DOG 1 EXPRESSION IN GASTRO-INTESTINAL STROMAL TUMORS REPORTED IN NAIROBI.

An Immuno-histochemical Evaluation of a Novel Antibody in Comparison to Morphology & CD117 Staining

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H58/71011/09

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF MEDICINE IN PATHOLOGY.

UNIVERSITY OF NAIROBI.

2012

DECLARATION

This dissertation report is my original work and has not been presented for any award in any University.

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ACKNOWLEDGEMENTS

I thank my supervisors, Dr. LW Muchiri, Dr. GW Kiarie and Dr. AY Kalebi who despite their busy schedule were always available to read through my work and make the necessary corrections. I am particularly grateful to Dr. LW Muchiri and Dr. AY Kalebi for the help they accorded me while reviewing the slides.

I acknowledge the technical assistance I received from Mr. Samuel Kasera and Mr. Brian Andebesa both technologists at Pathologists, Lancet Kenya. I also thank Willis and Kairu both technologists at the University of Nairobi Histopathology Laboratory for assisting me retrieve paraffin wax embedded blocks from the archives.

I am grateful to Dr. Bessie Byakika and Nancy from The Nairobi hospital. I thank my professional colleagues; without their support this work would not have been possible.

I thank The National Institute of Health (NIH) through AIDS Cancer Specimen Resource Kenya, at the University of Nairobi for awarding me a grant to carry out this study.

I thank my family for walking with me each step of the way and for always believing in me.

DEDICATION

This work is dedicated to my husband Dr.Tony Murithi Njoka and to our children Mark, Allan, Mariah and Terri for always believing in me.

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LIST OF ABBREVIATIONS

AJCC -American Joint Committee on Cancer

ANO-1-Anoctamin -1

ASCO -American Society of Clinical Oncologists

BCL-2 -B cell Lymphoma-2

CD -Cluster of Differentiation

DAB-Di-Amino Benzidine

DOG I -Discovered On Gastrointestinal Stromal Tumor Protein 1

eGIST -Extra Gastro Intestinal Stromal Tumor

ERC-Ethics Research Committee

HIER-Heat Induced Epitope Retrieval

hpf -high power field

HSP-Heat Shock Protein

IHC- Immuno-Histochemistry

GI- Gastrointestinal

GIST-Gastrointestinal stromal Tumor

KNH-Kenyatta National Hospital

KS-Kaposi's Sarcoma

KShs-Kenya Shillings

MRC-Medical Research Committee

ORAOV-Over -expressed In Oral Cancer

PKC-θ- Protein Kinase C Theta

PDGFRα/PDGFRA- Platelet Derived Growth Factor Receptor Alpha

SCF-Stem Cell Factor

SEER-Surveillance Epidemiological and End Result

TKI-Tyrosine Kinase Inhibitor(s)

TMEM16-A- Trans-Membrane Protein 16- A

ABSTRACT

Background: All Gastro-Intestinal Stromal Tumors (GISTs) should be diagnosed as they are amenable to treatment with Tyrosine Kinase Inhibitors (TKI). TKIs are a group of targeted therapies, which have revolutionalised treatment of tumors such as Chronic Myeloid Leukemia (CML) and more importantly GISTs. A commonly used TKI is Imatinib whose common brand name is Gleevec or Glivec. GISTs present diagnostic challenges on routine Hematoxylin and Eosin (H&E) stain and on Immuno-histochemistry with the commonly used markers in Kenya; CD117 and CD34. Due to these challenges a lot of research efforts are directed at markers that will identify all GISTS. Several markers have been shown to improve diagnostic sensitivity and specificity when used alone or together with CD117. Discovered on GIST 1(DOG 1) antibody is one such marker. Antibodies to DOG 1 protein have been shown to improve diagnostic sensitivity and specificity when included in the Immuno-Histo Chemical (IHC) panel for GIST.

Objective: To evaluate DOG 1 expression in tumors diagnosed as GISTs on routine histology and CD117 staining and in those suspected to be GISTs on morphology alone.

Design: Cross-sectional descriptive study

Setting: Kenyatta National Hospital, Nairobi Hospital and Pathologists, Lancet Kenya Laboratory.

Main outcome measures: Fifty three previously diagnosed GISTs were analyzed for DOG 1 expression by immunohistochemistry on paraffin wax embedded tumor tissue blocks. DOG 1 expression was correlated with age, sex, and anatomic site of tumor, mitotic counts and CD117 expression.

Results: The males were 25(47.2%) while the females were 28(52.8%).M: F was 9:10.Mean age was 50.5 years while the median age was 53 years. The odds of a tumor being DOG 1 positive

test in a male patient were 3.5 higher than a negative test .The odds of a tumor being DOG 1 positive are lower for tumors with more than 10 mitoses in 50 hpf than negative (OR =0.2). Tumors in males had more odds of being positive for CD 117(OR 4.6) when compared to those in females. Mitoses and tumor size had little correlation with CD117 positivity or negativity. The cases were stratified using DOG 1 and CD117 immunohistochemistry into GISTs and non-GISTs. The M: F in GIST cases is 1.3:1, median age was 52.3 years. Thirty three (62.3%) tumors were CD117 positive while 35(66%) tumors were positive for DOG 1 staining. Thirty one (58.5%) tumors were positive for both markers, 16 (30.2%) were negative for both markers. Six (11.3%) tumors were positive for only one marker; two (3.8%) with CD117 and 4(7.5%) with DOG 1. Using both CD117 and DOG1, 37(69.8%) 16(30.2%) found to be negative for both markers were classified as non-GIST.

Conclusion: GIST in Kenya occurs at an earlier age with an almost equal sex distribution.

DOG 1 immuno-positivity was positively correlated with the male sex as was CD117 expression. Although DOG 1 identified more cases than CD 117 the difference was not statistically significant. A significant proportion of tumors in this study were negative for both DOG 1 and CD117; these are thought to represent other biological entities. Extra gastro-intestinal GISTs were common in this study which may be attributed to late patient presentation when the tumor has already metastasized. All the EGISTs in this study were intra-abdominal; no extra-abdominal cases were described.

Recommendations: Epidemiological long term prospective studies should be done to ascertain the clinical behavior of GISTs including age of onset in Kenya. DOG 1 and CD117 should be used together to diagnose more GIST cases. An immuno-histochemical study using a broad

panel of antibodies should be done on the non- GIST cases in this study to determine their true diagnoses.

The apparent high frequency of metastatic GISTs needs to be addressed by advising clinicians to have a high index of suspicion and diagnose GISTs early when they are more amenable to cure.

1. BACKGROUND

The term GIST was first used by Mazur and Clark in 1983 to describe epithelial neoplasms without IHC features of Schwann cells or electron microscopy features of smooth muscle cells.(1) Although Gastrointestinal Stromal Tumors (GIST) are rare, accounting for less than 1% of all Gastro-Intestinal Tract (GIT) tumors, they are the most common mesenchymal tumors in the GIT.(2) It is thought that GIST arises from ICC cells in the GIT, and from ICC-like cells elsewhere, due to mutations in tyrosine Kinases. Mutations have been identified in Platelet Derived Growth Factor Receptor Alpha (PDGFRα) and C-KIT receptor in 90% of GISTs. (3) It is not clearly understood how the 10% of GIST without PDGFRα or C-KIT mutations develop. These cases are referred to as wild type GIST. Recently, BRAF mutations have been described in 7-11% of these cases.(4). The BRAF gene codes for a serine/threonine-protein kinase whose function is to control proliferation and differentiation of cells through the MAPK pathway.

Globally, epidemiological data is inaccurate and incomplete regarding the true incidence and prevalence of GIST. This is because; a diagnosis of GIST has become common only recently owing to lack of well-defined criteria for this entity. Before the year 2000 when the criteria for diagnosis of GIST was published this diagnosis was rarely rendered.(5) According to a Surveillance Epidemiological End Result (SEER) study that analyzed GIST data between the years 1978 to 2004, only 18.8% of GIST were diagnosed before the year 2000, 81.2 % were diagnosed in the year 2000 or later. (6)

In the year 2001, Tyrosine Kinase Inhibitors (TKIs) were introduced as targeted therapy for GISTs. Studies done since then have shown that almost all GISTs respond to TKIs to some

extent. Tyrosine kinase inhibitors are however very expensive; the approximate cost of Imatinib per month is, Kshs. 400,000 or US$ 5334. Novartis together, with Axios and the Max foundation have availed Tyrosine Kinase Inhibitors through the Glivec International Patients Assistance Programme (GIPAP) to Chronic Myeloid Leukemia and GIST patients in developing countries. This is done at little cost to the patients; they meet the confirmatory test for GIST e.g. CD117 or DOG 1, the cost of which is about Kshs.6000. As of 2009, there were 28 GIST patients enrolled in the GIPAP program in Kenya.(7) Confirming more GIST cases by using more sensitive IHC markers will avail targeted therapy to more patients. Conventional chemotherapy and radiotherapy are associated with a worse clinical outcome when compared to non-treated cases.(8)

Based on these findings all Gastro-Intestinal Stromal Tumors (GIST) should be diagnosed. They present diagnostic challenges on routine Hematoxylin and Eosin (H&E) stain and on Immuno-histochemistry with the commonly used markers in Kenya; CD117 and CD34. For instance CD117 is negative in up to 15 % of GIST cases and up to 30% of GIST are negative with CD34.(9) Due to these challenges a lot of research efforts are directed at markers that will identify all GISTS. Unfortunately, there is no IHC marker or a combination of IHC markers yet, that can identify all GIST patients. Several markers have been shown to improve diagnostic sensitivity when used alone or together with CD117. Discovered on GIST 1(DOG 1); an antibody against a chloride channel protein is one such marker. DOG 1 is coded for by the gene, FLJ10261 on chromosome 11. Antibodies to DOG 1 protein have been reported to improve diagnostic sensitivity and specificity when included in the IHC panel for GIST. DOG 1 has been of great utility in the diagnosis of GIST especially those in unusual location, those with unusual morphology and those which are CD117 negative.(10)

2. REVIEW OF LITERATURE

2.1 PATHOGENESIS

It is now assumed, that GIST originate from pluri-potent stem cells positive for the CD34 antigen, which differentiate towards an Interstitial Cell of Cajal (ICC) phenotype. The earlier theory that these tumors develop directly from (ICC) does not explain the occurrence of Extra Gastro-Intestinal Stromal Tumors (eGIST) at sites where ICC have not been found.(2) ICC-like cells that express CD34 and C-KIT have been described in the Fallopian tubes, urinary bladder, extra hepatic bile ducts, gallbladder, and pancreas and may explains the occurrence of GIST at these sites.(11)

The pathogenesis of GISTS is driven by mutations in Tyrosine Kinases (TK). Most of these mutations occur in in the C-KIT receptor and are detectable in 85-88% of all GISTS. Less frequent, are mutations in another tyrosine kinase, the PDGFRα, that are seen in 5 to 7% of GISTs. Ten % of GISTs have no detectable mutations in any of these two tyrosine kinases; these are referred to as wild type GIST. (4) Recently it has been shown that 7–11% of these wild-type GISTS have BRAF mutations. (4) Targeted therapy for these BRAF positive tumors has already been developed. The proportion of wild type GISTS without demonstrable mutations is likely to reduce from the current 10% in the coming years as more mutations in other tyrosine kinases other than KIT, PDGFRα & BRAF are identified. Mutations in TK in GISTs occur in 5 exons of c-kit receptor and 3 exons of PDGFRA. These mutations are deletions, substitutions, duplications, insertions or a combination of types. They cause constitutive dimerization of c-kit in the absence of the ligand –the Stem Cell Factor (SCF).(12)

DOG 1 is a calcium regulated chloride channel whose role in the pathogenesis of GIST is still uncertain. Based on the current evidence, this protein is unlikely to have a direct role in the pathogenesis of GISTs: Though widely expressed in GISTs, no mutations or extra copies of the gene have been detected in GISTs so far. Studies using broad spectrum chloride channel blocking agents have had little effect on proliferating GIST cell lines further casting doubt on a direct pathogenetic role for this protein. (13)

Other genes and proteins implicated in the pathogenesis of GIST include succinate dehydrogenase complex, carbonic anhydrase and the neuro-fibromin gene.(14,15 & 16).

2.2 EPIDEMIOLOGY

GISTs are rare tumors, in a large population based study done in Sweden between the year 1983-2000 estimated the average prevalence of GIST per annum to have been 129 per million of the population. The annual incidence of clinically detected primary GISTs within the region was estimated at 14.5 per million inhabitants and did not differ significantly over time.(17) This study was done at several hospitals with a catchment of approximately 1.5 million inhabitants.

Other studies elsewhere found a much lower prevalence and incidence; this may be attributed to much smaller sample sizes and inclusion of only symptomatic, clinically significant GISTs.

Prevalence in these studies range from 15-20 per million per year with an incidence of 6-10 per million per year.(2, 8 , 17)

The median age at which GIST occurs is 69 years in Europe, while it is 62 years in the USA, the M: F ratio is equal and does not vary much between the two continents. (17 & 19)

Most GISTs are sporadic but familial cases have been reported. These familial cases are usually associated with genetic disorders such as Neurofibromatosis (NF) and Carney's syndrome. The incidence of GISTs in NF patients is 5-25%.These GISTs tend to be of very low malignant potential, occur at a young age, show a female preponderance and are wild type for both CD117 and for PDGFRα mutations.(20)

Synchronous occurrence of separate GIST and adenocarcinoma is rare but has been reported more frequently than GIST/adenocarcinoma collision tumors that are exceedingly rare.(21)

GISTs most often arise from stomach (60%) or small gut (30%) and less frequently affect colo-rectum, duodenum, esophagus and appendix.(17) Only 0-8% of GISTs occur outside the GIT: at these sites they are known as extra intestinal GISTs (eGISTs).(22)

Mutations have been documented in 5 exons of the C-KIT receptor and 3 exons of PDGFRα.

C- KIT Mutations occur most commonly in exon 11 of the gene accounting for 65% of all GISTs. Ten % of c-kit GISTs have exon 9 mutations, 4% have either exon 13 or exon 17 mutations. While c-kit exon 11 mutations are found in GISTs from different locations, c-kit exon 9 mutations show a strong predilection to intestinal tumors.(23 & 24)

C-KIT and PDGFRα mutations appear to be mutually exclusive since GISTs with mutations in both of these genes do not exist or are very rare. The most common site of PDGFRα mutations in GISTs is in exon 18 accounting for 82.5%. Smaller numbers of mutations are found in exon 12 and in exon 14 accounting for 13.7% and 3.7% respectively.(24)

GISTs with PDGFRα exon 18 mutations represent a subset of tumors almost exclusively occurring in the gastric location. Histologically, these tumors often show epithelioid morphology and usually have low mitotic activity and most follow a benign course. Therefore, detection of

exon 18 PDGFRα mutations could be an additional feature that identifies gastric GISTs with a high probability of benign behavior.(25)

2.3 LABORATORY DIAGNOSIS

In routine practice, a diagnosis of GIST is arrived at on finding a monomorphic, spindle cell tumor or an epithelioid tumor in the tubular GI or abdominal cavity that expresses CD117 and/or CD34.

Tissue for diagnosis is usually obtained by surgical resection or incisional biopsy of the involved organ at laparotomy. Cytological diagnosis can also be made on FNA material obtained during endoscopy. Due to the sub-mucosal location of tumor, endoscopic biopsy using standard forceps is difficult to apply. (26)

2.3.1 DIFFERENTIAL DIAGNOSES

The differential diagnoses of GIST depend on, tumor location and cell type. GIST with an unusual morphology such as those with epithelioid or rhabdoid features are difficult to diagnose, as well as those in unusual locations.

Gynecological tumors both benign and malignant are particularly troublesome as they tend to resemble GIST histologically. In addition they may stain with one or the other of the commonly used markers. For instance the endometrial stromal sarcoma is commonly CD 117 positive while retroperitoneal leiomyomatosis may be DOG 1 positive. Both these tumors can be differentiated from GIST as they express estrogen receptor while GIST does not, in addition endometrial stromal sarcoma also expresses CD10.(4)

For epithelioid GIST in the peritoneal cavity, the differential diagnoses include primary and metastatic carcinomas, lymphomas and sarcomas that may have an epithelioid morphology.

With the first case of a primary pleural GIST recently reported by Long et al, GIST is an important differential diagnoses in the chest cavity and needs to be differentiated from solitary fibrous tumor. Unfortunately Solitary fibrous tumor like GIST is CD34 positive.(11) Other markers like DOG 1 and CD117 are required to make this distinction.

Melanoma, a common tumor in the rectum expresses CD117 in about half of the cases, leading to diagnostic difficulties in separating it from rectal GIST if CD117 alone is used. Furthermore rectal GISTs tend to be epithelioid and therefore resemble melanomas morphologically. DOG 1 resolves this dilemma.(27)

2.3.2 GROSS MORPHOLOGY

Grossly, tumors vary greatly in size, ranging from 1–2 cm to more than 20 cm in diameter. The tumors are usually well circumscribed and generally un-encapsulated, although a pseudo capsule may occasionally be seen. The lesions are sub-mucosal, intramural, or sub-serosal and may be ulcerated. On sectioning, the cut surface varies in color from grey/white to red/ brown, depending on the degree of hemorrhage, and may be solid, partially cystic, or necrotic.(4)

2.3.3 HISTO-MORPHOLOGY

Cellular features demonstrate a broad morphological spectrum but there are two principal histological patterns: a spindle cell pattern accounting for 60–70% of cases or an epithelioid pattern accounting for 30–40% of cases. Some cases exhibit a combination of both in variable proportions. (28)

GISTs of spindle cell type are composed typically of relatively uniform eosinophilic cells arranged in short fascicles or whorls. The tumor cells have a paler eosinophilic cytoplasm than smooth muscle neoplasms, often with a fibrillary and syncytial appearance. The nuclei of GIST

cells tend to have relatively pointed ends as compared with blunt-ended nuclei in the typical leiomyosarcomas. Striking juxta-nuclear cytoplasmic vacuoles are seen in up to 5% of all cases and are particularly a feature of gastric tumors.(29) Features reminiscent of Schwannoma are often seen these include; nuclear palisading, stromal lymphocytes and micro cystic stromal degeneration. Stromal collagen is minimal in most cases, but delicate thin-walled vessels may be prominent, and stromal hemorrhage is a common feature. (4)

GISTs of epithelioid type are composed of rounded cells with variably eosinophilic or clear cytoplasm. Epithelioid lesions, similar to spindle cell lesions, tend to have uniform round-to-ovoid nuclei with vesicular chromatin. This subset of tumors shows a nested architecture more often than spindle cell cases, enhancing the risk of confusion with an epithelial or melanocytic neoplasm.(29)

Majority of Small Intestine GISTs are composed of spindly cells arranged in loosely bound fascicles with variable amounts of intervening capillary-containing stroma. Many Small intestine and some colonic GISTs, especially the nonmalignant tumors, are distinctive for their microscopically distinctive, round aggregates of extracellular collagen fibers, which have a skein-like ultra-structural appearance and have therefore been named skenoid fibers.(30)

It is difficult to draw a sharp line between benign and malignant lesions based on gross and histologic findings alone. However, features that increase suspicion for malignancy include an extra-gastric tumor location, a large size, high mitotic counts and the presence of necrosis.(25) In addition various mutations can help to predict the biological behavior of a GIST.(5)

2.3.4 IMMUNOHISTOCHEMISTRY

2.3.4.1 CD34

Before CD117 Immuno-Histochemistry became available, CD34 was the most useful cell marker for GISTs staining 81% of cases. (31) However because of its low specificity, it has almost been abandoned as a GIST marker; false positives are seen in vascular tumors, in hemato-lymphoid tumors & in solitary fibrous tumors.

2.3.4.2 CD117

It has been shown that approximately 95% of GIST stain for CD117 exhibiting a diffuse, focal or mixed staining pattern with or without a dot like accentuation. Dot like accentuation is especially common in epithelioid GISTs. Up to 15% of GISTs lack CD117 expression by immunohistochemistry.(32) False positives sometimes occur with CD117, for instance Kaposi's sarcoma may show CD117 expression in a significant number of cases especially when antigen retrieval is used.(33) Other tumors with spindle cell morphology that may show CD117 staining include melanoma, and deep fibromatosis.(32) Cutaneous melanoma of the ano-rectal region is especially difficult to distinguish from GIST as it expresses CD117 in 48% of cases. This pitfall is less pronounced in metastatic melanomas which rarely express CD117.(27) Due to these pitfalls there is a move to develop more sensitive and specific markers. These markers are aimed at diagnosing most CD117 negative GIST and avoid false positives. Some of these markers include DOG 1, Nestin, Protein Kinase C Theta (PKCϴ), Carbonic Anhydrase II (CA-II) and Succinate Dehydrogenase Complex (SDHX).(34)

2.3.4.3 DOG1/FLJ10261/ANO1

The gene FLJ10261 was initially described by Van de Rijn as a gene highly expressed in GISTs, he named the gene product Discovered on GIST 1(DOG 1).(10) This protein was later renamed

as Trans-Membrane Protein 16A (TMEM16A).The official current name is Anoctamin 1(ANO 1), due to its chloride channeling activities. Nevertheless, the unofficial name DOG 1 has persisted and it is the name pathologists are most familiar with. They use the name to refer to antibodies targeting ANO1/TMEM16A/FLJ10261.

FLJ10261 is on chromosome 11q and has 26 exons. The gene product i.e. DOG 1 protein contains 960 amino acids, has 8 trans-membrane domains and is a calcium activated chloride channel. (10) DOG 1 is of optimal utility in the diagnosis of CD117 negative GISTs. These cases have been characterized clinically, genetically and morphologically. They tend to be epithelioid GISTs in the stomach, tumors in children ,PDGFRα mutants and in syndromic GIST that occur in Neurofibromatosis and in Carney's syndrome .(13)

The sensitivity of DOG 1 monoclonal antibodies varies, depending on the clone. DOG 1 Clone 9 (K9) is more sensitive than DOG 1.1,it labels half of CD117 negative GISTS while a third of c-kit negative GISTs are positive with DOG 1.I.(35) Moreover the combination of CD117 and DOG 1 in an Immuno-Histochemical panel can define GISTs in 99% of cases.(36) Furthermore DOG 1expression is not affected by treatment with tyrosine kinases which make CD117 fade and eventually turn negative after prolonged treatment.(13) However double negatives GISTs do occur and are estimated to occur at a frequency of 0.9%-1.6%.(31&37) For these cases mutation status and phosphorylation status of C-KIT can be used if GIST is still suspected.

3. PROBLEM STATEMENT

A diagnosis of GIST cannot be made on morphology alone as GISTs shares many morphological features with other mesenchymal tumors. At present, the 'gold-standard' for a GIST diagnosis on IHC is CD117 expression. However, a significant subset of GISTs does not express CD117 by IHC, this subset accounts for 4% to 15% of all GISTs. Negative GISTs may have mutations in the KIT, PDGFRα or be wild type for both tyrosine kinases. It is still important to correctly diagnose these cases since almost all subtypes of GIST have been shown to respond to tyrosine kinase inhibitors. In addition, various tumors that share morphology with GISTs also express CD117 which further confuses the diagnostic separation. These tumors include Kaposi's sarcoma, melanomas neurofibromatosis and desmoids tumors.

4. RATIONALE

With the discovery of the FLJ10261 gene, its protein product DOG 1 and an antibody against DOG 1, it appears that we may have a more specific and also highly sensitive IHC marker surpassing CD117 for diagnosis of GIST. DOG 1 expression is not dependent on the C-KIT mutation status, its cost is similar to that of CD117, and it is easier to interpret than CD117. Furthermore DOG 1expression is not affected by treatment with tyrosine kinases which makes CD117 fade and eventually turn negative after prolonged treatment. DOG 1 however, is only recently introduced in Kenya and there are no studies evaluating the utility of this antibody in the diagnosis of GISTs in Kenya.

For cases that are still negative with DOG 1 and CD 117 but still suspected to be GISTs, mutational analysis and phosphorylation status of C-KIT can be used. Mutational analysis has pitfalls as mutation negativity; in itself is not evidence against GIST: Several mutation-negative subgroups are known. These mutation negative GISTs include pediatric GISTs, GISTs in neurofibromatosis patients, and sporadic wild-type GISTs. Mutation analysis and phosphorylation studies are too expensive and cumbersome for use in routine clinical diagnosis. .

Novartis together, with Axios and the Max foundation have availed Tyrosine Kinase Inhibitors through the Glivec International Patient Assistance Programme (GIPAP) program to Chronic Myeloid Leukemia and GIST patients in developing countries. This is done at little cost to the patients; they meet the confirmatory test for GIST e.g. CD117 or DOG 1, the cost of which is about Kshs.6000. As of 2009, there were 28 GIST patients enrolled in the GIPAP program in Kenya. Confirming more GIST cases by using more sensitive markers such as DOG 1 will avail targeted therapy to more patients.

5. RESEARCH QUESTION AND OBJECTIVES

5.1 RESEARCH QUESTION

What is the pattern of DOG 1 expression in neoplasms diagnosed as GISTs in Nairobi?

5.2 BROAD OBJECTIVE

To determine, the pattern of DOG 1 expression in cases previously reported as Gastro-Intestinal Stromal Tumors in three major laboratories in Nairobi.

5.3 SPECIFIC OBJECTIVES

1. To examine the Histo-Morphology of all cases previously diagnosed as GISTs on light microscopy and/or CD117

2. To evaluate all cases previously reported as GIST for DOG 1 expression by IHC.

3. To correlate DOG 1 expression with Histo-Morphology and CD117 expression in tumors previously diagnosed as GIST.

6. STUDY DESIGN, MATERIALS AND METHODS

6.1 STUDY DESIGN

A laboratory based, descriptive, cross sectional study.

6.2 SETTING

The study was done at Kenyatta National Hospital, the Nairobi hospital and Pathologists, Lancet Kenya laboratory. Kenyatta National Hospital is the National Teaching and Referral Hospital in Kenya, it is a public hospital. Pathologists, Lancet, Kenya is a private referral Laboratory in Nairobi. The Nairobi hospital is a large private hospital in Nairobi.

Specimen processing was done at Pathologists, Lancet Kenya laboratories.

6.3 STUDY SUBJECTS

Fifty three cases reported as Gastrointestinal Stromal Tumor. The cases were from the three institutions stated above.

6.4 STUDY ELIGIBILITY CRITERIA

6.4.1 INCLUSION CRITERIA

1. Cases reported as Gastro-Intestinal Stromal Tumors (GISTs)

2. Cases reported as mesenchymal tumors favoring GISTs

3. Primary mesenchymal/spindle cell tumor of the GIT where GIST was not the initial diagnosis but on subsequent review was found to meet the clinic-pathological criteria of GIST.

6.4.2 EXCLUSION CRITERIA

1. Poorly processed tissues

2. Cases on review found to have insufficient tumor tissue

6.5 SAMPLE SIZE DETERMINATION

The standard statistical approach to determine sample size for a cross-sectional study was used; using the prevalence, the desired level of confidence and a tolerance error margin or width of the confidence interval a sample size was arrived at as follows.

$$n = \frac{z^2_{1-\alpha/2} P(1-P)}{D^2}$$

Where:

n = the required sample size

p = expected prevalence or proportion or estimated proportion of GISTs. In this study a prevalence of 129/mil was used.

D= degree of precision or a tolerance error margin or width of the confidence interval

(A measure of precision of the estimate which ranges from 1%- 20%).this study used 1%

Z= Z statistic for a level of confidence or is the normal distribution critical value for a probability of /2 in each tail.

For a 95% CI, z=1.96

Using this formula

$$n = \frac{(1.96)^2 (0.00129) (0.9998)}{(0.01)^2}$$

$$= 49.546728672 \qquad N= 50$$

6.6 SAMPLING METHOD

Cases that met the inclusion criteria were recruited into the study until the desired sample size was obtained. The recruitment started following ethical approval and was done retrospectively. Fifty three cases were recruited. All the cases that did not meet the inclusion criteria due to poorly processed tissues or insufficient tumor volume were replaced with other cases that met the inclusion criteria.

6.7 IDENTIFICATION OF CASES

The files containing histology reports at the Kenyatta National Hospital the Nairobi hospital and Pathologists, Lancet Kenya were perused to identify all the cases that met the inclusion criteria. The name, sex, ward, patients hospital number, hospital name and laboratory number were noted from the histology report as the cases were identified .This information was used to retrieve the archival specimen.

6.8. MATERIALS

6.8.1 EQUIPMENT

A microtome and a Leica bond max immuno -stainer were provided by Pathologists Lancet Kenya. An Olympus microscope was provided by the University of Nairobi department of Human Pathology.

6.8.2 REAGENTS AND OTHER CONSUMABLES

Gloves, cassettes, microtome blades, staining racks, slides, slide holder, labels, and reagents were supplied by Bactilab limited.

6.9 METHODS

6.9.1 PARAFFIN EMBEDDED BLOCK RETRIEVAL

Paraffin wax embedded blocks were retrieved from histology archives of the study sites using the laboratory numbers on the pathology reports. These were then transported to Pathologists, Lancet Kenya laboratory where the tests were done.

6.9.2 HISTOLOGICAL PREPARATION-H&E FOR LIGHT MICROSCOPY

A 5 micron section was cut from each of the blocks and stained with H&E as shown in Appendix 4.The slide was then reviewed to ascertain that the Histo- Morphology was compatible with a diagnosis of GIST and to establish that there was sufficient tumor tissue on the slide. The tumors were then classified into cell type depending on the predominant cell type into spindle cell, epithelioid or mixed type GIST. Mitoses were counted in 50(high power field) hpf which is equivalent to $5mm^2$ of tumor area. This information was then tallied into the data sheet. The slides were reported first by the principal investigator then reviewed together with two of the supervisors who are qualified pathologists.

6.9.3 IMMUNOHISTOCHEMISTRY CONTROLS

The controls were stained first to optimize the antibody dilution and other antigen retrieval conditions. The dilution for DOG 1 was optimized at 1:60 while that for CD117 was at 1:40. For DOG 1 staining, previously confirmed cases of GIST were used as the positive controls while cerebellar tissue was used as the negative control. Normal appendix was used as the IHC control tissue for CD117, the muscle layer was the negative control, while the ICC in the myenteric plexus and the mast cells in the lymphoid follicles were the positive control

6.9.4 IMMUNOHISTOCHEMISTRY STAINING

Two 5 micrometer sections were cut from each of the study blocks and placed on 2 negatively charged slides labeled with the patient's number and test using a diamond marking pencil. A positive and a negative control were also placed on each slide and were therefore subjected to the same test conditions as the test sample. For DOG 1 slides a section of the cerebellum and a section of a previously confirmed GIST were placed on one end of the slide as the controls. A section of the appendix was placed on each of the slides to be subjected to CD117 staining. The sections were then stained with CD117 & DOG1 using the automated Leica bond system as shown in the Appendix 2.

The slides were processed in batches of 18, 9 DOG 1 and 9 CD117 slides. H&E was used as the counter-stain and Diamino-Benzidine (DAB) as the chromogenic substrate. When the slides dried, a sticky label with the study number and test name was affixed on each slide for example, G/001/DOG 1 or G001/CD117 for case number one. A different color of label was used for each test; orange was used for CD117, while green was used for DOG 1.

The slides were then stored serially in trays according to study numbers.

6.9.5 IHC SCORING FOR CD117 & DOG 1

The Immuno-Histochemical reactions were visualized to localize DOG 1 & CD117 proteins in the cytoplasm and on the cell membrane. The interpretation of any staining or its absence was complemented by morphological studies and proper controls. All the scores were reviewed, first by the principal investigator and then by investigator together with the supervisors. This was scored and entered into the data collection sheet.

Reactions were scored as follows:

0: Absence of any staining; Less than 10% of tumor cells with membrane staining

1+: Equivocal staining; more than 10% of tumor cells with punctate, faint and incomplete staining of the cell membrane

2+: More than 10% of tumor cells with weak to moderate complete membrane staining.

3+: More than 10% of tumor cells with strong complete membranous staining, forming a fish net pattern.

Scores of 0 and 1+ were interpreted as being negative, while scores of 2+ and 3+ were interpreted as being positive.

Some cases displayed a granular cytoplasmic staining with CD117; this was disregarded if unaccompanied by membrane staining.

6.10 QUALITY ASSUARANCE

All reagents were prepared according to the manufacturer's instructions. Standard operating procedures were adhered to during all the procedures. The reagents were checked for expiry date, turbidity, odor and precipitates. If these were found or the reagent found to be expired it was not used. The recommended storage for all reagents was observed. Positive controls were used for all cases while doing immunohistochemistry staining and interpretation. The slides were well labeled before mounting the section and then arranged in order to avoid mix up of slides. All the scores were independently reviewed by the supervisors who are qualified pathologists. The two pathologists were blinded and each reviewed the slides independently.

6.11 DATA COLLECTION INSTRUMENT

A data collection sheet was used as the instrument in this study.

6.12 DATA ANALYSIS

Statistical analyses were performed using Statistical Package for Social Scientists (SPSS) version 19.0 .The correlation analysis of the expression of DOG 1 and CD117 with the clinic-pathologic variables were done using X^2 and Fischer's exact test. The comparison of means was done using the student t-test. A p-value less than 0.05 was considered to be statistically significant. The data is presented in form of graphs, tables and photomicrographs.

7. ETHICAL CONSIDERATIONS

Permission for records and specimen retrieval and use in this study was obtained from the ethical research bodies in the two hospitals. Authority for transfer of archival specimens was sought from the KNH and from the Nairobi hospital before their transfer to the Lancet laboratories for processing. All patient identifiers were protected to maintain confidentiality .Study numbers were used instead of original laboratory numbers to maintain confidentiality.

Any discrepancies or additional information that has been found in this study has been conveyed in the usual manner as an addendum to the primary care doctor wherever possible.

The findings of this research will also be disseminated to the two hospitals for use in the management of GIST patients in future. The findings will also be submitted to scientific journals for publication without patient identifiers.

8. RESULTS

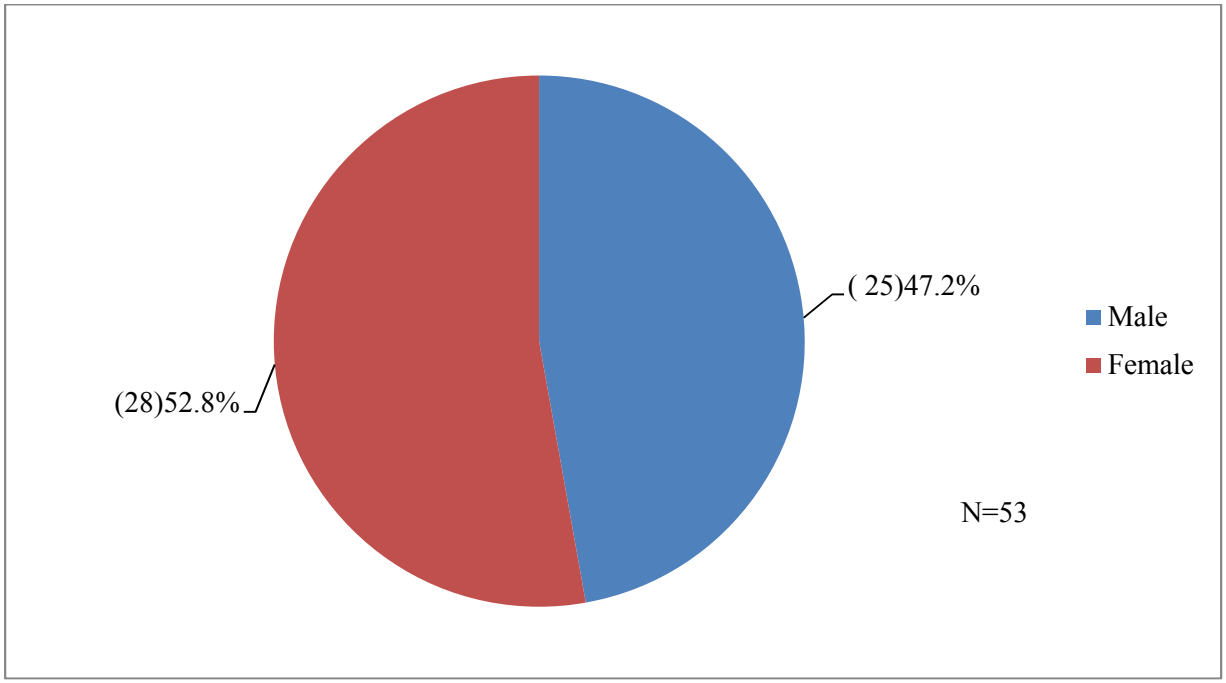
This study was carried out between October and November 2011. The total number of cases recruited was 53. The study participants were recruited from three facilities as shown below:

Table 1: Distribution of participants depending on study site

Study site	**Frequency (%) (n=53)**
KNH	31(58.5%)
Pathologists Lancet Kenya	15(28.3)
Nairobi Hospital	7 (13.2)

More than half of the cases were from Kenyatta National Hospital.

Figure 1: Sex Distribution



The males were 25(47.2%) while the females were 28(52.8%).M: F was 9:10.These results show a slight female preponderance in all the cases studied. When the cases were stratified using DOG 1 and CD117 Immuno-Staining a M: F of 1.3:1.emerged in the GIST cases.

AGE OF THE PARTICIPANTS

For the 51 cases in which age was provided, the mean age was 50.5 years while the median age was 53 years. The youngest patient was 9 years while the oldest patient was 76 years. Three patients (5.7%) were below 20 years.

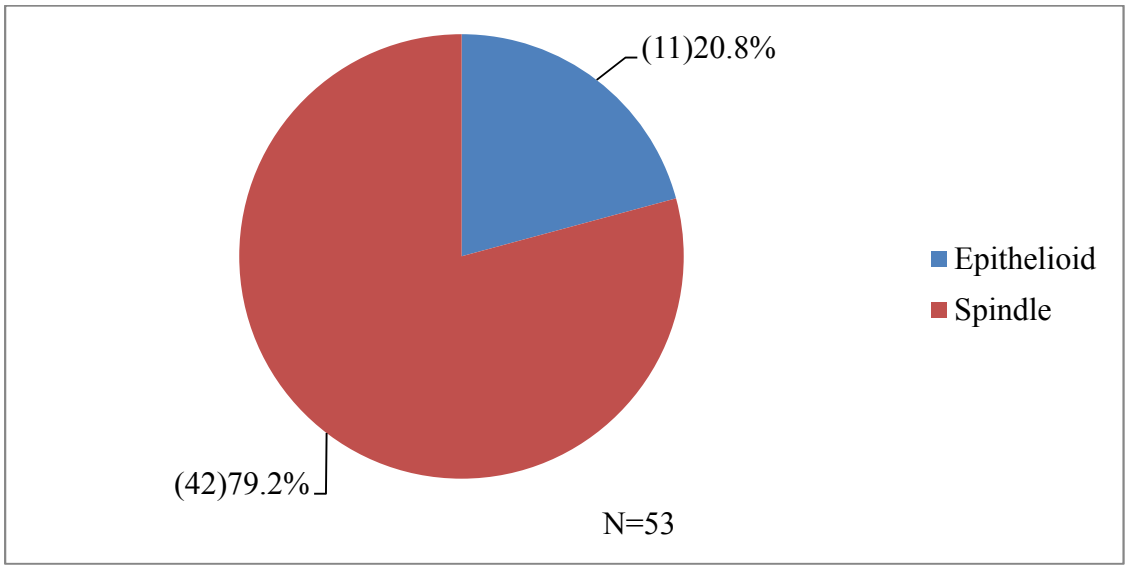
Age of the cases after they were stratified using Immuno-Histochemistry into GIST and non-GIST is shown in the table below

Table 2: Age of the participants

Variable	**GIST n=36**	**Non-GIST n=15**
Age Median (IQR)	52.3 (40.0-63.5)	56.0 (29.0-68.0)

GISTs occur at an earlier age than non-GISTs. The narrower interquartile range observed in GIST suggest a narrower age spectrum in GISTs when compared to non-GISTs.

Figure 2: Cell type



Most tumors i.e. forty two (79.2%) had spindle cell morphology while 11(20.8%) cases were epithelioid.

Table 3: The frequency of DOG 1 and CD117 expression as determined by Immuno-Histochemistry

Variable	**Frequency (%)(n=53)**
CD117 expression	
Positive	33 (62.3)
Negative	20 (37.7)
DOG 1 expression	
Positive	35(66.0)
Negative	18(34.0)
Variable	**Frequency (%)**
Both CD117&DOG 1 Positive	31 (58.5)
CD117 Positive, DOG 1Negative	2 (3.8)
CD117 Negative, DOG 1Positive	4 (7.5)
Both CD117 & DOG 1 Negative	16 (30.2)

Thirty three (62.3%) tumors were CD117 positive as compared to 35 (66%) tumors that were positive for DOG 1 staining. Thirty one (58.5%) tumors were positive for both markers, while16 (30.2%) were negative for both markers. Six (11.3%) tumors were positive for only one marker; two (3.8%) with CD117 and 4 (7.5%) with DOG 1.

Using both CD117 and DOG 1, 37(69.8%) of tumors were classified as GIST because they stained with one or both markers, while 16(30.2%) found to be negative for both markers were classified as non-GIST.

Table 4: DOG 1 expression as determined on immunohistochemistry

Variable	DOG1 expression		OR (95% CI)	P value
	Positive	Negative		
Age				
Median (IQR)	53.5 (41.0-66.0)	52.0 (29.0-63.0)	-	0.347
Sex				
Male	20 (57.1)	5 (27.8)	3.5 (1.0-11.9)	0.043
Female	15 (42.9)	13 (72.2)	1.0	
Anatomical site of tumor				
Esophagus	2 (5.7)	0		
Stomach	15 (42.9)	8 (44.4)	-	0.985
Small intestine	6 (17.1)	3 (16.7)		
Colon/rectum	3 (8.6)	1 (5.6)		
EGIST-Abdominal	9 (25.7)	6 (33.3)		
Size (cm)				
Median (IQR)	8.0 (4.5-13.0)	6.0 (5.5-13.3)	-	0.935
Size				
<5	5 (26.3)	1 (8.3)	1.0	
5-10	7 (36.8)	8 (66.7)	0.2 (0.0-1.9)	0.150
>10	7 (36.8)	3 (25.0)	0.5 (0.0-5.9)	0.556
Predominant cell type				
Epithelioid	5 (14.3)	6 (33.3)	0.3 (0.1-1.3)	0.154
Spindle	30 (85.7)	12 (66.7)	1.0	
Mitosis/50 per hpf				
Median (IQR)	2.0 (1.0-6.0)	6.5 (2.0-12.0)	-	0.100
Mitosis				
<5	21 (60.0)	7 (38.9)	1.0	
5-10	10 (28.6)	4 (22.2)	0.8 (0.2-3.5)	0.804
>10	4 (11.4)	7 (38.9)	0.2 (0.0-0.9)	0.030

DOG 1 EXPRESSION

Tumors less than 5cm, tumors in females, spindle cell tumors and those with less than 5 mitoses in 50hpf had equal odds of staining positive or negative for DOG 1(OR=1), however the p value was not statistically significant.

Tumors that were 5cm-10cm, epithelioid tumors, and those that had 5-10 mitoses in 50hpf had less odds of being DOG 1 positive than negative (OR<1), however the p value was not statistically significant.

The odds of a tumor being DOG 1 positive test in a male patient were 3.5 higher than a negative test and this was statistically significant. (p value=0.043). The odds of a tumor being DOG 1 positive are lower for tumors with more than 10 mitoses in 50 hpf than staining negative (OR =0.2) and this is statistically significant. (p value=0.03)

Table 5: CD 117 expression as determined by IHC on slides prepared from paraffin embedded wax blocks

Variable	CD117 expression		OR (95% CI)	P value
	Positive	Negative		
Age				
Median (IQR)	51.5 (39.5-63.5)	56.0 (32.0-68.0)	-	0.755
Sex				
Male	20 (60.6)	5 (25.0)	4.6 (1.4-15.8)	0.012
Female	13 (39.4)	15 (75.0)	1.0	
Anatomical site of tumor				
Esophagus	2 (6.1)	0	-	0.830
Stomach	13 (39.4)	10 (50.0)		
Small intestine	6 (18.2)	3 (15.0)		
Colon/rectum	2 (6.1)	2 (10.0)		
EGIST-abdominal	10 (30.3)	5 (25.0)		
Size (cm)				
Median (IQR)	9.0 (5.0-13.0)	6.0 (5.3-7.5)	-	0.231
Size				
<5	4 (21.1)	2 (16.7)	1.0	
5-10	7 (36.8)	8 (66.7)	0.4 (0.1-3.2)	0.413
>10	8 (42.1)	2 (16.7)	2.0 (0.2-20.0)	0.554
Predominant cell type	4 (12.1)	7 (35.0)	0.3 (1.0-1.0)	0.079
Epithelioid	29 (87.9)	13 (65.0)	1.0	
Spindle				
Mitosis/50 per hpf				
Median (IQR)	2 (2-7)	4.0 (1.5-9.0)	-	0.597
Mitosis				
<5	18 (54.5)	10 (50.0)	1.0	
5-10	9 (27.3)	5 (25.0)	1.0 (0.3-3.8)	1.000
>10	6 (18.2)	5 (25.0)	0.7 (0.2-2.7)	0.575

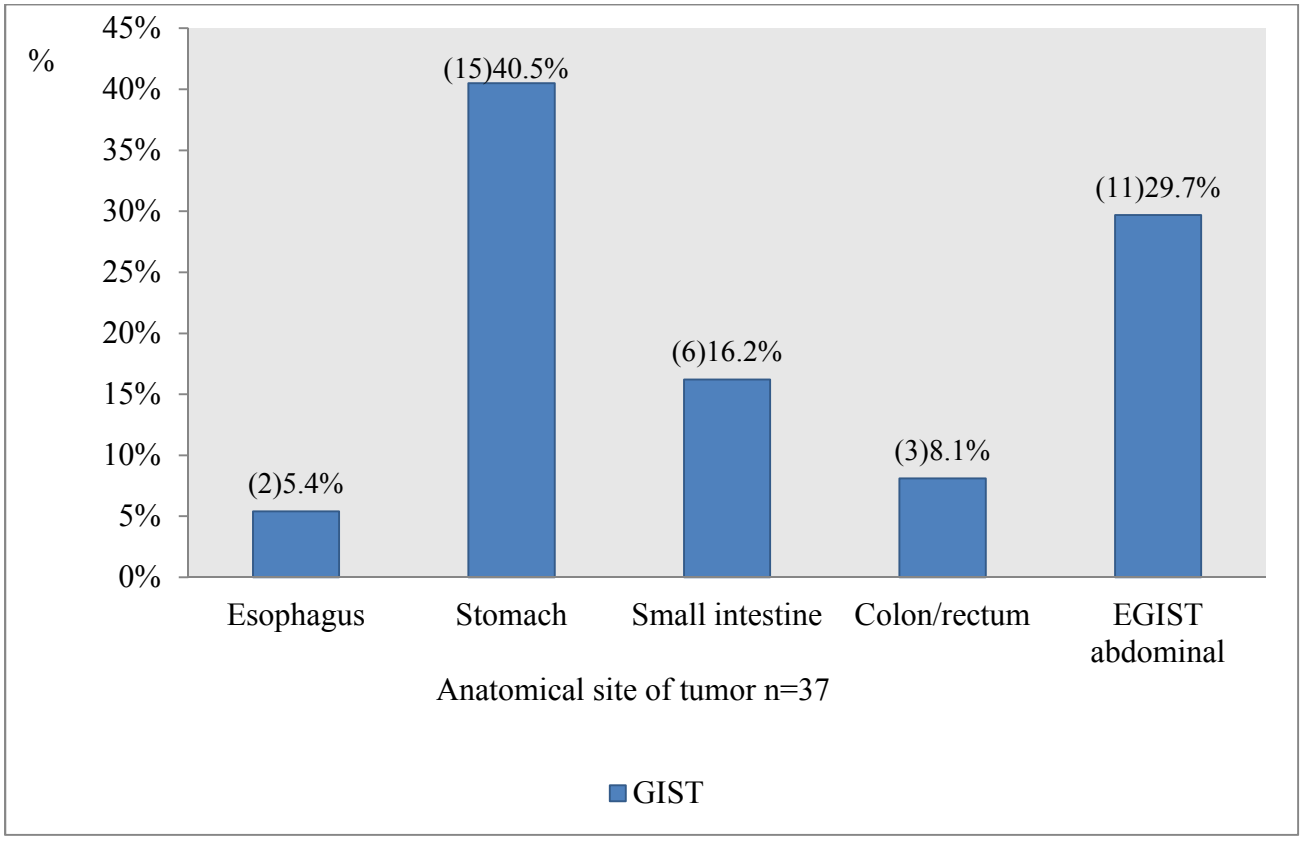
CD 117 EXPRESSION

Tumors in males had more odds of being positive for CD 117 (OR 4.6) when compared to those in females. This was statistically significant p value=0.012.

Epithelioid tumors had less odds of being CD 117 positive OR=0.3 when compared with spindle cell tumors that had equal odds, (OR1) of staining positive or negative with CD 117, however this was not statistically significant p value 0.079.

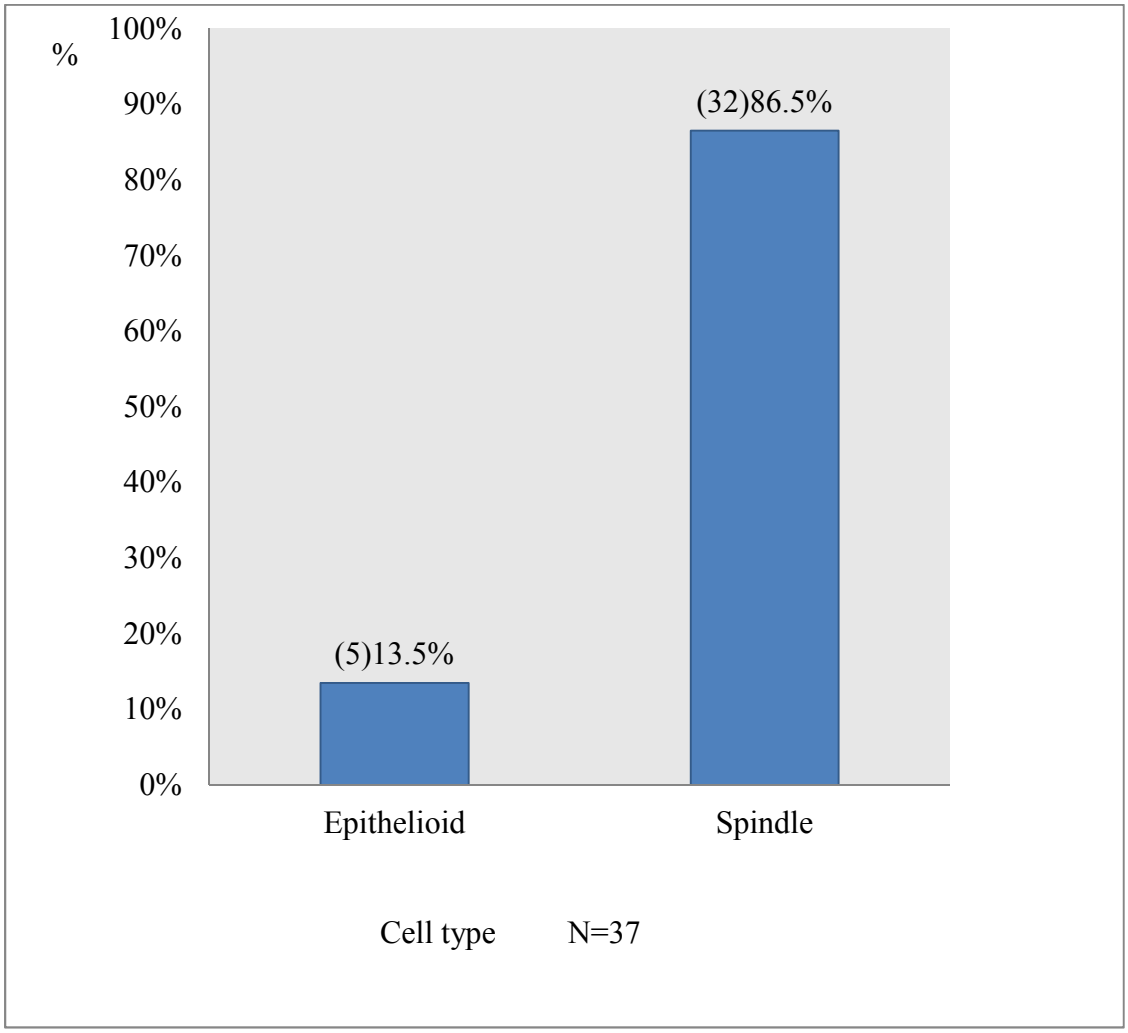
Mitoses and tumor size had little correlation with CD 117 positivity or negativity.

Figure 3: Anatomical site GIST Tumors



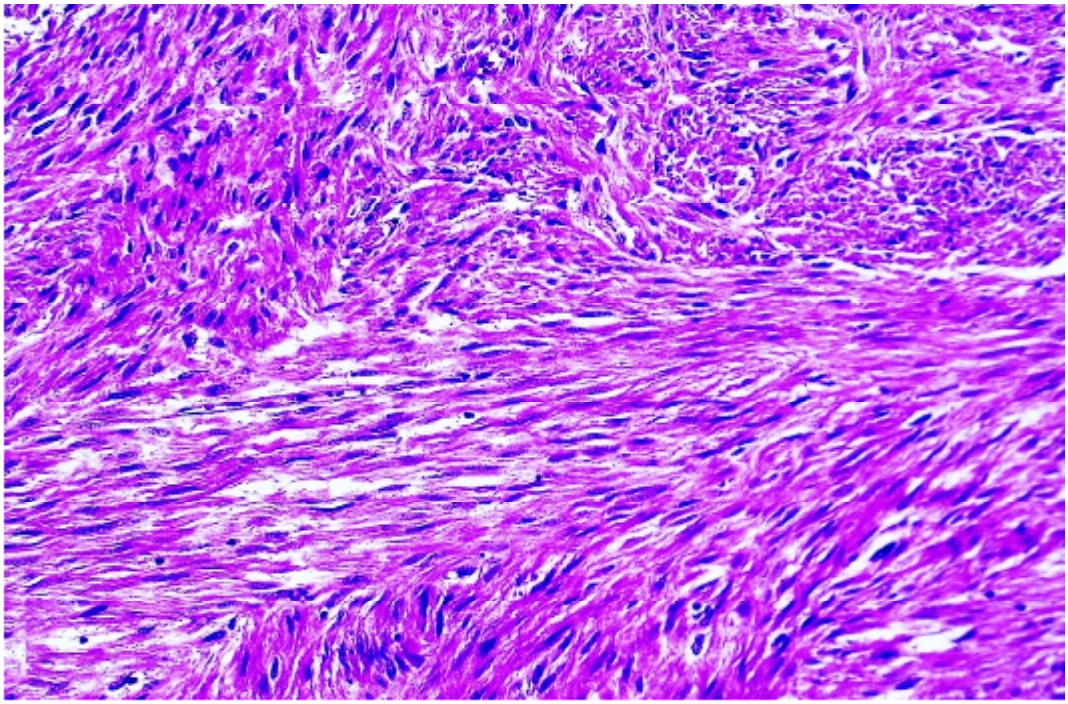
The most frequent anatomical site among GISTs was the stomach accounting for 15 (40.5%) of the cases, while esophageal tumors were the least frequent with only 2 (5.4%) cases.

Figure 4: Predominant cell type on H&E slides of GISTs (Cases positive for DOG 1, CD117 or both)



Most tumors had a spindle cell in morphology, 32 (86.5%), only 5 (13.5%) cases were epithelioid.

Figure 5: A Spindle Cell GIST

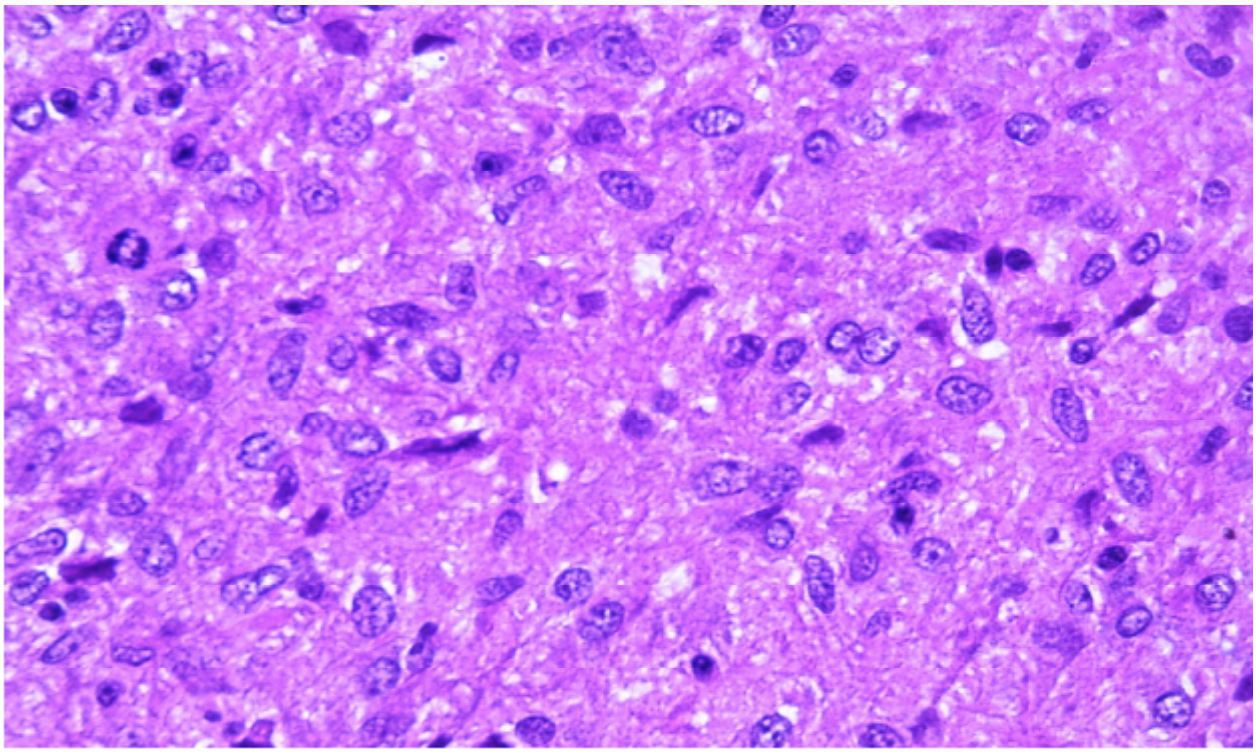


Case: G/029/11

Stain: H&E

Magnification: X40

Figure 6: An Epithelioid GIST

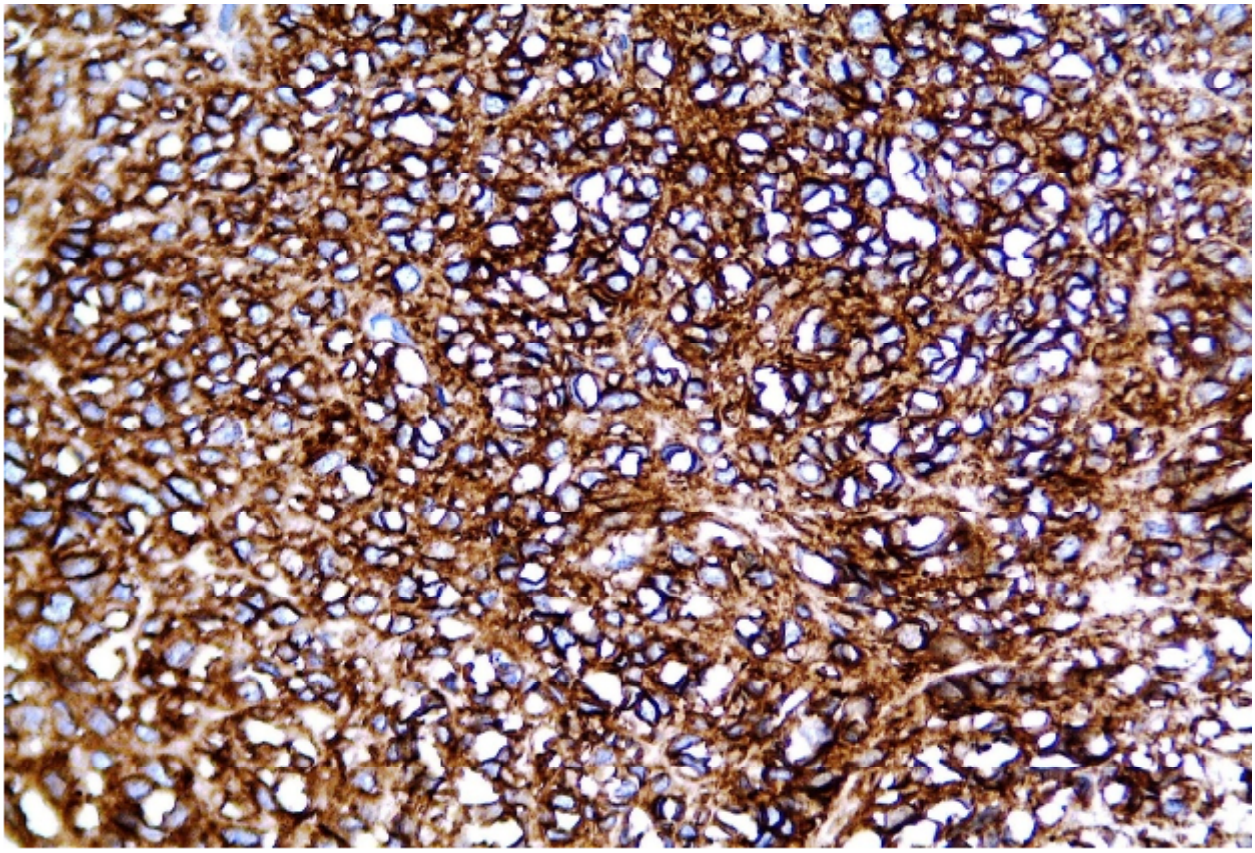


Case: G/003/11

Stain: H&E

Magnification: X63

Figure 7: A DOG 1 Positive Case



Case: G/003/11

DOG 1 IHC score of 3+ as shown here is characterized by complete and strong membranous staining.

IHC Stain: DOG 1(K9) antibody

Magnification X40

Table7: Characteristics of GISTS and non-GISTS

Variable	GIST n=37	Non-GIST n=16	OR (95% CI)	P value
Age				
Median (IQR)	52.3 (40.0-63.5)	56.0 (29.0-68.0)	-	0.812
Mean	51.6	41.1		
Sex				
Male	21 (56.8)	4 (25.0)	3.9 (1.1-14.5)	0.033
Female	16 (43.2)	12 (75.0)	1.0	
Anatomical site of tumor				
Esophagus	2 (5.4)	0		
Stomach	15 (40.5)	8 (50.0)	-	0.983
Small intestine	6 (16.2)	3 (18.8)		
Colon/rectum	3 (8.1)	1 (6.3)		
EGIST abdominal	11 (29.7)	4 (25.0)		
Size (cm)				
Median (IQR)	8.5 (4.8-13.0)	6.0 (5.5-8.5)	-	0.522
Size				
<5	5 (25.0)	1 (9.1)	1.0	
5-10	7 (35.0)	8 (72.7)	0.2 (0.0-1.9)	0.150
>10	8 (40.0)	2 (18.2)	0.8 (0.1-11.3)	0.869
Predominant cell type				
Epithelioid	5 (13.5)	6 (37.5)	0.3 (0.1-1.0)	0.068
Spindle	32 (86.5)	10 (62.5)	1.0	
Mitosis/50 per HPF				
Median (IQR)	2 (2-7)	5 (1.5-13.0)	-	0.322
Mitosis				
<5	21 (56.8)	7 (43.8)	1.0	
5-10	10 (27.0)	4 (25.0)	0.8 (0.2-3.5)	0.804
>10	6 (16.2)	5 (31.3)	0.4 (0.1-1.7)	0.220

CHARACTERISTICS OF NON GIST CASES

For the sixteen non-GIST cases half (8) were in the stomach, 4(25%) were in the mesentery/omentum, 3 in the small gut and 1 in the colo-rectum. Only one of the stomach non-GISTs was in a male, the other seven non-GISTs in the stomach were in females. Four (25%), of non-GISTs were in the mesentery or omentum.

9. DISCUSSION

Tyrosine Kinase Inhibitors are very expensive; the approximate cost of Imatinib; one of the most commonly used Tyrosine Kinase Inhibitors, per month is Kshs.400, 000 or US$ 5334. In Kenya Novartis together, with Axios and the Max foundation have availed Tyrosine Kinase Inhibitors through the Glivec International Patient Assistance Programme (GIPAP) to GIST and Chronic Myeloid Leukemia patients. This is done at little cost to the patients; patients pay the cost of a confirmatory test for GIST e.g. CD117 or DOG 1 only. The cost of CD 117 or DOG 1 in Kenya is about Kshs.6000. As of 2009, there were 28 GIST patients enrolled in the GIPAP program in Kenya.(7)

Therefore, availability of targeted treatment for GIST demands accurate tumor diagnosis in spite of relative rarity of this tumor. DOG 1 improves diagnostic sensitivity and specificity when included in an Immuno-Histochemical panel for GIST, DOG 1 Immuno-Histochemical stain alone is also the most specific and sensitive marker overall among the most commonly used Immuno-Histochemical markers. The expression of DOG 1 has been correlated with a number of factors among them tumor Histo-Morphological characteristics on a routine H&E histological preparation.(13, 38 & 4)

In this study most GISTs had a predominantly spindle cell morphology. This is the pattern most frequently described in other studies too.(34,38 & 25).However the relative frequency of spindle cell tumors in this study is higher when compared to that in the other studies while that of epithelioid tumors is lower. In this study spindle cell GISTs were 86.5%, while epithelioid GISTs were 13.5%. In the other studies the frequency of spindle cell GIST ranges between 65-70% while that of epithelioid GISTs ranges between 25-30%. The findings of this study come

closest to the study done by Kang et al. that found the frequency of spindle cell GISTs to be 88% and that of epithelioid GISTs to be 11.9%. (38). However, Kang et al. cases were GISTs confirmed by mutation status analysis and tyrosine kinase phosphorylation status. In the current study mutation status was not assessed. Mutation negative GISTs have been estimated to be very few with a frequency of 0.9%, (31) indicating that the findings of Kang et.al (38) are still comparable to the findings of this study.

The initial method used in recruiting the cases determines the relative frequency between the different cellular patterns. This study has a lower frequency of epithelioid tumors than expected in true GIST cases. This can be attributed to GISTs with an epithelioid morphology being wrongly categorized as other neoplasms especially those of epithelial origin. GISTs metastatic to the abdomen tends to have an epithelioid morphology which has been described to indicate malignant transformation, further causing confusion with metastatic carcinoma.(39) Spindle cell GISTs are thus disproportionately higher in this study due to the low index of suspicion of a GIST diagnosis in an epithelioid tumor. In this study, the median age of 52.3 and a mean age of 51.6 years in Gastro-intestinal stromal tumor patients at diagnosis are similar to that described by another study.(40) However, two, Surveillance End Epidemiological Result (SEER) based studies found a mean age of 62.6 and 63 years respectively, the median age was 64 years in both studies .(41& 42) These findings suggest that GISTs occur approximately a decade earlier in the population under study. This apparent early age at diagnosis may be attributed to the fact that tumor behavior, including the age at which it occurs, is determined by genetic differences that exist in people of different races and ancestry. . Early age of occurrence and a more aggressive clinical course in blacks is well known in other cancers for instance, prostatic carcinoma.

Prostatic carcinoma occurs in the fourth decade in American blacks as contrasted to the sixth decade in American whites.(43)

Although difference in age at diagnosis between races was not studied in the SEER data, a more aggressive course of disease was noted in the American blacks when compared to the American whites. The blacks had more tumor related deaths and a shorter survival. (41) However these SEER data based studies were done before widespread use of CD117 IHC for the diagnosis of GIST i.e. before the year 2000, and may have included non- GIST patients.

Non-GISTs tend to occur in older patients, these findings are supported by this study; the median age of 56 years is almost four years older than the GIST cases. GISTs seem to occur earlier than non-GISTs in this population; this however needs to be confirmed by large scale epidemiological studies

Females, 28(52.8%), predominated when GISTs were considered together with the non-GISTs. These proportions changed after doing CD117 and DOG 1 Immune-Histochemistry where the ratio of a slight male preponderance emerged. The males among GIST cases were 56.8% while females were 43.2%,this sex distribution that shows a slight male predominance is similar to that recorded by SEER-Males 52.8%, females 47.6%.(42) However those studies that recruited cohorts with special characteristics found significant differences in the sex distribution even in GIST confirmed cases. For instance in a cohort that recruited the armed forces veterans, males were markedly more than females,(25) while a study that studied syndromic GIST found that females outnumbered males by far (2). It is assumed that this cohort had no special attributes and the finding of a slight male preponderance agrees with that found by others and reflects the expected ratio in the general population.

The findings of females predominating before the cases were subjected to immunohistochemistry may be attributed to the fact that there is a wider range of possible diagnoses in the female abdomen when compared with male abdomen. Metastatic tumors in the abdominal cavity without a known primary are more common in the females than in the males.(44) This can be interpreted to mean that the females in this cohort with a non-GIST had a primary locally advanced tumor from nearby organs such as the ovary, and the uterus.

Mitoses were higher in both CD117 and DOG 1 positive cases as compared to the negative cases. These findings agree with those of other studies that found higher mitotic counts in other tumors such as carcinomas when compared to the GISTs.(6)

DOG 1 expression by immunohistochemistry described in this study is much lower than what most researchers have described. This may be attributed to aspects of sample storage and processing that may affect antigen stability. This is strongly felt to be the case as the expression of CD 117 was similarly affected. Aspects of sample storage and processing that may decrease the success of antigen retrieval include use of non-buffered formalin, inadequate or prolonged fixation periods and poor storage especially for prolonged periods.(45)

Among the factors shown to affect DOG 1 expression include anatomical site of tumor, cell type and tyrosine kinases mutations, but DOG 1 is not affected by treatment with tyrosine kinase inhibitors .(4)

DOG 1 expression is dependent on intrinsic tumor properties, specimen handling and on the type of antibody used. There are three DOG1 monoclonal antibodies used in the diagnosis of GIST namely: DOG 1.1, DOG 1.3 and DOG 1 Clone 9(K9). DOG 1 K9 antibodies used in this study are

the most sensitive among the available antibodies.(46)

DOG 1 positivity correlates with male gender, while negativity correlates with tumors with high mitotic counts of more than 10/50hpf. Non-GISTs are highly aggressive fast growing tumors with high mitotic counts. There is also the possibility that large tumors may have lost their immune-reactivity as part of tumor progression. The positive correlation of DOG 1 expression with the male gender may be due to the narrower spectrum of intra-abdominal tumors in males when compared to females.

Double negative GISTs in this study were 16 (30.2%), which is much higher than those found by Lopes et al. or by Novelli et al. of 0.9% and 1.6% respectively.(36 & 31) This can be interpreted to mean that most tumors reported as GISTs in this study represent other biological entities as double negatives GIST are rare. It may also mean that significant antigen deterioration had occurred as CD117 was found to be similarly affected.

In this study, the distribution of GISTs in tubular GI is similar to that reported in the USA and Europe, the most common sites being the stomach and the small gut. Tumors of the colon, rectum and esophagus are relatively rare. (4) The frequency of extra gastrointestinal stromal tumors diagnosed in this study was very high with (29.7%).This is contrasted to other studies that find rates of between 0-8%. (47) This may be attributed to the likelihood in this study setting of late presentation.. This means that most of these tumors are metastatic GISTs rather than true primary eGISTs. All the eGISTs found in this study were intra-abdominal. No extra-abdominal EGIST were reported in this study, this can be explained by the rarity of these tumors. Before the year 2010, when Long et al. described the first case of a pleural eGISTs, these tumors were virtually unknown. (11)

Mesenteric and omental tumors pose difficulties due to a wide range of differential diagnoses at these sites, including metastatic tumors. Four (25%) of tumors initially diagnosed as GISTs in the mesentery and omentum, were negative with both DOG1 and CD117.They are likely to be non-GIST metastatic to the abdomen. The most common metastatic tumors to the abdomen include ovarian in females and lung in males.(38)

LIMITATIONS OF THE STUDY

1. The study was done on archival tissues and specimen processing including fixation, and storage could have affected the stability of the antigen and therefore DOG 1 and CD117 expression.

2. Accrual of cases took too long due to rarity of GISTs.

CONCLUSIONS

The following conclusions can be drawn from the results of this study:

1. The spindle cell morphology was the most common morphological type amongst the GISTs studied.

2. Although DOG 1 labeled more cases than CD117 a significant subset of GISTs that had not been initially diagnosed were diagnosed when both markers were used together.

3. Almost a third of cases studied were negative with DOG 1 and with CD117.

RECOMMENDATIONS

1. Epidemiological long term prospective studies should be done to ascertain the clinical behavior of GISTs including age of onset in Kenya.

2. DOG 1 and CD117 should be used together to diagnose more GIST cases.

3. An immune-histochemical study using a broad panel of antibodies should be done on the non-GIST cases in this study to determine their diagnoses.

4. The apparent high rates of metastatic GISTs needs to be addressed by advising clinicians to have a high index of suspicion and suspect and biopsy GISTs early before the tumors metastasizes.

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APPENDICES

APPENDIX 1: DATA CAPTURE SHEET.

PROJECT TITLE:

DOG 1 EXPRESSION IN GASTROINTESTINAL STROMAL TUMORS REPORTED IN NAIROBI: AN IMMUNOHISTOCHEMICAL EVALUATION OF A NOVEL ANTIBODY IN COMPARISON TO MORPHOLOGY & CD117 STAINING

DATE……

PATIENTS BIO-DATA

1. PATIENT'S NAME----------

2. IN PATIENT NUMBER……

3. LABORATORY NUMBER……..

4. STUDYNUMBER……

5. STUDY SITE

THE KENYATTA NATIONAL HOSPITAL-	1
THE NAIROBI HOSPITAL	2
LANCET	3

6. AGE (specify in completed years)…….

7. SEX

M [1]

F [2]

REVIEW OF MORPHOLOGY

GROSS DESCRIPTION

PREVIOUS MICROSCOPIC FEATURES

CURRENT MICROSCOPIC FEATURES

9. ANATOMICAL SITE OF TUMOR

ESOPHAGUS [1]

STOMACH [2]

SMALL INTESTINE [3]

COLON/RECTUM [4]

eGIST ABDOMINAL [5]

eGIST EXTRA- ABDOMINAL [6]

10. SIZE (CM) --------

<5	1
5-10	2
>10	3

11. PREDOMINANT CELL TYPE:

EPITHELIOD	1
SPINDLE	2

12. MITOSIS/50 PER HPF----------

<5 [1]

5-10 [2]

>10 [3]

IMMUNO-HISTOCHEMISTRY

14. CD 117 EXPRESSION

POSITIVE [1]

NEGATIVE [2]

15. DOG -1 EXPESSION SCORE

0(absence of staining)	1
+1(equivocal staining)	2
+2(moderate membranous staining)	3
+3(strong complete staining)	4

0 &1 negative,2&3 positive

APPENDIX 2: DOG 1 AND CD 117 IHC PROCEDURE

Leica Bond -Max immuno- stainer Machine was be used. After mounting the sections negatively charged slidesand loading them into the machine, IHC staining for DOG 1 and CD117 consisting of a series of the following steps was carried out.

The only difference was in step number 8 where instead of incubating with DOG1 primary antibody the slides were incubated with CD 117 as the primary antibody.

1. Rinse slide twice with Bond wash solution and twice with tris EDTA buffer.

2. Incubate with tris EDTA buffer for 20 minutes at 100 ^{0}C.

3. Incubate further with the tris EDTA buffer for another 12 minutes at room temperature. Steps 2&3 are also referred to as heat induced epitope retrieval (HIER). HIER describes a process of heating formalin-fixed paraffin-embedded tissue sections for improved immunoreactivity of tissue antigens with their specific antibodies.

Following antigen retrieval;

4. Rinse three times with bond wash solution.

5. Wash with bond wash solution for three minutes.

6. Block with peroxide for 5 minute

7. Rinse three times using bond wash solution at 35^{0}C.

8. Incubated with DOG 1 clone K9 or CD117 as appropriate for 15 minutes.

9. Rinsed once with bond wash solution.

10. Applied post primary antibody for 8 minutes and washed with bond wash solution thrice each wash taking 2 minutes.

11. Applied Polymer for 8 minutes and washed with bond wash solution twice each wash taking 2 minutes.

12. Rinsed with deionized water.

13. Rinsed with mixed DAB refine then incubated the sections with mixed DAB refine for 10 minutes; DAB acts as the chromogen.

14. Rinsed with deionized water.

15 Stained with hematoxylin for 5 minutes.

16. rinsed with deionized water,

17Rinsed with bond wash solution,

18. Rinse with deionized water

19. Air-dry.

20. Visualize DOG1 and CD117 expression with a light microscope.

21. Scored DOG1 and CD117 expression on the data sheet

APPENDIX 3-CRITERIA FOR ESTIMATION OF MALIGNANT POTENTIAL OF GIST

Table 2

Modified Criteria for Estimation of Malignant Potential of GIST

National Institutes of Health	Modified
Very Low-Risk	**Risk Level I**
< 2 cm, < 5/50 HPF	< 5 cm, < 5/50 HPF
Low-Risk	**Risk Level II**
2–5 cm, < 5/50 HPF	< 5 cm, < 6–10/50 HPF 5–10 cm, < 5/50 HPF
Intermediate-Risk	**Risk Level III**
< 5 cm, > 5/50 HPF 5–10 cm, any mitosis	< 5 cm, > 10/50 HPF 5–10 cm, < 6–10/50 HPF > 10 cm, < 5/50 HPF
High-Risk	**Risk Level IV**
> 5, > 5/50 HPF > 10 cm, any mitosis > 10/50 HPF, any size	> 5 cm, > 10/50 HPF

GIST = gastrointestinal stromal tumor; HPF = high-power field.

Based on data from Huang et al.[17]

APPENDIX 4 -HARRIS HAEMATOXYLIN AND EOSIN STAINING PROCEDURE

Principle of the stain

The mordant forms a lake on the tissue. It is on the lake that the stain gets attached thus coloring the cell nuclei. The nuclei having an affinity for the basic radical in the dye retains the color even after treatment with 1%acid alcohol. Eosin stains the cytoplasm as a counter stain

Staining technique

1. Bring section to water

2. Stain in Harris haematoxylin for 5 minutes

3. Rinse in tap water

4. Differentiate in 1%acid alcohol, 3dips

5. Rinse in tap water

6. Blue in Scotts tap water for 30 seconds or in running tap water for 10 minutes

7. Counter stain in Eosin for 5 minutes

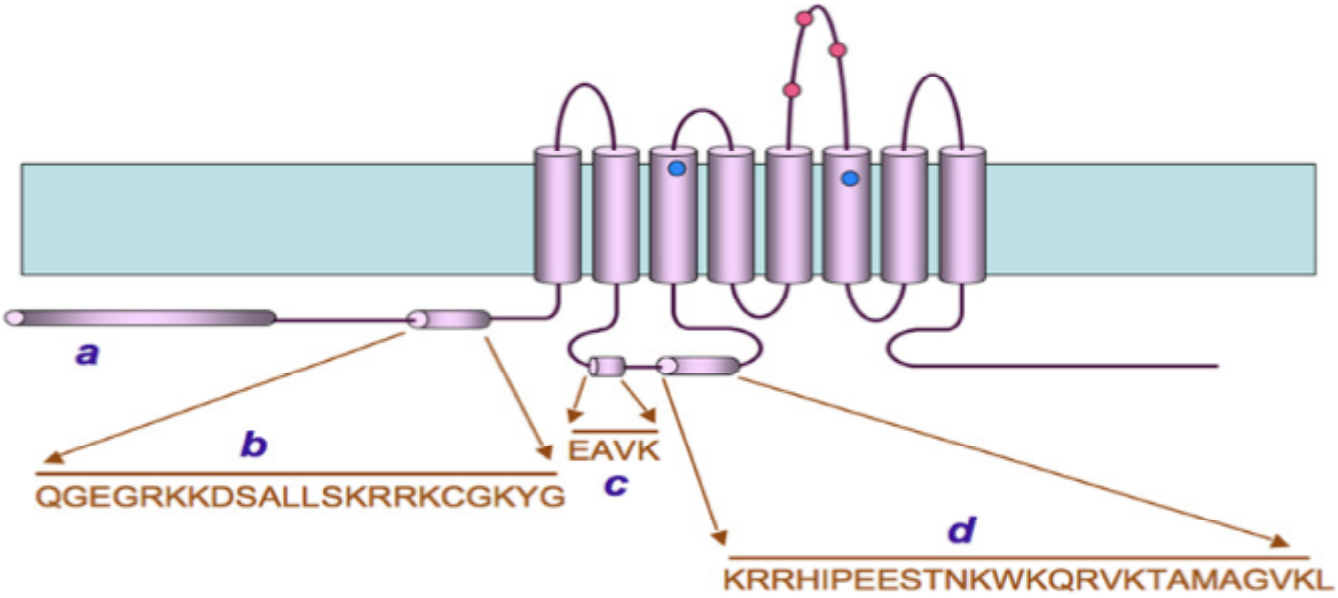
8. Rinse in tap water to remove excess eosin followed by 70%ethanol to obtain the desired shades of red and pink.

9. Dehydrate in the 3 changes of absolute alcohol

10. Clear in 3 changes of Xylene

11. Mount with D.P.X

APPENDIX 5: STRUCTURE OF DOG1



APPENDIX 6: ETHICAL BOARD APPROVALS

THE NAIROBI HOSPITAL

Our Ref: NH/ADMIN/CEO/24/02/11

24th February, 2011

Dr. Josephine Muthoni Muthami
University of Nairobi
Department of Human Pathology

Dear Madam,

RE: STUDY PROPOSAL: EXPRESSION IN GASTROINTESTINAL STROMAL TUMOURS REPORTED IN NAIROBI – AN IMMUNOHISTOCHEMICAL EVALUATION OF A NOVEL ANTIBODY IN COMPARISON TO MORPHOLOGY AND CD117 STAINING

Thank you for submitting your study proposal requesting for the services of our Pathology Laboratory Department.

I am happy to inform you that, we are willing to support you in this study. Please get in touch with our Chief of Pathology, Dr. Bessie Byakika on Ext. 5700 to progress this matter.

Yours faithfully,
For: KENYA HOSPITAL ASSOCIATION

Dr. Cleopa Mailu, EBS
CHIEF EXECUTIVE OFFICER

CC. Dr. Bessie Byakika
Chief of pathology

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Ref: KNH-ERC/ A/644

23rd November 2010

Dr. Josephine Muthoni Muthami
Dept.of Human Pathology
School of Medicine
University of Nairobi

Dear Dr. Muthami

Research proposal: "DOG-1 Expression in Gastrointestinal Stromal Tumors Reported in Nairobi: An imunohistochemical Evaluation of a Novel Antibody in Comparison to morphology & CD117 staining" (P300/09/2010)

This is to inform you that the KNH/UON-Ethics & Research Committee has reviewed and **approved** your above revised research proposal for the period 23rd November 2010 - 22nd November 2011.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimens must also be obtained from KNH/UON-Ethics & Research Committee for each batch.

On behalf of the Committee, I wish you a fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of the data base that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

PROF A N GUANTAI
SECRETARY, KNH/UON-ERC

c.c. The Deputy Director CS, KNH
The HOD, Records, KNH
The Dean, School of Medicine, UON
The Chairman, Dept. of Human Pathology, UON
Supervisors: Dr. L. W. Muchiri, Dept.of Human Pathology, UON
Dr. G.W. Kiarie, Dept.of Clinical Medicine & Therapeutics, UON
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