THE RELATIONSHIP OF SURVIVIN EXPRESSION WITH TUMOUR GRADE IN GASTROENTERO-PANCREATIC NEUROENDOCRINE TUMOUR (GEPNET) -USM EXPERIENCE-

BY

DR HILMA HAZMI

PUM 0116/14

Dissertation Submitted In Partial Fulfilment Of The Requirements For

The Degree Of Master Of Pathology (Anatomical Pathology)

UNIVERSITI SAINS MALAYSIA

2018

ACKNOWLEDGEMENT

In the name of Allah, the Most Beneficial, the Most Merciful.

I would like to express my utmost gratitude to my supervisor, Associate Professor Dr Md Salzihan Md Salleh for his advice and guidance in the completion of this research project.

My appreciation also goes to Dr Iskandar Zulkarnain Alias for the kind approval of usage of their in house survivin.

I would also like to extend my thanks to En Rosli Jusoh for their skilful expertise in materializing this project.

Lastly, to my ever loving, supportive and understanding husband, Dr Haniff Abdullah and my loving trio of children, Hadif, Hadiyya and Hadeera.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRAK

Pengenalan dan Objektif : Kanser neuroendokrin berpunca dari sistem sel neuroendokrin yang berada di semua organ di dalam badan manusia dan mempunyai pelbagai simptom, lokasi asal dan tahap agresif yg berbeza. Kanser neuroendokrin yg berasal dari sistem pencernaan dan pankreas (GEPNET) semakin mendapat perhatian berikutan simptom yang tidak spesifik, kekeliruan dalam sistem penamaan, pengredan dan pengenalan klasifikasi WHO 2010. Survivin adalah "inhibitor apoptosis' yang secara khusus hanya diekspresi di dalam sel-sel kanser dan bukan di sel-sel biasa. Kajian ini direka untuk melihat karakter klinikal dan patologi GEPNET, insiden GEPNET di HUSM dan nilai prognosa survivin di dalam GEPNET.

Metodologi : Kajian ' cross sectional' ini dijalankan ke atas semua kes-kes GEPNET dari tahun 1998 hingga 2016 di Hospital Universiti Sains Malaysia. Parameter klinikal and patologikal dan kiraan mitosis di catat. Blok-blok tisu parafin ini kemudiannya dilabel secara immunohistokimia dengan survivin dan Ki67. Berdasarkan kiraan mitosis dan Ki67, kes-kes ini telah dibahagi mengikut gred menggunakan klasifikasi WHO 2010.

Keputusan : 20 kes telah dikenalpasti. Terdapat sedikit peningkatan insiden GEPNET di HUSM. Kesemua kes ini mengalami simptom sakit perut. Simptom tidak spesifik lain yang dialami adalah perubahan dalam pola penyahtinjaan dan pendarahan dari dubur. Lebih separuh mengalami kurang selera makan dan berat badan (n=16). Kebanyakan GEPNET berasal dari sistem pencernaan (n=18) dan hanya 10% (n=2) berasal dari pankreas. 65% (n=13) mengalami rebakan kanser ke organ lain. Hanya 40% daripada kes kes ini telah diklasifikasikan mengikut WHO 2010 semenjak pengenalan klasifikasi ini

dan serum ‘Chromogranin A’ telah diuji pada 35% kes-kes ini. Kaedah pemeriksaan yang paling kerap digunakan untuk mengdiagnosa GEPNET adalah imbasan CT(n=16) diikuti endoskopi (n=10) dan ultrasound (n=6). 60% kes kes di HUSM didapati tergolong dalam gred 3, 15% gred 2 dan 25% gred 1 mengikut klasifikasi WHO 2010. Terdapat hubungan yang signifikan antara rebakan sel-sel kanser dan gred mengikut WHO 2010. Tiada hubungan signifikan antara ekspresi survivin dan gred GEPNET.

Kesimpulan: Penyelidikan ini menunjukkan terdapat peningkatan insiden GEPNET di HUSM dengan simptom-simptom yang tidak khusus. Tiada hubungan signifikan antara ekspresi survivin dan Gred mengikut klassifikasi WHO 2010. Kekurangan pangkalan data dan kesedaran mengenai GEPNET merupakan halangan utama dalam diagnosa dan pengendalian kes-kes ini.

ABSTRACT

Background and objective : Neuroendocrine tumours originate from the peripheral neuroendocrine cell system dispersed in all organs and is heterogenous in its presentation, localization and aggressiveness. Those arising from the gastrointestinal tract and pancreas are of interest due to its increase in incidence. The introduction of WHO 2010 classification for these tumours abates confusion on its nomenclature which depicts malignant behaviour . Survivin is an apoptosis inhibitor which are specifically expressed in tumour cells and not in normal cells. In this study we determine the clinicopathological characteristics of gastroenteropancreatic neuroendocrine tumour (GEPNET), the incidence in HUSM and the prognostic value of survivin in this tumour entity.

Materials and methods: This cross-sectional study was carried out on all cases of GEPNET from 1998 to 2016 in Hospital Universiti Sains Malaysia (USM). Data on the clinicopathological characteristics and mitoses counts were noted. We performed immunohistochemical staining for survivin and Ki67 in paraffin fixed paraffin embedded blocks. Gradings according to WHO 2010 classification were done based on Ki67 proliferation index and mitoses.

Results: Twenty cases of GEPNET were included in this study. There is a slight increase in GEPNET incidence in HUSM. All GEPNET presented with abdominal pain. Other vague symptoms include altered bowel habit and blood per rectum. More than half had constitutional symptoms (n=16). Majority of GEPNET arises from the gastrointestinal (n=18) and only 10% from the pancreas (n=2). 65% (n=13) demonstrated metastases. Only 40% of the cases were graded according to WHO 2010 classification since its

introduction. 35% had serum Chromogranin A done. The commonest investigation modality is CT scan (n=16) followed by endoscopy (n=10) and ultrasound (n=6). According to the WHO 2010 classification, 60% of the GEPNET diagnosed were in Grade 3 , 15% in Grade 2 and 25% in Grade 1. There was a significant association between occurrence of metastases and gradings of GEPNET (p=0.001). There was no association between the expression of survivin and the grades in GEPNET.

Conclusion: These results indicate there is an increasing trend in GEPNET incidences with variable vague presentations. There was no association found between the expression of survivin and the grades in GEPNET according to the WHO classification. Lack of database and awareness of the GEPNET and its latest classification is still a major hindrance in the diagnosis and management of the disease.

CHAPTER ONE : INTRODUCTION

Neuroendocrine tumours, NETs are relatively rare and highly malignant neoplasm (Diakatou *et al.*). According to the US surveillance Epidemiology and End Result (SEER), the incidence of NET worldwide is 1 in 1000 malignancies. However in recent large scale studies done by Yao *et al.* (2013) and Ellis *et al.* (2010), it is believed that there was an increase of incidences reported compared to what was believed in previous studies. This increase in both studies are said to be contributed by improvement in diagnostic techniques and increased survival span.

The term "karzinoide" was introduced by Oberndorfer in 1907, where he described six cases of the small intestine (Klöppel, October 2007). This term which means carcinoma-like implied that these tumours are benign, thus creating a lot of confusion of the nature of the disease. However, over the years, more and more understandings were discovered on the endocrine nature of the disease (Klöppel, October 2007) and it is now agreed that this tumour has a malignant potential.

The tumour constitutes a heterogenous group of neoplasm that may arise in virtually every topographic localization in the body (Pasaoglu *et al.*, 2015). They originate from the peripheral neuroendocrine cell system dispersed in all organs (Emma Elizabeth Ilett, 2015). Those arising from the gastrointestinal and pancreatic system are collectively known as gastroenteropancreatic neuroendocrine tumour or GEPNET in short. More than a dozen neuroendocrine cell types have been described in the human (Hofsli, 2006) and they share common morphological, biochemical and ultrastructural features. The neuroendocrine cells are capable of producing a variety of hormones and neuropeptides (neurotransmitters and growth factors).

Patients with GEPNET present with wide variety of clinical presentation. GEPNET can be divided into the functioning and non-functioning. The non-functioning group are not associated with symptoms and signs of hormone secretion. The functioning unit, is described as such, because they exhibit symptoms which are associated with the hormones and neuropeptides produced (Uccella *et al.*, 2015). For example, insulin produced in neuroendocrine tumour in pancreas (Insulinoma) causes hypoglycaemia and gastrin produced in gastrinoma may lead to Zollinger- Ellison syndrome Similarly serotonin secretion by intestinal and more rarely pancreatic NET lead to carcinoid syndrome (Raphael MJ, 2017) and many more different hormones. Thus, the occurrences of this disease are either discovered incidentally or by the non-specific symptoms related to the production of these hormones. This situation poses difficulty for clinicians to establish the diagnosis of neuroendocrine tumour and instead they may arrive at a different diagnosis.

The diagnoses of neuroendocrine tumour requires an integrated and multidisciplinary approach which includes medical oncologist, surgeons, radiologist and pathologist (Raphael MJ, 2017). There are many modalities utilized for the diagnosis of GEPNET which encompasses clinical evaluations, radio imaging, biochemical tests, morphological and immunohistochemical markers in attempt to identify the origin of the neuroendocrine tumours.

Plasma Chromogranin A is a reliable diagnostic and prognostic biomarker (Massironi *et al.*, 2015). It shows high sensitivity and specificity rate among Asian population (Chou *et al.*, 2012). Excess serotonin levels in serotonin producing NET can be measured via 24-hour urine 5-HIAA that has been excreted (Özaslan *et al.*, 2014). Histological recognition also plays an important role in the diagnosis of GEPNET, which

includes morphological and immunohistochemical staining. Most neuroendocrine tumours show reactivity to either synaptophysin or chromogranin A immunohistochemical tissue marker regardless of its histological subtypes (Kimiloglu Sahan *et al.*, 2015). Diagnostic imaging modalities used for the diagnosis of GEPNET include standard CT or MRI and functional imaging, which exploit the overexpression of somatostatin receptor seen in neuroendocrine tumour. Radiolabelled somatostatin analogues injected in the blood stream can bind to the tumour cells to help localized the tumour (Raphael MJ, 2017).

The grading and classification has gone through tremendous changes over the decades. This is with the realization of the importance of these gradings in predicting prognosis and outcome (Lee *et al.*, 2014). In 2000, the World Health Organization introduced its classification of GEPNET by integrating the concepts of tumour heterogeneity, tumour differentiation and malignancy (Hamilton SR, 2000). However, current recommendation is based on an updated WHO classification which was upgraded in 2010 where there are usage of a more well defined and meticulous classification (Bosman, 2010). In this new classification, Ki67 proliferation index and mitosis counts are the key indicator in determining the grades of GEPNET. Ki67 has been suggested as one of the reliable and reproducible indicator of malignant behaviour in GEPNET tumour and has the ability to discriminate between a less aggressive and a more aggressive tumour (Khan *et al.*, 2013; Miller *et al.*, 2014; Salama *et al.*, 2014).

There is an increasing interest in the utility of survivin as a diagnostic and prognostic marker in GEPNET. Survivin is a protein belonging to the family of apoptosis inhibitor. It suppresses apoptosis and regulates cell division (Altieri, 2008).The expression of survivin is said to be tumour specific (Velculescu *et al.*, 1999). Many

studies also showed that survivin is a novel prognostic factor especially in well differentiated tumour. This study will investigate the expression of survivin in GEPNET.

CHAPTER TWO : LITERATURE REVIEW

2.1 CLINICOPATHOLOGICAL PRESENTATION

Neuroendocrine tumours have always been regarded as a rare malignancy. However, there are more and more studies done on neuroendocrine tumour and being the highlights of many medical discussions proving that neuroendocrine tumour is not as rare as thought before. A large study by (Yao *et al.*, 2008) showed a significant increase in incidence and prevalence. However, most of these studies were done among Caucasian population which may not reflect the real situation in Malaysia. To date, there has been only one study done in Malaysia, describing the clinicopathology, diagnoses and management of GEPNET done (Gunavathy, 2014)

Neuroendocrine tumour consists of a spectrum of malignancies that can arise from neuroendocrine cells that exist in any parts of the body. These tumours are most commonly found located in the gastrointestinal tract than in the pancreas (de Miguel Novoa *et al.*, 2014; Pasaoglu *et al.*, 2015). However, study done in Malaysia by Gunavathy (2014) showed that 67.2% of the primary site of tumour originated from the pancreas. Again, this highlights the importance of initiating a local registry as our clinicopathological pattern may differ from other regions.

There are as many as 15 types of neuroendocrine cells in the pancreas and gastrointestinal tracts and each are capable of producing peptides that causes certain hormonal syndrome (Rindi, 1999). These cells share a number of antigens with nerve elements, thus, the term ‘ neuroendocrine’ was adopted (Rindi, 2000). Among the peptides discovered are insulin, serotonin, gastrin, histamine and others in different locations and concentrations along the gastrointestinal tract and pancreas as shown in

Table 2.1 (Solcia and Vanoli, 2014). Those capable of producing the different hormonal symptoms are regarded as functioning type causing hyperfunctional syndromes or secretory symptoms such as hypoglycaemia, diabetes mellitus and acid hypersecretion and carcinoid syndrome as described by de Miguel Novoa *et al.* (2014). In such cases, the tumour itself is labelled according to its associated hyperfunctional syndromes such as insulinoma, gastrinoma etc (Rindi *et al.*, 1998). Brzozowska *et al.* (2009) also documented a rare case of hypercalcaemia from excessive secretion of parathyroid hormone-related protein (PTHrP) in a lady with advanced GEPNET who had evidence of osteoporosis on bone mineral density scan.

Carcinoid syndrome encompasses another different sets of syndrome which includes vasomotor symptoms such as flushing, hypertension or hypotension, gastrointestinal hypermotility such as diarrhoea, right sided cardiac involvement, bronchospasm, pelagra, hepatomegaly, proximal myopathy and others (Melnyk, June 1997) . These constellation of symptoms is due to the secretion of serotonin, tachykinins, prostaglandins, catecholamines and histamines to varying extent in which serotonin is very prominent (de Herder, 2007).

To make things more complicated, it is unclear as why some of these cells do not have the propensity to develop into tumour especially those involving the upper intestine cells like secretin and cholecystokinin, while tumours like gastrinoma which is expected to develop in the gastric can occur in the pancreas (Solcia and Vanoli, 2014). The unpredictable behaviour of these functioning neuroendocrine cells leads to variability in the presenting symptoms resulting in the late management of the patient.

However, not all cases of GEPNET produces hormones, these we regard as non-functioning. Patients with non-functioning GEPNET mostly manifest with abdominal

symptoms such as abdominal pain, diarrhoea, constipation, abdominal pain, GI bleeding, jaundice, vomiting and constitutional symptoms such as anorexia and weight loss (de Miguel Novoa *et al.*, 2014). Abdominal symptoms are more frequently found in GEPNET patients in comparison to hyperfunctional syndromes (Niederle and Niederle, 2011; Birnbaum *et al.*, 2014).

Table 2.1: Different peptides and its concentration along the gastrointestinal tracts and pancreas

Table 1 Gut endocrine cells: types, distribution, and function

Cell	Main product	Pa	Stomach		Intestine					
					Small				Large	
			Cor	An	Du	J	I	Ap	Col	R
D1[a]	Ghrelin	f	+	f	f	r				
EC	5HT	r	+	+	+	+	+	+	+	+
D	Somatostatin	+	+	+	+	f	f	f	f	f
L	GLI/PYY				+	+	+	+	+	+
A	Glucagon	+	a							
PP	PP	+			a					
B	Insulin	+								
ECL	Histamine		+							
G	Gastrin			+	+					
CCK	CCK				+	+				
S	Secretin				+	+				
GIP	GIP				+	+	r			
M	Motilin				+	+	r			
N	Neurotensin				+	+	+			

Data from references [1–4]

Pa pancreas, *Cor* corpus, *An* antrum, *Du* duodenum, *J* jejunum, *I* ileum, *Ap* appendix, *Col* colon, *f* few, *r* rare, *a* fetal tissue only

[a] Corresponding to X (rabbit, dog, cat) or A-like (rat, mouse) cells of other species

*Adapted from Solcia and Vanoli (2014)

Such vast array of clinical symptoms which can be very nonspecific lead to delayed presentation and detection. Approximately 70% of patients who were diagnosed with GEPNET were found to have lymph node involvement and distant metastases in a study conducted by (Niederle *et al.*, 2010). Delayed presentation, delayed diagnosis, inadequate hormonal, biochemical and histopathological examination and the lack of

multidisciplinary approach (Gunavathy, 2014) causes inefficiency in the management of these patient.

2.2 WHO CLASSIFICATION

The grading and classification of neuroendocrine tumour is imperative in predicting the prognosis and determining the treatment options. This classification allows for a more structured process in diagnosing and thus allowing the clinicians and pathologist to speak in similar language in terms of neuroendocrine tumour.

The grading system that we use currently has undergone a massive and significant evolution. The first attempt to classify GEPNET was made in 1963 by Williams and Sandler where they divided these tumours according to their embryological origins: the foregut, midgut and the hindgut. However, this classification was not useful in clinical practice because of the diverse clinical behaviour (Kloppel, 2011). In 1980s and 1990s, the term 'carcinoid' and its largely incorrect benign implication, was widely employed for these neuroendocrine tumours (Rindi *et al.*, 2010).

In 2000, WHO emerged with a more practical classification and accepted the nomenclature of GEPNET and allowed the prognostic stratification of these neoplasm (Pasaoglu *et al.*, 2015). GEPNETS were classified as well differentiated and poorly differentiated. The well differentiated category constitutes benign, uncertain behaviour and carcinoma. Tumour size, depth of invasion, functionality and metastases were considered in the WHO 2000 classification (Solcia, 2000; Heitz, 2004). Apart from the well differentiated and the poorly differentiated, another group known as mixed exocrine-endocrine carcinoma was also introduced. However, the scheme was not widely

acknowledged. The reasons being 1) the inclusion of stage related information in the grading system; 2) complicated clinical-pathological classification scheme; and 3) the category of 'uncertain behaviour'(Rindi *et al.,* 2010). Furthermore, the usage of the term 'carcinoid' and its incorrect benign impression impeded the universal acceptance of the WHO 2000 classification.

To avoid these confusions, WHO endorsed a new classification in 2010 and recommended the term neuroendocrine tumours to be used generically to indicate the expression of neural marker in neoplastic cells, regardless of the origin. This new classification acknowledges the malignant potential of neuroendocrine tumour and this knowledge is used to organize the classification (Rindi *et al.,* 2010). The new WHO 2010 classification of GEPNET retains the concept of well differentiated and poorly differentiated tumours. The well-differentiated neuroendocrine neoplasm were graded as Grade I (mitotic count<2/10HPF and/or Ki67 index ≤2%) and Grade II (mitotic count 2-20/10HPFand or Ki67 index 3%-20%) All poorly differentiated tumours were termed neuroendocrine carcinomas and were graded as Grade 3 (mitotic count >20/10hpf and or Ki67 index>20%) as illustrated in Table 2.2 (Rindi *et al.*, 2010). The diagnosis of mixed neuroendocrine carcinoma (MANEC) was used when adenomatous or neuroendocrine components were malignant and at least 30% of each component can be identified. This classification is generally based on the mitotic count and Ki67 proliferation index. The usage of stage related information and clinic-pathological association were omitted thus creating clear and straightforward classification. WHO 2010 classification is said to be simple, easy to use and a repeatable classification (Pasaoglu *et al.*, 2015).

In view of the eminent qualities of the WHO 2010 GEPNET classification, it would be substantial if everyone could employ this classification in their daily practices.

This research uses the WHO 2010 GEPNET classification as it is the latest and only classification available.

Table 2.2 : WHO 2010 Gastroenteropancreatic Neuroendocrine tumour classification

	GRADE	MITOTIC COUNT	Ki-67 index
Neuroendocrine tumour /carcinoid	G1	<2	≤ 2%
Neuroendocrine tumour	G2	2-20	3-20%
Neuroendocrine carcinoma	G3	>20	>20%
MANEC (mixed adenoneuroendocrine carcinoma)	When adenomatous or neuroendocrine components were malignant and at least 30% of each component could be identified		

*Adapted from (Rindi *et al.*, 2010)

Ki67 was first identified in 1991. It is a protein antigen of 345 and 395 kDa found on the long arm of chromosome 10 in which it's expression is tightly related to the cell cycle (Gerdes *et al.*, 1983; Gerdes *et al.*, 1984; Gerdes *et al.*, 1991). More precisely, Ki67 was said to be expressed in the nucleus of proliferating G1 phase in the cell cycle, increases during the subsequent phases of the cell cycle and rapidly diminishes after mitosis (Gerdes *et al.*, 1984; Bruno and Darzynkiewicz, 1992) and absent in the dormant cells (Singh *et al.*, 2014).This knowledge of this special characteristic can be utilize to employ Ki67 as a marker of cell proliferation in histological material (Scott *et al.*, 1991) This protein antigen may also provide prognostic information in a range of other tumours as seen in lymphoma by Hall *et al.* (1988) and in breast cancer demonstrated by Bouzubar

et al. (1989). Ki67 was believed to be a good predictor of metastases and recurrence and was proven in many studies to be correlated with higher tumour grades as suggested by WHO 2010 (Erler *et al.*, 2011).

In GEPNET, Ki67 has a very significant prognostic value (Foltyn *et al.*, 2012) and was proven to be a reliable and reproducible marker by Salama *et al.* (2014)

2.3 SURVIVIN

2.3.1 Introduction to survivin

Predicting disease outcome is very important in understanding the natural history of tumours in general and specifically in GEPNET, planning treatment strategies and devising follow up plans. Many other prognostic markers are now being studied with the aim to discover a highly specific and sensitive marker to feed this purpose, apart from above mentioned Ki67. Studies done by Alexander *et al.* (2014) explored the usage of OCT 4 marker in neuroendocrine tumours in various sites and by Brunner *et al.* (2015) who experimented on E-cadherin, β-catenin, cyclin D1 and IL-17A. One potential marker exhibiting increasing interest in the diagnostic and prognostication in GEPNET is SURVIVIN.

Survivin in a protein belonging to the family of inhibitor of apoptosis (IAP) family of molecules. XIAP, NAIP, c-IAP1,c-IAP2, livin , ILPP2 and BRUCE are all the other members in this family (Deveraux and Reed, 1999; Cheung *et al.*, 2006).

Apoptosis, is a physiological cell suicide programme while avoiding inflammation and damage to the surrounding tissue. This is a crucial entity in the development and maintenance of a healthy and normal tissue. Dysregulation or

malfunction of this programme can lead to initiation of cancer, autoimmune and immunodeficiency disease, reperfusion injury after ischaemic episodes and in neurodegenerative disorders (Deveraux and Reed, 1999).

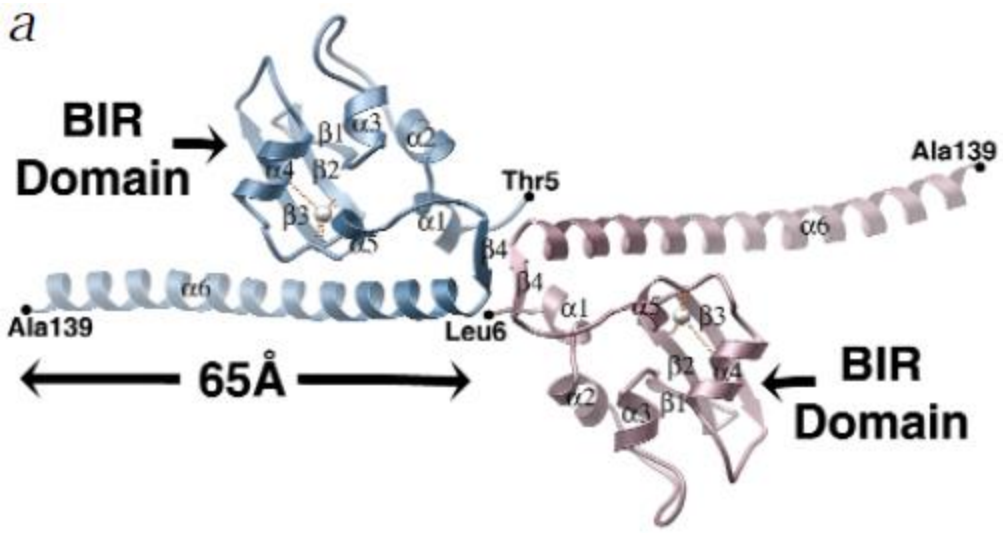
2.3.2 Structure of survivin

IAP was first discovered in baculoviruses, and was shown to be involved in suppressing cell death response to viral infection (Deveraux and Reed, 1999). IAP family protein are defined by a domain of 70 amino acids called baculovirus IAP repeat (BIR).Up to three copies of this BIR domain can be present in IAP. Other sequences that can be found in certain IAP includes RING finger domain which is defined by 7 cystein and 1 histidine residues, a caspase recruitment domain (CARD), an ubiquitin conjugative motif and a nucleotide P-loop sequence (Deveraux and Reed, 1999)

Survivin is the smallest protein in the IAP group, consisting of 142 amino acid residues and a molecular mass of 16.5 kDa. It possesses a single BIR domain, and instead of having a RING finger domain, it is replaced by a long amphipathic alpha-helical region as shown in Figure 2.1 (Verdecia, 2000). It suppresses apoptosis and regulates cell division (Altieri, 2008).

X-ray crystallography revealed the structure of human survivin, in which there are presence of an amino-terminal globular zinc finger domain (which includes the BIR motif) and a long carboxy-terminal helix separated by a short linked segment, important for dimerization (Rodríguez *et al.*, 2002) . This structure is closely related to its function as an apoptotic inhibitor. The amino terminal portion consists of 3 alpha helices and three beta sheets, which closely resembles the BIR domain that is conserved in the IAP family, which are crucially involved in their function in inhibiting apoptosis.

Figure 2.1 : Survivin structure



*Adapted fromVerdecia (2000)

2.3.3 Biological function

How does survivin inhibit apoptosis? In order to maintain a healthy tissue, there must be a balanced homeostasis between proliferation and cell death. The disruption of this balance by oncogenes are kept in check as strong proliferation stimulus also lead to the production of death signal. Caspases are a family of endoproteases. The activation of this enzymes in unfavourable cell environment can result in substrate inactivation and generate active signalling molecule to participate in apoptotic and inflammatory processes in maintaining the normal homeostasis.

Guo and Hay (1999) suggested that caspase activity occurs during each cell cycle and survivin acts to halt this activity. Alternatively, it was also suggested that survivin and caspase activity work hand in hand as part of the G2/M checkpoint. In such model,

survivin associated with microtubule is needed to block caspase activity during G2/M, in which disturbances to the microtubule will lead to a malfunction survivin and increase in death promoting activity. This finding was also similar to a study done by (Li and Altieri, 1999) that shows that survivin is upregulated in G2/M and that it is associated with spindle microtubule and seems to require this association for antiapoptotic activity.

Another implication of survivin apart from inhibition of apoptosis is its ability to enhance angiogenesis. Evidences linking survivin with angiogenesis include study done by 1) O'Connor *et al.* (2000) who demonstrated that survivin was upregulated in angiogenically stimulated endothelial cells compared to quiescent cells; 2) Tran *et al.* (2002), survivin expression was increased in cultures vascular endothelial cells following exposure to angiogenic factors such as VEGF and basic FGF; 3) Conway *et al.* (2003) found that following middle cerebral artery occlusion, mice with heterogenous deficiency of the survivin gene exhibited decreased blood vessels density. The mechanism by which survivin enhances angiogenesis and its ability to inhibit apoptosis will result in enhancement of cell survival.

2.3.4 Survivin and Cancer

IAP proteins have received a lot of attention in the last two decades, specifically in their ability to suppress apoptosis involving direct caspase inhibition. The identification of survivin as a structurally unique IAP protein and their role in cancer was brought to highlight with the fact that this protein is expressed during fetal development and in human cancers but not in normal adult tissues in vivo (Ambosini, 1997). Early work using SAGE (Serial analysis of gene expression) ranked survivin as the fourth most highly expressed transcript in a number of common cancers but rarely expressed in

normal tissue (Velculescu *et al.*, 1999). This unique entity of being expressed at high levels during fetal development, but rarely in normal healthy adult tissue, makes survivin very attractive target for tumour marker, prognostic marker and an anti cancer therapy target.

Multiple studies have been done to attest the potential of survivin as a tumour marker. Multiple studies were done to show that the expression of survivin was seen to be a good diagnostic potential in bladder tumour (Shariat *et al.*, 2004; Schultz *et al.*, 2006) where they were found positive in new and recurrent bladder cancer but not detected in patients who had undergone treatment and had negative cystoscopy findings (Smith *et al.*, 2001). A significant positive correlation was found between survivin and colorectal cancer, with emphasize on survivin levels and microvessel density, proving its involvement in tumour through angiogenesis (Kawasaki *et al.*, 2001).

Prognostic markers predict patient outcome and does have a significant influence in cancer management. Traditional prognostic factors for cancer include parameters such as tumour size, tumour grade and local or distant lymph nodes metastases. The ability of survivin to inhibit apoptosis and enhance angiogenesis makes it a good prospect as a prognostic marker. Because survivin were known to be involved in these processes, it is highly likely to be involved in tumour progression and consequently in leading to a higher level in aggressive tumours. This fact was repeatedly seen in breast cancer studies done by Span *et al.* (2004) and Ryan *et al.* (2006).

The association of survivin as a diagnostic and prognostic marker and GEPNET has also drawn an increasing interest. However, in comparison to other types of cancers, there were limited studies done to correlate both survivin and GEPNET. One study by Vikman (2005) exhibited that genes for survivin showed more restricted expression in

normal tissue but were found to be robustly expressed in midgut neuroendocrine tumour. Another study by Patricia Grabowski (2005) showed that nuclear survivin is a powerful prognostic marker in GEPNET.

Survivin is upregulated at G2/M phase of the cell cycle and downregulated at the G1 phase (Li *et al.*, 2005) , making it more specific in comparison to Ki67 which is expressed in G1, S, G2 and M phases but not in G0 phase. Ki67 antigen is expressed in every phase of the cell cycle and if the cell cycle becomes prolonged by G1 or S arrest, more mitoses may be visible without necessarily reflecting an accelerated growth rate. It is also an emerging area of interest where there is a possibility of using this gene product as target antigen for anti-cancer therapy (Ryan *et al.*, 2009) and T cell mediated therapy (Vikman, 2005). Because of the lack in studies correlating survivin and GEPNET and that survivin appears to be more specific than Ki67, we would like to explore more of the expression of survivin in our study.

CHAPTER THREE : AIMS AND OBJECTIVES

3.1 GENERAL OBJECTIVES

To observe the prevalence of Gastroentero-pancreatic Neuroendocrine Tumour (GEPNET) in HUSM in accordance to WHO 2010 classification with the added use of survivin as a prognostic tool

3.2 SPECIFIC OBJECTIVES

- To identify the prevalence of Gastroentero-pancreatic Neuroendocrine Tumour (GEPNET) in HUSM
- To reclassify all cases diagnosed as Gastroentero-pancreatic Neuroendocrine Tumour (GEPNET) under the WHO 2010 classification
- To determine the clinicopathological characteristics of Gastroentero-pancreatic Neuroendocrine Tumour (GEPNET) in HUSM
- To determine the association of prognosis (Grade) with expression of survivin in Gastroentero-pancreatic Neuroendocrine Tumour (GEPNET)

3.3 RESEARCH QUESTION

- What is the prevalence of Gastroentero-pancreatic Neuroendocrine Tumour (GEPNET) in HUSM?
- What are the clinicopathological presentations among those diagnosed with Gastroentero-pancreatic Neuroendocrine Tumour (GEP-NET)?
- Is survivin being expressed in Gastroentero-pancreatic Neuroendocrine Tumour (GEPNET) and does it have a role in terms of prognostic value in Gastroentero-pancreatic Neuroendocrine Tumour (GEPNET)?

3.4 RESEARCH HYPOTHESES

- H^0 – There is no relationship between survivin expression with tumour grade

CHAPTER FOUR : METHODOLOGY

4.1 STUDY DESIGN AND SAMPLES

This study was a cross sectional (retrospective) study. All samples were retrieved from the archived paraffin-embedded tissue blocks diagnosed by independent pathologists in the Department of Pathology, Hospital Universiti Sains Malaysia (USM) between 1998 to 2016. Selection were based on the confirmed diagnosis of neuroendocrine tumour or carcinoid of the gastroenteropancreatic region based on histopathological findings. The inclusion of the terminology ‘carcinoid” was used as the term was still widely utilized in previous years. All cases were diagnosed based on morphology and available immunohistochemical markers that were available at that time. The demographic data and histopathological examination reports were obtained from the archived LIS (Lab Information System) and PATHORS system of Hospital Universiti Sains Malaysia, Kelantan.

4.2 SAMPLE SIZE

Sample size for this study was calculated to fulfil the following objectives.

OBJECTIVE 1

To observe the prevalence of GEPNET in HUSM

- SINGLE PROPORTION FORMULA
    - $n = (z/\Delta)^2 p (1-p)$
    - n= sample size z= z distribution = 1.96 Δ= precision (0.005)
    - P= 0.00035 prevalence of GEPNET based on previous study is 35/100000 (Yao *et al.*, 2008)
    - Absolute precision (Δ) = ±0.005 (0.5%)
    - $n = (1.96 / 0.005)^2 (0.00035) (1- 0.00035)$

      $= 53$

Sample required according to calculation : 53 patients

OBJECTIVE 2 AND 3

To reclassify all cases diagnosed as GEPNET under the WHO 2010 classification

To identify the clinicopathological characteristics of GEP NET in HUSM

- SINGLE PROPORTION FORMULA (Gender-male)
    - $N=(z/\Delta)^2 p(1-p)$
    - n= sample size z= z distribution = 1.96 Δ= precision (0.15)
    - P= 0.47 prevalence of male in GEPNET based on previous study is 47% (Gunavathy, 2014)
    - Absolute precision (Δ) = ±0.15 (15%)
    - $n = (1.96 / 0.15)^2 (0.47)(1- 0.47)$

      $=42$

  Sample required according to calculation: 42 patients

- SINGLE PROPORTION FORMULA (Ethnicity)
    - $N=(z/\Delta)^2 p(1-p)$
    - n= sample size z= z distribution = 1.96 Δ= precision (0.15)
    - P= 0.39 prevalence of Malay in GEPNET based on previous study is 39% (Gunavathy, 2014)
    - Absolute precision (Δ) = ±0.15 (15%)
    - $n = (1.96 / 0.15)^2 (0.39)(1- 0.39)$

      $=40$

  Sample required according to calculation: 40 patients

- SINGLE PROPORTION FORMULA (Metastases)
    - $N=(z/\Delta)^2 p(1-p)$
    - n= sample size z= z distribution = 1.96 Δ= precision (0.15)
    - P= 0.70 prevalence of metastases in GEPNET based on previous study is 70% (Singh *et al.*, 2014 & Law, 2014)
    - Absolute precision (Δ) = ±0.15 (15%)
    - $n = (1.96 / 0.15)^2 (0.70)(1- 0.70)$

      $=35$

Sample required according to calculation: 35 patients

OBJECTIVE 4

To determine the association of expression of survivin with prognosis (grade) in GEPNET

PS software (2 proportion formula)(Dupont and Plummer, 1997)

- α= 0.05, Power = 0.8, p0 =? , p1 =? , m = 1
- P0= 0.75 (proportion of patient with poorer grade who has negative survivin expression) (Li *et al.*, 2005 Javle, & Tan, 2005)
- P1= 0.25 (proportion of patient with poorer grade who has positive survivin expression)
- Sample size = 12
- Sample size + 10% dropout= 14

4.3 INCLUSION CRITERIA

All patients diagnosed with gastroentero-pancreatic neuroendocrine tumour (GEPNET) or carcinoid of the gastroentero-pancreatic region based on histopathological and immunohistochemical marker findings.

4.4 EXCLUSION CRITERIA

Cases that fulfilled the inclusion criteria but were found to have missing corresponding blocks were excluded from the study. Other exclusion criteria include inadequate tumour tissue for serial section. Cases other than gastroenteropancreatic neuroendocrine or carcinoid tumour were also omitted.

4.5 SAMPLING METHOD

Cases from 1998 to 2016 that fulfilled the inclusion criteria were retrieved form archived LIS (Lab Information System) and PATHORS system of Hospital Universiti Sains Malaysia, Kelantan. Corresponding tissue blocks were then obtained from the tissue archive and cases were then excluded from the study based on the exclusion criteria. Sampling method cannot be applied in this study due to the limited number of cases.

4.6 TISSUE PROCESSING AND IMMUNOHISTOCHEMICAL STAINING.

4.6.1 Primary Antibodies

Primary antibodies are antibodies that are raised against an antigenic target of interest and are typically unconjugated (unlabelled). Primary antibodies that recognize and bind with high affinity and specificity to unique epitopes are available as high specificity monoclonal antibodies and/or polyclonal antibodies. This antibody can be very useful for the detection of biomarkers for diseases. Primary antibodies used in this study are as below:

a) Ki67

Ki 67 primary antibody that was used in this study is clone MIB-a, manufactured and marketed by Dako Denmark A/S. This antibody is a monoclonal antibody that is derived from a mouse. Human tonsil was used as positive control.

b) Survivin (Al-Joudi, 2004)

Survivin primary antibody was obtained and prepared in the research laboratory at the Chemical Pathology Department, Universiti Sains Malaysia. This was done by hyperimmunisation of rabbits with oligopeptides. These oligopeptides contain sequences that represent the C- and N-termini of the survivin amino acid sequence. The produced antibodies were tested for specificity by preabsorption test in competition ELISA and immunoblotting. These sera containing antibodies were tested and validated using colon cancer tissue and breast cancer tissue.