JOURNAL OF PROTEOMICS XX (2014) XXX–XXX

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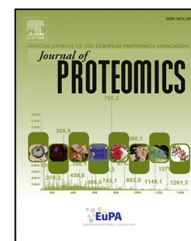

Q1 # Augmentation of multiple protein kinase activities associated with secondary imatinib resistance in gastrointestinal stromal tumors as revealed by quantitative phosphoproteome analysis

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ARTICLE INFO

Article history:
Received 28 August 2014
Accepted 16 December 2014

Keywords:
GIST
Imatinib
Phosphoproteomics
Acquired resistance
Targeted therapy

ABSTRACT

Mutations in the Kit receptor tyrosine kinase gene (*KIT*), which result in constitutive activation of the protein (KIT), are causally related to the development of gastrointestinal stromal tumors (GISTs). Imatinib, a targeted anticancer drug, exerts a therapeutic effect against GISTs by repressing the kinase activity of KIT. Long-term administration of this drug, however, causes the emergence of imatinib-resistant GISTs. We performed quantitative phosphoproteome analysis using a cell-based GIST model system comprising an imatinib-sensitive GIST cell line (GIST882), GIST882 under treatment with imatinib (GIST882-IM), and secondary imatinib-resistant GIST882 (GIST882-R). Phosphorylated peptides were purified from each cell line using titania-based affinity chromatography or anti-phosphotyrosine immunoprecipitation, and then subjected to LC-MS/MS based quantitative phosphoproteome analysis. Using this method we identified augmentation of the kinase activities of multiple elements of the signal transduction pathway, especially KIT and EGFR. Although, these elements were up-regulated in GIST882-R, no additionally mutated *KIT* mRNA was found in secondary imatinib-resistant GIST cells. Treatment of GIST882-R with imatinib in combination with gefitinib, an EGFR inhibitor, partially prevented cell growth, implying that EGFR may be involved in acquisition of secondary imatinib resistance in GIST.

Biological significance
In this study, we performed a quantitative phosphoproteome analysis using a cell culture-based GIST model system. The goal of the study was to investigate the mechanism of acquired resistance in GISTs against imatinib, a molecularly targeted drug that inhibits

Abbreviations: GIST, gastrointestinal stromal tumor; KIT, mast/stem cell growth factor receptor Kit; PDGFRA, platelet-derived growth factor receptor alpha; RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases.

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http://dx.doi.org/10.1016/j.jprot.2014.12.012
1874-3919/© 2014 Published by Elsevier B.V.

Please cite this article as: Nagata K, et al, Augmentation of multiple protein kinase activities associated with secondary imatinib resistance in gastrointestinal stromal tumor..., J Prot (2014), http://dx.doi.org/10.1016/j.jprot.2014.12.012

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kinase activity of the KIT protein and that has been approved for the treatment of GISTs. In imatinib-resistant GIST cells, we observed elevated expression of KIT and restoration of its kinase activity, as well as activation of multiple proliferative signaling pathways. Our results indicate that the effects of even so-called 'molecularly targeted' drugs, are broad rather than convergent, and that the mechanisms of action of such drugs during continuous administration are extremely complex.

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1. Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the digestive tract. GISTs may originate from the interstitial cells of Cajal (ICCs), which are mesenchymal pacemaker cells, and share ultrastructural and immunohistochemical features with ICCs [1–3]. The vast majority (85%) of GISTs have mutations in the gene encoding the Kit receptor tyrosine kinase (*KIT*), and 5–10% have mutations in the gene encoding platelet-derived growth factor receptor alpha (*PDGFRA*) [4–6]. KIT and PDGFRA are structurally homologous and belong to the type III receptor tyrosine kinase (RTK) family. KIT is activated upon binding to its ligand, stem cell factor (SCF), which leads to activation of signaling pathways including the PI3K/Akt/mTOR and Ras/Raf/MAPK cascades [7,8]. Thus, KIT prevents apoptosis and controls proliferation, adhesion, and differentiation [9]. These functions of KIT play important roles in the differentiation and proliferation of ICCs. Mutations of *KIT* found in GISTs result in constitutive activation of tyrosine kinase activity and downstream signaling pathways [1].

GISTs are generally resistant to conventional chemotherapy and radiation therapy; consequently, unresectable and/or metastatic cases are difficult to treat [10]. Based on recent findings regarding the biological relevance of KIT in GISTs, a selective tyrosine kinase inhibitor of BCR-ABL, KIT, and PDGFRA, imatinib mesylate (imatinib, STI571, or Gleevec; Novartis Pharma, Basel, Switzerland), has been administered to patients with advanced GISTs with the goal of determining its therapeutic effectiveness [11,12]. More than 40% of advanced GIST patients who received imatinib exhibited an objective response; among them, 85–90% exhibited improved control of disease [13]. Despite these encouraging early therapeutic results, resistance developed with a median time of 2 years after initiation of imatinib therapy; therefore, additional therapeutic strategies are desired [14]. GISTs may acquire secondary imatinib resistance by developing additional mutations in *KIT*, by activation of signal transduction pathways mediated by other kinases, and/or by activation of multidrug resistance genes [7,15].

In this study, we used a cell-based model system to investigate the molecular basis of the acquisition of secondary imatinib resistance in GIST. To this end, we generated a GIST cell line with secondary (acquired) imatinib resistance, GIST882-R, from the imatinib-sensitive line GIST882. To elucidate the cause of resistance, we performed MS-based quantitative phosphoproteome analysis, a robust method for the comprehensive elucidation of phosphorylation/dephosphorylation states of proteins that play key roles in the signal transduction cascades, especially in the context of biomedical science [16,17].

The cell-based model system that we developed for this study comprises GIST882 cell lines in three different states: GIST882, representing pretreatment; GIST882-IM, representing a tumor undergoing treatment; and GIST882-R, representing a tumor with acquired imatinib resistance. From each of these cell lines, we isolated phosphorylated peptides (phosphopeptides) and concentrated them by means of two distinct methods: titania-based affinity enrichment, which utilizes a titanium dioxide column, and immunoprecipitation (IP) enrichment, which utilizes an anti-phosphotyrosine antibody. The isolated phosphopeptides were then analyzed by LC-MS/MS.

Subsequently, we performed quantitative phosphoproteome analysis by direct comparison of phosphopeptide MS scan profiles. The results revealed that proteins involved in different signal transduction pathways exhibited distinct phosphorylation patterns, suggesting that the deviation of signal transduction pathways in GIST882-R from those in GIST882 caused the acquisition of its secondary imatinib resistance. Furthermore, we observed that the phosphorylation of epidermal growth factor receptor (EGFR) was upregulated in GIST882-R, suggesting that EGFR activation promotes proliferation in this cell line. Accordingly, we treated GIST882-R with an inhibitor for EGFR, gefitinib, together with imatinib, and found that this combination therapy partially suppressed cell growth.

2. Materials and methods

2.1. Generation of imatinib-resistant GIST cells

The GIST882 cell line was generous gift from Dr. Jonathan A. Fletcher (Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA). To generate an imatinib-resistant cell line (GIST882-R), GIST882 cells were exposed to increasing concentrations of imatinib. Imatinib concentrations were increased stepwise from 10 nM to 200 nM. The concentration was changed to the next higher value when the cells resumed growth kinetics similar to that of the untreated parental cells. Cell viability assays were performed to confirm the emergence of the imatinib-resistant cells (section 2.2.). The resultant GIST882-R cells were maintained in 200 nM imatinib. All cells were cultured in RPMI GlutaMAX medium (Gibco BRL/Life Technologies, San Diego, CA, USA) supplemented with 20% fetal bovine serum (Gibco BRL/Life Technologies) and 0.2% Fungizone (Gibco BRL/Life Technologies), in a humidified 5% incubator at 37 °C in an atmosphere containing 5% CO_2.

2.2. Cell-viability assay

GIST882 and GIST882-R cells were seeded in 96-well plates at an appropriate density to reach ~80% confluence at the end of the assay. After 2 days of incubation at 37 °C, the cells were exposed to imatinib at 16, 31, 63, 125, 250, 500, or 1000 nM for

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