An Immunohistochemical and Molecular Study of Gastrointestinal Stromal Tumours

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Summary
Gastrointestinal stromal tumour (GIST) is a rare but most common mesenchymal tumour in the gastrointestinal tract. Although GIST research has been carried out extensively worldwide, it has yet to be studied in Malaysia. To establish the immunohistochemical expression pattern of CD117 (c-KIT), CD34, S-100 and Desmin, the incidence of c-KIT and PDGFRα gene mutation in GISTs, and correlate it with clinicopathological parameters, eleven clinically diagnosed GISTs were stained for CD117, CD34, Desmin and S-100 protein by immunohistochemical technique, and c-KIT and PDGFRα gene mutations were studied by PCR-CGSE-DNA sequencing method. All GISTs (7 cases) stain positive for CD117, and co-expressed CD34. None of these cases express Desmin, and only one expressed S-100 protein focally. Fifty-seven percent (4/7) of GIST harboured mutations at exon 11 of c-KIT gene, and they were all high-risk and malignant cases. No mutation was detected at exons 9, 13 and 17 of KIT gene, and exons 12 and 18 of PDGFRα gene. Immunohistochemistry using a panel of antibodies shows consistent pattern of CD117 and CD34 expression in GIST, and mutational study may be a useful prognostic marker for kinase inhibitor treatment of GIST.

Key Words: Gastrointestinal stromal tumours, Immunohistochemistry, c-KIT, PDGFRα

Introduction
Gastrointestinal Stromal Tumour (GIST) is the most common mesenchymal tumour that occurs in the gastrointestinal tract. In the 1940s, GISTs were thought to be smooth muscle tumours. However, with the advent of immunohistochemistry in early 1980s, many of these lesions were found lacking immunophenotypic features of smooth muscle tumour cells. Recently, c-KIT mutation and c-KIT immunoreactivity in these spindle cell tumours revealed their origin from CD34-positive stem cells that differentiated towards the interstitial cells of Cajal (ICC). ICC expresses c-KIT and the c-KIT stem cell factor interaction is pivotal in ICC maturation. Since KIT expression is frequent in GISTs, it is now used as a marker for GIST.

C-KIT is a type III transmembrane tyrosine kinase receptor. The c-KIT receptor and its cognate ligand play a critical role in hematopoiesis, melanogenesis and gametogenesis by involving in different signaling pathways such as the Ras/Raf pathway, the JAK/STAT pathway and the phosphotidylinositol 3-kinase (PI-3 kinase)/Akt pathway, through its kinase domains. The activity and expression of KIT is tightly regulated in normal cells, but it is deregulated in cancer cells. Oncogenic activation and overexpression of KIT protein is closely related to mutations in KIT, the proto-oncogene.

C-KIT is the cellular homologue of the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene, and is located in the long arm of chromosome 4. There are
several types of c-KIT mutations being identified in the past, with mutation at exon 11 (associated with the juxtamembrane domain of receptor) being the most common type in GIST. Mutations at exon 9 (the extracellular domain), exon 13 (the kinase I domain) and exon 17 (activation loop) were also identified. The oncogenic activation of c-KIT protein in GIST is due to structural changes in the c-KIT proteins that make it favour receptor oligomerization and cross-phosphorylation (activation) even in the absence of its ligand.

Recently, mutations of an alternative oncogene, the platelet-derived growth factor receptor alpha (PDGFRA), was also observed in GIST. PDGFRA is also a type III transmembrane tyrosine kinase receptor. Mutations in exon 12 (the juxtamembrane domain) and 18 (tyrosine kinase domain) of PDGFRA are more common. Mutations of KIT and PDGFRA had been identified to be mutually exclusive. There was a study showing that GISTs with PDGFRA mutations have a better prognosis than those with c-KIT mutations.

Investigation on kinase expression and kinase gene mutation is important as c-KIT and PDGFRA are speculated to be a therapeutic target of imatinib, which is a biological inhibitor of kinases. GIST patients with c-KIT mutation at exon 9 and exon 11 are equally sensitive to imatinib, while those with mutations of other exons acquire resistance to imatinib. Different responses to imatinib treatment are also seen in patients with different mutant isoforms of PDGFRA.

Conventional histopathological assessments such as tumour size, mitotic index, histological subtype and immunohistochemical staining with a panel of antibodies have been used to confirm GIST and determine its malignant status. In the recent years, advancement in genetics has enabled the mutational study on GISTs, and as a consequence, higher accuracy of diagnosis and prognosis of GISTs can be achieved. The aim of this study is to establish and correlate the genetic alteration patterns of c-KIT and PDGFRA, and the expression pattern of CD117 (c-KIT), CD34, S-100 and Desmin in GIST.

Materials and Methods

Samples
Formalin-fixed, paraffin-embedded tissue blocks of clinically diagnosed GIST cases from year 2000 to 2004 were collected from the archive. Serial sections from the blocks of these cases were stained with hematoxylin-eosin and a panel of antibodies with immunohistochemical method. Pathological risk factor was recorded as very low risk, low risk, intermediate risk and high risk according to Fletcher classification, and GIST was histologically classified as epithelioid, spindle and mixed type (contain more than 20% of another type).

Immunohistochemical Analysis
Three μm thick sections were quenched in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity and subjected to microwave treatment to retrieve antigen for the staining procedure for CD117 (c-KIT), CD34 and Desmin. No antigen retrieval is needed for S-100. After conditioning in Tris-buffered solution (TBS, 8 g/L NaCl, 0.6 g/L Tris-base, 3.8 N HCl, pH 7.6), the sections were incubated with respective primary antibody: anti-c-KIT (CD117) (1:50; DakoCytomation, USA), anti-CD34 (1:50, QBEnd-10; DakoCytomation, USA), anti-Desmin (1:50, D33; Dako A/S, Denmark) and S-100 (1:1000, Dako A/S, Denmark). After incubation, the sections were first washed in TBS, and then immersed in ChemMate i (HRP, Rabbit/Mouse) solution, followed by visualization with Diaminobenzidine of ChemMate DAKO EnVision detection kit (DakoCytomation, Denmark) according to the manufacturer's instruction. The sections were slightly counterstained in Mayer's hematoxylin, rinsed with water, dehydrated in a series of alcohol and xylene and mounted. Positive expression of CD117 was used as the confirmative criterion for GIST. Intestinal cells of Cajal, endothelial cells of blood vessel and smooth muscle of intestine served as internal controls for CD117, CD34 and Desmin respectively.

DNA Extraction
The genomic DNA was extracted from formalin-fixed paraffin-embedded tissue blocks. Briefly, pieces of 5μm-thick sections were cut using sterilised microtome (Leica RM2135, Germany) and placed into 1.5 mL microcentrifuge tubes. The sections were deparaffinised two times with xylene, and washed twice with absolute ethanol. The tissue was then dried in the air for 30 minutes before subjecting to digestion. Proteinsase K digestion in 1X PCR buffer (200μg/mL) was carried out at 55°C overnight, and then inactivated at 95°C for 10 minutes. Any remaining tissue debris was eliminated by centrifugation at 12000 rpm for 10 minutes, and the supernatant was used directly for polymerase chain reaction (PCR).
Polymerase Chain Reaction
Exon 9, 11, 13 and 17 of c-KIT gene and exon 12 and 18 of PDGFRA gene were amplified by PCR, using the oligonucleotide primer pairs listed in Table I. The 25μL PCR reaction mixture contains 1X PCR buffer, 5 mM MgCl₂, 0.2 mM dNTPs mix, 1U HotStarTaq polymerase (Qiagen GmbH, Hildon, Germany), 1.5 μM each of forward and reverse primers and 1% DNA template. Cycling conditions for the PCR were as follow: 95°C for 15 minutes; followed by 40 cycles of: 95°C for 30 seconds, annealing temperature (52°C - 57°C, depending on the primers shown in Table I) for 30 seconds and 72°C for 30 seconds; and final extension at 72°C for 4 minutes. The PCR products were then kept at 4°C.

Conformation Sensitive Gel Electrophoresis
Mutational screening by Conformation Sensitive Gel Electrophoresis (CSGE) was performed according to Ganguly et al.29 and Korkko et al.30 with minor modifications. Briefly, 20 μl PCR product was mixed with EDTA to a final concentration of 10 mM, heated at 95°C for five minutes and incubated at 65°C for one hour to generate heteroduplex (if there is mutation). Samples were then mixed with same volume of loading dye (40% w/v sucrose, 0.25% xylene-glycol and 0.25% bromophenol blue) and electrophoresed on a 10% polyacylamide (29:1 acrylamide to bisacrylamide) with 1X TBE running buffer (8.8 mM Tris, 2.9 mM Taurine, 0.02 mM EDTA, pH 9.0) at constant 800 V, 45W for four hours, by using model S2 Sequencing gel electrophoresis apparatus (Life Technologies Inc., Gaithersburg, USA.). The gel was stained with SYBR Gold (Molecular Probe, Invitrogen, USA) and visualized in a gel documentation system (UVP).

DNA Sequencing
Samples with heteroduplexes observed in CSGE were subjected to automated sequencing. The PCR product was first purified with QIAquick PCR purification kit (Qiagen Inc, Valencia, CA) and sequencing of purified PCR products was performed using ABI PRISM BigDye Terminator Cycle Sequencing (Perkin Elmer, Foster City, CA, USA) in ABI 310 Genetic Analyzer (Applied Biosystem, Perkin Elmer, Foster City, CA, USA.). All mutations were confirmed with at least two times of independent sequencing reactions.

Results
Sample Data
From a total of 11 clinically-diagnosed GISTs, three cases were incisional biopsies and the remaining were excised tumors. The ages of patients at the time of diagnosis ranged from 25 to 76 years (mean = 57 years). There were six males and six females; five Chinese, three Malay and three Indian.

Immunohistochemical Expression Profile
Out of these 11 cases, 7 were confirmed as GIST, 3 were non-GIST, and 1 remained indeterminate (tissue was non-reactive to immunohistochemical stains). All GIST cases co-expressed CD117 and CD34 (Figures 1d, 1e), and there were 5 spindle, 1 epithelioid, and 1 mixed histology types (Table II and Figures 1a, 1b, 1c). The CD117 staining was strong, and was localized in cytoplasm and membrane (Figure 1d). Only one case (Case 7) expressed S-100 protein focally, and none of them expressed Desmin. All the non-GIST cases did not express CD117 and CD34, but had either strong Desmin or S-100 expression. They were re-diagnosed as inflammatory pseudo-tumor (Case 9), smooth muscle tumor (Case 10) and peripheral nerve sheath tumor (Case 11). All non-GIST cases were excluded from further study except case 9, which was used as negative control.

Clinicopathologic Features of GISTs
Among the confirmed GIST patients, four were females and three were males. Three of them were Chinese, two were Malay, and two were Indian. The mean age of patients at the age of diagnosis was 60 years (range: 29-76 years). The majority of them aged above 50 years (6/7), presented in the stomach (5/7), and spindle morphology was more common (5/7). Four out of the seven cases were high risk-GIST based on Fletcher classification.

Evaluation of Mutations in c-KIT and PDGFRA Genes
Mutation screening of c-KIT and PDGFRA genes were carried out on all confirmed GIST cases, the indeterminate (Case 8) and a non-GIST (Case 9) using PCR-CSGE-DNA sequencing method. A total of 54 PCR products were screened for the presence of heteroduplex in CSGE. Four heteroduplexes were observed for exon 11 of KIT and no heteroduplex was observed for other exons. DNA sequencing revealed four different types of mutation for the four heteroduplexes (Table III). Case 1 has a 9 base-pair deletion (75685_75693GAAGGTTGT) resulting in deletion of 3 amino acid residues, W557 to V560, and an insertion of Cysteine residue (W557-V560delinsC). Case 3 has a 3 base-pair in-frame deletion (75749_75751delGAT), resulting in a deletion of aspartic acid residue (D579 del). Case 5 has a 6 base-
pair deletion (75688_75693delGGTIGT), resulting in deletion of 3 amino acid residues, K558 to V560, and an insertion of Asparagine residue (K558_V560delinsN). Case 6 has a 52 base-pair deletion and a single T insertion (75679_75730delinsT), which results in a deletion of 17 amino acid residues, Q556 to D572 (Q556_D572del). Deletion is the common mutation among the four cases, and all of them had in-frame deletion in amino acid level.

Discussion

Molecular and immunohistochemical studies on GISTs had been extensively carried out in United States, Japan, China and many European countries. However, the study of this disease has not been carried out in Malaysia. Early studies aimed to characterize GIST and to distinguish it from smooth muscle tumours. Later, it proceeded to the assessment of prognostic criteria. As imatinib mesylate and other kinase inhibitors were proposed to be used as adjuvant therapy for GISTs that harbor c-KIT gene mutation, the studies of this tumour has shifted from prediction of malignancy to proper selection of patient for adjuvant chemotherapy.

There were only 11 cases of GISTs reported in University of Malaya Medical Centre from year 2000 to 2004, which indicates that GIST is an uncommon disease. After re-assessment by pathologist using standard criteria13, only seven of them are confirmed GISTs, and one of them remain indeterminate. All GISTs show strong expression of c-KIT protein regardless of tumour site, histological subtype, and benign or malignant, which is in agreement with previous study15. Most of the GISTs (86%, 6/7) examined co-expressed CD34, consistent with previous reports14,15,19. None of the cases expressed Desmin, and only one of them expressed S-100 protein focally, indicating that Desmin and S-100 expression are uncommon in GISTs, in accordance with other reports14,15,28.

Similar to previous studies13,34, our small series of cases showed 57% (4/7) of GIST cases harbored c-KIT gene mutation at exon 11. The reported mutation frequencies for c-KIT gene in GISTs range from 15-80%14,15,27,35,36 and the wide differences are believed to be mainly due to the proportion of malignant and high-risk cases in the studies. Our study showed that all GISTs that harbour mutations in exon 11 of c-KIT gene are high risk GISTs and malignant cases by histomorphological criteria, while none of the non-GIST or low-risk GISTs have mutation.

Previous studies show that codon 550 to 560 of c-KIT gene is the mutation hot spot for GISTs14,33,35. Two out of 4 mutations (Case 1 and 5) detected in our study were located at this hot spot. There is one mutation (Case 6), which starts at the hot spot and extends to codon 573. This mutation involved a large deletion (52bp) and a single T insertion, leading to the loss of 17 amino acids residues. We also found a GAT deletion

<table>
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<tr>
<th>Primer (5' -&gt; 3')</th>
<th>Annealing temperature (°C)</th>
<th>Fragment Size (bp)</th>
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<tr>
<td><strong>c-KIT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 TCCTAGAGTAAGCCAGGGGTCT</td>
<td>55</td>
<td>284</td>
</tr>
<tr>
<td>11 CTGAGACAATAATATTTTAAAGGTGATA</td>
<td>52</td>
<td>227</td>
</tr>
<tr>
<td>13 ACTGCATGCGCCTGGACATCGTGGTGT</td>
<td>52</td>
<td>203</td>
</tr>
<tr>
<td>17 ACAAGTTAAATGAATTTAATTGGT</td>
<td>52</td>
<td>224</td>
</tr>
<tr>
<td><strong>PDGFRA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 TCCAGTCTCTGTCGTGCTTC</td>
<td>65</td>
<td>260</td>
</tr>
<tr>
<td>18 ACCATGGATGACGCCAGTCTTG</td>
<td>56</td>
<td>250</td>
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Table I: Oligonucleotide Primers for c-KIT and PDGFRA genes

Abbreviation: PDGFRA, platelet-derived growth factor receptor alpha.
### Table II: Clinicopathological data of GISTS

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<thead>
<tr>
<th>No</th>
<th>Race</th>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
<th>Mitotic count</th>
<th>Tumour size (cm)</th>
<th>Risk Factor</th>
<th>Malignancy</th>
<th>CD117</th>
<th>CD34</th>
<th>Desmin</th>
<th>S-100</th>
<th>Histology</th>
<th>Diagnosis</th>
<th>Mutation</th>
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<tbody>
<tr>
<td>1</td>
<td>C</td>
<td>52</td>
<td>F</td>
<td>S</td>
<td>13/50</td>
<td>14 x 12 x 10</td>
<td>H</td>
<td>M</td>
<td>2+</td>
<td>3+</td>
<td>-</td>
<td>-</td>
<td>Mixed</td>
<td>GIST</td>
<td>9 bp del</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>61</td>
<td>M</td>
<td>S</td>
<td>4/50</td>
<td>4 x 3</td>
<td>L</td>
<td>B</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Spindle</td>
<td>GIST</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>67</td>
<td>F</td>
<td>S</td>
<td>9/50</td>
<td>10 x 6 x 2</td>
<td>H</td>
<td>M</td>
<td>3+</td>
<td>2-3+</td>
<td>-</td>
<td>-</td>
<td>Spindle</td>
<td>GIST</td>
<td>3 bp del</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>76</td>
<td>F</td>
<td>S</td>
<td>&lt;5/50</td>
<td>8cm (diameter)</td>
<td>I</td>
<td>B</td>
<td>2+</td>
<td>2-3+</td>
<td>-</td>
<td>-</td>
<td>Epithelioid</td>
<td>GIST</td>
<td>-</td>
</tr>
<tr>
<td>5A</td>
<td>I</td>
<td>66</td>
<td>M</td>
<td>R</td>
<td>44/50</td>
<td>3 x 3 x 3*</td>
<td>H</td>
<td>M</td>
<td>2+</td>
<td>3+</td>
<td>-</td>
<td>-</td>
<td>Spindle</td>
<td>GIST</td>
<td>6 bp del</td>
</tr>
<tr>
<td>5B</td>
<td>R</td>
<td>16/50</td>
<td></td>
<td></td>
<td>6 x 5 x 4</td>
<td>H</td>
<td>M</td>
<td>3+</td>
<td>3+</td>
<td>-</td>
<td>-</td>
<td>Spindle</td>
<td>GIST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>29</td>
<td>F</td>
<td>SI</td>
<td>6/50</td>
<td>5 x 3 x 3</td>
<td>H</td>
<td>M</td>
<td>3+</td>
<td>-/+</td>
<td>-</td>
<td>-</td>
<td>Spindle</td>
<td>GIST</td>
<td>52 bp del+</td>
</tr>
<tr>
<td>7</td>
<td>I</td>
<td>66</td>
<td>M</td>
<td>S</td>
<td>est.</td>
<td>0.5*</td>
<td>VL</td>
<td>B</td>
<td>3+</td>
<td>3+</td>
<td>-</td>
<td>+</td>
<td>Spindle</td>
<td>GIST</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>I</td>
<td>48</td>
<td>M</td>
<td>D</td>
<td>&lt;2/50</td>
<td>0.2*</td>
<td>N/A</td>
<td>B</td>
<td>NR</td>
<td>-</td>
<td>-</td>
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<td>Epithelioid</td>
<td>i.d.</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>C</td>
<td>25</td>
<td>F</td>
<td>S</td>
<td>N/A</td>
<td>8 x 6.5 x 7</td>
<td>N/A</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>-/+</td>
<td>N/A</td>
<td>IP</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>62</td>
<td>M</td>
<td>SI</td>
<td>N/A</td>
<td>6 x 5.5 x 3.5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>3+</td>
<td>-</td>
<td>N/A</td>
<td>SMT</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>C</td>
<td>71</td>
<td>M</td>
<td>S</td>
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<td>1 x 0.5 x 0.5</td>
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<td>N/A</td>
<td>-</td>
<td>-/+</td>
<td>3+</td>
<td>N/A</td>
<td>PNST</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: S, stomach; R, rectum; D, duodenum; SI, small intestine; †, high mitosis but little biopsy; * in aggregates; H, high; I, intermediate; L, low; VL, very low; N/A, not available; NR, not reactive; M, malignant; PM, potential malignant; B, benign; i.d., Indeterminate; IP, Inflammatory Pseudotumor; SMT, smooth muscle tumour; PNST, peripheral nerve sheath tumour.

### Table III: Amino acid sequence encoded by wild type and mutant c-KIT exon 11.

<table>
<thead>
<tr>
<th></th>
<th>550</th>
<th>560</th>
<th>570</th>
<th>580</th>
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<tr>
<td>Wildtype</td>
<td>KPMYEVQWKV</td>
<td>VEEINGNNYYV</td>
<td>YIDPTQLPYD</td>
<td>HKWEFPNRNL</td>
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<tr>
<td>Case 1</td>
<td>KPMVEVQC -</td>
<td>-</td>
<td>YIDPTQLPYD</td>
<td>HKWEFPNRNL</td>
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<tr>
<td>Case 3</td>
<td>KPMYEVQWKV</td>
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<td>YIDPTQLPY -</td>
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<tr>
<td>Case 5</td>
<td>KPMYEYQWN -</td>
<td>-</td>
<td>YIDPTQLPYD</td>
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<tr>
<td>Case 6</td>
<td>KPMYEV- - -</td>
<td>-</td>
<td>-</td>
<td>PTQLPYD</td>
</tr>
</tbody>
</table>
An Immunohistochemical and Molecular Study of Gastrointestinal Stromal Tumours

Fig 1: Histological subtype of GIST. (a) spindle type, (b) epithelioid type, (c) mixed spindle-epithelioid type; immunohistochemical staining profile of GIST showing (d) CD117 expression, and (e) CD34 expression.

(codon 579) that located beyond the hot spot area of c-KIT gene, which led to the loss of an aspartic acid residue (case 3) for the protein. As described in previous study, KIT<sup>codon 579</sup> was a gain-of-function mutation that eventually resulted in constitutive activation of c-KIT protein, hence tumorigenesis of GIST.

No mutation was detected in other exons of c-KIT gene. This suggested that mutation in these exons was less common than that in exon 11. Due to the small sample size, correlation of mutation spectrum with clinical data cannot be determined.

c-KIT and PDGFRA gene mutations were claimed to be an important predictive value of clinical response of imatinib mesylate on GISTs. GIST patients with mutations in exon 11 of c-KIT were reported to have better response and better survival under imatinib mesylate treatment when compared to those with mutations in exon 9 of c-KIT, or with no detectable mutations. Our study showed exon 11 c-KIT mutation rate of 57% among the GIST cases examined, and hence it may be more beneficial to our local GIST patients if imatinib mesylate is instituted for the treatment of GIST with exon 11 c-KIT mutation.

In conclusion, immunohistochemical staining using a panel of antibodies are helpful for making the diagnosis of GIST. Not all c-KIT-positive GISTs harboured c-KIT mutation, and all of these cases with mutations (57%) were of poor prognostic morphology when compared to those without mutation.


