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KIT exon 10 variant (c.1621 A>C) germ line characteristics as predictor of GIST patient outcome

Mehdi Brahmi

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UNIVERSITE JOSEPH FOURIER
FACULTE DE MEDECINE DE GRENOBLE

Année 2015

N°

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OF GIST PATIENT OUTCOME**

**POLYMORPHISME NUCLEOTIDIQUE (c.1621 A>C) DE L'EXON 10 DU GENE *KIT* ET
PREDICTION DU RISQUE METASTATIQUE DANS LES GIST**

THESE PRESENTEE POUR L'OBTENTION DU DOCTORAT EN MEDECINE
DIPLOME D'ETAT

Mehdi BRAHMI

Né le 16 août 1985 à Givors

THESE SOUTENUE PUBLIQUEMENT A LA FACULTE DE MEDECINE DE GRENOBLE

Le 28 avril 2015

DEVANT LE JURY COMPOSE DE

Président du jury : Madame le Professeur Mireille MOUSSEAU

Directeur de thèse : Monsieur le Professeur Jean-Yves BLAY

Membres

Monsieur le Docteur Benoît BUSSER

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Monsieur le Docteur Axel LE CESNE

Madame le Docteur Anne MCLEER

UFR de Médecine de Grenoble

DOMAINE DE LA MERCI
38706 LA TRONCHE CEDEX – France
TEL : +33 (0)4 76 63 71 44
FAX : +33 (0)4 76 63 71 70



Affaire suivie par Marie-Lise GALINDO sp-medecine-pharmacie@ujf-grenoble.fr

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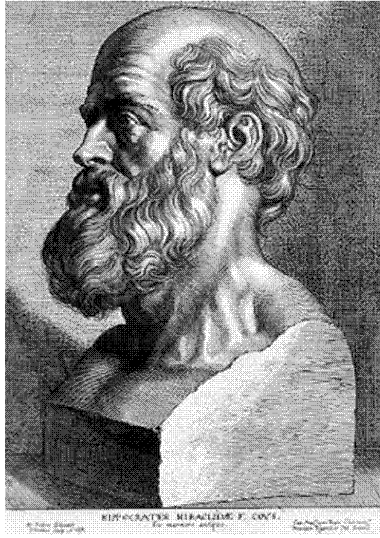
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SERMENT D'HIPPOCRATE

En présence des Maîtres de cette Faculté, de mes chers condisciples et devant l'effigie d'HIPPOCRATE,

Je promets et je jure d'être fidèle aux lois de l'honneur et de la probité dans l'exercice de la Médecine.

Je donnerais mes soins gratuitement à l'indigent et n'exigerai jamais un salaire au dessus de mon travail. Je ne participerai à aucun partage clandestin d'honoraires.

Admis dans l'intimité des maisons, mes yeux n'y verront pas ce qui s'y passe ; ma langue taira les secrets qui me seront confiés et mon état ne servira pas à corrompre les mœurs, ni à favoriser le crime.

Je ne permettrai pas que des considérations de religion, de nation, de race, de parti ou de classe sociale viennent s'interposer entre mon devoir et mon patient.

Je garderai le respect absolu de la vie humaine.

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SOMMAIRE

Ce travail est présenté sous forme d'article scientifique :

***KIT* exon 10 variant (c.1621 A>C) germ line characteristics as predictor of GIST patient outcome**

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***KIT* exon 10 variant (c.1621 A>C) germ line characteristics as predictor of GIST patient outcome**

Mehdi Brahmi¹, Laurent Alberti², Armelle Dufresne¹, Isabelle Ray-Coquard¹, Philippe Cassier¹, Pierre Meeus¹, Anne-Valérie Decouvelaere¹, Dominique Ranchère-Vince¹, Jean-Yves Blay¹.

Author Affiliations

¹Medical Oncology Department, Centre Leon Berard, 28 rue Laënnec, Lyon, France

²Cancer Research Center of Lyon, INSERM UMR 1052, CNRS UMR 5286, Centre Leon Berard, 28 rue Laënnec, Lyon, France

* *Correspondence to:* Mehdi Brahmi, Department of medical oncology, Centre Léon Berard, 28 rue Laënnec, Lyon, France

Key words : GIST, KIT, Single nucleotide polymorphism.

Abstract

BACKGROUND: GIST are the most common mesenchymal tumors of gastro-intestinal tract, characterized by driver mutations in *KIT* or *PDGFRA* gene. Tumor genotype may play a crucial role in clinical management of those tumors. Thus, it is of interest to evaluate the role of inherited genetic polymorphism in GIST patient outcome. The *KIT* exon 10 variant (c.1621 A>C) has been described in several diseases (aggressive fibromatosis, CML, pediatric mastocytosis), with a controversial clinical impact. We investigated a potential biological and clinical significance of the variant in transfected cell lines and in 2 retrospective series of GIST.

METHODS: Transfected NIH3T3 cells were used, stably expressing different expression vectors of *KIT*: WT, mutated and polymorphic (L541). We investigated in our cell lines *KIT* functionality using western blotting analyses. Then, a retrospective translational research program was performed on tumor and blood samples of patients with GIST. The detection of the SNP was performed by Competitive Allele-Specific Taqman® PCR technology. Chi-2 analyses were performed to search for a correlation between *KIT* status, metastatic status at diagnosis and rate of relapse. Progression free (PFS) and overall survival (OS) were compared by log-rank test after Kaplan-Meier analysis.

RESULTS: In L541 cell lines, *KIT* protein has a spontaneous phosphorylation status comparable to WT, but display an activation pattern of AKT and ERK1/2 similar to that of the mutated forms of the receptors in the presence of CSF. *KIT*^{L541} share some of the biochemical properties of the mutated *KIT* receptors. Overall, 24 of 109 patients (22%) harboured *KIT*^{L541}, showing no statistical difference with healthy blood donors. The variant

KIT^{L541} was correlated to metastatic status at diagnosis and lower RFS at 5 years. PFS and OS at 5 years of the 2 groups were not different.

CONCLUSION: Our results support a poor prognosis value of *KIT*^{L541} in patients with GIST.

Key message

This is the first case-control study that explore the prevalence and the potential biological and clinical significance of *KIT* exon 10 variant (c.1621 A>C) in GIST. Our results support that the variant form of KIT share some of the biochemical properties of the mutated KIT receptors. Moreover, the presence of the variant seems associated to a poor prognosis value in patients with GIST. Thus, *KIT*^{L541} may be a new prognostic tool for GIST.

Introduction

GIST (gastrointestinal stromal tumors) are the most common mesenchymal tumors of gastrointestinal tract, and account for 15 to 20 % of soft tissue sarcomas and 1 to 3% of all malignant digestive tumors [1]. Their annual incidence is approximately 12-15 cases per million inhabitants [2]. GIST are paradigmatic models of cancers with a driver mutation of an oncogene. These driver mutations may occur in different genes but are mutually exclusive: most affect the proto-oncogenes *KIT* (75-80%) and *PDGFRA* (5-10%), which encode for proteins with ligand independent constitutive activation of tyrosine kinase receptor. There are a variety of *KIT* and *PDGFRA* mutations, on different portions of the receptors. These different mutations are associated with different risk of relapse after resection of the primary tumor, but also impact on the activity of imatinib (selective inhibitor of KIT and PDGFR- α). About 10%-15% of GIST do not harbor any mutations in the *KIT* and *PDGFRA* genes. Previously called “wild-type” (WT), these GIST are now known to present with mutations on *SDHA/B/C/D* [3, 4], *BRAF* [5-7], *NFI* [8]. These mutations are also associated with specific clinical presentations and clinical behavior.

As tumor genotype plays a crucial role in clinical management of GIST, it is of interest to evaluate whether single nucleotide polymorphism (SNP) may influence the clinical behavior of GIST. In the literature, variants have been found associated with soft tissue sarcoma incidence [9] and prognosis [10]. In GIST, O'Brien et al. [11, 12] investigated the role of inherited genetic SNP in tumor development and have identified several SNP associated with GIST mutation subtypes (for example, CYP1B1 SNP and deletion at *KIT* exon 11 codons 557-8).

A SNP of *KIT* exon 10, encoding for the substitution of a methionine in position 541 by a leucine (*KIT*^{L541}) in the mature KIT protein, has already been reported to be associated with

several diseases, including aggressive fibromatosis (AF) [13-15] chronic myelogenous leukemia (CML), pediatric mastocytosis [16] and more recently in chronic eosinophilic leukemia [17]. The potential clinical impact of this variant, on GIST biology, clinical presentation, and response to treatment has never been explored.

In the present work, we have investigated the possible biological significance of the presence of the variant protein on the biology of GIST using 2 approaches: first exploring KIT^{L541} signalling in transfected cell lines and response to imatinib and then exploring the prevalence and prognostic value of KIT^{L541} in GIST patients, investigating 2 distinct series of patients.

Materials and methods

Transfected NIH3T3 cell lines

Murine fibroblast cell lines (NIH3T3) were stably infected with oncoviral vectors containing the full-length human *KIT* cDNA wild-type (**WT**), or with either homozygous mutations (del557-558=**D6** or del564-581=**D54**) or heterozygous mutations (**WT/D6** or **WT/D54**) or homozygous polymorphic *KIT* (1621 A>C = **L541**), or with the empty vector (**MIGR**) [18]. The stably infected NIH3T3 cells expressed the KIT protein in two forms: the mature and the immature proteins of 145 and the 125 kDa, respectively.

Cell culture

Cells were cultured at 37°C humidified atmosphere containing 5% CO₂ in DMEM with 10% newborn calf serum (Life Technologies), 2% penicillin/streptomycin, 1mM L-Glu. For transfected cells, medium was supplemented with 1/1000 G418 (Invitrogen) to maintain a selective pressure for vectors' expression. Fifty ng/mL of Chinese hamster ovary–mouse rhSCF (recombinant human Stem Cell Factor, R&D systems) were added for cells expressing WT *KIT*, so that both the expression and the activation state of WT and mutant forms of *KIT* were equivalent. Treated cells medium was supplemented with 1µM of Imatinib (Novartis) 24 hours before lysis.

Protein extraction and Western blotting

Cells were lysed in Petri plate using RIPA buffer (10mM Tris pH 7.4, 150mM NaCl, Triton 1%, DOC 0.5%, SDS 0,1%, EDTA 1mM, 1mM ortho vanadate) plus protein inhibitors 30

minutes at 4°C. Proteins were quantified using a Dc protein assay kit (Biorad). Migration was performed using gradient gel (Invitrogen® NuPAGE® 4-12% Bis Tris Midi Gel, 1.0 mm) in NuPAGE® MES SDS running buffer (Invitrogen®). After electrophoretic separation, proteins were electrotransferred on a polyvinylidene difluoride membrane (Millipore). The membrane was then blocked for 1h at room temperature with ECL Advance™ Blocking Agent 0.2% (GE healthcare Limited) in TBS/Tween 0.1% and primary antibody incubated for one night at 4°C. The membrane was washed three times in TBST 0.1%, and secondary antibodies were incubated for one hour at room temperature. After washing, revelation was performed using Lumigen™ TMA-6 reagents (GE healthcare Limited) and Amersham Hyperfilm™ ECL (GE healthcare Limited).

Tumor and blood samples

This study was performed as a retrospective translational research program on tumour and blood samples of 109 patients with GIST. The first series included 87 resected primary GIST untreated until recurrence and the second series included 22 metastatic or locally advanced/unresectable at diagnosis GIST. A control group consisted of 60 blood samples from healthy donors collected at the French department of blood transfusion.

All samples of the 109 patients were obtained from pathology department:

- Paraffin-embedded tissues samples of 98 GIST, at initial diagnosis, obtained by biopsy or surgical excision to establish the diagnosis of the disease
 - 44 included in “EMS” project from February 2006 to July 2007 [19]
 - 35 patients “off-protocol” treated in Centre Léon Bérard from May 2004 to June 2012

- 19 patients included in “BFR14” trial from June 2002 to April 2006 (15 patients in whom dry pellet PBMC corresponding were available)
- Dry pellets of peripheral blood mononuclear cells (PBMC) frozen of 11 patients included in “BFR14” trial.

DNA/RNA extraction

Total DNA and RNA was extracted from tumours and PBMCs, using *QIAamp DNA FFPE Kit*, *QIAamp RNeasy FFPE Kit* and *Universal Kit* (Qiagen[®], France) according to the manufacturer’s instructions, and quantified by spectrophotometry (NanoDrop ND-100 instrument, Thermo Fisher Scientific, Waltham, MA). After several washing with toluene and ethanol, FFPE tumours were lysed using ATL buffer and proteinase K and incubating at 56°C for 24-48 hours and 90 °C 1 hour. DNA and RNA extraction was carried out using *QIAampMiniElute Spin Column* and *RNeasy MiniElute Spin Column*.

PBMCs were lysed using RLT buffer and proteinase K. DNA and RNA extraction was carried out using *AllPrep DNA spin column* and *Rneasy Mini spin column*.

Competitive Allele-Specific Taqman[®] PCR (CAST[®]-PCR) and sequencing

The detection of the SNP was performed by Competitive Allele-Specific Taqman[®] PCR technology provided by Applied Biosystems[®], using the method extensively described by Dufresne et al. [15]. We both performed a CAST[®]-PCR and a classical sequencing in 13 cases. Polymerase chain reaction (PCR) was used to amplify *KIT* exon 10. The primers used were designed by Primer Express and generated by Beckman Coulter Genomics: 5’-GTA-ATC-GTA-GCT-GGC-ATG-ATG-TG-3’ (sense) and 3’-AAC-CAT-TTA-TTT-GTT-CTC-TCT-CCA-GAG-5’ (antisense). PCR was carried out using Taq DNA polymerase (Transgenomic, Omaha, NE, USA) with the following cycle conditions: denaturation at 95°C,

followed by 10 cycles at decreasing temperatures between 66°C and 61°C with a decrement of 0.5°C per cycle, and additional extension at 72°C. The primers used for sequencing were the same as those used for amplification. Before sequencing, PCR products were purified using Centriprep centrifugal filter device (Millipore, Billerica, MA, USA). The purified PCR product was then subjected to automated sequencing performed by Beckman Coulter Genomics. Results of that double detection of the SNP, by sequencing and CAST[®]-PCR, were always consistent.

Statistical analysis

Statistics were performed using R software (version 3.1.1). Chi-2 test, Fisher's exact test and multivariate analysis (logistic regression and Cox model) were performed in order to:

- Compare *KIT*^{L541} prevalence in GIST and in the control series.
- Study the distribution of known prognostic factors (tumor size, mitotic index and location) according to the presence of the SNP.
- Investigate the possible correlation between *KIT*^{L541} and clinical and biological characteristics of GIST (disease stage, mutations, relapse).

Relapse free survival (RFS) and overall survival (OS) of patients harboring or not *KIT*^{L541} variant were plotted using the after Kaplan-Meier method and compared by log-rank test.

Results

KIT^{L541} has a similar maturation and activation than WT KIT in NIH3T3 cells

We investigated KIT function and phosphorylation status using western blotting analyses (**Figure 1A**). No basal expression or phosphorylation of KIT was detected in MIGR cell control. The two forms of the receptor, 125 kD (immature) and 145 kD (mature) were found expressed in WT transfected cells. The 145 kD form was found phosphorylated on both Y-823 and Y-703, at a basal level. The L541 cell line (expressing the polymorphic form of KIT, *KIT^{L541}*) displayed the same expression and phosphorylation pattern as WT cell line after SCF stimulation. D6 and D54 transfected mutants showed an increased phosphorylation status on both residues. Their immature 125 kD form was also phosphorylated on both tyrosines, in contrast with WT or L541. In heterozygous cell lines (ie, cells transfected with WT and D6 or D54 mutated *KIT*), phosphorylation status of both Y-703 and Y-823 was found increased, in line with previous report on KIT activation status in tumors exhibiting homo/heterozygous mutations of the receptor [18]. Overall, in WT and L541 cell lines, only membrane mature KIT is phosphorylated upon SCF exposure, while both mature and immature KIT are constitutively activated in D6 and D54 cell lines. In all cell lines, Y-823 phosphorylation is completely lost after imatinib treatment, while Y-703 phosphorylation remaining marginally detectable for mature KIT only.

Intracellular signaling in KIT-expressing cell lines with or without imatinib treatment

We then investigated the activation status of KIT signaling pathways using western blotting analyses (**figure 1 B**). We assessed the phosphorylation status of AKT (PI3K-AKT pathway), RAF, ERK1 and ERK2 (MAPK pathway).

- The PI3K/AKT pathway

L541 cell line was found to activate AKT at a similar level than the D6 and D54 mutants. After imatinib exposure, P-AKT increased in MIGR cell line, but remain stable in WT and D54 cell lines, and decreased in D6, L541 and heterozygous (WT/D6 and WT/D54) cell lines.

- The *MAPK* pathway

In all cell lines, RAF phosphorylation was found only marginally different in WT, D6 and D54 cell lines and not affected by imatinib exposure. In contrast, the downstream ERK proteins were found to display phosphorylation variations largely depending upon KIT mutation status. High levels of spontaneous phosphorylation are detectable in MIGR control, D54, D54/WT and mainly in L541 cell lines, in particular ERK2 (42kDa). Lower levels are observed in D6 cell lines cells whereas is undetectable in WT cell lines. Exposure to imatinib was found to increase ERK phosphorylation in WT, D6, D54 and WT/D6 cell lines but interestingly, not in WT/D54 and L541 cell lines.

Overall, in L541 cell line, KIT had a spontaneous phosphorylation status comparable to WT, but displays an activation pattern of AKT and ERK ½ similar to that of the mutated forms of the receptors in presence of SCF. We conclude that KIT^{L541} share some of the biochemical properties of the mutated KIT receptors.

We then investigated the association between the presence of the variant *KIT*^{L541} and clinical presentation and prognostic in GIST patients.

Clinical and biological characteristics of the series

This retrospective case-control study was conducted on 2 different series: 87 resected primaries GIST untreated until recurrence, 22 metastatic at diagnosis (or locally advanced/unresectable) GIST.

Characteristics of the 2 series of GIST are presented in **Table 1**.

Within the first series, clinical and biological characteristics are similar to those described in literature, with a median age at diagnosis at 65 years old (range = 22-84), a sex ratio about 1 (42/45), a majority of gastric location ($n=52$, 60%) and a majority of *KIT* exon 11 mutation ($n=40$, 54%). With a median follow-up of 68 months, there were 17 (20%) local or metastatic relapse at 5 years: 7 of 12 (59%) *KIT* exon 11 mutated, 1 of 12 (8%) *KIT* exon 9 mutated, 1 of 12 (8%) *KIT* exon 13 mutated, 2 of 12 (17%) with mutations in *PDGFRA* (exon 18, D842V), 1 of 12 (8%) WT and 5 with an undetermined mutational status.

As expected, tumours' parameters of the second series (size, location and mitotic rate) were worse according to Miettinen classification : higher median size (90 mm, range 45-240, versus 58 mm, range 17-400), higher mitotic rate and more small bowel location ($n=11$, 50%, versus $n=24$, 28%).

In the control group of 60 healthy blood donors, the sex ratio was 1 (30/30) and the median age was 45 years old (range 19-63).

Clinical and biological impact of the variant

The variant was always detected in both tumors and dry pellets PBMC when both samples were available ($n=15$), confirming that it is not a mutation but a somatic genetic variant.

Distribution of the clinical and biological characteristics of GIST harbouring or not *KIT*^{L541} are detailed in **Table 2**, showing no differences between those 2 groups regarding to the size, the location, the mitotic index and the distribution of *KIT* and *PDGFRA* mutations. Importantly, prevalence of *KIT*^{L541} was statistically higher in this metastatic GIST series ($n=9/22$, 41% versus $n=15/87$, $n=17\%$; Fischer's exact test, $p=0.02$).

In the first series, 15 patients had a *KIT*^{L541} (17%), a prevalence similar to that observed in healthy blood donors ($n=10/60$, 17%; Fischer's exact test, $p=1$). *KIT*^{L541} was significantly correlated to a higher rate of local or metastatic relapse at 5 years ($n=7/15$, 47%, versus $n=10/72$, 14%; Fisher's exact test, $p=0.008$), resulting in a lower relapse free survival (RFS) at 5 years (**Figure 2A**; Logrank test, $p=0.001$).

Importantly, in multivariate analysis (*KIT*^{L541} status, tumor size, location, mitotic index and mutation status) using Cox model, *KIT*^{L541} was independently and directly associated to a lower RFS at 5 years ($p=0.004$), together with tumor size ($< \text{ or } \geq 50 \text{ mm}$, $p=0.03$) and mitotic rate ($< \text{ or } \geq 5 \text{ per } 50 \text{ HPF}$, $p=0.006$) (**Table 3**).

Overall survival of the 2 groups was not different (**Figure 2B**; Logrank test, $p=0.3$). In the subgroup of WT and *PDGFRA* mutated GIST, *KIT*^{L541} was associated to a lower RFS at 5 years (Logrank test, $p=0.02$). In the subgroup of *KIT* mutated GIST, RFS of the 2 group was not different (Logrank test, $p=0.09$).

In the second series, PFS and OS at 5 years of the patients harbouring or not *KIT*^{L541} were not different (Logrank test, $p=0.2$ and $p=0.6$). Pooling this series of patients with patients from the first series who relapsed yield a group of 46 patients. In this pooled series as well, PFS and OS at 5 years of the patients harbouring or not *KIT*^{L541} were not different (Logrank test, $p=0.6$ and $p=0.6$).

Discussion

In this work, we investigated the biochemical significance of the SNP *KIT*^{L541} in GIST, the potential role in carcinogenesis and the potential predictive value of this variant in GIST patients outcome.

Our biological data highlight that *KIT*^{L541} has a similar maturation and activation than WT *KIT*, but that it induces a mutant-like intracellular signalling. The substitution of a methionine into a leucine in position 541, at the end of the transmembranous domain, does not change the unpolar status of the amino acid. However, it adds a positive charge in the amino-acid sequence and increases space crowding. This might change the ability of the receptor to activate *PI3K/AKT* and *MAPK* signalling pathways in comparison with WT *KIT*. However, this region, upstream from the ATP-binding site, does not affect the ATP-binding site conformation, as imatinib is still effective. All these results suggest that *KIT*^{L541} might have a clinical significance.

Then we investigated the prevalence of this variant in 2 series of GIST and in a control group. *KIT*^{L541} was both detected in tumors and PBMCs DNA; it is a germinal variant unlike activating mutations of *KIT* and *PDGFRA*. As *KIT*^{L541} prevalence in general population does not differ from patients, as it is associated with activating mutations of *KIT* or *PDGFRA*, and as proportion of *KIT*^{L541} does not differ between WT GIST and other, we conclude that *KIT*^{L541} is not involved in carcinogenesis of GIST. Otherwise, *KIT*^{L541} seems to promote tumor progression. Indeed, *KIT*^{L541} is associated with a higher risk of relapse, resulting in lower relapse-free survival at 5 years. Importantly, this impact of *KIT*^{L541} is observed in multivariate analysis (*KIT*^{L541}, Miettinen risk factors, and mutation status), suggesting that it is an independent prognostic factor. Moreover, *KIT*^{L541} seems to be correlated to metastatic

status at diagnosis. All these results are consistent with our previous biological results. Results on overall survival and progression free survival at 5 years were not significant, but we can mention here the low event rates (death), a longer follow up needed, but mainly existence of confounding factors like treatments received. Interestingly, subgroups analysis showed a poor prognosis value of the variant in GIST not *KIT* mutated. These results suggest that the variant could be a useful tool in management of WT or PDGFRA GIST. In our study, *KIT*^{L541} was not found to be a predictive biomarker for the efficacy of imatinib. However, it must be noted that the power of the study remained limited by the small size of the cohort treated by imatinib, as well as subgroup analysis in subgroup of WT and PDGFRA mutated GIST (6 cases) could not be performed .

In GIST, *KIT* or *PDGFRA* mutations are not always identified, suggesting that there must be other mechanisms of carcinogenesis. Other mutations have been described (BRAF, etc...). Together with those activating mutations, sequence variations (SNP) of *KIT* have been described. Rare SNP on *KIT* exon 10 (*A533D* and *F522C*), identified in systemic aggressive mastocytosis [20], lead to constitutive activation of the receptor. Our study focused on the variant (c.1621 A>C) in exon 10 of *KIT* (*KIT*^{L541}). This SNP has previously been described in CML, aggressive fibromatosis (AF), systemic mastocytosis and Merkel cell carcinoma. In CML, Inokuchi and al. [21] showed a higher proliferation in response to SCF in cells expressing *KIT*^{L541}. Moreover, in a cohort of 80 patients with CML, *KIT*^{L541} was associated to a statistically lower median survival. Thus, *KIT*^{L541} may be a poor prognosis factor in CML. In vitro studies of Foster et al. also demonstrated that the polymorphic form of *KIT* confers an enhanced proliferative response to low levels of SCF. Grabellus et al. [22] showed that prevalence of *KIT*^{L541} in aggressive fibromatosis did not differ from the general population, suggesting that this variant was not involved in carcinogenesis of these tumors. In 2006,

Goncalves et al. [13] published the case of a major tumor response of imatinib, corresponding to a desmoid tumor expressing *KIT*^{L541}. Results of Dufresne et al. did not support a predictive value of *KIT*^{L541} in efficacy of imatinib for patients with AF. Currently, the significance of the *KIT*^{L541} variant in AF remains unclear. More recently, Iurlo et al. [17] suggest that the presence of *KIT*^{L541} in chronic eosinophilic leukemia could be a predictive factor of imatinib sensitivity. In GIST, *KIT*^{L541} has never been explored before.

We investigated the prevalence of this variant using CAST[®]-PCR technology. Currently, sequencing is the gold standard. However, sequencing showed a limited sensitivity for mutations detection in FFPE tumors especially with low representation of tumor DNA [23]. CAST[®]-PCR is an interesting approach in the detection of mutations, easily reproducible, fast, and reliable. *KIT*^{L541} prevalence in control group corresponds to literature [22, 24], confirming the CAST[®]-PCR technology. Using this technology, we were not able to determine if the SNP was always heterozygous and if it was located in the mutated allele of *KIT*. Moreover, the L541 cell line was stably infected with oncoviral vectors containing a homozygous polymorphic *KIT*^{L541}, and it would be interesting to study a heterozygous model (WT/L541 and D54/L541 or D6/L541).

Currently, GIST recurrence potential after complete surgery resection is measured by “Miettinen risk table”. This classification is based on several prognosis factors: size, site and mitotic index. Cutoff values define risk groups: very low / low (adjuvant imatinib treatment not recommended), high (adjuvant imatinib recommended) and a moderate risk group in which adjuvant imatinib treatment is controversial. Other risks factors exist, like tumor rupture, incomplete resection, genetic factors (type of mutation). In order to select patients likely to benefit from imatinib, identifying new biomarkers predictive of GIST patient

outcome is crucial. Our results suggest that KIT^{L541} is a predictor of metastasis in patients with GIST. Thus, KIT^{L541} may be a new prognostic tool for GIST: its detection could be useful for clinical management of GIST patients, especially in the context of localized GIST, so as to decide for an adjuvant therapy.

Limitations of our study included the retrospective design. Otherwise, a prognostic biomarker can also be a biomarker predictive of response to treatment, like KIT exon 11 deletion in GIST, associated to a poorer prognosis but a better response to imatinib. KIT^{L541} variant is located on imatinib binding site. Our results are not sufficient to support or not a predictive value of KIT^{L541} in efficacy of imatinib for patients with GIST. A large prospective study is necessary to confirm the interest of the variant as a prognostic marker in GIST and to search for a potential predictive value on the efficacy of imatinib for patients with GIST, especially WT and $PDGFRA$ mutated.

In conclusion, our results suggest that an association between KIT^{L541} and GIST tumorigenesis is unlikely. However, our analysis supports a poor prognostic value of KIT^{L541} in GIST. The predictive value of KIT^{L541} in efficacy of imatinib for patients with GIST remains undetermined.

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Footnotes

Competing interests

JYB and IRC received research support and honoraria from Novartis, Pfizer Bayer and GlaxoSmithKline. The others authors have declared no conflicts of interest.

Authors' contributions

MB, LA, and AD carried out the molecular genetic studies, collected and analyzed the data. MB drafted the manuscript and performed the statistical analysis. JYB conceived of the study, participated in its design and coordination, and critically revised each draft of the manuscript. All authors read and approved the final manuscript.

Contributor Information

Mehdi Brahmi, Email : mehdi.brahmi@hotmail.fr

Laurent Alberti, Email : laurent.alberti@lyon.unicancer.fr

Armelle Dufresne, Email : a.dufresne@hotmail.fr

Isabelle Ray-Coquard, Email : isabelle.ray-coquard@lyon.unicancer.fr

Philippe Cassier, Email : philippe.cassier@lyon.unicancer.fr

Pierre Meeus, Email : pierre.meeus@lyon.unicancer.fr

Anne-Valérie Decouvlaere, Email : anne-valerie.decouvlaere@lyon.unicancer.fr

Dominique Ranchère-Vince, Email : dominique.ranchere-vince@lyon.unicancer.fr

Jean-Yves Blay, Email : jean-yves.blay@lyon.unicancer.fr

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Tables

Table 1. Baseline characteristics of the 2 retrospective series of GIST.

Characteristics	First series of resected primary GIST	Second series of metastatic or locally advanced at diagnosis GIST	<i>p</i> value (Fischer's exact test)
N	87	22	0.5
Male	42	13	
Female	45	9	
Age (years)	65 (22-84)	64 (39-79)	
Median size (mm)	58 (17-400)	90 (45-240)	
Location			
Stomach	52 (60%)	8 (36%)	0.06
Small bowel	24 (28%)	11 (50%)	0.07
Other locations	11 (12%)	3 (14%)	1
Mitotic index per 50 HPF			
≤ 5	51 (59%)*	5 (26%)*	0.01
6-10	12 (14%)*	4 (21%)*	0.5
>10	23 (27%)*	10 (52%)*	0.05
Unknown	1	3	
Mutation status			
KIT ex 11	40 (54%)*	12 (75%)*	0.2
KIT ex 9	5 (7%)*	3 (19%)*	0.1
KIT ex 13	1 (1%)*	1 (6%)*	0.3
KIT ex 17	2 (3%)*	0	
PDGFRA ex 18	11 (15%)*	0	
D842V	9	0	
No mutation on <i>KIT</i> or <i>PDGFRA</i> hotspots	15 (20%)*	0	
Unknown	13	6	
Prevalence of the variant <i>KIT</i>^{L541}	15 (17%)	9 (41%)	0.02

* percentages are determined on the evaluable cases

Table 2. Clinical and biological characteristics of the two series according to *KIT*^{L541}

Clinical and biological characteristics	<i>KIT</i> ^{L541}	<i>KIT</i> ^{M541}	p
N	24	85	X
Median size (mm)	50 (17-240)	60 (20-400)	
Location			
Stomach	12 (50%)	48 (56%)	0.6
Small bowel	7 (29%)	28 (33%)	1
Mitotic index			
≤ 5	11 (50%)*	46 (54%)	0.8
6-10	2 (9%)*	15 (18%)	0.5
> 10	9 (41%)*	24 (28%)	0.3
Mutation status			
<i>KIT</i> Mutations	15 (75%)*	49 (70%)*	0.8
<i>KIT</i> exon 11	10 (50%)*	42 (60%)*	0.5
<i>KIT</i> exon 9	3 (15%)*	5 (7%)*	0.4
<i>KIT</i> exon 13	2 (10%)*	0	
<i>KIT</i> exon 17	0	2 (3%)*	
<i>PDGFRA</i> Mutations	2 (10%)*	9 (13%)*	1
<i>PDGFRA</i> exon 18 D842V	1	8	
Wild Type	3 (15%)*	12 (17%)*	1
Undetermined	4	15	
Stage of the disease at diagnosis			
Localized	15 (62%)	72 (85%)	0.02
Metastatic (or locally advanced/unresectable)	9 (38%)	13 (15%)	

* percentages are determined on the evaluable cases

Table 3. Impact of KIT^{L541} , tumor size, location, mitotic index and mutational status on RFS at 5 years in multivariate analysis.

Variable	HR (95% CI)	<i>p</i> value
KIT^{L541}	6.1 (1.8-21)	0.004
Tumor size (< 50 mm versus \geq 50 mm)	3.6 (1.1-12)	0.03
Location (gastric versus non gastric)	0.5 (0.1-2)	0.06
Mitotic index per 50 HPF (< 5 versus \geq 5)	6.2 (1.7-23)	0.006
Mutation status (all other versus KIT11)	1.5 (0.4-5)	0.6

HR, Hazard ratio; CI, confidence intervals.

Figures

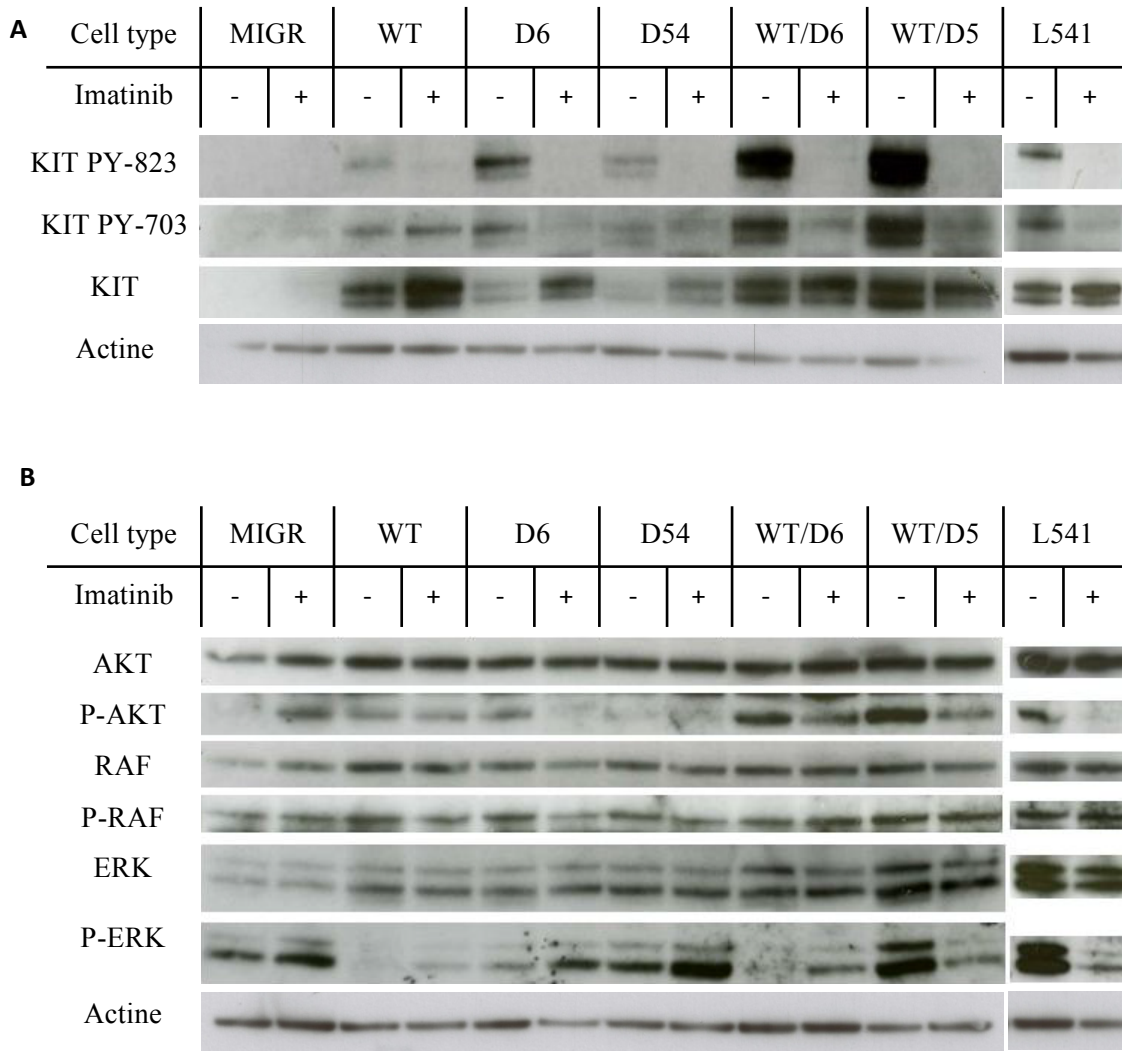


Figure 1: *KIT* maturation and activation status and related intracellular signaling in *KIT*-expressing cells treated or not with imatinib. Western blotting analysis after a 24-hours exposure to 1 μ M of imatinib of the expression and the phosphorylation of **A.** mature (145 kDa) and immature (125 kDa) forms of *KIT* on residues Tyr⁷⁰³ and Tyr⁸²³ and **B.** *AKT*, *RAF* and *ERK1/2*.

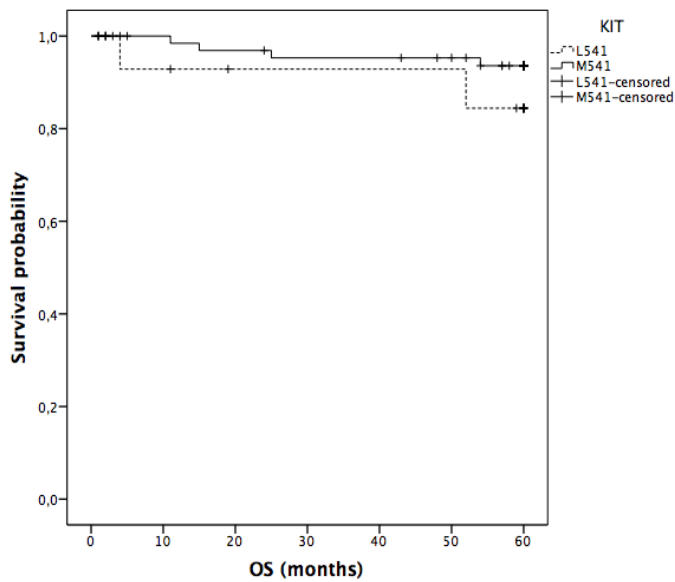
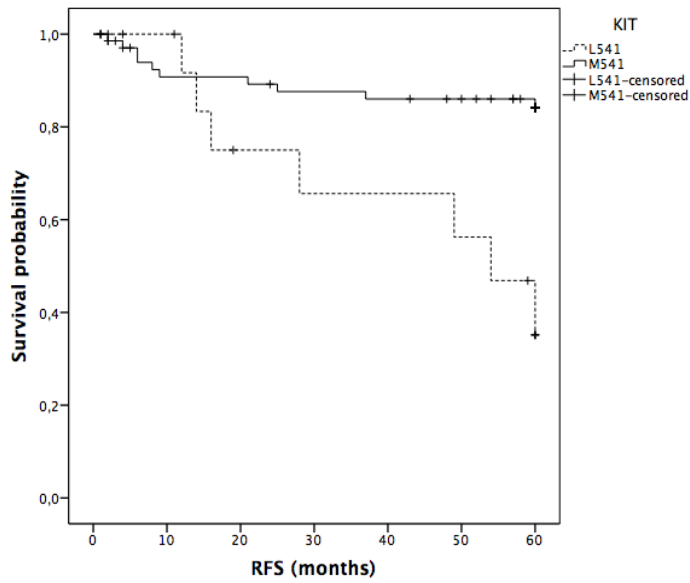


Figure 2.

- A. Kaplan–Meier analysis of RFS at 5 years of the first retrospective series of primary resected GIST stratified by KIT^{541} status ($p=0.001$); x -axis, time in months.
- B. Kaplan–Meier analysis of OS at 5 years of the first retrospective series of primary resected GIST stratified by KIT^{541} status ($p=0.3$); x -axis, time in months.

THESE SOUTENUE PAR : M. BRAHMI MEHDI

TITRE : POLYMORPHISME NUCLEOTIDIQUE (c.1621A>C) DE L'EXON 10 DU
GENE *KIT* ET PREDICTION DU RISQUE METASTATIQUE DANS LES GIST

CONCLUSION

Les GIST constituent un très bel exemple du changement récent de stratégie diagnostique et thérapeutique en oncologie. La découverte d'une anomalie moléculaire spécifique et « causale » (mutation sur les gènes *KIT* ou *PDGFRA*) associée à l'utilisation d'une thérapie « ciblée » sur cette anomalie (Imatinib, Glivec®) a permis d'améliorer considérablement le pronostic de ces tumeurs. Aujourd'hui, en cas de suspicion de GIST, une analyse par biologie moléculaire est systématiquement réalisée à la recherche des mutations suivantes: mutations sur l'exon 9, 11, 13 et 17 de *KIT* et mutations sur l'exon 10, 12, 14 et 18 de *PDGFRA*. En plus de constituer une aide au diagnostic, ces différentes mutations guident notre prise en charge thérapeutique puisqu'elles sont corrélées au pronostic et à la réponse aux traitements. Dans 10-15% des cas, aucune mutation n'est mise en évidence, suggérant qu'il existe d'autres mécanismes de carcinogénèse. Des mutations sur d'autres oncogènes ont été plus récemment décrites (*BRAF*, *NF1*, *SDHA/B/C/D*) et les recherches doivent se poursuivre en ce sens.

Comme le statut mutationnel des GIST est un élément essentiel pour leur prise en charge, il semble pertinent d'étudier également les caractéristiques génétiques germinales des patients. De précédentes études ont notamment montré que des polymorphismes génétiques étaient associés à l'incidence et au pronostic de certains sarcomes. Dans notre étude, nous nous

sommes intéressés au polymorphisme nucléotidique (SNP) c.1621A>C de l'exon 10 de *KIT* (*KIT*^{L541}).

Sur le plan biologique, nous avons mis en évidence que la forme du récepteur avec le SNP présentait des similitudes avec la forme mutée du récepteur, notamment en ce qui concerne l'activation des voies de signalisation. Bien que le récepteur *KIT*^{L541} ait une maturation et une présentation similaire à un récepteur sauvage (sans mutation du gène *KIT*), l'analyse des voies de signalisation par Western Blot a montré une activation des kinases des voies « MAP-Kinases » et « PI3-Kinase » semblable à celle d'un récepteur muté. Ces résultats suggèrent que ce SNP entraîne une activation constitutionnelle du récepteur et des voies de signalisation en aval, favorisant ainsi la progression tumorale.

Sur le plan clinique, nous avons montré que la prévalence de *KIT*^{L541} ne différait pas de la population générale et que ce SNP était associé aux mutations activatrices de *KIT* ou *PDGFRA* en proportion habituelle. Ces résultats suggèrent que ce SNP n'est pas impliqué dans la carcinogenèse des GIST. Par ailleurs, la prévalence du SNP était significativement plus importante chez les patients présentant une maladie d'emblée métastatique. De plus, le SNP était corrélé à un plus grand risque de rechute de la maladie à 5 ans, avec une survie sans rechute à 5 ans statistiquement plus faible. Les résultats sur la survie globale et la survie sans progression à 5 ans n'étaient pas significatifs. Nous pouvons évoquer ici le faible taux d'évènements (décès) mais surtout l'existence d'autres facteurs pronostiques et prédictifs de la réponse aux traitements masquant l'effet du SNP.

En conclusion, nos résultats suggèrent que *KIT*^{L541} entraîne une activation du récepteur à activité tyrosine kinase KIT. Ce SNP ne semble pas être impliqué dans la carcinogenèse des GIST mais semble être un facteur de mauvais pronostic, corrélé à l'évolution métastatique des


GIST. La détection de KIT^{L541} pourrait donc être un élément important dans la décision thérapeutique.

VU ET PERMIS D'IMPRIMER

Grenoble, le 24/03/2015

LE DOYEN

LE PRESIDENT DE LA THESE



J.P. ROMANET


PROFESSEUR MOUSSEAU

Mehdi BRAHMI

POLYMORPHISME NUCLEOTIDIQUE (c.1621 A>C) DE L'EXON 10 DU GENE *KIT* ET PREDICTION DU RISQUE METASTATIQUE DANS LES GIST

42 pages, 2 figures, 3 tableaux

RESUME

INTRODUCTION : Dans les GIST, la découverte d'une anomalie moléculaire spécifique et « causale » (mutation sur les gènes *KIT* ou *PDGFRA*) associée à l'utilisation d'une thérapie « ciblée » sur cette anomalie (Imatinib, Glivec®) a permis d'améliorer considérablement le pronostic de ces tumeurs. Le statut mutationnel des GIST est un élément essentiel pour leur prise en charge thérapeutique et il semble pertinent d'étudier également les caractéristiques génétiques germinales des patients. Le polymorphisme nucléotidique (SNP) c.1621A>C de l'exon 10 de *KIT* (*KIT*^{L541}) a été décrit dans plusieurs pathologies (tumeurs desmoides, LMC) avec un impact clinique controversé. Nous avons étudié son potentiel impact biologique et clinique dans les GIST.

MATERIELS ET METHODES : Des cellules transfectées ont été utilisées, exprimant de manière stable les différents vecteurs d'expression de *KIT*: sauvage, muté et polymorphique (L541). Nous avons étudié dans chaque lignée cellulaire les fonctionnalités du récepteur à tyrosine kinase *KIT* en utilisant des analyses de Western-Blot. Ensuite, un programme de recherche translationnelle rétrospective a été réalisée sur des échantillons de tumeurs et le sang de patients atteints de GIST. La détection de *KIT*^{L541} a été réalisée par la technologie CAST-PCR®.

RESULTATS : Sur le plan biologique, la forme du récepteur avec le SNP présentait des similitudes avec la forme mutée du récepteur, notamment en ce qui concerne l'activation des voies de signalisation. Sur le plan clinique, la prévalence de *KIT*^{L541} ne diffère pas de la population générale, ce SNP est associé aux mutations activatrices de *KIT* ou *PDGFRA* en proportion habituelle, mais sa prévalence est significativement plus importante chez les patients présentant une maladie d'emblée métastatique. De plus, le SNP est corrélé à un plus grand risque de rechute de la maladie à 5 ans, avec une survie sans rechute à 5 ans statistiquement plus faible.

CONCLUSION : *KIT*^{L541} entraîne une activation du récepteur à activité tyrosine kinase *KIT*. Ce SNP ne semble pas être impliqué dans la carcinogénèse des GIST mais semble être un facteur de mauvais pronostic, corrélé à l'évolution métastatique des GIST.

Mots clés : GIST, *KIT*, Polymorphisme nucléotidique

Jury : Président : **Madame le Professeur Mireille MOUSSEAU**

Directeur : **Monsieur le Professeur Jean-Yves BLAY**

Assesseurs : **Monsieur de Docteur Benoit BUSSER**

Monsieur le Docteur Axel LE CESNE

Madame le Docteur Anne MCLEER

Date de soutenance : 28 avril 2015

Adresse de l'auteur : 31 rue Béchevelin 69007 LYON