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RESEARCH ARTICLE

A COMPARITIVE AND PROGNOSTIC STUDY OF CIRCULATING TUMOR CELLS AND VEGFR-2 IN DIFFERENT HISTOLOGICAL GRADES OF ORAL SQUAMOUS CELL CARCINOMA

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Abstract

Background And Objectives: Oral squamous cell carcinoma (OSCC) is the fourth leading cancer and the eighth leading cause of cancer - related death worldwide. Despite improvements in diagnosis and therapeutic concepts, the 5 year overall survival rate has not improved significantly over the last 25 years and remains around 56%. Circulating tumor cells are cancer cells detached from a primary or secondary tumor and entered the circulation, these serve as a clinical biomarker for diagnostic, prognostic and pharmacological purposes. Neoangiogenesis is essential for the growth and metastasis of solid tumors.VEGFR-2 is one of the major mediator of endothelial cell mitogenesis, proliferation and survival. Inhibition of angiogenesis by disruption of VEGFR-2 pathways, this could suppress tumors` growth by limiting their blood supply. Hence, the present study was carried out to isolate and study circulating tumor cells in different grades of oral squamous cell carcinoma from the peripheral venous blood of the patient. To assess the histological grading of oral squamous cell carcinoma by using H and E sections of the biopsy sample and to assess the immunohistochemical expression of VEGFR-2 / FLK1 in circulating tumor cells and in biopsy samples.

Material and Methods: The present study consisted of 60 paraffin embedded blocks of histologically diagnosed cases of 15 well differentiated OSCC, 15 moderately differentiated OSCC, 15 poorly differentiated OSCC and 15 cases of normal epithelium. Out of 45 OSCC cases, 20 OSCC patients' blood samples were collected and subjected to CTCs isolation using Pluriselect beads. Tissue sections of 4µm thickness of paraffin embedded tissue blocks were subjected to VEGFR-2 staining. Expression of VEGFR-2, were counted randomly in 5 high power fields(40X).Quantitative analysis of VEGFR-2 was done using image analysis (image progress software) and data obtained was subjected to statistical analysis using ANOVA and Unpaired t test.

Results: Higher mean expression for VEGFR-2 was seen in PDSCC followed by MDSCC, WDSCC and the control group. The difference in mean VEGFR-2 expression was found to be statistically significant between WDSCC & MDSCC (P< 0.049) as well as between WDSCC &PDSCC (P<0.000) and between MDSCC& PDSCC (P<0.001).The circulating tumor cells (CTCs) were found in 3/20(15%) patients of OSCC.

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Conclusion: The expression of the VEGFR-2 was higher in PDSCC as compared to MDSCC, WDSCC and normal epithelium respectively.Circulating tumor cells were found in 3 patients of OSCC.

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Introduction:-

Oral squamous cell carcinoma (OSCC) is one of the leading cause of cancer - related death worldwide. It is the most frequent histologic type representing 95% of head and neck cancers. The estimated incidence worldwide is approximately 5,50,000 cases and almost 50% of the patients die of the disease. Only about one third of the patients present with early-stage disease, whereas two thirds show already advanced disease (UICC stage III–IV) with poor outcome. The prognosis of patients remains poor and the risk to develop relapse is higher than 50%. Furthermore, the development of distant metastasis occurs in 15% to 25% of the patients. The most important prognostic indicator for relapse of OSCC is the presence of metastatic spread to lymph nodes in the neck.[1]

OSCC is a major health problem in India, the major risk factor being, chronic exposure of oral mucosa to betel quid along with areca nuts chewing a practice that is highly prevalent in different parts of India.[2]Despite improvements in diagnosis and therapeutic concepts, the 5year overall survival rate has not improved significantly over the last 25 years and remains around 56%. Therefore an early detection of metastases acts as an important indicator of survival, prognosis and relapse and a better understanding of mechanisms underlying the metastasis is crucial.[3]

According to various studies it has been established that the circulating tumor cells (CTCs) in the peripheral blood and disseminated tumor cells (DTCs) detected in the bone marrow of OSCC patients can serve as prognostic markers. These cells are defined as cancer cells, detached from a primary or secondary tumor and entered the circulation.[4] In cancer diseases including in OSCC, these cells eventually detach from the primary site and disseminate in the blood, the lymphatic fluid, the bone marrow or even in the cerebrospinal fluid. Circulating tumor cells (CTC) designate mainly the ones circulating in the peripheral blood. Under certain circumstances, like immune escape or immunoediting, these cells can establish a new tumor in distance sites, a process known as metastasis.[5,6]

Neoangiogenesis is essential for the growth and metastasis of solid tumors. The factors responsible for neoangiogenesis are vascular endothelial growth factors and their receptors.VEGFR-2 being one of the major mediator of endothelial cell mitogenesis, proliferation and survival.[7,8]A fundamental approach to inhibit angiogenesis during tumorigenesis is the disruption of VEGFR-2 pathways, this could suppress tumors` growth by limiting their blood supply; by changing their morphology, the vascular wall structure, and rendering VEGF and VEGFR-2 expression in tumor vasculature to a more normal pattern. Thus helps in improving the drug penetration in tumors, and also by blocking the VEGF autocrine pathways there is reduced uncontrolled neoplastic cell proliferation.[9]

Materials and Methods:-

This study included patients diagnosed with Oral Squamous cell carcinoma, both clinically and histologically
60 paraffin embedded tissue blocks of OSCC, were retrieved from the archives of Department of Oral Maxillofacial Pathology and Microbiology.

1. 15 cases of normal mucosa
2. 15 cases of well differentiated OSCC
3. 15 cases of moderately differentiated OSCC
4. 15 cases of poorly differentiated OSCC

Out of 45 OSCC cases, 5ml of peripheral venous blood sample from cubital fossa and biopsy from 20 histopathologically diagnosed patients was collected after obtaining informed consent.

Relevant information viz. age, sex, site of the lesion, hisopathological grading of oral squamous cell carcinoma (Annexure) was obtained from the records of the patients and was tabulated.

Inclusion criteria

Histopathologically diagnosed cases of

1. Well Differentiated Oral Squamous Cell Carcinoma.
2. Moderately Differentiated Oral Squamous Cell Carcinoma
3. Poorly Differentiated Oral Squamous Cell Carcinoma
4. Normal epithelium of the oral cavity from biopsies of benign connective tissue pathologies.

Exclusion Criteria
1. Patients with other simultaneous primary tumors
2. Patients receiving any form of treatment for oral squamous cell carcinoma.

Evaluation of the IHC (VEGFR-2) Stained Slides
The IHC stained sections (normal mucosa and different grades of OSCC cases) were analysed and assessed in 5 random high power fields under 40X magnification using research microscope (Olympus-BX53-progress software) and the number of the tumoral epithelial cells showing positivity for VEGFR-2(membranous stain) were assessed and subjected to appropriate statistical analysis.

The stored sample was retrived and a smear of the same was done using a cytospin on a charged slide. IHC was carried out using Pancytokeratinconjugated with FITC (fluorescein isothiocyanate) in order to demonstrate CTCs.

Evaluation of slides for CTCs
The stored sample was retrieved and a Cytosmear of the same was made. A standard IHC procedure as mentioned before was followed, omitting the antigen retrieval step.

To demonstrate the epithelial cells (captured CTCs), Pan cytokeratin (IHC marker) conjugated with FITC (fluorescent antibody) was used.On evaluation under a magnification power of 10X, the epithelial cells showed a green fluorescence under a research microscope (Olympus-BX53-progress software) indicating the presence of circulating tumor cell.

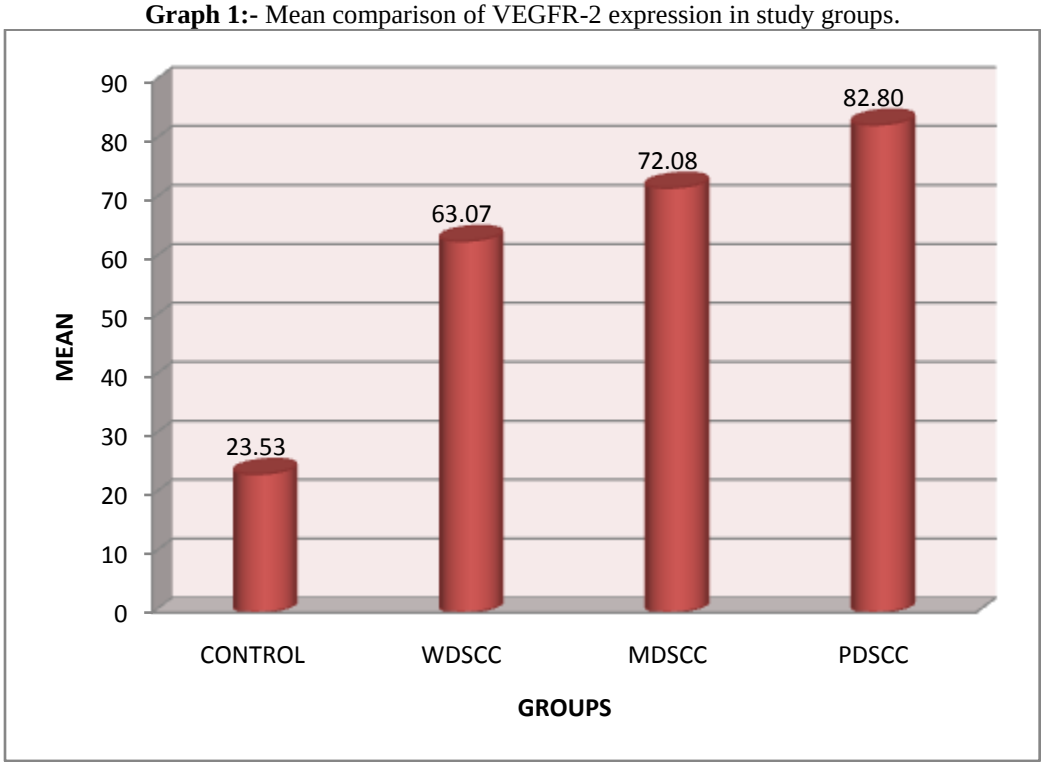
Results:-

Table 1:- Mean comparison of VEGFR-2 Expression in study groups.

GROUP	MEAN	SD	P VALUE
CONTROL	23.53	5.55	0.000
WDSCC	63.07	14.36	S
MDSCC	72.08	9.04	
PDSCC	82.80	5.44	

Statistical Analysis:
ANOVA test. Statistically significant if $P<0.05$

Graph 1:- Mean comparison of VEGFR-2 expression in study groups.



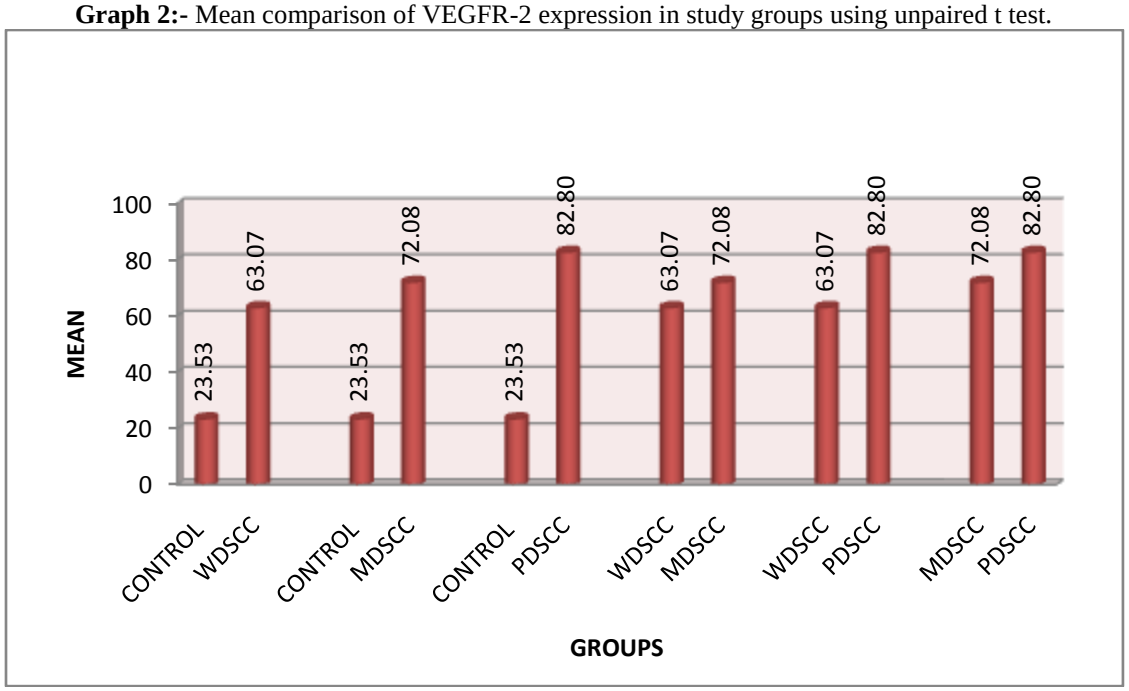
On evaluating the study group we found the mean expression of vascular endothelial growth factor receptor-2 (VEGFR-2) immunohistochemically, in control group to be 23.53% using ANOVA. In different histological grades of OSCC mean expression of VEGFR-2, immunohistochemically was moderate in WDSCC (63.07%),more than moderate in MDSCC (72.08%) and the highest expression was noted in PDSCC (82.80%). The difference in mean VEGFR-2 expression, immunohistochemically among the groups was statistically found to be significant (p value 0.000).In order to find out among which pair of groups there existed a significant difference, we carried out multiple comparison using Unpaired t test. The results are given below:

Table 2:- The difference inmean comparison of VEGFR-2 expression in study groups.

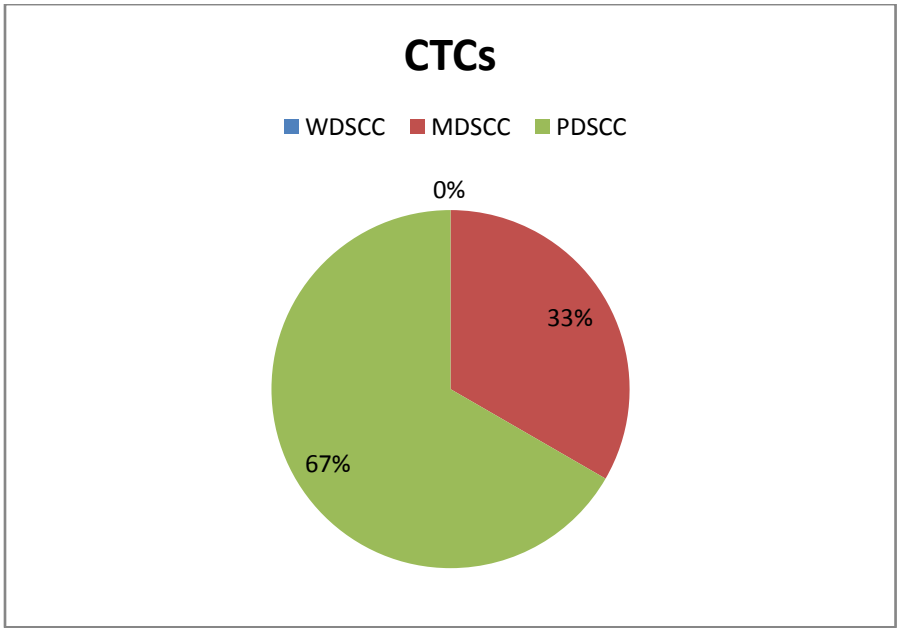
NGROUP	MEAN	SD	difference MEAN±SD	P VALUE
CONTROL	23.53	5.55	39.54±8.81	0.000
WDSCC	63.07	14.36		S
CONTROL	23.53	5.55	48.55±3.49	0.000
MDSCC	72.08	9.04		S
CONTROL	23.53	5.55	59.27±0.11	0.000
PDSCC	82.80	5.44		S
WDSCC	63.07	14.36	9.01±5.32	0.049
MDSCC	72.08	9.04		S
WDSCC	63.07	14.36	19.73±8.92	0.000
PDSCC	82.80	5.44		S
MDSCC	72.08	9.04	10.72±3.60	0.001
PDSCC	82.80	5.44		S

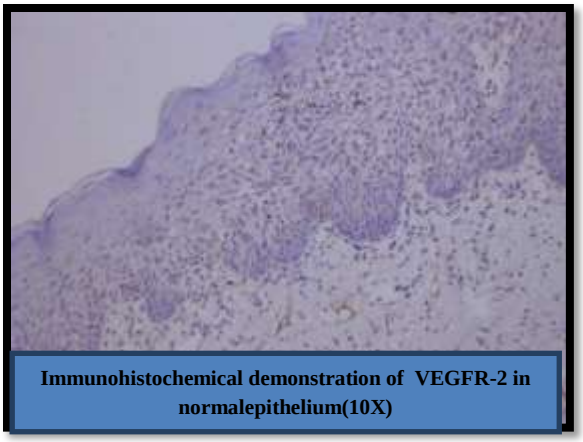
Statistical Analysis: Unpaired t test. Statistically significant if P<0.05

Graph 2:- Mean comparison of VEGFR-2 expression in study groups using unpaired t test.



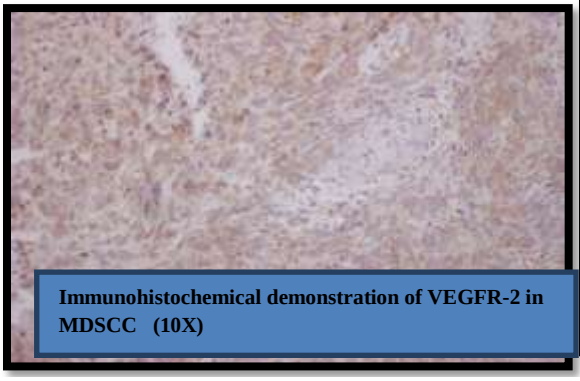
The difference in mean VEGFR-2 expression was found to be statistically significant between WDSCC& MDSCC (P< 0.000) as well as between WDSCC &PDSCC (P<0.000) and between MDSCC& PDSCC (P<0.000).



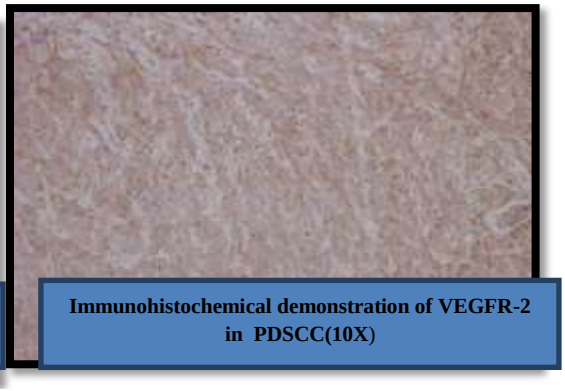


Immunohistochemical demonstration of VEGFR-2 in normalepithelium(10X)

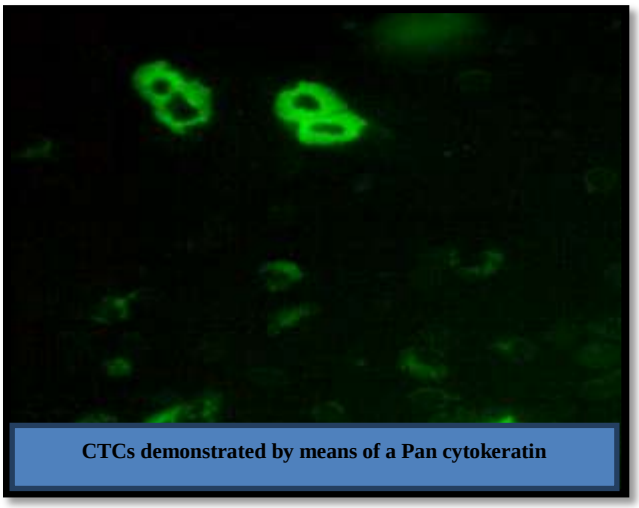
Immunohistochemical demonstration of VEGFR-2 in WDSCC (10X)



Immunohistochemical demonstration of VEGFR-2 in MDSCC (10X)



Immunohistochemical demonstration of VEGFR-2 in PDSCC(10X)



CTCs demonstrated by means of a Pan cytokeratin

Circulating tumor cell isolationkit-Pluri-Select

Discussion:-

Metastatic disease is responsible for 88% of HNSCC patient deaths within 12 months ofdiagnosis, with median time to death from diagnosis of metastatic disease ranging from 1 to 12months. Therefore, there is an urgent need for new and better prognostic markers to improve theprediction of loco regional recurrence and distant metastases in HNSCC patients. The ability toidentify high risk patients with disseminated disease prior to the presentation of clinicallydetectable metastases, holds remarkable potential for individualized treatments enablingimproved rates of survival. [10,11]

Vascular endothelial growth factor (VEGF), acts as a both potential vascular permeabilityinducing factor and a selective endothelial mitogen.

VEGF, besides playing a role inphysiological vessel formation, also acts as a regulator of tumor neovascularisation and itsexpression has been reported in various human solid cancers. VEGFR2 (Vascular EndothelialGrowth Factor Recetor-2)/ FLK1 (Fetal Liver Kinase) is known to be the most essentialreceptor for the actions of VEGF on permeability and growth.[11,12]

CTCs are described as cells shed by a primary tumor into vasculature and they are found in the blood even before the cancer metastasizes to various parts of the body[13,14] .The enumeration of circulating tumor cells (CTCs) represents an effective prognostic and predictive biomarker, which is able to monitor efficacy of adjuvant therapies, detect early development of (micro) metastases and at last, assess therapeutic responses of advanced disease earlier than traditional imaging methods. Moreover, since repeated tissue biopsies are invasive, costly and not always feasible, the assessment of tumor characteristics on CTCs, by a peripheral blood sample as a 'liquid biopsy', represents an attractive opportunity.[15,16]

In our study, we attempted to isolate and quantify circulating tumor cells and assessed the immunohistochemical expression of VEGFR-2 by the tumor cells in various histopathological grades of OSCC. Assessment of the immunohistochemistry using VEGFR-2 antibody was done on sections obtained from the paraffin blocks and was seen as a membrane stain, using a research microscope and was an intra-observer study. In our study, we found that the mean expression of VEGFR-2 between normal epithelium (Control group) and all grades of oral squamous cell carcinoma was statistically significant. There was also statistically significant difference in mean expression of VEGFR-2 between WDSCC and MDSCC (p<0.049), between WDSCC and PDSCC (p<0.000) and between MDSCC and PDSCC (p<0.001).

Based on immunohistochemical findings, highest VEGFR-2 expression in tumor cells was observed in less-differentiated/poorly differentiated invasive oral squamous cell carcinoma(82.80%) followed by MDSCC(72.08%) and finally the least expression was seen in WDSCC(63.07%).These results suggest that the degree of VEGF expression is correlated with the degree of differentiation or invasiveness of carcinoma. This was seen in accordance with studies by Kyzas PA et al.(2014) [17] and Sato H et al(2009) [12].Thus The VEGFR-2 positive cell count was found to be higher in poorly differentiated tumors. This was further supported by Kim S K et al.(2015) [18],who conducted a study to evaluate the role of VEGF`s in OSCC and concluded that, VEGF expression was increased in insufficiently differentiated invasive carcinomas and was overexpressed in invasive oral squamous cell carcinoma and a study by Stinga AC et al.(2011)[19] which stated that VEGFR1 and VEGFR2 are expressed in OSCC tumoral epithelial cells, with higher expression of these receptors in the center of the tumor, and correlations with pathologic parameters such as localization, grade, and stage, suggesting their involvement in a sequential manner in VEGF signalling regulation.

VEGFR-2 could be implicated in tumor progression from dysplastic to poorly differentiated tumors. This phenomenon can be explained by the differing regulation and processing of VEGF protein and its receptor VEGFR-2. Regulation studies revealed that VEGF upregulation is predominantly and directly caused by hypoxia, which is common within a growing tumor. Dependent on splice variants, VEGF can be kept membrane or extracellular matrix-bound, or diffuses to cells bearing VEGFR-2. Receptor binding of VEGF is additionally modulated by cell surface structures like neuropilins or heparin sulfate proteoglycans like CD44v3, which is in accordance with Neuchrist C et al. (2011)[20]alsoMargaritescu C et al. (2009)[21] suggested an autocrine loop signalling pathway of VEGF, expressed by both tumoral and the stromal cells in cases of metastasis in OSCC.

Sugiura T et al(2009) also emphasized the role of Vascular endothelial growth factors and their relationship with metastasis via lymph nodes in OSCC.[22]

A fundamental approach to inhibit angiogenesis during tumorigenesis is the disruption of VEGFR-2 pathways, which could hamper the tumor growth by limiting their blood supply; by depriving them of oxygen, by changing their morphology, the vascular wall structure and

rendering the VEGFR-2 expression in tumor vasculature to a more normal pattern, and thus improving the drug penetration in tumors. This was supported by Yazici YD et al . In 2005, WHO showed for the first time that PTK897, which inhibits the VEGF-R tyrosine kinases and specifically targets the VEGFR-2 receptors, decreased the tumoral growth and its vascularisation in case of squamous cell carcinoma of the tongue, grown orthotopically in mice. [23]

These findings are in accordance with our study and suggest that VEGFR-2 can be used in the diagnosis, prognosis and in the treatment of oral squamous cell carcinomas.

In this present study, out of 60 cases, 20 samples were subjected to isolation and quantification of CTCs. 5 cases in each group. Only 3(15%) samples showed pellet formation. This is in accordance with the study conducted by Grobe A et al. in which they isolated CTCs in peripheral blood of 80 histopathologically diagnosed oral squamous cell carcinoma patients. CTCs could be detected in 10/80(12.5%) patients.[1]

Tinhofer I et al. (2012) conducted a study, and monitored the level of CTCs during combined radiotherapy regimens in locally advanced SCC? 9 out of 31(29%) patients showed positivity for CTCs in peripheral venous blood before treatment and 11 out of 23 patients showed CTCs after the treatment.[24] Winter SC et al.(2009)[25] conducted a study titled Long term survival followingthe detection of circulating tumor cells in HNSCC which showed that almost all patients with advanced head and neck cancers had CTCs at the time of surgery.

Schneck H et al. conducted a study to isolate Circulating tumor cells (CTCs) that express no/low levels of EpCAM, (undergoing epithelial-to-mesenchymal transition (EMT). [26]

Summary & Conclusion:-

Summary

Our present study was undertaken to isolate circulating tumor cells, from different histological grades of diagnosed OSCC cases and to find the expression of VEGFR-2 in tumor cells, by immunohistochemical method in different histological grades of OSCC. Twenty peripheral blood samples were collected from patients histologically diagnosed as OSCC, based on the biopsy reports, from the out-patient department of M.R Ambedkar Dental College And hospital. These samples were then subjected to Pluriselect-Anti-Human-CD326-pluri beads (EpCAM), based on antigen-antibody affinity. A pellet was obtained as a result of this procedure, and cytosmear of the same was made, on which IHC staining using pan cytokeratin cojugated FITC antibody was done. Captured CTCs showed a green fluorescence on examination of the smear under a research microscope. Sixty paraffin-embedded blocks of histologically diagnosed cases of OSCC, were retrieved from the archives. 4μm thick sections were obtained and were subjected to immunohistochemical staining with antibody for VEGFR-2/FLK-1 by following the IHC protocol. The expression shown by tumor cells (membranous stain) was noted and counted in high power (40 X) by selecting 5 random fields, in each tissue section. The expression shown by tumoral epithelial cells was compared among different groups (control, WDSCC, MDSCC and PDSCC) and reviewed.

Conclusion:-

From the study, it was concluded that:

1. Expression of VEGFR-2 was found to be more in OSCC when compared to normal epithelium.
2. Expression of VEGFR-2 was significantly higher in PDSCC when compared to MDSCC, WDSCC, which showed lesser staining intensity
3. Thus the mean expression was significantly highest in PDSCC (82.80%), followed by MDSCC (72.08%) and least in WDSCC (63.07%).The difference in the mean expression of VEGFR-2, among the groups was found to be statistically significant.
4. The difference in mean VEGFR-2, expression was found to be statistically significant between WDSCC &MDSCC ($P<0.049$) and between WDSCC & PDSCC ($P<0.000$) as well as between as MDSCC &PDSCC ($P<0.001$).
5. Circulating Tumor Cells (CTCs) were found in 3/20(15%) patients of OSCC.

Hence from this study it can be concluded that VEGFR-2 expression was more in OSCC, when compared to normal epithelium. Increased expression was seen in PDSCC, when compared to MDSCC & WDSCC. Neoangiogenesis, plays a significant role in progression of the tumor and survival of tumor cells by providing sufficient blood flow which delivers nutrients and oxygen to the growing tumor mass.Various studies have showed that VEGFR-2 / FLK-1 is the predominant RTK (Receptor Tyrosine Kinase) that mediates VEGF signalling in endothelial cells, that drives VEGF-mediated angiogenesis. Tumour cells and the stromal cells (endothelial cells) have been known to express VEGFR-2 and it has a prime role in mediating VEGF signalling.

In our study, Circulating Tumor Cells (CTCs), were found in 3/20 (15%) cases of OSCC.

Considering the vast literature and studies on CTCs, these have been seen to show immense potential as a diagnostic tool and their bio molecular characterization offers new perspectives to identify potential targets for tailor made therapies. Therefore, detecting tumor cell dissemination early and understanding the underlying mechanisms are crucial for predicting prognosis, relapse and survival.

Our study needs to be further carried out with a larger sample size, and the various limitations during CTCs enrichment and isolation such as EpCAM negative/low expressive CTCs have to be taken into account.

Finally from this study we would like to concludeand suggest that VEGFR-2/FLK-1 is a predominantly functional marker for the early onset of angiogenesis and tightly associated with rapid tumor growth, thus can be used as a tool in diagnosis and prognosis of OSCC.

CTCs have potential to aid in the entire course of a patient's cancer journey starting from diagnosis, treatment selection, post-treatment/surgery monitoring, and follow-up, thus can be used as a crucial diagnostic and prognostic tool in OSCC patients.

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