cKIT Mutations in GISTs and Other Malignancies in Thailand

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ABSTRACT

Objective: 1) To further identify tumors other than GISTs; 2) To study cKIT gene mutation for proper Imatinib treatment.
Methods: Fresh tumor tissue samples were obtained from: 2 tissue samples of GISTs; 1 parotid cancer; 1 soft tissue sarcoma; 1 chondrosarcoma; 1 liposarcoma and 1 pure seminoma. The fresh tissues were divided into two portions: one for histopathological diagnosis and cKIT protein study, the other for DNA extraction and cKIT gene mutation study.
Results: The authors described the gastrointestinal stromal tumors (GISTs) and other malignancies associated with positive cKIT protein in Thailand. We also describe briefly the methodology and molecular techniques used in the study of the mutation of cKIT gene in our laboratory using DNA sequencing method. Examples of positive cKIT protein and deletion mutation as well as missense (point mutation) mutations from our own patients are shown. Mutation study of exons 9, 11, 13 and 17 of cKIT gene in all 7 patients was carefully done to assist us in treating those patients with Imatinib therapy.
Conclusion: Mutation study of cKIT gene could be done routinely in our molecular laboratory in Thailand. We recommend cKIT gene mutation study be done in every case of GIST and tumor with positive cKIT protein other than GIST prior to initiation of Imatinib therapy.

Keywords: cKIT; cKIT mutation; cKIT protein; GISTs; Imatinib and non-GISTs

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KIT is a normal cellular gene with function relating to embryogenesis and growth of many stems cells.[1,2] Human KIT gene is located at 4q11-21 site on long arm of chromosome 4 and KIT gene has 21 exons with mRNA of 5Kb size and producing 976 amino acid residues.[3] KIT protein is a 145 Kilodalton receptor protein located on the surface of cell and is one of many members of the subclass III family of receptor tyrosine kinases. KIT is closely related to PDGFR (Platelet-Derived Growth Factor Receptor) and M-CSF (Macrophage Colony Stimulating Factor).[1-4] The ligand for KIT is stem cell factor (SCF: also known as Steel Factor), which promotes the dimerization and autophosphorylation of KIT receptor. The resulting phosphorylated tyrosine residues provide binding sites for signal molecules that contain SH2 domain, including Phosphatidylinositol 3- Kinase (PI-3K) (chemical molecule, participating in a process of signal transduction). In turn, these signaling molecules activate a variety of downstream targets. KIT signaling is critical for normal development and survival of hematopoietic progenitor cells, mast cells, melanocytes, interstitial cells of Cajal (intestinal pacemaker cells) and germ cells.[1-4] Activating mutations of KIT tyrosine kinase are common in GISTs (Gastro-Intestinal Stromal Tumors) and mast cell leukemia.[5-9] Specific mutation at a specific site of KIT gene in a tumor appears to be associated with Imatinib's response of the tumor.[9-12] We have a special interest in KIT gene and its activating mutations in GISTs as well as other malignancies because it may represent the only way to offer the appropriate and targeted treatment for patients with such malignancies.

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MATERIALS AND METHODS

Patients with tissue diagnosis of GIST who came to us for consultation of the treatment in Thailand will be subject to:
1) histopathological diagnosis of GIST;
2) following the pathological diagnosis, the tissue paraffin block will be tested for immunohistochemical study; and for our purpose, they will be tested for cKIT gene protein overexpression.

Fresh tumor tissues were obtained from: 2 samples with GIST (Gastro-intestinal Stromal Tumor), 3 sarcomas (chondrosarcoma, soft tissue sarcoma and liposarcoma), 1 malignant parotid tumor and 1 seminoma. Each tumor tissue was divided into two portions: one for tissue tumor diagnosis, followed by immunohistochemical study of cKIT, and the other for DNA extraction.

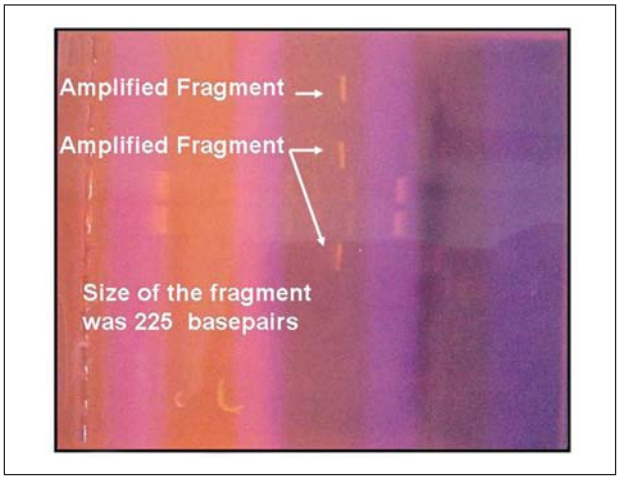


Fig 1. Picture of 2% agarose gel showing three bands of amplified fragments. Each fragment has 225 basepairs of exon 9 of cKIT gene.

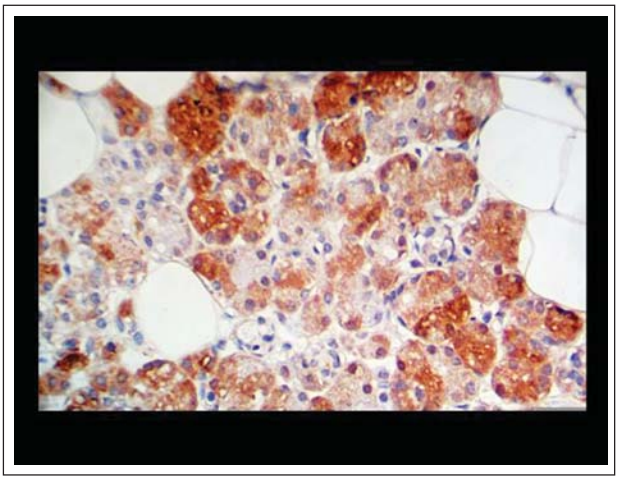


Fig 3. Picture of adenocarcinoma of the parotid gland stained by immunohistochemical study. The appearance of the cancer showed glandular feature with prominent nuclei and cytoplasmic stained dark brown, indicative of positive cKIT protein (CD117).

Method

1) DNA preparation We were able to obtain fresh tumor tissue following the biopsy or operation from 7 patients. We applied a standard technique for DNA extraction from fresh tissue without any difficulty. The method for DNA preparation has been published elsewhere.[13]

2) Polymerase Chain Reaction (PCR) We used PCR machine by Perkin Elmer 2400 throughout the study.[13] Taq DNA polymerase was purchased from Amersham Biosciences Thailand or Bio-Active Co. As for cKIT gene, we have already known both the forward and reverse primers for all 21exons, but for practical purpose of the study, PCR would be performed only on exon 9, exon 11, exon 13 and exon 17. Using proper primer[8,15] and proper technique applicable to Perkin-Elmer thermal cycler, PCR reagents and disposables, we applied 35 cycles of 30 seconds at 95C, 45 seconds at 66C and 90 seconds at 72C to the samples. The PCR products were always tested for amplified fragments on 2% agarose gel containing ethidium bromide. A clean single band of amplified PCR product was usually seen as shown in Fig 1.

3) DNA Sequencing The strategy for Thermal Cycle sequencing involved the removal of leftover primers and the application of enzymatic method of Sanger et al.[14] Today, we have been using automated DNA sequencer with the help of fluorescent dye terminator labeling method. Two automated DNA Sequencers, ABI Prism system and ALF Express II system have been used throughout our study.

Specific coding regions of exon 9, 11, 13 and 17 of cKIT gene have been studied in details. From many previous reports, it would seem unlikely to find any activating mutations of cKIT gene in exons other than that mentioned above.[7-9]

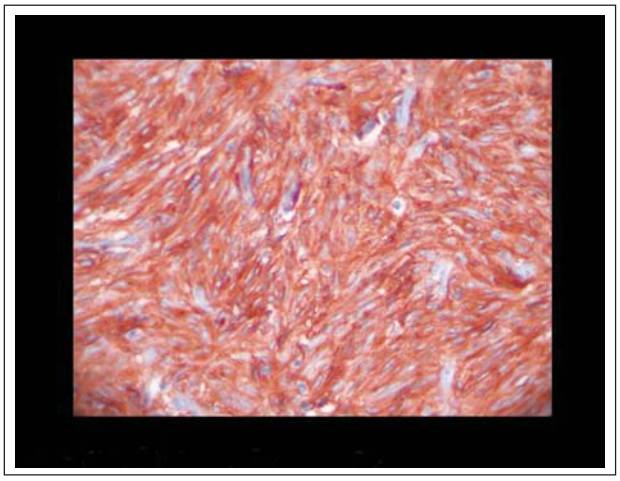


Fig 2. Picture of GIST case 2. The tumor is characterized by spindle cells with large blue nucleoli. The cytoplasm stained deeply brown, characteristic of cKIT protein (CD117) by immunohistochemical staining.

RESULTS

1) Immunohistochemical study

Tissues obtained from all 7 patient-samples were first histopathologically studied with Hematoxilin and Eosin

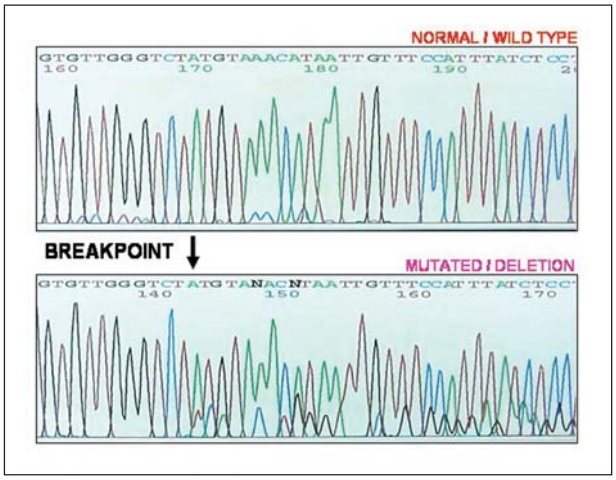


Fig 4. Picture of DNA sequence of part of exon 11 of cKIT gene. Upper panel showed DNA sequence of normal DNA. Lower panel showed DNA sequence of DNA from GIST case 1. Observe the mutation deletion type at the dark line indicated. Base sequence **G** (Guanine) showed black color: Base sequence **T** (Thymine) showed red color: Base sequence **A** (Adenine) showed green color: Base sequence **C** (Cytosine) showed blue color.

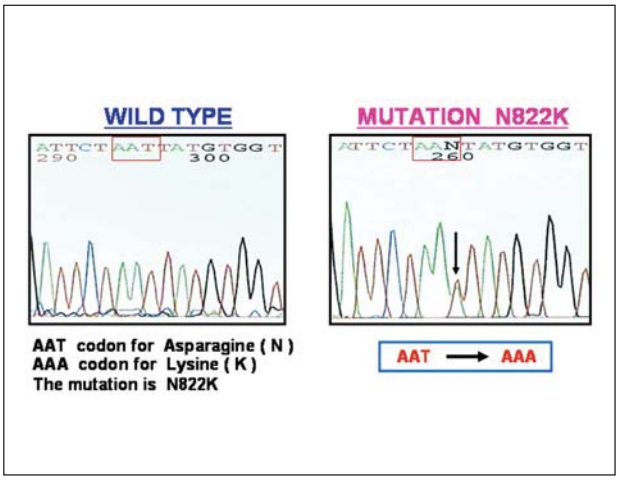


Fig 5. Picture of DNA sequence of part of exon 17 of cKIT gene. Left panel showed normal DNA. Right panel showed DNA from GIST case 2. Observe codon AAT from wild type which codes for asparagines amino acid (N), while codon AAN, showing the N could be A or T. See right panel AAA will code for lysine amino acid (K). AAT changes to AAA. The mutation is called N822K

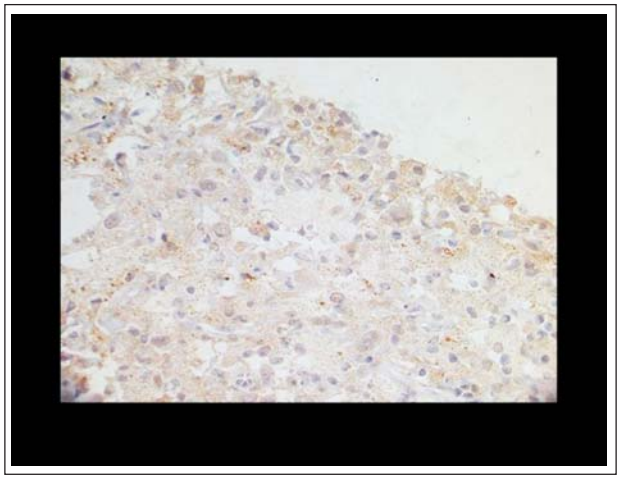


Fig 7. Picture of tumor tissue biopsied from case in figure 6. The tumor tissue was stained by immunohistochemical study of cKIT (CD117). Light brown color was indicative of positive cKIT protein.

stain for diagnosis of malignancy. As shown in Table 1, there were two samples of GISTs, one of adenocarcinoma of the parotid salivary gland, 3 sarcomas and one case of pure seminoma. Once the diagnoses of malignancy were made, the same tumor tissue paraffin blocks would be further studied using immunohistochemical technique with the aid of monoclonal antibody against cKIT protein. They were recognized as positive or negative cKIT protein (CD117). Fig 2 shows a classic example of strongly positive cKIT protein. The cytoplasm and cellular membrane stained dark brown while nuclei stained bluish-gray. Fig 3 shows positive cKIT protein from adenocarcinoma of the parotid salivary gland. Table 1 also shows all tumor samples tested positive for cKIT except liposarcoma sample.

2) cKIT Mutation study

As mentioned earlier, we used DNA sequencing method to identify mutation of the cKIT gene. We examined exon 9 (located at the ECD - extra cellular domain), exon 11 (juxtamembrane part), exon 13 (tyrosine kinase I domain) and exon 17 (tyrosine kinase II domain). Table 1 shows the results of all the 7 samples. All four exons 9, 11, 13 and 17 were studied in all 7 samples.

3) Patients and clinical course

First patient had his GIST tumor diagnosed and removed some three years ago with positive cKIT protein study. The tumor was not totally removed and therefore he was placed on Imatinib 400 mg/day since then. The tumor was carefully studied using DNA sequence as a method to detect the mutation. Exon 9, 11, 13 and 17 were studied and we found a mutation at exon 11. It was deletion type - P548 - N567. Fig 4 shows deletion mutation of this patient.

The second GIST showed strong cKIT protein overexpression in Fig 2. The mutation study shows deletion mutation at exon 11 - Del V555 - I 751 and point mutation at exon 17 - N822K. Fig 5 shows mutation N822K.

The third case was diagnosed as adenocarcinoma of the parotid gland. The tumor was removed and recurred in a short period of time. Fig 3 shows positive tumor staining cKIT. The cKIT mutation study was performed on exon 9, 11, 13 and 17, but it failed to show any mutation

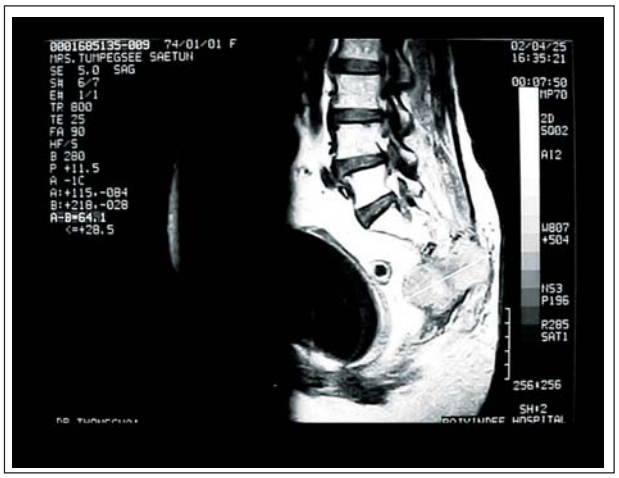


Fig 6. Picture showed MRI study of soft tissue sarcoma case. Observe the large tumor mass at the tip of coccyx.

cKIT MUTATION

TUMOR	Positive cKIT/IHC	Ckit MUTATION				Clinical Response
		Exon 9	Exon 11	Exon 13	Exon 17	
GIST	YES	del P548- N567				YES
GIST	YES	del V555- I571			N822K	YES
Parotid Cancer	YES	NO	NO	NO	NO	NO
Chondro-Sarcoma	YES	-	-	-	K818R	YES
Soft tissue Sarcoma	YES	-	-	-	N822K	YES
SEMINOMA	YES	-	-	-	D816V	YES
Liposarcoma	NO	NO	NO	NO	NO	N/A

TABLE 1. Tabulation of cKIT gene mutation study from 7 cases of various malignancies. Two tissue samples of GISTs. One case of parotid cancer and one case of Seminoma. One of each soft tissue sarcoma, chondrosarcoma, and liposarcoma.

at the exons. The patient was placed on Imatinib 400 mg/day, and failed to show any response.

The fourth case was chondrosarcoma of the right ilium with extension into the right pelvis and metastasis to the lung. The patient had her tumor tested positive for cKIT protein overexpression. The mutation study of cKIT gene showed point mutation at exon 17 codon 818-K818R type. There was no mutation found at other exon 9, 11 or 13. The patient tumor began to show some response with Imatinib (400 mg/day) plus other therapy.

The fifth case had a long history of cancer of the cervix stage III B previously treated by radiation, both external beam therapy and brachytherapy, some 8 years ago. She developed pain in the back while sitting. MRI showed a large 7cm tumor at the coccyx (Fig 6) and biopsy showed malignant fibrous histiocytoma with positive cKIT protein overexpression (Fig 7). She was placed on Imatinib 400 mg/day and had an excellent response. The tumor DNA was further studied for cKIT mutation and it was found that there was a point mutation type N822K only at exon 17.

The sixth case involved a large mediastinal seminoma, proven by biopsied sample, showing pure seminoma. The cKIT protein was found to show overexpression and cKIT DNA study showed D816V mutation only at exon 17. No other mutation was found at other exons. The response of the tumor after Imatinib treatment was excellent.

The seventh case involved a liposarcoma originated from multiple lipomas diagnosed and observed for many years. The tissue from the nape of the posterior part of the neck was studied for positive cKIT protein; but mutation study of tumor DNA failed to show any mutation of cKIT gene, exon 9, 11, 13 and 17. So far, no data regarding clinical response are currently available.

DISCUSSION

GISTs (Gastro-intestinal Stromal Tumors) are the most common mesenchymal tumors of the gastro-intestinal tract. Histopathologically, they contain mostly spindle cells type but sometimes they also have some epitheloid features.[3,4] Previously, they were put together with leiomyomas and leiomyosarcoma, but they are now known to represent a discrete neoplastic entity after the discovery of KIT protein overexpression in this tumor.[4-6] It was believed also that the origin of cells forming GIST should come from interstitial cells of Cajal. It is now known that most GISTs express the KIT receptor tyrosine kinase protein and the majority of GISTs also contain activating mutations within KIT juxtamembrane region (exon 9 and exon 11). Our first GIST case represented a true GIST disease, overexpressing cKIT protein by immunohistochemical study and had a classic deletion mutation at exon 11-del P548 - N567 and should respond to Imatinib 400 mg/day treatment.[10-11,15,17-19] The clinical response was very good and effective. On the other hand, the second patient showed mutation type deletion at exon 11 - del V555 - I571 as well as point mutation at exon 17 - N822K. The deletion mutation represented activating mutation of the cKIT that should show a good response to Imatinib, but N822K is known to represent resistance to the Imatinib treatment.[12,15,16,18] Many reports appear to suggest resistance or late resistance to Imatinib, if and when, mutation such as N822K occurred at exon 17 in the tyrosine kinase region II.[9,12,15,16,18]

The second patient did receive a complete tumor resection and was placed on Imatinib 400 mg/day maintenance dose until we have evidence of recurrence.

In most cases of GISTs reported in the literatures to date, based on a large-scale genetic study, there have been no GISTs with activating mutation in more than one exon of the cKIT gene.[7-9,11,15,18] The findings of two mutations in GIST sample 2 appear to be unique and suggestive of the loss of sensitivity to Imatinib by N822K mutation rather than two types of gain-of-function mutation. It was also reported in many literatures that mutation at exon 17 or exon 13 always represent a loss of sensitivity to Imatinib.[9,12,15,18]

Other KIT-Positive Malignancies[20-24]

Expression of KIT protein has been studied not only in GISTs, but also in other malignancies, such as neuroblastic tumors,[22] pure seminoma,[16] and others.[20-24] It must also be realized that expression of KIT protein alone of the tumors other than GISTs may not be adequate to justify Imatinib treatment. It is our opinion that mutation study of cKIT gene, using DNA sequencing or other methods is an absolute requirement to decide whether or not Imatinib be used for treatment. Our own data here appear to suggest that soft tissue sarcoma, chondrosarcoma and even seminoma have tumor response to Imatinib, if there is a mutation at one of cKIT exons. When there was no cKIT mutation in at least exons 9, 11, 13 and 17, one should not expect a clinical response. Examples of our data showed in adenocarcinoma in the parotid gland and liposarcoma cases. Interestingly, one case had strong cKIT protein in their cancer cells.

CONCLUSION

1) GISTs occur in Thailand often enough and should be regarded as a special class of tumor that require special attention to identify them mainly by immunohistochemical study - cKIT or CD117.
2) Identifying cKIT protein alone may not be adequate to understand and treat this tumor. We recommend those who involve in treating GISTs to identify the activating mutation of cKIT gene, particularly exons 9, 11, 13 and 17 in order to plan Imatinib therapy.
3) Activating mutation of exon 11 or sometimes exon 9 in cKIT gene appear to indicate the sensitivity of GISTs to Imatinib treatment. Likewise, additional missense mutation at exons 13 or 17 appear to indicate resistance to Imatinib therapy.
4) Positive KIT protein by immunohistochemical study alone of tumors, other than GISTs, may not be adequate to suggest Imatinib treatment. We recommend mutation study of cKIT gene before prescribing Imatinib therapy.

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บทคัดย่อ

การกลายพันธุ์ของยีน cKIT ใน GIST และมะเร็งชนิดอื่น ๆ ในประเทศไทย

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**ศาสตราจารย์และผู้อำนวยการ, **ผู้จัดการ, ศูนย์มะเร็งและพันธุกรรมบำบัด, โรงพยาบาลปิยะเวท, กทม. 10320, ประเทศไทย.*

วัตถุประสงค์: โปรตีน cKIT ผลบวกใช้ในการวินิจฉัยโรค GIST (Gastrointestinal Stromal Tumor) ซึ่งเป็นโรคที่พบได้บ่อยที่สุดของ Mesenchymal Tumor ของระบบทางเดินอาหาร การตรวจพบโปรตีน cKIT นี้จะเป็นข้อบ่งชี้ว่าเนื้องอกชนิดนี้ควรจะได้รับการรักษาด้วยยา Imatinib วัตถุประสงค์ของการศึกษานี้ 1. เพื่อตรวจวิเคราะห์ยีน cKIT ในเนื้องอกชนิดอื่น ๆ ที่ไม่ใช่ GIST 2. เพื่อศึกษาการกลายพันธุ์ของยีน cKIT สำหรับการพิจารณาให้การรักษาด้วยยา Imatinib ได้อย่างถูกต้องและเหมาะสม

วิธีการ: ตัวอย่างชิ้นเนื้อที่ได้จากผู้ป่วยประกอบด้วย GIST 2 ราย, มะเร็งต่อมน้ำลาย 1 ราย, มะเร็งของ soft tissue 1 ราย, chondrosarcoma 1 ราย, liposarcoma 1 ราย และ pure seminoma 1 ราย ตัวอย่างชิ้นเนื้อจะถูกแบ่งเป็น 2 ส่วน ส่วนหนึ่งจะนำไปตรวจทาง histopathological และตรวจหาโปรตีนของ cKIT อีกส่วนหนึ่งจะนำไปตรวจหา DNA และศึกษาการกลายพันธุ์ของยีน cKIT

ผลการศึกษา: ผู้เขียนได้อธิบายเกี่ยวกับ GIST และมะเร็งชนิดอื่น ๆ ที่มีความเกี่ยวข้องกับโปรตีน cKIT ที่มีผลบวกในประเทศไทย และยังได้อธิบายถึงวิธีการตรวจหาการกลายพันธุ์ของยีน cKIT อย่างย่อ ๆ ในห้องปฏิบัติการวิจัยที่ใช้เครื่อง DNA sequencing มีตัวอย่างของโปรตีน cKIT ที่มีผลบวกและยีน cKIT ที่มีการกลายพันธุ์ ในผู้ป่วยทั้ง 7 ราย ได้ศึกษาการกลายพันธุ์ของยีน cKIT ที่ Exon 9, 11, 13, และ 17 เพื่อใช้เป็นแนวทางในการรักษาผู้ป่วยเหล่านั้นด้วยยา Imatinib

สรุป: การศึกษาการกลายพันธุ์ของยีน cKIT ควรจะทำทุกรายในห้องปฏิบัติการทางโมเลกุลในประเทศไทย เราแนะนำให้ศึกษาการกลายพันธุ์ของยีน cKIT ในผู้ป่วย GIST ทุกราย และผู้ป่วยเนื้องอกที่มีโปรตีน cKIT เป็นผลบวกก่อนที่จะพิจารณาให้การรักษาด้วยยา Imatinib