SRI VENKATESWARA INTERNSHIP PROGRAM FOR RESEARCH IN ACADEMICS (SRI-VIPRA)

SRI-VIPRA

PROJECT REPORT - 2332

Evaluation of Eyelid Tumours And Their Metastatic Potential Using CAM Assay

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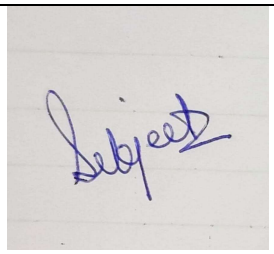
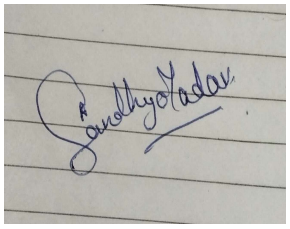
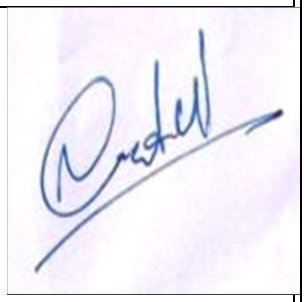
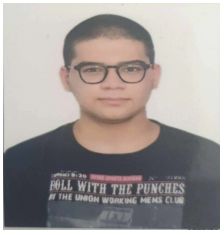
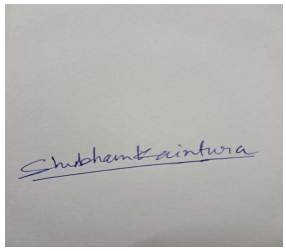
1	Debjeet Ray	B.Sc (H) Zoology	2021007		
2	Sandhya Yadav	B.Sc.(P) Life Science	1121112		
3	Mridul	B.Sc.(H) Biological Science	1321036		
4	Shubham kaintura	B.Sc. (P) Life Science	1121174		
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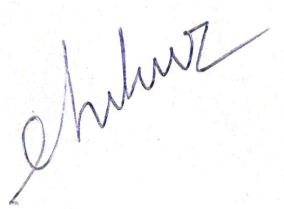
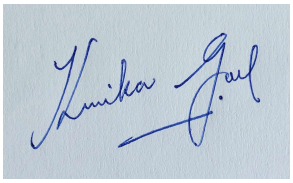
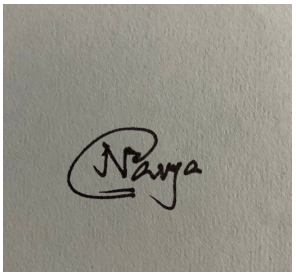
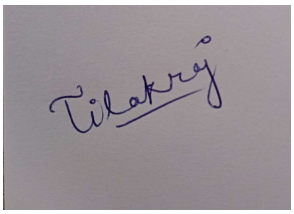
6	Ankur Verma	B.Sc.(H) Biological Science	1321039		
7	Kevika Goel	B.Sc.(H) Biological Science	1321029		
8	Navya Nandini Tripathi	B.Sc.(H) Biochemistry	1221028		
9	Tilak Raj	B.Sc.(H) Zoology	2021035		

Signature of Mentor

Certificate of Originality

This is to certify that the aforementioned students from Sri Venkateswara College have participated in the summer project SVP-2332 titled ***EVALUATION OF EYELID TUMOURS AND THEIR METASTATIC POTENTIAL USING CAM ASSAY.***

The Participants have carried out the research project work under my guidance and supervision from 15 July,2023 to 15 September 2023.

The work was carried out is original and carried out in an online/ offline/ hybrid mode.

Signature of Mentor

ACKNOWLEDGEMENT

We would like to express our heartfelt gratitude to Sri Venkateswara College for providing us with this opportunity. We also thank the **Department of Zoology, Sri Venkateswara College** and **Shivaji College** for their support. We would like to express our great appreciation to our mentors **Dr. Perumal Jayaraj** and **Dr. Seema Sen** for their patient guidance, enthusiastic encouragement and useful critiques during this project. Their valuable and constructive suggestions during the planning and development of this research work along with their willingness and dedication to give their generously has molded this project perfectly.Our appreciation also goes out to ***Dr. R.P Centre for Ophthalmic Sciences, AIIMS Delhi***. We are immensely grateful to **Ms. Pooja, JRF, AIIMS Delhi,** for all her efforts in patiently guiding us though the laboratory procedures.

CONTENT

ABSTRACT

Background:

The eyelids are composed of four layers: skin and subcutaneous tissue including its adnexa, striated muscle, tarsus with the meibomian glands, and the palpebral conjunctiva. Benign and malignant tumors can arise from each of the eyelid layers. Most eyelid tumors are of cutaneous origin, mostly epidermal, which can be divided into epithelial and melanocytic tumors. Benign epithelial lesions, cystic lesions, and benign melanocytic lesions are very common. The most common malignant eyelid tumors are basal cell carcinoma in Caucasians and sebaceous gland carcinoma in Asians. Adnexal and stromal tumors are less frequent. The present review describes the more important eyelid tumors according to the following groups: Benign and malignant epithelial tumors, benign and malignant melanocytic tumors, benign and malignant adnexal tumors, stromal eyelid tumors, lymphoproliferative and metastatic tumors, other rare eyelid tumors, and inflammatory and infections lesions that simulate neoplasms. Histopathology is essential for the success of clinical trials. Histopathologists have a detailed understanding of tumour biology and mechanisms of disease, as well as practical knowledge of optimal tissue handling and logistical service requirements for study delivery, such as biomarker evaluation, tissue acquisition and turnaround times. As such, histopathologist input is essential throughout every stage of research and clinical trials, from concept development and study design to trial delivery, analysis and dissemination of results using H/e staining. Majority of cancer and tumours are studied using CAM assay. It have been widely used to study angiogenesis and tumor invasion of colorectal, prostate and brain cancers. However, there have been limited studies that have used CAM assays to assess eyelid tumour invasion and metastasis. We have therefore developed a CAM assay protocol to monitor the metastatic properties of Eyelid Tumour (SCC, SGC) and potential molecule(cyclin B1) study. Immunohistochemistry (IHC) is a powerful technique that exploits the specific binding between an antibody and antigen to detect and localize specific antigens in cells and tissue, most commonly detected and examined with the light microscope. A standard tool in many fields in the research setting, IHC has become an essential ancillary technique in clinical diagnostics in anatomic pathology with the advent of antigen retrieval methods allowing it to be performed conveniently on formalin fixed paraffin embedded (FFPE) tissue and automated methods for high volume processing with reproducibility . IHC is frequently utilized to assist in the classification of neoplasms, determination of a metastatic tumor's site of origin and detection of tiny foci of tumor cells inconspicuous on routine hematoxylin and eosin (H&E) staining. Cyclin B1 is a key mitotic cyclin in the G2-M phase transition of the cell cycle and is overexpressed in various malignant tumors. Numerous studies have reported contradictory evidences of the correlation between cyclin B1 expression and prognosis in human solid tumors.

Materials and Methods: For Histopathological analysis of the Eyelid SCC formalin fixed , paraffin Tissue section undergo certain gradient of Alcohol for paraffin wax removal , Hydration then nuclear stain by Hematoxylin followed by removal of excess background stain in weak acid acid and counter staining with Eosin depending of preference . The tissue section was then dehydrated with xylene ,

cleaning and mounted with DPX mountant for microscopic analysis.

To development of CAM we used fertilized chicks egg and incubated at 37'C and Humidity 70-80% providing suitable condition their development and at EDD 17 we extracted the CAM from the egg and in another egg the processed Eyelid tumour was allowed to develop.

TO study Cyclin B1 expression the in following tissue of Eyelid SCC Immunohistochemistry was performed on Formalin fixed, paraffin tissue section of SCC (eyelid) which was deparaffinized with xylene and hydrated using Gradients of Alcohole. Then the Antigens were Retrived using Antigen Retrival machine (EDTA Steam) and Blocking, Antigen masking (Primary, Secondary, Amplifier etc.). The Chromogenic Staining was done By DAB followed by counterstaining and mounting with DPX.

Result and Discussion

The Histopathology of eyelid tumour SCC was study show pleomorphism, which includes anaplastic cells, abnormal mitotic pattern, little or no evidence of keratinization, and loss of intercellular bridges. The IHC of the Cyclin B1 the expression was observed on various sub-cellular staining locations, namely nucleus,cytoplasm and perinuclear region of the cell, both cytoplasmic and nuclear reagion were apprecated.

The CAM was established At EDD7 and extracted upon H/E staining it show dense muscular fibers and rich blood vessels.Futher metastatic potential cannt be studied using the CAM certain unfavourable reasons.

INTRODUCTION

1. Eyelid Tumour

Human Eyelid and Function:

The function of the eyelids is crucial for proper tear film distribution throughout the ocular surface, tear drainage, ocular surface protection, and cosmesis. Additionally, the eyelids have glands that secrete lubricants for the ocular surface. Although the eyelid is a small part, it has many histological features that can be the source of benign and malignant lesions. The eyelid consists of four layers: skin and subcutaneous tissue, striated muscle (orbicularis oculi), tarsus and conjunctiva.

The eyelid is the thinnest part of the body and does not contain subcutaneous fat, but all other skin structures are seen. The skin epithelium is a keratinized stratified squamous epithelium. Melanocytes are scattered throughout the basal layer of the epithelium.

The dermis consists of fibrous tissue, blood vessels, lymphatic vessels, and blood vessels.

The eye is rich in glandular tissue: eccrine glands - sweat glands of the eyelids and accessory lacrimal glands of Claus and Wolflin; Moore's apocrine gland; and sebaceous glands - meibomian and Zeiss glands.

All entry lines are covered by the orbicularis oculi muscle; The striated muscles include the pretarsal and preseptal portion and the orbital portion above the extraorbital bone. The tarsus is a dense plate of tissue in which the meibomian glands are embedded.

The posterior surface of the eyelid is covered by the palpebral conjunctiva consisting of epithelium and subepithelial stroma (lamina propria).

The epithelium of the palpebral conjunctiva is usually cuboidal and contains goblet cells. Melanocytes are scattered throughout the basal layer of the epithelium. The stroma consists of fibrovascular tissue.

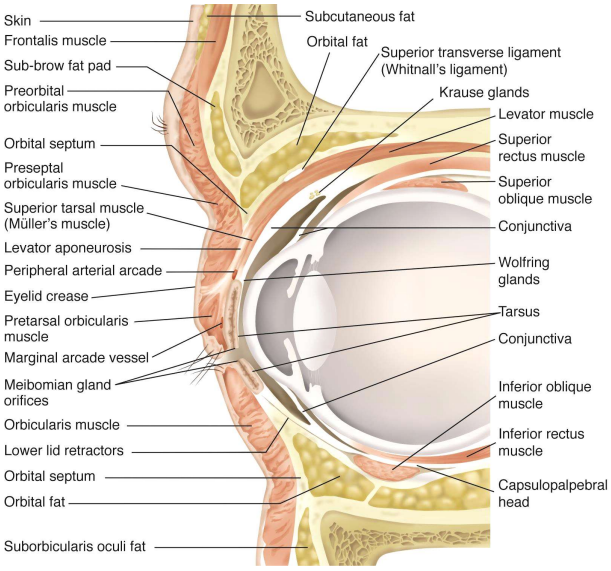


FIGURE 1- Modified from Stewart WB. Surgery of the Eyelid, Orbit, and Lacrimal System. Ophthalmology Monograph 8, vol 2. San Francisco: American Academy of Ophthlamology; 1994:23,85. Illustration by Cyndie C. H. Wooley

The main task of the eyelids is to protect the eyes and preserve vision. Glands in the eyelids form a complex tear film that nourishes, lubricates, and protects the ocular surface. Eyelids also help to pump tears, and blinking helps to remove tears from the tear reservoir. The eyelids play an important role in protecting the ocular surface through the eye's defense mechanism. These are normal physiological responses to protect the precorneal tear film and protect the eye.

The main protective mechanisms provided by the eyelids are to protect the eye surface from drying out and include the stability of precorneal tears by reducing tear formation, closure of the eyelids, frequent and adequate blinking, and tear loss.

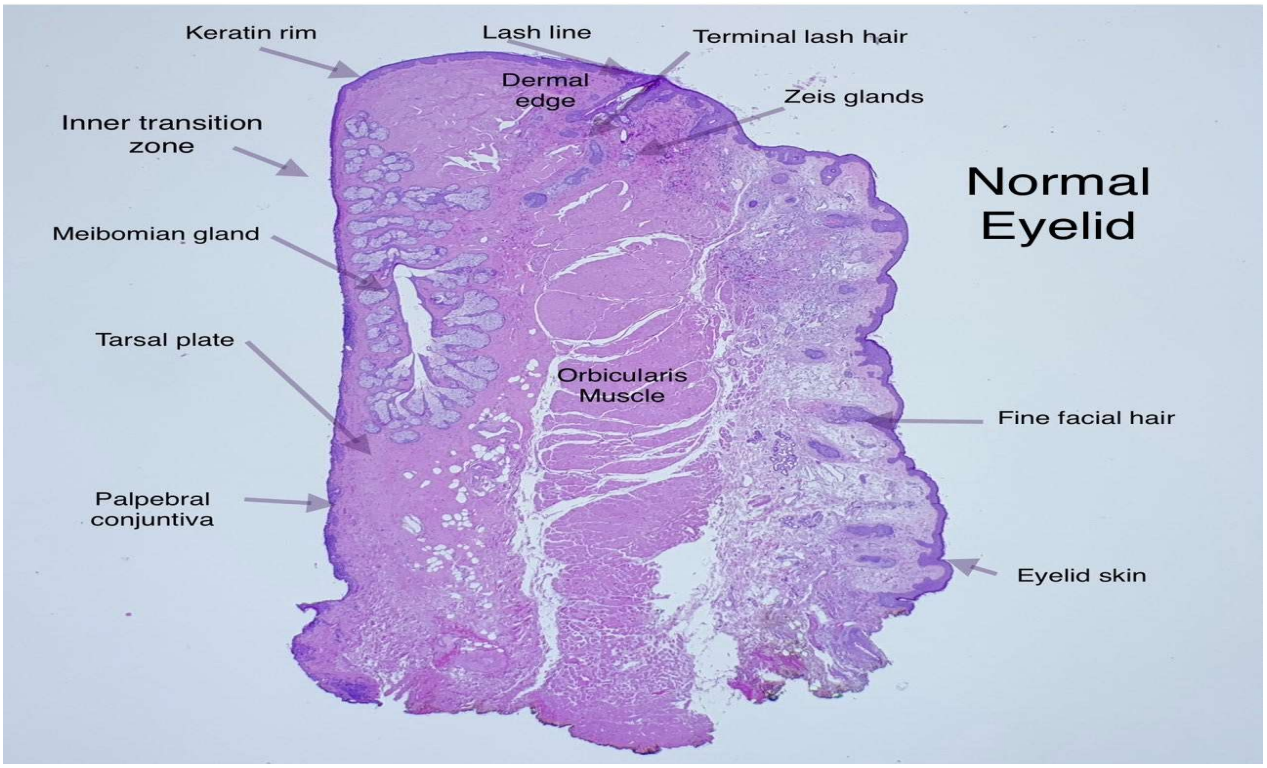
Classification of eyelid tumors:[1]

The eyelid consists of four layers: the skin and subcutaneous tissue, its appendages, striated muscle, meibomian gland and palpebral conjunctiva. Benign and benign tumors can occur in all layers of the eyelid. Eyelid tumors consist mostly of the skin, especially the epidermis, and can be divided into epithelial tumors and melanocytic tumors. Benign epithelial, cystic, and benign melanocytic lesions are common. The most common eyelid cancers are basal cell carcinoma in Caucasians and sebaceous gland carcinoma in Asians. Adnexal tumors and stromal tumors are less common.

Like other organ tumors, eye tumors can be classified as benign or malignant depending on their tissue or cellular origin. Eyelid tumors originate from the skin, mostly the epidermis, and can be divided into epithelial tumors and melanocytic tumors. Benign epithelial lesions, basal cell carcinoma (BCC), cystic lesions, and melanocytic lesions account for approximately 85% of all eyelid neoplasms. Cutaneous squamous cell carcinoma (SCC) and melanoma are rare. Adnexal tumors and stromal tumors are less common. Other tumors of the eyelid are lymphomas, hamartomas, and tumors. Cancer-like swelling and infection are common. This review includes only the more benign and malignant eyelids. Palpebral conjunctival cancers are included in the analysis of conjunctival diseases. Skin tumors can be divided into three main groups according to their clinical and histological features: benign, precancerous, and malignant.

The following table represents the majority of the tumors found in the human eyelid and the associated structures with the eyelid. But as per our research work we will only discuss those we have worked upon.

Category	Subtypes
Epidermal tumors	Nonmelanotic tumors
	Melanocytic tumors
Adnexal tumors	Sebaceous gland tumors
	Sweat gland tumors
	Hair follicle tumors
	Cystic lesions
Stromal tumors	Fibrous tissue tumors
	Fibrohistiocytic tumors
	Lipomatous tumors
	Smooth muscle tumors
	Skeletal muscle tumors
	Vascular tumors
	Perivascular tumors
	Neural tumors
	Lymphoid, plasmacytic, and leukemic tumors
	Cartilage and bone tumors
	Hamartoma and choristoma
	Palpebral conjunctival tumors
Secondary tumors	
Metastatic tumors	
Inflammatory and infections lesions that simulate neoplasma	



Histological Image : Normal Human Eyelid

2. Squamous cell carcinoma

What is squamous cell carcinoma? Squamous cell carcinoma (SCC) of the skin is the second most common form of skin cancer, characterized by abnormal, accelerated growth of squamous cells. When caught early, most SCCs are curable[2]. Squamous cell carcinoma (SCC), is a type of invasive malignancy arising from the squamous cell layer of the skin epithelium [2]. It can be found in various locations on the body including the skin, anus, cervix, head/neck, vagina, esophagus, urinary bladder, prostate, and lungs. In the ocular and periocular region, it can affect the conjunctiva, cornea, and eyelid. Squamous cell carcinoma (SCC) of the eye is an invasive epithelial malignancy and involves the periocular skin, ocular surface and lacrimal apparatus [1]. Over the past four decades, there has been a progressive rise in the global incidence of SCC on account of increased exposure to carcinogens such as ultraviolet (UV) radiation, cigarette smoking, immunosuppressive drugs or human papillomavirus (HPV) infection [2]. The tumor comprises a large and diverse spectrum of conditions and threatens both vision and life.

Epidemiology

Eyelid SCC is the second most common periocular skin malignancy, far exceeded by basal cell carcinoma (BCC) which is 10–13 times more common [3]. The reported incidence of SCC of the eyelid is 0.09 to 2.42 cases per 100,000 persons per year, representing 3.4–12.6% of all types of malignant eyelid neoplasms [6]. A longitudinal study in England has shown that the age-standardized incidence of SCC has increased approximately 2% per year between 2000 and 2014 [4]. It mainly presents in the seventh decade of life with a male predominance (1.83:1)

Ocular surface SCC is the most common primary ocular neoplasm with reported incidence from 0.3 to 1.9 per 100,000 persons per year, accounting 4–29% of all oculo-orbital tumors.

Primary lacrimal sac/duct malignancies are very rare with SCC being the most frequently reported neoplasm. In one study, only 38 out of 3865 (0.98%) specimens of lacrimal sac biopsy showed malignant

Pathogenesis and pathology

SCC may arise de novo or from preexisting actinic keratosis or carcinoma in situ (Bowen disease) [2]. Conjunctival intraepithelial neoplasia (CIN) refers to varying degrees of conjunctival epithelial dysplasia. CIN that involves the entire epithelium is referred to as carcinoma in situ. In some cases of squamous cell papilloma, they have been found to grow quite large, covering the surface of the cornea and simulating a squamous cell carcinoma. Most cases of squamous cell papilloma are benign tumor, but its potential for malignant transformation has yet to be studied .

Squamous cell carcinoma is characterized by full thickness atypia with increased mitotic activity of the squamous cells (Figure 1). The tumor may be graded on the amount of dedifferentiation. More differentiated tumors produce keratin. The formation of keratin decreases in less well differentiated tumors and is not seen in poorly differentiated SCC. Nests and strands are also characteristic of well differentiated SCC. However, characteristic intercellular bridges are generally maintained in all SCC (Figure 2).[3]

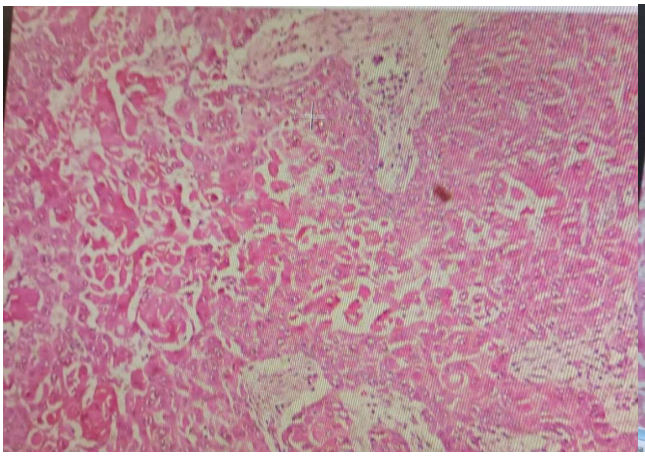


fig.1 Represents squamous cell carcinoma. The keratin pearl. (hematoxylin-eosin, original magnification x 40).

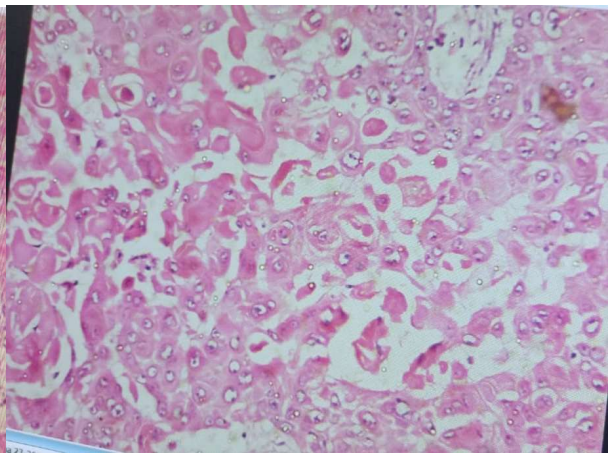


fig.2 Represents squamous cell carcinoma. Keratin is seen in the center and intercellular bridges are present throughout the image. (original magnification x 400)

Clinical and pathophysiological features[3]

SCC typically manifests as a spectrum of progressively advancing malignancies, ranging from a precursor actinic keratosis (AK) to squamous cell carcinoma in situ (SCCIS), invasive SCC, and finally metastatic SCC. It is classified as in situ when it is superficial to the basal membrane, and it is considered invasive when it extends deep to the basal membrane layer of the skin. It is also possible to find in the literature others types of skin lesions classified as SCC variants, such as keratoacanthoma and cutaneous horn.

A full thickness biopsy is the gold standard to diagnose SCC as it can determine the depth of invasion and extent of invasion of the cancer. Patients with regional lymph node alterations should undergo Fine Needle Aspiration (FNA) biopsy to determine if cancer cells have spread to these nodes. Invasive SCC is commonly associated with perineural spread.

In situ carcinoma

In situ carcinoma is the term for epithelial lesions in which cells have cytological abnormalities characteristic of malignancy (hyperchromatism, pleomorphism, mitoses) and have lost their typical architecture but lack evidence of local invasion or distant metastases. In the skin, the squamous cell carcinoma in situ (SCCIS) is known as Bowen's disease. It appears as a persistent brown/red spot which may be confused with psoriasis or eczema. There is a strong association of Bowen's disease with HPV (human papilloma virus) infection, mainly type 16.

Cutaneous horn

A cutaneous horn (CH) is a lesion with a papular or nodular base and a keratotic cap of various shapes and lengths resembling an animal horn. Usually , it represents hypertrophic solar keratoses. Clinically, CHs vary in size from a few millimeters to several centimeters. The horn may be white, black, or yellowish and can be straight, curved, or spiral in shape. Histologically, there is usually hypertrophic actinic keratosis, SCC in situ, or invasive SCC at the base. Because of the possibility of invasive SCC, a CH should always be excised.

Actinic keratosis:

Actinic keratosis is the most common precancerous skin lesion, affecting about 60% of fair-skinned people over the 4th decade of life. Actinic keratosis (AK) appears as a hyperkeratotic lesion. In general, they are round or oval, commonly present in sunlight exposed skin areas, and may or may not have an erythematous base. AK is a direct precursor to squamous cell carcinoma and a risk factor for other skin cancers. Although progression to invasive malignancy is rare, actinic keratoses are squamous cell carcinomas in situ. The main histological feature of actinic keratosis is dysplasia of keratinocytes, or disordered maturation of these cells.

Figure 3. Keratoacanthoma in the right upper eyelid Image Courtesy by: Patricia Henriques Lyra Frasson M.D. , MSc - Federal University of Espirito Santo, Brazil.

Keratoacanthoma:

This type of lesion, shown in the figure 3, has recently been classified as a variant of SCC, but there is still a long-standing debate as to whether those lesions are benign reactive lesions or a variant of SCC. Keratoacanthoma typically presents as a cup-shaped nodule with a central keratin crater and elevated, rolled margins. It usually develops over a short period of weeks to a few months and may regress spontaneously. Histopathologically, these dome shaped lesions have thickened epidermis containing islands of well-differentiated squamous epithelium that may be infiltrated by neutrophils surrounding a central mass of keratin. The lesion base is often well-demarcated from the adjacent dermis by inflammatory reaction.

Squamous cell carcinoma:

The clinical types of carcinoma are variable and there are no pathognomonic characteristics. The tumor may be clinically indistinguishable from a basal cell carcinoma (BCC), but usually it does not have superficial vascularization, it grows more rapidly, and hyperkeratosis is more frequent. The nodular SCC is characterized by a hyperkeratotic nodule that may develop with the presence of crusts and fissures. The ulcerating SCC (Figure 4) has a red base with well defined, hardened and everted edges. The histopathologic features of SCC depend on the degree of differentiation of the tumor. In well-differentiated tumors, the cells are polygonal with abundant acidophilic cytoplasm and hyperchromatic nuclei with ɔtic cells, and intercellular bridges. Poorly differentiated lls, abnormal mitotic figures, little or no evidence of Variants of SCC are spindle and adenoid SCC.

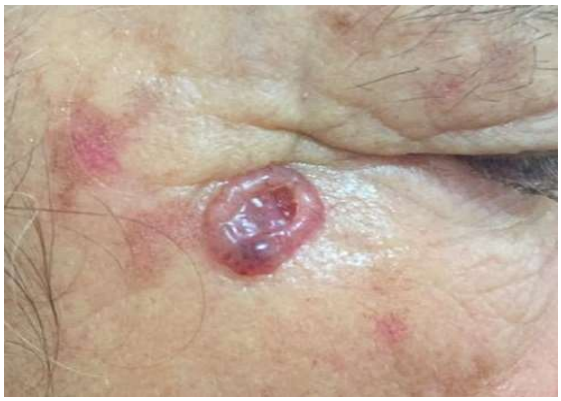


Figure 4. A Caucasian patient with actinic keratosis and a lesion suspicious for SCC between the lower lid and the malar area. Courtesy by: Patricia Henriques Lyra Frasson M.D. , MSc - Federal University of Espirito Santo, Brazil.

A clinical, histologic, and molecular comparison of AKs, cSCC, and metastatic cSCC is shown in the figure [5]

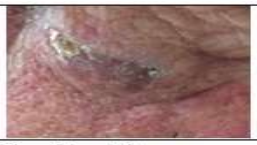
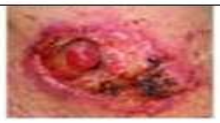
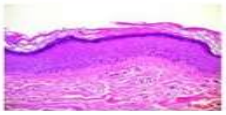
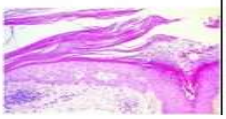
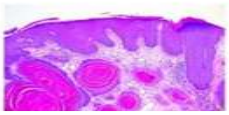
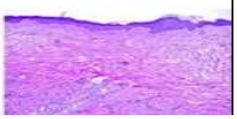
	Normal Skin	AK	cSCC	Metastatic cSCC
				
Clinical Description		Scaly skin colored /pink macule or papule	Persistent firm or scaly papule or red nodule which may spontaneously bleed	Multiple nodular lesions in skins or other organs
Histopathology	Well-defined stratum basalis spinosum, and granulosum with orthokeratotic scale	Enlarged atypical keratinocytes confined to the epidermis	Enlarged atypical keratinocytes invading the dermis	Enlarged atypical keratinocytes in the dermis lympho nodes or internal organs
				

Figure 5.A clinical, histologic, and molecular comparison of AKs, cSCC, and metastatic cSCC Adapted from Ratushny, V., Gober, M. D., Hick, R., Ridky, T. W., & Seykora, J. T. (2012). From keratinocyte to cancer: the pathogenesis and modeling of cutaneous squamous cell carcinoma. The Journal of Clinical Investigation, 122(2), 464–47 Images kindly lent by Patricia Henriques Lyra Frasson M.D. , MSc - Federal University of Espirito Santo.

Adapted from Ratushny, V., Gober, M.D., Hick, R., Ridky, T. W., & Seykora, J. T. (2012). From keratinocyte to cancer: the pathogenesis and modeling of cutaneous squamous cell carcinoma. The Journal of Clinical Investigation, 122(2), 464–47, Courtesy by: Patricia Lyra M.D. , MSc - Federal University of Espirito Santo, Brazil.

3. Histopathology[9]

Histopathology is the diagnosis and study of diseases of the tissues, and involves examining tissues and/or cells under a microscope. Histopathologists are responsible for making tissue diagnoses and helping clinicians manage a patient's care.

Histopathologists provide a diagnostic service for cancer; they handle the cells and tissues removed from suspicious 'lumps and bumps', identify the nature of the abnormality and, if malignant, provide information to the clinician about the type of cancer, its grade and, for some cancers, its responsiveness to certain treatments.

With the help of sophisticated imaging techniques, biopsy tissue can now be obtained from previously inaccessible sites such as the pancreas or retroperitoneum (behind the peritoneum, the membrane lining the abdominal cavity). Tissue is then processed, usually overnight, before being examined under a microscope. In certain limited circumstances using special techniques, the specimen can be examined immediately.
With rapidly changing developments in molecular pathology, pathologists are leading the way with new techniques such as fluorescence in-situ hybridization (FISH) and polymerase chain reaction (PCR), to map the genetic material in tissues or tumours, which are essential in the management of many cancers.

Role Of Hematoxin and esoin staining[9]

The H&E staining procedure is the principal stain in histology in part because it can be done quickly, is not expensive, and stains tissues in such a way that a considerable amount of microscopic anatomy is revealed, and can be used to diagnose a wide range of histopathologic conditions. The results from H&E staining are not overly dependent on the chemical used to fix the tissue or slight inconsistencies in laboratory protocol, and these factors contribute to its routine use in histology.

H&E staining does not always provide enough contrast to differentiate all tissues, cellular structures, or the distribution of chemical substances, and in these cases more specific stains and methods are used.

The H&E stain provides a comprehensive picture of the microanatomy of organs and tissues. Hematoxylin precisely stains nuclear components, including heterochromatin and nucleoli, while eosin stains cytoplasmic components including collagen and elastic fibers, muscle fibers and red blood cells. In a high-quality H&E stain, there are subtle differences in the shades of color produced by the stains, particularly eosin, and this aids in the detection and interpretation of morphological changes associated with disease.

4. Cell Cycle and Cyclin B1:-

Cell division is one of the most fundamental properties of a cell and is essential to maintain and sustain life. It is the very property owing to which , a single fertilised egg has the capability to development into approximately 10^14 cells that make up the human body. Owing to its vital role in survival , cell cycle must be carefully regulated and coordinated with both cell growth and replication to ensure progeny cells with intact genomes. Progression between the discreet stages of the cell division cycle is is under the control of a regulatory apparatus. This regulatory apparatus also helps in coordinating the cell cycle with extracellular signals that control cell proliferation.

Normal Cell Cycle:

The cell cycle is broadly divided into two distinct phases , interphase and mitosis. 95% of the duration of cell cycle is spent with the cell being in interphase. The interphase stage is characterised by the present of a morphologically uniform nucleus and condensed chromosomes present in the nucleus. Cell growth and DNA replication occur during the interphase. Most dividing cells double in size between one mitosis cycle and the rest. Mitosis on the other hand is the most dramatic stage of cell cycle characterised by separation of daughter chromosomes followed by cytokinesis. The M phase of the cycle corresponds to mitosis. The M phase is followed by G1 or gap 1 phase which is characterised by the cell being metabolically active and continuously grows in size. This is followed by the S phase or synthesis phase wherein DNA replication happens. Completion of DNA synthesis is followed by G2 or gap 2 phase during which cell growth continues and cell synthesises proteins. The duration of phases of cell cycles depends on one cell to another. For a typical human cell having a cell cycle which is 24 hours long , G1 phase might last for about 11 hours, S phase about 8 hours, G2 about 4 hours and M about 1 hour.On the other hand , yeast cells have cell cycle that is 90 minutes long while early embryo cells after fertilisation of the egg has a cell cycle of duration 30 minutes or less. On the contrary , certain animal cells like nerve cells cease cell division altogether while some other animal cells like fibroblasts divide only occasionally , as needed to replace cells that have been lost because of injury or cell death. These cells exit G1 stage to enter quiescent stage G0 where they remain metabolically active but no longer proliferate unless called on to do so by appropriate extracellular signals. Animal cells have DNA content 2n in G1 stage. In S phase , DNA content of cell increases from 2n to 4n and remains as 4n in G2 phase and M phase , further decreasing back to 2n post cytokinesis.G2 checkpoints prevent mitosis initiation before S phase completion.This prevents distribution of incompletely replicated DNA to daughter cells.On completion of DNA replication in S phase , certain control mechanisms come into picture to prevent re-initiation of DNA replication. This ensures that the genome is replicated only once per cell.MCM proteins acts as licensing factors and helps in ensuring that replication happens only once per cycle by binding to the DNA in G1 phase. The MCM proteins binds to the replication origins in G1 phase and allows initiation of DNA replication as cell enters the S phase.As DNA replication initiates , MCM proteins displaces from the DNA preventing a second round of DNA replication until the cell enters the G1 of a new cell cycle. Protein kinases prevent the association of MCM complexes with the DNA in S , G2 and M phase

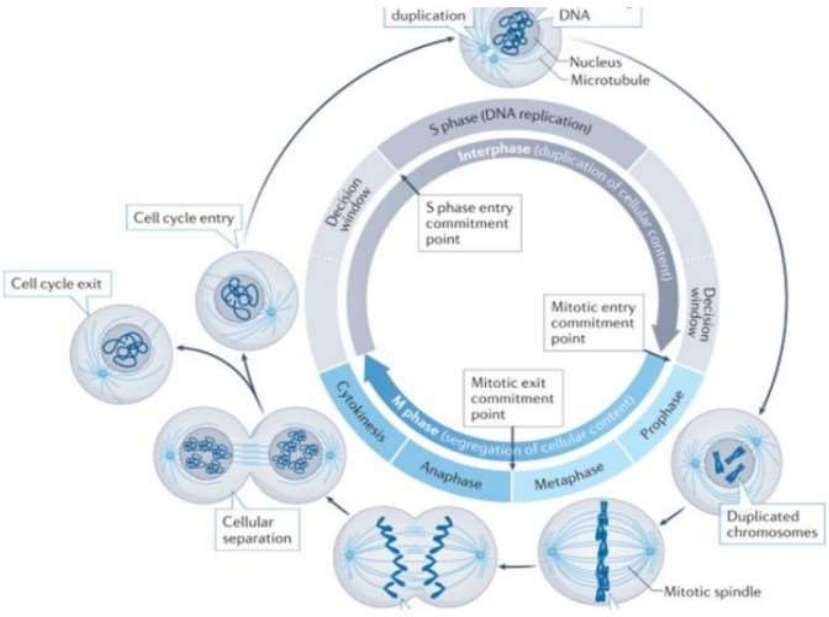


Figure4.1 Stages in cell cycle

Cyclin Expression in Cell Cycle:

One of the regulatory mechanisms of cell cycle progression is by the sequential activity of various cyclins. These cyclins act in coordination with serine threonine kinases , also called as cyclin dependent kinases or Cdks. Cyclins bind to and activate various Cdks.The activity of cyclin-Cdk complexes is tightly regulated by a complex network of proteins that function as activators and inhibitors and influences

their transcription, sub-cellular localisation and degradation. Several different classes of cyclins have been described in mammalian cells and they are designated from A to I along with T. During the cell cycle, cyclins from the D-type family (D1, D2 and D3) regulate progression of cells through the G1 phase by binding and activating Cdk4 and Cdk6. These cyclin D-Cdk4/6 complexes phosphorylate and functionally inactivate the retinoblastoma protein, pRB and Stages of cell cycle pRB-related proteins p107 and p130, thereby contributing to cell cycle progression. E-type family cyclins(E1 and E2) are expressed during late G1 and during S-phase progression. These cyclins activate majorly Cdk2 along with association with Cdk1 and Cdk3. During the S-phase cyclin A2 becomes activated which associates with both Cdk1 and Cdk2, and phosphorylates substrates. M_phase progression is driven by the B-type cyclins which activate Cdk1. Cyclins also play kinase-independent functions. In particular, D-type cyclins and E-type cyclins have been reported to have Cdk-independent roles as co-activators or co_repressors of tissue-specific transcription factors. Deregulation in the function of these cyclin-cdk complexes results in virtually the whole spectrum of human tumors and this comes from the fact that tumor_associated alterations in cyclins help to sustain proliferation independently of external mitogenic or anti-mitogenic signals. Multilevel controls on expression and activation of cyclin/CDK complexes controls the necessary coordination of the cell cycle stages and thereby prevents the formation of tumor cells

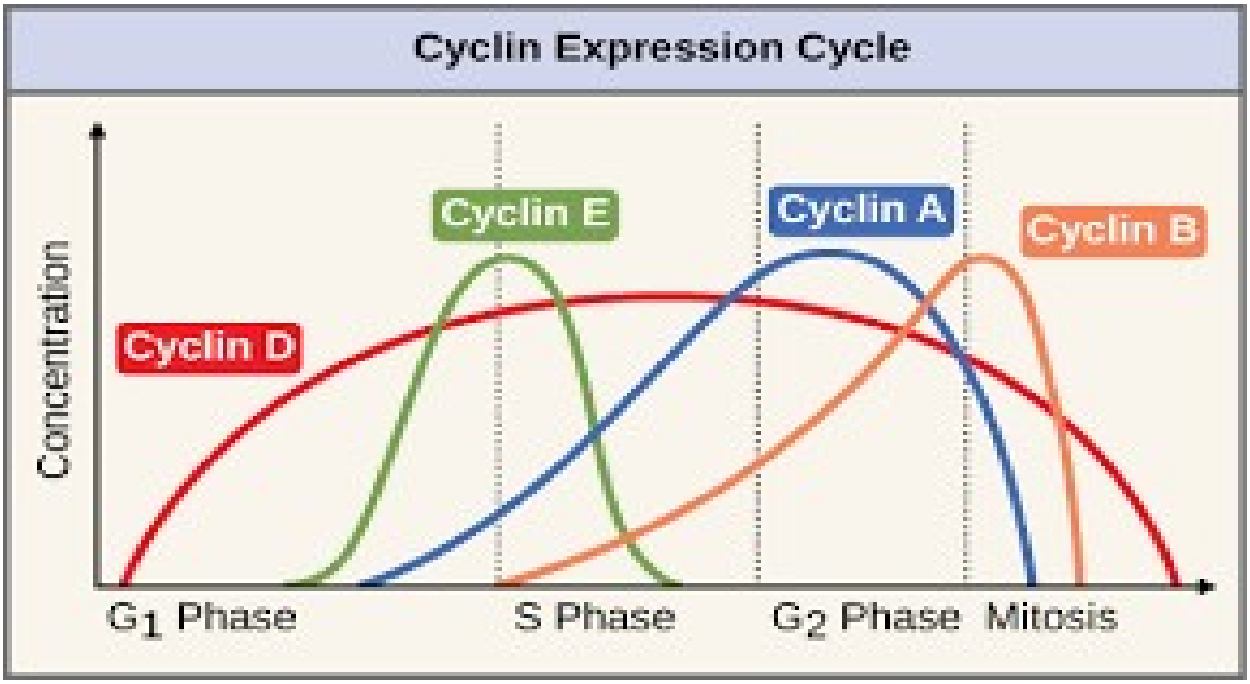


Figure 4.2 showing: Cyclin Concentrations at Checkpoints: The concentrations of cyclin proteins change throughout the cell cycle. There is a direct correlation between cyclin accumulation and the three major cell cycle checkpoints. Also, note the sharp decline of cyclin levels following each checkpoint (the transition between phases of the cell cycle) as cyclin is degraded by cytoplasmic enzymes.

checkpoints that monitor completion of critical events and delay progression to the next stage if necessary. Expression of cyclins during various stages of cell cycle The first type of control involves a highly regulated kinase family. Kinase activation generally requires association with a second subunit that is transiently expressed at the appropriate period of the cell cycle; the periodic “cyclin” subunit associates with its partner “cyclin-dependent kinase” (CDK) to create an active complex with unique substrate specificity. Regulatory phosphorylation and dephosphorylation fine-tune the activity of CDK–cyclin complexes, ensuring well-delineated transitions between cell cycle stages. A second type of cell cycle regulation is with the help of ‘checkpoint control’ . Cell cycle checkpoints helps in sensing flaws

in critical events such as DNA replication and chromosome segregation. When checkpoints are activated, for example by under-replicated or damaged DNA, signals are relayed to the cell cycle_progression machinery. These signals cause a delay in cycle progression, until the danger of mutation has been averted. Because checkpoint function is not required in every cell cycle, the extent of checkpoint function is not as obvious as that of components integral to the process, such as CDKs.

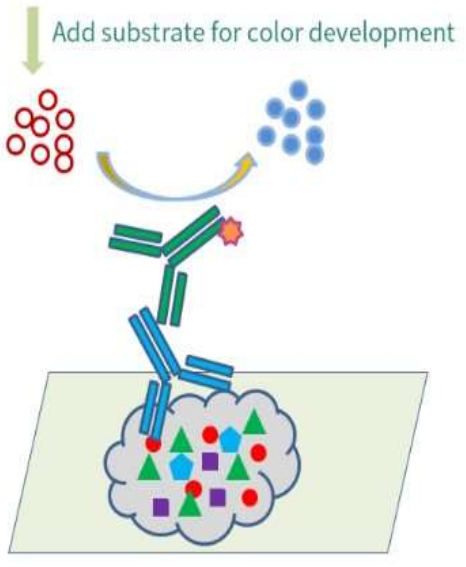
Cyclin B1:

Cyclin B1 is a regulatory protein of mitosis. It is encoded by the cyclin B1 gene , also represented as CCNB1 gene. The gene product of this gene complexes with Cdk1 to form maturation promoting factor or MPF. The encoded protein is necessary for proper control of the G2/M transition phase of the cell cycle. Cyclin B1 activity imitates an all or none phenomenon in terms of helping the cell to decide to commit to mitosis. Positive feedback loops helps prevent deactivation of cyclinB1-CDK complexes once activated. This cyclin B1-CDK complex is involved in regulating the early events of mitosis , like chromosome condensation, nuclear envelope breakdown, and spindle pole assembly. In mammalian cells , cyclin B1 complexes with CDK 1 during G2 phase. On the formation of these complex, CDK1 is phosphorylated at two critical regulatory positions. One of the phosphorylations occurs at threonine-161 and is required for CDK kinase activity. The second is the phosphorylation of tyrosine-15 and of the the adjacent threonine-14 in vertebrates. Phosphorylation of tyrosine-15 by protein kinase called Wee-1 , inhibits CDK1 activity and leads to accumulation of inactive cyclin B1 and CDK1 throughout G2 phase. The transition from G2 to M is then brought about by activation of the cyclinB1-CDk1 complex by dephosphorylation of threonine-14 and tyrosine-15 by a protein phosphatase called Cdc25c. On activation , the CDK1 protein kinase phosphorylates many target proteins that initiate the events of M phase. CDK1 activity also causes degradation of cyclin B1 occurring due to ubiquitin mediated proteolysis. Destruction of cyclin B1 further causes CDK1 inactivation leading to the cell to exit mitosis , undergo cytokinesis and return to interphase. The role of cyclin B1 is to transition the cell from G2 to M phase but becomes unregulated in cancer cells where over expression of cyclin B1 can lead to uncontrolled cell growth by binding to its partner Cdks. Binding of Cdks can lead to phosphorylation of other substrates at inappropriate time and unregulated proliferation.This is a consequence of p53, tumor suppressor protein, being inactivated. Wild-type p53 have been shown to suppress cyclin B1 expression

5. Immunohistochemistry:-

Immunohistochemistry (IHC) is the process whereby antibodies are used to detect proteins (antigens) in cells within a tissue section (for instance liver, pancreas or the heart).

When a tissue sample is passed to a lab to be examined for disease, there are several details that cannot be determined easily. Several diseases or disease sub-types may look alike or appear to have similar size cells under a microscope but have different behaviors and necessary treatments.



The best way to differentiate them is to detect specific molecules on these cells that act as markers. Immunohistochemistry (IHC) is a technique that uses antibodies (matching molecules) that can seek out, identify and attach themselves to these markers on cells. The antibodies themselves can be seen under the microscope, which helps the technician make a precise identification.

Immunohistochemistry (IHC) has found numerous applications in medicine, especially in cancer diagnosis. Lymphomas are among the cancers most dependent on IHC for correct diagnosis and treatment decisions.[7]

Principle of Immunohistochemistry

At its core, immunohistochemistry capitalizes on the specific interactions between antibodies and antigens within tissue specimens. Antibodies, produced by the immune system in response to foreign substances (antigens), possess an innate ability to recognize and selectively bind to unique epitopes on these antigens. This recognition process operates with high specificity, akin to a lock and key mechanism. In the context of IHC, the primary objective is to employ these antibodies to target and highlight specific proteins of interest within formalin-fixed tissue sections.[8]

Application

IHC is used for disease diagnosis, biological research, and in drug development. For example, using specific tumor markers, physicians use IHC to diagnose if a tumor is benign or malignant, to determine its stage and grade, and to identify the cell type and origin of a metastasis in order to find the site of the primary tumor. A variety of other non-neoplastic diseases and conditions are diagnosed using IHC as a primary tool or as a confirmatory procedure. In a research

context, IHC can be used alone or in conjunction with other analytical techniques to study, for example, normal tissue and organ development, pathological processes, wound healing, cell death and repair, and many other fields. IHC is also used in drug development to test drug efficacy by detecting either the activity or the up- or down-regulation of disease markers in the target tissues and elsewhere.

Traditional IHC is based on the immunostaining of thin sections of tissues attached to individual glass slides. Multiple small sections can be arranged on a single slide for comparative analysis, a format referred to as a tissue microarray.

Typically, IHC slides are prepared, processed, and stained manually or in small groups. However, current technology provides automation options for high-throughput sample preparation and staining. Samples can be viewed by either light or fluorescence microscopy, and advances in the last 15 years have improved our ability to capture images, quantitate multiparametric IHC data, and increase the collection of that data through high content screening. Below are some striking examples of IHC staining results obtained with Thermo Scientific Invitrogen antibodies and other IHC reagents.

Immunohistochemical Analysis

Immunohistochemistry (IHC) analysis is a method for demonstrating the presence and location of proteins in tissue sections. Though less sensitive quantitatively than immunoassays such as western blotting or ELISA, it enables the observation of processes in the context of intact tissue. This is especially useful for assessing the progression and treatment of diseases such as cancer. In general, the information gained from IHC combined with microscopy literally provides a "big picture" that can help make sense of data obtained using other methods.

Immunohistochemical staining is accomplished with antibodies that recognize the target protein. Since antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction is then visualized using either chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate at the location of the protein, or fluorescent detection, in which a fluorophore is conjugated to the antibody and can be visualized using fluorescence microscopy.

IHC-P refers to the staining of tissues that have been fixed (usually in neutral buffered formalin) and then embedded in paraffin before being sectioned. The basic steps of the IHC-P protocol are as follows:

- Fixing and embedding the tissue
- Cutting and mounting the section
- Deparaffinizing and rehydrating the section
- Antigen retrieval
- Immunohistochemical staining
- Counterstaining (if desired)
- Dehydrating and stabilizing with mounting medium
- Viewing the staining under the microscope

6. Choriollantoic Membrane (CAM) Assay[8]

The chick chorioallantoic membrane (CAM) model has emerged to be a good experimental system to establish a patient-derived model. It provides a unique biological microenvironment appropriate for cancer cells. The CAM is a structure fusing the mesoderm layer of allantois and chorion membranes with a rich vascular network [Ribatti, 2016]. The rich vascular plexus within the mesoderm layer of CAM supported by allantoic arteries and veins, offers tumor cells a supplement of growth-essential nutrients. The secretion of tumor angiogenic factors enables penetration of proliferating host vessels which bring oxygen to the graft [Folkman, 1971]. The time-course and onset of vascularization inside the CAM tumor graft has been clearly demonstrated [Knighton et al., 1977]. There is an initial avascular phase during the first 72 h of grafting [Knighton et al., 1977], during which the tumor growth is highly restricted [Ausprunk et al., 1975; Ausprunk and Folkman, 1976; Knighton et al., 1977]. In the meantime, the endothelial cells also undergo dynamic changes during the avascular phase. Due to ischemic damages, the endothelial cells in tumor grafts disintegrate and undergo lipid accumulation, vacuolation and degeneration at 2 h, 8 h, and 2 days post-implantation, respectively [Ausprunk et al., 1975; Ausprunk and Folkman, 1976]. The avascular tumor grafts on CAM eventually undergo necrosis and autolysis during the pre-vascular phase [Ribatti, 2008]. The neovascularization begins approximately 72–96 h after implantation, which is followed by a rapid growing phase [Knighton et al., 1977]. The reperfusion of vessels occurs through the release of angiogenic factors by tumor cells at the later phase and rescues the necrosis of tumor cells subsequently. The existence of this microvasculature inside the CAM system plays a crucial role in tumor cell proliferation and survival for the CAM xenograft.

The chick embryo development lasts for 21 days after which it hatches. The innate immune system starts developing during early embryo incubation starting from embryonic day (ED) 3 till ED16 when it is well-developed. The adaptive immunity develops later from around ED10 to ED18, which is when chick embryos are immunocompetent to react to pathogens via both innate and adaptive immune systems [Hincke et al., 2019]. Therefore, the developing chick embryo is naturally in an immunodeficient state until ED18 making the CAM an ideal setting for tumor grafting. From ED3 onwards, the chick embryo starts to express toll-like receptors, which prepare the embryo to defend pathogens through its innate immunity [Meade et al., 2009; Kannaki et al., 2015]. Macrophages can be observed inside the chick circulation from ED4 and could be detected in liver after ED12. The liver and spleen residing macrophages are functional as early as ED12 and ED16 [Janse and Jeurissen, 1991; Qureshi et al., 2000]. These embryonic macrophages have been shown to not only phagocytose dead cells [Cuadros et al., 1992], but also to guide the development of the lymphoid system [Houssaint, 1987; Houssaint et al., 1987]. Another inflammatory cell type existing during the early chick embryo incubation period are heterophils, which are analogs of the mammalian neutrophils contributing to the production of matrix metalloproteinase-9 (MMP-9) [Zijlstra et al., 2006]. Similar to mammalians, the chick adaptive immune system consists the T cells and B cells. T cells are first released from the thymus on around ED6. The second wave of release happens around ED11, which is when they develop functionally [Jankovic et al., 1975; Davison, 2003; Schilling et al., 2018; Hincke et al., 2019]. On the other hand, pre-bursal B cell precursors originating from the bursa of Fabricius are released on ED8 [Ratcliffe and Härtl, 2014], fully developed by ED12, and are differentiated after ED15 [Davison, 2003; Schilling et al., 2018; Hincke et al., 2019]. Therefore, the chick embryo becomes fully immunocompetent in both the innate and adaptive immune system only after ED18. This immature immune system before ED18 allows a higher rate of successfully accepting tumor transplantation, either allograft or xenograft [Davison, 2003; Schilling et al., 2018; Hincke et al., 2019]. Moreover, this immature immune environment has been shown to allow the preservation of immunogenic characteristics from human cells. The expression of human antigens has been shown to be retained after transplantation and re-transplantation of human tumors on CAM for several passages [Korngold and Lipari, 1955]. This suggests that the immunogenic property to recruit

immune cells to the human tumor grafts would still be preserved. This further gives strong indications that the related functionality and biological behaviors of the tumor grafts could also be retained, which is an important consideration for the application of PDXs.

Metastasis in the CAM Model

Both spontaneous and experimental models can be established to study tumor metastasis in CAM. The spontaneous model is to examine metastasis after grafting tumor cells or patient-derived samples on the surface of CAM. The experimental model is to study metastasis by inoculating tumor cells by direct injection into the allantoic vein. Since the metastasis of tumor cells includes a series of processes, these 2 different settings are to distinguish different functionalities of nonmetastatic and metastatic cells. Penetration of tumor cells through the CAM and the ability of cells to intravasate (the invasion of tumor cells into blood vessels) can be examined by the spontaneous model. Extravasation, docking and growth of tumor cells to secondary organs, is usually studied by the experimental model. Both models can be used to quantify survival and arrestment of tumor cells in the vasculature. Interestingly, cancer cells tend to arrest in the microcirculation of CAM with a high survival and extravasation rate [Chambers et al., 1982]. While in mice, circulating cancer cells perished rapidly and underwent significant cell damages with a low extravasation rate [Massagué and Obenauf, 2016]. The choice cellular arrest or dormancy and proliferation of cancer cells in the CAM system could be contributed by the urokinase plasminogen activator receptor [Landree et al., 2004] and the activation of ERK and p38 pathways [Ossowski et al., 1999; Aguirre-Ghiso et al., 2001, 2003, 2004]. Colonization at various metastatic sites could also be established by the CAM model. Dagg et al. [1956] demonstrated that squamous cell carcinoma implanted on the surface of CAM metastasized to the eye, brain, liver and myocardium of the chick embryo. The ability to model metastasis to a diverse range of embryonic chick organs makes the CAM model more desirable to study the organ-specific metastatic process. Human leukemia cells have been shown to metastasize to the chick brain, displaying a more coherent outcome in human leukemia progression, as sites of metastasis in mice are sometimes restricted [Taizi et al., 2006].

Several methods have been developed to examine tumor morphology at the metastatic sites. Classic techniques used in the murine models such as the hematoxylin and eosin (H&E) or IHC staining of tumor sections are generally applicable for the CAM tumor grafts . This allows the identification of different cell morphologies and cell types and to assess the invasion of inoculated cells into the underlying CAM tissue. Cell-labeling with fluorescent tags would further facilitate the examination of metastatic steps in the CAM model. By using fluorescence-tagged human HT-1080 fibrosarcoma cells and the concurrent labeling of vasculature with fluorescence-tagged Lens culinaris agglutinin, real-time imaging has elegantly demonstrated the intravasation, dissemination as well as extravasation of cancer cells with high (HT-hi/diss) and low (HT-lo/diss) disseminating potential [Deryugina et al., 2005; Deryugina and Quigley, 2008]. This double labeling technique allowed clear distinction between the intravasating cancer cells and the endothelial cells to assess the dynamic interaction with the microenvironment during metastasis. From this study, HT-hi/diss cells were found to display a behavior called vasculotropism, which is the wrapping of tumor cells by blood vessels instead of being randomly distributed within the CAM mesoderm. The attraction of HT-hi/diss cells to blood vessels enables intravasation. In contrast, only few HT-lo/diss cells appeared to escape from the primary site of inoculation, and thereby maintained a relatively clear tumor-stroma border

In addition to the evaluation of metastatic ability, quantitative studies of each step within the metastatic cascade can be done by the CAM assay [Zijlstra et al., 2002]. This could be done by tracking of mRNA levels of metastasizing cancer cells in chick embryos [Shioda et al., 1997] or detecting the human-specific Alu sequences in the metastatic sites. PCR-mediated amplification and quantification of

human Alu sequences helps correlate the fraction of human tumor cells intravasating and disseminating into the chick embryo through the CAM [Kim et al., 1998; Schneider et al., 2002]. High expression of metastasis-related genes in the circulating cancer cells has suggested that the host-cancer cell interactions alter gene expressions in early phases of metastasis [Shioda et al., 1997].

Application

Functional assessments of tumor growth, angiogenesis, and metastasis have been studied by using the CAM platform [Shioda et al., 1997; Cimpean et al., 2008; Deryugina and Quigley, 2008]. Both immortalized cancer cell lines (CCLs) and patient-derived samples have been successfully inoculated onto the surface of CAM [Karnofsky et al., 1952; Dagg et al., 1956; Gronau et al., 2006; Lugassy et al., 2009; Klingenberg et al., 2014; Xiao et al., 2015; Zabielska-Koczywas et al., 2017] or been injected into the veins of CAM [Ho et al., 2010; Leong et al., 2012]. Various types of CCLs or patient-derived tumors have been tested in the CAM model

Advantages

The CAM model could overcome limitations encountered in the murine models to establish PDXs. The cost-effectiveness, simplicity, high reproducibility, visibility, and shorter experimental duration make the CAM model a suitable alternative [Tufan and Satiroglu-Tufan, 2005]. The lower cost of the embryonic eggs, the elimination of forage and reduced facility access costs makes the CAM model more cost-effective compared to the immunodeficient mice. The manipulation to the eggs is simpler than handling the mice. The access to the CAM simply requires an opening of a window on the eggshell. The lower cost and the simplicity of CAM makes scaling up the experiments in multiple repeats feasible. Therefore, the chick CAM model could deliver experimental data with higher reproducibility. As the CAM is positioned right underneath the eggshell and on top of the developing embryo, no further surgery is required to examine the CAM tumor grafts. This allows the application of several imaging methods which could greatly enhance the visualization. As the experiments are always synchronized with the embryonic development of the chick before hatching, the duration to establish the graft only requires 14–18 days which is significantly shorter than what is usually needed for the mouse model. These advantages, together with the ability to inoculate multiple samples of interest onto individual CAMs [Bertossi et al., 1999; Deryugina, 2016], position the CAM as a good model to set up a large scale in vivodrug screening platform. It has been reported that experimental compounds have a longer half-life in the enclosed CAM system compared to other animal models [Lokman et al., 2012]. This unique feature could make drug testing in the CAM system more stable in pharmacodynamics and pharmacokinetics.

AIM : ***To Understand the histopathology features of Eyelid carcinoma and development of Chick Embryo for CAM Membrane establishment.***

OBJECTIVES:-

- **Histology of Normal Eyelid and Histopathological study of Squamous Cell Carcinoma of the Eyelid origin.**
- **Preparation of CAM Membrane for Potential use in Evaluation of Eyelid Tumour Metastasis.**
- **Immunohistochemical Evaluation of Cyclin B1 in Squamous Cell Carcinoma.**

Materials And Methodology :-

1.HEMATOXYLIN AND EOSIN STAINING:-

Material : Formalin fixed, Paraffin tissue sections

Reagents: Mayer's hematoxylin stain or hematoxylin solution

aqueous Eosin Y solution 0.5%, alcoholic Eosin Y solution 0.5% or 1%, or alcoholic eosin-phloxine solution

Methodology:-

1.1 **Deparaffinization or Removal of wax**

Following the preparation of a paraffin section, all the elements are infiltrated with and surrounded by paraffin wax which is hydrophobic and impervious to aqueous reagents. The majority of cell and tissue components have no natural color and are not visible. The first step in performing an H&E stain is to dissolve all the wax away with *Xylene* (a hydrocarbon solvent).

1.2 **Hydrate the Section**

After thorough de-waxing, the slide is passed through several changes of alcohol to remove the xylene, The introduction of hydration into the tissue section was done by passing the slides slowly through a series of decreasing concentrations of alcohol .The first alcohol used after the clearant was anhydrous (100%). To prevent the carryover of the clearant into lower alcohol concentrations, three changes of anhydrous alcohol were used followed by alcohols of lower concentrations: 95% (1-2 changes), 70% (1 change), and sometimes 50% (1 change). Immersion times should be sufficient to assure the complete removal of the *Xylene* solutions, then thoroughly rinsed in water. The section is now hydrated so that aqueous reagents will readily penetrate the cells and tissue elements.

1.3 **Hematoxylin Nuclear Stain**

The slides were stained with a nuclear stain hematoxylin, which consists of a dye (oxidized hematoxylin or hematein) and a mordant or binding agent (an aluminum salt) in the solution. Initially this stains the nuclei and some other elements a reddish-purple color. The metallic firm was removed using whatsman paper 1 and slides were immrsed in hematoxylin for 3-5 seconds.

1.4 **Complete the Nuclear Stain by "Blueing"**

The slides was washed in running water 2-3 times for 2 minutes each . The section was rinsed and checked to see if the nuclei are properly stained, showing adequate contrast and to assess the level of background stain.

***1.5 Differentiation*: Remove Excess Background Stain**

After hematoxylin is employed, a differentiation (destaining) step is required to remove non-specific background staining and to improve contrast. A weak acid alcohol was used for selective removal of excess dye from the section [*Dip in 1% acid alcohol (1% HCl in 70% alcohol) for a 20-30 seconds*]. Staining methods that include a destaining or differentiation step are referred to as "regressive" stains.

1.6 **Counterstain (using Eosin)**

The section was stained with an aqueous or alcoholic solution of eosin (depending on personal preference) for 45 seconds . This color's many nonnuclear elements in different shades of pink.

1.7 **Dehydration, Clearing and Mounting**

Following the eosin stain, the slides were passed through increasing concentration of *alcohols:* 70%, 95% for 1 minute each to remove all traces of water, then rinsed in bath of xylene for 2 minutes which "clears" the tissue and renders it completely transparent. A drop of DPX mountant was applied, followed by glass cover slip. Observe under compound microscope

2. Establishment of CAM Assay:-

Materials : Fertilised Eggs, Eggs Incubator, Paraffin Wax, Ethanol swabs etc.

Sample : Saline treated Tumour

Tumour procurement

Fresh tumour tissues of melanoma and squamous cell carcinoma were obtained from patients undergoing surgery Dr. R.P. Centre for Ophthalmic Sciences, All India Institute of Medical Sciences (AIIMS), New Delhi, India. The samples were kept in saline solution and an ambient temperature was maintained using through an ice pack. They were then transferred from AIIMS to the laboratory in Shivaji College, University of Delhi.

Methodology

2.1. *Procuring fertilized eggs and Candling*

Fertilised eggs were procured. Details of day0 (the day of egg laying) were estimated and the eggs were maintained in a warm, conducive environment. Each egg was analysed above a light source (a flash light) inside a dark room. The presence of the air sac was noted, the opacity in the centre of the egg was marked and recognised as the developing embryo. Fertilised eggs were separated from the unfertilised ones by this process

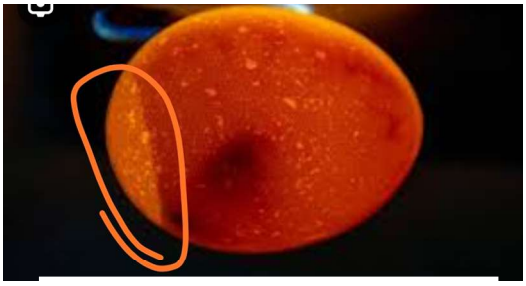


Image.Showing Air Space in Egg

2.2 **Incubation**

Fertilised chicken eggs (2- 3 days old) were procured from a local farm and cleaned. The presence of the embryo was confirmed by the process of egg candling. The eggs were then placed in an incubator in an upright position under the conditions of 37°C and relative humidity of 55-60% to allow stable conditions for embryogenesis until EDD 7- 9 (EDD- embryonic development day).

Image Showing Incubator Temperature at 36'C

2.3 **CAM Dropping**

The CAM assay was performed on EDD 7. The air sac was located by candling and a hole was made using a pin. Air was sucked out using a rubber bulb and the sucking force allowed the CAM to drop.

2.4**. Windowing and tumour placement**

The egg was cleaned using absolute ethanol. A square was drawn on the shell, just above the located embryo. A needle was used to finely chip away the corners of the square. A sturdy tape (electric tape/thick cello tape) could also be used for careful removal of cut piece.

Fig2.a

Fig2.b

Fig2.c

A sterile silicone o- ring was then placed on the CAM (fig 2.a) to place the tumour sample

Melted wax was used to ensure the placement of the coverslip (Fig.2.b) well above the CAM layer and seal the egg .The developing embryo could be viewed (Fig2.c) , as and when required..

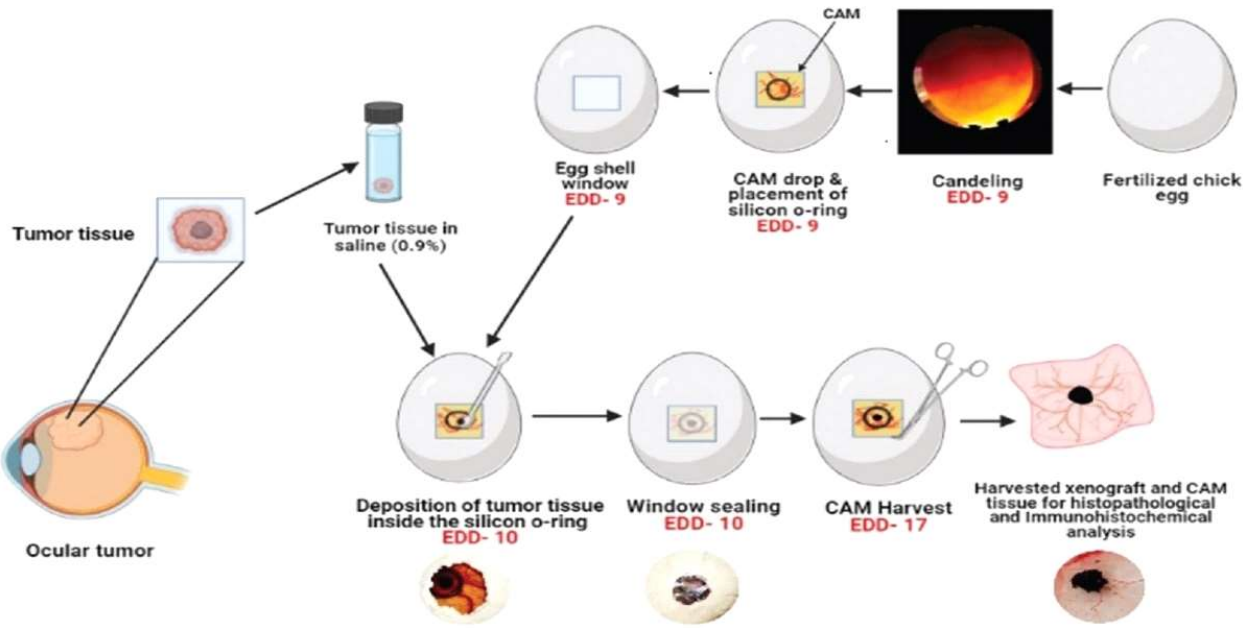


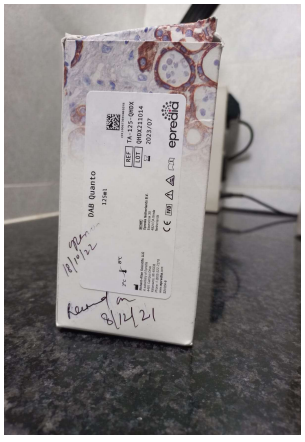
Fig.- Schematic representation of CAM assay protocol

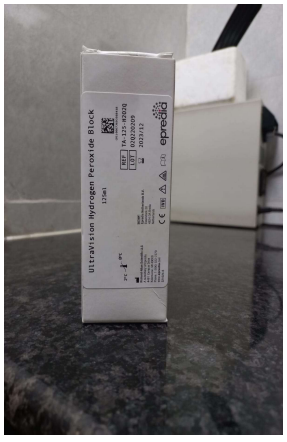
3. Immunohistochemistry For Cyclin B1:-

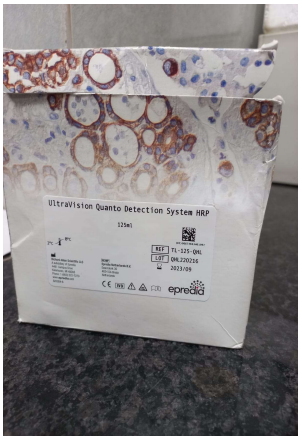
Materials : Formalin fixed, Paraffin tissue sections

Reagents: Xylene, Alcohol (vary concentration) or absolute Ethanol, EDTA buffer, TBST buffer Epredia IHC Detection sydtem kit : Ultra vision Peroxide Block, Ultra Vision Quanto Detection System HRP, DAB Quanto, Primary Antibody [REF: TL-125-QPB],

Instruments: Antibody Retrival Machine, Refrigerator (;as per need)







Methodology

3.1 **Deparaffinization and Rehydration**

- Immerse the slides in xylene (mixed isomers) 2 times for 10 minutes each.
- Immerse the slides in 100% acetone for 3 minutes
- Immerse the slides in 100% alcohol for 3 minutes
- Washed the slides in running water for 5 minutes.
- The slides were then washed with distilled water 3 times for 3 minutes each.

3.2 **Retrival of Antigens**

In the antigen retrieval we add EDTA buffer in the retrieval steam machine and set the temperature of retrieval machine at 98.C. and when the temperature in the machine rises to 65^{0}C we placed the slides such that they totally get submerged in the buffer and after restarted the machine and increased the stared the process of retrieval and and set the timer for retrival to 30 minutes in sterile condition. Afterwards we carefully removed the slides from the retrieval machine with the help of forceps and rinsed it with distilled water 3 times for 3 minutes each.

Antigen Retrival Machine

Most formalin-fixed tissues require an antigen retrieval step prior to immunohistochemical staining. Methylene bonds formed during the repair of the broad bean protein cover the antigenic surface. The antigen retrieval method disrupts methylene bonds and exposes antigenic sites, allowing antibodies to bind.

Cleaned the slides with tissue carefully and Surround the tissue with a hydrophobic barrier using a barrier pen.

3.3 **Blocking**

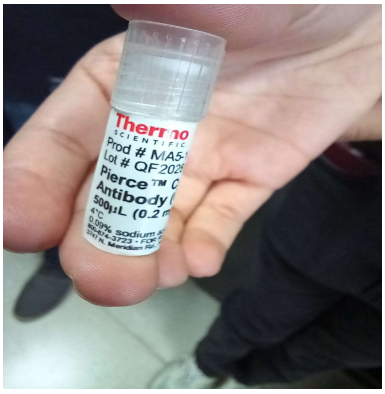
To Quench Peroxidase activity, The sample was Incubated with 2-3 drops of Peroxidase blocking reagent for 10- 12 minutes in humid chamber.

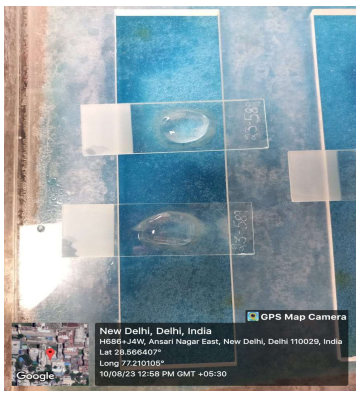
Showing Hydrophobic circle and peroxidase over tissue

This prevent non specific binding of Antibody- Antigens.

After that cyclin B1 was added in the slides with protein block (UV blocker)and left for 10 minutes and then washed with TPBS buffer 3 times for 3 minutes each. Then the primary antibodies were added to slides and after that slides were isolated at 4^{0}C for a whole night.

Next day the slides were kept in room temperature for 1hr prior the addition of secondary antibodies and washed with the buffer 3 times for 5 minutes each.





Then primary antibody amplifier was added and then kept undisturbed for next 10 minutes and again washed with buffer 3 times for 5 minutes. and then secondary antibodies were added to the slides in absence of light as it is light sensitive and kept in dark for 30 minutes. then the slides were washed with buffer 3 times for 5 minutes.

3.4 **Chromogens Staining**

the slides were then washed in distilled water and wiped up with tissue paper and then the sections were simply incubated with the DAB solution for a few minutes. DAB (3,3'-diaminobenzidine) is a benzene derivative. It is commonly used as a chromogen for immunohistochemical (IHC) staining. It is also used in in situ hybridization (ISH) and sometimes in dot blots and Western blots. In DAB staining, DAB is oxidized by hydrogen peroxide in a reaction that is usually catalyzed by horseradish peroxidase (HRP). Oxidized DAB forms a brown precipitate on the HRP surface, which can be visualized using light microscopy

3.5 **Counterstaining and Mounting**

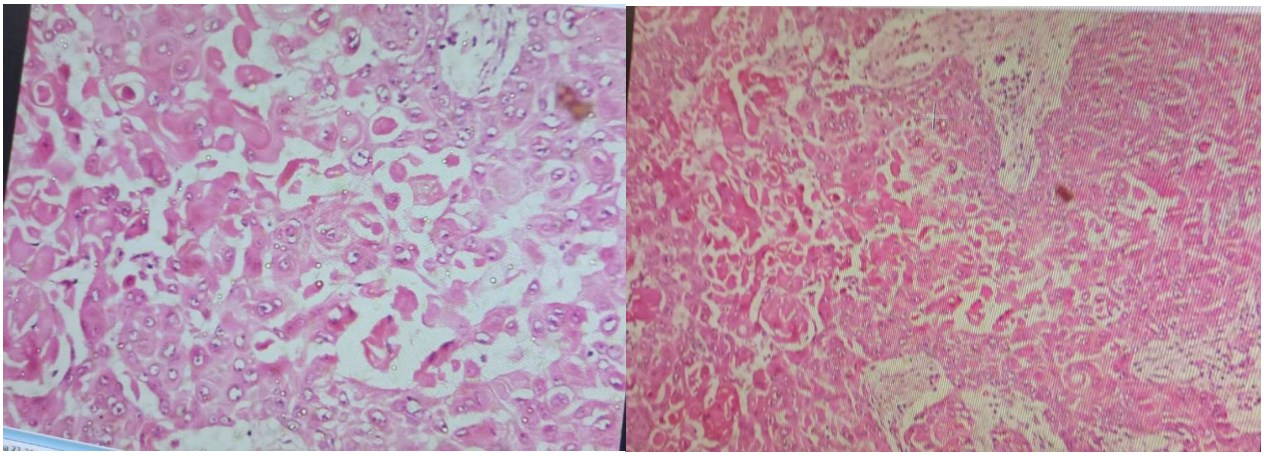
After staining the target antigen by IHC, a secondary stain Hematoxylin is usually applied to provide contrast that helps the primary stain more distinct.

A mounting medium drop of DPX mountant was used to attach a coverslip and observed under microscope.

Results :-

1.Histology of SCC

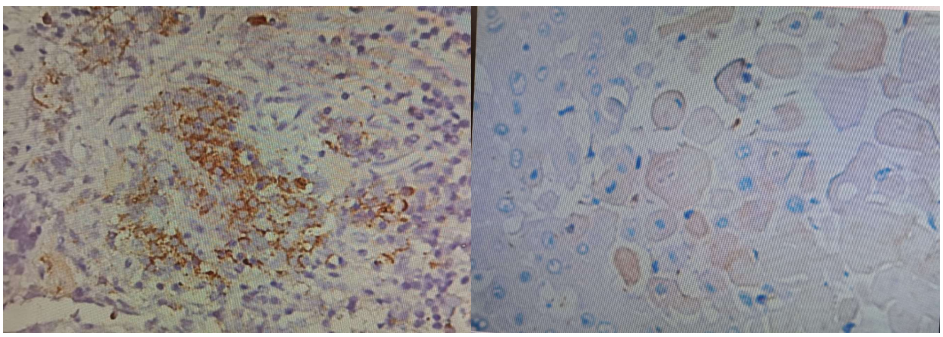
The Histology SCC has nests of squamous epithelial cells arising from the epidermis and extending into the dermis. The malignant cells are often large with abundant eosinophilic cytoplasm and a large, often vesicular, nucleus. Variable keratinisation (keratin pearls) is present.



2.Immunohistochemistry of Cyclin B1

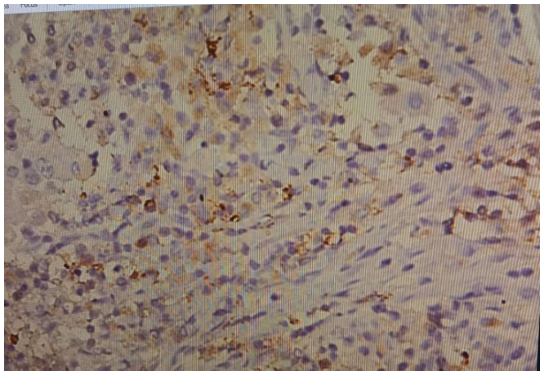
Cyclins bind to and regulate the activity of the Cyclin dependent protein kinases (CDKs). Cyclin B1 or CCNB1 is a regulatory protein involved in mitosis. It is essential for the control of the cell cycle at the G2/M transition. Cyclin B1 complexes with p34 (cdc2) to form the maturation-promoting factor (MPF). Two alternative transcripts have been found, a constitutively expressed transcript and a cell cycle-regulated transcript, that is expressed predominantly during G2/M phase. Cyclin B1 is not ubiquitinated during G2/M phase, resulting in its steady accumulation during G2 phase, followed by abrupt APC dependent destruction at the end of mitosis. Destruction of Cyclin B1 is required for cell cycle progression. Cyclin B1 is overexpressed in various cancers, including breast, prostate, and non_small cell lung cancer.

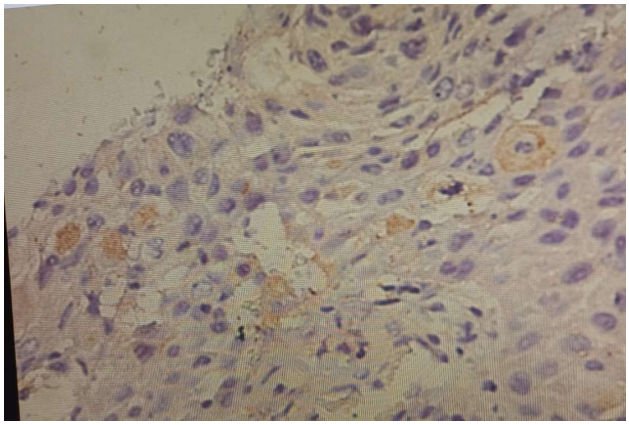
On observation of various sub-cellular staining locations , namely , nucleus , cytoplasm and perinuclear region of the cell , both cytoplasmic and nuclear regions were apprecated.

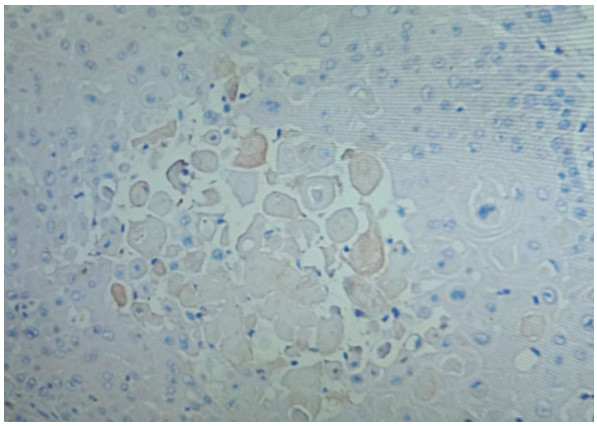


Cell cycle related proteins are localised at various sub cellular locations and aid in progression of cell cycle. Cyclin B1 is associated with mitotic asters during the prophase and thereby is located in the nucleus. In some cells , the localisation of cyclin B1 to the interphase was due to the fact that those cells were in interphase (S-G2 phase). This cytoplasmic localisation of mitochondria was due to its Section of tissue stained with cyclin B1 antibody association with the microtubule. This association prepares the establishment of mitotic spindle.

It has been documented widely in the literature that G2 arrest is accounted in cells after DNA damage. Such an arrested cell seem to possess near maximal Cyclin B1 concentration and high levels of Cyclin B1-CDC2 association. DNA damage in human cells also seems to arrest cells in G2 by stabilization of inhibitory phosphorylation sites of CDC2. This is done by inhibiting the phosphatase, CDC25c. There is also a possibility that DNA damage has stabilised the cytoplasmic location of cyclin B1.





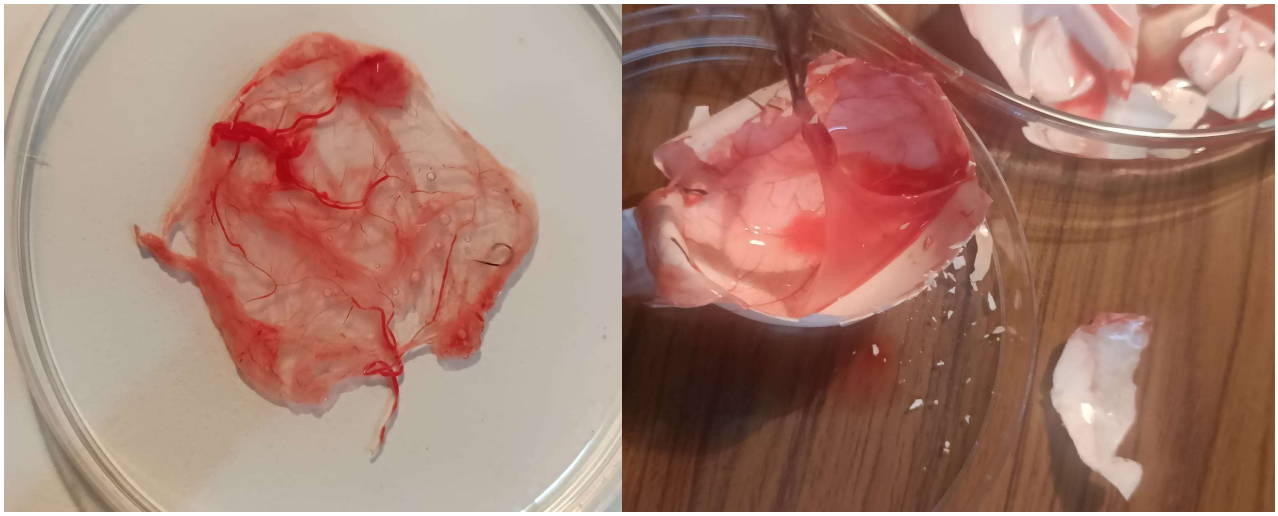


It is declared that spontaneous or constant appearance of Cyclin B1 throughout the cell cycle would direct the activation of already existing CDC2 which forces the cell in to mitosis. This variety of over expression might be the result of amplified synthesis or impaired degradation, or because of its inappropriate localization owing to failure in nuclear/cytoplasmic homeostasis.

3. Development of Choriollantoic Membrane

Choriollantoic membrane is developed after allowing the eggs to incubate for the next Next days (Embryonic Development Day or EDD 17)munder stable cotitions providing temperature 37’C and Humidity 70-80%.

The CAM tissue layer was extracted from the Egg. then tissue was processed, fixed followed with Hemotoxylin and eosin {H/E) staining.



The Section were Mounted on a slide and observed under the microscope

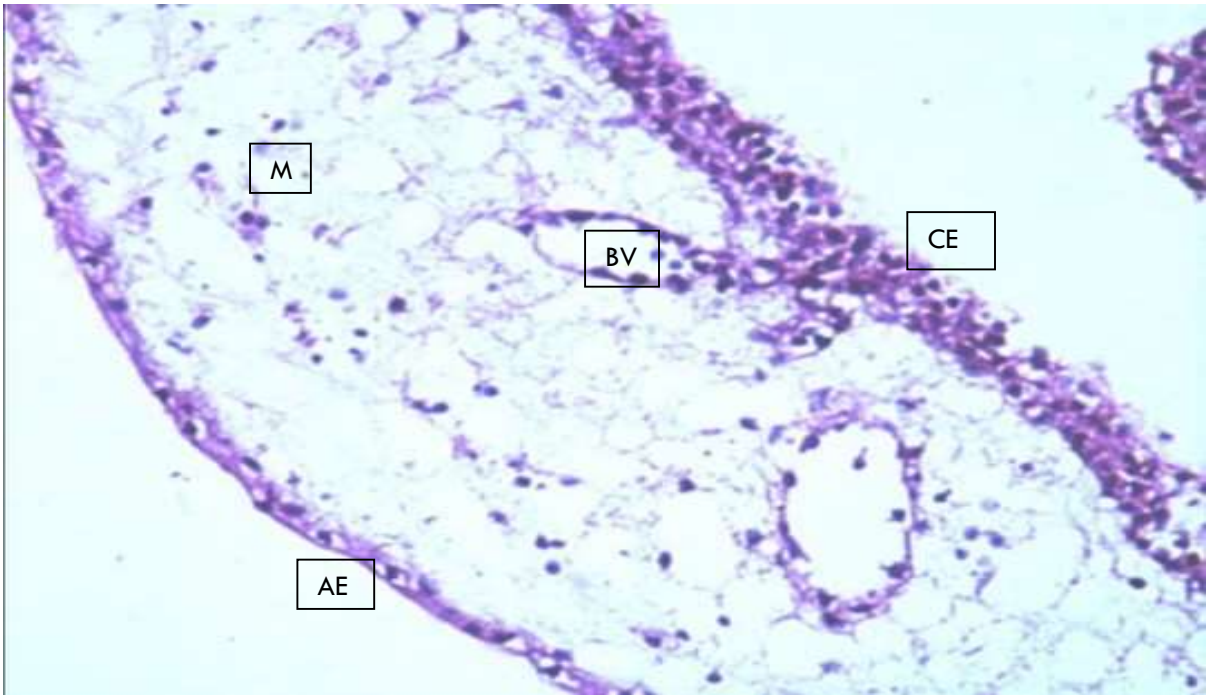


Figure Showing hematoxylin-eosin staining of Choriollantoic Membrane;

CAM, showing the trilayered structure consisting of the CE, central mesenchyme layer(M) rich in BV, and AE. The figures is original work by our research groups. CAM, chorioallantoic membrane; CE, chorionicectoderm; AE, allantoic endoderm; BV,blood vessels.

Discussion :-

Hematoxylin and Eosin Staining (H&E Staining) :

H&E staining, considered the cornerstone of histopathological analysis, offers detailed insights into tissue architecture and cellular morphology. Its widespread adoption is a testament to its reliability and efficiency. While the primary visual cues—blue nuclei and pinkish cytoplasm—are quite straightforward, the interpretation demands a deeper understanding of cellular structures and pathological changes. For instance, certain diseases manifest as alterations in nuclear size, shape, or staining intensity, which can be discerned clearly through H&E staining. One limitation, however, is that this staining technique does not allow for the differentiation of all cellular subtypes or specific protein expressions, which is where specialized immunohistochemical stains come into play.

CAM Assay :

The chick embryo chorioallantoic membrane (CAM) has been used as a relatively simple, time-, and cost-efficient model to study different cancer-associated processes, including angiogenesis, cancer cell invasion, metastasis formation, and tumor progression.

As concerns the use of the CAM assay in the study of metastatic process, many fundamental applications have benne introduced in the last fifty-sixty years . In 1949, Bender et al., showed that cell suspensions obtained

The use of CAM has continued to increase in the last decade as it has been shown to be a good growth platform for a wide range of cancer cell lines. CAM has been successfully used in the experimental metastasis studies, providing comprehensive information about critical steps of metastatic process including survival of cancer cells in the systemic circulation and transport to target organs; arrest of cancer cells in the microcirculation; migration of cancer cells through the vessels wall into

Since the chick embryo is naturally immunodeficient, the CAM can support the engraftment of tumor cells, and their growth therein can faithfully recapitulate most of the characteristics of the carcinogenic process including: growth, invasion, angiogenesis and colonization of distant tissues.

We used this set-up study various Developmental stages and extracted CAM for the examination.

Unfortunately certain circumstances doesn't allow to get the metastasis study.

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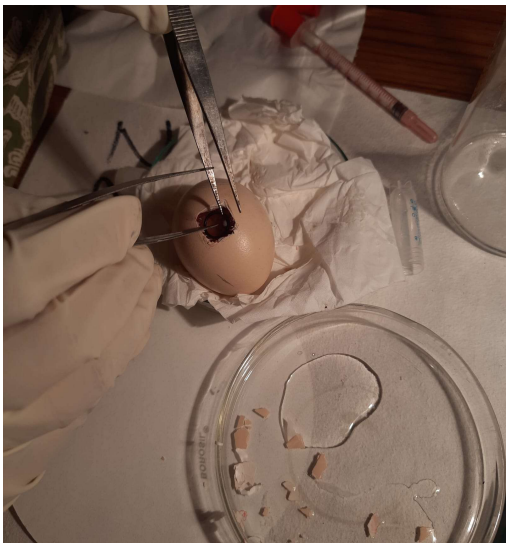
Immunohistochemistry (IHC) for Cyclin B1 :

IHC is an indispensable method that marries histological techniques with immunological detection. Targeting Cyclin B1, a regulator of the G2/M phase of the cell cycle, provides essential information about cellular proliferation within a tissue. Dysregulation of Cyclin B1 has been implicated in numerous cancers, and its overexpression can be indicative of a poor prognosis. The precise visualization of Cyclin B1 through IHC can guide therapeutic decisions, especially in oncology. However, the reliability of IHC is contingent upon numerous factors: the quality of the primary antibody, the specificity of the secondary antibody, and even the stringency of the washing steps. A misstep or inconsistency in any phase can lead to false results, underscoring the meticulous precision required in this method.

In wrapping up, these methods—H&E staining, CAM assay, and IHC for Cyclin B1—while seemingly distinct, are intertwined in the tapestry of biomedical research and diagnostics. Each offers a unique lens through which we can unravel the complexities of biology, and together, they provide a more holistic understanding of tissue morphology and function.

OUR VISIT AND EXPOSURE IMAGES



References:

1 .https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4869455/

2. https://www.skincancer.org/skin-cancer-information/squamous-cell-carcinoma/

3. Squamous Carcinoma of the Eyelid ; Article initiated by: Ana Vega Carreiro de Freitas, MD, Marcus M. Marcet, MD, Patricia Henriques Lyra Frasson M.D. https://eyewiki.aao.org/Squamous_Carcinoma_of_the_Eyelid

4. Squamous Cell Carcinoma of the Eyelid and Ocular Surface;WRITTEN BY : Jin-Jhe Wang, Yueh-Ju Tsai and Chau-Yin Chen https://www.intechopen.com/chapters/81221

5. Figure4.1 **Cell cycle control in cancer | Nature Reviews Molecular Cell Biology**

6..Figure4.2
https://bio.libretexts.org/Bookshelves/Introductory_and_General_Biology/Book%3A_General_Biology_%28Boundless%29/10%3A_Cell_Reproduction/10.03%3A_Control_of_the_Cell_Cycle/10.3C%3A_Regulator_Molecules_of_the_Cell_Cycle

7. https://www.sinobiological.com/category/ihc-introduction

8.https://www.thermofisher.com/in/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-immunohistochemistry.html

8 Applications of the Chick Chorioallantoic Membrane as an Alternative Model for Cancer Studies . https://karger.com/Article/FullText/513039

9.Histopathology
https://www.rcpath.org/discover-pathology/news/fact-sheets/histopathology.html#:~:text=Histopathology%20is%20the%20diagnosis%20and,clinicians%20manage%20a%20patient's%20care.

10.https://www.abcam.com/content/immunohistochemistry-the-complete-guide#:~:text=Immunohistochemistry%20(IHC)%20uses%20antibodies%20to,detection%20with%20a%20fluorescent%20dye.