THE WNT SIGNALLING PATHWAY IN EWING SARCOMA / PRIMITIVE NEUROECTODERMAL TUMOUR: AN IMMUNOHISTOCHEMICAL INVESTIGATION

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DECLARATION

I, Dr Hue-Tsi Wu, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. Furthermore, this work has not been published prior to registration for the abovementioned degree.

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LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli
CK	cytokeratin
CRD	cysteine-rich domain
CTBP	C-terminal binding protein
CREBBP	CREB (cAMP response element-binding)-binding protein, also known as CBP
CTNNB1	β-catenin
CTNNBIP1	beta-catenin, interacting protein 1
dsh	dishevelled (in *Drosophila*)
DVL	dishevelled (in human)
EMA	epithelial membrane antigen
ES	Ewing sarcoma
EWS	Ewing sarcoma gene/protein
FISH	fluorescent in-situ hybridisation
FLI1	Friend leukaemia virus integration 1
FZD	Frizzled
GSK3β	glycogen synthase kinase 3 beta
HMWCK	high molecular weight cytokeratin
IGFBP3	insulin growth factor binding protein 3
LEF	lymphoid enhancer-binding factor
LRP	low-density lipoprotein receptor-related protein
MMTV	mouse mammary tumour virus
NSE	neuron-specific enolase
PCP	planar cell polarity
PCR	polymerase chain reaction

PNET	peripheral neuroectodermal tumour
RB	retinoblastoma
RT-PCR	reverse transcriptase polymerase chain reaction
sFRP	secreted frizzled-related protein
TCF	T cell factor
TDT	terminal deoxynucleotidyltransferase
TGF	transforming growth factor
TLE1	transducin-like enhancer of split 1
VEGF	vascular endothelial growth factor
WIF	WNT inhibitory factor
WLS	wntless
WNT	Wg and Int; approved name is wingless-type MMTV integration site

PART A: PROTOCOL

1. PROJECT TITLE

The WNT signalling pathway in Ewing sarcoma/primitive neuroectodermal tumour: an immunohistochemical investigation

2. SHORT DESCRIPTION OF THE PROJECT

The WNT pathway is a major developmental pathway that plays an important role in the development of many tumours, including neuroectodermal and bone tumours. Ewing sarcoma (ES) / primitive neuroectodermal tumour (PNET) shows varying degrees of neuroectodermal differentiation and is the second commonest bone malignancy in childhood. A recent study on ES cell lines using RT-PCR analysis and biological response assays suggests that an intact WNT pathway exists in ES and that addition of exogenous WNT ligands enhances cell motility. Based on this we hypothesize that the WNT pathway may play a role in the biology of ES/PNET and we aim to investigate this by immunohistochemical stains on archival tissue.

3. COLLABORATORS

Nil

4. PROJECT

4.1. AIM

To identify alterations in the WNT signalling pathway in ES/PNET and to determine if this holds any role in the pathobiology of ES/PNET.

4.2. OBJECTIVES

To perform immunohistochemical staining of ES/PNET cases using antibodies against multiple components of the WNT pathway: WNT1, WNT5A, dishevelled, phosporylated GSK3β (glycogen synthase kinase 3 beta), β-catenin, MYC, cyclin D1, E-cadherin extracellular domain and E-cadherin cytoplasmic domain.

To correlate expression patterns of the immunohistochemical stains with clinical data and histological features:

1. To discuss the possible reasons for the patterns of immunohistochemical expression in ES/PNET
2. To elucidate the role of WNT pathway in ES/PNET

4.3. BACKGROUND

ES/PNET are primary malignant small round cell tumours of bone and soft tissues. They are the second most common malignant bone tumours of childhood (osteosarcoma being the commonest) and account for 6% of all malignant bone tumours. Traditionally ES and PNET have been differentiated from each other based on the absence and presence of neuroectodermal differentiation respectively. However, immunohistochemical and molecular studies indicate that they are the same entity, so they are currently often referred to as ES/PNET. ES/PNET are characterised by chromosomal translocations involving the *EWS* gene and a member of the *ETS* family of transcription factors, usually Friend Leukaemia virus Integration 1 (*FLI1*). They range morphologically from small undifferentiated cells to larger, less uniform cells with varying degrees of neuroectodermal differentiation.[1]

The WNT pathway is a highly conserved pathway occurring in many animals, including the fruitfly *Drosophila* (Wingless pathway), mouse (Int pathway) and humans. It controls cell fate, proliferation, migration, tissue architecture and organogenesis during embryonic development and is a critical pathway in neuroectodermal development. In the adult this pathway regulates haematopoiesis, osteogenesis, angiogenesis, as well as adipogenesis, and its ligands, the WNT proteins, are normally secreted in bones, muscle and soft tissues.[2]

Abnormalities of this pathway are present in many cancers, for example: *APC* gene mutation is one of the first mutations to occur in the development of colon cancer;[3] abnormal WNT5A expression is an indicator of poor prognosis in neuroblastomas (underexpression)[4] and malignant melanomas (over expression);[5] nuclear β-catenin expression is a significant indicator of favourable prognosis in medulloblastomas[6] but associated with poor differentiation in hepatocellular carcinomas.[7]

A RT-PCR study on nine ES cell lines found that most cell lines express certain components of the WNT pathway (such as WNT10B, WNT5A, WNT11, WNT13, FZD2, FZD3, FZD4, FZD7, FZD8 and LRP5/6) but not other components (WNT1, WNT2, WNT3, WNT3A, WNT7A, FZD1, FZD6, FZD9 and FZD10). It was reported that an intact WNT pathway was present in these cell lines, and that exogenous stimulation by WNT3A ligand caused markedly increased cytoplasmic β-catenin expression, 3-5 fold increase in cell chemotaxis, but no increase in cell proliferation. Immunohistochemical staining for β-catenin was then done on seven cases but all cases were negative.[8] Although this investigation on ES cell lines suggested a potential pathway for metastasis, it was not correlated with clinicopathological parameters and was limited by the small number of cases.

Neuroblastomas, malignant melanomas, medulloblastomas and ES/PNET all belong to the group of neuroectodermal tumours. The findings in these tumours raise the possibility that the WNT pathway may play a role in the biology of ES/PNET. However, there is a relative lack of immunohistochemical testing and clinicopathological correlation in ES/PNET. We would therefore like to investigate the expression of WNT pathway proteins in ES/PNET by immunohistochemistry and to correlate the results with available clinicopathological parameters. This may lead to a better understanding of the pathobiology of ES/PNET and potentially aid in its management.

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4.5. DETAILED METHODOLOGY

4.5.1. Cases

1. This is a retrospective study.
2. The databases of the Division of Anatomical Pathology, University of Cape Town, Groote Schuur Hospital, Red Cross War Memorial Children's Hospital, and Anatomical Pathology Laboratory Greenpoint, Cape Town will be searched for all cases of ES/PNET.
3. Cases will be allocated study numbers and patients' names and other identification details will be not be used.
4. All stained slides of the cases will be retrieved and reviewed.
5. The diagnosis in each case will be confirmed and the morphological data (including results of special stains) recorded.
6. Blocks from the confirmed cases will be retrieved and additional sections cut for immunohistochemistry.

4.5.2. Antibodies

The following primary antibodies will be used (**Table 1**): WNT1, WNT5A, DVL1, phosporylated GSK 3β, β-catenin, MYC (also known as c-myc), cyclin D1, E-cadherin extracellular domain (36B5) and E-cadherin cytoplasmic domain (36/E-cadherin).

Table 1: Primary antibodies.

Primary antibody	Clone	Supplier
WNT1	ab15251 (polyclonal)	Abcam
WNT5A	ab72583 (polyclonal)	Abcam
DVL1	ab21062 (polyclonal)	Abcam
GSK3β	ab30619 (polyclonal)	Abcam
β-catenin	17C2 (monoclonal)	Novo-castra
MYC	9E11 (monoclonal)	Novo-castra
Cyclin D1	SP4 (monoclonal)	Lab Vision
E-cadherin (extracellular domain)	36B5 (monoclonal)	Novo-castra
E-cadherin (cytoplasmic domain)	36/E-cadherin (monoclonal)	BD Biosciences

4.5.3. Staining procedure

Paraffin wax embedded tissue sections will be stained using standard techniques.

1. Paraffin wax embedded sections will be cut onto 3-aminopropyltriethoxysilane (APES) coated slides and heat-fixed overnight at 60°C to adhere sections to slides.
2. Sections will be dewaxed through xylene, rehydrated in graded ethanols, and washed in water.
3. Endogenous peroxidase activity will be blocked by treating slides with a 1% H_2O_2 in water solution for 15 minutes.
4. Slides will be washed well in water.
5. Antigen retrieval will be performed by pressure-cooking slides in either citrate buffer at pH 6 for 2 minutes or EDTA (pH8) for 1 minute at full pressure.
6. Slides will be immediately immersed in water.
7. Slides will be rinsed with phosphate buffered saline solution (PBS pH 7.6).
8. Non-specific binding will be blocked by treating slides with a 5% Goat Serum Solution (DAKO #X0907).
9. Serum will be drained off.

10. Sections will be incubated with primary antibody at room temperature at specified times and dilutions.
11. Sections will be washed well with PBS Buffer.
12. Sections will be incubated with DAKO Envision labelled Polymer, HRP (DAKO #K4001) for 30 minutes at room temperature.
13. Sections will be washed well with PBS buffer.
14. Positivity will be developed by applying 3.3 – diaminobenzidine (DAKO K3466) for 5-10 minutes.
15. Slides will be washed in water.
16. Slides will be immersed in a 1% CuSO4 solution for 5 minutes.
17. Slides will be washed in water.
18. Slides will be counterstained in haematoxylin, blued in Scott's tap water.
19. Slides will be washed in water, dehydrated using graded alcohols, cleared with xylene and mounted with rapid mountant medium.

4.5.4. Grading of staining

β-catenin immunostaining will be scored according to the protocol used by Jass et al.[9] This protocol scores 1 for loss of cell membrane staining, 1 or 2 for slight and pronounced increase in cytoplasmic staining, respectively, and 1 or 2 for slight and pronounced nuclear staining, respectively, giving a maximum score of 5. Cases scoring 4 or more will be regarded as positive for abnormal β-catenin immunolocalisation.

For the other antibodies the immunostaining score will be based on the protocol used by Chetty et al.[10] The scoring will be as follows: moderate and/or strong immunolabelling will be regarded as positive, with quantification of the positivity as follows: a score of 0 (or negative), less than 5% tumour cells stained; a score of 1 (or focal positivity), 6% to 50%; and a score of 2 (or diffuse positivity), more than 50%. In addition, the cellular location of the immunoreactivity will be noted.

4.6. CLINICAL CORRELATION

Results of immunostaining of each antibody will be correlated with available clinical data, tumour morphology, and with immunostaining of other antibodies. The clinical and morphological features to be compared include: age (≤ 12 / >12 years of age), gender (male / female), site (skeletal / extraskeletal), location (central / peripheral), metastasis (present / absent), mitotic count (≤ 10 / > 10 mitoses per 10 high power fields), nucleoli (inconspicuous / prominent) and neuroectodermal differentiation (present / absent).

4.7. STATISTICAL ANALYSIS

For each clinical and morphological feature, immunostaining median scores will be divided into two subgroups and compared using the two-sample Wilcoxon rank-sum (Mann-Whitney) test since these data were not normally distributed. For analysing the relationship between scores of the antibodies (comparing more than two medians, the median immunostaining score of a given antibody by scores of another antibody), the Kruskall-Wallace equality of rank test (with ties) will be employed. A p-value of less than 0.05 will be used as the standard cut-off for statistical significance. All analyses will be performed using Stata 11.1 (StataCorp LP, 4905 Lakeway Drive, College Station, TX 77845, USA).

PART B: LITERATURE REVIEW

1. OBJECTIVES OF LITERATURE REVIEW

- To review the current literature on Ewing sarcoma (ES)/Primitive neuroectodermal tumour (PNET).
- To review the current literature on the WNT signalling pathway.
- To review the current literature on the WNT signalling pathway in ES/PNET.
- To identify gaps or needs for future research.

2. LITERATURE SEARCH STRATEGY

2.1. STUDY SEARCH

Journal articles were searched for using various internet search engines, including: PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), Google Scholar (http://scholar.google.co.za) and Scirus (http://www.scirus.com). The search terms used included: Ewing sarcoma, PNET, WNT and names of individual WNT pathway components such as WNT, E-cadherin, cyclin D1 and MYC (also known as c-myc). Names of individual genes were verified using the "Entrez Gene" database (http://www.ncbi.nlm.nih.gov/gene) and the "Human Gene Nomenclature" database (http://www.genenames.org/cgi-bin/hgnc_search.pl).

Other resources used to retrieve further journal references included standard surgical and specialized pathology textbooks and "The Wnt Homepage" by Roel Nusse (http://www.stanford.edu/~rnusse/wntwindow.html).

The full text of the journal articles were retrieved electronically from the journal website, PubMed, and by using the electronic journal portals (http://www.tdnet.com/rlc/and http://uctsfx.hosted.exlibrisgroup.com/) of the library of the University of Cape Town (UCT). When the articles were not available electronically, they were retrieved physically from the UCT Faculty of Health Sciences library, and if not available from the UCT library, they were obtained via interlibrary loans.

Selected article titles and their abstracts were reviewed to identify articles relevant to the topics discussed. No attempt was made to provide an exhaustive review of either ES/PNET or the WNT pathway in general.

2.2. INCLUSION CRITERIA

1. Review articles on ES/PNET.
2. Review articles on the WNT pathway.
3. Original research articles investigating the WNT pathway in human diseases.
4. Original research articles investigating the WNT pathway in human neoplasms, particularly ES/PNET.

2.3. EXCLUSION CRITERIA

Non-English language literature was excluded.

2.4. QUALITY CRITERIA

1. The more recent review articles were preferred over the older review articles.
2. The original articles describing the first discovery of an entity were preferred.
3. Studies with large numbers of cases were preferred.
4. Articles in journals with a high impact factor were preferred (impact factor is looked up via http://admin-apps.isiknowledge.com/JCR/JCR).
5. For WNT pathway, articles by eminent authors with multiple publications in the field of WNT research were preferred.
6. For ES/PNET, articles by eminent authors and those that were referenced in surgical pathology textbooks and in the WHO manual (Pathology and Genetics of Tumours of Soft Tissue and Bone) were preferred.

3. LITERATURE REVIEW

3.1. EWING SARCOMA / PNET

Ewing sarcomas (ES) / primitive neuroectodermal tumours (PNET) are round cell sarcomas with consistent *EWS* gene re-arrangements[1] and varying but usually minimal degrees of neuroectodermal differentiation.[2] Despite the presence of neuroectodermal differentiation, current evidence favours the cell of origin in ES/PNET to be CD133+ bone marrow-derived mesenchymal stem cell.[3, 4]

ES/PNET includes ES, PNET and Askin tumours. While immunohistochemical and cytogenetic studies indicate that these tumours are the same entity, Ewing sarcoma and PNET have traditionally been distinguished from each other based on the absence or presence of neuroectodermal differentiation on light microscopic, immunohistochemical, or ultrastructural studies respectively.[5]

3.1.1. History

PNET was first described in 1918 by Arthur Purdy Stout in a case report of a 42 year old man with an ulnar nerve tumour composed of undifferentiated round cells that formed rosettes [6]. ES was first described in 1921 by James Ewing in a case report of a 14-year old girl with a round cell neoplasm in the radius; in that report he proposed the term "diffuse endothelioma of bone".[7] Askin tumour was first described in 1979 by Frederic Askin in a study on malignant small cell tumour of the thoracopulmonary region in 20 children and adolescents.[8]

3.1.2. Epidemiology

In the United States, the overall incidence of ES/PNET appears to be stable and is 2.93 per million people for the period 1973-2004.[9] In the general population, ES/PNET accounts for 6-8% of primary bone malignancies.[5] In children, it is the second commonest primary bone malignancy after osteosarcoma. In people older than 20 years it is the fourth commonest primary bone malignancy, after myeloma, osteosarcoma and chondrosarcoma. ES/PNET most commonly occurs between the ages of 5 and 20 years, the peak incidence being the second decade and median age being 14 years (Figure 1). ES/PNET is rare before the age of 5 years and over the age of 40 years.[10] Below the age of 5 years, metastatic neuroblastoma is more common.[11]

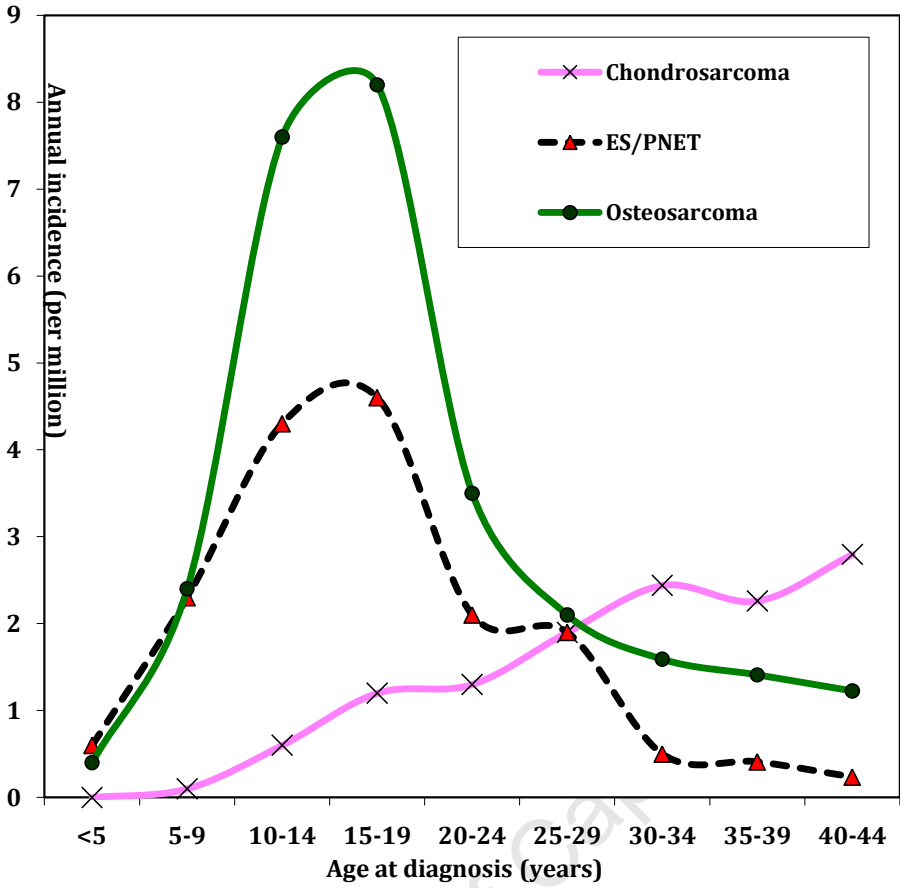

Figure 1: Incidence of bone sarcomas by type from the Surveillance Epidemiology and End Results (SEER) data (1975-2000) (modified from Mascarenhas *et al*., 2006).[12]

The male : female ratio is 1.4:1. In the United States, ES/PNET is commonest in Whites and least common in African Americans, who are 6 times less likely to develop ES than White Americans.[13, 14]

3.1.3. Sites of involvement

Approximately 76% of cases of ES/PNET are skeletal in origin.[14] Skeletal ES/PNET has a predilection for the diaphysis and metadiaphyseal regions of long bones. Involvement of the pelvis is also common. Ribs, scapula and vertebra (thoracic, lumbar and coccygeal) are occasionally involved. Skull or short tubular bones of hands and feet are rarely involved (**Table 1**).[5, 12, 15]

Table 1: Skeletal distribution of ES/PNET compared to chondrosarcoma and osteosarcoma from SEER data (1992-2002) (modified from Mascarenhas *et al*., 2006).[12]

	ES/PNET	Chondrosarcoma	Osteosarcoma
Lower limb long bones	22%	22%	68%
Pelvis	28%	23%	5%
Clavicle, rib, sternum	14%	9%	3%
Vertebra	12%	6%	1%
Upper limb long bones & scapula	11%	14%	10%
Feet	4%	1%	1%
Skull	3%	15%	9%
Hand	1%	1%	<1%

Extraskeletal ES/PNET commonly involves the deep soft tissues of extremities, such as the upper thigh and buttock, followed by the upper arm and shoulder. Less commonly they may be paravertebral or close to the ribs. They may be attached to a major nerve. Although rare, virtually any organ site can be involved.[16] In a study of 130 extraskeletal ES/PNET, the commonest primary sites were: trunk (32%), extremity (26%), head and neck (18%), and retroperitoneum (16%).[17]

3.1.4. Clinical features

The commonest symptoms are localised pain and swelling, usually less than a year in duration. Common signs include fever, leukocytosis and anaemia. There may be local erythema and the clinical differential diagnosis often includes osteomyelitis. Even at surgery, the tumour may have a yellow semi-liquid appearance resembling pus. Patients may also present with complications such as pathological fracture and metastatic disease. Metastases are present in 25% of the patients at the time of presentation; with 38% to the lungs, 31% to other bones particularly the skull, and 11% to the marrow. Other sites include the central nervous system and rarely regional lymph nodes.[5, 9, 16]

3.1.5. Imaging

Classically imaging shows a poorly defined permeative ("moth-eaten" or "rotten-wood") area of radiolucency with surrounding multilayered ("onion-skin") periosteal reaction

(**Figure 2**). Expansile bone destruction with soap-bubble appearance and sclerotic lesions with bone expansion has also been described.

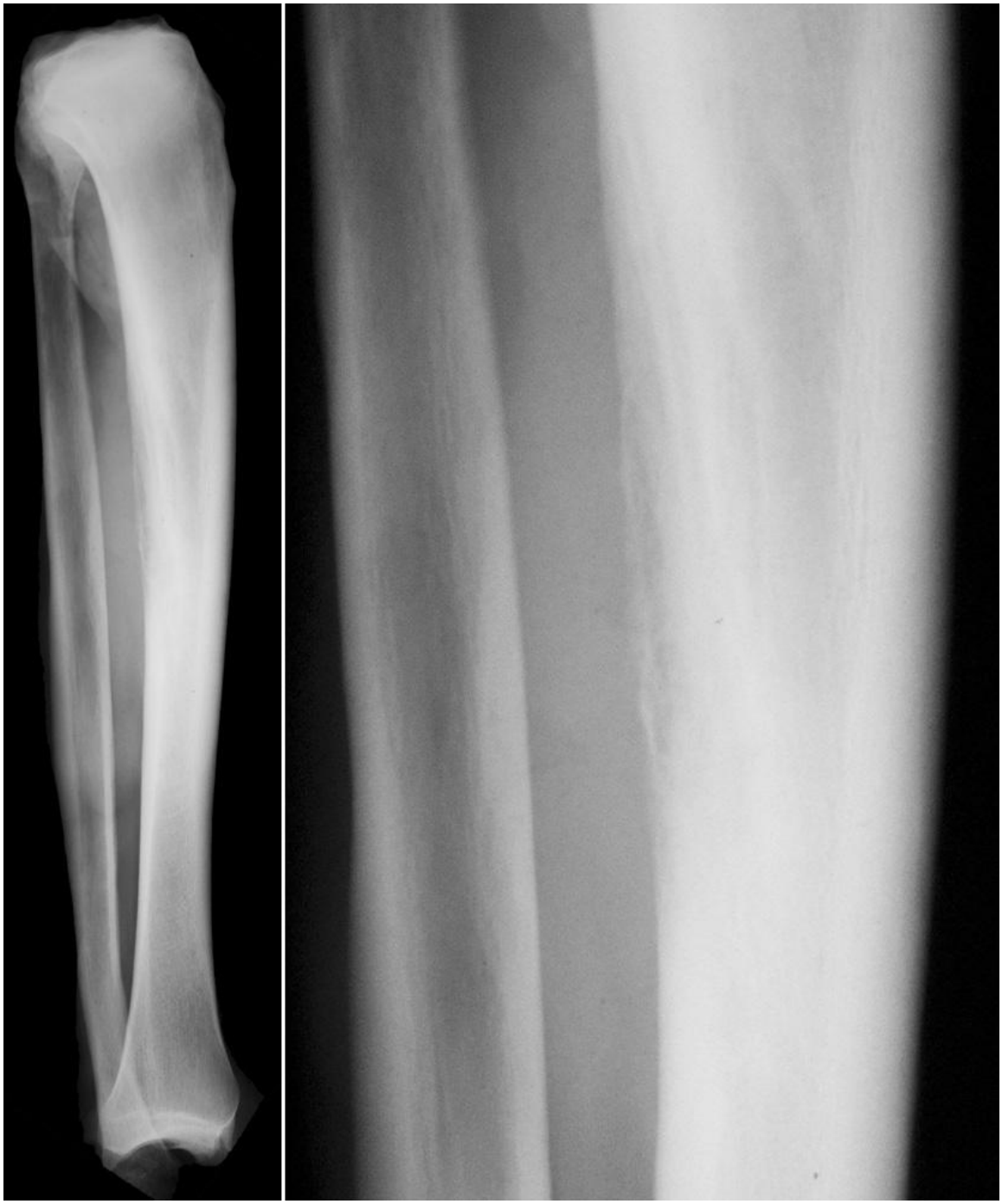

Figure 2: X-ray of a case of Ewing sarcoma involving the tibia showing extensive soft tissue involvement with subtle extension into the cortex of the diaphysis and minimal periosteal reaction.

Computed tomography is useful to demonstrate the extent of bone destruction. Magnetic resonance imaging is used to define the soft tissue involvement, the tumour being

hypointense on T1-weighted images (compared to marrow), and hyperintense on T2 (compared to muscle). On bone scan, ES shows increased uptake.[5, 16, 18]

3.1.6. Macroscopy

ES/PNET are poorly demarcated, tan-grey, soft, often necrotic and haemorrhagic tumours (**Figure 3**). Intramedullary skeletal ES/PNET may have a yellow semi-liquid appearance.[5]

Figure 3: Forearm of a 17 year old white female (amputated in 1938) showing on bisection a metadiaphyseal tumour in the proximal ulnar (extent demarcated by arrows) with permeative growth through the bone to involve the soft tissues (arrow head). Tumour cut surface was tan with areas of haemorrhage.

3.1.7. Histopathology

ES/PNET is the prototypical small round blue cell tumour of childhood, characteristically consisting of lobules or sheets of densely packed small cells with round hyperchromatic

nuclei, scanty cytoplasm, and no matrix production. Nucleoli are usually inconspicuous. Cytoplasm is clear to eosinophilic and may contain glycogen. Cell borders are poorly defined. Necrosis is common and the tumour may show a peritheliomatous growth pattern with viable cells surrounding blood vessels. A light-and-dark pattern of viable (light) and apoptotic (dark) tumour cells may also be seen.

Homer-Wright rosettes may be seen and indicates a diagnosis of PNET traditionally. Atypical ES or large cell ES refers to the presence of large pleomorphic nuclei and prominent nucleoli (**Table 2**).[19, 20] Cytoplasm also tends to be more abundant and the reticulin framework more prominent in atypical ES.[21] Atypical ES has also been used to describe ES with non-conventional morphology, such as ES with spindle-cell sarcoma-like (synovial sarcoma-like), sclerosing, clear cell (hypernephroid), or vascular-like with haemangioendothelioma-like features.[19, 22] An adamantinoma-like variant of ES with nests of epithelioid cells that are positive for high molecular weight cytokeratin has been described in 5% of cases.[19, 23] Isolated cases of ES/alveolar rhabdomyosarcoma (ectomesenchymoma),[24] ES/desmoplastic small round cell tumour[25] and ES/small cell osteosarcoma[26] have been described.

Table 2: Light microscopic features in ES/PNET (modified from Folpe *et al*., 2005; Navarro *et al*., 1994).[19, 27]

Feature	Classical ES	Atypical ES	PNET
Cell shape	Uniform, round	Pleomorphic, spindled	Irregular
Cell size	Small	Large	Medium
Chromatin	Fine	Coarse	Coarse
Cytoplasm	Scanty	Abundant	Moderate
Glycogen	Abundant	Moderate	Scanty
Nucleoli	Inconspicuous	Prominent	Prominent
Rosettes	Absent	Absent	Present

3.1.8. Immunophenotype

ES/PNET typically show immunoreactivity for vimentin, CD99 (O13, HBA-71, mic-2), Friend leukaemia virus integration 1 (FLI1), and variable positivity for neuron-specific

enolase (NSE), CD57, synaptophysin, cytokeratin and CD117.[19] Caveolin 1 (CAV1) is a novel marker reported to be present in 96% of cases of ES/PNET and may be a potential diagnostic marker.[22]

CD99 is a sensitive marker present in 88-100% of ES/PNET cases and can also be used on fine needle aspirate specimens.[19, 22, 28, 29] However, CD99 is also seen in other tumours, some of which may display a small round blue cell morphology, including: mesenchymal chondrosarcoma (100%), lymphoblastic lymphoma (93%), granulosa cell tumour (88%), synovial sarcoma (70%), malignant melanoma (60%), granulocytic sarcoma (55%), Merkel cell carcinoma (55%), rhabdomyosarcoma (up to 40%), desmoplastic small round cell tumour (35%), osteosarcoma (32%) and small cell carcinoma (6%).[30-40]

FLI1 staining is nuclear and is a more specific but less sensitive (74-91%) marker for ES/PNET than CD99. FLI1 expression is limited to ES/PNET with *EWS-FLI1* translocations. FLI1 is expressed in normal endothelial cells and lymphocytes. It is often expressed in vascular tumours, desmoplastic small round cell tumours, olfactory neuroblastomas, and is less often expressed in lymphomas and neuroendocrine carcinomas.[30, 41, 42]

An antibody to EWS has been developed and staining is seen in approximately 90% of ES/PNET but because it is non-specific, EWS immunohistochemistry is not routinely used for diagnosis.[30]

NSE expression is non-specific, but may indicate neuroectodermal differentiation. Neuroectodermal differentiation carries no prognostic significance.[20]

KIT (also known as c-kit or CD117) expression occurs in approximately 20-40% of ES/PNET, but its therapeutic and prognostic significance is uncertain as mutations in the *KIT* gene are rare in ES/PNET.[19, 43]

Pancytokeratin (such as AE1/AE3) may be positive in 30% of cases. The adamantinoma-like variant of ES/PNET typically expresses high molecular weight cytokeratins (HMWCK), such as 34βE12, which is otherwise not seen in ES/PNET.[19]

Expression of p53 is seen in approximately 10% of ES/PNET and appears to be associated with an extremely poor outcome.[44] Loss of immunoexpression of cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as p16 or p16INK4a) has been associated with metastatic disease at presentation.[45]

3.1.9. Ultrastructure

Electron microscopy reveals primitive cells containing intracytoplasmic glycogen granules and few organelles. Neuroectodermal differentiation is characterised by fine cytoplasmic processes (neuritic or dendritic processes), primitive intercellular junctions, neurosecretory granules (50-150nm), and microtubules (neurotubules) (**Table 3**).[21, 27, 28]

Table 3: Ultrastructural features in ES/PNET (modified from Llombart-Bosch *et al*., 1978; Navarro et al., 1994).[21, 27]

Feature	**Classical Ewing sarcoma**	**Atypical Ewing sarcoma**	**PNET**
Glycogen	Abundant	Moderate	Rare
Organelles	Scarce	Moderate	Abundant
Dense-core granules	Absent	Rare	Abundant
Neurotubules	Absent	Rare	Abundant
Neuritic processes	Absent	Rare	Abundant

3.1.10. Genetics

Virtually all ES/PNET are characterised by the translocation of *EWS* gene (22q12) to one of the *ETS* family members. The resulting fusion protein is formed by the N-terminal transcriptional activation domain of *EWS* fused to the C-terminal DNA binding domain of the *ETS* partner. The *ETS* partner is *FLI1* (11q24) in 85% of cases and *ERG* (21q22) in 10-15% of cases. In the remainder it is either *ETV1* (7p22), *E1AF* (17q12), *FEV* (2q33), or *PATZ1* (*ZSG*, 21q12.2).[1, 46, 47] Interestingly, isolated cases resembling ES/PNET morphologically and immunophenotypically and characterised by functionally similar fusion proteins not involving *EWS* (e.g. *FUS-FEV*), not involving *ETS* (e.g. *EWS-SP3*), or not involving either (e.g. *CIC-DUX4*) have been reported.[48-51]

Molecular diagnostic methods can be used to confirm a diagnosis of ES/PNET, especially in atypical cases.[19] The molecular methods include fluorescent in-situ hybridisation (FISH), chromosome banding, reverse transcriptase polymerase chain reaction (RT-PCR), and Southern blotting. RT-PCR can be used to detect minimal residual disease by detecting fusion transcripts in the peripheral blood or marrow.[5] In formalin-fixed paraffin-embedded tissues, RT-PCR has a sensitivity of 54% and a specificity of 85%, whereas FISH has a sensitivity of 91% and a specificity of 100%, using either fusion probes (EWS and FLI1) or the commercial EWS break-apart probes.[52]

The EWS break-apart probes use two probes that flank the *EWS* break-point to detect t(22q12) translocations. However, it is not specific for ES/PNET, as *EWS* translocations may also occur in other tumours (**Figure 4**).[53, 54]

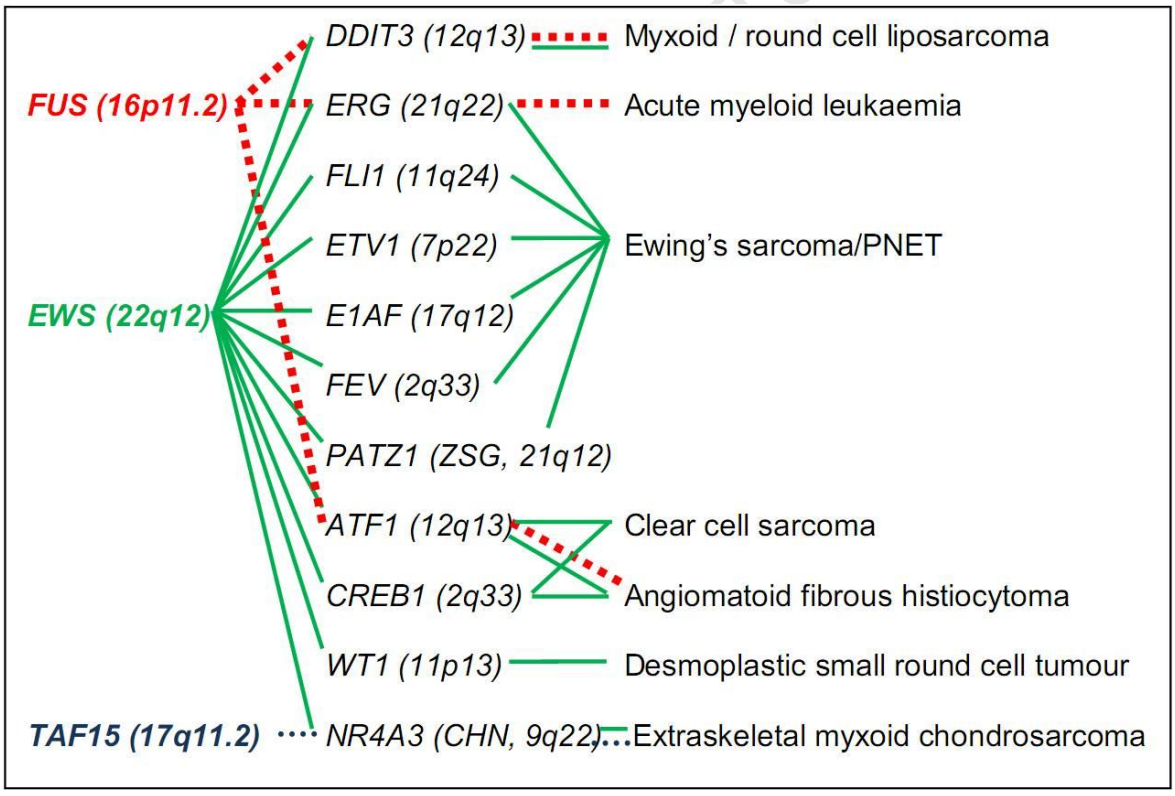

Figure 4: Tumours which may show *EWS* translocations (modified from Shing & Coleman, 2003).[54]

The EWS-ETS chimeric proteins function as transcription factors that bind to ETS targets, and based on integrative analysis of gene expression profiling experiments more than 200 genes have been found to be up-regulated by the EWS-FLI1 fusion product.[55] Many of the

upregulated genes involve cell cycle and proliferation control such as cyclin D1 promoter, cyclin E promoter, *NR0B1* (*DAX1*) and VEGF promoter.[56-59] Other upregulated genes include genes involved in intracellular signalling (protein kinase C β, caveolin 1), neuronal development (*NKX2-2*), and neural crest development (*MYC*, cholecystokinin and microtubule associated protein tau).[55, 60-62]

EWS-ETS fusion proteins also repress many genes, including cyclin-dependent kinase inhibitor 1A (*CDKN1A*, also known as *p21*) promoter, *TP53*, *GADD45A*, *GADD45B*, TGFβ type II receptor promoter, IGFBP3 promoter, *FOSL* and *TCF7L*.[55, 63-66]

The commonest secondary genetic mutation is inactivation of *p16*.[67] This allows stable expression of EWS-FLI1 by preventing EWS-FLI1 induced apoptosis and is also associated with a poor outcome.[67, 68] Other abnormalities include increased expression of *MYC*, *MYB*, *SRC* and *RAF1*.[69] Mutations in *TP53* and *RB* are rare.[70] Chromosome copy number changes are frequent (particularly gains at 1q, 2, 8 and 12 and losses at 9p and 16q), with higher copy number changes associated with worse prognosis.[71]

3.1.11. Prognostic factors

Historically, overall survival is poor (41%) but with modern therapy prognosis has improved for non-metastatic disease, which occurs in approximately 75% of patients. In this group, the overall survival is approximately 70%, whereas the overall survival for metastatic disease is approximately 20 to 30%.[72, 73]

High stage, recurrent disease, size of tumour >8cm, central location, older age (age ≥18 years worse than age 10-17 years worse than age <10 years) and elevated lactate dehydrogenase (LDH) levels at presentation are statistically significant indicators of worse prognosis.[72, 74, 75] Non-Hispanic Caucasians are reported to have the best outcome, and African Americans the worst.[14] Histologically, lobular growth pattern, atypical ES/PNET morphology and higher mitotic activity (5 or more mitoses per 10 high power fields) correlate with worse survival but none are independently prognostic on multivariate analysis.[22, 28]

Molecular indicators of poor prognosis include *TP53* mutations, *p16* alterations and elevated levels of six-transmembrane epithelial antigen of the prostate 1 (STEAP1), cyclin

D1, or NKX2-2 transcription factor.[44, 75, 76] With regards to *EWS-ETS* fusion status, although 2 earlier retrospective studies reported that patients with type-1 fusion (*EWS* exon 7 fused to *FLI1* exon 6) perform better than those with larger, less common fusion transcripts.[77, 78] Two recent large scale prospective studies failed to confirm this.[79, 80]

3.1.12. Differential diagnosis

The differential diagnosis includes other small round cell malignant neoplasms (**Table 4**).

Table 4: Immunohistochemical profile of ES/PNET and its mimics (modified from Hameed, 2007; Rossi *et al*., 2004).[81, 82]

Tumour	CD99	FLI1	LCA	TDT	CK	Chr	S100	NB84
ES/PNET	+	+	-	-	+/-	-	+/-	+/-
DSRCT	+	+/-	-	-	+	+/-	+/-	+/-
LBL/ALL	+	+	+/-	+	+/-	-	-	-
Other lymphomas	+/-	+	+	-	-	-	-	-
Mesenchymal CS	+	-	-	-	-	-	+	-
Neuroblastoma	-	-	-	-	-	+	+/-	+
Rhabdomyosarcoma	+/-	-	-	-	-	-	+/-	+/-
Synovial sarcoma	+/-	-	-	-	+/-	-	+/-	ND
Small cell OS	+/-	ND	-	-	+/-	-	+/-	+/-
Melanoma	+/-	+	-	-	-	-	+	-
Small cell carcinoma	-	ND	-	-	+	+	-	-
Merkel cell carcinoma	+/-	+	-	-	+	+	-	ND

LCA, leukocyte common antigen; TDT, terminal deoxynucleotidyltransferase; CK, pan-cytokeratin; Chr, chromogranin; DSRCT, desmoplastic small round cell tumour; LBL/ALL, lymphoblastic lymphoma/acute lymphoblastic leukaemia; CS, chondrosarcoma; OS, osteosarcoma; ND: no data; + positive; - negative; +/- variable positivity.

Both neuroblastomas and ES/PNET are small round cell malignant neoplasms which may contain Homer-Wright rosettes. Neuroblastomas and approximately 20% of ES/PNET also express NB84.[83] However, neuroblastomas occur in a younger age group, often have elevated urinary catecholamine metabolite levels, may show ganglionic differentiation, and may form schwannian stroma, particularly at the periphery of the tumour. Neuroblastomas

also rarely contain glycogen, and are CD99 and t(11;22) negative.[83] Dystrophic calcification in necrotic areas, common in neuroblastomas, is rare in ES/PNET.[16]

Alveolar rhabdomyosarcoma often shows an alveolar growth pattern with central discohesion. Multinucleated giant cells with marginally placed nuclei and eosinophilic rhabdomyoblasts with or without cross striations may also be present. Solid forms of alveolar rhabdomyosarcomas can resemble ES/PNET. Depending on the study reported, different rates of CD99 positivity has been reported, ranging from 0 to 40% of rhabdomyosarcomas, and seems to be more common in the embryonal subtype.[30, 84-86] Desmin may rarely be expressed in ES/PNET.[19] Myogenin and MyoD1 are specific markers for rhabdomyosarcomas. Approximately 80% of cases of alveolar rhabdomyosarcoma also harbour specific translocations between the forkhead box O1 gene (*FOXO1*) on chromosome 13 and the paired box (*PAX*) family of genes. The specific translocations are: t(2;13)(q35;q14) *PAX3-FOXO1* translocation in 60% of cases, and t(1;13)(p36;q14) *PAX7-FOXO1* translocation in 20% of cases.[87] The remaining cases are fusion negative and are often seen in the solid form of alveolar rhabdomyosarcomas.[88]

Non-Hodgkin's lymphoma, particularly lymphoblastic lymphoma can mimic ES/PNET, CD99 being positive in most T cell lymphoblastic lymphomas, and CD45 being negative in a proportion of lymphoblastic lymphomas.[89] However, TDT is positive in lymphoblastic lymphomas, and lymph node enlargement is rare in ES/PNET.

Small cell carcinoma and other neuroendocrine carcinomas such as Merkel cell carcinoma tend to occur in the older age group (age >45 years). ES/PNET can express cytokeratin and neuroendocrine markers,[19] but CD99 is only positive in 6% of cases of small cell carcinomas.[34] Merkel cell carcinoma can show immunoexpression of CD99 and FLI1 in up to 55% and 90% of cases respectively.[90] However, dot-like CK20 positivity and vimentin negativity aids in differentiating Merkel cell carcinoma from ES/PNET.[31]

Mesenchymal chondrosarcoma occurs in young adults and like ES/PNET is CD99 positive. The diagnosis depends on the presence of well differentiated cartilage intimately admixed with undifferentiated round cells, in a vascular background with haemangiopericytic vessels. The cartilage may be absent in small biopsies. They are NSE and S100 positive and may rarely be t(11;22) positive.[91]

Small cell osteosarcoma occurs in young adults. Diagnosis requires the presence of small malignant cells forming osteoid. Isolated cases with osteosarcomatous phenotype (osteoid production) and ES/PNET genotype (*EWS-FLI1* translocations) have been reported.[26]

Poorly differentiated synovial sarcomas occur in adults and may have a small round cell appearance. CD99 is often positive but cytokeratin, uncommon in ES/PNET, is expressed in around 50%. Diagnosis may require the demonstration of the t(X;18) *SSX1-SYT* or *SSX2-SYT* fusion transcript characteristic of synovial sarcomas.

Desmoplastic small round cell tumour (DSRCT) occurs in young adults. CD99 is seen in up to 35% of cases but DSRCT is characteristically associated with abundant desmoplastic stroma and shows polyphenotypic immunoexpression of cytokeratin, EMA and desmin (often with perinuclear dot-like positivity)in more than 95% of cases.[33] DSRCT also immunolabels with WT1 in 90% of cases due to underlying t(11;22)(p13;q12) *EWS-WT1* translocation.[33, 92] However, up to 20% of ES/PNET (and up to 100% of rhabdomyosarcomas) also show WT1 expression.[86]

Malignant melanoma expresses CD99 in 60% of cases.[32] Small cell malignant melanoma is a rare variant that typically arises in a giant congenital naevus and behaves aggressively.[93] When present, melanin pigment and background naevus aid in the diagnosis, which can be confirmed by immunopositivity for melanocytic markers (such as Melan-A and HMB45) and melanosomes on electron microscopy.

3.2. WNT PATHWAY

From the hydra to humans, WNTs form a family of evolutionarily conserved secreted protein ligands that bind to neighbouring cell surface receptors to guide the fate of the cells, from cell migration and morphogenesis in the embryo to tissue homeostasis and regeneration in the adult. *WNT*, pronounced "wint", was coined by Nusse *et al* as a combination of the fruit fly *wingless* (*wg*) gene and the mouse *Int1* gene.[94] *Wg* was discovered in the fruit fly *Drosophila menalogaster* as a recessive mutation causing absent wings and haltere.[95] *Int1* was discovered in the mouse as an oncogenic integration site for the mouse mammary tumour virus (MMTV), which, as the name indicates, causes mammary carcinomas in mice.[96] Subsequently *wg* and *Int1* were found to be homologs based on their encoded amino-acid sequences.[97] *Int1* in the mouse has been renamed *Wnt1* as *Int2* and *Int3* are unrelated genes.

3.2.1. Members

WNTs are defined according to their amino acid sequences rather than their functions, which are complex and pleiotropic.[98] WNTs are glycolipoproteins containing a signal sequence followed by a highly conserved distribution of 21 specifically spaced cysteines.[99] They bind to receptors with cysteine-rich domains (CRDs); these receptors include Frizzled (FZD), low-density lipoprotein receptor-related protein (LRP), and the transmembrane tyrosine kinases RYK and ROR2.

There are currently 19 *WNT* genes in humans and mice, 14 in the sea anemone *Nematosteall vectensis*, 7 in *Drosophila*, and 5 in *Caenorhabditis elegans.* This broad diversity indicates that *WNT* genes have evolved since at least 600 million years ago.

The names of the *WNT* genes in humans are: *WNT1, WNT2, WNT 2B (WNT13), WNT3, WNT 3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT 9A (WNT14), WNT9B (WNT15), WNT10A, WNT10B (WNT 12), WNT11* and *WNT16*. Those in brackets are the initial names which have been subsequently renamed based on sequence homology.[99-101]

FZD receptors belong to the family of G-protein coupled receptors (GPCRs). FZD has an extracellular long N-terminal extension (cysteine-rich WNT binding domain), a serpentine seven-transmembrane spanning region, and an intracellular C-terminal tail. The secreted

forms of FZDs are called secreted frizzled-related proteins (sFRPs). Secreted FRPs contain CRDs but no transmembrane segments; they bind to WNT and act as WNT inhibitors. There are currently 10 FZD receptors in humans, numbered from FZD1 to FZD10.

LRPs are members of the low density lipoprotein receptor family. LRP 5/6 are single-pass transmembrane molecules and are required for WNT signalling when WNTs bind to FZD.[102, 103]

3.2.2. WNT secretion

In the endoplasmic reticulum, WNTs undergo post-translational modification by glycosylation at the amino-termini and palmitoylation at the first conserved cysteine and on a serine in the middle of the protein.[104] Palmitoylation renders WNTs lipid soluble and water insoluble. Inhibiting palmitoylation inactivates WNTs[105]. Secretion requires a dedicated transport protein, wntless (WLS), which is localised to the Golgi, cell surface and endosomes.[99, 106] After WNT is transported to the surface by WLS and secreted, the retromer complex, a multi-protein complex, recycles WLS back into the Golgi apparatus by retrograde transport in endosomes. If the retromer complex is absent, WLS is mistrafficked to the lysosome and degraded.[107]

Once extracellular, WNT moves onto adjacent cells and activates target genes in a concentration-dependent manner, a mechanism by which it provides positional information and regulates morphogenesis. Short range diffusion is facilitated by glypicans on apical cell surfaces, whereas WNTs released on the basal lateral aspects of epithelial sheets undergo long range diffusion by binding to lipoproteins (**Figure 5**).[108]

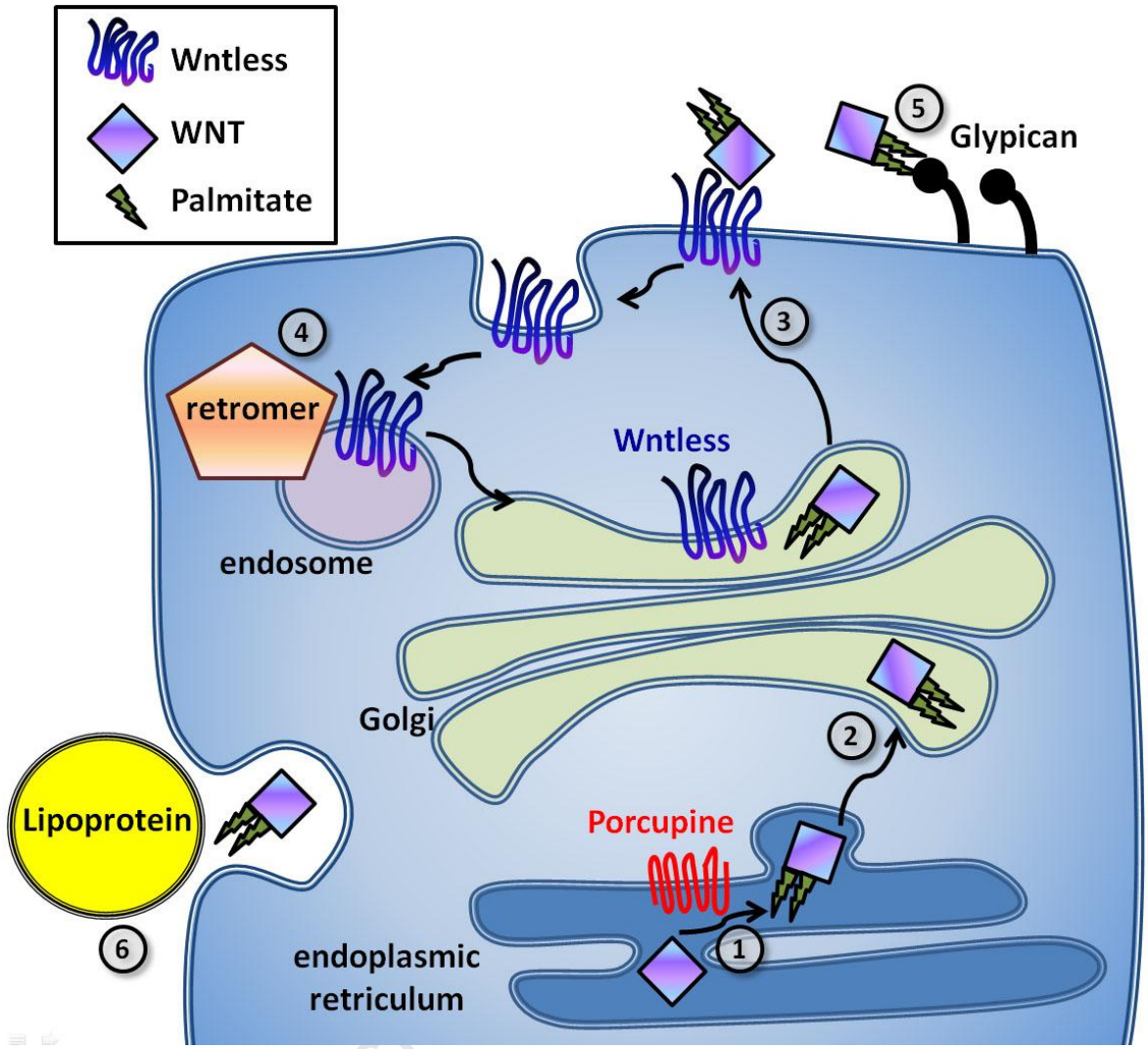

Figure 5: WNT secretion. After glycosylation, WNT is palmitoylated by the porcupine acyltransferase (1) and exported from the ER into the Golgi (2). Wntless transport WNT to the cell membrane (3) and is recycled by the retromer complex (4). Extracellular transport of WNT is facilitated by glypicans (5) and lipoproteins(6) (modified from Cadigan, 2008).[99]

3.2.3. WNT signalling pathways

WNT signalling involves several pathways. The canonical pathway involves stabilisation of β-catenin, primarily affects cell fate, and includes WNT1, 2, 3, 3A, 7A, 8A and 8B. The non-canonical pathways are β-catenin independent, mainly affect cytoskeletal organisation, and include WNT 5A and 11. However, there is extensive cross-talk between the pathways so now the WNTs are thought not to be intrinsically "canonical" or "non-canonical", but rather the pathway initiated depends on the receptor to which the WNTs bind (**Figure 6**), and the response of the cell depends on the integration of the signals from

all the pathways.[100, 109] Nevertheless, the pathways are described separately here, as is done in the majority of the literature.

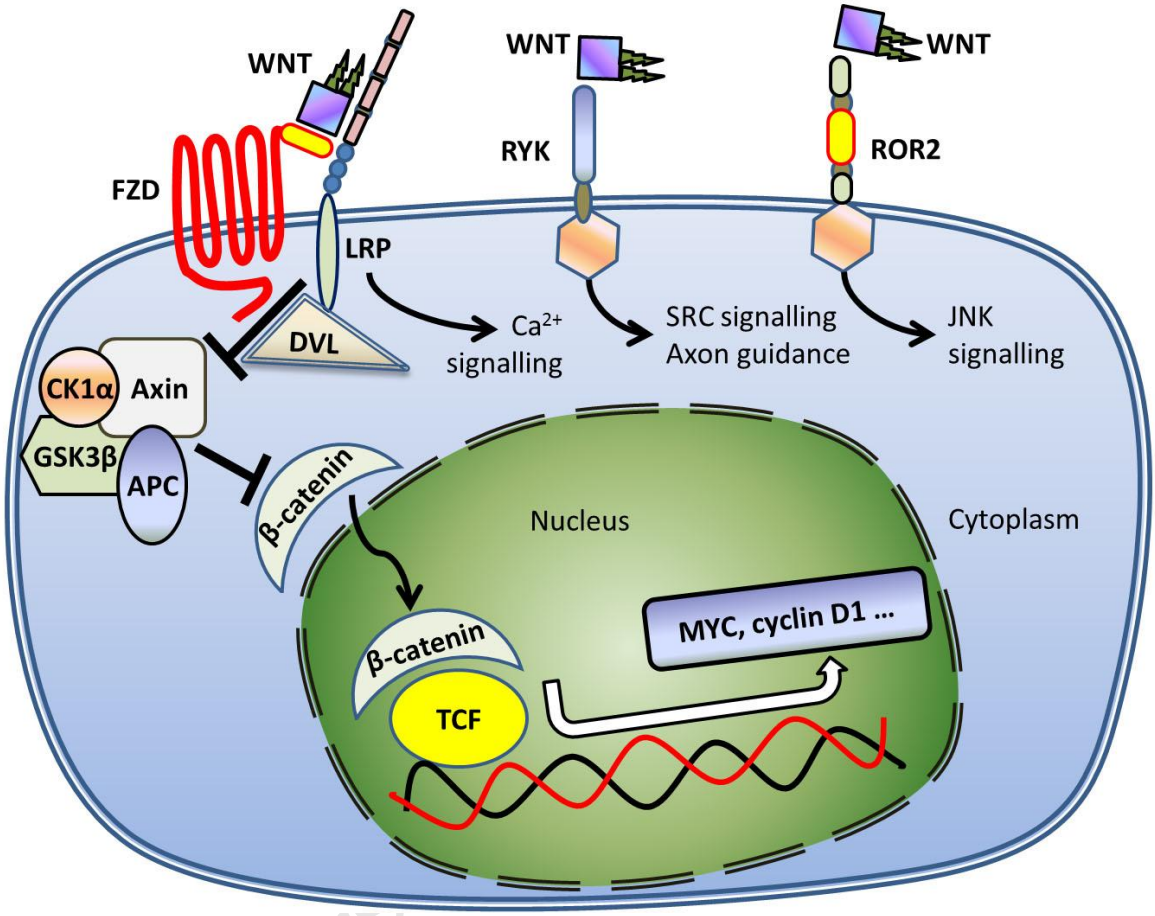

Figure 6: Canonical and non-canonical pathways. The canonical (β-catenin dependent) pathway involves binding to the receptors FZD and LRP, which rescues β-catenin from APC complex mediated destruction with subsequent translocalisation of β-catenin into the nucleus to bind TCF and activate downstream genes such as *MYC* and cyclin D1. The non-canonical (β-catenin independent) pathways include calcium, SRC, and JNK signalling pathways.

3.2.4. WNT-β catenin signalling pathway (canonical pathway)

In the cell there are two β-catenin pools. One pool is membrane bound and connects E-cadherin, a cell adhesion molecule, to the actin cytoskeleton. The other pool is cytoplasmic and is regulated by WNT. In cells not exposed to WNT, cytoplasmic β-catenin has a half-life of less than an hour[110] and undergoes phosphorylation, which triggers ubiquitination by F-box-containing E3 ubiquitin ligase β-TrCP and degradation in the proteasome.[111] Phosphorylation is mediated by casein kinase 1α (CK1α) and glycogen

synthase kinase 3β (GSK3β). These 2 enzymes are complexed to the scaffolding proteins axin and adenomatous polyposis coli (APC) that position CK1α and GSK3β for phosphorylation of β-catenin. APC-axin-CK1α-GSK3β is often referred to as the β-catenin destruction complex.[112]

When WNT binds to FZD and the co-receptor LRP5/6, it brings FZD and LRP into proximity, forming a trimer. The cytosolic domain of FZD then binds dishevelled (dsh in the fruitfly; DVL in humans). FZD, DVL and axin together then allow CK1α and GSK3β to phosphorylate the cytosolic tail of LRP. Phosphorylated LRP binds axin and inactivates the axin-APC-CK1α-GSK3β complex. As a result, nonphosphorylated β-catenin accumulates in the cytoplasm and translocates across the nuclear pores into the nucleoplasm, where it acts as a transcriptional co-regulator by binding to specific DNA-binding transcription factors (**Figure 6**).[98, 109] Because the WNT/β-catenin pathway relies on the stabilisation of β-catenin and protein synthesis, it acts over the course of hours and the context it acts in is often cell fate determination and tissue homeostasis.[99]

The commonest DNA-binding transcription factors that β-catenin binds are the members of the T cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) family. Other nuclear proteins that β-catenin can bind include forkhead box O (FOXO),[113] paired-like homeodomain 2 (PITX2),[114] SRY (sex determining region Y)-box 9 (SOX9)[115] and SOX17.[116]

The TCF/LEF family is a group of transcription factors that include TCF1, TCF3, TCF4, and LEF1. In the nucleus, TCF/LEF is normally bound to members of the transducin-like enhancer of split (TLE) family and recruits histone deacetylases (HDAC) to form a repression complex that silences WNT target genes. When sufficient levels of β-catenin are present in the nucleus, β-catenin replaces the TLE repressors and binds to TCF to activate transcription of WNT target genes.[117, 118]

Other co-activators that are recruited by β-catenin include: BCL9 (legless in *Drosophila*), pygopus (PYGO), CREB-binding protein (CREBBP), hyrax/parafibromin and SMARCA4 (also known as BRG1). BCL9 and PYGO bind to the amino-terminal half of β-catenin; CREBBP and parafibromin bind to the carboxy-terminal half.[99, 102].

To prevent inappropriate activation of WNT targets by low levels of β-catenin, several proteins act as intranuclear "buffers".β-catenin interacting protein 1 (CTNNBIP1, also known as ICAT), chibby, SOX9 and CTBP (C-terminal binding protein) bind to and inhibit β-catenin, whereas TLE binds to and inactivates TCF.[99]

Extracellular inhibitors are also present. Secreted FRP directly binds to WNT and inhibit WNT signalling. Dickkopf (DKK) is a multigene family comprising DKK1, DKK2, DKK3 and DKK4, each with two cysteine-rich domains. If KREMEN1 or KREMEN2 is present, DKK1 is a potent WNT inhibitor by binding to LRP and promoting DKK-mediated internalisation of LRP5/6, thus preventing WNT signalling. If KREMEN is absent, DKK1 inhibition is weak. If DKK1 is absent, KREMEN promotes localisation of LRP5/6 to the cell surface, thus promoting WNT signalling.[100] DKK2 inhibits WNT signalling but is a weak activator of LRP5/6, so it can be an agonist if levels of LRP5/6 are high.[119] DKK3 does not appear to modulate WNT signalling. DKK4 is a WNT antagonist.[120]

The β-catenin pathway can be activated without WNT. Norrin, which does not show sequence similarity with WNT, binds to FZD4 and activates the β-catenin pathway.[121]

3.2.5. Non-canonical pathways

The planar cell polarity (PCP) pathway mediates the polarisation of epithelial cells that occurs in orientations of hairs, fur, scales, feathers and cilia (such as the stereocilia in the cochlea), as well as convergent extension (narrowing and elongation of the tissue by cell movement). It involves DVL and FZD but not LRP, β-catenin, or TCF. FZD, as the name suggests, was discovered by its role in organising the hair and bristle pattern in the *Drosophila*. WNTs do not play a role in PCP in the *Drosophila*, but there is evidence that WNTs may be involved in PCP in higher order vertebrates.[122, 123]

The WNT-Ca^{2+}pathway is a recently proposed pathway based on experiments in mainly *Xenopus* and zebrafish, where WNT binding to FZD may activate G proteins with release of intracellular calcium and activation of Ca^{2+} dependent effector molecules such as the transcription factor, nuclear factor associated with T cells (NFAT).[122, 124]

WNT can also signal via tyrosine kinase receptors, particularly RYK and ROR2. These receptors have a cytoplasmic tyrosine kinase motif and have been found to interact with FZD. RYK has a surface WNT inhibitory factor (WIF) ligand binding-domain that sequesters WNT away from binding to FZD. It mediates axonal guidance in *Drosophila* and mammals, via WNT5-mediated activation of the *SRC* family of tyrosine kinases (in *Drosophila*)[125] and/or WNT1-mediated FZD interaction with TCF activation (in mammals).[126] ROR2 has a cysteine rich domain (CRD) which binds to WNT5A. It influences convergence extension movements and inhibits TCF/β-catenin signalling in the nucleus, possibly through activating the mitogen-activated protein kinase JUN N-terminal kinase (JNK) (**Figure 6**).[127]

3.2.6. WNT target genes

The WNT pathway targets more than 30 genes. The genes activated depend on the type of WNT, the receptor it binds to, and the cross-talk that occurs between the different pathways. Targeted genes include WNT pathway components for feedback control e.g. WNT signalling downregulates *FZD* and upregulates *AXIN2* to inhibit WNT signalling. Genes involved in oncogenesis are also activated, such as *MYC*, cyclin D1, *JUN*, *FOSL1* (*fra-1*), matrilysin and members of the AP-1 family of genes (*FOS*, *FOSB* and *JUNB*).[128]

MYC is a proto-oncogene encoding a nuclear transcription factor that activates genes involved in cell proliferation. It can also reprogram somatic cells into pluripotent stem cells, enhance self-renewal, and block differentiation. Persistent *MYC* expression is seen in many tumours. *MYC* translocation is characteristic of Burkitt lymphoma.[129] Cyclin D1 is a cell cycle protein that binds to cyclin dependent kinases to form a complex that phosphorylates RB and allows the cell cycle to pass the G1-to-S transition. Cyclin D1 is characteristically translocated in mantle cell lymphomas.[130] Matrilysin is a matrix metalloproteinase, and may be involved in tumour invasiveness. *JUN* and *FOS* are proto-oncogenes whose products dimerise to form activator protein 1 (AP-1), a transcription factor involved in cell proliferation and apoptosis.[131]

3.2.7. Functions

WNTs control organized cell movements, axis polarity, tissue polarity, stem cell maintenance, cell proliferation and cell fate.[100] Mutations in *WNT* result in specific

developmental defects, while abnormalities in WNT pathways are implicated in cancers and degenerative diseases (**Table 5**).

Table 5: WNT pathway genes and associated human diseases (modified from Logan & Nusse, 2004; Nusse, 2009)[101, 102]

Gene (locus)	Human disease
WNT3 (17q21)	Tetra-amelia[132]
WNT4 (1p36)	Müllerian-duct regression and virilisation (WNT4 inactivation) [133] SERKAL (SEx Reversion, Kidneys, Adrenal and Lung dysgenesis) syndrome[134]
WNT5B (12p13)	Associated with susceptibility to type 2 diabetes mellitus[135]
WNT7A (3p25)	Fuhrmann and Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndromes[136]
WNT10A (2q35)	Odonto-onycho-dermal dysplasia (severe oligodontia, nail dystrophy, palmoplantar hyperkeratosis and hyperhidrosis)[137]
WNT10B (12q13)	Early onset obesity[138] Split-Hand/Foot Malformation[139]
LRP5 (11q13)	Abnormal bone densities[140, 141] Vascular defects in the eye: osteopetrosis-pseudoglioma syndrome (OPPG),[140, 142] familial exudative vitreoretinopathy (FEVR)[143] Association with obesity[144]
FZD4 (11q14.2)	FEVR[143]
AXIN2 (17q23-q24)	Tooth agenesis[145] Predisposition to colorectal cancer[145]
APC (5q21)	Polyposis coli, colon cancer[146]

3.2.7.1. Role in development

During development, WNTs control gastrulation (formation of the 3 germ layers), early pattern formation and organogenesis.[99] In the *Xenopus*, after fertilisation, WNT/β-catenin signalling is relocated posteriorly (dorsally), allowing the establishment of the primary body axis pattern.[147] Morphogenesis requires the coordinated regulation of both canonical and non-canonical pathways.[109]

In humans, WNT4 is critical in female gonadal differentiation and in the formation of the kidneys, adrenals, pituitary gland and mammary tissues. Homozygous loss of function mutations of *WNT4* causes various developmental abnormalities in humans.[133, 134] *WNT5B* and *WNT10B* regulate adipogenesis and mutations in these genes have been associated with diabetes mellitus[135] and obesity.[138]

LRP5 is expressed in osteoblasts and is important in establishing bone mass. *LRP5* mutations cause the autosomal recessive condition osteoporosis-pseudoglioma syndrome (with reduced bone mass in carriers)[140] and various autosomal dominant conditions with increased bone density (such as osteopetrosis).[141] LRP5 is also required for glucose and cholesterol metabolism and *LRP5* polymorphisms are also associated with obesity.[144]

DVL is important in cardiac organogenesis and neural tube closure and DVL-deficient mice display cardiac outflow tract defects and neural tube defects.[148]

3.2.7.2. Role in stem cell maintenance

In general, WNT signalling prevents stem cell differentiation. Whereas growth factors such as fibroblast growth factor and epithelial growth factor activate stem cell proliferation via tyrosine kinase receptors.[104] Leucine-rich repeat-containing G protein-coupled receptor 5 (*LGR5*) is a WNT target gene. LGR5 is an adult stem cell marker that is expressed in the intestines, stomach, hair follicles and mammary gland and may be a universal marker for adult stem cells.[149] WNT also plays important roles in wound repair.

3.2.7.3. Role in Cancer

Constitutive activation of the WNT pathway is present in human cancers, particularly colorectal carcinomas.[146] In colorectal carcinomas, mutations in *APC* was first identified in the familial adenomatous polyposis syndrome (FAP) but was subsequently found to be also present in up to 80% of sporadic colorectal carcinomas.[150] Of the remaining 20%, half shows β-catenin mutations.[151] Less commonly mutations in *AXIN1* and *AXIN2* have been identified.[152, 153]

APC and β-catenin mutations are mutually exclusive and this phenomenon has also been observed in desmoid fibromatosis, an FAP-associated tumour.[154, 155] Other sporadic tumours occurring with increased incidence in FAP patients, such as intestinal type gastric

adenocarcinomas, hepatoblastomas, medulloblastomas and pancreatoblastomas do not show *APC* mutations, but do show β-catenin mutations in a subset of cases.[154, 156]

β-catenin mutations in colorectal carcinomas were associated with hereditary non-polyposis coli syndrome (HNPCC).[152] β-catenin mutations were also seen in ovarian and endometrial carcinomas (often of the endometrioid type), which are also associated with HNPCC.[157-159] Approximately 90% of solid pseudopapillary tumours of the pancreas harbour β-catenin mutations, particularly in exon 3.[160] Other tumours associated with mutations in β-catenin include pilomatricomas, ovarian borderline endometrioid tumours, anaplastic thyroid carcinomas, hepatocellular carcinomas and nephroblastomas.[154, 161]

Upregulation of WNT/β-catenin pathway is seen in neuroblastomas,[162] cervical squamous cell carcinomas[163, 164], prostate carcinomas[165, 166], lung adenocarcinomas[128] and malignant melanomas[128]. *DVL1* is located on chromosome 1p36. It has been proposed as a candidate gene for neuroblastoma, Charcot–Marie–Tooth disease type 2A and Schwartz-Jampel syndrome.[167]

During malignant progression in carcinomas where the tumour shows increased invasiveness, E-cadherin is downregulated during epithelial-mesenchymal transition, a process whereby individual carcinoma cells change from a cohesive epithelial phenotype to a motile mesenchymal phenotype and detach from the tumour mass to lie independently in the interstitium.[168] Membrane E-cadherin loss occurs in breast lobular carcinomas, diffuse type gastric adenocarcinomas and a high proportion of renal cell carcinomas.[169] Membrane loss and/or nuclear expression is observed in solid pseudopapillary tumours of the pancreas. Nuclear expression in pancreatic neuroendocrine tumours is associated with a more aggressive potential.[170]

3.3. WNT AND ES/PNET

The first study investigating WNT signalling in Ewing sarcoma was a RT-PCR study on nine Ewing sarcoma cell lines.[171] In that study, it was determined that most cell lines express certain components of the WNT pathway (such as WNT5A, WNT10B, WNT11, WNT13, FZD 2, FZD3, FZD4, FZD7, FZD8 and LRP5/6) but not other components (WNT1, WNT2, WNT3, WNT3A, WNT7A, FZD1, FZD6, FZD9 and FZD10). It was discovered that an intact WNT pathway is present in these cell lines, and that exogenous stimulation by WNT3A ligand causes markedly increased cytoplasmic β-catenin expression, 3-5 fold increase in cell chemotaxis, but no increase in cell proliferation. β-catenin immunohistochemical staining was also done on seven cases, all of which were negative.[171] In view of the increased mobility, the authors of that study suggested that activation of WNT signalling may influence the metastatic potential of ES.

Later on, WNT3A, DKK1, and treatment with LRP5/LRP6 short interfering RNA (siRNA) were found to enhance JNK activity and stimulate formation of neurites in ES cell lines; whereas neurite extension was reduced by treatment with FZD3 siRNA as well as knockdowns of DVL2 and DVL3.[172]

Recent evidence shows that the EWS-FLI1 fusion protein activates the promoter region of DKK2 and represses the DKK1 promoter.[173] EWS-FLI1fusion protein also binds to LEF1 to inhibit β-catenin/TCF mediated transcription.[174]

ES/PNET can display epithelial differentiation morphologically (adamantinomatous variant), immunophenotypically (cytokeratin expression) and ultrastructurally (cell junctions). The nature of the cell junctions has been investigated immunohistochemically in 30 ES/PNET cases and appears to be tight junctions with frequent expression of claudin 1 (63%), zonula occludens 1 (59%), and negativity for E-cadherin.[175]

Markedly increased MYC expression has been reported in 10 of 13 ES/PNET cell lines using Western blot. Expression of MYC appears to be modulated by the EWS-FLI1 fusion protein.[176]

Cyclin D1 expression has been reported in 13 of 13 ES/PNET cell lines[176] and 13 of 31 paraffin wax-embedded tissue specimens.[59] Increased cyclin D1 expression may be due to

the EWS-FLI1 fusion protein. Using cell lines, Matsumoto *et al* [58] demonstrated decreased cyclin D1 expression after repression of the *EWS-FLI1* gene by using *EWS-FLI1* antisense oligonucleotides, and increased cyclin D1 expression after *EWS-FLI1* cDNA transfection. Cyclin D1 promoter was also demonstrated to be a downstream target of the *ETS* family members.[59]

4. MOTIVATION FOR CURRENT RESEARCH

There are currently three immunohistochemical studies on WNT pathway components in ES/PNET reported in the current English language literature, including β-catenin in 7 cases of ES which did not show any significant cytoplasmic or nuclear staining[171], E-cadherin in 30 cases, all of which lack membranous staining[175], and cyclin D1 immunoexpression in 13 of 31 cases which did not show prognostic significance.[59] All three studies targeted only isolated components of the WNT pathway without correlation with other components of the WNT pathway. There was also very little, if any, clinicopathological correlation.

Most investigations of the WNT pathway in ES/PNET attempted in the past were mainly performed on ES cell lines. These studies suggested a repressed β-catenin pathway, although parts of the WNT pathway that are β-catenin independent may be active.[171-174] Past investigations also showed that the EWS-FLI1 fusion protein interacts with components of the WNT pathway.[59, 173, 174, 176]

In view of the current literature suggesting a role for WNT signalling in ES/PNET, and the lack of comprehensive immunohistochemical studies of WNT components in ES/PNET with clinicopathological correlation, there exists a need for a comprehensive investigation of the expression of WNT pathway proteins in ES/PNET. This may lead to a better understanding of the pathobiology of ES/PNET and potentially aid in its management.

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PART C: JOURNAL MANUSCRIPT

IMMUNOEXPRESSION OF WNT SIGNALLING PATHWAY COMPONENTS IN EWING SARCOMA / PRIMITIVE NEUROECTODERMAL TUMOUR

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ABSTRACT

Aim: To examine the immunohistochemical expression of the WNT pathway components in Ewing sarcoma (ES) / primitive neuroectodermal tumour (PNET)

Method and results: Twenty five cases originally diagnosed with ES/PNET were retrieved from the archives and stained with antibodies against WNT1, WNT5A, DVL1, GSK3β, β-catenin, MYC, cyclin D1, E-cadherin (extracellular domain) and E-cadherin (cytoplasmic domain). Of the 25 cases analysed, 23 cases were confirmed as ES/PNET on review. Of the 23 cases, WNT1 was positive in 7 cases (30%), WNT5A in 12 cases (52%), DVL1 in 11 cases (48%), MYC in 11 cases (48%), cyclin D1 in 20 cases (87%), and nuclear localisation of the cytoplasmic domain of E-cadherin in 11 cases (48%). Nuclear β-catenin localisation was seen in 2 cases (8%). Membranous E-cadherin staining was absent in all cases. Except for an association of age with DVL1 and nuclear E-cadherin expression there was no statistically significant difference in the mean scores of the antibodies when segregated by clinicopathological parameters.

Conclusion: WNT pathway components are expressed in a subset of ES/PNET, but seldom involve β-catenin nuclear localisation. Cyclin D1 was frequently expressed. It may contribute to the proliferative potential of ES/PNET and may lead to the development of new therapeutic agents. Lack of membranous E-cadherin is postulated as a contributory factor in the micrometastatic behaviour of ES/PNET. The significance of nuclear E-cadherin expression remains to be elucidated.

INTRODUCTION

Ewing sarcoma (ES) / primitive neuroectodermal tumours (PNET) are round cell sarcomas of probable CD133+ bone marrow-derived mesenchymal stem cell origin.[1, 2] They are characterised by varying but usually minimal degrees of neuroectodermal differentiation and almost always harbour a chromosomal translocation involving the Ewing sarcoma breakpoint region 1 gene (*EWS* or *EWSR1*) and a member of the *ETS* family of transcription factors (*FLI1* in 85% of cases).[3-9] They can occur in soft tissues and virtually any organ site, but are commonest in the bone, where they account for 6% of all bone malignancies in the Mayo Clinic files,[6] and after osteosarcomas, are the commonest bone malignancy of childhood.[10]

ES is traditionally distinguished from PNET based on the absence of neuroectodermal differentiation, which has no prognostic significance.[4, 11,10] Based on cytogenetic findings, ES, PNET and Askin tumour, a childhood malignant small cell tumour of thoracopulmonary origin,[12] are grouped together as the ES family of tumours.[10] PNET should be distinguished from supratentorial PNET, which is morphologically similar but lacks CD99 immunoexpression and *EWS/FLI1* translocation.[13, 14]

The differential diagnosis of ES/PNET includes other small round cell malignancies. Because numerous *EWS-ETS* fusion combinations are possible, reverse transcriptase polymerase chain reaction (RT-PCR) testing for the fusion transcripts may be less sensitive than fluorescent in situ hybridisation (FISH) testing for *EWS* translocations using *EWS* break-apart probes.[15] However, *EWS* translocations also occur in myxoid round cell liposarcoma, acute myeloid leukaemia, clear cell sarcoma, angiomatoid fibrous histiocytoma, desmoplastic small round cell tumour, and extraskeletal myxoid chondrosarcoma.[4, 16, 17]

ES/PNET is regarded as a micrometastatic disease from the outset,[18] as without systemic chemotherapy fewer than 10% of patients survive even though the tumour is radiosensitive.[19] With current combination of surgery, chemotherapy, and/or radiotherapy, the overall survival rate is 34% for patients with metastatic disease and 72% for those without metastatic disease on presentation.[20]

WNT proteins are a family of evolutionarily conserved, secreted ligands that guide the fate of the cells, from cell migration and morphogenesis in the embryo to stem cell maintenance and tissue homeostasis in the adult.[21, 22] The complex functions are accomplished by having multiple WNT members (19 in humans)[23-25] that bind to multiple receptors with activation of multiple interacting pathways.[24, 26] WNT receptors include Frizzled (FZD), low-density lipoprotein receptor-related protein (LRP), and the transmembrane tyrosine kinases RYK and ROR2.[22, 27] WNT pathways include the canonical and the non-canonical pathways.

In the canonical pathway, secreted WNT binds to FZD and LRP 5/6 and recruits the cytoplasmic protein dishevelled (DVL) to prevent the adenomatous polyposis coli (APC)-glycogen synthase kinase 3β (GSK3β)-axin-casein kinase 1α (CK1α) protein complex from degrading cytoplasmic β-catenin. Cytoplasmic β-catenin subsequently accumulates and translocates to the nucleus to induce transcription of WNT target genes such as MYC (also known as c-myc) and cyclin D1.[23, 28] β-catenin also exists as a membrane bound form that links E-cadherin to α-catenin and the actin cytoskeleton.[29, 30]

Abnormalities of the WNT/β-catenin pathway are present in many cancers, most notably colorectal carcinomas, in which *APC* mutation is the initiating event in more than 80% of sporadic colorectal carcinomas.[31-33]

WNT pathway components have been investigated in neuroectodermal tumours. Thirty six percent of supratentorial PNET have been reported to exhibit nuclear β-catenin immunolocalisation consistent with an active WNT/β-catenin signalling pathway, although β-catenin (*CTNNB1*) mutation was only identified in 4% of supratentorial PNET.[34] Of note, nuclear β-catenin expression is associated with a trend towards longer patient survival in supratentorial PNET.[34] In other neuroectodermal tumours, WNT5A underexpression in neuroblastomas[35] and overexpression in malignant melanomas was found to be an indicator of poor prognosis.[36] WNT proteins also play critical roles in bone formation[37] and stem cell functioning.[38] These findings raise the possibility that the WNT pathway may play a role in the biology of ES/PNET.

To our knowledge, there are only three studies reported in the current English language literature that investigated the WNT pathway in ES/PNET using immunohistochemistry.

The components tested include β-catenin in 7 cases of ES which did not show any significant cytoplasmic or nuclear staining,[39] E-cadherin in 30 cases, all of which lack membranous staining,[40] and cyclin D1 immunoexpression in 13 of 31 cases.[41] The last study also found that cyclin D1 immunoexpression did not correlate with survival.

With the exception of one study,[39] the studies to date targeted only isolated components of the WNT pathway. Most of the studies did not correlate the expression of the individual components of the WNT pathway with each other. There was also very little, if any, clinicopathological correlation. In view of the relative lack of immunohistochemical testing and clinicopathological correlation in ES/PNET, this study was conducted to investigate more comprehensively the expression of WNT pathway proteins in ES/PNET by immunohistochemistry in order to understand the pathobiology of ES/PNET and potentially aid in its management.

MATERIALS AND METHODS

From 1972 to 2008, 62 cases of ES/PNET were retrieved from the archives of the Division of Anatomical Pathology, University of Cape Town, Groote Schuur Hospital, Red Cross War Memorial Children's Hospital and Anatomical Pathology Laboratory Greenpoint, Cape Town. All haematoxylin and eosin (H&E) and immunohistochemically stained slides were reviewed to confirm the original diagnosis. Of the 62 cases, 25 cases with sufficient tissue available were selected and an appropriate tumour block selected for immunohistochemical staining. Blocks with no or minimal tumour necrosis were preferred.

Antibodies

The following primary antibodies were used (**Table 1**): WNT1, WNT5A, DVL1, GSK3β, β-catenin, MYC, cyclin D1, E-cadherin extracellular domain (36B5), and E-cadherin cytoplasmic domain (36/E-cadherin).

Staining procedure

Paraffin-wax embedded tissue sections were stained according to the following procedure: 2-3μm sections were floated onto 3-aminopropyltriethoxysilane coated glass slides and incubated overnight at 60 °C to adhere sections to slide. Sections were dewaxed in xylene, rehydrated in graded ethanol and washed in water. Sections were

then blocked for endogenous peroxidase in 3% H_2O_2 water solution for 5 minutes, and after washing in water, heat-induced antigen retrieval was performed by pressure cooking slides at full pressure in either 0.01M citrate buffer (pH 6) for 2 minutes or EDTA (pH8) for 1 minute (**Table 1**). Antigen retrieval was optimised for optimal intensity and clarity of staining. After antigen retrieval sections were rinsed in phosphate buffered saline (PBS) pH 7.6 and then blocked for non-specific binding using a 5% goat serum solution at room temperature for 10 minutes (Dako X0907). The goat serum was drained off and the sections were incubated with the primary antibody at room temperature for specified durations (**Table 1**).

After incubation, the sections were washed in PBS and then treated with goat anti-mouse immunoglobulin labelled with horseradish peroxidase (Envision: Dako K4001) at room temperature for 30 minutes. Sections were washed in PBS. Positivity was developed by applying 3,3'-diaminobenzidene (DAB, Dako K3466) at room temperature for 5-10 minutes. Sections were washed in water and immersed in a 1% $CuSO_4$ solution for 5 minutes to enhance DAB staining. Slides were counter-stained in an aqueous haematoxylin solution with "bluing" of nuclei performed using Scott's tap water, followed by dehydration using graded ethanols, clearing with xylene before mounting with rapid mountant medium.

A negative control, in which the primary antibody was replaced by PBS (buffer), was run simultaneously. A positive external control was used for each antibody using tissues recommended by the manufacturer when possible (**Table 1**).

Grading of staining

β-catenin immunostaining was scored according to the protocol used by Jass et al.[42] This protocol scores 1 for loss of cell membrane staining, 1 or 2 for slight and pronounced increase in cytoplasmic staining, respectively, and 1 or 2 for slight and pronounced nuclear staining, respectively, giving a maximum score of 5. Cases scoring 4 or more were regarded as positive for abnormal β-catenin immunolocalisation.

For the other antibodies the immunostaining score was based on the protocol used by Chetty et al.[43] The scoring was as follows: moderate and/or strong immunolabelling was regarded as positive, with quantification of the positivity as follows: a score of 0 (or

negative), less than 5% tumour cells stained; a score of 1 (or focal positivity), 6% to 50%; and a score of 2 (or diffuse positivity), more than 50%. In addition, the cellular location of the immunoreactivity was noted.

Clinical and pathological correlation

The immunoscores of each antibody were segregated into 2 subgroups in each clinical and morphological category as follows: age (≤ 12 / > 12 years of age), gender (male / female), site (skeletal / extraskeletal), location (central / peripheral), metastasis (present / absent), mitotic count (≤ 10 / > 10 mitoses per 10 high power fields [HPF]), nucleoli (inconspicuous / prominent), and neuroectodermal differentiation (present / absent). The subgroups were then compared using the two-sample Wilcoxon rank-sum (Mann-Whitney) test since these data were not normally distributed.

The relationship between the antibodies was analysed by comparing the mean immunoscores of an antibody by those of another antibody. The Kruskall-Wallace equality of rank test with ties was used since the comparisons involved more than two means.

A p-value of less than 0.05 was used as the standard cut-off for statistical significance for all the analyses. All analyses were performed using Stata 11.1 (StataCorp LP, 4905 Lakeway Drive, College Station, TX 77845, USA).

RESULTS

On review 2 cases were found to be misdiagnosed as ES/PNET. One case showed a fibrillary background, CD99 negativity, and diffuse and strong synaptophysin positivity consistent with a neuroblastoma. The other case showed a linear pattern of infiltration suspicious for lymphoblastic lymphoma, which was confirmed by positive immunostaining for terminal deoxynucleotidyltransferase (TDT).

Of the remaining 23 cases, 22 cases were biopsy specimens and 1 case a resection specimen. The age range was 2-43 years (mean age = 11 years). There was a male preponderance (male : female = 1.3:1). Fourteen of the 23 cases (61%) were of mixed race, 8 (35%) were Caucasian, and 1 (4%) African.

Eleven cases had an osseous primary, 11 extraosseous (including 2 cases of Askin tumours), and 1 undetermined (metastatic ES/PNET without information on the primary site). Ten cases were located in the trunk, 2 in the head and neck, 10 in the extremities, and 1 unknown. The biopsy specimen was taken from the primary tumour in 21 patients, and from the metastatic deposit in 2 (one of which had a known primary in the femur). At least 3 cases received chemoradiation prior to histological examination. At least 4 cases were known with metastatic disease.

Six cases (26%) showed neuroectodermal differentiation (Homer-Wright rosettes) and were classified as PNET. Of those without neuroectodermal differentiation, 2 cases (9%) were classified as Askin tumour based on their thoracopulmonary location. The remaining 15 cases (65%) were classified as ES. The highest mitotic count was 19 mitoses per 10 HPF with a mean of 2 mitoses per 10 HPF. Nucleoli were absent or inconspicuous in 17 cases (74%), and distinct or prominent in 6 cases (26%). CD99 positivity was present in all cases, usually in a membranous distribution (**Figure 1A**).

WNT1 immunoscores were 0 in 16 cases (70%), 1 in 4 cases (17%), and 2 in 3 cases (13%). The mean score was 0.43 (95% confidence interval being 0.12-0.75). Of the cases scoring 1 or more, the staining was membrane and cytoplasmic in 4 cases, and cytoplasmic only in 3 cases (**Figure 1B**). Nuclear membrane staining was noted in 6 cases and was regarded as aberrant staining that did not count towards scoring.

WNT5A immunoscores were 0 in 11 cases (48%), 1 in 3 cases (13%), and 2 in 9 cases (39%). The mean score was 0.91 (0.50-1.32). Staining was cytoplasmic in all cases (**Figure 1C**).

DVL1 immunoscores were 0 in 12 cases (52%), 1 in 3 cases (13%), and 2 in 8 cases (35%). The mean score was 0.83 (0.42-1.23). Staining was cytoplasmic in all cases (**Figure 1D**).

GSK3β immunoscores were 0 in 13 cases (52%), 1 in 2 cases (13%), and 2 in 8 cases (35%). The mean score was 0.78 (0.37-1.19). Staining was cytoplasmic in all cases (**Figure 1E**).

β-catenin immunoscores were 1 in 6 cases (26%), 2 in 9 cases (39%), and 3 in 8 cases (35%). None scored 4 or more (the positive cut-off point).[42] The mean score was 2.09 (1.74-2.43). Cytoplasmic only staining was present in 13 cases (**Figure 1F**), membranous and cytoplasmic staining in 3 cases, nuclear and cytoplasmic staining in 1 case, and nuclear only staining in 1 case (**Figure 1G**). The remaining 5 cases (22%) were negative for β-catenin. Membranous staining was lost in 20 cases (87%).

MYC immunoscores were 0 in 12 cases (52%), 1 in 7 cases (31%), and 2 in 4 cases (17%). The mean score was 0.65 (0.32-0.99). Staining was cytoplasmic with paranuclear localisation (**Figure 1H**).

Cyclin D1 immunoscores were 0 in 3 cases (13%), 1 in 3 cases (13%), and 2 in 17 cases (74%). The mean score was 1.61 (1.30-1.92). Staining was nuclear in all cases (**Figure 1I**).

There was no membranous E-cadherin staining with both the 36B5 clone and the 36/E-cadherin clone in all cases (100%). Unlike the 36B5 clone, the 36/E-cadherin clone showed nuclear staining (**Figure 1J**), with a score of 0 in 12 cases (52%), a score of 1 in 1 cases (4%), and a score of 2 in 10 cases (44%). The mean score was 0.91 (0.48-1.34).

DVL1 scores were significantly increased in cases from patients older than 12 years of age, with a mean score of 0.42 in patients ≤ 12 years of age, and a mean score of 1.3 in patients > 12 years of age (p=0.032). The mean nuclear E-cadherin score was significantly higher in cases from patients younger than 12 years of age, being 1.41 in patients <12 years of age and 0.4 in patients ≥ 12 years of age (p=0.017). There was no statistically significant difference in the mean scores of the other antibodies when correlated with clinicopathological parameters

The mean scores of WNT1 were increased with increasing DVL1 scores (p=0.0315) and with increasing MYC (p=0.0201) scores. WNT5A scores were increased with increasing GSK3β scores (p=0.0247). DVL1 scores were increased with increasing WNT5A (p=0.0182) and GSK3β (p=0.0255) scores. GSK3β scores were increased with increasing WNT5A (p=0.0231) and DVL1 (p=0.0203) scores. Cyclin D1 scores were increased with increasing nuclear E-cadherin scores (p=0.0167). MYC, β-catenin, and

E-cadherin immunoscores do not show a statistically significant correlation with other antibodies, although the 2 cases with β-catenin nuclear translocalisation both showed a high WNT5A score of 2.

DISCUSSION

The sample population had a mean age of 11 years. Although this is less than the mean age of 15 years reported by the United States Surveillance Epidemiology and End Results (SEER) program,[44] our study population was not consecutive and was biased by paediatric cases. The male : female ratio of 1.3:1 of the sample population is similar to the male : female ratio of 1.4:1 reported by the SEER program. The majority (61%) of the sample population was of mixed ancestry, probably related to local population demographics. African race was uncommon (4%), a finding also reported by the SEER program, but our study population was biased by spanning a period of time where the hospitals were limited to certain population groups.

CD99 expression was 100% in our sample, comparable with the CD99 positivity of 90 to 100% reported by the literature.[4, 45-47] It is acknowledged that the lack of molecular confirmation is a short-coming in this study.

Most investigations of the WNT pathway in ES/PNET were on ES/PNET cell lines. A RT-PCR study on 9 ES/PNET cell lines found expression of certain components of the WNT pathway (WNT5A, WNT10B, WNT11, WNT13, FZD 2, FZD3, FZD4, FZD7, FZD8 and LRP5/6) but not others (WNT1, WNT2, WNT3, WNT3A, WNT7A, FZD1, FZD6, FZD9 and FZD10). The investigators of that study concluded that there was evidence that an intact WNT signalling pathway existed in ES/PNET cell lines and suggested that WNT appeared to modulate cell motility.[39] Unlike that study, our study showed WNT1 immunostaining in 7 cases, possibly because of larger sample size.

WNT1 immunostaining in our sample correlated with DVL1 and MYC immunostaining. This may suggest an intact canonical WNT pathway in a subset of ES/PNET cases, but there was no β-catenin nuclear translocalisation in the WNT1 positive cases. One of the WNT1 negative cases with nuclear β-catenin expression showed nuclear membrane expression of WNT1 in 5% of the tumour cells. The cause for the nuclear membrane staining is unexplained at this stage.

The anti-tumoural effect of WNT1 blockade has been reported in a human neuroblastoma cell line (using WNT1 RNAi)[48] and human hepatoma cell lines (using anti-WNT1 antibody).[49] Investigation of the effects of WNT1 blockade in ES/PNET has not been done but may be of some value.

WNT5A has been reported to modulate GSK3β to phosphorylate ROR2 to activate the WNT-JNK pathway (a non-canonical pathway).[50] In our study, WNT5A immunostaining correlated with GSK3β immunostaining. This suggests the presence of non-canonical WNT signalling in a subset of ES/PNET. WNT1 and WNT5A immunostaining did not seem to correlate with each other. The canonical and non-canonical WNT signalling pathways may not be dependent on each other and may be mutually exclusive in our study population. WNT5 mediates axonal guidance in *Drosophila* via the RYK receptor[51] but there was no association between WNT5A scores and neuroectodermal differentiation in this study.

Antitumoural effects of WNT5A blockade has been reported in pancreatic carcinoma and melanoma xenografts in mice (using phenylmethimazole)[52] and also in melanoma cell lines (using Wnt5a-derived N-butyloxycarbonyl hexapeptide).[53]

DVL1 has not been investigated in ES/PNET previously, but knockdown of DVL2 and DVL3 in ES cell lines has been reported to suppress neurite outgrowth.[54] There was, however, no statistically significant correlation between DVL1 expression and lack of neuroectodermal differentiation in our study. DVL1 overexpression has been observed in lobular carcinoma of the breast.[55] Interestingly, lobular carcinomas, similar to ES/PNET, characteristically lack membranous E-cadherin expression.

GSK3β immunostaining correlated with WNT5A and DVL1 expression. This may indicate inhibition of the canonical WNT signalling in a subset of ES/PNET cases. GSK3β affects both the canonical and non-canonical pathways. In the canonical pathway it phosphorylates β-catenin and marks β-catenin for degradation. In the non-canonical pathway GSK3β activates the WNT-JNK pathway and/or inhibits β-catenin signalling.[50]

None of our cases had a β-catenin score of ≥ 4. EWS-FLI1 represses β–catenin signalling[56] and may account for the rarity of nuclear β–catenin expression as well as the lack of association between β–catenin and WNT1 and DVL1 expression in this study. The 2 cases with nuclear β-catenin translocalisation both showed strong WNT5A immunostaining. WNT5A can inhibit β-catenin signalling if the receptor ROR2 is present. It can also activate β-catenin signalling if FZD4 is present,[57] and FZD4 expression has been reported in ES/PNET cell lines.[39] It is possible that FZD4 may play a role here.

Cyclin D1 immunostaining was not significantly associated with WNT1, WNT5A, DVL1, GSK3β or β-catenin immunostaining. This may be because cyclin D1 promoter is a downstream target of the *ETS* family members,[41] with repression of the *EWS-FLI1* fusion gene using *EWS-FLI1* antisense oligonucleotides resulting in decreased cyclin D1 expression, and *EWS-FLI1* cDNA transfection resulting in increased cyclin D1 expression.[58]

Cyclin D1 is a cell cycle protein that allows the cell cycle to pass the G1-to-S transition.[59] However, despite high cyclin D1 immunostaining in cases with a high mitotic count, the association was not statistically significant (p=0.16). WNT5A and nuclear E-cadherin were also weakly associated with increased proliferation (p=0.10 and p=0.11 respectively), but the underlying mechanisms are yet to be unravelled. The 4 cases with metastatic disease all showed a cyclin D1 immunoscore of 2, but the metastatic group was small and statistical analysis failed to show significance. Cyclin D1 immunostaining was associated with nuclear E-cadherin staining in our study. Of interest, solid pseudopapillary neoplasms of the pancreas also co-express nuclear E-cadherin and cyclin D1.[60, 61] However, unlike our sample, most solid pseudopapillary neoplasms show nuclear β-catenin expression.

Flavopiridol (alvocidib) is a cyclin dependent kinase inhibitor. Administration has been found to result in significant clinical response in a phase II trial for relapsed chronic lymphocytic leukaemia[62] and in a phase I trial for multiple myeloma and mantle cell lymphoma.[63]

MYC was associated with WNT1 immunostaining. It is a downstream target of the canonical pathway but is also upregulated by EWS-FLI1.[64] *MYC* is a proto-oncogene encoding a nuclear transcription factor that activates genes involved in cell proliferation, self-renewal, and reprogramming of somatic cells into stem cells. Increased MYC has been reported in 10 out of 13 ES/PNET cell lines using Western blot.[64] However, in our study, MYC expression was only seen in 48%, possibly because the intensity of MYC immunostaining was only moderate at best. There was no significant association between MYC expression and mitotic activity in our study; possibly because cyclin D1 was already upregulated.

Drugs targeting MYC are under development and testing.[65] Quarfloxin is a G-quadruplex (four-stranded DNA structure) that targets the MYC promoter and was in phase 2 clinical trials for neuroendocrine/carcinoid tumours, but has now been withdrawn from clinical studies. The cause for the withdrawal has not yet been published.[66]

Membranous E-cadherin expression was absent in our cases, confirming the finding of a previous study which reported negative E-cadherin immunoexpression in ES/PNET.[40] That study also reported variable expression of tight junction markers (claudin-1, occludin, zonula-occludens-1) in ES/PNET, consistent with the ability of ES/PNET to show partial epithelial differentiation. Although E-cadherin competes with APC for binding to β-catenin, loss of E-cadherin does not normally cause nuclear β-catenin accumulation because cytoplasmic β-catenin is normally degraded by the APC-GSK3β-axin-CK1α complex. However, if canonical WNT signalling is present, β-catenin is no longer degraded, and a lack of membranous E-cadherin may then increase cytoplasmic β-catenin.[67] This may account for the frequency of cytoplasmic β-catenin staining in our study. Membranous β-catenin is rare in our sample, possibly due to the lack of membranous E-cadherin to which β-catenin is normally anchored to. The lack of membranous E-cadherin expression has been postulated to play a role in the micrometastatic propensity of carcinomas[68] and may potentially also play a role in that of ES/PNET. Overexpression of membranous E-cadherin has been reported to cause G1-S phase arrest and apoptosis by interfering with β-catenin signalling in epithelial cells.[69] In ES tumour cells induction of membranous E-cadherin is associated with

downregulation of cyclin D1 and reduced proliferative activity[70] but is also associated with reduced apoptosis and increased chemoresistance.[71]

Nuclear expression of E-cadherin in ES/PNET has not been reported previously in the English language literature. With regards to other tumours, nuclear E-cadherin expression has been seen in synovial sarcomas,[72] Merkel cell carcinomas,[73] various carcinomas (colorectal, oesophageal, clear cell renal cell carcinomas) and pancreatic endocrine tumours, and all solid-pseudopapillary tumours of the pancreas.[74, 75] The mechanism for nuclear localisation of E-cadherin is uncertain and appears to be independent of β-catenin[75] but dysregulation of p120 is a postulated mechanism.[76] While nuclear E-cadherin expression did not correlate with β–catenin immunostaining in our study, nuclear E-cadherin may bind nuclear β-catenin and prevent it from binding to transcription factors.[77]

CONCLUSION

This study demonstrated expression of the WNT pathway components in a cohort of patients with ES/PNET, but nuclear β–catenin immunolocalisation was rare, suggesting a repressed canonical pathway. Non-canonical pathways may still have a role to play in ES/PNET. Despite rare nuclear localisation of β-catenin, expression of the targets of the canonical pathway such as cyclin D1 and MYC were common, most likely related to alternative upregulation of these targets by the ETS family of transcription factors. Cyclin D1, MYC, WNT5A and WNT1 may be potential targets for therapy. All cases of ES/PNET were negative for membranous E-cadherin, which may be a possible mechanism contributing to the micrometastatic ability of ES/PNET. A subset of ES/PNET also showed nuclear E-cadherin expression, the significance of which remains to be elucidated.

COMPETING INTERESTS

None to declare.

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FIGURE LEGEND

Figure 1: Immunohistochemical results. **A**: Membranous CD99 expression, 200x. **B**: WNT1 cytoplasmic expression, 200x. **C**: WNT5A cytoplasmic expression, 200x. **D**: DVL1 cytoplasmic expression, 200x. **E**: GSK3β cytoplasmic expression, 200x. **F**: Cytoplasmic β-catenin, 200x. **G**: Nuclear β-catenin expression, 200x. **H**: MYC paranuclear expression, 200x. **I**: Cyclin D1 nuclear expression, 200x. **J**: Nuclear E-cadherin, 200x.

Table 1: Primary antibodies.

Primary antibody	Clone	Supplier	Antigen retrieval	Dilution	Incubation time (min)	Positive control
WNT1	ab15251 (polyclonal)	Abcam	EDTA	1:200	45	Breast carcinoma
WNT5A	ab72583 (polyclonal)	Abcam	EDTA	1:200	45	Breast carcinoma
DVL1	ab21062 (polyclonal)	Abcam	EDTA	1:250	45	Breast carcinoma
GSK3β	ab30619 (polyclonal)	Abcam	EDTA	1:200	45	Breast carcinoma
β-catenin	17C2 (monoclonal)	Novo-castra	EDTA	1:100	45	Breast carcinoma
MYC	9E11 (monoclonal)	Novo-castra	EDTA	1:400	120	Burkitt lymphoma
Cyclin D1	SP4 (monoclonal)	Lab Vision	EDTA	1:50	45	Mantle cell lymphoma
E-cadherin	36B5 (monoclonal)	Novo-castra	Citrate	1:50	45	Normal prostate
E-cadherin	36/E-cadherin (monoclonal)	BD Biosciences	EDTA	1:100	45	Normal prostate

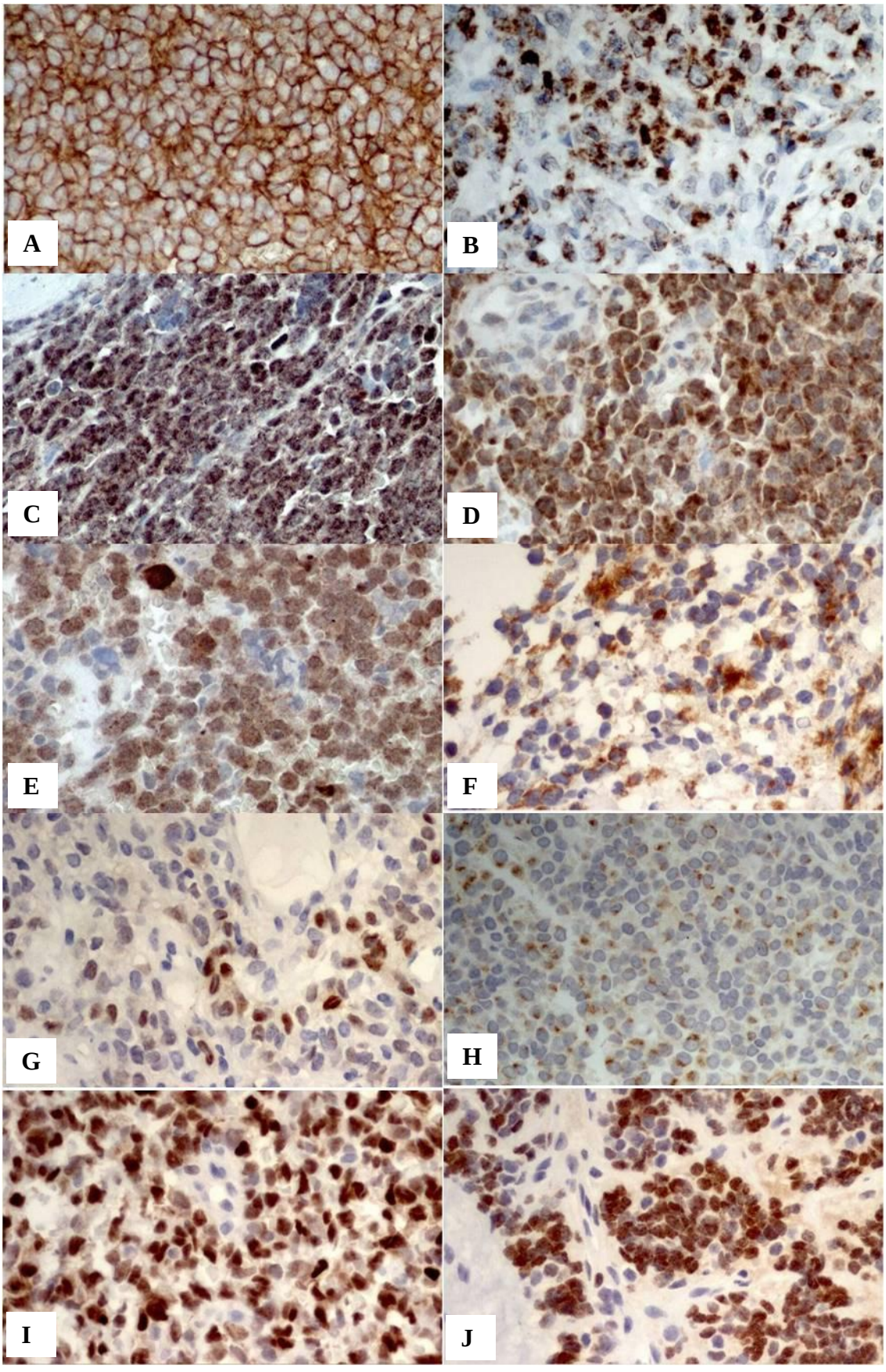

Figure 1

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PART D: SUPPORTING DOCUMENTS

1. TABLES

Table 1: Clinical features.

No.	*Race*	*Sex*	*Age*	*Site*	*Diagnosis*	*Clinical data*
1	W	F	10	Unknown	Metastatic ES (from skeletal ES of right proximal femur)	Presented at age 4 with tumour right thigh, biopsy diagnosis favoured neuroblastoma, differential diagnosis ES. Lung biopsy 2 year and 1 month later confirmed metastatic tumour. Hindquarter amputation performed 8 month later (probably post-radiation) showed 90% necrosis, tumoural calcification and negative lymph nodes. Lymph node biopsy 8 month later showed metastatic tumour. Biopsy of a metastatic deposit (current case) 3 years later at the age of 10 confirms the diagnosis of a metastatic ES.
2	W	M	6	Chest wall	Askin tumour	Anterior mediastinal tumour eroded through anterior chest wall – probably arising in the chest wall.
3	C	F	2	Dorsum R foot	Extraskeletal ES	Tumour dorsum right foot said to be present since birth. Excisional biopsy of large tumour within extensor digitorum brevis.
4	C	M	10	Left leg (tibia diaphysis)	Skeletal PNET	No clinical data
5	C	M	6	Bone	Skeletal ES	No clinical data
6	C	F	6	Left tibia	Neuroblastoma	Osteitis not settling. ? tumour left tibia
7	W	F	11	Right thigh	Extraskeletal ES	Rigth thigh mass; cyst excised from subcutaneous tissue
8	W	F	6	Left leg	Extraskeletal ES	Sent in as recurrent ES
9	C	M	2	Post neck	Extraskeletal PNET	Four month swelling in subcutaneous tissue of low occipital region
10	W	M	8	Left tibia	Lymphoblastic lymphoma	Query chronic osteomyelitis left tibia
11	C	M	11	Right lung	Metastatic ES	Multiple coin-shaped lesions in right lung, also multiple lytic bone lesions. Primary unknown.
12	C	M	2	Extradural space	Extraskeletal PNET	Thoracic extradural mass; urinary HVA levels normal; received 5 cycles of

						chemotherapy; case used = pre-therapy biopsy; post-therapy biopsy not available for review
13	W	F	7	Left rib	Skeletal PNET	Neoadjuvent therapy given Moderately pleomorphic small ovoid cells with distinct nucleoli;
14	C	M	19	Left proximal fibula	Skeletal ES	MRI favoured Ewing sarcoma. Last blood result in 2003. Patient deceased. Folder withdrawn in 2008.
15	C	F	10	Right ileum	Skeletal ES	Large tumour right ileum
16	C	M	31	Spinal dura mater	Extraskeletal ES	Spinal tumour, progressive back pain for 1 month associated with lower limb weakness and loss of perianal sensation. MRI showed spinal cord compression at T10-11 by extradural mass. Pleural biopsy 2 years later confirmed metastatic disease.
17	C	M	33	Right lung apex	Extraskeletal ES	Small cell carcinoma diagnosed at around age 28, had chemotherapy, now large lesion right lung apex invading vertebral body and dura
18	C	M	19	Left proximal femur	Skeletal PNET	Query osteosarcoma with bone destruction. Biopsy in 2001. Last blood result in 2003 showed hypercalcaemia. Patient's folder withdrawn in 2007.
19	C	M	24	Tibial diaphysis	Skeletal ES with soft tissue extension	Painful swelling R calf for 1 year; 10 x 8cm hard, fixed mass medial aspect of right calf. MRI shows calf mass encasing peroneal and posterior tibial neurovascular bundles. Initial CT chest showed four small lung nodules interpreted as metastatic disease (post-chemotherapy CT showed decrease in size of the nodules). Bone scan and marrow biopsy negative for metastasis. Neo-adjuvant chemotherapy (ifosfamide, etoposide) with good clinical response (significant decrease in primary tumour size on MRI); through knee amputation 3 months after biopsy showed tumour involving soft tissues and tibial cortex without medullary involvement. Vascular invasion was present. There was no post-chemotherapy tumour necrosis. Patient alive but lost to follow up 1 year 5 months (last discharged 27/10/2009 TCB in 3/12) after initial presentation (07/05/2008).
20	B	F	14	Ankle	Extraskeletal ES	Subcutaneous ankle tumour

21	C	M	32	Chin	Extraskeletal ES	Rapidly enlarging mass on tip of chin
22	C	F	43	Left femur	Skeletal ES	Pathologic fracture left femur
23	W	F	Unknown	Pelvis	Skeletal ES	Pelvic mass, query Ewing sarcoma
24	W	M	27	Right chest wall	Askin tumour	Right chest wall mass, 10 x 10cm
25	W	F	17	Acetabulum	Skeletal PNET	Tumour right hemipelvis; MRI query ES / chondrosarcoma / osteosarcoma

No., case number; W white, B black, C coloured (mixed ancestry), F, female; M, male; Age, age in years; NA, not available.

Table 2: Pathologic characteristics.

No.	*Diagnosis*	*Morphology*	*Nucleoli*	*MF*	*PAS*	*CD99*	*Other investigations*
1	Metastatic ES	Uniform small cells	none	0	None	+	EMA-, NB-, CD68-, LCA-.
2	Askin tumour	Alveolar growth pattern	distinct	0	Abundant	+	myoglobin+ (reported-), desmin weak + (reported-), CK-, EMA-, LCA-, S100-, SMA-, MSA-
3	Extraskeletal ES	Uniform medium sized cells	inconspicuous	15	Scant	+	EM desmosome-like structures, focal glycogen, abundant polyribosomes, poorly developed organelles, no secretory granules.
4	Skeletal PNET	Homer-Wright rosettes present	none	16	Scant	+	NB-, desmin-, myoglobin-, SMA-; EM: neurosecretory granules.
5	Skeletal ES	Uniform small cells	none	0	None	+	
6	Neuroblastoma	Moderately pleomorphic, fine chromatin	inconspicuous	1	None	- (CD99 repeated)	Synaptophysin strongly and diffusely positive
7	Extraskeletal ES	Moderately pleomorphic, coarse chromatin	distinct	8	Scant	+	LCA-, CD30-, CD3-, CD20-, S100-, desmin-, MYF4-
8	Extraskeletal ES	Medium sized uniform cells	inconspicuous	1	Moderate	+	Electron microscopy confirms ES
9	Extraskeletal PNET	Moderately pleomorphic; perivascular pseudo-rosettes	inconspicuous	14	None	+	NSE+ vimentin+, S100+; CK-, LCA-
10	Lymphoblastic lymphoma	Uniform small cells	none	0	None	+	TDT+
11	Metastatic ES	Moderately pleomorphic, vesicular chromatin	inconspicuous	12	Scant	+	LCA-, SMA-, CD68-, NB-, EMA -
12	Extraskeletal PNET	Uniform medium sized cells, dispersed chromatin	none	0	Not done	+	NSE weak +, neurofilament +; chromogranin+/-; NB-, S100-, LCA-, CD68-; EM microtubules, primitive junctional complexes
13	Skeletal PNET	Moderately pleomorphic small ovoid cells Homer-Wright rosettes present	distinct	2		+	S100+, vimentin+; GFAP-, NSE-, desmin-; EM shows long cytoplasmic processes, neurotubules, abundant glycogen in some cells, no neurosecretory granules
14	Skeletal ES	Small round to oval cells, dense chromatin	inconspicuous	0	Scant	+	Scanty glycogen; CD56-, LCA-, desmin-, LEDER-

15	Skeletal ES	Uniform small cells	distinct	4	Scant	+	desmin-, NB84-, NF-
16	Extraskeletal ES	Uniform medium sized cells	inconspicuous	17	None	+	CD56+, S100+; NF-, MNF-, AE1/AE3-, EMA-, CK7-, Melan A-, HMB45-, LCA-, TDT-, inhibin-, CD34-, CD43-, MYF4-, CD30-, neurofilament-, chromogranin-, PGP9.5-
17	Extraskeletal ES	Uniform medium sized round cells	inconspicuous	11	Not done	+	Vimentin+; Cam 5.2-, MNF-, TTF1-, CD10-, CD56-, desmin-, calponin-, MYF4-, synaptophysin-.
18	Skeletal PNET	Moderately pleomorphic angular to spindled cells, vesicular chromatin, Homer-Wright rosettes.	inconspicuous	19	Scant	+	vimentin+, CD117+; LCA-, desmin-, MSA-, MyoD1-, NSE-, S100-, synaptophysin-, NB74-, chromogranin-, CD56-. EM: primitive cells with neuritic processes and occasional glycogen
19	Skeletal ES	Uniform small cells	inconspicuous	0	Abundant	+	CK7-, EMA-, LCA-, desmin-, MyoD1-, NSE weakly +
20	Extraskeletal ES	Uniform medium sized cells, stippled chromatin	inconspicuous	11	Scant	+	vimentin+, Bcl2+, EMA-, MNF116-, CK7-, E-cadherin-, S100-, synaptophysin-, CD56-
21	Extraskeletal ES	Uniform medium sized cells, vesicular chromatin	prominent	19	Moderate	+	vimentin+, Cam5.2-, MNF116-, CD20-, NSE-, desmin-
22	Skeletal ES	Moderately pleomorphic, vesicular chromatin	distinct	3		+	Vimentin+, NSE+; Cam5.2-, CK7-, CK20-, TTF1-, CD43-
23	Skeletal ES	Uniform small cells, dispersed chromatin	none	2	None	+	AE1/AE3-, desmin-, myf4-, LCA-, S100-
24	Askin tumour	Uniform small cells, dense chromatin	none	0	None	+	Vimentin focally +; Cam5.2-, desmin-, MyoD1-, LCA-, S100-, chromogranin-
25	Skeletal PNET	Uniform medium to large cells, fine chromatin, Homer-Wright rosettes are prominent	inconspicuous	0	Abundant	+	MNF116 isolated +, S100 isolated +, synaptophysin-, NB84 +

No., case number; MF, mitoses per 10 high power fields; +, positive; -, negative; +/-, equivocal; CK, pancytokeratin; EMA, epithelial membrane antigen; LCA, leukocyte common antigen; MSA, muscle specific antigen; NB, neuroblastoma; NF, neurofilament; SMA, smooth muscle antigen; EM, electron microscopy.

Table 3: Immunohistochemical scores and immunolocalisation. Case 6 and case 10 were not ES/PNET on review and were excluded from statistical analysis.

No.	WNT1	WNT5A	DVL1	GSK3β	β-catenin	MYC	Cyclin D1	E-cadherin
1	0 C	0 C	0 A	0 C	1 A	1 P	2 N	0 A
2	0 NM	1 C	2 C	2 C	2 C	1 P	2 N	2 N
3	0 M	0 A	0 A	0 A	1 A	2 P	2 N	2 N
4	1 M,C	2 C	0 A	2 C	3 C	1 P	2 N	2 N
5	1 C	0 C	0 A	0 C	1 M,C	0 A	1 N	0 N
6	0 A	0 A	0 A	1 C	1 A	0 A	0 A	0 A
7	0 NM	2 C	2 C	2 C	2 C	0 P	2 N	2 N
8	0 NM	0 A	0 C	0 A	3 C	0 P	0 N	1 N
9	0 NM	2 C	0 C	0 C	3 N,C	0 P	2 N	2 N
10	0 A	0 C	0 A	0 A	1 A	0 A	0 A	0 N
11	0 A	0 C	0 A	1 C	2 C	0 A	2 N	2 N
12	0 A	1 C	1 C	0 A	2 C	0 A	1 N	0 A
13	0 A	0 A	0 A	0 A	2 C	0 A	2 N	2 N
14	0 A	0 A	0 A	0 C	3 C	2 P	2 N	0 A
15	0 M	0 C	0 A	0 C	2 C	0 A	2 N	2 N
16	1 M,C	2 C	2 C	2 C	3 C	1 P	2 N	2 N
17	2 C	2 C	2 C	2 C	2 M,C	1 P	2 N	0 A
18	0 A	2 C	0 C	0 A	1 A	0 A	2 N	0 A
19	2 M,C	2 C	2 C	2 C	3 C	2 P	2 N	0 A
20	0 NM	2 C	2 C	1 C	2 C	0 A	2 N	2 N
21	2 C	0 A	2 C	0 C	2 M,C	1 P	1 N	0 A
22	0 C	2 C	1 C	2 C	3 N	2 P	0 N	0 A
23	0 NM	0 A	1 C	2 C	1 A	0 P	2 N	0 A
24	0 A	0 A	0 A	0 A	1 A	0 A	0 A	0 A
25	1 M,C	1 C	2 C	0 C	3 C	1 P	2 N	0 A

No., case number; NM nuclear membrane; A completely absent staining; C cytoplasmic; M membrane; N nuclear; P paranuclear. Membranous E-cadherin was negative in all cases. Nuclear E-cadherin staining is only seen with the antibody against the cytoplasmic domain of E-cadherin (clone 36/E-cadherin).

Table 4: Comparing immunohistochemical scores using clinical parameters (two-sample Wilcoxon rank-sum test). A p-value of less than 0.05 is statistically significant.

Antibody	Age	Gender	Skeletal site	Peripheral location	Metastatic disease
WNT1	0.0651	0.0558	0.9284	0.8994	0.3418
WNT5	0.1212	0.3406	0.3557	0.2639	0.8587
DVL1	0.0315	0.9452	0.1133	0.5138	0.7193
GSK3β	0.4278	0.7252	0.8601	0.7108	0.2504
β-catenin	0.3409	0.6439	0.8080	0.3972	0.6348
MYC	0.0777	0.6822	0.5078	0.1713	0.2842
Cyclin D1	0.7044	0.7473	0.4832	0.7527	0.2062
E-cadherin	0.0171	0.4185	0.1195	0.8522	0.8540

Table 5: Comparing immunohistochemical scores using pathologic features (two-sample Wilcoxon rank-sum test). A p-value of less than 0.05 is statistically significant.

Antibody	Neuroectodermal differentiation	Nucleolar prominence	Mitotic activity
WNT1	0.9312	0.5171	0.2284
WNT5A	0.1914	0.8177	0.0970
DVL1	0.3324	0.2952	0.8615
GSK3β	0.1648	0.5517	0.5922
β-catenin	0.3710	0.8521	0.9199
MYC	0.2808	0.9386	0.8083
Cyclin D1	0.4670	0.6494	0.1643
E-cadherin	0.8117	0.2335	0.1079

Table 6: Comparing immunohistochemical scores with each other (chi-squared test with ties). A p-value of less than 0.05 is statistically significant.

Antibody	**WNT1**	**WNT5A**	**DVL1**	**GSK3β**	**β-catenin**	**MYC**	**Cyclin-D1**	**E-cadherin**
WNT1	0.0001	0.4578	0.0315	0.2632	0.4200	0.0201	0.2131	0.3511
WNT5A	0.4579	0.0001	0.0621	0.0247	0.1226	0.6545	0.4341	0.4969
DVL1	0.0557	0.0182	0.0001	0.0255	0.1066	0.1301	0.6372	0.6092
GSK3β	0.4727	0.0231	0.0203	0.0001	0.3772	0.3672	0.2649	0.4585
β-catenin	0.3602	0.1151	0.4795	0.4448	0.0001	0.2325	0.5283	0.361
MYC	0.1414	0.7378	0.4108	0.1474	0.107	0.0001	0.7553	0.4111
Cyclin-D1	0.9747	0.5031	0.1926	0.3625	0.8308	0.6223	0.0001	0.0167
E-cadherin	0.2306	0.7323	0.2278	0.2186	0.1622	0.5803	0.0989	0.0001

2. FIGURES

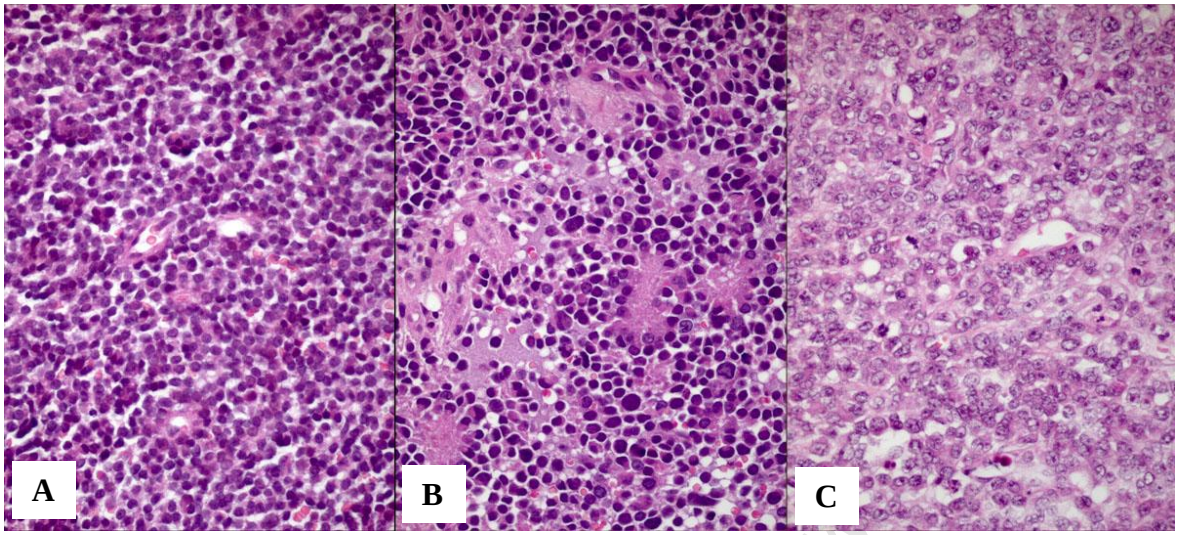


Figure 1: Morphologic spectrum of ES/PNET. **A**: Case 19: Classic ES with sheets of uniform, small, round, blue cells. **B**: Case 25: PNET with Homer-Wright rosettes. **C**: Case 21: Atypical ES with larger cells that often have a prominent nucleolus. Haematoxylin and eosin stain, 200x.

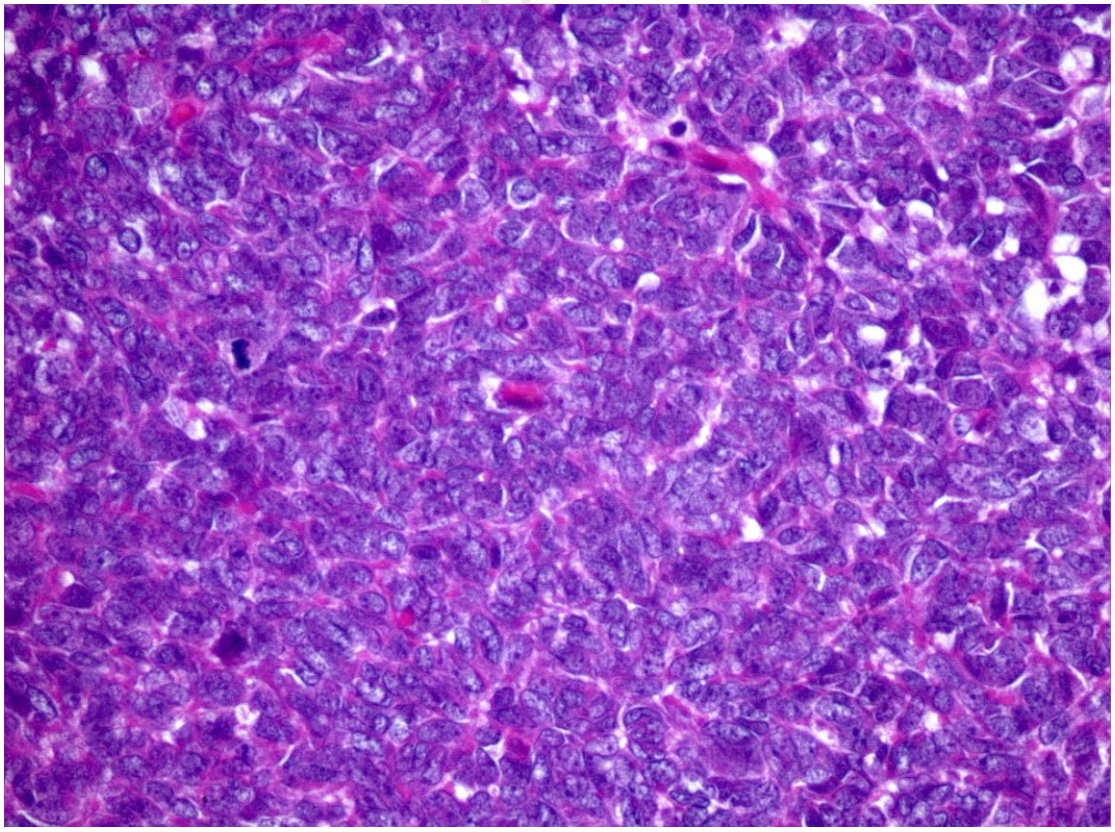


Figure 2: Ewing sarcoma, haematoxylin and eosin stain (case 8), 200x.

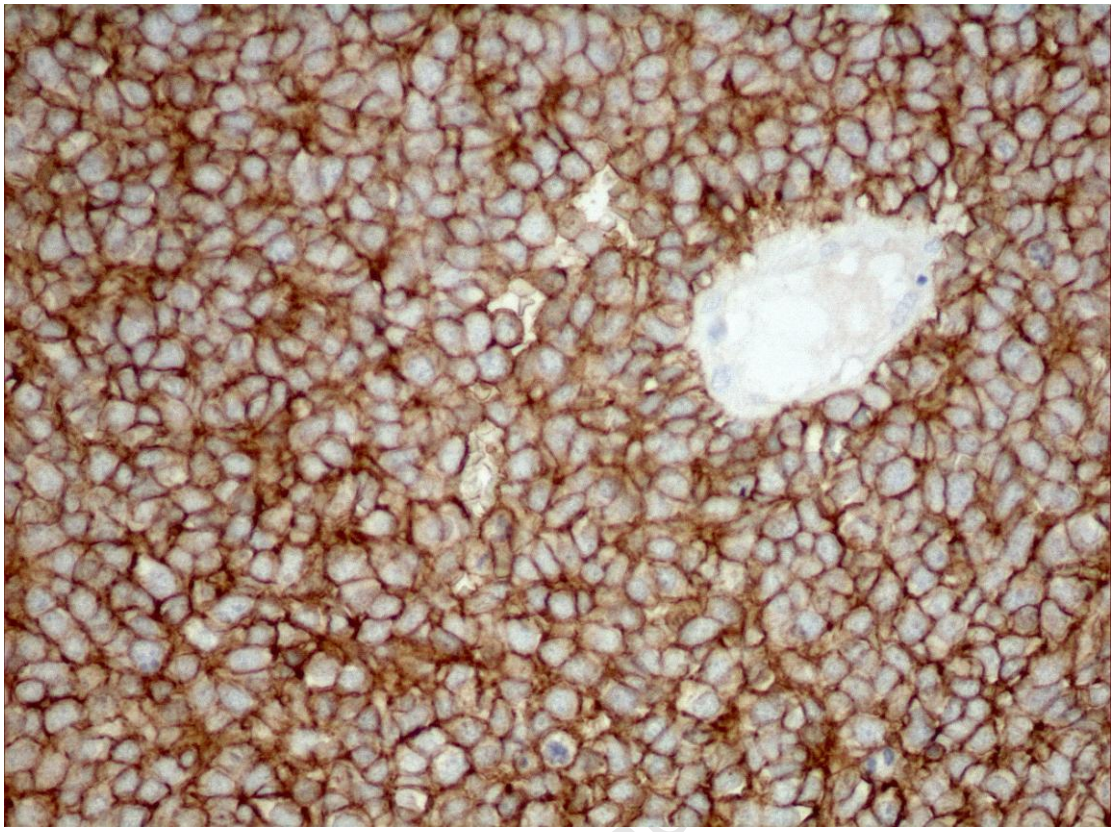


Figure 3: Membranous CD99 expression (case 1), 200x

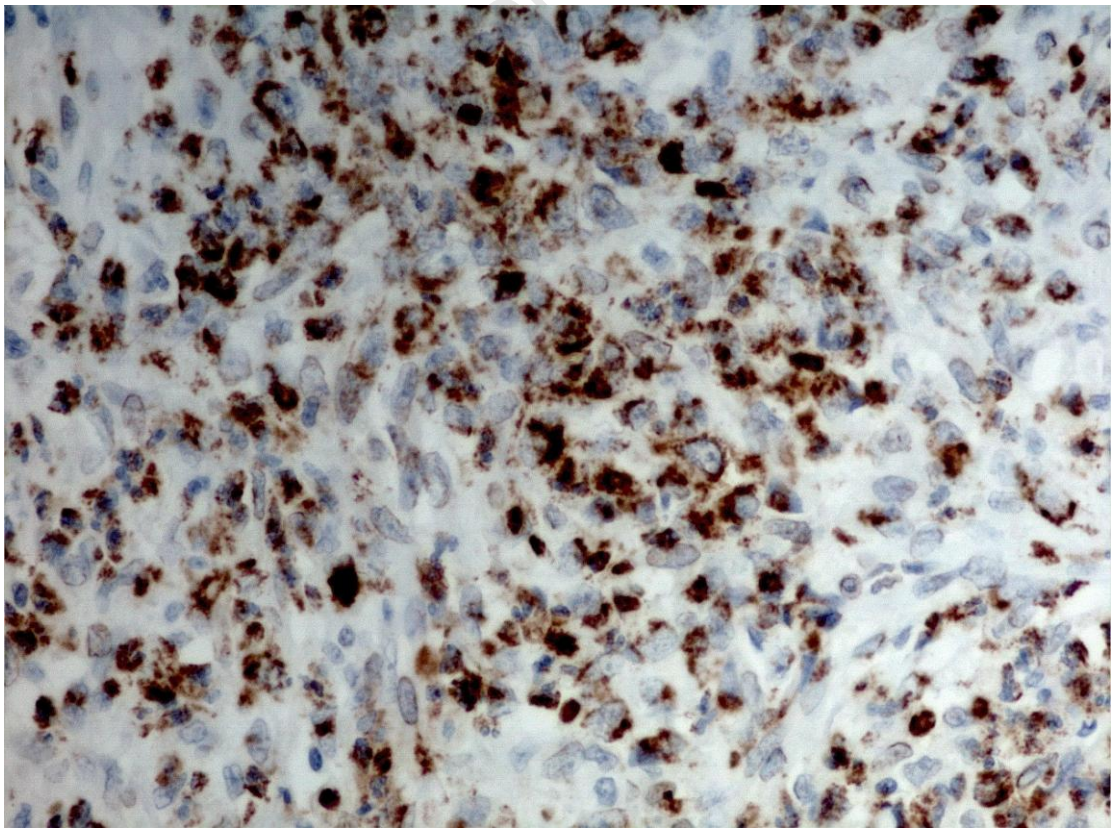


Figure 4: WNT1 cytoplasmic expression (case 21), 200x.

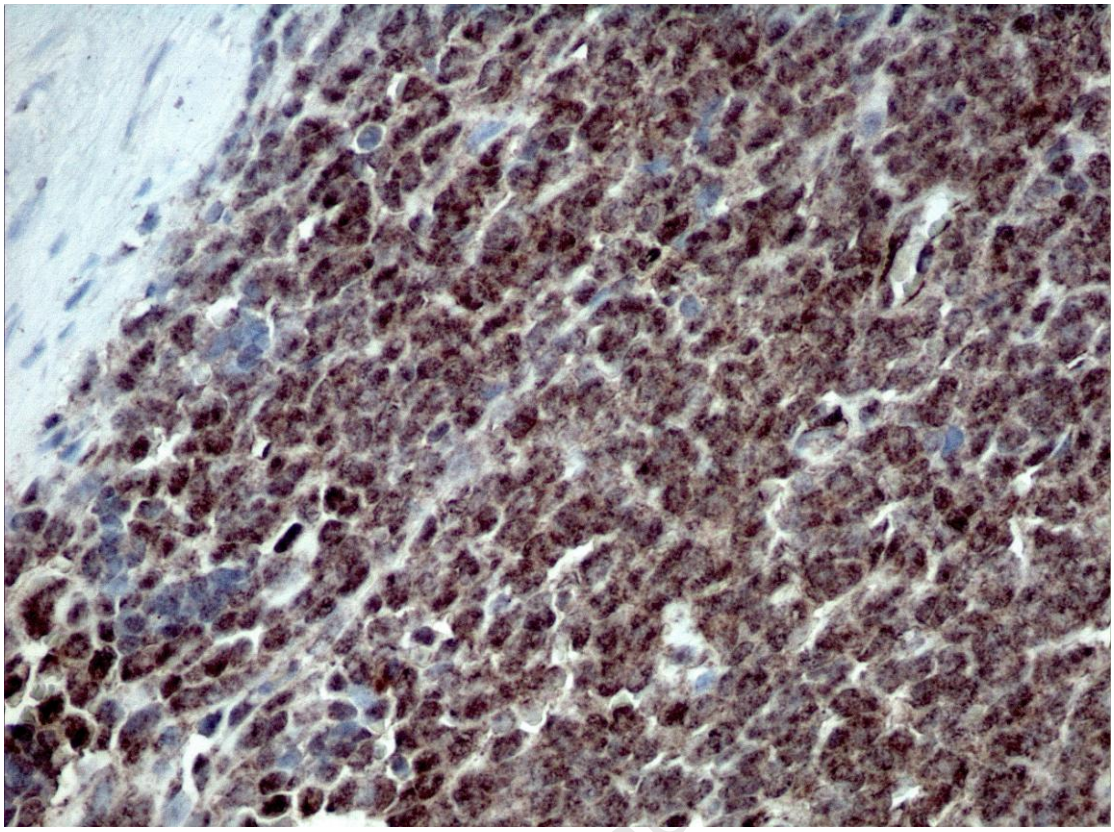


Figure 5: WNT5A cytoplasmic expression (case 7), 200x

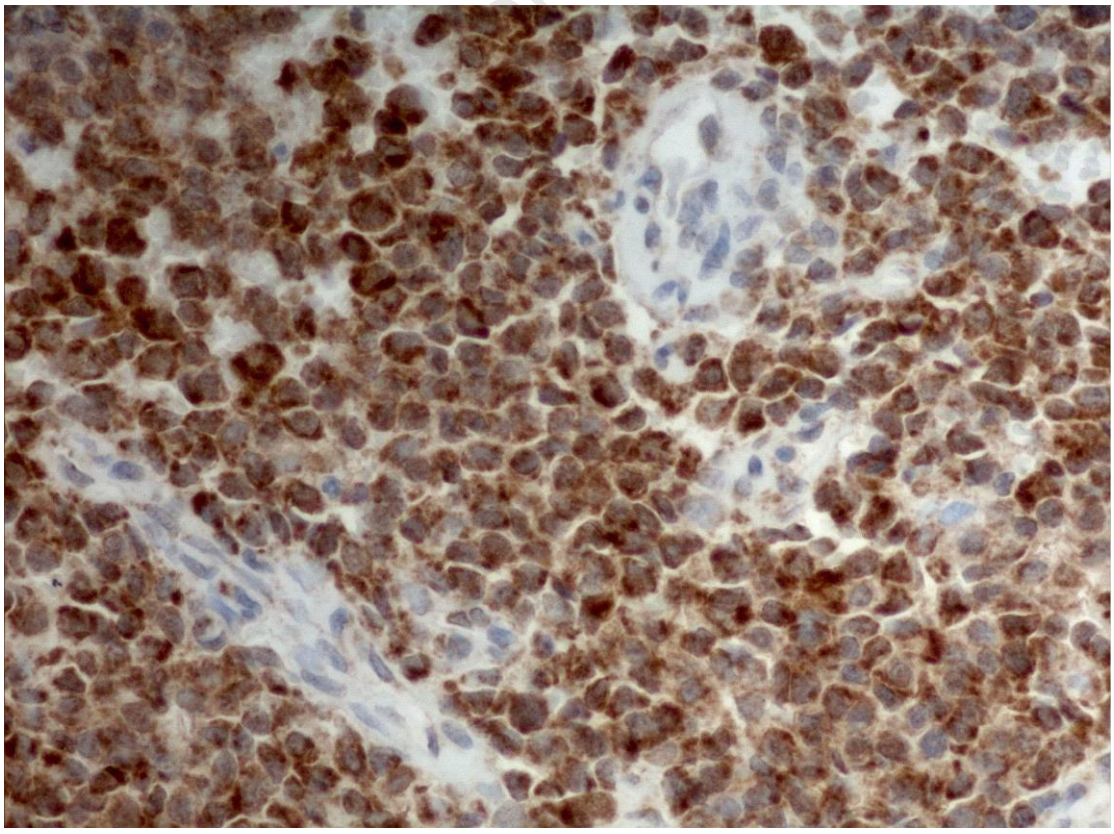


Figure 6: DVL1 cytoplasmic expression (case 17), 200x.

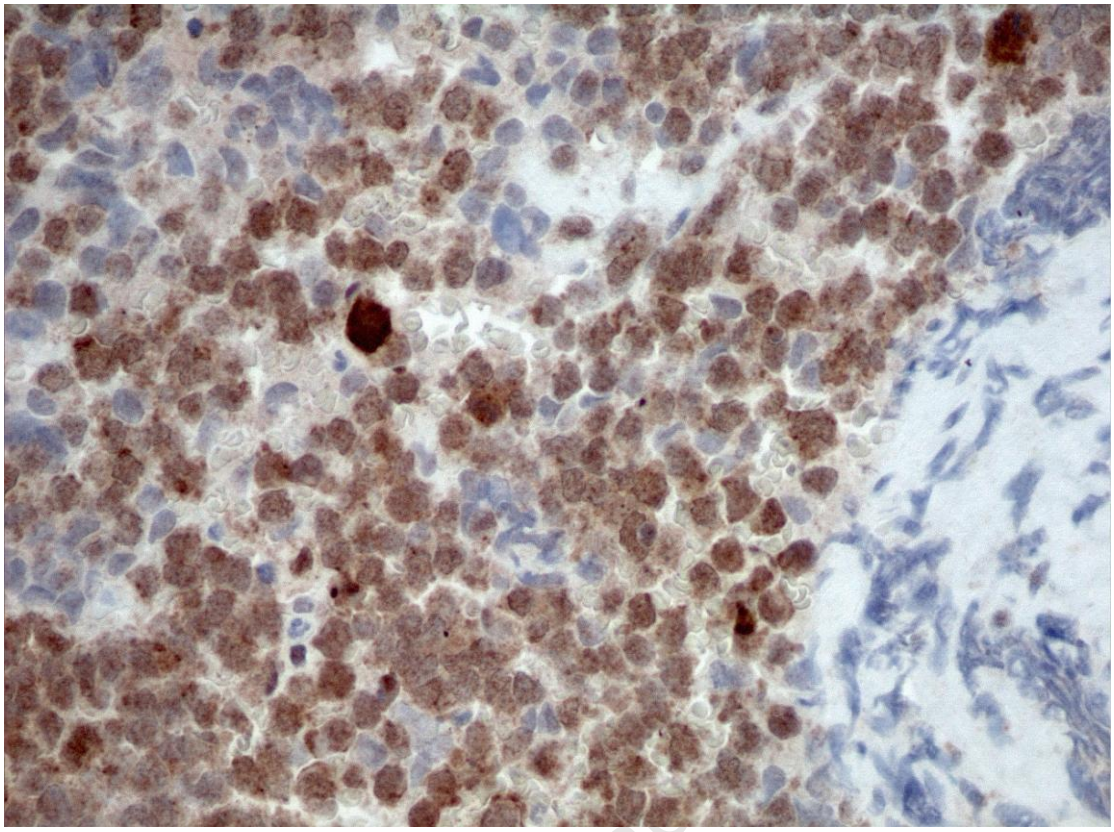


Figure 7: GSK3β cytoplasmic expression (case 16), 200x

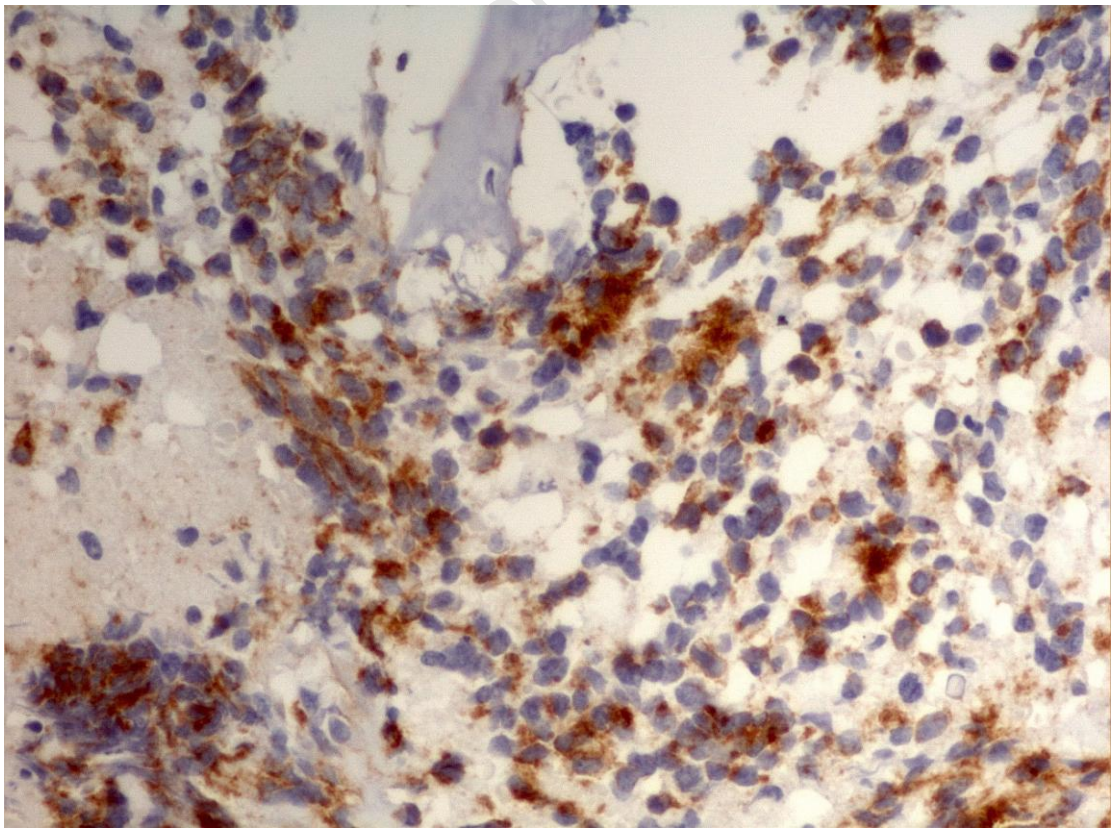


Figure 8: Cytoplasmic β-catenin (case 14), 200x

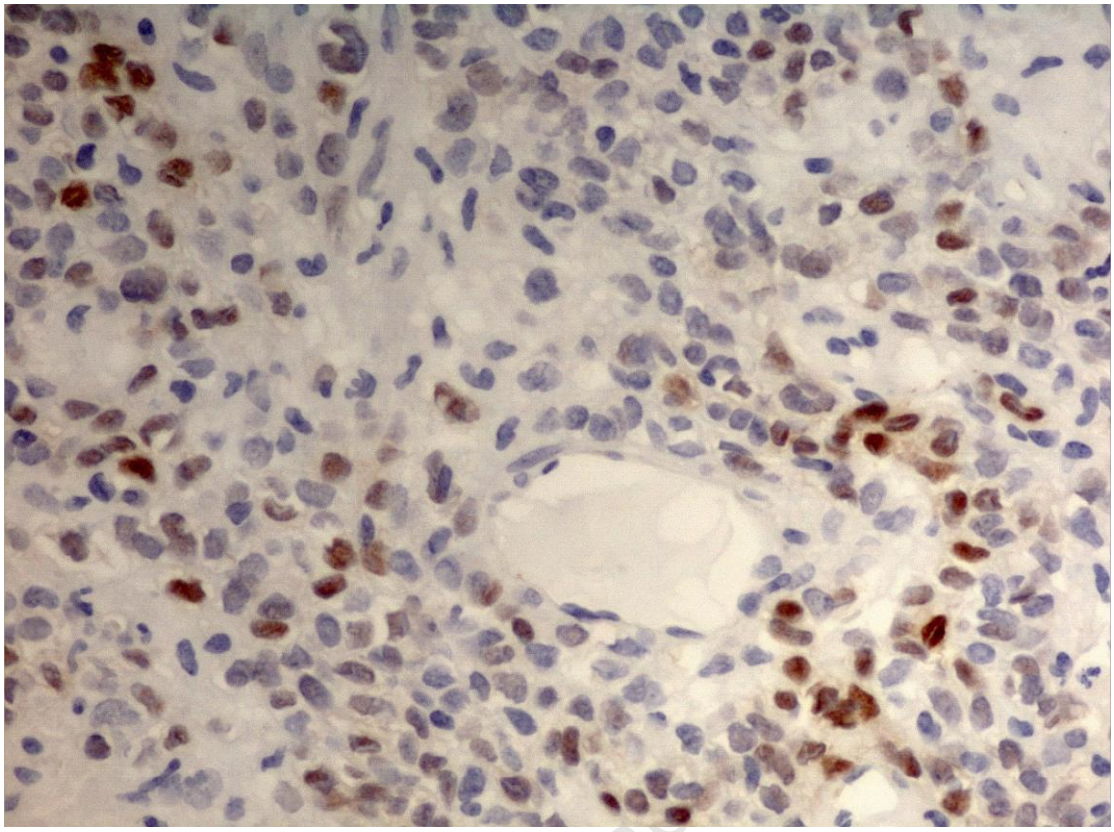


Figure 9: Nuclear β-catenin expression (case 22), 200x.

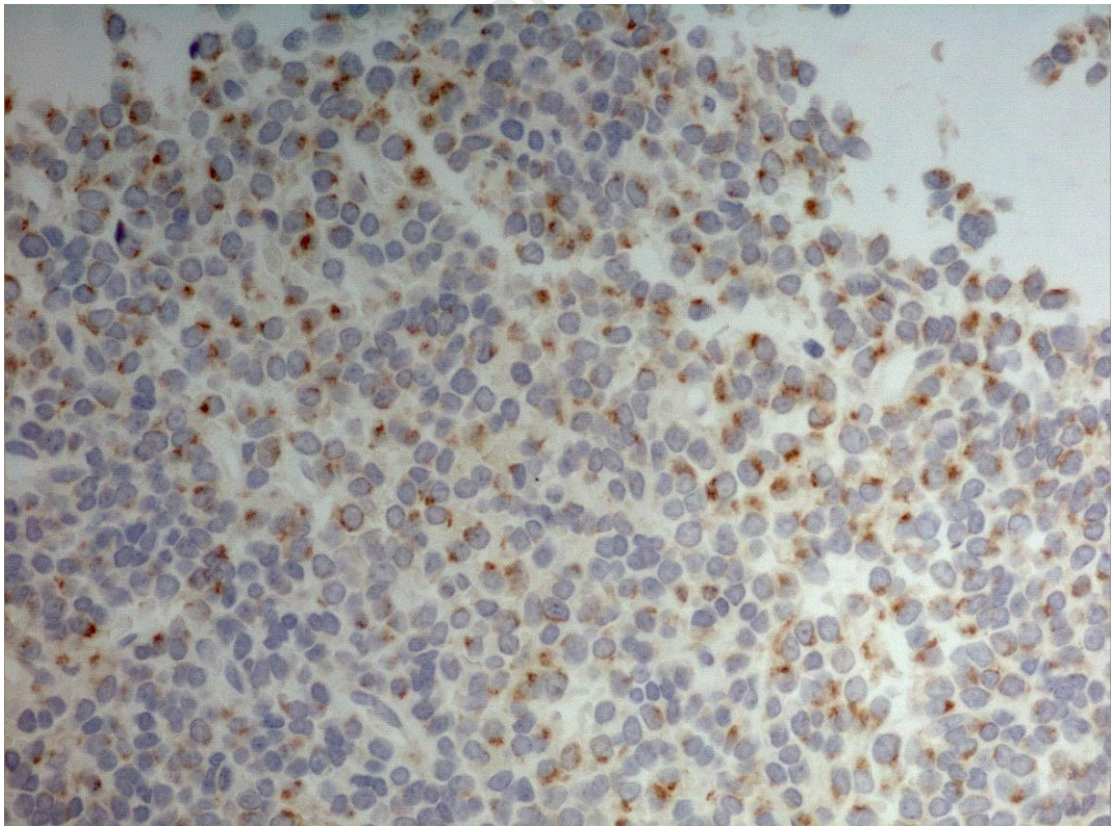


Figure 10: MYC paranuclear expression (case 19), 200x.

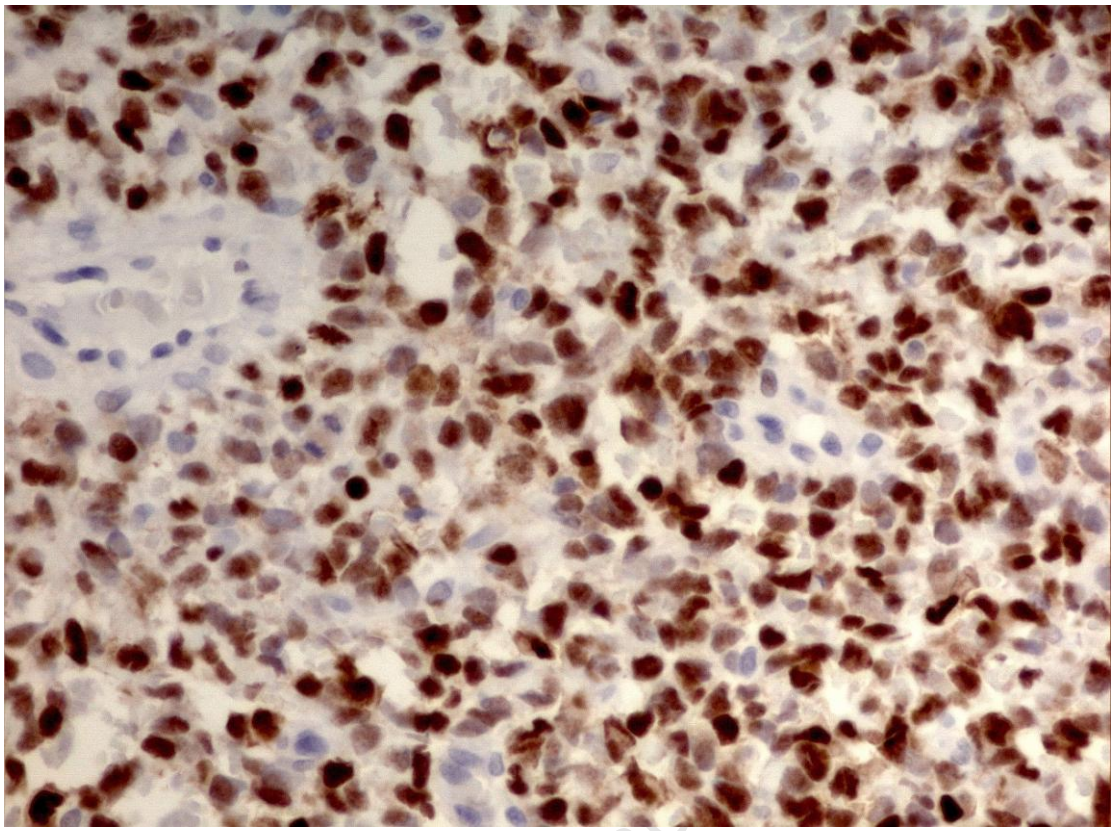


Figure 11: Cyclin D1 nuclear expression (case 20), 200x.

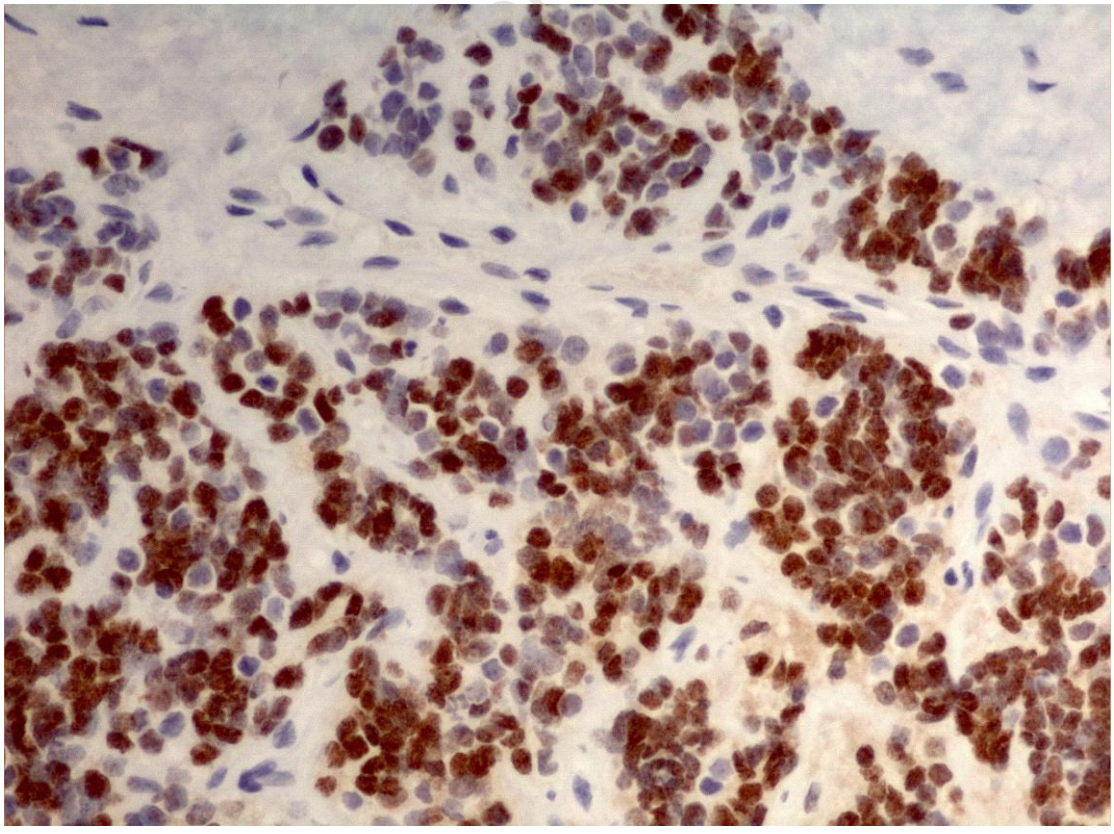
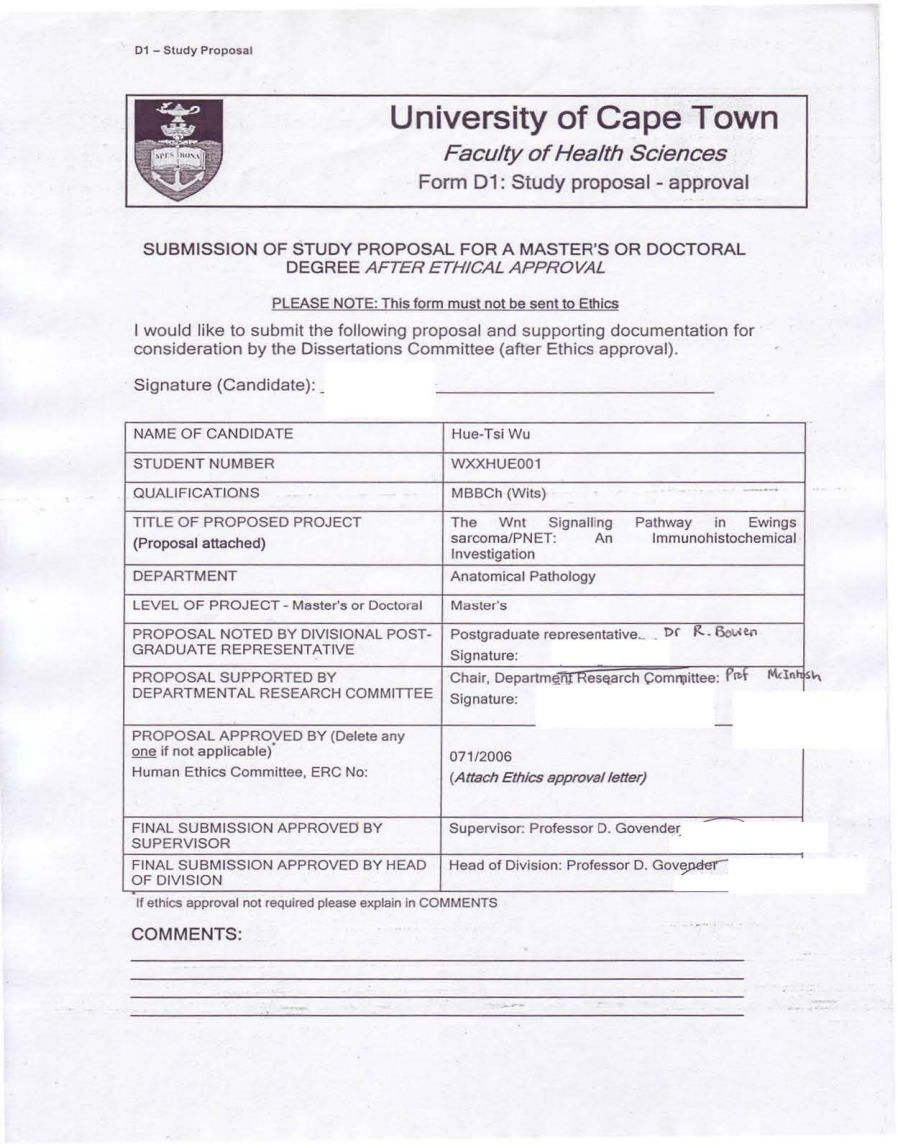


Figure 12: Nuclear E-cadherin (case 15), 200x.

3. SCIENTIFIC APPROVAL FORM



D1 – Study Proposal

University of Cape Town

Faculty of Health Sciences

Form D1: Study proposal - approval

SUBMISSION OF STUDY PROPOSAL FOR A MASTER'S OR DOCTORAL DEGREE *AFTER ETHICAL APPROVAL*

PLEASE NOTE: This form must not be sent to Ethics

I would like to submit the following proposal and supporting documentation for consideration by the Dissertations Committee (after Ethics approval).

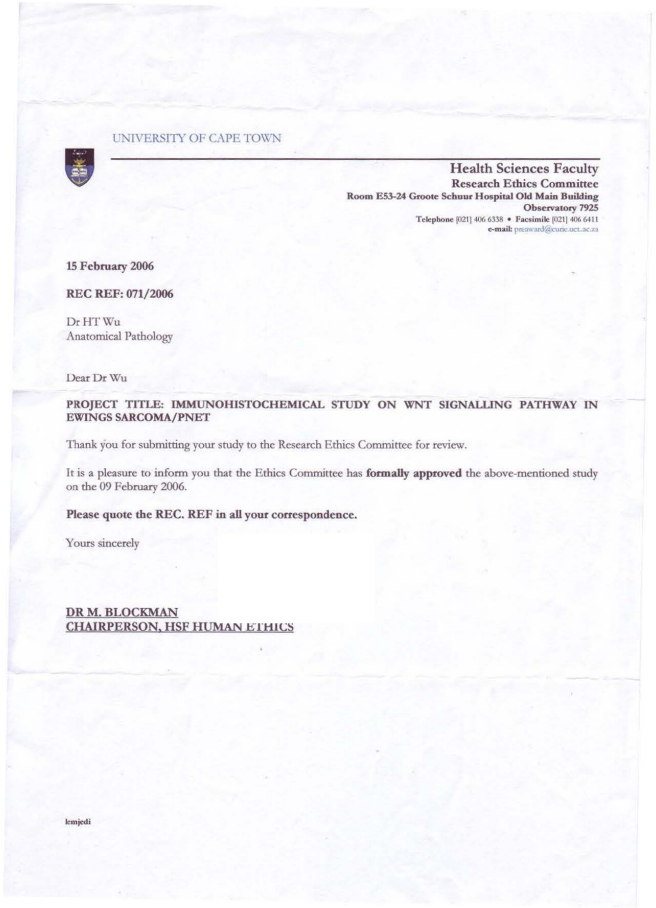
Signature (Candidate): _____

NAME OF CANDIDATE	Hue-Tsi Wu
STUDENT NUMBER	WXXHUE001
QUALIFICATIONS	MBBCh (Wits)
TITLE OF PROPOSED PROJECT **(Proposal attached)**	The Wnt Signalling Pathway in Ewings sarcoma/PNET: An Immunohistochemical Investigation
DEPARTMENT	Anatomical Pathology
LEVEL OF PROJECT - Master's or Doctoral	Master's
PROPOSAL NOTED BY DIVISIONAL POST-GRADUATE REPRESENTATIVE	Postgraduate representative: Dr R. Bowen Signature:
PROPOSAL SUPPORTED BY DEPARTMENTAL RESEARCH COMMITTEE	Chair, Department Research Committee: Prof McIntosh Signature:
PROPOSAL APPROVED BY (Delete any one if not applicable) Human Ethics Committee, ERC No:	071/2006 *(Attach Ethics approval letter)*
FINAL SUBMISSION APPROVED BY SUPERVISOR	Supervisor: Professor D. Govender
FINAL SUBMISSION APPROVED BY HEAD OF DIVISION	Head of Division: Professor D. Govender

If ethics approval not required please explain in COMMENTS

COMMENTS:

4. OFFICIAL ETHICS APPROVAL LETTER



UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E53-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone (021) 406 6338 • Facsimile (021) 406 6411
e-mail: prezward@curac.uct.ac.za

15 February 2006

REC REF: 071/2006

Dr HT Wu
Anatomical Pathology

Dear Dr Wu

PROJECT TITLE: IMMUNOHISTOCHEMICAL STUDY ON WNT SIGNALLING PATHWAY IN EWINGS SARCOMA/PNET

Thank you for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study on the 09 February 2006.

Please quote the REC. REF in all your correspondence.

Yours sincerely

DR M. BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

lemjedi