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Insights into ligand stimulation effects on gastro-intestinal stromal tumors signalling

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ABSTRACT

Mutations in KIT or PDGFRA are responsible for >85% of gastrointestinal stromal tumors. The introduction of imatinib in the GIST therapy scheme revolutionized the patient outcome. Unfortunately, the therapy allows the disease stabilization instead of curation. Furthermore the resistance to the inhibitor arises in most cases within two first years of therapy. A thorough investigation of the signalling pathways activated by the major PDGFRA and KIT mutants encountered in the GIST landscape allowed to identify striking differences between the two receptor tyrosine kinases. PDGFRA mutants were not responsive to their ligand, PDGFAA, and displayed a high constitutive kinase activity. In contrast, all KIT mutants retained, in addition to their constitutive activation, the ability to be stimulated by their ligand. Kit mutants displayed a lower intrinsic kinase activity relative to PDGFRA mutants, while the KIT Exon 11 deletion mutant exhibited the highest intrinsic kinase activity among KIT mutants. At the transcriptomic level, the MAPK pathway was established as the most prominent activated pathway, which is commonly up-regulated by all PDGFRA and KIT mutants. Inhibition of this pathway, using the MEK inhibitor PD0325901, reduced the proliferation of GIST primary cells at nanomolar concentrations. Altogether, our data demonstrate the high value of MEK inhibitors for combination therapy in GIST treatment and more importantly the interest of evaluating the SCF expression profile in GIST patients presenting KIT mutations.

1. Introduction

Gastro-intestinal stromal tumors (GIST) represent the most common mesenchymal neoplasm of the gastrointestinal tract. They arise from the interstitial cells of Cajal and are found essentially in the stomach (60% of the cases), in the small intestine (25%) but also in rectum, oesophagus and other locations outside the gut wall [1]. GIST are mainly due to mutations in KIT (75–85%) [1] or PDGFRA (10%) genes [2]. KIT and PDGFRA are type III receptor tyrosine kinases (RTKs), composed of three regions: (i) an extracellular domain responsible for ligand binding; (ii) a single transmembrane region with a juxtamembrane (JM) domain responsible for the kinase auto-inhibition properties; and (iii) a cytoplasmic domain that carries the kinase activity [3,4]. In the non-activated configuration, the JM domain blocks the kinase in a closed conformation impeding ATP molecules to access the catalytic site. After ligand binding, the wild type (WT) receptor dimerizes and tyrosine residues within the juxtamembrane domain are phosphorylated [3]. This induces conformational changes and releases the inhibitory function of this region towards the cytoplasmic domain [4]. Finally intermolecular phosphorylation at tyrosine residues serves as docking sites for SH2 domain containing molecules for further downstream signaling, which leads essentially to the activation of MAPK and PI3K pathways [4].

Mutations responsible for GIST occur mainly in the JM domain for KIT and in the kinase domain for PDGFRA, leading to constitutively activated receptors [2]. The nature of the mutation plays such an important role for the treatment schedule that the ESMO recommendations include a systematic molecular analysis in the diagnosis of GIST [5]. If surgery remains the treatment of choice for resectable tumors, adjuvant therapy is highly recommended for intermediate and high risk patients since >50% of patients relapse even after complete tumor resection [6]. In case of KIT Exon 9 or PDGFRA D842V mutations that confer resistance to imatinib, it is recommended either to start with high doses of imatinib or to switch to the second line therapy sunitinib [5]. Both drugs target the receptor tyrosine kinases themselves, competing with ATP for binding within the catalytic site. In addition to the primary resistance of certain types of mutations to imatinib, a appears frequently within the two first years of therapy as a result of the acquisition of secondary mutations within the kinase domain of the receptor [7]. Since the landscape

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of secondary mutations is quite broad within a single patient, the development of new generation of RTK inhibitors is not expected to cope with this issue. Therefore, the discovery of new targets for GIST therapy is essential. Hence we propose a thorough comparison of the signalling capacities, proffered by the main mutations found in GIST patients, in a homogenized background in order to identify common druggable targets.

2. Material and methods

2.1. Cell culture

The 293FR host cell line was generated from Hek293 cells by the co-transfection of the Flp-In™ target site vector (pFRT/lac Zeo, Invitrogen) and Tetracyclin repressor (pcDNA™6/TR, Invitrogen) as previously reported [8]. KIT mutant expressing cell lines were generated by the transfection of the Flp recombinase expression plasmid (pOG44, Invitrogen) together with the transgene expression plasmid (pcDNA5/FRT/TO-based, Invitrogen). Stably transfected cells were then selected and cultivated in the presence of 100 μg/ml Hygromycin and 10 μg/ml Blasticidin. Experiments were conducted under serum reduced (1%) conditions for 11 h and for additional 3 h under serum free (0% FBS) conditions using 5 ng/ml doxycycline (Sigma), unless differently stated in the figure legends. The stimulation of KIT and PDGFRA expressing cells was performed with SCF (100 ng/ml) and PDGF-AA (100 ng/ml), respectively (Immunotools).

GIST primary cell line GIST882 [9], presenting a homozygous missense mutation in KIT exon 13, and GIST48, a homozygous primary exon 11 missense mutation and a secondary heterozygous exon 17 missense mutation were generously provided by Dr. Sebastian Bauer (WTZ, Essen) and cultivated in RPMI and IMDM, respectively with 15% FBS in humidified atmosphere containing 5% CO_2. PD0325901 and XL-184 (Selleck Chemicals) were added to the medium 24 h after cell seeding for 30 h at the indicated concentrations. The stimulation of primary cell lines was performed after 6 h starvation to a final concentration of 100 ng/ml SCF. Cell viability was assessed with PrestoBlue (Thermofischer) in 96 well plate format following the manufacturer's recommendations. The fluorescence was monitored using the CLARIOstar microplate reader (BMG LABTECH).

2.2. Mutagenesis

KIT wild type cDNA was reverse transcribed from Mel501 RNAs (cells kindly provided by R. Halaban, [10]) and cloned into pCDNA5/FR/To plasmid after insertion of *Asc*I and *Sgr*AI restriction sites by PCR. Plasmid was sequenced and compared to the c-KIT variant 2 mRNA sequence (NM_001093772.1). Two mutations were identified and fixed using Quik-change site-directed mutagenesis KIT (Stratagene) following the manufacturer's recommendations. The single point mutation V559D was prepared using the same procedure, while KIT Ex9 duplication mutant (AY502–503) and KIT Ex11 deletion mutant (553–557) were prepared by Life Technologies using GeneArt™Gene Synthesis. All plasmids were sequenced prior transfection to 293FR cells.

2.3. Flow cytometry analysis

Cell surface and total protein expression of KIT wild type and mutants were analyzed by flow cytometry using a FACS CantoII Instrument (Becton Dickinson, Heidelberg, Germany). 10^5 cells were harvested in the presence of PBS/10 mM EDTA and then washed with FACS-buffer (PBS/5%FCS/0.1%NaN3). Afterward, the cells were either directly incubated with 10 microL KIT primary antibody (anti-CD117-APC conjugated; C7244; Dako, Belgium) for cell surface expression, or first incubated with 0.1% saponin permeabilizing buffer prior KIT antibody incubation for total KIT expression. The specificity was controlled using an isotype-matched/ APC conjugated antibody (IgG1 kappa; ×0968, Dako, Belgium).

2.4. Model building and refinement with CHARMM

Since the kinase domain of KIT is located in the cytoplasmic region, the extracellular region was removed from the model building and the kinase insert region (residues 693 to 755) was replaced by Glycine residues. The initial models of KIT regions were built by homology modeling using MODELER9v7 on Linux based operating environment based on the crystal structures (PDB ID: 2EC8 and 2E9W) as templates. The primary 3-dimensional structure of KIT WT model was further improved by using energy minimization, followed by equilibration methods both in a vacuum and in an implicit membrane with implicit water solvation methods (EEF1) with CHARMM35 parameters. Finally, 20-ns molecular dynamics (MD) simulations were run with Langevin dynamics with a time step of 2 fs.

2.5. Western blot analysis

Cell lysis was performed on ice, using 1× Laemmli buffer. Proteins were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Roth) and then probed with the respective antibodies. The last are listed in the corresponding "Data in Brief article" [11]. Signals were detected on a Fusion-FX7 chemiluminescence detection device (Vilber) using a home-made ECL (Enhanced ChemiLuminescence) solution [12]. Signal intensities were quantified using the Bio1D analysis package (Vilber).

2.6. Microarray analysis

293FR cells expressing KIT-WT, KIT Ex11 deletion mutant and KIT Ex9 mutant were treated with 5 ng/ml doxycycline and 100 ng/ml SCF for 21 h in DMEM with 1%FCS. Cells were then starved for 3 h (without FCS) and further stimulated with SCF. Gene Expression analysis was performed using GeneChip Human Gene ST 2.0 arrays (Affymetrix). The raw data in the form of Affymetrix CEL files were imported into Partek® Genomics Suite™ software (Partek GS). The Robust Multichip Average (RMA) with GC correction was applied to the data set resulting in expression values for Affymetrix transcript clusters. Quality control and data normalization were performed as previously reported [13,14]. We focused on differentially expressed genes (DEG) across the mutants comparing them with non-stimulated KIT-WT. To exclude non-relevant lowly expressed transcript clusters, only those showing $\log_2$ expression above 4.5 were considered for further analysis. Transcript clusters were further summarized in order to obtain a single expression value for each gene in each experiment. The differentially expressed genes were statistically evaluated by two-factor linear model with empirical Bayes statistical approach using *limma* package of R/Bioconductor [15]. In order to correct for the false discovery rate (FDR), the Benjamini & Hochberg step-up method correction was applied. Probe-sets with FDR <0.05 and absolute fold change >0.5 were considered to be significantly differentially expressed (DEGs). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4548.

2.7. Rank-Rank analysis

In order to compare the different KIT mutants and wild-type signalling without any pre-defined cutoffs, we used the nonparametric rank-rank geometric overlap analysis (RRHO) [16] to identify the statistically significant overlap between the gene signatures. The probe sets were first ranked based on their signed log-transformed *p*-values of ANOVA results to compare between the subgroups and the control (non-stimulated wild-type KIT). The results of the analysis are represented as a group of two plots: 1. RRHO heat map. The heat map value, visualized