"EVALUATION OF IMMUNOEXPRESSION OF AJUBA PROTEIN IN NORMAL ORAL MUCOSA AND ORAL SQUAMOUS CELL CARCINOMA".

By
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Dissertation

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(BRANCH - IV)

Under the Guidance of
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*"**An investment in knowledge pays the best interest" – Benjamin Franklin***

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*I **Dr. Priyanka Desai** would like to extend my sincere thanks to all of them.*

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LIST OF ABREVATIONS

%	Percentage
i.e.,	That Is
No.	Number
SL	Serial
Akt	AKR Mouse Thymoma
APC	Adenomatous Polyposis Coli
APES	Amino Propyl Triethoxysilane
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-Related
BM	Basement Membrane
CAN	Copy Number Alterations
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
DAB	Diaminobenzidine
DDR	DNA Damage Response
DJub	Drosophila Associated Jub
DLL	Delta-Like Protein 1
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-Dependent Protein Kinase
DOI	Depth Of Invasion
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
EIF3E	Eukaryotic Translation Initiation Factor 3 Subunit E

EGFR	Epidermal Growth Factor
FAT1	Protocadherin
FFPE	Formalin Fixed Parafin Embedded
Grb2	Growth Factor Receptor-Bound Protein 2
Greb1	Growth Regulating Estrogen Receptor Binding 1
CRC	Colorectal Cancer
GSK-3b	Glycogen Synthase Kinase-3
GSTM1	Glutathione S-Transferase Mu 1
GLUT1	Glucose Transporter
H & E	Haematoxylin And Eosin
HCC	Hepatocellular Carcinoma
HNSCC	Head And Neck Squamous-Cell Carcinoma
HCT116	Human Colorectal Carcinoma Cell Lines
HPV	Human Papillomavirus
IARC	International Agency for Research on Cancer
IGF	Insulin-Like Growth Factor
IHC	Immunohistochemistry
Isl1	Insulin Gene Enhancer Protein
JAG1	Jagged1
JNK- pathway	C-Jun N-Terminal Kinases
LATS	Long-Acting Thyroid Stimulator.
LIMD1	LIM Domain Containing Protein 1
LNCaP	Lymph Node Carcinoma of The Prostate
LPA	Lipoprotein (A)

Lpp	Lipoma-Preferred Partner
MAPK	Mitogen-Activated Protein Kinase
MDSCC	Moderately Differentiated Squamous Cell Carcinoma
MM	Malignant Mesothelioma
MMP 10	Matrix Metallopeptidase 10
miR-1184	Microrna 1184-1
mRNA	Messenger Ribonucleic Acid
Mst	Macrophage Stimulating 1
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyl Tetrazolium Bromide
Myc	Myelocytomatosis Oncogene
NES	Nuclear Export Sequence
NLS	Nuclear Localisation Site
NICD	Notch Intracellular Domain
Nrf2	Nuclear Factor-Like 2
NCI-N87	Hypotriploid Human Cell Line
OSCC	Oral Squamous Cell Carcinoma
pSin-EF2-puro	Plasmid-Ef2-Nanog-Puro
PCR	Polymerase Chain Reaction
PDK	Pyruvate Dehydrogenase Kinase
PDSCC	Poorly Differentiated Squamous Cell Carcinoma
Pc	Prostate Cancer
PIKK	Phosphatidylinositol 3-Kinase-Related Kinases
PI-3-Kinase	Phosphoinositide-3kinase
PRMT	Protein Arginine Methyltransferases

RAS	Retinoic Acid Signaling
Ras- MAPK	Mitogen-Activated Protein Kinase
RNAs	Ribonucleic Acid
RND	Radical Neck Dissection
RPA	Replication Protein A
RT-PCR	Reverse Transcription–Polymerase Chain Reaction
SAV	Salvador
SGC-7901	Human Gastric Cancer Cell Line
Sgk3	Serum/Glucocorticoid Regulated Kinase Family Member 3
SH2	Src Homology 2
SH3	Src Homology 3
SP1	Specificity Protein 1
siRNA	Small Interfering RNA
SNAIL	Drosophila Homolog, Zinc Finger Protein
SW480	Primary Adenocarcinoma Colon Cell Lines
SMAD	S (Small Body Size) + Mothers Against Decapentaplegic (MAD)
SWH	Salvador-Warts-HIPPO Pathway
TAZ	Transcriptional Coactivator With PDZ-Binding Motif
TFF1	Trefoil Factor 1
TGFβ	Transforming Growth Factor Beta
TNM	Tumor (T), Nodes (N), And Metastases (M)
TP53	Tumor Protein P53
TRIP6	Thyroid Hormone Receptor Interacting Protein 6
WDSCC	Well Differentiated Squamous Cell Carcinoma

WILMS	Wilms Tumor 1 Interacting Protein
Wnt	Wingless-Related Integration Site
WTIP	Wt1-Interacting Protein
YAP	Yes-Associated Protein

ABSTRACT

Introduction:

OSCC is a major public health problem and it ranks among the top ten cancer worldwide. Though there are several modalities for treatment of OSCC but the prognosis remains to be poor. The researchers have reported several molecules in these steps of OSCC which undergo mutations. One such molecule is from the Zyxin family of proteins, named AJUBA. The AJUBA is a cytosolic protein and has the ability to shuttle between the nucleus and cytoplasm. It is a multifunctional scaffold protein which takes part in several physiological conditions. Recent studies on various malignancies have noted its association in tumor progression, growth, migration and in few malignancies as a tumor suppressor. There is a sparse literature available on its role in OSCC and normal oral mucosa. The present study aims to evaluate the role of AJUBA in normal oral mucosa and OSCC.

Objective:

- To assess and compare the immunoexpression of AJUBA in normal oral mucosa and oral squamous cell carcinoma.
- To corelate the immunoexpression of AJUBA in oral squamous cell carcinoma with demographic and clinicopathological parameters.

Materials and Method:

In the present study, FFPE blocks of 42 each, normal oral mucosa and OSCC were obtained. Two sections of each with 3µm thickness were obtained. One section was stained with hematoxylin and eosin and other section was stained with Anti- AJUBA polyclonal antibody. The normal oral mucosa slides were analysed for

immunoexpression of AJUBA among the different layers of the epithelium and deeper part of the connective tissue component. In OSCC cases we analysed the immunoexpression of AJUBA at the superficial and invasive front of the tumor, localization in tumor islands, cellular location within the tumor cells and percentage of positivity. Further the intensity of immunoexpression of AJUBA in normal oral mucosa and oral squamous cell carcinoma was compared. We also evaluated the association of immunoexpression of AJUBA with demographic and clinicopathological parameters in all cases of oral squamous cell carcinoma.

Statistical analysis:

In normal oral mucosa and OSCC frequency percentage was calculated both in the epithelium and connective tissue. Comparison of immunoexpression of AJUBA in normal oral mucosa and OSCC with intensity of staining and corelation of immunoexpression of AJUBA with the clinicopathological parameters was done using Chi square test.

Results:

Immunoexpression of AJUBA in normal oral mucosa was found predominantly in basal and supra basal layer of the epithelium and also in the deeper part of the connective tissue component. The cellular location in majority of the cases was seen both in nucleus and cytoplasm. In all the OSCC cases an enhanced immunoexpression of AJUBA was noted and percentage of positivity was 50-75% with localization of AJUBA at the periphery of the tumor islands and tumor cells showing nuclear and cytoplasmic location. Intensity of expression of AJUBA in OSCC cases was enhanced when compared with normal oral mucosa. Among the demographic and clinical pathological parameters, statistically significant association with AJUBA

immunoexpression was found only in two parameters i.e, histological tumor grade and inflammatory response.

Conclusion:

To conclude, this is the first study of its kind showing immunoexpression of AJUBA in normal oral mucosa. AJUBA is known as a key regulatory protein involved in epithelial proliferation and stratification in the normal physiological context and thus its enhanced expression in the normal oral epithelium validates its role in normal epithelium. We also hypothesize that the upregulation of immunoexpression of AJUBA in the OSCC cases when compared to the normal oral mucosa may suggests its possible role in tumor growth and progression.

Key words:

Normal oral mucosa, Oral squamous cell carcinoma, AJUBA

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INTRODUCTION

Oral Carcinoma (OSCC) an entity of Head and Neck Carcinomas (HNSCC) is a neoplasm particularly prevalent in developing countries across the world, affecting more than 40,0000 individuals annually[1]. This disease is seen to affect males more than females with an increased mortality and morbidity rate[2]. These rates have remained unchanged though the research in oncology has evolved as the patients are unaware of oral cancer and its risk factors[3].

A multifactorial etiology like tobacco use, alcohol consumption, dietary factors, immunodeficiency and viral infections can lead to the occurrence of this malignancy. Various histopathological parameters play a key role during the diagnosis of the disease and in understanding the patient prognosis and survival[4,5].

This epithelial neoplasm disrupts several molecules and causes alterations in physiological signalling pathways[1], such as Wnt[6]- involved in cell differentiation, proliferation and migration, NOTCH[7]- cell proliferation, HIPPO[8]- determination of an organ growth and size, SNAIL/SNAG[9]- epithelial mesenchymal transition etc.

Tumour infiltration and metastasis in the cancer cells occurs due to dysregulation of these pathways resulting in uncontrolled neoplastic cell growth. These pathways involve series of tumor host interactions and also are mediated by certain proteins present in the cellular and sub cellular components of the cell[10,11].

Thus, it is important for onco-biologists to understand these proteins mechanisms underlying this neoplastic disease of the oral cavity so that new therapies may be rationally applied[11].

A genetic atlas[12] of HPV associated HNSCC revealed that AJUBA protein mutations are seen in 0- 6% cases and their findings showed that overexpression of AJUBA led to increased cellular growth and proliferation of cells.

AJUBA[13] belongs to the Zyxin family which include LIMD1, AJUBA, LPP, WTIP etc. "The Ajuba adaptor proteins are characterized by the presence of three highly related tandem LIM domains at their carboxyl terminus (the LIM region), and a variable proline-rich amino-terminal preLIM region." AJUBA takes part in various physiological and pathological conditions.

In physiological conditions it participates in embryonic development, mediating various cellular processes like, cell matrix organization, cell adhesion, cell migration, proliferation and also in mitosis. In cell- cell adhesion it takes part in intracellular interactions mediated by cadherin family of proteins, it interacts with filamentous actin and helps in actin mediated cytoskeleton organization. In the G2 phase of cell cycle AJUBA prevents activation of Aurora- A and depletes at the centrosomes in late G2 phase and inhibits mitotic entry[14,15].

In pathological conditions AJUBA is seen to act on systemic malignancies like ESCC[16,17], colorectal cancer[18-21], cervical[22], breast[23,24], prostate cancer[25] etc. by negatively regulating the "Hippo pathway by activation of the Ras- MAPK pathway", it takes part in transcriptional regulation by "Snail- dependent repression of E-cadherin[9]" and helps in epithelial mesenchymal transmission and also known to takes part in response to DNA damage by repressing the ATR- mediated DNA damage response[26].

AJUBA has a multifunctional role in several cancer and act as a key regulator in signaling pathways involved in oncogenesis, tumor growth and promotion. However, its mechanism as tumor suppressor has been studied[27,28] in few cancers also. Thus, AJUBA has a dual role in cancer as a tumor promoter and tumor suppressor. Its upregulation and down regulation are diverse in various cancer and is tissue dependent.

Although, the role of AJUBA protein has been reported in carcinomas, sparse literature is available on its expression in normal oral mucosa and OSCC. Further, its possible role in oral carcinogenesis remains unexplored. Hence the aim of our study is to evaluate the immunoexpression of AJUBA protein in normal mucosa and OSCC.

AIM AND OBJECTIVES

AIM:

Evaluation of Immunoexpression of AJUBA protein in Normal Oral Mucosa and Oral Squamous Cell Carcinoma using Immunohistochemistry.

OBJECTIVES:

1. To assess the immunoexpression of AJUBA in normal oral mucosa and oral squamous cell carcinoma.
2. To compare the immunoexpression of AJUBA in normal oral mucosa and oral squamous cell carcinoma.
3. To associate the immunoexpression of AJUBA in oral squamous cell carcinoma with demographic and clinicopathological parameters.

REVIEW OF LITERATURE

I. ORAL SQUAMOUS CELL CARCINOMA

i. Epidemiology and incidence

Oral Squamous Cell Carcinomas are a group of malignant neoplasms[29] comprising about 10% of HNSCC[30] and are ranked as the 6th most common neoplasms among all the cancers[29] affecting the body. OSCC are associated with uncontrolled cell growth[3,31] and are multi-factorial in origin and aetiology[32]. This disease is found most commonly in communities with low-income and effects older men, 90% being more than 45-year-age group[1]. Males and female ratios are of 2:1 and mean age being 57.1 to 52.5 respectively[1].

OSCC most commonly occurs in the buccal mucosa[33] followed by other sites in oral cavity like tongue, gingiva, lip, floor of mouth and hard palate[3,32]. These lesions clinically appear to proliferate in to ulcerative, papillary, infiltrative or verrucous types[32,33].

ii. Etiology

Use of Tobacco and consumption of alcohol[2], dietary and nutrition factors[34], viral infections[5] etc are few etiological factors responsible for its occurrence[2,5,34]. Consumption of tobacco in a chewable or non-chewable[34] form is considered to be one of the major risk factors in OSCC development[5].

Intake of alcohol acts as a local[2] and systemic risk factor[34] and has a synergistic effect[3] along with areca nut on its etiology. Thus, its effects alone do not account for cancer associated deaths[3]. All these etiological factors dependently or independently[5] damage the host immunity, and also affect the genes by DNA mutation[1] leading to tumor formation and progression of cancer.

iii. Histological parameters and genetic alterations

With all this clinical considerations, certain histological parameters are also assessed which aid in diagnosing the patient survival and prognosis. One such grading was given by Bryne in 1992 and revised in 1998[35]. This grading system includes the analysis of the Histograde present whether WDSCC, MDSCC or PDSCC, depth of invasion, the connective tissue stroma, the inflammation, lympho node metastasis, neural invasion, muscle invasion etc. Thus, continuous revision of this histological features helps us to determine the most efficient method to predict patients' outcome.

Along with this, research has also done various studies on molecules of different family groups, such as, cell-cycle proteins[23], growth factors receptors[36], angiogenic signals[37] etc have identified various pathways like cell cycle[23], HIPPO[38], Myc[39], Notch[7], Nrf2[40], PI-3-Kinase/Akt[41], RAS[42], TGFβ signaling[43], p53[44] and β-catenin/ Wnt[45] which aid in understanding the process and various molecules involved in oral carcinogenesis[46].

In OSCC, alterations of genes (p53 gene, p14, p16, RB1 hypermethylation etc.[44]) in genetic and epigenetic[47] manner offer different predisposing factors. These gene mutations aid in tumor determination, to understand the metastatic potential, treatment and prognosis.

Sequencing of the whole-exome was performed on HPV associated HSCC[12] which identified certain somatically mutated genes. Most of them were located in regions of CAN (copy number alterations). Further, the RNAs were interrogated to check the expression of the mutated alleles. Two were linked to cell cycle and survival[23], CDKN2A[48] and TP53[44], and two were linked to Wnt/b-catenin signaling[45],

FAT1[49] and AJUBA[13], among the four genes that segregated exclusively or were mostly seen.

II. AJUBA PROTEIN

i. Introduction

"AJUBA is a scaffold or an adapter protein[50], present on Human Chromosome 14q11.2[13] encoded by the JUB gene. It is a part of the AJUBA/ Zyxin family[51] of proteins which includes AJUBA, LIM Domain containing protein 1 (LIMD1)[52], Wilms Tumor 1 Interacting Protein (WTIP)[53], Lipoma Preferred Partner (LPP)[54] and Thyroid Hormone Receptor Interacting Protein 6 (TRIP6)[55]."

It is one of the important regulators of many cellular processes[25]. "It is as a key gene which links the multitude of signaling pathways such as HIPPO[38], Notch signaling[7], Wnt[45], SNAG/ SNAIL[9] etc. It acts as a signaling hub and strategically regulates, selectivity and forming cross-talks between signaling pathways[56]."

ii. History

Goyal et al*[13] *in 1998 1st isolated and characterized AJUBA cDNA using a "Yeast two Hybrid screen" and identified four clones of proteins of which two were overlapped partial cDNAs and further on DNA sequencing they found that it contained an "open reading frame" which encoded for two LIM Domains. Further a partial piece of cDNA was used and a single open reading frame of 1638 nucleotides was obtained which encoded for 547 AA proteins of 58kDa[28]. This was designated as AJUBA meaning "Curiosity in Urdu- an Indian dialect".

Further in vivo and in vitro studies[13] were performed for identifying the functional role and significance of AJUBA. These studies, demonstrated that it associates with Grb2[57] which is "an adapter protein that couples signals from activated cell surface growth factor receptors or other activated cytosolic signaling intermediates" and further causes activation of Ras and MAPK[42]. Through this pathway AJUBA helps in meiotic progression within developing Xenopus oocytes.

Thakur et al*[58] *in 2010 studied AJUBA protein in Drosophila Melanogaster i.e., "Fruit flies". and identified dJUB gene "Drosophila AJUBA LIM protein" which is an orthologue (single) of Mammalian AJUBA. In this study it was noted that when dJUB was deleted from the eye and wing of drosophila there was a defect and reduction in the size of these organs, which concluded that dJUB plays a critical and important role in organ size regulation.

Gregory et al*[59] *in 2015 in his review on AJUBA protein suggested that dJUB is an essential gene in the embryonic development in Zebra fish and in the authors hypothesized that in mammals AJUBA protein may also take part in tumor development.

iii. Structure (Figure 1)

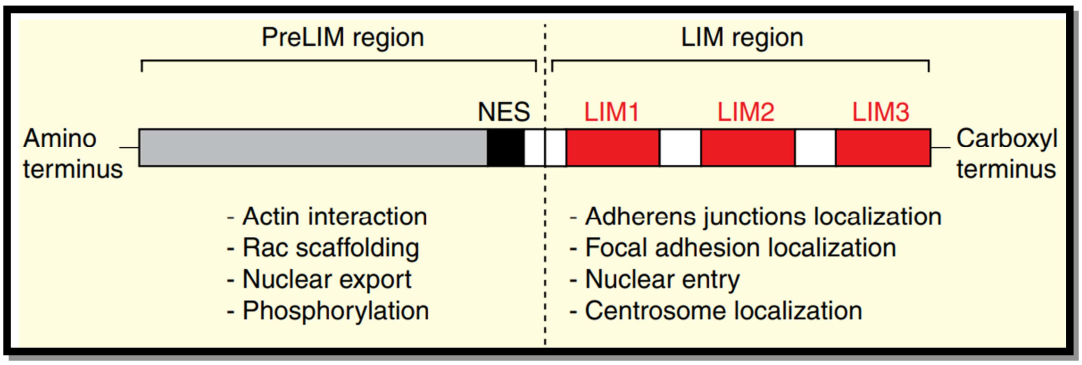


Figure 1: Demonstrates the AJUBA protein structure. Nuclear localization sites (NLS) are depicted d by red color is towards nucleus and nuclear export Sequence depicted by grey color is towards cytoplasm

REF: Schimizzi GV, Longmore GD. Ajuba proteins. Curr Biol. 2015 Jun 1;25(11): R445-6.

The AJUBA is a cytosolic protein and has the ability to shuttle between the nucleus and cytoplasm[13,56]. It is "characterized by the presence of three highly related tandem LIM domains at their carboxyl terminus i.e, the LIM region- directed towards the nucleus[13], and a variable proline-rich amino-terminal i.e, N-terminal non-LIM or the preLIM region[56] towards the cytoplasm".

a. Pre-LIM region[13]

The two third of pre-LIM region of AJUBA protein is rich with proline and glycine and has a consensus with SH3 recognition sites. Also, there is a NES area present in the pre-LIM region which has a similar function like another AJUBA family protein Zyxin. The NES maintains the protein concentration in the cytoplasm by eliminating the expressed protein there by exporting nuclear proteins to cytosol similar to zyxin protein.

b. LIM DOMAINS[13]

Discovered 25yrs ago the "LIM domains are cysteine and histidine1[13,42] rich double zinc-finger domains found in nuclear homeobox transcription factors LIN-11[60], Isl1[61], and MEC3[62], which govern cell destiny during development". Protein–protein interaction domains, such as SH2 and SH3 domains[63], are now regarded as being as frequent as common protein-interaction motifs.

Because LIM domains are modular and many proteins have several LIM domains, they can interact with a wide range of proteins in different locations[23] (cell surface, actin cytoskeleton, cytosol, and nucleus). "LIM domains can also bind to other proteins LIM domains, because there is no consensus LIM domain recognition sequence in proteins, these domains are thought to recognize a common structural property of proteins".

LIM domains have been divided into three groups.

- LIM1

"LIM domains are connected to a homeodomain and a putative transcription activation domain in LIM 1 proteins. LIN-11[60], Isl1[61] and MEC3[62] are three of the original LIM proteins, and they represent a developing set of nuclear transcription factors involved in determination of cell fate[64] and cellular differentiation."

- LIM2

"They are LIM-only (LMO) proteins, meaning they only have one to five LIM domains and no other structural or functional features. These proteins can be nuclear, cytosolic or both."

- LIM3

"LIM 3 proteins contain three to four tandem LIM domains at the C terminus in association with distinct N-terminal domains. Members of this group include zyxin[51], Paxillin[65], LPP[54], TRIP6[55] etc. these proteins can sometimes form a group of four."

III. LOCALIZATION OF AJUBA IN HUMAN CELL[66,67] (Figure 2)

As AJUBA is a cytosolic protein, but has the ability to shuttle between the nucleus and cytoplasm[56] and thus it is found in various cellular components such as in cytoplasm, membrane, at sites of focal adhesion, cell-cell junctions, cytoskeleton, nucleus, golgi in cytosol, plasma membrane, etc. Through distinct interactions[13] interacts with the LIM and pre- LIM regions AJUBA has the ability to couple with various signalling pathways.

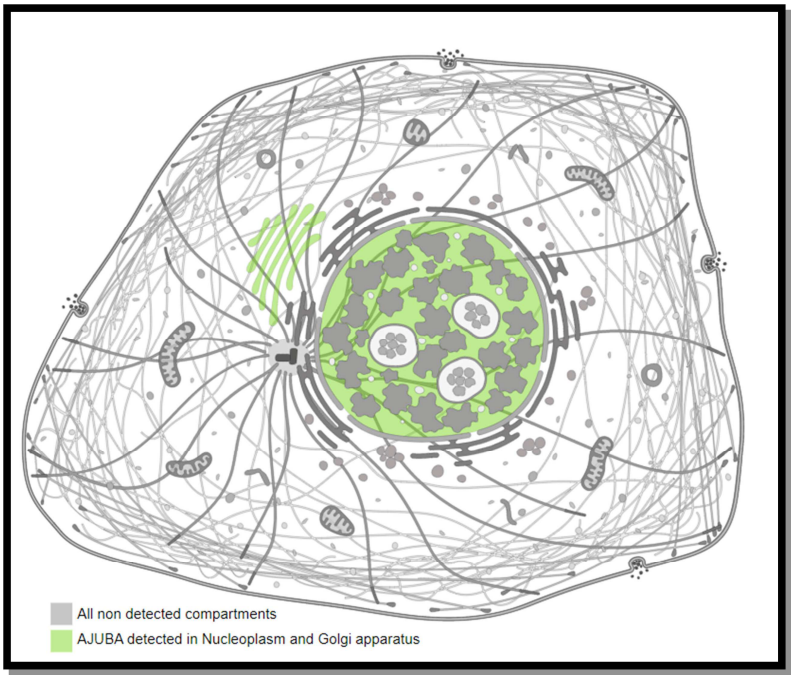


Figure 2: Demonstrating subcellular location of AJUBA, Localized to the Nucleoplasm and Golgi apparatus
REF: Human protein atlas. https://www.proteinatlas.org/ENSG00000129474-AJUBA/tissue

IV. AJUBA ROLE IN PHYSIOLOGICAL FUNCTIONS-

AJUBA is a multifunctional protein that takes part in development of embryo[64], various physiological and also in pathological conditions. It helps in the transduction of external signals and is involved in several cellular and in various biological processes. There are more than 25 biological functions where AJUBA protein acts, few among them include cell-cell adhesion, cytoskeleton organisation[45] and localisation of cellular proteins[23]. Differentiation and proliferation of cell[9] is also controlled by AJUBA protein. Apart from these major roles it is identified as a migration growth factor and as a negative regulator of HIPPO signaling pathway[58].

i. EMBRYONIC ROLE OF AJUBA PROTEIN

Development of an organism is a crucial process. It occurs by proliferation and differentiation at various cellular and subcellular[68] sites of all three germ layers in the Embryo.

Jyotshnabala Kanungo et al*[64] *in 2000, performed various tests including cell culture, cell transfection etc. on embryonic cell lined to identify the role of AJUBA protein in them. The study results suggested that AJUBA protein was expressed during the early murine fetal development stages and was seen in all the three germ layers and also in placenta.

Another study authors showed the presence of AJUBA protein[56] in different stratification layers of the epidermis during the murine development[69].

In a study by ***Petit et al*[54] *in 2000*** it was noted that AJUBA does not localize at focal adhesion sites but was found at sites of cell-cell adhesion and in absence of the cell-cell adhesion it was found in the cytoplasm. It was also observed that when

AJUBA was accumulated in the nucleus of the P19 embryonic cells it resulted in the inhibition of proliferation and altered the fate of the cell. This feature was only specific to AJUBA protein in the LIM domain family. It was also noted that AJUBA affects the activity of the MAPK enzymes and also increases the JNK- pathway activity.

Alexander W. Lange et al*[8] *in 2014 conducted a study on the embryonic lung to show the proliferation and differentiation of the epithelial cells by using both embryonic and adult lung cell lines. The study concluded that during maturation and morphogenesis of the lung Mst1/2 and YAP play important role. When Mst1/2 was deleted or YAP was activated, AJUBA was upregulated. Thus, AJUBA arbitrates the effects of YAP on tissue organisation and cell proliferation.

All the above-mentioned studies[8,54,56,64,69] concluded that AJUBA's intracellular trafficking in embryonal cells has functional effects on cell proliferation and differentiation decisions, unlike the other LIM domain family proteins, and may provide a new approach to understand its function.

ii. CELL - SURFACE AND CYTOPLASMIC FUNCTIONS OF AJUBA PROTEIN

In the cytoplasm AJUBA takes part in cell-cell adhesion, cytoskeleton organization, cell growth and differentiation etc. which are mediated by various signaling molecules

a. Cell-cell adhesion and cytoskeleton organisation

Cell adhesion[70] is defined as a, “process by which cells interact and attach to neighbouring cells through specialised molecules of the cell surface. This process can

occur either through direct contact between cell surfaces such as cell junctions or indirect interaction." This is primarily done by intracellular interactions mediated by the catenin and cadherin protein families. These proteins are weakly associated to actin filaments. AJUBA acts as a significant protein which links the adherence junctions to actin cytoskeleton[14] thereby, enabling mechano-transduction.

Helene Marie et al.[14] ***in 2003*** conducted a study on AJUBA protein in human keratinocytes to understand its functional role in cell junctions and cytoskeleton organisation. The study concluded AJUBA is recruited to cadherin-dependent cell adhesion complexes in primary epithelial cells in a controlled way, according to the researchers. At the amino terminal of AJUBA, it acts on the membrane of E-cadherin which is bound to α-catenin.

In the same study they also observed that AJUBA primarily co-localizes with cadherin adhesion complexes during cell contacts, but not at focal adhesions. It directly interacts with filamentous actin and contributes to bridging of the cadherin adhesive complexes to the actin cytoskeleton[14]. One of the important observations in the study was that adhesion was abnormal in AJUBA Null Primary Keratinocytes. Their results indicate that AJUBA aids in the formation of cell-cell junctions, the maintenance of newly formed cell junctions, or both.

In addition to catenin, they also found that in in vitro cell extracts, AJUBA interacts with F-actin directly. As a result of AJUBA's association with F- actin and catenin at cadherin adhesive complexes, it aids cytoskeleton organization.

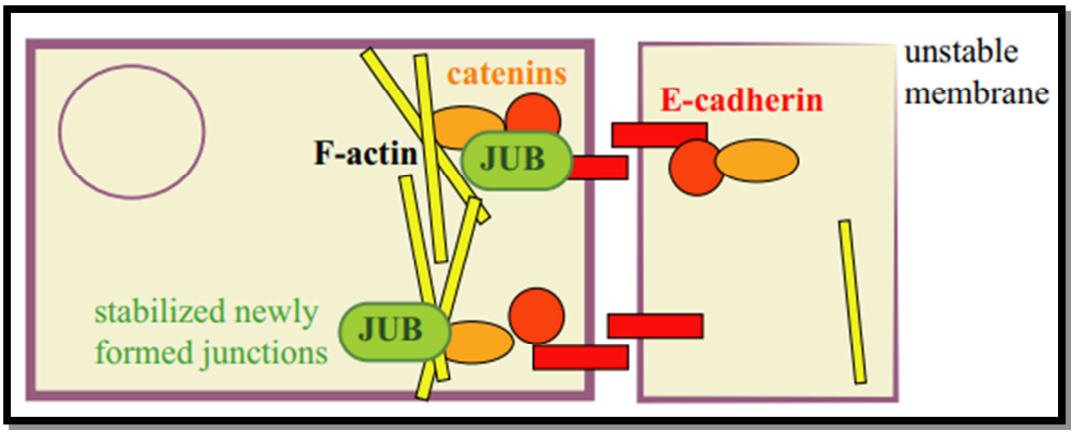


Figure 3: Demonstrating AJUBA protein in cytoskeleton organization
REF: Schleicher K, Schramek D. AJUBA: A regulator of epidermal homeostasis and cancer. Exp Dermatol. 2021 04;30(4):546-59.

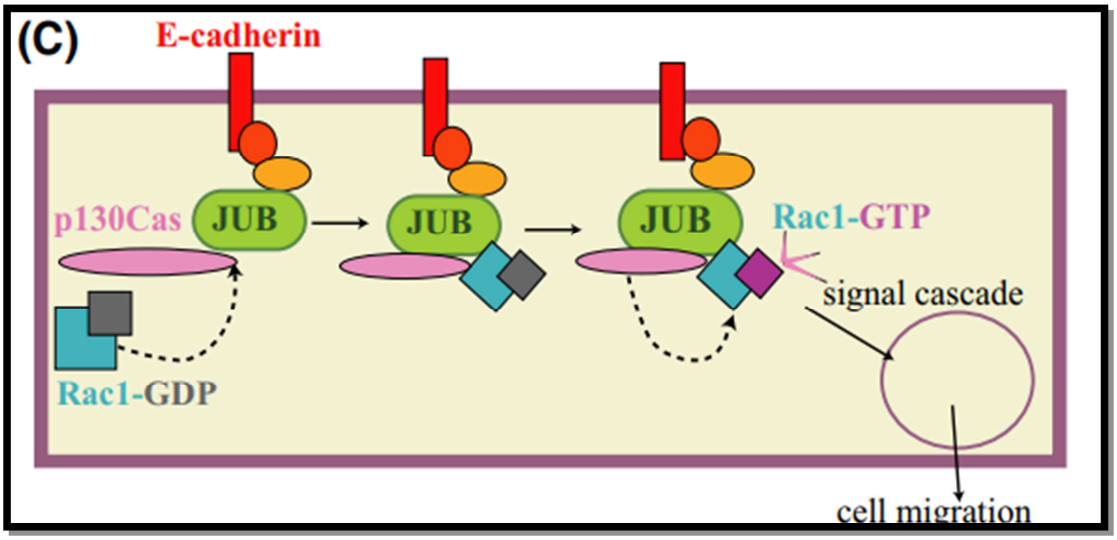


Figure 4: Demonstrating AJUBA protein in cell cell adhesion and communication
REF: Schleicher K, Schramek D. AJUBA: A regulator of epidermal homeostasis and cancer. Exp Dermatol. 2021 04;30(4):546-59.

b. Cell growth and differentiation

Growth of a cell[71] and its differentiation are biological processes which refers to an increase in the cell structure including all its organelles and transformation of a one cell type to another cell type respectively[72,73]. AJUBA is also expressed in human tissues during adulthood[56]. It is abundantly seen in skin and it maintains adult epidermal homeostasis[74].

It affects the cellular differentiation through Wnt pathway[45], regulates basal cell proliferation in keratinocytes through HIPPO signaling pathway[58] and promotes kinase-dependent mitotic[15] commitment.

c. Wnt pathway

The Wnt site pathway is an important pathway which plays a role in various developmental process and takes parts in cellular differentiation[45]. This pathway is a termed as the "Hallmark pathway for stabilization and nuclear location of β- catenin in cells"[6].

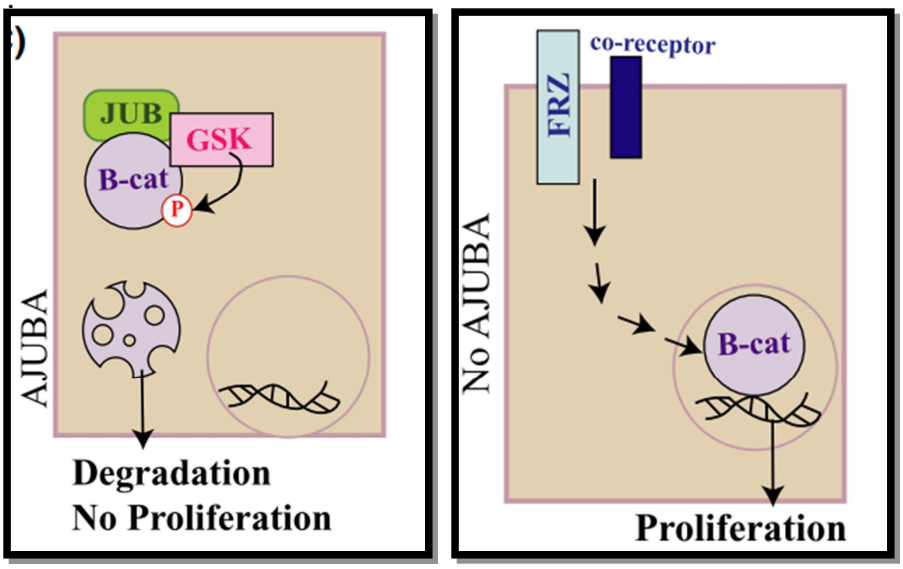


Figure 5: Demonstrating AJUBA in cell proliferation via Wnt pathway
REF: Schleicher K, Schramek D. AJUBA: A regulator of epidermal homeostasis and cancer. Exp Dermatol. 2021 04;30(4):546-59.

In a study conducted by ***Haraguchi et al.***[75] ***in 2007*** on Wnt pathway, it was noted that AJUBA protein negatively regulated this pathway. AJUBA interacts with b-catenin which resulted in phosphorylation of GSK-3b. This process intern causes B-catenin phosphorylation and degradation. Along with this it was noted that as the b-catenin was suppressed, these was a suppression of Cyclin D1 gene (regulator of cell growth in Wnt pathway).

It was also observed that when AJUBA protein was absent there was a decrease of cell in the G0/G1 phase, whereas in S-phase the cell population increased. Thus, suggesting that it plays a salient role in cell differentiation and growth by the inhibition of the Wnt-signaling pathway.

d. HIPPO pathway

The HIPPO pathway[76] in animals controls the size of an organ via cell proliferation regulation and apoptosis. This is also called as the "Salvador-Warts-HIPPO (SWH) pathway". This pathway takes part in proliferation as well as apoptosis, molecules involved in this pathway are LATS, MST, YAP, YORKIE, SAV etc which take part in determining the size of an organ.

Barry M. Gumbiner et al.[77] ***in 2014*** studied the HIPPO pathway and Zyxin family proteins to understand its association in growth. During growth of the cell various growth factors are expressed e.g., EGF, IGF ETC. when these growth factors are absent in the HIPPO pathway a complex is formed by the PDK1 along with the AJUBA family proteins like LATS, LIMD1 etc. this causes activation of the HIPPO signalling. But in the cytoplasm of the cell the LATS are phosphorylated by MST, further causing phosphorylation of YAP (yes associated protein). Thus, entry of YAP in the nucleus does not occur and the growth of cell is arrested.

When HIPPO is on

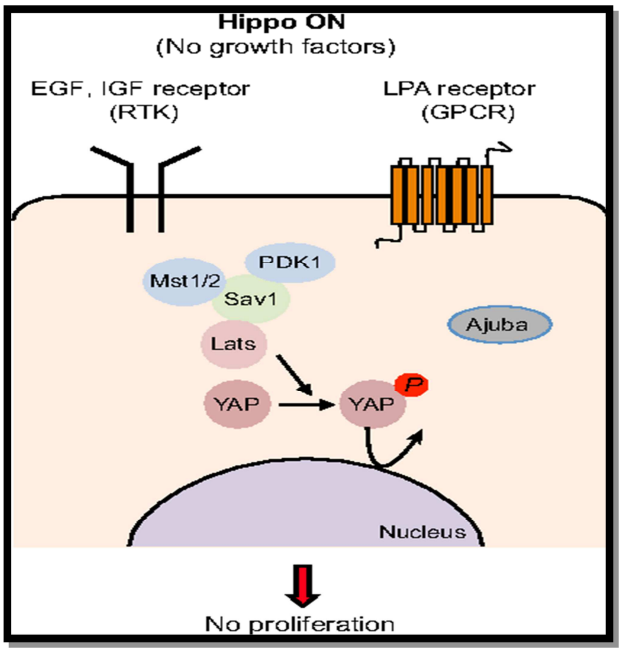


Figure 6: Demonstrating AJUBA in Hippo pathway(on)

REF: Gumbiner BM, Kim N G. The HIPPO-YAP signaling pathway and contact inhibition of growth. J Cell Sci. 2014 Feb 15;127(Pt 4):709-17.

iii. NUCLEAR FUNTIONS OF AJUBA PROTEIN

AJUBA with the ability of shuttling takes part in various signalling pathways in the nucleus such as Mitosis, transcriptional repression, EMT. It also maintains the stratification of epidermis by NOTCH signalling pathway[7].

a. Mitosis

Mitosis[78] is defined as a, "Part of the cell cycle in which replicated chromosomes are separated into two new nuclei" and Meiosis[79] is a "a special type of cell division of germ cells in sexually-reproducing organisms used to produce the gametes" these processes are carried out by certain molecules and signally pathways. One such is Aurora- A kinase.

Aurora A kinase is a "centrosome-localized serine/threonine kinase", it takes part in mitosis and meiosis process of the cell cycle[18] i.e, in the late G2 phase. Here cyclin B1–Cdk1 complex is recruited by Aurora to the centrosomes, which causes mitotic entry[80].

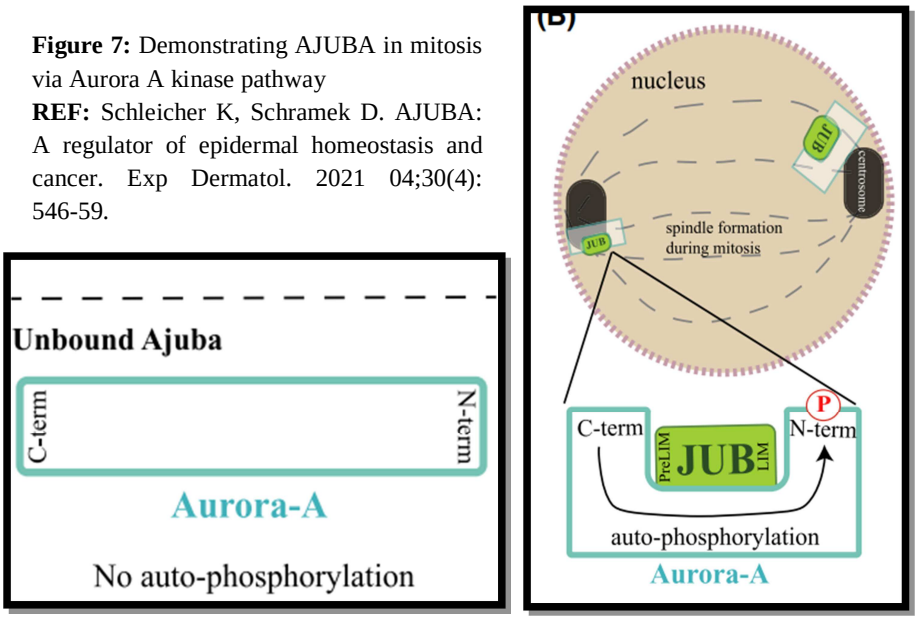


Figure 7: Demonstrating AJUBA in mitosis via Aurora A kinase pathway
REF: Schleicher K, Schramek D. AJUBA: A regulator of epidermal homeostasis and cancer. Exp Dermatol. 2021 04;30(4): 546-59.

Meirong bai et al*[15] *in 2014 conducted a study to understand the novel regulatory function of AJUBA protein on aurora-A kinase and to know its significant role in cell cycle process. This study suggested that aurora regulated certain functions like assembling of the bipolar spindle, segregation of chromosomes etc. it was also noted that excess entry of aurora A kinase during the G2 phase caused instability in the genomic sequence. The study concluded that when AJUBA interacted with aurora-A in mitosis, it caused autophosphorylation of Aurora. Thus, understanding a newer mechanism of Aurora-A via its action with AJUBA.

b. Transcriptional corepressors in EMT as SNAIL corepressors

EMT plays a major fundamental role in different biological processes like embryogenic development, wound healing etc. In this process the epithelial cells which are immotile attain filopodia and thus giving rise to mesenchymal cells which migrate[81].

Zhaoyuan Hou et al.[9] ***in 2008*** assembled Snail1 repressor complex initiates an epithelial–mesenchymal transition molecule that governs neural crest development in Xenopus by linking Snail1 (through the LIM area) with the chromatin-remodeling enzyme PRMT5 (preLIM region).

AJUBA binds retinoic acid signalling to Isl1 in developing zebrafish hearts to suppress Isl1 transcriptional activity and limit progenitor cell specification and proliferation. Retinoic acid signals also boost AJUBA's expression and nuclear entrance.

Zhaoyuan Hou et al[82] ***in 2010***, studied Retinoic Acid (RA) signalling and demonstrated that AJUBA modulates and negatively regulates RA signalling and probably acts as a classic corepressor. Nonetheless, the discovery of AJUBA as a corepressor for a subset of nuclear receptors gives light on a method requiring LIM-domain containing proteins for nuclear receptor-mediated repression. The researchers suggested that investigating AJUBA's corepressor activity could lead to the development of more effective cancer treatments.

c. NOTCH PATHWAY

The formation and maintenance of distinct squamous layers is controlled by Notch signalling[7]. “NOTCH receptors are transmembrane proteins” that are cleaved

by proteases when they interact to the "JAG1/2 or DLL1/3/4 ligands". The NICD is cleaved and enters into the nucleus, where it interacts to the "DNA-binding protein RBPJ" and controls gene expression. In primary mouse keratinocytes, AJUBA was recently demonstrated to directly bind "NOTCH1/2, NICD1/2, and NUMB[83], a negative regulator of Notch signaling[7]."

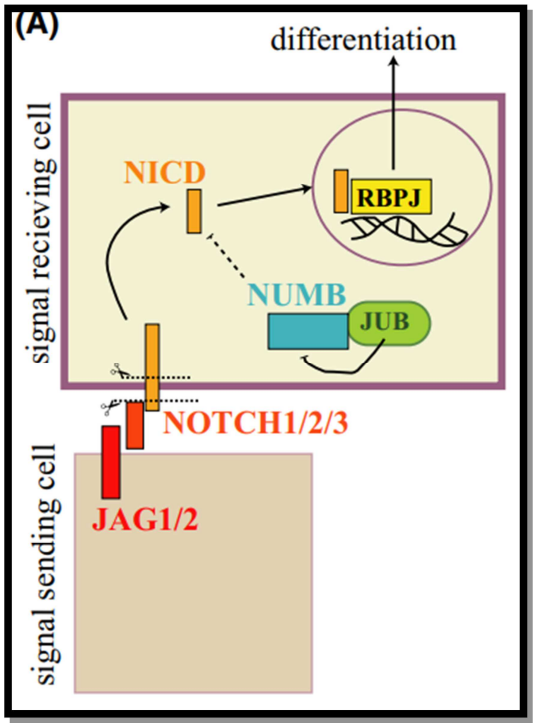


Figure 8: Demonstrating AJUBA in cell differentiation via NOTCH pathway
REF: Schleicher K, Schramek D. AJUBA: A regulator of epidermal homeostasis and cancer. Exp Dermatol. 2021 04;30(4):546-59.

Sequestering of NUMB is promoted by AJUBA, preventing NUMB's interaction with NOTCH1/NICD and NUMB- mediated recruitment occurs[83]. Both of which are necessary for NOTCH/proteasomal NICD's destruction[84]. AJUBA mutant keratinocytes demonstrated decreased nuclear translocation of NICD1/2 and transcription of canonical NOTCH downstream targets after ligand-induced NOTCH activation.

These findings show that AJUBA allows cells to differentiate by sequestering NUMB and allowing transcriptional targets of NOTCH to be activated. The lack of correlation between NOTCH, Hippo, and WNT signalling could be linked to AJUBA control, and it would be fascinating to investigate cross-talk between AJUBA[13], NOTCH[7], HIPPO[58] and WNT[45], particularly in cellular decision-making during epidermal stratification.

V. ROLE IN PATHOLOGICAL FUNCTIONS-

Carcinogenesis or cancer formation is a multi-step process. In this process the normal cells undergo cellular and nuclear alterations. These are caused due to mutations in various genetic signaling molecules and pathways. As a result, cell division becomes uncontrolled. AJUBA a LIM protein is seen to take part in various cancers via pathways like HIPPO, EMT, ATR etc.

i. HIPPO pathway

HIPPO pathway is important in "tissue generation and repair in response to injury and repair in adult organism and its deregulation appears to contribute for both tumor development and suppression"[22]. Cancer cells are often thought to have lost contact inhibition of growth, and can proliferate despite the normal limitations of the tissue structure they reside in. The HIPPO pathway, which mediates contact inhibition may be dysregulated in cancer cells, enabling differentiated cells to revert to a more embryonic and/or developmental stage, as well as acquiring additional embryonic cell qualities such as enhanced cell motility.[22,38,58,77]

When HIPPO off

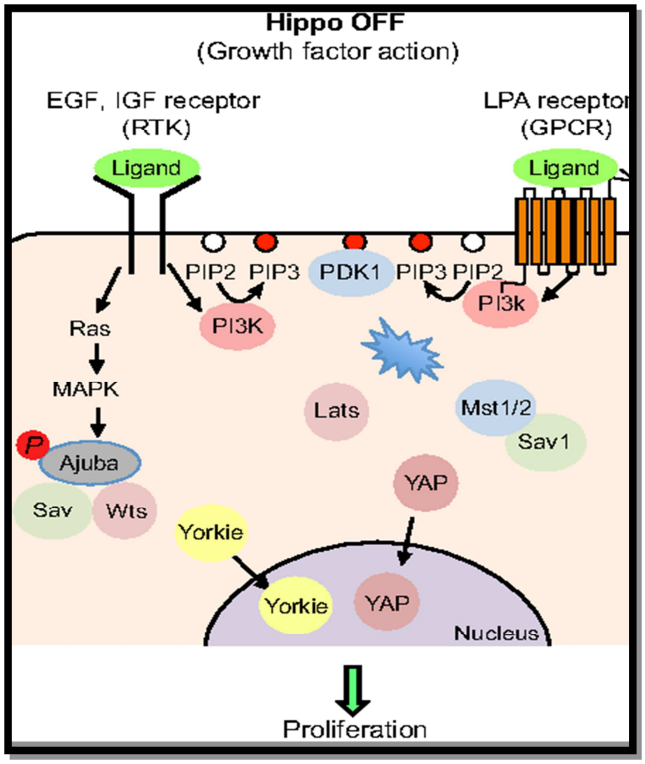


Figure 9: Demonstrating AJUBA in Hippo pathway(off)

REF: Gumbiner BM, Kim NG. The HIPPO-YAP signaling pathway and contact inhibition of growth. J Cell Sci. 2014 Feb 15;127(Pt 4):709-17.

EGF signalling activates the Ras-MAPK pathway, studied ***Barry M. Gumbiner et al.***[77] ***in 2014*** which phosphorylates AJUBA. This inhibits the action of the Sav-Wts complex, resulting in Yorkie dephosphorylation, nucleoplasm accumulation, and enhanced cell proliferation. Growth factors (EGF, LPA, or serum) can activate PI3K18 and attract PDK1 to the membrane, causing the PDK1–HIPPO complex to dissociate. As a result, Mst's regulation of Lats is disrupted, resulting in YAP accumulation in the nucleus and cell proliferation.

Reddy et al*[42] *in 2013 studied HIPPO signaling and EGFR in drosophila. The study concluded that EGFR activated a transcription factor Yorkie, which is required for EGFR based cell proliferation in Drosophila in the Ras-MAPK signaling. AJUBA a LIM protein causes phosphorylation and enhances EGFR-Ras-MAPK. Their observation implicated that HIPPO pathway which is mediated by EGFR-RAS-MAPK signaling contributes to tumorigenesis and also has been identified that there is a molecular link between these pathways. Thus, they also demonstrated that AJUBA is a "key target of MAPK signaling within the HIPPO pathway". Thereby, it negatively regulates the HIPPO pathway.

ii. Transcriptional regulation-EMT pathway

EMT is the first step in "tumour metastasis, during which cancer cells develop a mesenchymal phenotype, spindle-like shape, high motility, and invasiveness"[85]. EMT is characterized by the downregulation of E-cadherin. Downregulation of E-cadherin is mostly caused by transcriptional control of the gene[9,85]. Several transcription factors have been found to inhibit E-cadherin expression, with Snail being the first to be discovered. SNAIL causes EMT in mammalian cells, at least in part, by suppressing the E-cadherin gene[9], which alters cell adhesion. Snail has reported to be frequently up regulated in various carcinomas. AJUBA protein interact with SNUG domain of the SNAIL family and thus act as a corepressor in regulating EMT in cancer and promoting metastasis.

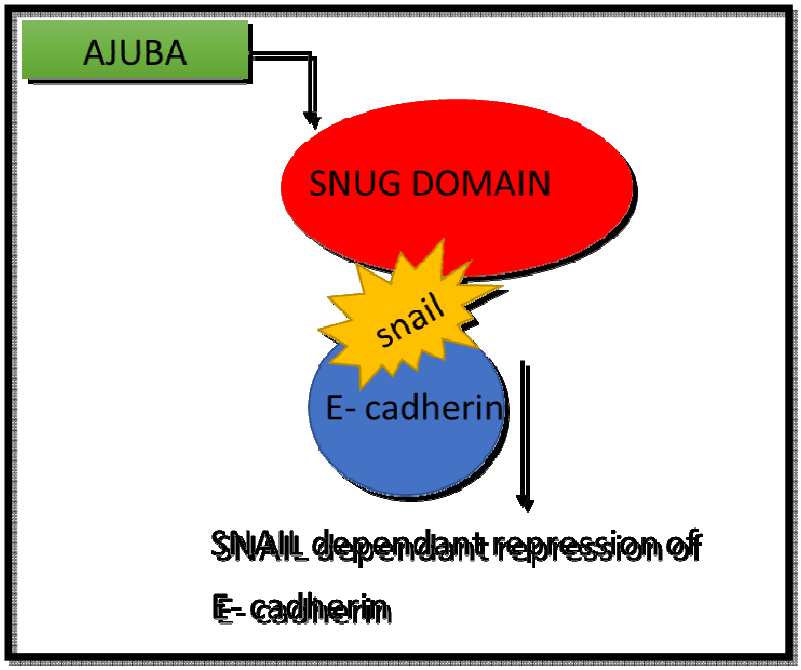


Figure 10: Demonstrating AJUBA in SNAIL dependent transcription repression

Zhaoyuan Hou et al*[9] *in 2008 conducted a study to understand the transcriptional repression by the action of AJUBA and SNAIL. The study concluded that The AJUBA family of LIM proteins functions as functional Snail corepressors by interacting with the SNAG domain. It interacts with Snail on endogenous E-cadherin promoters in the nucleus, contributing to Snail-dependent E-cadherin suppression. PRMT5 is a key component of the SNAIL-silencing complex through binding to AJUBA.

Because of their functional association with Snail proteins in the nucleus, AJUBA LIM proteins appear to be essential regulators of epithelia dynamics, bridging the gap between surface and nuclear responses. Thus, repression of E-cadherin takes place and thus helps in EMT.

iii. Response to DNA damage-

Endogenous and exogenous chemicals can cause DNA damage, which can lead to genomic instability, which is a cause of early human tumorigenesis[86]. Cells have specific checkpoints that detect damaged or irregularly organised DNA and allow repair mechanisms or apoptosis to be activated[87]. Checkpoints check for DNA lesions at different times in the cell cycle and work to delay transitions from G1 to S phase and G2 to M phase[88].

A DNA damage response[86] is elicited by the cell in attempt to counteract and repair DNA damage (DDR). ATM, ATR, and DNA-PK are signalling kinases that belong to the PIKK family. These DDRs are structured pathways that include damage detection, damage signal transduction, and activation and recruitment of repair proteins to damaged sites[86,26].

AJUBA and similar compounds may have carcinogenic capabilities during the early stages of cellular transformation by decreasing the protective or tumour suppressive activities of ATR[26].

In a study conducted by ***Sampada kalan et al.***[26] ***in 2013*** demonstrated that ATR-mediated DNA Damage Response is repressed by the AJUBA protein. Depletion of AJUBA resulted in cell cycle delays, as well as enhanced Rb phosphorylation and Chk1 phosphorylation. AJUBA was identified in a complex with RPA, and its absence resulted in RPA phosphorylation, which is known to be a key step in ATR activation. AJUBA and related compounds have a broader impact in that they have oncogenic qualities at the early stages of cellular transformation by blocking ATR's protective or tumour suppressive activities.

In carcinogenesis AJUBA protein- Acts like a double-edged sword. Studies on HNSCC, ESCC, cervical cancer, colorectal cancer etc. have been done regarding its expression suggest the dual role i.e., promoter and suppressor of tumor by Its action on HIPPO pathway as its negatively regulator, it takes part in transcriptional regulation by Snail- dependent repression of E- cadherin and helps in epithelial mesenchymal transmission. AJUBA also represses the ATR- mediated DDR.

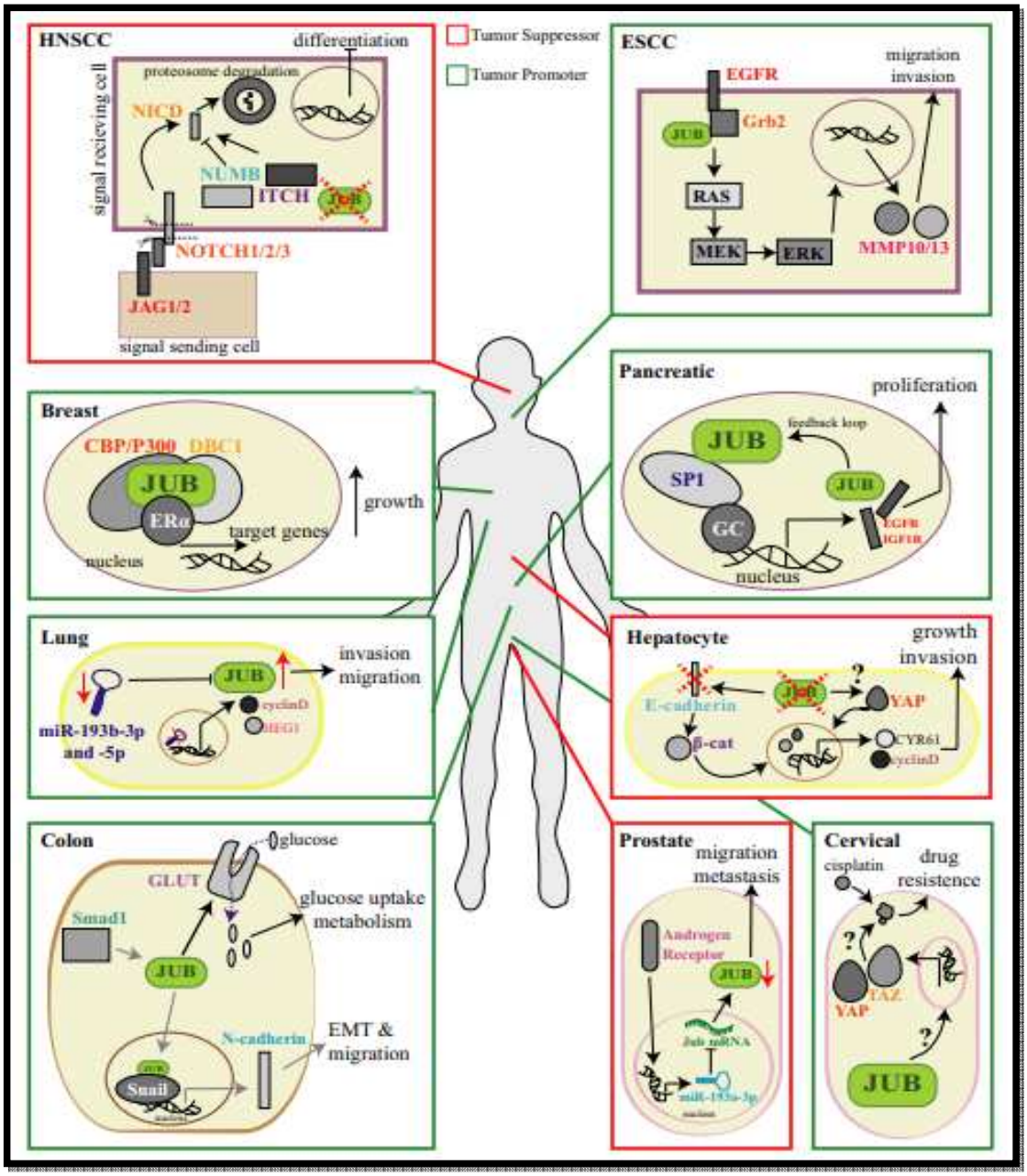


Figure 11: Demonstrating the dual role of AJUBA- Tumor promoter and suppressor
REF: Schleicher K, Schramek D. AJUBA: A regulator of epidermal homeostasis and cancer. Exp Dermatol. 2021 04;30(4):546-59.

VI. AJUBA AS A TUMOR PROMOTER

The cancer Genomic atlas[44] ***in 2015***, which profiled genomic alterations in 279 HNSCC cases, suggested mutation of a gene involved in the WNT pathway i.e, AJUBA. The analysis of this genomic report concluded that AJUBA inactivation may converge to uncheck WNT signaling, implicated in deregulation of cell polarity and differentiation. These findings provide a new insight into HNSCC and suggest that shared and unique alterations might be leveraged to accelerate progress in prevention and therapy across tumour types.

Xu et al.[24] ***in 2019*** in his study used T47D cell lines and reported that AJUBA acted as a co-activator of estrogen in breast carcinoma. The high expression of AJUBA suggested that it took part in the activation of estrogen receptor alpha, thereby increasing the levels of TFF1, SGK3 and Greb1. The high levels of these molecules promoted growth of the tumor and lead to resistance of tamoxifen. Also, when the AJUBA expression was low there was decrease in drug toxicity. Thus, AJUBA acts as a breast carcinoma promoter and its drug resistant action on tamoxifen suggests a newer therapeutic approach.

Lihong Bi et al.[22] ***in 2017***. studied HIPPO pathway in cervical cancer cell lines and its association with cisplatin resistance. mRNA levels of AJUBA were elevated in tumor than the adjacent tissue which was confirmed by RT-PCR. Overexpression of AJUBA was noted in patients with cervical cancer. They also showed cisplatin resistance when compared to patients sensitive to cisplatin. The overall survival was reduced when AJUBA expression was high. They concluded that in cervical cancer is enhanced with increased AJUBA levels.

Xing-Hua Liang et al.[19]***in 2014*** studied EMT in colon cancer in a pSin-EF2-puro retroviral vector, cancer cell lines etc. The study results suggested that an upregulation was noted in cell lines and tissues of AJUBA and its family proteins. This suggested that SNAIL by acting as a corepressor of AJUBA aids in EMT and increases the ability of tumor cells motility and invasiveness. Their study indicated for metastatic CRC and concluded that AJUBA is a novel target.

Yang et al.[20] ***in 2017*** studied colorectal adenocarcinoma with cell lines of human cancer colon i.e HCT116 etc. It was noted that AJUBA was promoted along with SNAIL by Smad1 which is a TGF-β receptor in cell- cell adhesion sites by acting on the cadherins i.e E-cadherin. The study concluded that AJUBA and Smad1 newer therapeutic targets for assessing the prognostic factors of CRC.

Dommann et al.[89] ***in 2020*** in colon cancer used lentiviral constructs to knockdown or overexpress Ajuba protein in SW480 human colon cancer cells. RNA sequencing was used to examine the transcriptome of the transformed cell lines. The study suggested that cells lacking Ajuba were less proliferative, more sensitive to irradiation, moved less, and were less efficient in colony formation. The study concluded that Ajuba increases colon cancer development, migration, and metastasis, and hence could be a marker for targeted therapy**.**

Wang et al.[21] ***in 2020*** studied colon cancer using circulating RNA and AJUBA. It was noted that both highly up-regulated in colorectal cancer tissues, while miR-1184 was significantly down-regulated relative to healthy tissues. Also, circ 0128846 was found to contribute to the formation of CRC by lowering miR-1184 expression, boosting AJUBA expression, and inactivating Hippo/YAP signalling pathway leading to tumor promotion.

Li et al [90]***in 2019*** studied gastric cancer and noted that AJUBA was overexpressed and siRNA knocked down in the SGC-7901 and NCI-N87 cell lines, respectively. When Ajuba was overexpression, it increased the rate of colony formation and proliferation rate, but siRNA (AJUBA) suppressed this action, according to MTT and colony formation assays. According to the findings, AJUBA playing a role as a potential therapeutic target in gastric cancer progression via the YAP-GLUT1/Bcl-xL axis.

Shi et al.[16] ***in 2016*** studied AJUBA protein in esophageal squamous cell carcinoma in cells lines via Western blot and RT-PCR and IHC via RNA microarray. AJUBA was highly expressed (IHC) in ESCC with statistically significant results. Also in vivo & in vitro studies of AJUBA showed that it took part in promoting growth of the cell, as well as invasion and migration. They also concluded that two downstream targets of AJUBA are MMP10 & 13. Thus, acting as an oncogene and a new target in ESCC therapy.

Qing Zhang et al.[17] ***in 2018*** studied AJUBA, MMP14 and YAP in ESCC. The study results concluded that an increased immunoexpression of AJUBA was seen in cancer tissue than compared to the adjacent. These results were statistically significant with the TNM stage and lymph node metastasis. Thus, AJUBA along with MMP14 and YAP1 can act as an oncoproteins. Further as a novel cancer targeted therapy in ESCC.

Bosen Zhang et al.[91] ***in 2019*** studied the molecular mechanism of transcription factor SP1 and AJUBA in cell lines and human Pancreatic ductal adenocarcinoma tissue. As, AJUBA shuttles in the nucleus and cytoplasm, it takes part in various cellular processes. The study results concluded that AJUBA is a target

gene of SP1 and this forms a complex at one of the functional tandems. Thus, AJUBA acts as a co-activator of SP1 and further SP1 gene undergoes transcription and there-by takes part in tumor cell proliferation and an increase was corelated to poor prognosis and decreased survival.

Xiaofeng Yao et al[92] ***in 2019*** studied AJUBA in OSCC by PCR and IHC methods, also they studied AJUBA in Snail pathway by western blotting methods. It was noted that in OSCC expression of AJUBA was high in contrast to the adjacent normal oral mucosa. The study results were correlated to various clinicopathologic parameters and it was found significant with T stage, recurrence etc. the study concluded that increased immunoexpression of AJUBA in OSCC and may influence cell invasion and proliferation via the Snail/E-cadherin pathway.

Le et al.[84] ***in 2018*** discovered that AJUBA expression was elevated in HCC (Hepatocellular Carcinoma) compared to normal tissue. According to the findings, patients with high AJUBA expression had a bad prognosis. Furthermore, re-expression of AJUBA in AJUBA-deficient cells may be able to restore the AJUBA-deficient cells' phenotype. The authors showed that AJUBA is elevated in HCC and enhances HCC cell proliferation and migration and finally suggested that AJUBA could be a potential target for HCC detection and treatment.

VII. AJUBA AS A TUMOR SUPPRESSOR

Loganathan et al[7] in 2020 studied mutations in 484 “Long tail” genes in HNSCC via the NOTCH signaling pathway and suggested that 7% cases of HNSCC were mutated by AJUBA, whereas 18% and 7% were mutated in cutaneous SCC and ESCC respectively. All these mutations were associated with tumor suppressor activity of the AJUBA gene.

Li Jia et al.[25] ***in 2017*** conducted a study in LNCaP and C4-2B cells and observed an increase in migration and metastasis of Pc (Prostate cancer) when AJUBA protein was downregulated. Also, mRNA studies showed that there was a low expression of AJUBA in PC. PCa progression is linked to a new "AR/miR-193a-3p/AJUBA pathway". MiR-193a-3p has been identified as a possible therapeutic target for patients with metastatic PCa.

I Tanaka et al.[27] ***in 2015*** in the HIPPO pathway studied malignant mesothelioma. Frequent activation of the YAP was noted in MMs. This led to dysfunction of HIPPO pathway and in turn caused malignant transformation of cells. IHC studies in MMs suggested that AJUBA was frequently downregulated. AJUBA inactivation in MM, as well as its tumor-suppressive activity linked to the Hippo signalling system. Despite the fact that most MM cells display inactivation of the Hippo pathway, resulting in constitutive activation of YAP, new therapy techniques targeting this pathway may possibly be developed to cure patients with this very aggressive cancer.

Du et al.[28] ***in 2017*** conducted a thorough genetic investigation on the largest ESCC cohort. 18 Significantly mutated genes, mutational signatures were found in ESCC cases. Poor survival and prognosis of ESCC was corelated to mutations of AJUBA gene leading to dysfunction of WNT, NOTCH, AKT pathways. Thus, the study concluded these investigations could lead to an improvement in the treatment and prognosis of ESCC patients.

VIII. NEED FOR STUDY

Although, the role of AJUBA protein has been reported in various other malignant tumors, sparse literature is available on its expression in normal oral mucosa and OSCC. Further, its possible role in oral carcinogenesis remains unexplored. Hence the aim of our study is to evaluate the immunoexpression of AJUBA protein in normal mucosa and OSCC.

MATERIALS AND METHODOLOGY

ETHICAL APPROVAL

Ethical approval for the study was taken from institutional ethical review committee. Ethical clearance number: 1322 **(Annexure I)**

TISSUE SAMPLE

The retrospective study used 42 paraffin embedded tissue blocks of clinically and histologically proven cases of normal oral mucosa and oral squamous cell carcinoma. The cases used in the study were retrieved from the Archives of Department of Oral and Maxillofacial Pathology and Oral Microbiology, KLE Academy of higher education and research (KAHER)'s VK Institute of Dental Sciences, Belagavi where the tissue has been stored in the form of paraffin embedded blocks which were then subjected to immunohistochemical analysis. For control, normal human oesophagus was taken.

Three tissue sections of 4 µm each were cut from each block and taken onto "amino propyl triethoxysilane (APES)" coated slides **(Annexure IV)**. One slide was stained with Hematoxylin and eosin **(Annexure V)**.While the other slides were stained immunohistochemically using Anti- AJUBA antibody.

SAMPLE SIZE ESTIMATION:

$N = \frac{2pq(Z\alpha+Z\beta)^2}{}$ $p = \frac{p_1+p_2}{2}$ $p_1 = 60\%$ $q_1 = 100 - p_1$

$p_2 = 30\%$

$q = 100-p$ $q_2 = 100 - p_2$

N= 42 $d = p_1 - p_2$

$Z\alpha = 1.96$ at 5% α- error

$Z\beta = 0.84$ at 20% β- error

Normal	**= 42**
Oral Squamous Cell Carcinoma	**= 42**
Total	**= 84**

METHODOLOGY

DEMOGRAPHIC DATA

Demographic regarding age, sex, site, size, habit history, TNM stage, histopathological grade and lymph node status was retrieved from the departmental wherever available for OSCC cases. The normal oral mucosa tissues were collected during procedures of periodontal crown lengthening and dys-impaction of third molars.

STAINING PROCEDURE

Immuno-staining was done using antibody against AJUBA. Using PolyExcel HRP/DAB Detection System Two Step Universal Kit (PathnSitu Catalogue no #PEH002/USA).

PRINCIPLE OF IMMUNO-STAINING

The PolyExcel HRP/DAB Detection System Two step universal kit is based on the principle of antigen/antibody reaction in tissues. Primary AJUBA antibody combines with its corresponding antigen in tissues. Secondary antibodies which have

a dextran polymer backbone conjugate with the primary antibody. DAB (3'3'diaminobenzidine) chromogen combines with antigen-antibody complex and demonstrate a coloured reaction product.

REGENTS USED

1. Primary antibody:

Company: Thermofisher scientific (Invitrogen)

Catalog # PA5-52264: AJUBA polyclonal antibody

Species Reactivity: Human

Host/Isotope: Rabbit / IgG

Class: Polyclonal

2. PolyExcelHRP/DAB detection system two step kit (PathnSitu) contains

- Peroxide block (H_2O_2): This contains 3% hydrogen peroxide in water for blocking the endogenous peroxidase activity.
- PolyExcel target binder: This is a universal protein that helps in binding to primary antibody.
- PolyExcel HRP Reagent: This contains anti-mouse or anti rabbit antibody that is labelled with IgG and enzyme polymer in phosphate buffered saline, along with stabilizers and proclin 300.
- Liquid DAB Chromogen: DAB chromogen that has enhanced sensitivity with HRP as colorimetric agent.

- Stable DAB substrate buffer: This buffer contains Tris-buffer along with proxide as well as stabilizers. It is used along with DAB chromogen.

3. Buffers:

- Tris buffer: for antigen retrieval **(Annexure VI).** This was used for heat induced epitope retrieval (HIER) to unmask antigen binding sites in the tissues with pH 9.
- Phosphate buffer saline: wash buffer **(Annexure VI).** This was used as wash buffer with pH ranging from 7.2-7.6.

4. Xylene for clearing/ dewaxing.
5. Graded alcohol solution (100%, 90%, 80%, 70%, and 50%) for dehydrating
6. Distilled water- wash
7. Harris Hematoxylin- counter stain
8. Mounting medium, DPX
9. Other equipment's used:

- APES coated glass slides
- Humidifying chamber
- Wash bottles
- Absorbent wipes (tissue papers)
- EZ retriever system V.2.1 (for HIER)
- Calibrated test tube
- Plastic Pasteur pipette (provided with detection kit to mix DAB chromogen & buffer)
- Cover slips

- Micropipettes
- Refrigerator (4°C, -20°C)
- Semi-automatic microtome (Leica RM 2145)
- Slide warmer
- Water bath
- Multi-viewer Microscope

IHC STAINING PROTOCOL:

1. Sectioning: Formalin fixed paraffin embedded tissues was sectioned at 4µ and mounted on APES coated slides.
2. Deparaffinization: Slides were deparaffinized by heating on a slide warmer at 60°C for 1 hour and treated with two changes of xylene for 15 minutes each.
3. The slides were treated with one change each of 100% alcohol followed by graded alcohol 90%, 80% and 70% for 10 min each.
4. Slides were then rinsed with distilled water.
5. Heat Induced Epitope Retrieval:

- The prepared tris buffer 1000 ml was poured in the tank which contained slots for holding the slides. The sections were submerged and placed apart properly in the tris buffer.
- The slides were then placed in the pressure cooker- 15psi and 120 degrees Celsius.

6. After completion of antigen retrieval, the sections were allowed to cool at room temperature for minimum of 45 mins prior to next step.
7. After cooling to room temperature, the slides followed distilled water wash for 5 minutes and later followed by PBS rinse for 5 minutes.

IMMUNOHISTOCHEMICAL STAINING

1. Blocking of Endogenous peroxidase activity was done by incubating with peroxidase block for 15 minutes. Slides were washed with wash buffer (PBS) for 5 minutes.
2. Then the slides were incubated with primary polyclonal antibody against AJUBA for overnight incubation at 4 degrees Celsius in a humidified chamber. After that slide were washed with wash buffer (PBS) for 5 minutes each.
3. Target binder was added to promote Ag-Ab reaction and incubated for 15 minutes in humidifying chamber. This was followed by 2 changes of PBS rinse for 5 minutes each.
4. Slides were then incubated with Poly HRP for 30 minutes and it was followed by 2 changes of in PBS for 5 minutes each.
5. Incubation with was done with fresh substrate/ chromogen mix of 3,3'Diaaminobenzidine (DAB) mixed with buffer (i.e 25µl concentrated DAB in 500µl of substrate buffer for 10 slides) for up to 10 minutes. This step enables visualization of antigen-antibody reaction as a brown colored end product. After that slide were dipped in distilled water.
6. Slides were counterstained with Harris hematoxylin up to 1-2 minutes.
7. Under running tap water, bluing was carried out for up to 10mins.
8. After that slide were dehydrated and mounted with DPX.

DATA ANALYSIS:

The clinical data of the cases was collected and tabulated from the archives. The H & E-stained slides of OSCC were assessed according to the Bryne's grading system[35]. The immuno-stained slides were evaluated by two oral pathologists and the observations were tabulated in excel sheet. Any disparity was assessed again in the penta-headed multi-viewing microscope.

The two groups were analyzed for immunoexpression of AJUBA, localization, intensity and percentage of positivity**. (Table 6 - 15)**

- Criteria used for evaluating AJUBA immunoexpression for localization, cellular location and intensity in **Normal oral mucosa**

Table 6: Criteria used for Level of expression of AJUBA in epithelium layers

Level of expression of AJUBA in epithelium layers	GRADE
Basal	1
Basal + Supra basal	2
Basal +Supra Basal +Superficial	3

Table 7: Criteria used for Level of expression of AJUBA in the connective tissue

Level of expression of AJUBA in the connective tissue	GRADE
Juxta epithelial	1
Submucosal	2
Deeper epithelium	3

Table 8: Criteria used for Cellular location of AJUBA epithelium

Cellular location of AJUBA epithelium	GRADE
Nucleus (N)	1
Cytoplasm (C)	2
Membrane (M)	3
N+C	4
N+C+M	5

Table 9: Criteria used for Intensity of AJUBA

Intensity of AJUBA	GRADE
No staining	0
Light brown	1
Dark brown	2

- Criteria used for evaluating AJUBA immunoexpression for localization, cellular location and intensity in **OSCC**

Table 10: Criteria used for Expression of AJUBA in tumor

Expression of AJUBA in tumor	GRADE
Superficial front	1
Invasive front	2
Superficial + Invasive	3

Table 11: Criteria used for Expression of AJUBA in tumor islands

Localization of AJUBA in tumor islands	GRADE
Peripheral	1
Central	2
Peripheral + Central	3

Table 12: Criteria used for Cellular location of AJUBA in the tumor cells

Cellular location of AJUBA in the tumor cells	GRADE
Nuclear (N)	1
Cytoplasm (C)	2
Membrane (M)	3
Nucleus + Cytoplasm	4
Nucleus + Cytoplasm + Membrane	5

Table 13: Criteria used for Intensity of AJUBA

Intensity of AJUBA	GRADE
No stain	0
Light brown	1
Dark brown	2

Table 14: Criteria used for Percentage of Positivity

Percentage of Positivity	GRADE
<25% immunoreactive cells	1
25-50% immunoreactive cells	2
50-75% immunoreactive cells	3
>75% immunoreactive cells	4

ABSENT was considered as zero (0) in both normal oral mucosa and OSCC cases

ASSESSMENT OF VARIOUS HISTO-PATHOLOGICAL PARAMETERS IN OSCC CASES:

Various histopathological parameters were assessed such as, depth of invasion (DOD), type of invasive front, type of stroma, extent of inflammation, lympho-vascular invasion (LVI), peri-neural invasion (PNI). All these parameters were analysed using a H&E-stained section of OSCC cases.

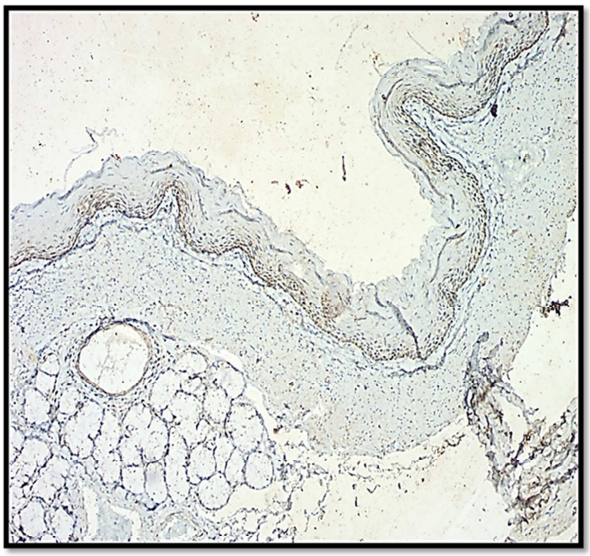
Table 15: Table showing histopathological criteria for evaluation of H & E-stained slides according to Bryne's grading system[35].

Depth of invasion	1- CA in situ
	2- Invasion involving lamina propria
	3- Invasion below lamina propria involving muscle, gland, periosteum
	4- Deep invasion involving jaw bone
Histo grade	1- WDSCC
	2- MDSCC
	3- PDSCC
Invasive front	1- Pushing border
	2- Infiltrative solid cords
	3- Small groups or cords of infiltrative cells (n >15)
	4- Wide spread cellular dissociation (n < 15)
	5- Tumor satellite's
Type of Stroma	1- Abundant
	2- Dense
	3- Delicate
	4- None
Extent of inflammation	1- marked
	2- moderate
	3- slight
	4- none
Lymph vascular invasion	0- absent
	1- present
Surgical margins	0- absent
	1- present
Lymph node metastasis	0- absent
	1- present

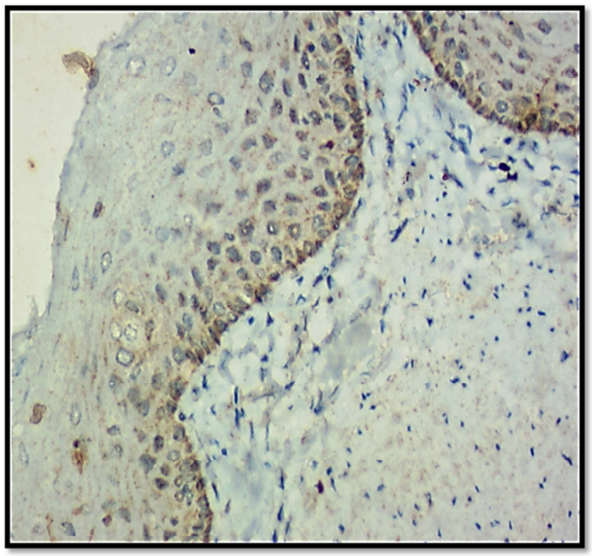
STATISTICAL ANALYSIS: (Annexure-III)

1. Frequency percentage was done for Immunoexpression of AJUBA in normal oral mucosa with localisation, cellular location and intensity.
2. Frequency percentage was done for Immunoexpression of AJUBA in OSCC with localisation, cellular location, intensity and percentage of positivity.
3. Comparison was done for immunoexpression of AJUBA in normal oral mucosa and OSCC with intensity of staining
4. Association between various clinicopathological parameters and the immunoexpression of AJUBA in OSCC was done using Chi square test.

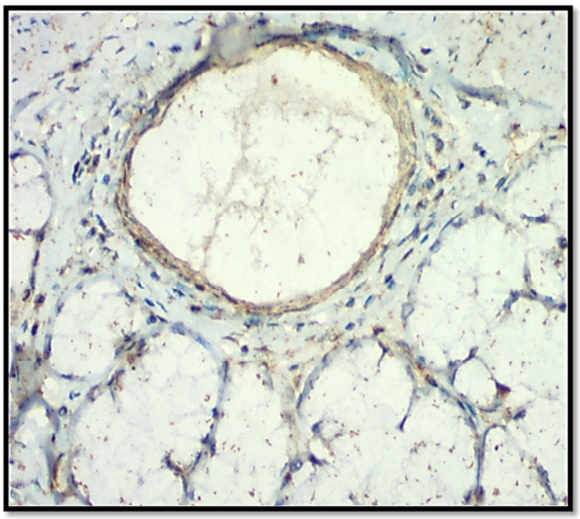
1.Photomicrographs of AJUBA immunoexpression in normal human esophagus



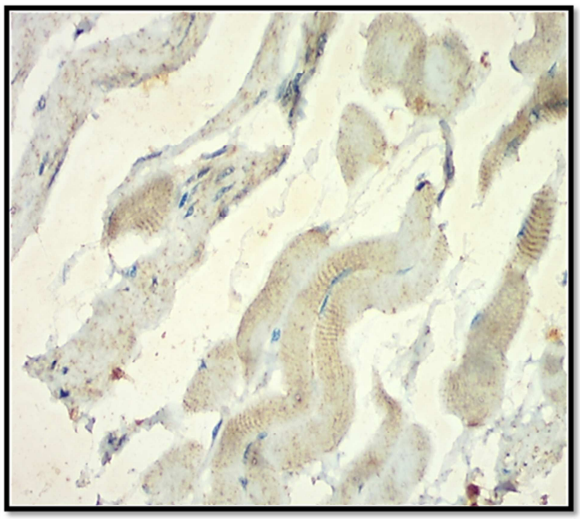
Photomicrograph 1: Normal human oesophagus- Positive control (10x)



Photomicrograph 2: Normal human oesophagus showing nuclear and cytoplasmic staining in basal and supra-basal layers- Positive control (40x)

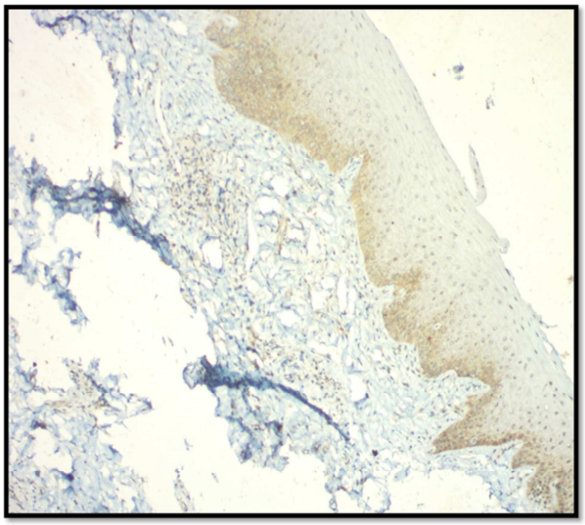


Photomicrograph 3: Normal human oesophagus Positive expression in ductal lining of mucosal glands in submucosal layer (40x)

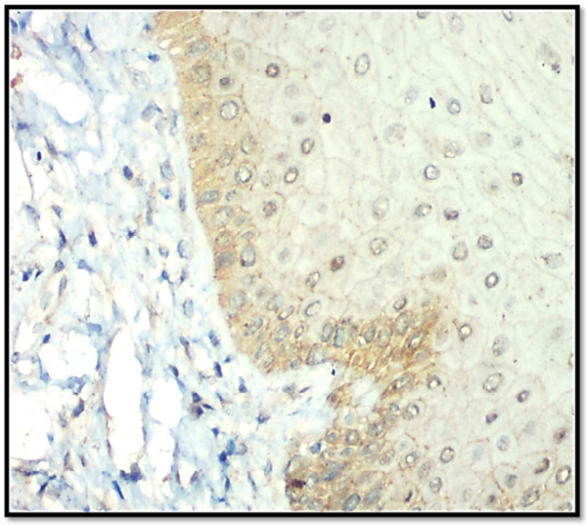


Photomicrograph 4: Normal human oesophagus Positive expression in muscle of submucosal layer (40x)

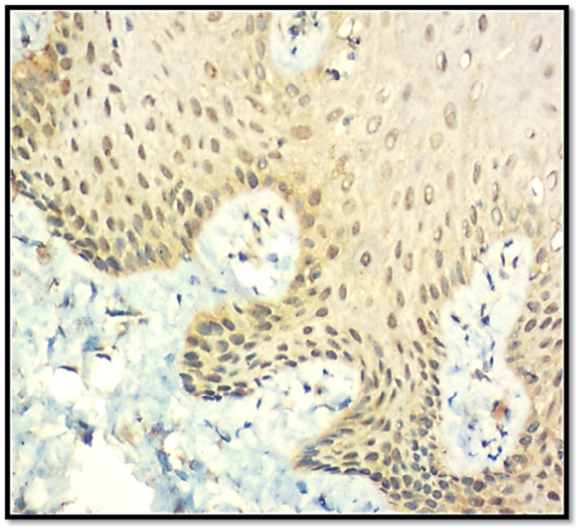
2.Photomicrographs of AJUBA immunoexpression in normal oral mucosa



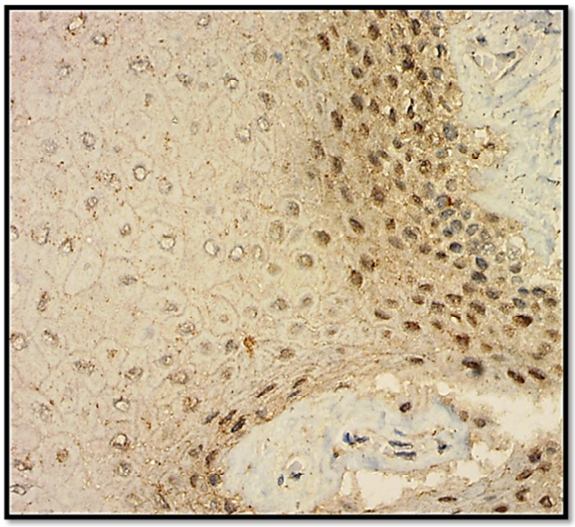
Photomicrograph 5: Normal oral mucosa showing immunoexpression of AJUBA in basal and supra-basal layers. (10x)



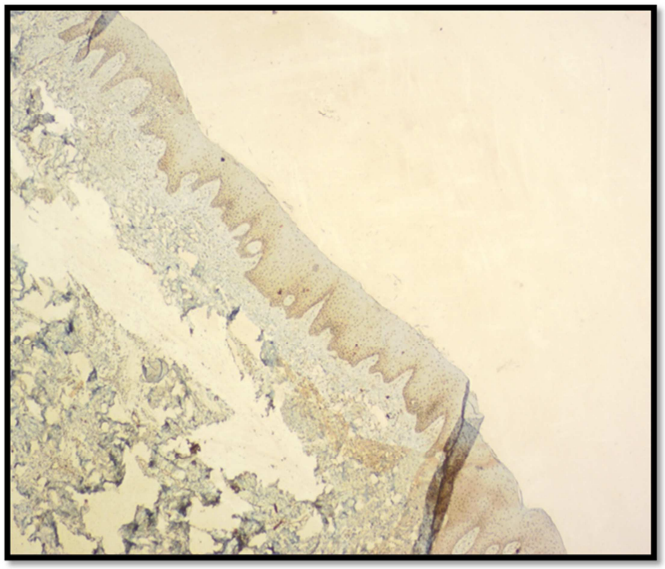
Photomicrograph 6: Normal oral mucosa showing immunoexpression of AJUBA in basal and supra-basal layers. (40x)



Photomicrograph 7: Normal oral mucosa showing nuclear and cytoplasmic cellular location of AJUBA in epithelium. (10x)

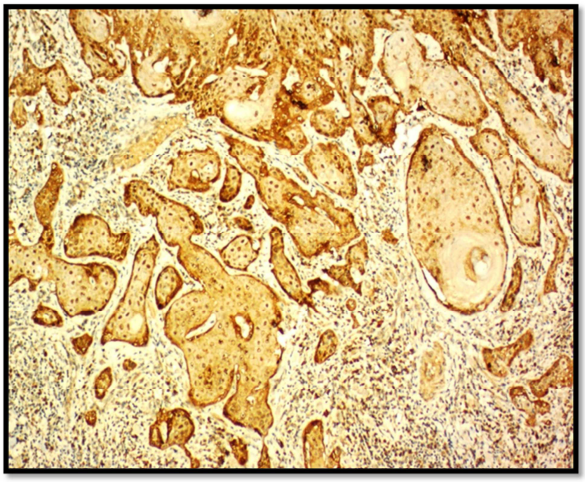


Photomicrograph 8: Normal oral mucosa showing immunoexpression of AJUBA in basal and supra-basal layers. (40x)

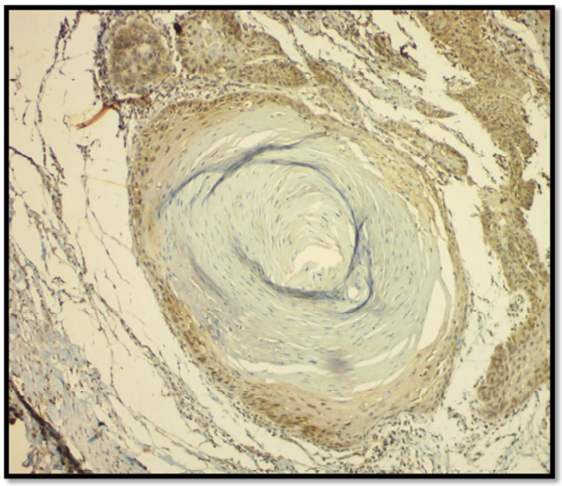


Photomicrograph 9: Photomicrograph of normal oral mucosa showing expression of AJUBA in submucosal layer of connective tissue stroma (10x)

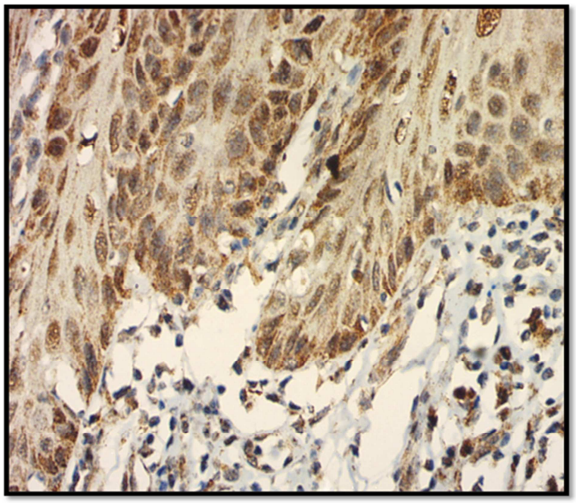
3.Photomicrographs of AJUBA immunoexpression in oral squamous cell carcinoma



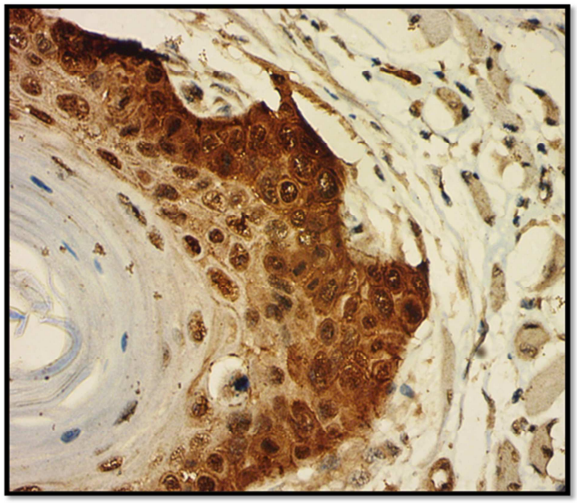
Photomicrograph 10: Well differentiated squamous cell carcinoma showing High intense (dark brown) immunoexpression of AJUBA both in superficial and invasive front of the tumor (10x)



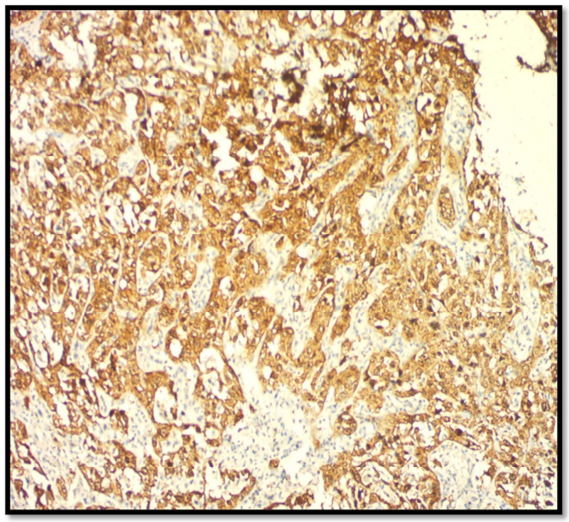
Photomicrograph 11: Well differentiated squamous cell carcinoma showing High intense dark brown immunoexpression of AJUBA in peripheral cells of tumor island (40x)



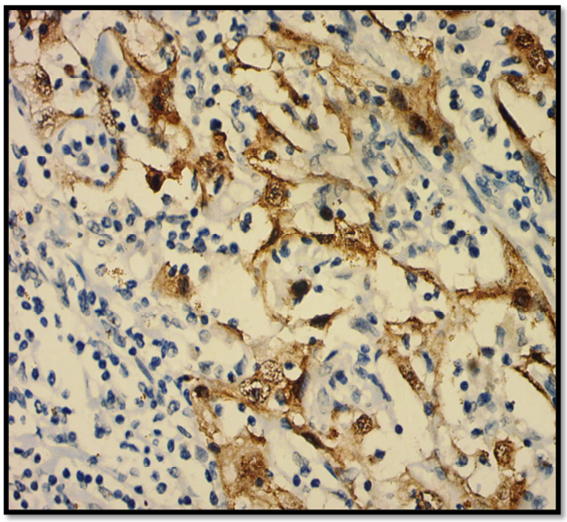
Photomicrograph 12: Oral squamous cell carcinoma showing High intense (dark brown) immunoexpression of AJUBA both in nuclear and cytoplasm in tumor cells (10x).



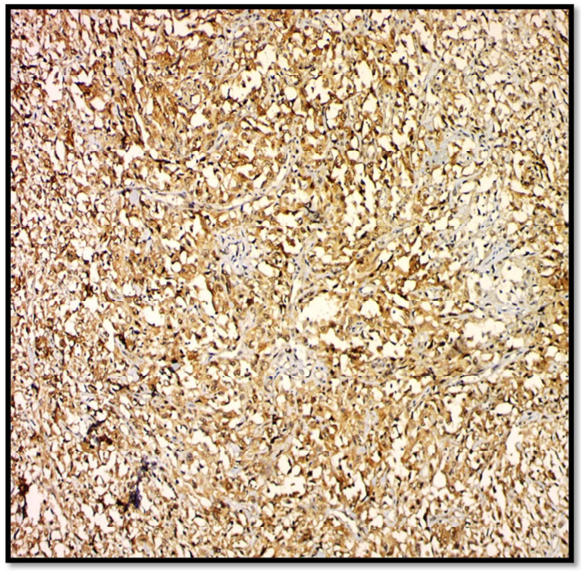
Photomicrograph 13: Well differentiated squamous cell carcinoma showing High intense dark brown immunoexpression of AJUBA in peripheral cells of tumor island (40x)



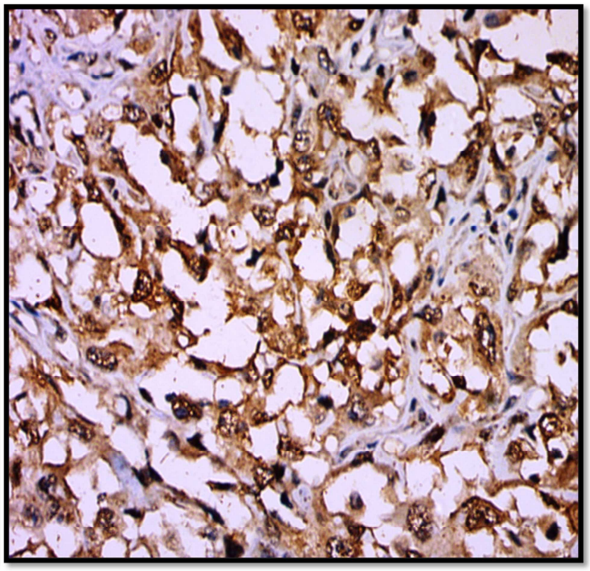
Photomicrograph 14: Oral squamous cell carcinoma showing High intense (dark brown) immunoexpression of AJUBA both in MDSCC (10x)



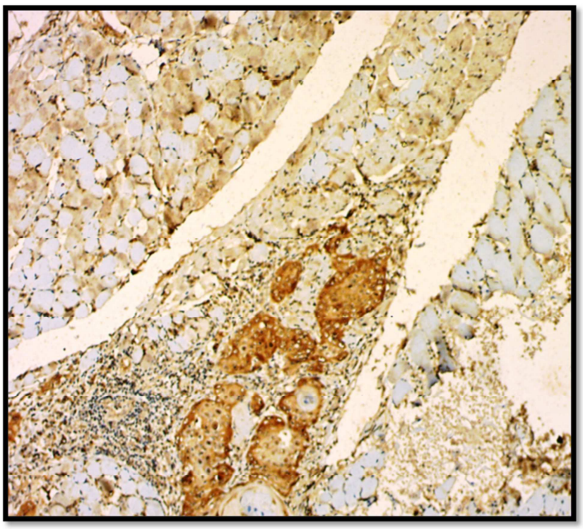
Photomicrograph 15: Oral squamous cell carcinoma showing High intense (dark brown) immunoexpression of AJUBA both in MDSCC (40x)



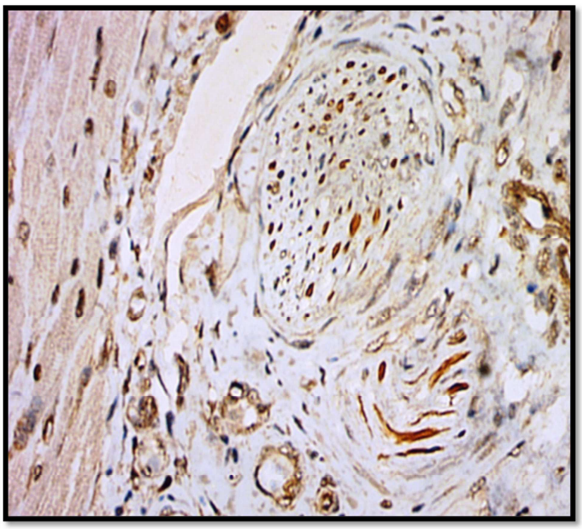
Photomicrograph 16: Oral squamous cell carcinoma showing High intense (dark brown) immunoexpression of AJUBA in PDSCC (10x)



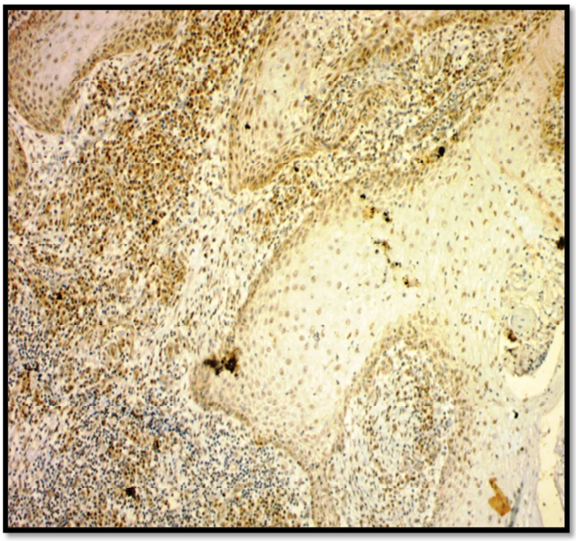
Photomicrograph 17: Oral squamous cell carcinoma showing High intense (dark brown) immunoexpression of AJUBA in individual cells of PDSCC (40x)



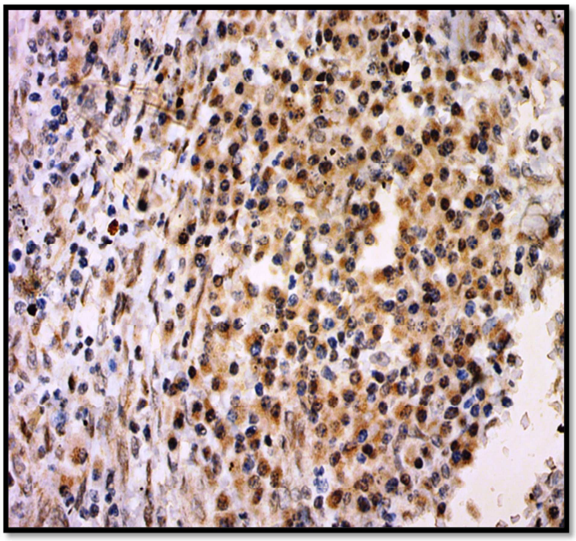
Photomicrograph 18: Oral squamous cell carcinoma showing AJUBA positive tumor cells invading the muscle (10x)



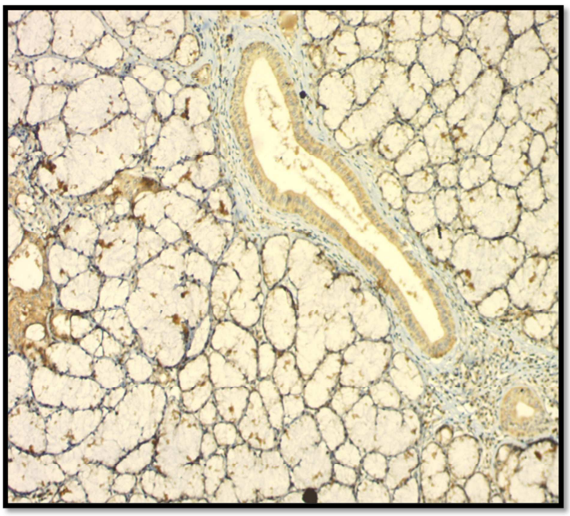
Photomicrograph 19: Oral squamous cell carcinoma showing AJUBA positive expression in the nerve (40x)



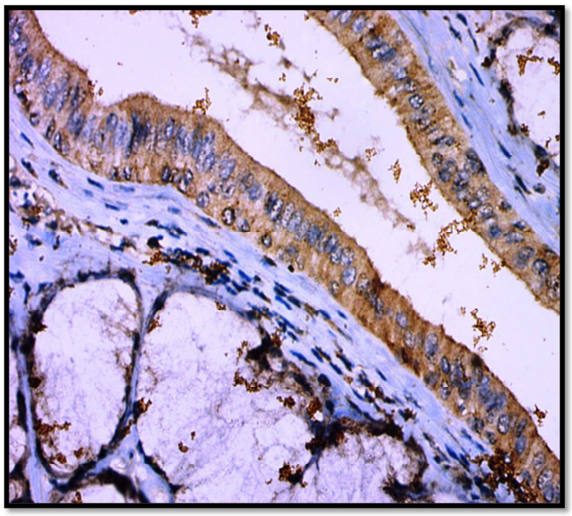
Photomicrograph 20: Oral squamous cell carcinoma showing AJUBA expression in the inflammatory cells (10x)



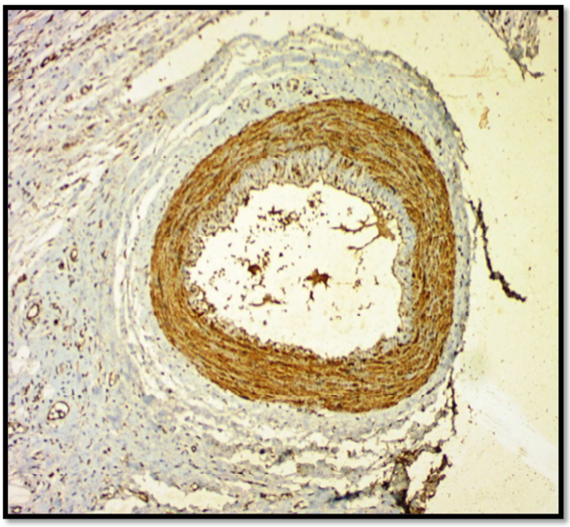
Photomicrograph 21: Oral squamous cell carcinoma showing AJUBA expression in the cytoplasm of plasma cells (40x)



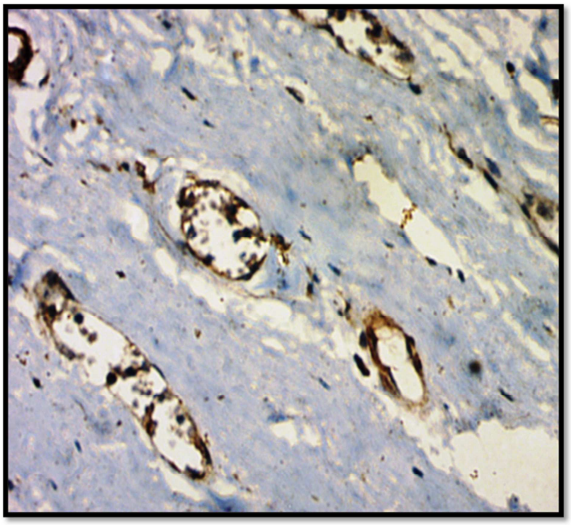
Photomicrograph 22: Oral squamous cell carcinoma showing AJUBA expression in the salivary gland ducts (10x)



Photomicrograph 23: Oral squamous cell carcinoma showing AJUBA expression in the salivary gland ductal lining (40x)



Photomicrograph 24: Oral squamous cell carcinoma showing AJUBA expression in the Large blood vessels (10x)



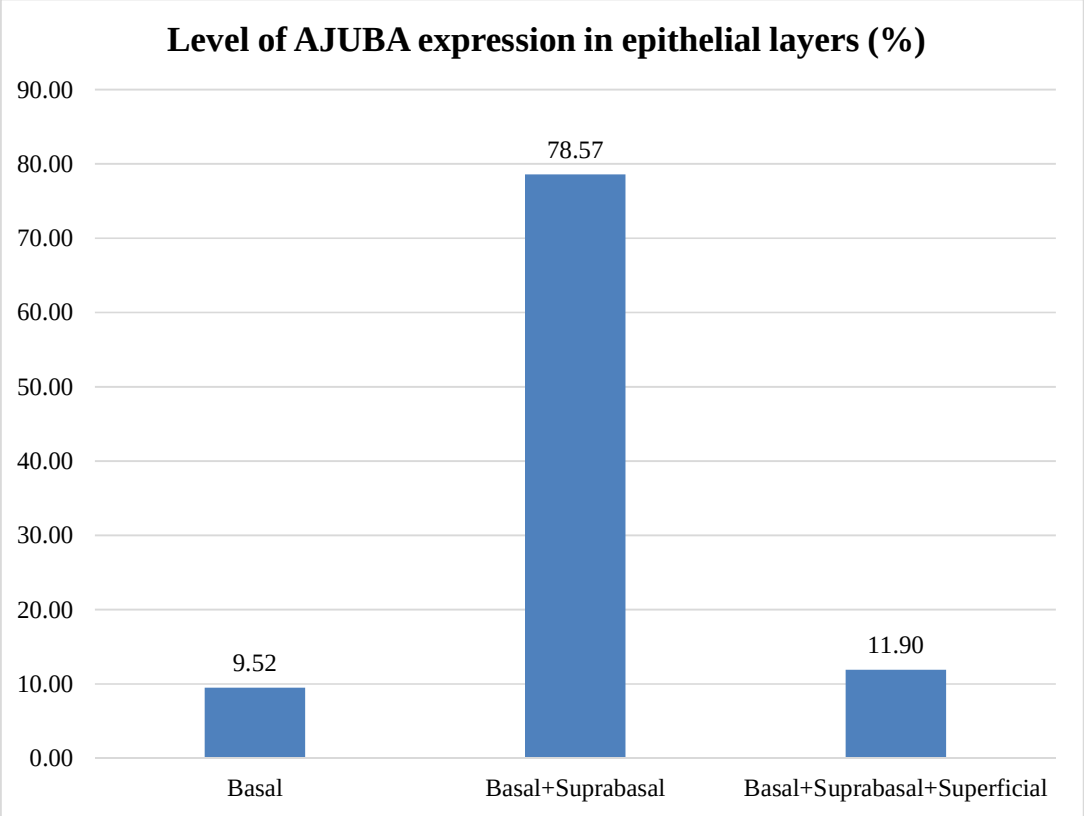
Photomicrograph 25: Oral squamous cell carcinoma showing AJUBA expression in the endothelial lining of capillaries (10x)

RESULTS

I. EVALUATION OF AJUBA IN NORMAL ORAL MUCOSA

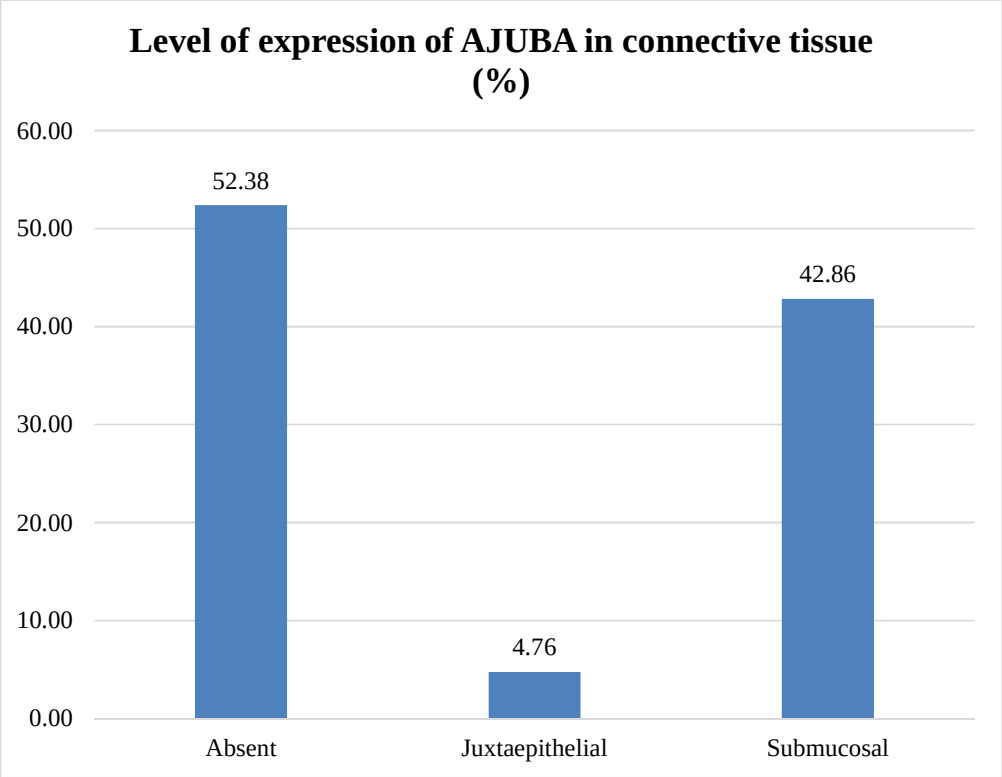
Bar graph 1: Bar graph showing Evaluation of Immuno expression of AJUBA in Epithelium Of Normal Oral Mucosa (NOM) in Frequency percentage:

- **Level of AJUBA expression in epithelial layers of normal oral mucosa.**



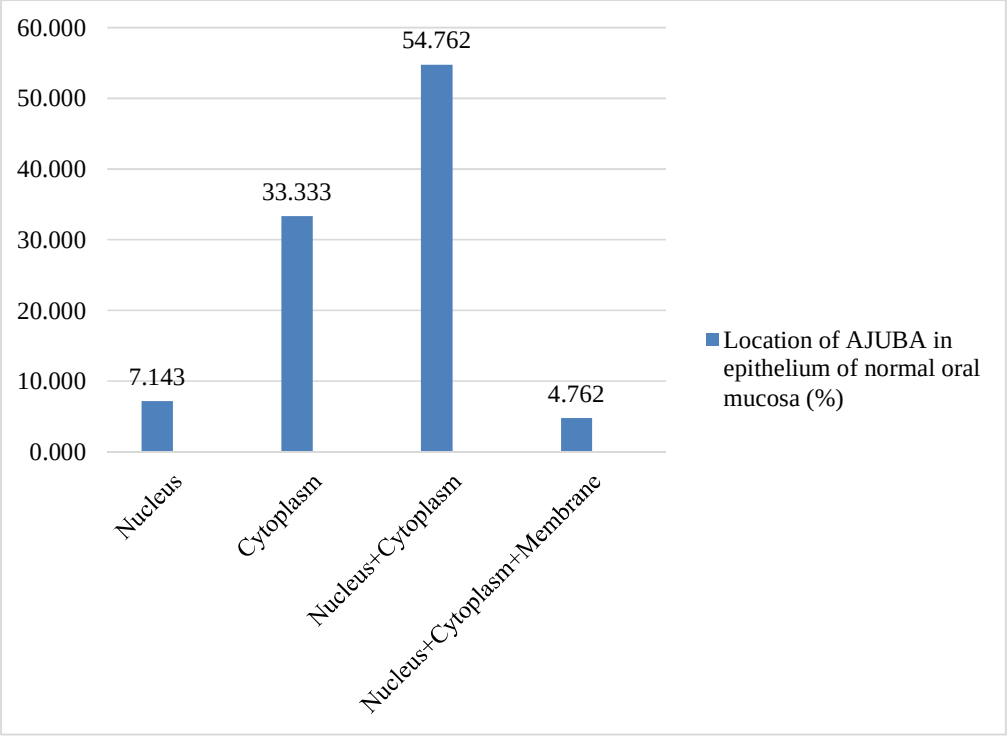
Inference: Expression of AJUBA in the epithelial cell layers of normal oral mucosa revealed that among 42 cases included in our study i.e., majority of cases showed expression of AJUBA in Basal layer along with supra-basal layers of the epithelium **78% (n=33)**. However, only few of our cases showed AJUBA expression in basal cell layer (9.52%) and we also noted only few of our cases showing AJUBA expression in all the three cell layers (11.90%).

Bar graph 2: Bar graph showing evaluation of Immuno expression of AJUBA in connective tissue of Normal Oral Mucosa (NOM) in frequency percentage:



Inference: Expression of AJUBA in the connective tissue of normal oral mucosa revealed that majority of the cases i.e., **52.38% (n= 22)** showed negative expression of AJUBA However, we observed that the submucosal component of connective tissue expression of AJUBA was found to be 42.86% (n= 18). Only few of our cases showed juxta-epithelial immunoexpression of AJUBA i.e., **4.76% (n= 2)**.

Bar graph 3: Bar graph showing evaluation of cellular location of Immuno expression AJUBA in epithelium of normal oral mucosa(NOM) in frequency percentage.



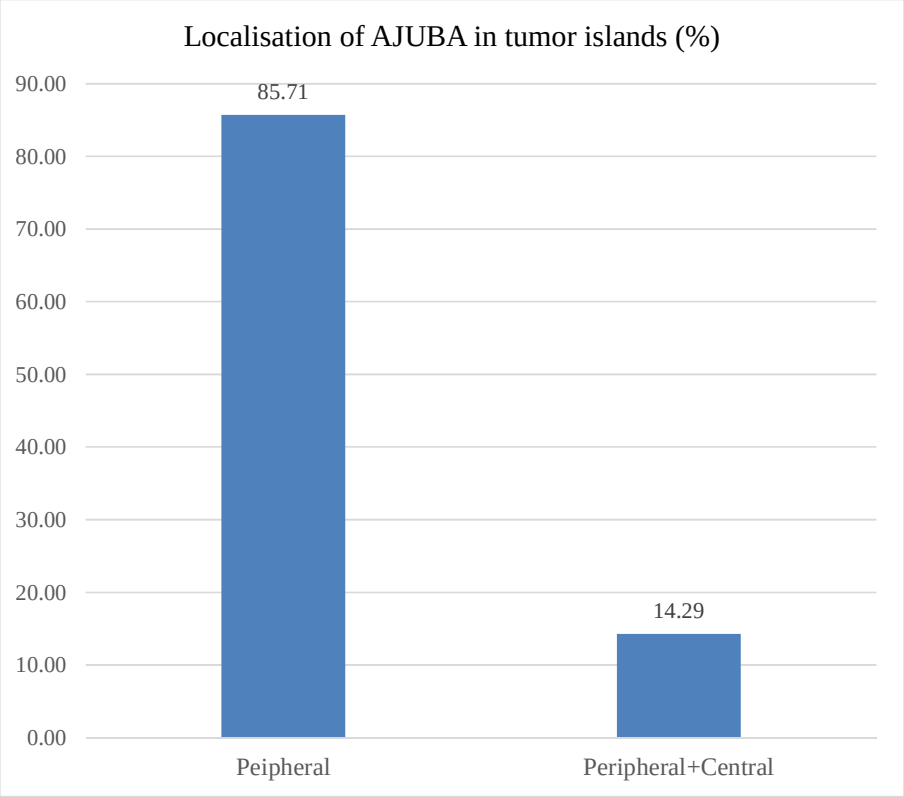
Inference: On noting down the cellular location of immunoexpression of AJUBA in the epithelium of normal oral mucosa, we observed that majority of the cases i.e., **54.76% (n= 23)** showed nuclear and cytoplasmic location. Cytoplasmic immunoexpression of AJUBA was seen in **33.33% (n= 14).** Only few of our cases showed nuclear immunoexpression **7.14% (n= 3) and 4.76% (n= 2)** nuclear, cytoplasmic and membrane expression respectively.

Enhanced immunoexpression of AJUBA in Normal Oral Mucosa was found predominantly in basal and supra basal layer of the epithelium and deeper part of the connective tissue component. The cellular location in majority of the cases was seen both in nucleus and cytoplasm.

II. EVALUATION OF AJUBA IN ORAL SQUAMOUS CELL CARCINOMA

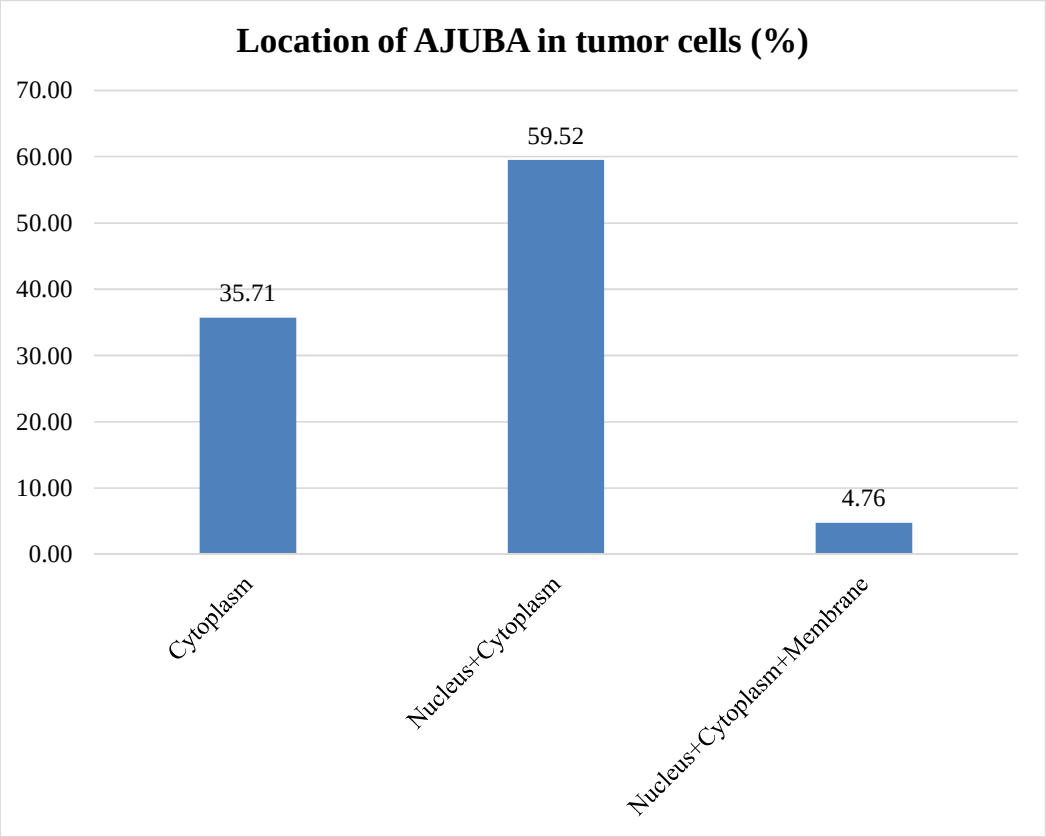
Enhance immunoexpression of AJUBA was noted in all the cases of OSCC and we also observed its expression to be same at both superficial and invasive front of the tumor. We also noted that all our cases of OSCC revealed same percentage of positivity of AJUBA expression i.e., **50-75% (n= 42)**

- **Bar graph 4: Bar graph showing localization of Immuno expression AJUBA in the tumor islands of Oral Squamous Cell Carcinoma (OSCC) in frequency percentage.**



Inference: Expression of localization of AJUBA in the tumor islands revealed that majority of the cases i.e., **85.71% (n= 36)** showed expression in the periphery of the tumor Islands. It was also noted that few tumor islands showed immunoexpression in the peripheral and center of the islands i.e, **14.29% (n= 6).**

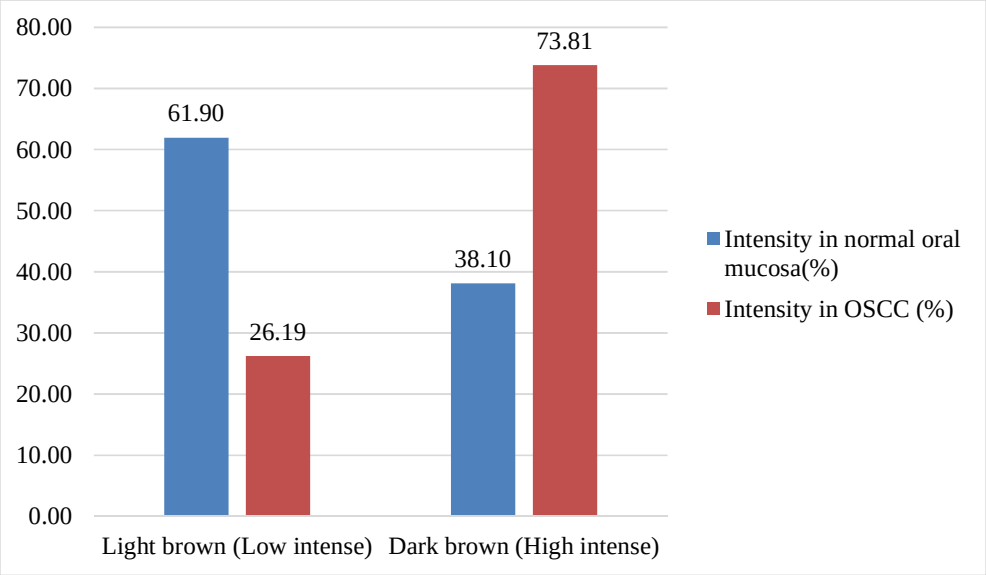
- **Bar graph 5: Bar graph showing cellular location of Immuno expression AJUBA In Oral Squamous Cell Carcinoma In frequency percentage.**



Inference: Immunoexpression of AJUBA in tumor cells revealed that majority of cases showed cellular location in both nucleus and cytoplasm i.e., **59.52% (n= 25)**. Cytoplasmic immunoexpression of AJUBA was seen in **35.71% (n= 15).** However, we observed that only few of our cases showed nuclear, cytoplasmic and membrane immunoexpression of AJUBA in tumor cells of OSCC i.e., **4.76% (n= 2)**.

Enhance immunoexpression of AJUBA in OSCC revealed predominantly at periphery of the tumor islands. We also found cellular location of AJUBA predominantly within the nucleus and cytoplasm of the tumor cells of OSCC.

- **Bar graph 6 and Table 1: Bar graph showing the comparison of Intensity of immunoexpression of AJUBA between Normal Oral Mucosa (NOM) and Oral Squamous Cell Carcinoma (OSCC) in frequency percentage.**



	Intensity in normal oral mucosa (%)	Intensity in OSCC (%)	z-value	p-values
Light brown	61.90	26.19	2.18	<0.05
Dark brown	38.10	73.81	-2.46	<0.05

Inference: On noting down the intensity of immunoexpression of AJUBA, we observed that light brown expression which is considered to be low intense expression was seen in majority of cases in normal oral mucosa **(61.9%)** than compared to OSCC cases **(26.19%)** and this difference of expression was found to be statistically significant with chi-square test **($p<0.05$).** We observed enhanced immunoexpression of AJUBA with dark brown considered to be high intense was seen in majority of cases of OSCC **(73.81%)** than compared to normal oral mucosa **(38.10%)** and this was found to have a statistically association with chi-square test **($p<0.05$)**

Enhanced immunoexpression of AJUBA was seen in OSCC cases when compared with normal oral mucosa.

- **Table 2: Table showing the association of Demographic Data parameters with AJUBA localization in tumor islands of OSCC.**

Demographic Data		Localisation of AJUBA In Tumor Islands			
		Peripheral	Peripheral + Central	Total	P-Value
Age	Less Than 40yrs	6 (14%)	1 (2%)	7 (16%)	1
	More Than or Equal To 40yrs	30 (71%)	5 (12%)	35 (83%)	
Sex	Male	33 (78%)	6 (14%)	39 (92%)	0.463
	Female	3 (7%)	0	3 (7%)	
Habit	Present	36 (85%)	6 (14%)	42 (100%)	-
Site	Buccal Mucosa	11 (26%)	3 (7%)	14 (33%)	0.564
	Tongue	2 (4%)	1 (2%)	3 (7)	
	Gingivo Buccal Sulcus	10 (23%)	1 (2%)	11 (26%)	
	Retro Molar Trigone	8 (19%)	0	8 (19%)	
	Hard Palate	1 (2%)	0	1 (2%)	
	Alveolus	1 (2%)	1 (2%)	2 (4%)	
	Mandible	2 (4%)	0	2 (4%)	
	Labial Mucosa	1 (2%)	0	1 (2%)	
Size	Absent	3 (7%)	1 (2%)	4 (9%)	
	Less Than 10cm	17 (40%)	2 (4%)	19 (45%)	0.730
	More Than 10cm	16 (38%)	3 (7%)	19 (45%)	
TNM	Absent	26 (61%)	5 (11%)	31 (73%)	0.895
	Stage 2	1 (2%)	0	1 (2%)	
	Stage 4 A	7 (16%)	1 (2%)	8 (19%)	
	Stage 4 B	2 (4%)	0	2 (4%)	

- **Table 2a: Table showing the association of Histological Parameters with AJUBA localization in tumor islands of OSCC.**

Histo Pathological Parameters		Localisation Of AJUBA In Tumor Islands			
		Peripheral	Peripheral + Central	Total	P-Value
Depth Of Invasion	2- Invasion Involving Lamina Propria	10 (23%)	0	10 (23%)	0.177
	3- Invasion Below Lamina Propria Involving Muscle, Gland, Periosteum	16 (38%)	5 (11%)	21 (50%)	
	4- Deep Invasion Involving Jaw Bone	10 (23%)	1 (2%)	11 (26%)	
Histograde	1- WDSCC	20 (47%)	3 (7%)	23 (54%)	0.966
	2- MDSCC	11 (26%)	2 (4%)	13 (30%)	
	3- PDSCC	5 (11%)	1 (2%)	6 (14%)	
Invasive Front	1- Pushing Border	1 (2%)	0	1 (2%)	0.899
	2- Infiltrative Solid Cords	6 (14%)	1 (2%)	7 (16%)	
	3- Small Groups or Cords of Infiltrative Cells (N >15)	19 (45%)	4 (9%)	23 (54%)	
	4- Wide Spread Cellular Dissociation (N < 15)	10 (23%)	1 (2%)	11 (26%)	
Stroma	1- Abundant	3 (7%)	1 (2%)	4 (9%)	0.114
	2- Dense	27 (64%)	2 (4%)	29 (69%)	
	3- Delicate	6 (14%)	3 (7%)	9 (21%)	
Inflammation	1- Marked	21 (50%)	4 (9%)	25 (59%)	0.9
	2- Moderate	9 (21%)	1 (2%)	10 (23%)	
	3- Slight	6 (14%)	1 (2%)	7 (16%)	
Lympho Vascular Invasion	Negative	28 (66%)	4 (9%)	32 (76%)	0.554
	Positive	8 (19%)	2 (4%)	10 (23%)	
Surgical Margin	Negative	32 (76%)	6 (14%)	38 (90%)	0.391
	Positive	4 (9%)	0	4 (9%)	
Lymph Node	Negative	20(47%)	2 (4%)	22 (52%)	0.313
	Positive	16 (38%)	4 (9%)	20 (47%)	
Neural Invasion	Negative	25 (59%)	5 (11%)	30 (71%)	0.486
	Positive	11 (26%)	1 (2%)	12 (28%)	

Inference: We didn't not find any statistically significant association of demographic parameters and histological parameters with localization of immunoexpression of AJUBA in tumor islands of OSCC.

- **Table 3: Table showing the association of Demographic Data parameters with cellular location of Immuno expression of AJUBA in tumor cells of OSCC**

Demographic Data		Location of AJUBA In Tumor Cells				
		Cytoplasm	Nucleus + Cytoplasm	Nucleus + Cytoplasm + Membrane	Total	P-Value
Age	Less Than 40yrs	4 (9%)	3 (7%)	0	7 (16%)	0.392
	More Than or Equal To 40yrs	11 (26%)	22 (52%)	2 (4%)	35 (83%)	
Sex	Male	14 (33%)	24 (57%)	1 (2%)	39 (92%)	0.052
	Female	1 (2%)	1 (2%)	1 (2%)	3 (7%)	
Habit	Present	15 (35%)	25 (59%)	2 (4%)	42 (100%)	-
Site	Buccal Mucosa	8 (19%)	6 (14%)	0	14(33%)	0.227
	Tongue	0	3 (7%)	0	3 (7%)	
	Gingivo Buccal Sulcus	3 (7%)	8 (19%)	0	11 (26%)	
	Retro Molar Trigone	2 (4%)	4 (9%)	2 (4%)	8 (19%)	
	Hard Palate	0	1 (2%)	0	1 (2%)	
	Alveolus	0	2 (4%)	0	2 (4%)	
	Mandible	1 (2%)	1 (2%)	0	2 (4%)	
	Labial Mucosa	1 (2%)	0	0	1 (2%)	
Size	Absent	3 (7%)	1 (2%)	0	4 (9%)	0.481
	Less Than 10cm	5 (11%)	13 (30%)	1 (2%)	19 (45%)	
	More Than 10cm	7 (16%)	11 (26%)	1 (2%)	19 (45%)	
TNM	Absent	11 (26%)	18 (42%)	2 (4%)	31 (73%)	0.784
	Stage 2	1 (2%)	0	0	1 (2%)	
	Stage 4 A	2 (4%)	6 (14%)	0	8 (19%)	
	Stage 4 B	1 (2%)	1 (2%)	0	2 (4%)	

- **Table 3a: Table showing the association of Histopathological Parameters with cellular location of Immuno expression of AJUBA in tumor cells of OSCC.**

Histological Parameters		Location Of AJUBA In Tumor Cells				
		Cytoplasm	Nucleus + Cytoplasm	Nucleus + Cytoplasm + Membrane	Total	P-Value
Depth Of Invasion	2- Invasion Involving Lamina Propria	2 (4%)	7 (16%)	1 (2%)	10 (23%)	0.647
	3- Invasion Below Lamina Propria Involving Muscle, Gland, Periosteum	9 (21%)	11 (26%)	1 (2%)	21 (50%)	
	4- Deep Invasion Involving Jaw Bone	4 (9%)	7 (16%)	0	11 (26%)	
Histograde	1- WDSCC	5 (11%)	16 (38%)	2 (4%)	23 (54%)	**0.007**
	2- MDSCC	4 (9%)	9 (21%)	0	13 (30%)	
	3- PDSCC	6 (14%)	0	0	6 (14%)	
Invasive Front	1- Pushing Border	0	1 (2%)	0	1 (2%)	0.207
	2- Infiltrative Solid Cords	0	7 (16%)	0	7 (16%)	
	3- Small Groups or Cords of Infiltrative Cells (N >15)	9 (21%)	12 (28%)	2 (4%)	23 (54%)	
	4- Wide Spread Cellular Dissociation (N < 15)	6 (14%)	5 (11%)	0	11 (26%)	
Stroma	1- Abundant	0	4 (9%)	0	4 (9%)	0.131
	2- Dense	9 (21%)	18 (42%)	2 (4%)	29 (69%)	
	3- Delicate	6 (14%)	3 (7%)	0	9 (21%)	
Inflammation	1- Marked	6 (14%)	18 (42%)	1 (2%)	25 (59%)	**0.041***
	2- Moderate	3 (7%)	6 (14%)	1 (2%)	10 (23%)	
	3- Slight	6 (14%)	1 (2%)	0	7 (16%)	
Lympho Vascular Invasion	Negative	11 (26%)	19 (45%)	2 (4%)	32 (76%)	0.707
	Positive	4 (9%)	6 (14%)	0	10 (23%)	
Surgical Margin	Negative	14 (33%)	22 (52%)	2 (4%)	38 (90%)	0.767
	Positive	1 (2%)	3 (7%)	0	4 (9%)	
Lymph Node	Negative	6 (14%)	15 (35%)	1 (2%)	22 (52%)	0.470
	Positive	9 (21%)	10 (23%)	1 (2%)	20 (47%)	
Neural Invasion	Negative	10 (23%)	18 (42%)	2 (4%)	30 (71%)	0.615
	Positive	5 (11%)	7 (16%)	0	12 (28%)	

Inference: Most of the parameters included in our study did not find any statistically significant association with cellular location of immunoexpression of AJUBA. However, only two of the parameters like tumor grade and inflammatory response was found to be statistically significant. On observing the histological tumor grade, we found that majority of **WDSCC cases (n= 23)** showed both **nuclear and cytoplasmic** immunoexpression **(38%, n= 16)** than compared to the MDSCC **(n= 13)** and PDSCC **(n= 6)** and this was found to be statistically significant with **p= 0.007**. We also found that majority of OSCC cases showed marked inflammatory cell response **(59%, n= 25)** and furthermore this was found to have significant association with nuclear and cytoplasmic expression of AJUBA with **p= 0.041**.

- **Table 4: Table showing the association of Demographic Data parameters with intensity of Immuno expression of AJUBA in OSCC.**

Demographic Data		Intensity			P-Value
		Low Intense	High Intense	Total	
Age	Less Than 40yrs	3 (7%)	4 (9%)	7 (16%)	0.272
	More Than or Equal To 40yrs	8 (19%)	27 (64%)	35 (19%)	
Sex	Male	10 (23%)	29 (69%)	39 (92%)	0.770
	Female	1 (2%)	2 (4%)	3 (7%)	
Habit	Present	11 (26%)	31 (73%)	42 (100%)	-
Site	Buccal Mucosa	3 (7%)	11 (26%)	14 (33%)	0.608
	Tongue	1 (2%)	2 (4%)	3 (7%)	
	Gingivo Buccal Sulcus	4 (9%)	7 (16%)	11 (26%)	
	Retro Molar Trigone	2 (4%)	6 (14%)	8 (19%)	
	Hard Palate	0	1 (2%)	1 (2%)	
	Alveolus	0	2 (4%)	2 (4%)	
	Mandible	0	2 (4%)	2 (4%)	
	Labial Mucosa	1 (2%)	0	1 (2%)	
Size	Absent	3 (7%)	1 (2%)	4 (9%)	0.50
	Less Than 10cm	3 (7%)	16 (38%)	19 (45%)	
	More Than 10cm	5 (11%)	14 (33%)	19 (45%)	
TNM	Absent	8 (19%)	23 (54%)	31 (73%)	0.660
	Stage 2	0	1 (2%)	1 (2%)	
	Stage 4 A	3 (7%)	5 (11%)	8 (19%)	
	Stage 4 B	0	2 (4%)	2 (4%)	

- **Table 4a: Table showing the association of Histopathological Parameters with intensity of Immuno expression of AJUBA in OSCC.**

Histological parameters		Intensity			P-Value
		Low Intense	High Intense	Total	
Depth of Invasion	2- Invasion Involving Lamina Propria	10 (23%)	0	10 (23%)	0.373
	3- Invasion Below Lamina Propria Involving Muscle, Gland, Periosteum	16 (38%)	5 (11%)	21 (50%)	
	4- Deep Invasion Involving Jaw Bone	10 (23%)	1 (2%)	11 (26%)	
Histograde	1- WDSCC	6 (14%)	17 (40%)	23 (54%)	0.810
	2- MDSCC	4 (9%)	9 (21%)	13 (30%)	
	3- PDSCC	1 (2%)	5 (11%)	6 (14%)	
Invasive Front	1- Pushing Border	1 (2%)	0	1 (2%)	0.361
	2- Infiltrative Solid Cords	2 (4%)	5 (11%)	7 (16%)	
	3- Small Groups or Cords of Infiltrative Cells (N >15)	6 (14%)	17 (40%)	23 (54%)	
	4- Wide Spread Cellular Dissociation (N < 15)	2 (4%)	9 (21%)	11 (26%)	
Stroma	1- Abundant	0	4 (9%)	4 (9%)	0.430
	2- Dense	8 (19%)	21 (50%)	29 (69%)	
	3- Delicate	3 (7%)	6 (14%)	9 (21%)	
Inflammation	1- Marked	4 (9%)	21 (50%)	25 (59%)	0.117
	2- Moderate	5 (11%)	5 (11%)	10 (23%)	
	3- Slight	2 (4%)	5 (11%)	7 (16%)	
Lympho Vascular Invasion	Negative	9 (21%)	23 (54%)	32 (76%)	0.610
	Positive	2 (4%)	8 (19%)	10 (23%)	
Surgical Margin	Negative	10 (23%)	28 (66%)	38 (90%)	0.955
	Positive	1 (2%)	3 (7%)	4 (9%)	
Lymph Node	Negative	20 (47%)	2 (4%)	22 (52%)	0.470
	Positive	16 (38%)	4 (9%)	20 (47%)	
Neural Invasion	Negative	9 (21%)	21 (50%)	30 (71%)	0.375
	Positive	2 (4%)	10 (23%)	12 (28%)	

Inference: We did not find any statistically significant association with intensity of immunoexpression of AJUBA with all the demographic and histological parameters included in our study. Though we did not find SS association but we observed that high intense (dark brown) staining was seen in majority of cases belonging to more than or equal to 40yrs **(64%, n= 27),** in males patients **(69%, n= 29),** chewing type of tobacco habit **(73%, n= 31),** buccal mucosa **(26%, n= 11)** Gingivo buccal sulcus **(16%, n= 7)** and tumor size less than 10cm **(38%, n= 16).** On analyzing the histopathological parameters, we found high intense immunoexpression of AJUBA in all histological grade, small groups or cords of infiltrative cells in the invasive front, dense stroma and marked inflammation. However, these results were not statistically significant with immunoexpression of AJUBA. Similarly, we did not find any significant association of AJUBA expression with other histological parameters like DOI, LN metastasis, surgical margins and neural invasion.

- **Table 5: Table showing the association of Demographic Data parameters with percentage of positivity of Immuno expression of AJUBA in OSCC.**

Demographic Data		% Of Positivity			p- Value
		50-75%	>75%	Total	
Age	Less Than 40yrs	6 (14%)	1 (2%)	7 (16%)	0.195
	More Than or Equal To 40yrs	34 (80%)	1 (2%)	35 (83%)	
Sex	Male	37 (88%)	2 (4%)	39 (92%)	0.688
	Female	3 (7%)	0	3 (7%)	
Habit	Present	40 (96%)	2 (4%)	42 (100%)	-
Site	Buccal Mucosa	13 (30%)	1 (2%)	14 (33%)	0.946
	Tongue	3 (7%)	0	3 (7%)	
	Gingivo Buccal Sulcus	11 (26%)	0	11 (26%)	
	Retro Molar Trigone	7 (16%)	1 (2%)	8 (19%)	
	Hard Palate	1 (2%)	0	1 (2%)	
	Alveolus	2 (4%)	0	2 (4%)	
	Mandible	2 (4%)	0	2 (4%)	
	Labial Mucosa	1 (2%)	0	1 (2%)	
Size	Absent	4 (9%)	0	4 (9%)	0.281
	Less Than 10cm	17 (40%)	2 (4%)	19 (45%)	
	More Than 10cm	19 (45%)	0	19 (45%)	
TNM	Absent	29 (69%)	2 (4%)	31 (73%)	0.863
	Stage 2	1 (2%)	0	1 (2%)	
	Stage 4 A	8 (19%)	0	8 (19%)	
	Stage 4 B	2 (4%)	0	2 (4%)	

- **Table 5a: Table showing the association of Histopathological Parameters with percentage of positivity of Immuno expression of AJUBA in OSCC.**

Histological parameters		% Of Positivity			P-Value
		50-75%	>75%	Total	
Depth Of Invasion	2- Invasion Involving Lamina Propria	10 (23%)	0	10 (23%)	0.620
	3- Invasion Below Lamina Propria Involving Muscle, Gland, Periosteum	20 (47%)	1 (2%)	21 (50%)	
	4- Deep Invasion Involving Jaw Bone	10 (23%)	1 (2%)	11 (26%)	
Histograde	1- WDSCC	22 (52%)	1 (2%)	23 (54%)	0.758
	2- MDSCC	12 (28%)	1 (2%)	13 (30%)	
	3- PDSCC	6 (14%)	0	6 (14%)	
Invasive Front	1- Pushing Border	1 (2%)	0	1 (2%)	0.383
	2- Infiltrative Solid Cords	6 (14%)	1 (2%)	7 (16%)	
	3- Small Groups or Cords of Infiltrative Cells (N >15)	23 (54%)	0	23 (54%)	
	4- Wide Spread Cellular Dissociation (N < 15)	10 (23%)	1 (2%)	11 (26%)	
Stroma	1- Abundant	4 (9%)	0	4 (9%)	0.574
	2- Dense	28 (66%)	1 (2%)	29	
	3- Delicate	8 (19%)	1 (2%)	9 (21%)	
Inflammation	1- Marked	23 (54%)	2 (4%)	25	0.383
	2- Moderate	10 (23%)	0	10 (23%)	
	3- Slight	7 (16%)	0	7 (16%)	
Lympho Vascular Invasion	Negative	31 (73%)	1 (2%)	32 (76%)	0.373
	Positive	9 (21%)	1 (2%)	10 (23%)	
Surgical Margin	Negative	36 (85%)	2 (4%)	38 (90%)	0.638
	Positive	4 (9%)	0	4 (9%)	
Lymph Node	Negative	21 (50%)	1 (2%)	22 (52%)	0.945
	Positive	19 (45%)	1 (2%)	20 (47%)	
Neural Invasion	Negative	29 (69%)	1 (2%)	30 (71%)	0.492
	Positive	11 (26%)	1 (2%)	12 (28%)	

Inference: We did not find any statistically significant association with percentage of positivity of immunoexpression of AJUBA with all the demographic and histological parameters included in our study.

No statistically significant association of immunoexpression of AJUBA in regarding to localisation, cellular location, intensity and percentage of positivity with most of the demographic and histological parameters. Only two of the parameters like histological tumor grade and inflammatory response found to have Statistically Significant association with cellular location of AJUBA

DISCUSSION

OSCC is a major public health problem and it ranks among the top ten cancer worldwide[2]. A variety of risk factors such as, poor oral hygiene, chronic irritation, viral infection, occupational exposure, malnutrition and genetic factors, have been proposed as etiological factors for the cause of oral cancer[93,94]. Though there are several modalities for treatment of OSCC but the prognosis remains to be poor with postoperative complications leading to difficulty in mastication, dysphagia, as well as speech and aesthetic issues, all of which can further leads to poor patient's quality of life.[94]

Morbidity and mortality rates are exceedingly high in OSCC despite the attainments achieved in its diagnosis and therapy. The overall survival rate has also remained to be only five years, this could be due to field cancerization, inadequate clearance of the tumor margins leading to local recurrence and metastasis and decreased in host immune response[2].

Thus, oral oncobiologist explored the reason for poor prognosis of oral cancer by understanding its molecular biology. Douglas Hanahan and Robert Weinberg published “The Hallmarks of Cancer in January 2000”, to understand the exact molecular mechanism as to how the cancer cells get mutated though the normal controlling mechanisms govern and how the cancer progression occurs. These hall marks include, sustaining proliferative signaling potential, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis.[96]

The researchers have reported molecules in these steps which are specific to genetic variations in tumor suppressor genes (APC, p53), proto-oncogenes (Myc), oncogene (Ras) and genes controlling normal cellular processes (EIF3E, GSTM1) that in turn undergo processes such as mutations in chromosomes, alterations in genomic copy number, loss of heterozygosity, telomere instabilities, dysregulations of cell-cycle checkpoints, DNA damage repairs and defects in notch signaling pathways are involved in causing oral cancer.[5]

One such molecule which has been recently discovered and known to have both tumor suppressor and tumor promoter role is AJUBA. AJUBA are a group of proteins which belong to "Zyxin/AJUBA family (AJUBA, WTIP, LIMD1) and are characterized by 3 tandem C-terminal LIM domain and unique n-terminal LIM region". The LIM-domains of AJUBA interacts with a large number of enzymes, cytoskeletal proteins, receptors etc. and known to participate in several signalling pathways.

AJUBA is a multifunctional protein involved in the development of embryo via taking part in epidermal germ layer stratification and differentiation.[56] In physiological conditions AJUBA participates in a diverse array of cellular processes such as cell-cell adhesion, cytoskeleton organization, localization of various cytosolic proteins, mitosis and cell cycle. Apart from the normal physiological functions AJUBA takes part in various pathological conditions such as in development and progression of cancer. The role of AJUBA in cell proliferation, tumorigenesis, tumor progression, invasion and metastasis and its oncogenic properties have been shown in various pathways like the Hippo pathway, Ras- MAPK pathway, Snail- dependent

repression of E- cadherin pathway, EMT and in pathways involved in DNA damage response.

Studies has shown oncogenic role of AJUBA as a tumor promoter in different types of human cancers including ESCC, colorectal carcinoma, gastric cancer, small cell lung cancer, breast cancer, cervical cancer and pancreatic cancer. However, others have reported it as a tumor suppressor in small cell lung cancer, malignant mesothelioma, prostate cancer and hepatocellular carcinoma and a multitude of mechanisms have been explained highlighting this complexity of the functions of AJUBA in the cancer context.

However, the literature regarding the role of AJUBA is limited to HNSCC, on literature search we found that in comprehensive genomic atlas of HNSCC, mutations has been noted in zyxin family of proteins linked to the AJUBA protein. It has been observed that 0- 6% of cases showed mutation of AJUBA protein and overexpression had led to increased cellular growth and proliferation of tumor cells of HNSCC[12]

AJUBA has been implicated in epithelial carcinomas such as SCC of skin and carcinoma involving the upper aerodigestive tract. ***Shi et al***[16]***, Zhang et al***[17] in ESCC and ***Xiaofeng Yao et al***[92] in OSCC showed that AJUBA was significantly overexpressed compared to the adjacent normal epithelium. They also corelated the high level of expression with various clinicopathologic parameters such as TNM staging, lymph node metastasis and recurrence. Thus, their findings revealed that AJUBA is a new biomarker for cancer targeted therapy.

The above studies mentioned is of other population with the individuals having more of smoking form of tobacco habit. As our study participants belong to

tobacco belt area and the common etiology being chewing form of tobacco. Thus, the expression profile of AJUBA in our population with OSCC cases still needs to be explored. Hence, the present study aimed to investigate and compare the immunoexpression of AJUBA in normal oral mucosa and OSCC and further we focused on evaluation of its association with various clinicopathological parameters.

In the present study 42 cases of normal mucosa and OSCC were evaluated for immunoexpression of AJUBA. On noting down the demographic parameters, we found that most of the patients were above the age of 40 years with male predominance. All the patients had habit history of chewing tobacco and the most common site of occurrence seen was in buccal mucosa and gingivobuccal sulcus followed by retromolar region, tongue etc.

The data suggested by IARC for peak age of incidence is 5^{th} to 6^{th} decade of life. However, ***Cathy babu et al***[97] ***in 2021*** has reported that a large number of OSCC cases develop a decade earlier in between the 4^{th} to 6^{th} decade of life. They also found age group belonging to above the age of 40yrs with males representing the highest occurrence rate than females. This study was in concordance with the demographic findings mentioned in our study. ***Malay Kumar et al***[34] in their study reported that OSCC is associated with tobacco history- smokeless (chewable) or smoked form, which is an important causative factor of cancer, followed by the most usual site of occurrence i.e., buccal mucosa and gingivo-buccal sulcus, tongue, gingiva etc. We also observed the tobacco habit being the main etiology of OSCC in our cases, moreover we found that all our cases had a history of tobacco chewing habit. Due to continuous keeping of tobacco in the form of quid in the buccal vestibule the common site noted in our cases being buccal mucosa and gingivo buccal sulcus.

As the role of AJUBA has been studied in cancer but its expression profile in normal tissue is limited. On noting down the immunoexpression of AJUBA in normal oral mucosa, we observed a low intense (light brown) immunoexpression of AJUBA in all our cases. Among different layers of the epithelium, immunoexpression was found predominantly in basal and supra basal layer of the epithelium and deeper part of the connective tissue component. Majority of the cases showed cellular location of AJUBA in the cytoplasm and nucleus in both the layers of the epithelium, however, few cases also showed membrane location of AJUBA. **(Photomicrograph 5 – 9 & Bar-graph: 1- 3**)

Concurring to our study the gene and protein expression of AJUBA in different human tissues including its molecular and biological functions have been described in a Human protein atlas.[66,67] Here, a low intense immunoexpression of AJUBA was seen, with intracellular location, which possibly might be suggesting its cytoplasmic and nuclear expression. Also, it was noted that the immunoexpression of AJUBA was limited to the basal and supra basal keratinocytes of oral mucosa.

AJUBA which is a cytosolic protein is found in several cellular components and has been implicated in taking part in various normal physiological role at cellular and biological events. Coupling with different signaling pathways through distinct interactions[13] with the pre-LIM and LIM regions it has the ability to shuttle between the nucleus and cytoplasm since its contains both NES and NLS regions[56].

Several studies[8,9,14,15,54,64,70,71,75,77,82,83] have shown functions of AJUBA as a key regulator in cell proliferation and differentiation through various signalling pathways. In a review by ***Krista Schleicher et al***[56] the regulatory mechanism of AJUBA in maintaining the epidermal homeostasis and also in cancer has been

described. AJUBA promotes cellular differentiation and stratification in basal layers by participating in the assembly of various signalling pathways such as NOTCH and NUMB. AJUBA causes sequestration of NUMB and thus, permits nuclear translocation of NICD to promote differentiation of the keratinocytes. One more pathway has been explained in the proliferating basal keratinocyte is the shutting down of the HIPPO signalling pathway. AJUBA acts as a negative regulator of HIPPO pathway by allowing the nuclear translocation of YAP/TAZ. Thus, all these pathways could possibly explain AJUBA's immunoexpression in basal and supra basal cell layers of the epithelium in normal physiological context.

In a study conducted by ***Helene Marie et al.***[14] on human keratinocyte the functional role of AJUBA in cell junction and cytoplasm has been explained where they showed that there is a regulated recruitment of LIM domain protein AJUBA to cadherin-dependent cell-cell adhesive complexes and it acts on the membrane of E-cadherin which is bound to α-catenin by its amino terminal. They also showed that AJUBA, through its association with catenin to cadherin adhesive complexes interacts with F-actin. Thus, suggesting a possible immunoexpression of AJUBA in cytoplasm and cell membrane.

AJUBA shuttling into the nucleus has been explained in several studies which have demonstrated that it has a key role in nuclear events such as in Mitosis and transcriptional repression. ***Meirong bai et al***[15] studied the novel regulatory function of AJUBA protein and its significant role in cell cycle process and suggested that when AJUBA interacted with Aurora-A it causes autophosphorylation of Aurora in a regulated manner and thus preventing excess entry of Aurora-A kinase during the G2 phase of cell cycle. In a study conducted by ***Zhaoyuan Hou et al***[82] it was

demonstrated that AJUBA by acting as a classic corepressor of RA signalling pathway modulates and negatively regulates the pathway. They also observed that when AJUBA protein was absent, there was a decrease of cell population in the G0/G1 phase, whereas in S-phase the cell population increased. Hence its nuclear expression may be justified by the fact that it plays a key role in cell cycle regulation through this function. Based on the above literature support we hypothesize that AJUBA do have physiological role in normal oral mucosal epithelium and is required for its prime role in epithelial proliferation and differentiation for maintaining its stratification status. The expression of AJUBA is less intense this could be due to downregulation of AJUBA molecule after completion of its physiological function. Our observation regarding expression profile of AJUBA is first of its kind and proved to have a role in normal physiological function of normal oral mucosal epithelium.

On evaluation of AJUBA immunoexpression in OSCC cases we observed an enhanced immunoexpression in all the cases. We found that there was no difference in the immunoexpression at the superficial and invasive front of the tumor. Majority of our cases showed immunoexpression of AJUBA in the periphery of the tumor islands compared to the central portion and percentage of positivity was noted as 50-75%. Our results were in concordance with various other studies[16,17,21,22] conducted on systemic malignancies where they noted overexpression of AJUBA in the tumorous tissue. All these have suggested that AJUBA plays a key role in tumor promotion and tumor growth. **(Photomicrographs 10 – 17 & Bar-graph: 4 & 5**)

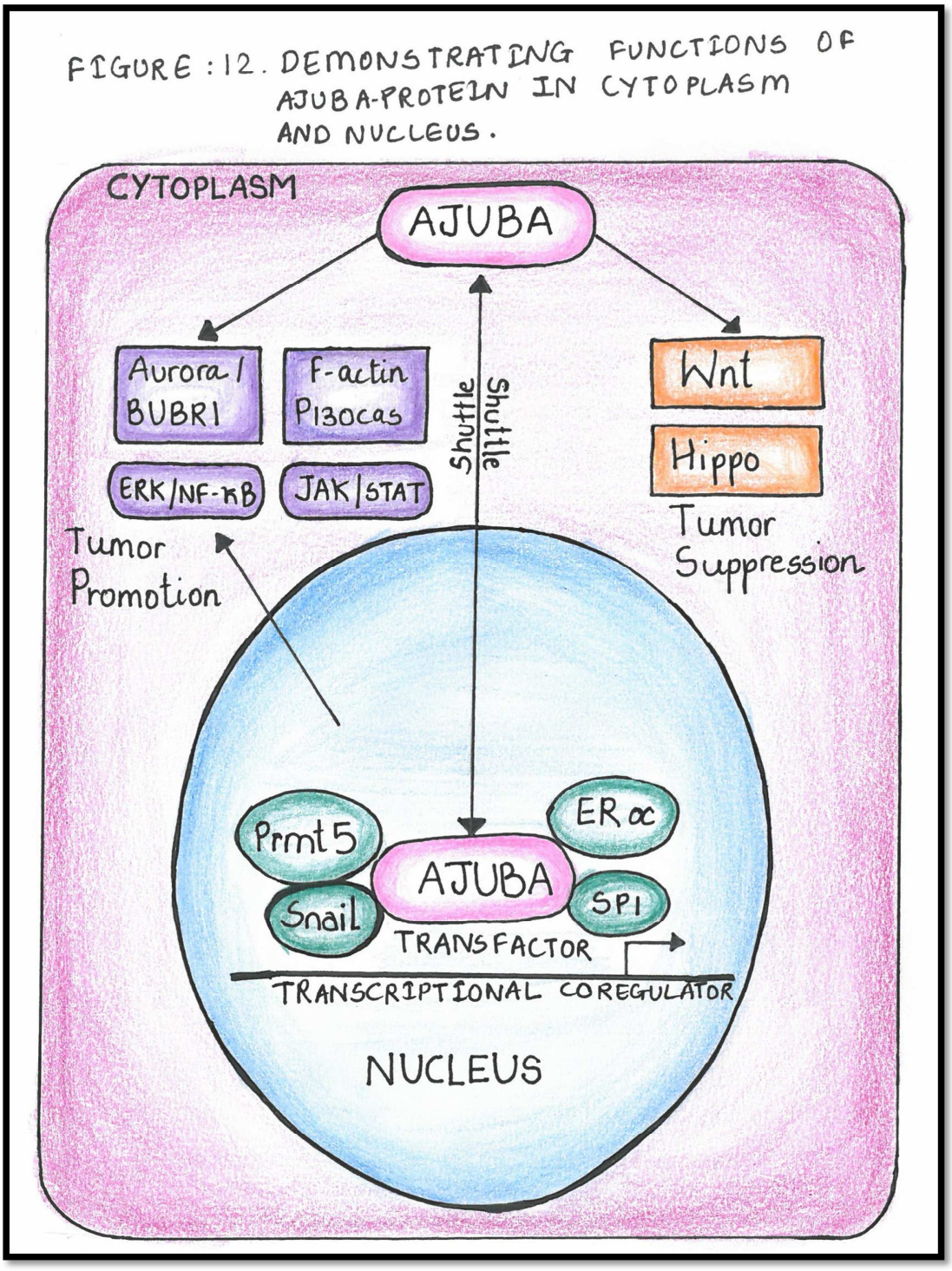
Role of AJUBA in cancer as an oncogene or a tumor suppressor remains controversial although majority of the studies supports tumor promotion action and a few studies are in favor of AJUBA as a tumor suppressor. In different cancer types varied functional role of AJUBA has been explained.

AJUBA acts as an oncogene in ESCC[16] by activation of the MAP/ERK 1/2 pathway by upregulating the levels of MMP 10 and MMP13 thus, promoting ESCC growth. It also acts as a newer therapeutic target. Role of AJUBA is implicated in EMT pathways, in a study done on colorectal cancer[19] role of AJUBA has been demonstrated on SNAIL dependent EMT, SNAIL acts as a classic corepressor of AJUBA which further interacts with the SNUG domain of SNAIL thereby causing E-cadherin downregulation which is an important molecule of EMT. Thus, by regulating the EMT pathway in colorectal carcinoma it promotes tumor cell migration and can also act as a cancer targeted therapy in metastatic CRC.

It was also noted that in malignant mesothelioma[27] AJUBA acts as a tumor growth suppressor by its action on the HIPPO pathway. It was seen to interact with LATS1/2 there by prevention phosphorylation of YAP. In contrary to this, YAP gets translocated in the nucleus and causes transactivation of CD1 and cause development of various tumors and aids in tumor progression[27]. Hence based on the above literature support the overexpression of AJUBA in OSCC could be due to its dual role of tumor promoter and tumor suppressor activity. But the exact role of AJUBA in OSCC still needs to be validated with other molecular techniques.

On analyzing cellular location of AJUBA in tumor cells majority of our cases showed both nucleus and cytoplasmic location. However, we observed that only few of our cases showed nuclear, cytoplasmic and membrane immunoexpression of

AJUBA in tumor cells of OSCC. These findings are similar to the studies conducted by ***Shi et al.***[16] and ***Zhang et al.***[17] on ESCC, where they noted a enhance nuclear and cytoplasmic immunoexpression of AJUBA.



Nuclear and cytoplasmic expression of AJUBA in cancer may be attributed to its various adaptive and multifunctional role and its property of shuttling between cytoplasm and nucleus. In cytoplasm of tumor cell AJUBA has an oncogenic role as it regulates major signalling pathways such as Wnt, RAS/ERK and HIPPO. Thus, propagates signals from cytoplasm to nucleus. In the nucleus of Cancer cell, it acts as a key co-regulator of various transcriptional factor such as "SNAIL, SP1 and nuclear hormone receptors." Thus, AJUBA's role in all these pathways, could possibly explain its enhanced immunoexpression in nucleus and cytoplasm of the tumor cells.

On assessing clinicopathological parameters with AJUBA expression in OSCC we did not find any statistically significant association of AJUBA in most of the parameters regarding to its localization, cellular location, intensity and percentage of positivity, this could be due to unequal distribution of sample**. (Table: 2- 5)**

These results were similar to the studies conducted on other systemic cancers like ESCC[16,17] breast cancer and gastric cancer[90] where similar results were found suggesting that the expression profile of AJUBA did not have any impact on demographic data.

On evaluation of clinical data such size of the tumor and TNM staging with all the parameters of immunoexpression of AJUBA, in contrary to our results studies done[16,17,19,92] showed that there is a statistically significant association with increased tumor size and stage III & IV of TNM staging. These results suggest that overexpression of AJUBA is corelated with decreased survival and poor prognosis.

We found only two of the histological parameters like inflammatory response and histological tumor grade to have statistically significant association with cellular

location of AJUBA expression. We observed marked inflammatory response and found to be significantly associated with cellular location of immunoexpression of AJUBA. In a study conducted by ***Grivennikov et al***[98] in colorectal cancer and chronic inflammation their findings suggested that AJUBA is a specific suppressor of interferon signalling and has a targeted role in inflammation. This possibly explains the association of AJUBA with inflammatory response in cancerous tissues. Further, role of AJUBA and tumor associated inflammation in OSCC needs to be explored. **(Table: 3a)**

In histological tumor grade, we found that majority of **WDSCC cases** showed both **nuclear and cytoplasmic** immunoexpression than compared to the MDSCC and PDSCC and this was found to be statistically significant. This was in contrary with ***Shi et al.***[16] who corelated increased immunoexpression of AJUBA with high histological grade of ESCC. The role of AJUBA in tumor differentiation still needs to be validated in OSCC as our sample size had an unequal distribution.

It has been observed that overexpression of AJUBA was significantly associated with depth of invasion and LN metastasis, decreased survival rate and poor prognosis[16,17,90]. Though we did not find any statistically significance with these prognostic parameters, but as per the literature the molecule AJUBA has the propensity in upregulation of MMP 10 or 13 and YAP molecules for the tumor cells to pay the way for lymph node metastasis. But surprisingly we also observed node negative cases of OSCC also revealed enhance expression of AJUBA. Thus, for the mechanism of role of AJUBA probably requires several other molecular interactions which was out of context for this study.

We compared immunoexpression of AJUBA in OSCC cases with NOM, an enhanced immunoexpression of AJUBA was observed in all cases of OSCC which showed dark brown staining suggestive of high intensity compared to NOM which showed low intense staining (light brown). These results were statistically significant and were in concordance with various other systemic malignancies[16,17,19,20,21,22,24], which showed overexpression of AJUBA in the cancerous tissue when compared with the adjacent non- tumors tissue or the normal epithelium. **(Bar-graph: 6 & Table: 1)**

In a study done on hepatocellular carcinoma it was shown that overexpression of AJUBA inhibits cancer cell proliferation by diminishing YAP levels and β-catenin. A study on prostate cancer metastasis it was shown that loss of AJUBA resulted in enhanced migration of the tumor cells. Suggestive of its tumor protective action.

In contrary to our results few studies have also shown negative expression of AJUBA[7,25,27,28]. In all these it has been demonstrated that over expression of AJUBA causes inhibition of the cellular growth, differentiation and migration by modulating several cellular components and proteins.

All these findings possibly hypothesize that the diverse role of AJUBA is more of tissue specific and cancer context dependent, where over or under expression of AJUBA leads to either tumorigenesis, tumor promotion, metastasis or tumor suppression.

Moreover, we also observed AJUBA expression in other connective tissue components of normal oral mucosa and OSCC like muscle, endothelial lining of small and large blood vessels, nerve and salivary gland component **(Photomicrographs 18 - 25).** Similarly other authors have also found its expression in the above-mentioned

connective tissue component. ***William Razzell et al***[98], observed expression of AJUBA in the Muscle and located its expression at the nucleus of the myocytes and stated that AJUBA junctional localization is regulated by actomyosin contractility in the muscle. In a study conducted by ***Masayuki Tsuneki et al***[100] on infantile hemangiomas an increased immunoexpression of AJUBA was noted in the endothelial cells in the proliferative phase of the tumor. In concordance with these finding we also noted increased expression of AJUBA in the endothelial lining of capillaries and blood vessels within the tumor. Thus, possibly suggesting its role in tumor angiogenesis and this needs to be further explored.

Hence, we hypothesize that as AJUBA is upregulated in OSCC cases compared to Normal Mucosa and is known to be a novel biomarker having a multifunctional role in cancer and a potential target for cancer targeted therapy, future studies should be conducted focusing on exploring molecular mechanism of AJUBA in cancer with various other techniques.

SUMMARY AND CONCLUSION

Oral squamous cell carcinoma is a major public health problem and it ranks among the top ten cancer worldwide. Cancer is associated with uncontrolled cell growth and are multi-factorial in origin and aetiology. Though there are several modalities for treatment of oral squamous cell carcinoma but the prognosis remains to be poor. The researchers have reported certain molecules which specifically mutated or dysregulated like the p53, APC, Myc, Ras etc. One such novel protein known to be mutated belongs to Zyxin family of proteins, named AJUBA.

AJUBA is a cytosolic protein and has the ability to shuttle between the nucleus and cytoplasm. It is a multifunctional scaffold protein which takes part in several physiological conditions such as cell-cell adhesion, cytoskeleton organization, localization of various cytosolic proteins, mitosis and cell cycle. Apart from the normal physiological functions recent studies have also noted its role in various systemic malignancies. AJUBA is seen to be associated with tumor progression, growth, migration and in few malignancies also as a tumor suppressor.

However, the literature regarding the role of AJUBA is limited to HNSCC. As per the report on comprehensive human genomic atlas it has been observed that 0- 6% of HNSCC cases showed mutation of AJUBA protein and overexpression had led to increased cellular growth and proliferation of tumor cells of HNSCC, however the literature regarding its immunoexpression profile and significance is limited in normal oral mucosa and oral squamous cell carcinoma. Hence, the present study aimed at evaluating the role of AJUBA in normal oral mucosa and oral squamous cell carcinoma.

In our study we included histologically proven cases of normal oral mucosa (n=42) were taken as a control group and the study group comprised of oral squamous cell carcinoma (n=42) cases and all the cases were subjected to IHC for detection of AJUBA. The parameters considered for evaluation of expression of AJUBA in normal oral mucosa was level of expression in epithelium and connective tissue, cellular location and intensity and in oral squamous cell carcinoma cases localization in tumor islands, cellular location in tumor cells, intensity and percentage of positivity. After assessing the immunoexpression of AJUBA, frequency percentage was considered for some parameters and Chi square test was done considering the CI 95% with $p<0.05$.

The current investigation revealed that:

- **In normal oral mucosa**, we found light brown indicating low intense immunoexpression of AJUBA in all our cases. The expression was limited to basal and supra basal layer **(78%, n= 33)** of the epithelium and also in the deeper part of the connective tissue component **(42.86%, n= 18)** The cellular location in majority of the cases was seen both in nucleus and cytoplasm (**54.76%, n= 23)**. Based on this observation we would like to hypothesize that AJUBA do have physiological role in normal oral mucosal epithelium and is required for its prime role in epithelial proliferation and differentiation for maintaining its stratification status. The expression of AJUBA is less intense this could be due to downregulation of AJUBA molecule after completion of its physiological function. Our observation regarding expression profile of AJUBA is first of its kind and proved to have a role in normal physiological function of normal oral mucosal epithelium.

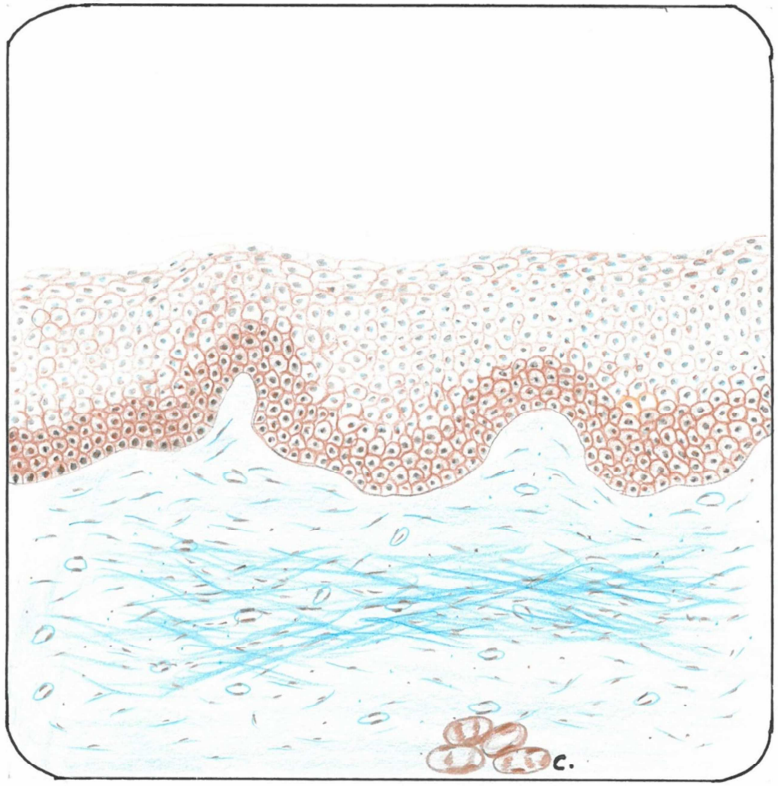
- **In oral squamous cell carcinoma** case, we found enhance dark brown staining indicating high intense immunoexpression of AJUBA than compared to normal oral mucosa and this found to be statistically significant with $p<0.005$. We also observed that in all our cases of oral squamous cell carcinoma we found 50-75% of positivity of expression of AJUBA. We also noted that expression of AJUBA predominantly seen at the periphery of the tumor islands **(85.71%, n= 36)** and cellular location of AJUBA was seen within the nucleus and cytoplasm of the tumor cells **(59.52%, n= 25).** We did not find statistically significant of AJUBA expression with most of the demographic parameters and histological parameters (**Table 2 - 5a**), this could be due to unequal distribution of sample. Only histological grade and inflammatory response revealed statistically significant with cellular location of AJUBA expression (**Table 3a**). Based on our observation we would like to hypothesize that the molecule AJUBA which is known to have double sword role in its tumor suppression and tumor progression is also true in oral cancer.

CONCLUSION:

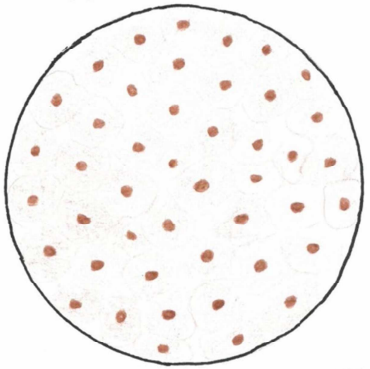
Our study is first of its kind in detecting AJUBA expression in NOM. Thus, the molecule AJUBA do have physiological role in normal oral mucosal epithelium and is required for its prime role in epithelial proliferation and differentiation for maintaining its stratification status. It enhances expression profile in all our cases of oral squamous cell carcinoma signifies that the molecule AJUBA do have significant role in oral carcinogenesis. Though we found significant association of AJUBA with histological tumor grade and inflammatory response, but the exact mechanism of its role in tumor differentiation and tumor immune response needs to be further explored. Hence, we would like to conclude that the molecule AJUBA has a definitive role in oral cancer growth and progression and also, we would like to propose that the AJUBA is novel biomarker for future cancer targeted therapy.

FIGURE.13: LOW INTENSE [LIGHT BROWN]

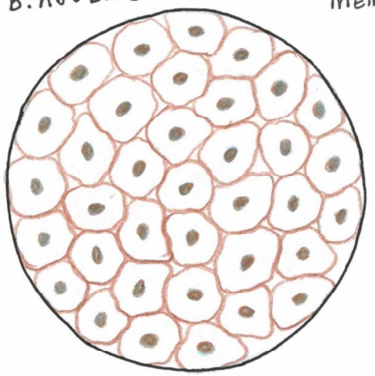
IMMUNOEXPRESSION OF AJUBA IN NORMAL ORAL MUCOSA



A. AJUBA IMMUNOEXPRESSION IN NUCLEUS AND CYTOPLASM



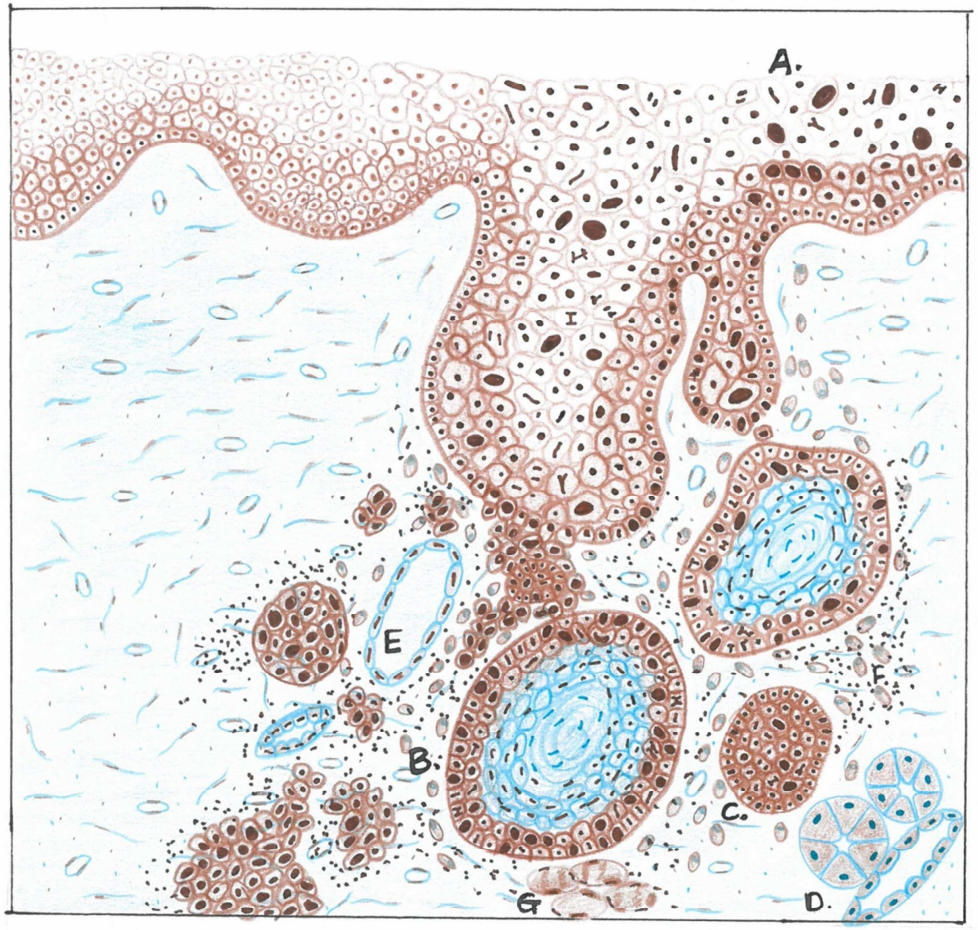
B. AJUBA IMMUNOEXPRESSION IN MEMBRANE.



C. IMMUNOEXPRESSION OF AJUBA IN THE MUSCLE

FIGURE 14:

IMMUNO EXPRESSION OF AJUBA IN ORAL SQUAMOUS CELL CARCINOMA



A. OSCC TISSUE SHOWING HIGH INTENSE EXPRESSION OF AJUBA

B. PERIPHERAL CELLS OF TUMOR ISLANDS SHOWING AJUBA EXPRESSION

C. CELLULAR LOCATION OF AJUBA IS SEEN IN NUCLEUS & CYTOPLASM OF TUMOR CELLS.

D. SALIVARY GLAND DUCT SHOWING AJUBA EXPRESSION

E. ENDOTHELIAL LINED BLOOD VESSELS SHOWING AJUBA EXPRESSION

F. INFLAMMATORY CELLS SHOWING AJUBA EXPRESSION

G. IMMUNO EXPRESSION OF AJUBA IN MUSCLE.

LIMITATIONS

The main limitation of our study is unequal distribution of sample between the parameters, due to this we were not able to establish association of AJUBA expression with most of the clinicopathological parameters. As the immunoexpression of AJUBA was found to be shuttling between nucleus, cytoplasm and membrane of normal and tumor cells, we were not able to quantify its expression with the available software's like ImageJ. Though we could observe enhance expression profile of AJUBA in all our OSCC cases, but we were not able to derive its exact role in oral cancer.

FUTURE SCOPE

The expression profile of AJUBA has been detected in normal oral mucosa and OSCC, but still our observation needs to be further validated with larger sample size with equal distribution of sample between all the parameters. As there is no literature regarding the expression of AJUBA, further studies should be carried out in evaluating the expression of AJUBA in all the potentially malignant disorders, so as to detect the cancer progression at early stage and to aid accurate treatment approaches. Moreover, the role of molecule AJUBA still needs to be established through other advanced molecular techniques so as to reach this molecule as a target for therapy in OSCC.

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ANNEXURE I- ETHICAL CLEARANCE LETTER

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
Nehru Nagar, Belagavi - 590 010, Karnataka State
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Web: http://www.kledental-bgm.edu.in
E-mail:principal@kledental-bgm.edu.in

Sl. No. : 1322

CERTIFICATE

This is to Certify that the synopsis titled

IMMUNOHISTOCHEMICAL EVALUATION OF AJUBA PROTEIN IN ORAL SQUAMOUS CELL CARCINOMA AND NORMAL MUCOSA.

Submitted by

Dr. PRIYANKA · DESAI *P. G. Student /*

Staff, Guided by DR · NANJULA · M · *from Department of* ORAL PATHOLOGY & MICROBIOLOGY *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date :

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

MEMBER SECRETARY
Research and Ethical Committee
KLEVK Institute of Dental Sciences
BELAGAVI.

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Research and Ethical Committee
KLE VK Institute of Dental Sciences
Belgaum

ANNEXURE II- WAIVER FORM

Waiver form

Department of Oral and Maxillofacial Pathology and Oral Microbiology, KAHER VK

Institute of Dental Sciences, Nehru Nagar, Belagavi.

"EVALUATION OF IMMUNOEXPRESSION OF AJUBA PROTEIN IN NORMAL ORAL MUCOSA AND ORAL SQUAMOUS CELL CARCINOMA"

Waiver of informed consent form

It is not feasible to obtain individual informed consent of donors of specimens used in this study. However, I assure that confidentiality of the participant information will be ensured and no identifying information related to the study participants will be disclosed in any report/ publication arising from the study.

Post Graduate
Dr. Priyanka Desai
REG NO: IH0219002
Department of Oral and Maxillofacial Pathology and Oral Microbiology

Guide
Dr. Manjula M M.D.S
Reader
Department of Oral and Maxillofacial Pathology and Oral Microbiology

ANNEXURE III- BIOSTATISTICS CLEARANCE CERTIFICATE

KLE V.K. Institute of Dental Sciences
(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA
Re-Accredited 'A' grade by NAAC (2nd Cycle) & Placed in Category 'A' by MHRD (GoI)
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Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation / Research work of **Dr. Priyanka Desai, Post Graduate Student**, under the guidance of **Dr. Manjula** M.D.S, **Reader, Department of Oral and Maxillofacial Pathology and Oral Microbiology** entitled **"Evaluation of immunoexpression of AJUBA in normal oral mucosa and oral squamous cell carcinoma"** has been done under my guidance and considered satisfactory.

04/01/22

Place: Belagavi
Date:

Name & Signature of Biostatistician

DEPARTMENT OF EPIDEMIOLOGY & BIOSTATISTICS*KAHER'S BELAGAVI

ANNEXURE IV- PREPARATION OF APES (3- AMINO PROPYL TRIETOXYSALINE) COATED GLASS SLIDES:

1. Clean dried glass were dipped in 1 % APES in acetone.
2. Slides were drained and dipped in acetone
3. Slide were drained again and dipped in dipped in distilled water.
4. Slides were then placed in a rack and allowed to dry.
5. Slides can then be stored and used as required.

ANNEXURE V- HEMATOXYLIN AND EOSIN STAINING TECHNIQUE (REGRESSIVE)

1. Sections were deparrafinized by warming on slide warmer for 10 min and passed through Xylene I and Xylene II for 10 min each.
2. Slides were passes through 90 % and 70% alcohol for 5 min each.
3. Slides were rehydrated by keeping in running water for 10 min.
4. Slides were dipped in Harris hematoxylin for 3 minutes 30 seconds.
5. Slides were kept in water wash for 2-3 min.
6. Slides were differentiated by 1 dip in 1% acid alcohol
7. Slides were kept in water wash for 10 min.
8. Bluing was done by keeping the slides in lithium carbonate for 5-8 min and then water wash for 10 min
9. Slides were stained with eosin 10 sec.
10. Slides were passed through increasing grades of alcohol, 70% and 90% for 5 sec each.
11. Slides were dried, cleared in xylene and mounted.

ANNEXURE VI- BUFFER PREPARATION METHOD

Tris Buffer: This was used for heat-induced epitope retrival (HIER) to unmask the antigen binding sites in the tissues.

1. Tris buffer- 1.21gm
2. EDTA- 0.37gm

The salts were dissolved and the volume was made up to 1000ml by adding distilled water. The ph of the solution was maintained at 8.5 to 9.

Phosphate buffered saline: It is used as wash buffer with pH ranging from 7.2-7.6. The preparation formula is as follows:

1. Sodium Chloride (NaCl) – 3.2 gm
2. Dipotassium hydrogen phosphate (K_2HPO_4): 0.484 gm
3. Potassium dihydrogen phosphate ($KH_2\ PO_4$) :0.144 gm
4. Dissolve the salts in 500 ml of distilled water.
5. The solution can be stored in a clean amber colored bottle in the refrigerator for a week.

ANNEXURE VII- DEMOGRAPHIC DATA OF OSCC CASES

		DEMOGRAPHIC PARAMETERS					
Sl. No	**Biopsy No**	**Age**	**Sex**	**Habit**	**Site**	**Size**	**TNM**
1	3716/11	2	1	1	3	2	5
2	3828/12	2	1	1	3	2	0
3	3840/12	2	1	1	3	2	0
4	3885/12	2	1	1	1	2	5
5	4003/12	2	1	1	3	1	5
6	4072/12	2	1	1	6	2	0
7	4088/12	2	2	1	6	1	5
8	4179/13	2	1	1	1	2	3
9	4281/13	1	1	1	4	1	0
10	4373/13	2	1	1	3	1	5
11	4410/14	2	1	1	3	0	5
12	4443/14	2	1	1	1	2	0
13	4448/14	1	1	1	3	2	0
14	4455/14	2	2	1	4	1	0
15	4585/14	2	1	1	7	1	0
16	4587/14	2	1	1	1	2	0
17	4598/14	2	1	1	1	1	0
18	4694/14	2	1	1	1	0	0
19	4706/15	1	1	1	3	0	0
20	4711/15	2	1	1	4	0	0
21	4712/15	1	1	1	1	2	5
22	4759/15	2	1	1	3	2	0
23	4807/15	2	2	1	3	2	0
24	4814/15	2	1	1	7	2	0
25	4820/15	1	1	1	1	2	0
26	4914/15	2	1	1	1	1	0
27	4951/15	1	1	1	8	1	0
28	5009/16	2	1	1	1	1	0
29	5036/16	2	1	1	5	1	0
30	5045/16	2	1	1	2	1	0
31	5109/16	2	1	1	4	1	0
32	5250/17	2	1	1	1	2	5
33	5341/17	2	1	1	1	2	0
34	5420/17	2	1	1	4	1	0
35	5440/17	2	1	1	3	1	0
36	5441/17	2	1	1	4	2	0
37	5454/17	2	1	1	1	2	0

38	5696/18	2	1	1	2	1	6
39	5913/19	2	1	1	1	1	6
40	6048/20	1	1	1	4	1	0
41	6138/20	2	1	1	2	1	0
42	6213/21	2	1	1	4	2	0

Age	**Grade**
Less than 40 yrs	1
More than 40 yrs	2
Sex	**Grade**
Male	1
Female	2
Site	**Grade**
Buccal mucosa	1
Tongue	2
Gingivo buccal sulcus	3
Retro molar trigone	4
Hard palate	5
Alveolus	6
Mandible	7
labial mucosa	8
Habit	**Grade**
Present	1
Absent	0
TNM staging	**Grade**
Stage 0	1
Stage 1	2
Stage 2	3
Stage 3	4
Stage 4 A	5
Stage 4 B	6
Stage 4 C	7

CINICOPATHLOGICAL DATA OF OSCC CASES

Sl. No	Biopsy No	DOI	Histo	Invasive front	Stroma	Inflam	Lympho vascular invasion	Surgical margin	LN	Neural invasion
1	3716/11	2	1	3	1	1	0	0	1	0
2	3828/12	2	2	3	3	2	0	0	1	0
3	3840/12	2	2	2	2	2	0	0	1	0
4	3885/12	2	1	1	2	1	0	0	0	0
5	4003/12	4	1	3	2	1	1	0	1	1
6	4072/12	3	2	3	2	1	1	0	1	0
7	4088/12	4	2	4	2	2	1	1	0	1
8	4179/13	4	3	4	2	3	1	0	1	1
9	4281/13	3	2	3	2	1	0	0	1	1
10	4373/13	3	1	2	2	1	0	0	0	0
11	4410/14	4	1	2	2	1	0	0	0	0
12	4443/14	3	1	3	2	3	1	1	0	1
13	4448/14	3	1	3	2	1	0	0	0	0
14	4455/14	2	1	3	2	2	0	0	0	0
15	4585/14	3	3	4	2	1	0	0	0	0
16	4587/14	3	2	3	3	2	0	0	1	0
17	4598/14	3	3	3	2	2	1	0	1	1
18	4694/14	3	2	3	2	1	0	0	0	0
19	4706/15	3	1	3	2	1	0	0	1	0
20	4711/15	2	1	3	2	3	0	0	0	0
21	4712/15	4	3	4	3	3	1	0	1	1
22	4759/15	4	1	4	2	1	0	0	1	1
23	4807/15	3	1	3	2	1	0	0	1	0
24	4814/15	4	1	2	1	1	0	0	0	0
25	4820/15	4	1	4	3	2	0	0	0	0
26	4914/15	4	1	2	3	1	0	0	0	0
27	4951/15	4	2	4	2	1	0	0	0	0
28	5009/16	2	1	2	1	1	0	0	0	0
29	5036/16	3	1	3	2	1	0	0	0	0
30	5045/16	3	1	3	2	2	1	1	0	1
31	5109/16	2	1	3	3	3	0	0	0	0
32	5250/17	3	3	4	3	3	1	0	1	1
33	5341/17	3	2	3	3	2	0	0	1	0
34	5420/17	3	1	4	2	1	0	0	1	0
35	5440/17	3	2	3	2	1	0	0	0	0
36	5441/17	2	1	2	2	2	0	0	0	0
37	5454/17	3	2	3	2	1	0	0	1	0

38	5696/18	2	2	3	2	1	0	0	0	0
39	5913/19	4	3	4	3	3	0	1	1	1
40	6048/20	3	2	4	2	1	1	0	1	1
41	6138/20	3	1	3	1	1	0	0	0	0
42	6213/21	3	1	3	2	1	0	0	1	0

Depth of invasion	1- CA in situ 2- Invasion involving lamina propria 3- Invasion below lamina propria involving muscle, gland, periosteum 4- Deep invasion involving jaw bone
Histo grade	1- WDSCC 2- MDSCC 3- PDSCC
Invasive front	1- Pushing border 2- Infiltrative solid cords 3- Small groups or cords of infiltrative cells (n >15) 4- Wide spread cellular dissociation (n < 15) 5- Tumor satellite's
Type of Stroma	1- Abundant 2- Dense 3- Delicate 4- None
Extent of inflammation	1- marked 2- moderate 3- slight 4- none
Lymph vascular invasion	1- positive 0- negative
Surgical margins	1- positive 0- negative
Lymph node metastasis	1- positive 0- negative

ANNEXURE VII- MASTER CHART OF NORMAL ORAL MUCOSA CASES

Sl. No	OP No	Level of AJUBA expression in epithelial layers	Level of expression of AJUBA in connective tissue	Cellular location of AJUBA In epithelium	Intensity
1	1711	2	0	2	1
2	1715	2	2	4	1
3	1716	2	0	4	1
4	1803	2	2	2	1
5	2638	2	2	4	2
6	3262 A	2	0	4	1
7	3262 B	2	2	4	1
8	3268	2	0	4	1
9	3848	2	0	4	1
10	3849 A	1	0	2	1
11	3849 B	1	0	2	1
12	3855	2	2	4	1
13	3856	2	1	4	2
14	3860	2	0	4	2
15	4227	2	0	4	1
16	4228	2	0	2	1
17	4235	2	2	2	1
18	4470	1	0	4	1
19	4471	1	0	4	2
20	4472 A	2	0	4	2
21	4472 B	2	0	2	1
22	4507	2	2	4	1
23	4510	2	0	4	2
24	4529	2	1	1	1
25	4571	2	0	4	2
26	4771 A	3	2	1	2
27	4771 B	3	2	1	2
28	4807	2	2	2	1
29	5383	2	2	4	2
30	6236	2	2	2	1
31	6248	2	0	2	1
32	6249	2	0	2	1
33	6257 A	2	0	5	1
34	6257 B	3	2	4	2

35	6258	2	2	2	1
36	6270	3	0	2	2
37	6271	2	0	5	2
38	6276	3	0	4	2
39	6313	2	2	4	2
40	6331	2	2	4	1
41	6350	2	2	2	1
42	6364	2	2	4	2

Level of expression of AJUBA in epithelium layers	**GRADE**
Basal (B)	1
B+Suprabasal (SB)	2
B+SB+Superficial (SU)	3
Level of expression of AJUBA in the connective tissue	**GRADE**
Juxta epithelial	1
Submucosal	2
Deeper epithelium	3
Localization of AJUBA in epithelium	**GRADE**
Nucleus (N)	1
Cytoplasm (C)	2
Membrane (M)	3
N+C	4
N+C+M	5
Intensity of AJUBA	**GRADE**
No staining	0
Light brown	1
Dark brown	2
Absent	0

ANNEXURE VII- MASTER CHART OF OSCC CASES

	IHC ANALYSIS				
Sl. No	**Expression of AJUBA in Tumor**	**Localisation of AJUBA in tumor islands**	**Cellular location of AJUBA in tumor cells**	**Intensity**	**Percentage of Positivity**
1	3	1	4	2	3
2	3	1	4	1	3
3	3	1	4	1	3
4	3	1	4	1	3
5	3	1	4	2	3
6	3	3	4	2	3
7	3	1	4	2	3
8	3	1	2	2	3
9	3	1	4	2	3
10	3	1	4	2	3
11	3	1	4	1	3
12	3	1	4	2	3
13	3	1	2	2	3
14	3	1	5	1	3
15	3	1	2	2	3
16	3	3	2	1	3
17	3	1	2	2	3
18	3	1	2	2	3
19	3	3	2	1	3
20	3	1	2	1	3
21	3	1	2	1	3
22	3	1	4	2	3
23	3	1	2	2	3
24	3	1	4	2	3
25	3	1	4	2	3
26	3	3	4	2	4
27	3	1	2	1	3
28	3	1	4	2	3
29	3	1	4	2	3
30	3	1	4	1	3
31	3	1	2	2	3
32	3	3	2	2	3
33	3	1	2	2	3

34	3	1	4	2	3
35	3	1	4	2	3
36	3	1	4	2	3
37	3	1	4	2	3
38	3	1	4	2	3
39	3	1	2	2	3
40	3	1	4	2	4
41	3	3	4	2	3
42	3	1	5	2	3

Expression of AJUBA in tumor	**Grade**
Superficial front	1
Invasive front	2
S+I	3
Localization of AJUBA in tumor islands	**Grade**
Peripheral	1
Central	2
P+C	3
Cellular location of AJUBA in the tumor cells	**Grdae**
Nuclear (N)	1
Cytoplasm (C)	2
Membrane (M)	3
N+C	4
N+C+M	5
Intensity	**Grdae**
No stain	0
Light brown	1
Dark brown	2
% of Positivity	**Grade**
<25%	1
25-50%	2
50-75%	3
>75%	4