=0.05) (Table 3).

Table 3: Correlation of Circulating NK cells with tumor infiltrating NK cells and EMT markers

Marker	Circulating CD56 ^{Dim}	Circulating CD56 ^{Bright}
Spearman's correlation (*R _s Correlation coefficient)		
Tumor infiltrating NK cells	0.422*	-0.188
E-Cadherin	-0.281	-0.035
Vimentin	0.14	-0.15
Disease Stage	0.340*	-0.035*

Moreover, a high incidence of Tumor infiltrating NK cells expression was associated with lower E-cadherin expression (59%, p value=0.094) and a higher Vimentin expression (50%, p value=0.018) (Table 4 Supplementary material).

Table:4 Correlation of Peripheral Blood NK cells along with Tumor infiltrating NK cells

	Tumor infiltrating NK cell	
	Negative	Positive
Low expression group	12 (70%)	5 (30%)
Higher expression group	6 (46%)	7 (54%)
X ²	1.833	
r	0.247	
p	0.176	
p value ≤ 0.05 was considered significant, X ² = Chi-square value, r = Correlation coefficient		

Discussion

It is conceived that NK cells mount response against haematological malignancies which are more easily accessible through circulation, in contrast to solid tumours, as NK cells and Cytotoxic T cells are functionally exhausted due to cancer “immunoediting” that is mainly observed during EMT⁽¹¹⁾. It has to be considered that peripheral blood NK cells might display many differences with regards to phenotype and function compared to infiltration of NK cells in the tumor tissue. Only few studies address the issue of the potential changes in tumor immunogenicity during EMT mediated by immune cells like NK cells and T cells via the induction of altered cell-cell signalling changes of TGFβ, FGFR receptors or E-cadherin loss⁽¹²⁾.

In present study, we have tried to understand NK cell changes during EMT process in OSCC. In our study, a significant increased WBC with decreased Lymphocytes count in peripheral blood circulation of patients suggests a systemic inflammatory response, which can be associated with alterations in circulating WBC's which due to increase in number of circulating Neutrophils or due to the presence of Lymphopenia as suggested by Gabay et al. (1999)⁽¹³⁾ and Zahorec et al. (2001)⁽¹⁴⁾.

Our study shows a higher incidence of NK cells expression both in circulation and at tumor front which is in resemblance to the findings suggested by Céspedes et al. (2011) in cervical cancer⁽¹⁵⁾, suggests a feed forward infiltration of NK cells from circulation to tumor microenvironment.

Analysis done by Vural et al. in 2000⁽¹⁶⁾ suggests that infiltration of NK cells in perineural spaces of up to 52% of OSCC cases marks as a predictor for metastasis and loco regional recurrence. Similar to

this result, in present study, higher Tumor infiltrating NK cells was observed with the presence of Perineural invasion.

NK cells mainly have two functions, one is of having cytotoxic activity against tumor antigens and other is the secretion of IFN-γ. CD56^{Dim} subsets are highly cytotoxic and produce less IFN-γ, while CD56^{bright} have opposite characteristic⁽¹⁷⁾. A positive correlation of CD56^{Dim} with tumor infiltrating NK cells in our study shows migration of cytotoxic NK cells at tumor front which is mainly occurs through chemo attractants present at tumor site. However, in relation to disease status, inverse correlation suggest that NK cells initially have control on tumor but in advance stage it supports tumor for invasion.

Subsequently, the inter marker-correlation not only acknowledges a trend of decreased expression of E-cadherin but also a significant increase of Vimentin expression in accordance with Tumor infiltrating NK cells expression promoting a pro-invasive EMT phenotype in oral cancer which hasn't been reported earlier as per the best knowledge.

Although the role of NK cells in the control of primary tumors remains debatable, our current study highlights that Tumor infiltrating NK cells might have a role in development of EMT and this might be due to its dynamic association with focal adhesion molecules⁽¹⁸⁾.

This is a preliminary study performed on smaller sample size to ascertain the dynamics of NK cells in correlation with EMT markers. One possible limitation of this study is the shorter follow-up period. Nevertheless, in future we would like to undertake more patients and continue with cytokine study and patient's diseased status.

Conclusion

The present investigation features the influence of NK cells on EMT by mediating an aberrant loss of E-cadherin and an upregulation of Vimentin, thereby inducing transient properties for tumor cells which prompt pro-invasive tumor phenotype. Early changes associated with EMT are hard to envision morphologically in tumor sections therefore early detection of EMT may anticipate likely invasive behaviour and could aid in advancement of enhanced anticancer immunotherapeutic strategies in the coming years.

Acknowledgments

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Conflicts of Interests

All authors confirm that there are no conflicts of interest associated with this publication.

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