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

PROJECT REPORT – SVC 2245

Immunohistochemical detection of anti-apoptotic marker and differential mRNA network analysis of Rhabdomyosarcoma

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(Sri Venkateswara College Internship Program in Research and Academics)

This is to certify that this project on "**Immunohistochemical detection of anti-apoptotic marker and differential mRNA network analysis of Rhabdomyosarcoma "**was registered under SRIVIPRA and completed under the mentorship of Dr.P. Jayaraj during the period from 21st June to 7th October 2022.

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AIM:

To study the immunohistochemical expression of Desmin in ERMS

OBJECTIVES:

Specific objectives of the study were as following:-

1. To examine the differential upregulation of Desmin and XIAP in Embryonal Rhabdomyosarcoma, using sebaceous cell carcinoma as control by immunohistochemical techniques.
2. To correlate their expression with clinicopathological outcomes in ERMS.
3. To perform various biochemical techniques, including but not limited to, microtomy, protein blocking and brightfield microscopy.
4. To perform various bioinformatics analysis using tools like KEGG, Human Protein Atlas, miRDb, STRING, GEO2R etc.

INTRODUCTION:-

Cancer remains as a leading cause of worldwide deaths since the past decade which in turn has encouraged multi-level scientific research in this field. This genetic and inheritable malady is characterized by uncontrolled cell proliferation and tumorigenesis that can go on to invade healthy tissues and induce malignancy, making it highly dangerous and difficult to localize. There are multiple types of neoplasms present based on a number of criteria like site of origin, intensity of metastasis etc.

Rhabdomyosarcoma (RMS) is the most widely known pediatric and childhood soft tissue sarcoma originating from juvenile mesenchymal cells involved in the differentiation of skeletal muscles. Although soft tissue sarcomas are rare malignancies accounting for roughly 1% adult cases and 7% pediatric reportings, RMS itself represents almost 50% of such pediatric cancers with a frequency of 4.3 million occurrences per year in the 0-10 year age group [1],[2]. This cancer affecting skeletal myoblast-like cells has numerous histological manifestations as it is a heterogeneous tumor based on site of incidence, age, prognosis etc. RMS has 4 significant subtypes, that are embryonic, alveolar, spindle cell/sclerosing and pleomorphic, out of which only two are the most frequently occurring ones- embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS). Both of these sarcomas have distinct clinical manifestations and biochemical basis resulting in different prognosis and even treatment strategies. In our study as well, we aim to explore the immunohostochemical aspects of ERMS based on collected patient samples.

ERMS accounts for approximately 80% of the entire RMS occurrences affecting a vulnerable age group of 0-4 pediatric ages with common sites being head, neck as well as the urino-genital tract. The morphology of tumor cells has resemblance to the normal skeletal myocytes. Conversely, ARMS is representative of roughly 20% of all RMS cases but has a high lethality with prevalence in young adults and adolescents. Its typical origin is observed in the peripheries or limbs, trunk region, and is many times involved with lymph nodes, either regionally or metastatically,

resulting in a much more severe and fatal manifestation. In ARMS, the cancer cells are arranged such that a pattern is created with small spaces in between, known as "pseudo-alveoli", hence, termed as alveolar rhabdomyosarcoma. Further details and contrast between ARMS and ERMS is tabulated in the following table.

S. No.	Embryonal Rhabdomyosarcoma	Alveolar Rhabdomyosarcoma
1.	Mostly seen in children under the age of 5.	Seen in all age groups.
2.	Affects the head, neck, bladder, prostate or vagina and testicles.	Affects the large muscles of arms, legs and trunk.
3.	More common in males than females.	Occurs equally in both males and females.
4.	Cells are small, round with variable degrees of differentiation.	Cells are small, round and undifferentiated.
5.	Prognosis is favorable.	Prognosis is worse.
6.	Myogenin expression is strong in the nucleus.	Myogenin expression is diffused.

Orbital Rhabdomyosarcoma:-

In around 10% of rhabdomyosarcoma cases, the orbit is the primary location. An estimated 350 new instances of RMS are identified each year in the US, of which 35 have orbital RMS. 8 years is the average age at diagnosis. Boys are more frequently impacted than girls. Ocular RMS consists of tumors in the orbit, or less rarely in other ocular adnexal tissues or inside the eye.

Patients with orbital RMS typically appear with exophthalmos, which increases quickly over weeks (80-100%), or globe displacement (80%). Although orbital RMS rarely spreads metastatically, if untreated, RMS has a tendency to do so, mostly by

hematogenous migration, to the lung, bone, and bone marrow.Locally, orbital RMS can extend intracranially and infiltrate the orbital bones

Histology of erms :

Embryonal rhabdomyosarcoma has been informally classified as a "small round blue cell tumor" because of the characteristic microscopic appearance of its cells after histological staining with hematoxylin and eosin. Histologically,embryonal rhabdomyosarcoma commonly presents as alternating loose and dense patches of cells, including round cell and spindle cell components. The heterogeneous structure resembles striated muscle at various embryonal developmental stages.

Erms are composed of primitive mesenchymal cells that show variable degrees of skeletal muscle differentiation.They are moderately cellular but in the typical pattern often contain both hypocellularity and hypercellular areas with a loose, myxoid stroma. Perivascular condensations of tumor cells in the less cellular regions are common. Sheets of small, stellate, spindled or round cells with scant or deeply eosinophilic cytoplasm and eccentric, small oval nuclei with a light chromatin pattern and inconspicuous nucleoli.Can occasionally identify tumor cells that contain generous amounts of eosinophilic cytoplasm, a feature of rhabdomyoblastic differentiation (so called strap cells).These may become more prominent with chemotherapy (chemotherapeutic induced cytodifferentiation). May have cells with elongated tails of cytoplasm (tadpole cells). If densely cellular, it may resemble solid alveolar rhabdomyosarcoma. Botryoid variant frequently shows a cambium layer: a hypercellular zone immediately beneath the epithelial surface. Cells are undifferentiated, round or spindled with minimal cytoplasm, frequent mitotic figures. Deeper layers of the tumor are typically less cellular but overall conform to the histology of embryonal rhabdomyosarcoma (ERMS) with variation by region.

- Rare morphological patterns:
    - Rhabdomyosarcoma with rhabdoid features (epithelioid or rhabdoid-like RMS)
    - Clear cell change

- Cartilaginous metaplasia
- Do not confuse with the following different entities:
    - Ectomesenchymoma: melanocytic, neuroblastic / ganglionic or Schwannian differentiation in the setting of background ERMS.
    - Malignant triton tumor: rhabdomyosarcomatous differentiation in a malignant peripheral nerve sheath tumor; very bad prognosis.

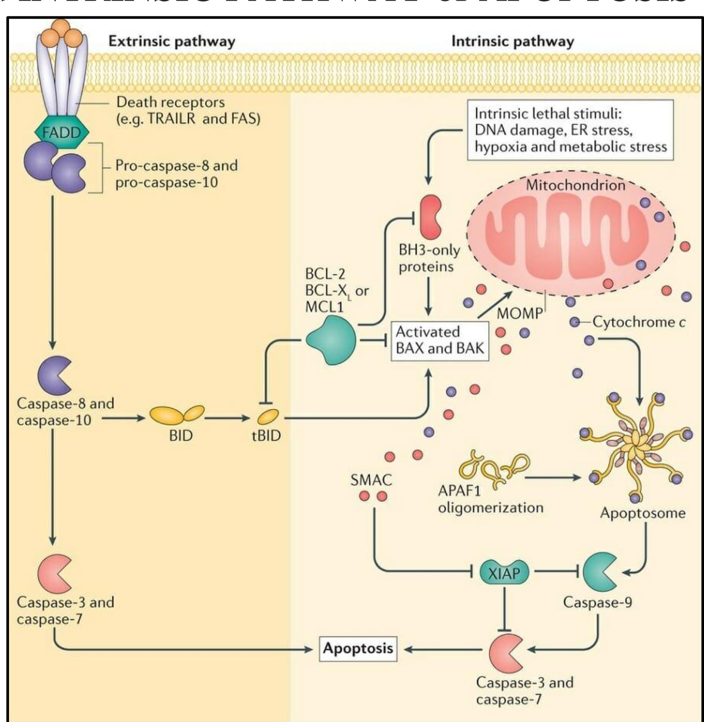
Histologically, three subgroups could be distinguished: primitive (<10% rhabdomyoblasts), intermediate (10–50% rhabdomyoblasts) and well differentiated (>50% rhabdomyoblasts) eRMS. Vimentin-positive cells predominated in the primitive eRMS. Intermediate eRMS showed large proportions of desmin-positive cells but vimentin-containing cells were also numerous. Myoglobin could only be demonstrated in well differentiated eRMS. Primitive and well differentiated eRMS mainly occurred in the head and neck area, whereas intermediate eRMS were predominantly located in the abdomen.

ERMS are characterized by frequent loss of heterozygosity at the 11p15 locus, a region harboring the genes for insulin-like growth factor 2 (*IGF2), H19,* $p57^{kip2}$, and associated with alterations of imprinting.

Role of Apoptosis in Cancer:-

Apoptosis is the mechanism for programmed cell death. It is a highly regulated process and ensures elimination of any unnecessary or unwanted cells. The apoptotic pathway is activated by both intracellular and extracellular signals. There are two different pathways that lead to apoptosis: the intrinsic and extrinsic pathways that correlate with the signal type. The intracellular signals include DNA damage, growth factor deprivation and cytokine deprivation, whereas the most common extracellular signals are death-inducing signals produced by cytotoxic T cells from the immune system in response to cells that are damaged or infected.

EXTRINSIC and INTRINSIC PATHWAY of APOPTOSIS



The prevention of cancer is one of the main functions of apoptosis. Typically, it is the intrinsic pathway that is inhibited in cancer. The loss of apoptotic

control allows cancer cells to survive longer and gives more time for the accumulation of mutations.
Loss of Apoptosis can be induced by inhibiting caspase function or the trigger for apoptosis can be disabled. The upregulation of antiapoptotic BCL-2 proteins and loss of BAX and/or BAK are the predominant methods of evasion. Mutation in *BCL-2* enhance tumor onset. The overexpression of BCL-2 protein is present in over half of all cancers, regardless of type. This results in tumor cells that are resistant to any intrinsic apoptotic stimuli which includes some anticancer drugs.

Few known apoptotic/anti-apoptotic genes in RMS

GENE	**NATURE OF GENE**	**REGULATION IN RMS**
PAX3/*FKHR* fusion gene	Anti-apoptotic	Upregulation
wild-type *PAX* genes	Anti-apoptotic	Upregulation
BCL2	anti-apoptotic	Upregulation
BAX	Proapoptotic	Downregulation

IHC MARKERS FOR RHABDOMYOSARCOMA AND OTHER SARCOMAS

Rhabdomyosarcoma is one of the most common soft tissue sarcomas in pediatric studies which accounts for about for 5-10 percent of all pediatric cancers and broadly they are of two types Alveolar Rhabdomyosarcoma ARMS) and embryonal rhabdomyosarcoma (ERMS) and out of two ARMS have much worse prognosis and is more malignant than the later. Therefore, it is relevant to distinguish the two from each other so that treatment methods can be chosen accordingly. To do that we use a technique known as immunohistochemistry (IHC) and it is a globally accepted tool for cancer diagnosis.(5)

IHC MARKERS:-

Most widely used IHC markers to distinguish RMS from other types of tumors/sarcomas are MyoD, Desmin and myogenin.(5,6)

Myogenin - It belongs to myogenic regulator proteins whose expression would determine commitment of primitive mesenchymal cells to skeletal muscle and therefore myogenin expression has been used as a specific diagnosis marker for RMS from other tumors as it is specific for rhabdomyoblastic differentiation.

Desmin – It is an intermediate filament and is a characteristic of skeletal muscles and certain visceral muscles and is usually expressed early in muscle development. We prefer the use of desmin as a marker than myoglobin because relatively few of tumor cells came to be positive in comparison to desmin in pediatric patients.(4)

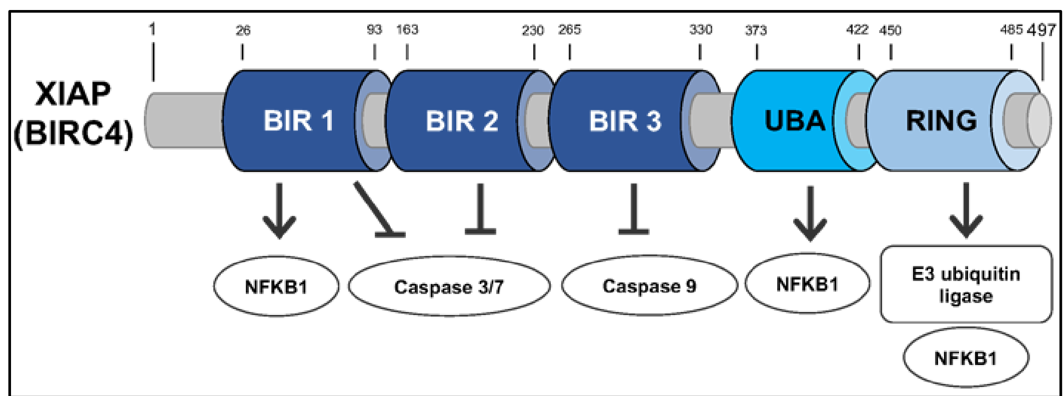
Some of the other IHC markers can be seen in tabular form below– (4,5,6,7,8,9)

IHC MARKER	ERMS	ARMS	OTHER SARCOMAS
Desmin	Positive	Positive	Positive in Desmoplastic small cell tumor, leiomyosarcoma
Myogenin	Positive	Positive (usually stronger stanning than ERMS)	
MyoD1	Positive	Positive	
AP2β	Negative	Positive (Fusion positive)	
P-cadherin	Negative	Positive (Fusion Positive)	
EGFR	Positive	Negative – FP (Low level expression in fusion negative ARMS)	
fibrillin-2	Positive	Negative -FP (Low level expression in fusion negative ARMS)	

Dax-1	Negative (slight expression can be seen)	Positive (fusion positive)	Positive in Ewing sarcoma
HMGA2	Positive	Negative- FP (Positive in Fusion negative-ARMS)	
TFAP2β	Negative	Positive- FP	
NOS-1	Negative	Positive- FP	

XIAP (X-linked inhibitor of apoptosis protein)

XIAP (X-linked inhibitor of apoptosis protein), regulates cell death signalling pathways by binding to and inhibiting caspases. It is involved in cancer initiation, promotion and progression. It belongs to the IAP family and is also known as inhibitor of apoptosis protein 3 (IAP3). The entire XIAP gene is approximately 54.2 Kb. The polymerase chain reaction (PCR) approach was used to detect XIAP utilizing homologous IAP sequences from Cydia pomonella's X chromosome and therefore it was named as X-linked inhibitor of apoptosis protein. The Xiap gene codes for XIAP and is located at Xq24–25.

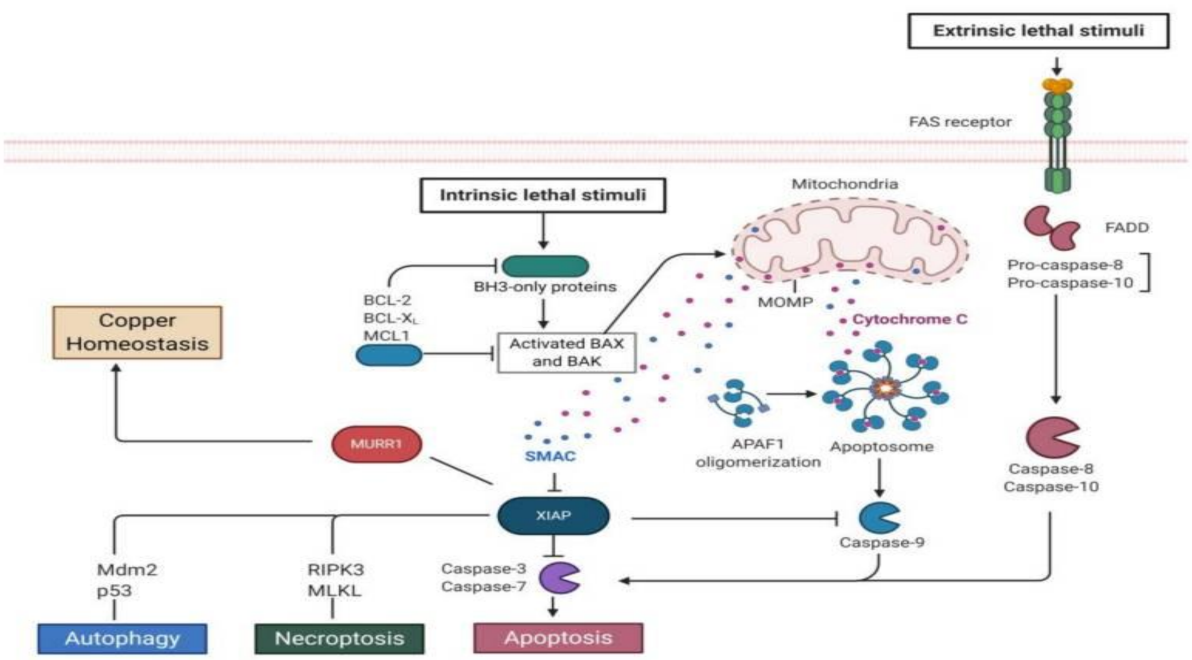


Structural representation of XIAP. Three BIRs, a UBA, and a RING finger domain are all present in XIAP. Activated caspase-3 and -7 bind to a groove in BIR2 that also requires amino terminal flanking residues, which can be completed by IAP-binding motif (IBM) containing proteins. TAB1 binds the BIR1 domain. It has been demonstrated that the RING domain is crucial for heterodimerization with h c-IAP1[10].

XIAP consists of three major types of structural domains: A baculoviral IAP repeat (BIR) domain (approximately 70 amino acids), which characterizes all IAP (inhibitor of apoptosis), a UBA domain, which allows XIAP to bind to ubiquitin and a zinc-binding domain, or a "carboxy-terminal RING Finger". XIAP has been characterized with three amino-terminal BIR domains followed by one UBA domain and finally one RING (Really Interesting New Gene) domain. Between the BIR-1 and BIR-2 domains, there is a linker-BIR-2 region that is thought to contain the only element that comes into contact with the caspase molecule to form the XIAP/Caspase-7 complex. The BIR2 domain of XIAP inhibits caspase 3 and 7, while BIR3 binds to and inhibits caspase 9. BIR2/3 domains are the major components responsible for the anti-apoptosis activity of XIAP. IAPs can catalyze self-ubiquitination, ubiquitination of caspase-3, or caspase-7 by degradation via proteasome activity by utilizing the RING domain, which makes use of

E3 ubiquitin ligase activity. In solution the full length form of XIAP forms a homodimer of approximately 114 kDa.

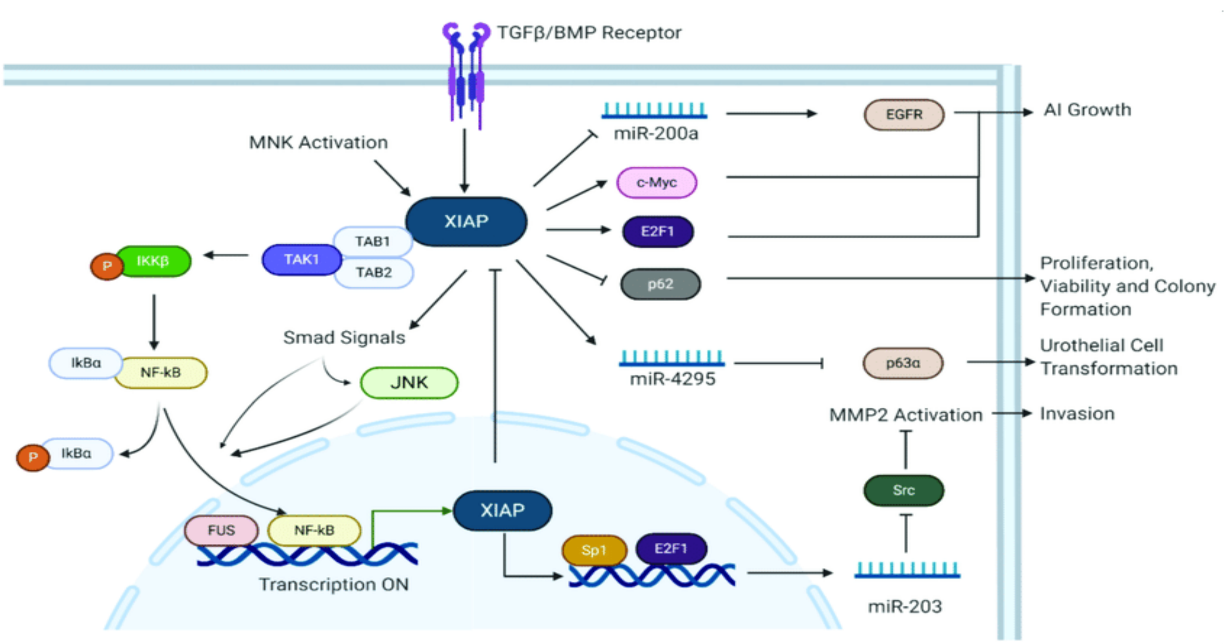
XIAP and Cellular Functions



XIAP is a multifunctional protein as it is involved in multiple cellular signaling pathways and cellular processes, including cell death, inflammation, cell cycle, and cell migration. The multifunctionality of this protein may be due to the presence of different domains..The major function of XIAP is to prevent apoptosis. It is also involved in the regulation of necroptosis, a programmed form of necrosis, or inflammatory cell death. According to research on mouse neutrophils, the transition to necroptosis involves the proteins RIPK3 (Receptor-Interacting Serine/Threonine-Protein Kinase 3) and MLKL (Mixed Lineage Kinase domain Like pseudokinase).apart from playing a role in apoptosis and necroptosis, it is also a modulator of autophagy. Additionally, XIAP regulates innate immunity through NOD2 (Nucleotide-binding Oligomerization Domain-containing Protein 2) signalling and restricts caspase-8 dependent IL-1β processing. It has been observed that XIAP participates in copper homeostasis maintenance by inhibiting MURR1, a recently discovered copper homeostasis factor. MURR1 is bound to by XIAP and ubiquitinated, which causes MURR1 to degrade without impairing XIAP's anti-apoptotic properties.

XIAP in Cancer;-

The National Cancer Institute panel of 60 human tumor cell lines was used to evaluate the expression of human IAPs, and XIAP was discovered to be expressed in the majority of these cell lines.
XIAP gene encodes a protein that belongs to a family of apoptotic suppressor proteins. All members of this family share a conserved motif called baculovirus IAP repeat, which is essential for their anti-apoptotic function. This protein suppresses apoptosis caused by menadione, a powerful inducer of free radicals, and interleukin 1-beta converting enzyme via binding to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2. Additionally, this protein inhibits at least two cell-death proteases from the caspase family, namely caspase-3 and caspase-7. Caspases are important initiators and effectors of apoptosis, and XIAP predominantly prevents it by blocking their activation and maturation.
XIAP prevents the activation of caspases 3, 7, and 9 that promote apoptosis. It has been demonstrated that increased XIAP expression in cell culture confers resistance to radiation and chemotherapy that induce apoptosis. XIAP is expressed in a variety of human tissues, although at low levels in normal tissues and more strongly in cancer cells. In cells from cancers such ovarian cancer, glioma, prostate cancer, rectal cancer, and childhood acute leukemia cells, XIAP can inhibit apoptosis of tumor cells and reduce their sensitivity.
XIAP is believed to be the most potent anti-apoptotic protein till date. Cancer, autoimmune illnesses, and neurological diseases are all closely correlated with apoptotic dysregulation or inhibition. As a result, aberrant XIAP expression is a helpful biomarker for identifying cancer and other disorders early in life.



XIAP signalling pathways in human cancers. Through TGF and bone morphogenetic protein (BMP) receptors, XIAP detects outside signals. RPS3 (ribosomal protein S3) and MNK activation boost XIAP expression levels. The XIAP-TAB1-TAB2-TAK1 complex, which is formed when TAB1 and XIAP bind, further activates NF-B and downstream pathways connected to NF-B. NF-B and JNK activation are both increased by XIAP activating Smad signals, while JNK activation can also activate the NF-B pathway on its own. The FUS protein and activated NF-B translocate into the nucleus where they bind to and start the transcription of XIAP. Intranuclear XIAP binds to Sp1 and E2F1 transcription factors, enhancing miR-203 production in the process. By blocking Src, miR-203 then promotes MMP2 activation and invasion. AI growth is aided by miR-200a inhibition, which also stabilizes c-Myc and interacts with E2F1 and XIAP. By inhibiting p62, XIAP enhances cell viability, proliferation, and colony formation.

ROLE OF miRNA

- **What are microRNAs?**

  MicroRNAs (miRNAs) are small, noncoding RNAs approximately 22 nucleotides (nt) in length that regulate gene expression. It has been evaluated that miRNAs regulate ~30% of human genes.

  They are usually convoluted in regulating various biological processes, including cell cycle, differentiation, development, and metabolism, as well as human diseases, such as diabetes, immuno- or neurodegenerative disorders, and cancer.

  De-regulation of a single or small subset of miRNAs was reported to have a profound effect on the expression pattern of several hundred mRNAs. MicroRNAs usually function by pairing with mRNAs of protein-coding genes to control their posttranscriptional repression.(12)

- **Biogenesis of microRNA.**

  The genes of miRNA are evolutionarily conserved and may be located either inside the introns or exons of protein-coding genes (70%) or in intergenic areas (30%). MiRNAs are favourably transcribed by polymerase II into long primary transcripts, up to several kilobases (pri-miRNA) that are subsequently processed in the nucleus by the enzyme Drosha to become ~70-nt-long precursor strands (pre-miRNA).

  During miRNA biogenesis, miRNAs are subject to intense transcriptional and post-transcriptional regulation, and the elucidation of these mechanisms has improved our understanding of miRNA deregulation in disease.(12,13)

Different mechanisms for the aberrant expression of miRNA were documented.

Three of them, viz.,

1. **Genetic alterations and single nucleotide polymorphism (SNP)**
2. **Epigenetic silencing**
3. **Defects in the miRNA biogenesis pathway**
4. **Transcriptional control changes**

5. **Chromosomal abnormalities(13)**

- ## miRNA and apoptosis

Tumor cells evolve a variety of strategies to limit or circumvent apoptosis. Among them, the loss of p53 tumor suppressor function is the most common. The alternative ways to evade apoptosis include upregulation of anti-apoptotic regulators, suppression of proapoptotic factors, and inhibition of the death pathway induced by extrinsic ligands. The components involved in anti-apoptosis are broadly inhibited or activated by miRNAs. Anti-apoptotic regulators (Bcl-2 and Bcl-xL) and proapoptotic factors (Bax, Bim, and Puma) are potential targets of some miRNAs, which have an important role in cell death. (13,14,15)

- ## miRNA can act as a Tumor suppressor or an Oncogene.

A particular miRNA may have exhibited its oncogenic function in some types of cancers whereas the same miRNA was reported to act as a tumor suppressor in other cancers. Some such examples include: (i) miR-29, specifically miR-29a/-b/-c was reported as an oncogene in breast cancer while the same miRNA-29 acted as a tumor-suppressor gene in lung tumors.

Some miRNAs can act as an oncogene or a tumor suppressor coherently because the same miRNA can participate in distinct pathways, having different effects on cell survival, growth, and proliferation that are dependent on the cell type and pattern of gene expression. Furthermore, the potential for miRNA-mediated regulation of gene expression is enormous since ~60% of mRNAs are predicted to be under the control of miRNAs.

Like a protein-coding gene, a miRNA can act as a tumor suppressor when its function loss can initiate or contribute to the malignant transformation of a normal cell.

Many other miRNAs are believed to act as tumor suppressors, although the evidence supporting those claims is merely correlative. Substantial experimental data are lacking, and miRNA knockout mice that develop or are predisposed to cancer have not been yet reported.

It is noteworthy that most of the miRNAs with a clear tumor suppressor role (e.g., miR-15- a/16-1, miR-29s, and let-7) have more than one genomic location, and

although they are transcribed from different precursors, the mature miRNA is identical.(11,13,14,15)

- **miRNA and its role in Rhabdomyosarcoma**

Normal physiological contribution of myogenic differentiation in RMS:-

The differentiation of stem cells into skeletal muscle tissue occurs through a tightly controlled spatial and temporal molecular cascade that involves miRNAs. The importance of these non-coding regulatory small RNAs in myogenesis has been recently highlighted by studies on mice conditionally deleted in a Dicer allele in skeletal muscle progenitors. These mice show severe muscle hypoplasia associated with perinatal death thereby, Micro-RNAs (miRNAs) are promising diagnosis biomarkers with their tissue specificities and their involvement in the oncogenic process.(17,18,19)

A total of ninety-seven distinct miRNAs were deregulated in ARMS and ERMS when compared to Normal skeletal muscle.

Related MiR-378 were dramatically decreased in RMS tumor tissue and cell lines. MiR-378 family showcased a possible target to the insulin-like growth factor receptor 1 (IGF1R), a key signaling molecule in RMS. Over-expression of miR-378-3p in an RMS-derived cell line repressed IGF1R expression and affected phosphorylated-Akt protein levels. Ectopic expression of miR-378a-3p caused differential changes in apoptosis, cell migration, cytoskeleton organization as well as a reformation of the muscular markers MyoD1, MyoR, desmin, and MyHC.(16,18)

Recently, some miRNAs acting as important regulators of skeletal muscle cell fate determination have been shown to be deregulated in both alveolar and embryonal RMS.

Gain-of-function experiments have proved that re-expression of selected"tumor-suppressor"miRNAs impairs the tumorigenic behavior of RMS cells. Moreover, miRNA expression profiling appears to be a propitious strategy for differentiating specific variants among RMS subsets and for providing useful potent information, especially for what concerns fusion-negative alveolar and embryonal forms.(19,20,21,

Materials and methods

- **IMMUNOHISTOCHEMISTRY (IHC):-**

Immunohistochemistry (IHC) is a highly sensitive and specific technique which exploits the binding between an antibody and antigen to detect specific antigens in cells and tissue which are then examined with the light microscope. Immunohistochemical (IHC) staining of formalin-fixed and paraffin-embedded tissues (FFPE) is extensively used in diagnostic surgical pathology.Here, the secondary antibody is labelled, allowing for signal amplification with primary antibody. There are various labels, one of which is horseradish peroxidase which produces a colored product after incubation with a chromogenic substrate such as diaminobenzidine (DAB).
DAB (3,3'-diaminobenzidine) is oxidized in the presence of peroxidase and hydrogen peroxide resulting in the deposition of a brown, alcohol-insoluble precipitate at the site of enzymatic activity.DAB (3, 3'-diaminobenzidine) produces a dark brown reaction product and can be used for both immunohistochemical and blotting applications.
Primary antibody used: Monoclonal antibody against XIAP (A-7 clone, Santa Cruz Biotechnology)

Two kits, UltraVision Quanto Detection System 125 ml and DAB Quanto 125 ml were used in the process.

- **KIT DETAILS:**

  Kit: UltraVision Quanto Detection System 125 ml (epredia)

  Kit components:

QUANTITY	COMPONENT	USE

1	UltraVision Protein Block (125 ml)	Block non-specific antibody binding (block endogenous peroxidase) which otherwise would have led to background staining.
1	Primary Antibody Amplifier Quanto (125 ml)	Amplifies the signal/reaction
1	HRP Polymer Quanto (125 ml)	secondary antibody

Kit: UltraVision Quanto Detection System HRP DAB

Kit components:

QUANTITY	COMPONENT	USE
1	DAB Quanto Substrate 125 ml	H_2O_2; oxidizes DAB in presence of peroxidase
1	DAB Quanto chromogen 4 ml	Required for color development

Figure- kit used in IHC

- **PROTOCOL:**

1. **Tissue processing:** Formalin-fixed paraffin-embedded sections were cut on poly-L-lysine coated microscope slides.
2. **Deparaffinization and Rehydration:** Deparaffinized the slides in xylene followed by rehydration in acetone and ethanol washing with running tap water.
3. **Antigen Retrieval:** Microwave antigen retrieval was performed using citrate buffer solution at pH 6.0 for 45 minutes.
4. **H_2O_2 blocking:** After cooling tissue sections for 20-30 minutes, the slides were incubated with 0.3% H_2O_2 for 30 minutes to inactivate the endogenous peroxidase, after washing thrice with TBS buffer pH 7.5, then the slides were washed with buffer thrice.
5. **Protein blocking:** Incubated for 5 minutes after adding UltraVision Protein Block.
6. **Primary antibody binding**:- The slides were first incubated with the monoclonal antibody against XIAP (*A-7 clone, Santa Cruz Biotechnology*) at dilution (*1:50*). Then the slides were incubated at 4°C overnight in a humidified chamber and washed the slides with buffer thrice.
7. **Amplification:** Incubated with Primary Antibody Amplifier Quanto for 10 minutes.
8. **Secondary antibody binding**:- Secondary antibody polymer was added and incubated in dark for 30 minutes.
9. **Color development:-**Washed with buffer thrice and developed by diaminobenzene (DAB) as chromogen for 2 min.
10. **Counter staining:**Sections were counterstained with hematoxylin,

11. **Mounting and detection:-** Mounted with DPX (DibutylPhthalate Polystyrene Xylene), and examined using a light microscope.

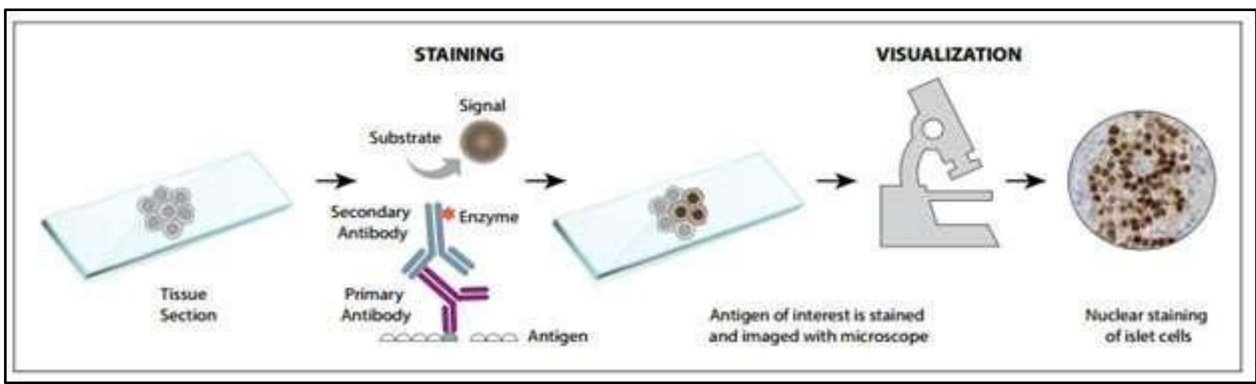


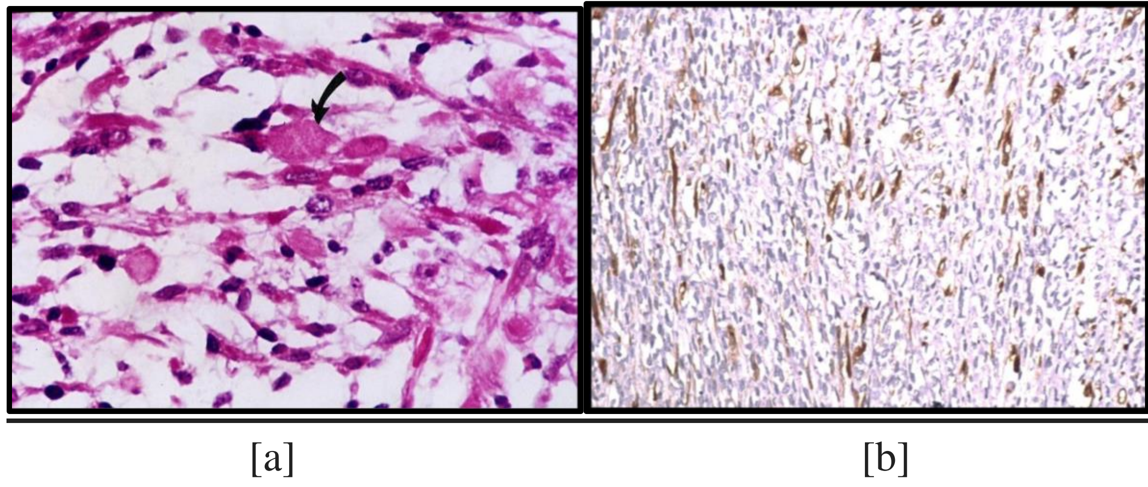
Figure 4: Schematic illustration of steps involved in Immunohistochemistry (IHC)

- **Immunohistochemical evaluation of XIAP:**
  The immunohistochemical staining results were negative for used sample case using Epithelium layer of eyelid sebaceous carcinoma was used as positive control in XIAP staining. Desmin is proven to be an excellent marker for Rhabdomyosarcoma samples[1], which is used in combination with MyoD and myogenin to confirm myogenic differentiation. Generally exhibits cytoplasmic staining with dot positivity in perinuclear regions.
  Desmin staining was studied using IHC and cytoplasmic regions exhibited positive staining.

RESULTS

Immunohistochemical staining for rhabdomyosarcoma came out to be positive for desmin as seen in figure 1[a].

We also investigated the expression level of XIAP in ERMS through immunohistochemical staining, using the epithelium layer of eyelid sebaceous carcinoma as a positive control. The results came out to be negative.



[a] [b]

Figure 1: *Representative immunohistochemistry images of ERMS positivity (a)ERMS showing Tadpole like cells (b) ERMS showing Desmin positivity*

DISCUSSION

Desmin is the intermediate filament protein which is associated with skeletal muscle and certain visceral muscle differentiation(myogenic). In skeletal muscles, it is found in Z bands between myofibrils where it acts as binding source for contractile proteins.

We used IHC to study the expression level of Desmin in the RMS samples and after IHC staining, samples came out to be positive.

Hence, Desmin has been used as one of the sensitive markers for rhabdomyosarcoma but it cannot differentiate between the specific types of RMS and it could also come positive in certain other sarcomas too like leiomyosarcoma, neuroblastoma, etc. Therefore, we can say that desmin is a sensitive marker for RMS but not specific.

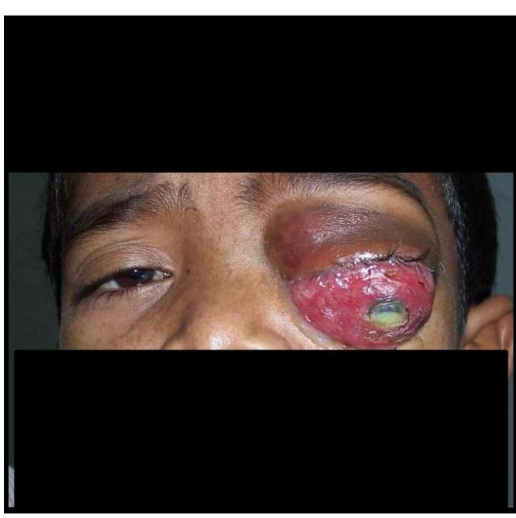
Therefore to conduct the proper diagnosis of RMS, additional markers like MyoD1 and myogenin have to be used along with desmin.

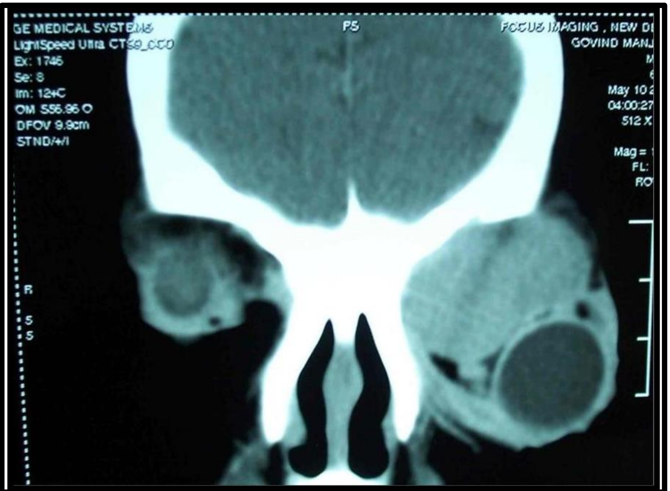
XIAP (X-linked inhibitor of apoptosis protein), regulates cell death signaling pathways by binding to and inhibiting caspases. It is involved in cancer initiation, promotion and progression. It belongs to the IAP family and is also known as an inhibitor of apoptosis protein 3 (IAP3). XIAP prevents the activation of caspases 3, 7, and 9 that promote apoptosis.

XIAP upregulation has been associated with various types of cancers like breast cancer, colon cancer, eyelid sebaceous carcinoma, etc.

Its expression level in ERMS came out negative by using IHC staining.

Hence XIAP cannot be used as a diagnostic or prognostic marker in ERMS.





[a] **[b]**

Figure: *Representative images showing (a)Extraocular embryonal rhabdomyosarcoma growth as observed in a patient (b) MRI scan of the head, offering a medial view of the growing tumor in the afflicted eye*

*(*The above added pictures were taken with patient's consent)*

ANNEXURE

- **GEO2r:-** GEO2R is an interactive web tool that allows users to compare two or more groups of Samples in a GEO Series in order to identify genes that are differentially expressed across experimental conditions. Results are presented as a table of genes ordered by significance. Page | 39 It is an online public repository for high-throughput expression data sets containing over 2 million samples grouped into over 90,000 individual series. GEO is maintained by the National Center for Biotechnology Information (NCBI) and has data organized into 4 components: platforms (GPL), samples (GSM), series (GSE), and DataSets (GDS) Geo2R performs comparisons on the original submitter supplied processed data tables using the GEO query and limma R packages from the Bioconductor project. Bioconductor is an open-source software project based on the R programming language that provides tools for the analysis of high throughput genomic data. The limma (linear models for microarray analysis) R package has emerged as one the most widely used statistical tests for identifying differentially expressed genes. It handles a wide range of experimental designs and data types and applies multiple-testing corrections on the p-value to help correct for the occurrence of false positives. GEO2R provides a simple interface that allows users to perform R statistical analysis without command line expertise. GEO2R does not rely on curated Datasets and interrogates the original series matrix data file directly. This allows a greater proportion of GEO data to be analyzed in a timely manner. However, it is important to realize that this tool can access and analyze almost any GEO series, regardless of data type and quality.

  Reference:- https://academic.oup.com/nar/article/41/D1/D991/1067995

- **KEGG (Kyoto Encyclopedia of Genes and Genomes):-** KEGG is a database resource that integrates genomic, chemical and systemic functional information. In particular, gene catalogs from completely sequenced genomes are linked to higher-level systemic functions of the cell, the organism and the ecosystem. It helps in understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high throughput experimental technologies. KEGG was developed by Kanehisa Laboratories. It is a computer representation of the biological system, consisting of molecular building blocks of genes and proteins (genomic information) and chemical substances (chemical information) that are integrated with the knowledge on molecular wiring diagrams of interaction, reaction and relation networks (systems information). It also contains disease and drug information (health information) as perturbations to the biological system. Page | 43 Major efforts have been undertaken to manually create a knowledge base for such systemic functions by capturing and organizing experimental knowledge in computable forms; namely, in the forms of molecular networks called KEGG pathway maps, BRITE functional hierarchies and KEGG nodules. Continuous efforts have also been made to develop and improve the cross-species annotation procedure for linking genomes to the molecular networks through the KEGG Orthology (KO) system. KEGG NETWORK represents a renewed attempt by KEGG to capture knowledge on diseases and drugs in terms of network variants caused by not only gene variants, but also viruses and other factors. As a result, KEGG is widely used as a reference knowledge base for integration and interpretation of large-scale datasets generated by genome sequencing and other high throughput experimental technologies. In addition to maintaining the aspects to support basic research, KEGG is being expanded towards

more practical applications integrating human diseases, drugs and other health-related substances.

References-
https://www.genome.jp/kegg/kegg1a.html
https://www.genome.jp/kegg1.html

- **miRNet:-** Support for varioistics: miRNet accepts a list of miRNAs, miR-SNPs, genes, transcription factors, small molecules, ncRNAs, diseases, epigenetic modifiers, any of their combinations or a data table from microarray, RNAseq or RT-qPCR experiments. Page | 44 miRNet supports differential analysis using limma, edgeR and HTqPCR methods; enrichment analysis using standard hypergeometric tests and unbiased random sampling. Comprehensive functional annotation: miRNet integrates data from 14 different miRNA databases - TarBase, miRTarBase, miRecords, miRanda (S mansoni only), miR2Disease, HMDD, PhenomiR, SM2miR, PharmacomiR, EpimiR, starBase, TransmiR, ADmiRE, and TAM 2.0. It currently supports Human, Mouse, Rat, Cattle, Pig, Chicken, Zebra fish, Fruit fly, C. elegans, and S. mansoni. Exploring xeno-miRNAs and their potential targets: miRNet currently supports six hosts (Human, Mouse, Chicken, Fruit fly, and C. elegans) with xeno-miRNAs reported from over 50 species. It contains over 400 experimentally detected xeno-miRNAs supplemented with 1000 computational predicted transportable miRNAs. Their potential gene targets are predicted using two algorithms - miRanda and TarPmiR. Creation of miRNA-target interaction networks: miRNet provides a wide array of options to allow researchers to build miRNA-target interaction networks at different confidence levels. The resulting network can be further optimized using different algorithms to improve visualization and understanding. The network algorithm includes Force Atlas, Fruchterman-Reingold, Graphopt, Large Graph, Random, Reduce Overlap, Bipartite/Tripartite, Concentric Circle, and Backbone

algorithm. High-performance network visual analytics: miRNet allows users to easily create miRNA-centric networks consisting of different molecules or phenotypes of interest: genes, diseases, small molecules, SNPs (affecting miRNAs or their binding sites), ncRNAs (lncRNA, sncRNA, circRNA or pseudogene), epigenetic modifiers, and transcription factors. The system supports zooming, batch highlighting, point-and-click, Page | 45 drag-and-drop, enrichment analysis, etc. to enable users to intuitively explore miRNAs, targets and functions

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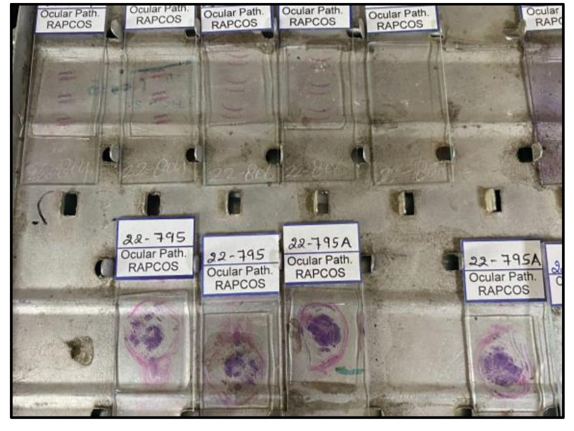
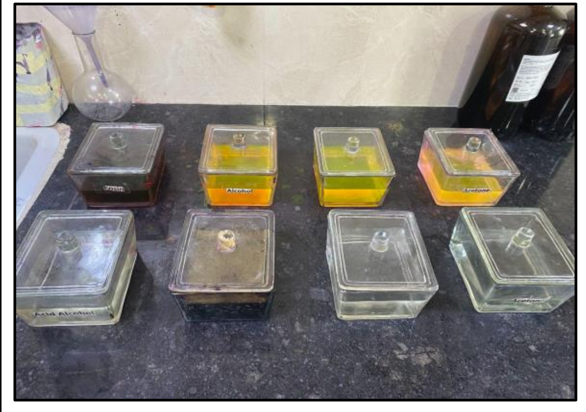
*The following pictures were taken during our AIIMS Delhi visit.

	Tissue processor for paraffin infiltration
	***Ocular Tissue Samples***
	Observing tissue samples under light microscope

	Rotary Paraffin Microtome
	***Dehydration reagent setup***
	FFPE Tissue Block

Pathology Lab

Dr. R P Center for Ophthalmic Sciences, AIIMS Delhi

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