Amino Acids (2004) 27: 335–337
DOI 10.1007/s00726-004-0140-2

Amino Acids
Printed in Austria

Differential protein expression profile in gastrointestinal stromal tumors

Short Communication

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Received October 4, 2004
Accepted October 4, 2004
Published online November 12, 2004;

Summary. Gastrointestinal stromal tumors (GISTs) arise from the interstitial cells of Cajal through gain of function mutations of the oncogene *KIT*. Imatinib offers the first effective treatment for patients with GISTs, but the therapeutic outcome strongly depends on the type of *KIT* mutation. We used ProteinChip technology to investigate whether GISTs with different *KIT* mutations express different proteins. In total, 154 proteins were significantly differentially expressed in GISTs with exon 9 *KIT* mutation compared to GISTs with exon 11 *KIT* mutation.

Keywords: GIST – KIT – Imatinib – SELDI TOF MS – ProteinChip

Introduction

Gastrointestinal stromal tumors (GISTs) originate in the interstitial cells of Cajal (Kindblom et al., 1998), and are the most common nonepithelial tumors of the digestive tract. The majority of GISTs express the transmembrane receptor tyrosine kinase for the Stem Cell Factor (KIT) (Kindblom et al., 1998), which is normally involved in hematopoiesis, gametogenesis and intestinal motility. Up to 90% of all malignant GISTs harbour gain of function mutations in the *KIT* oncogene (Hirota et al., 1998) and another 5% have a mutated Platelet Derived Growth Factor Receptor alpha gene (*PDGFR*a) (Heinrich et al., 2003a, b). Point mutations in the intracellular juxtatransmembrane coding exon 11 of *KIT* are the most frequent (70%), followed by mutations in the extramembrane coding exon 9 (Heinrich et al., 2002).

Imatinib mesylate (STI571, Glivec®, Gleevec™) specifically inhibits the tyrosine kinases KIT, PDGFRa, PDGFRb, ABL, ARG, and BCR-ABL by competing with ATP in the intracellular kinase domain (Buchdunger et al., 1996; Druker et al., 1996). Imatinib is the first effective drug in the treatment of GISTs. In about two thirds of GIST patients treated with Imatinib, a reduction of more than 50% in tumor size can be achieved, and in about 90% symptoms can be relieved (Demetri et al., 2002; van Oosterom et al., 2001). This response to Imatinib strongly depends on the type of *KIT* mutation. In a randomised phase II trial 84% of GIST patients with exon 11 mutation responded to Imatinib, compared to only 48% of patients with exon 9 mutation (Heinrich et al., 2003). Thus far, nothing is known about the underlying mechanisms of the differential Imatinib response of GISTs with *KIT* mutations in exons 9 and 11. Gene expression profiling has revealed differential gene expression in GISTs with *KIT* mutation in exon 9 compared to exon 11 (Antonescu et al., 2004; Subramanian et al., 2004), but it is not clear whether these differences are also present at the proteome level. Insights into differential protein expression are essential to the development of novel signal transduction inhibitors, because these molecules act on protein and not mRNA. We therefore performed a pilot proteomic study in 7 patients with GISTs to investigate whether GISTs with *KIT* mutation in exon 9 express or process different proteins than GISTs with *KIT* mutation in exon 11. Surface Enhanced Laser Desorption/Ionization Time-of-flight Mass Spectrometry (SELDI TOF MS) is a valuable method for the analysis of

differential protein expression in tissue extracts and body fluids (Bischoff and Luider, 2004), and we used this technology to monitor protein expression in the GIST tissues.

Material and methods

Buffers and solutions

Chemicals were purchased from Sigma-Aldrich (Saint Louis, MO), and all solutions were prepared with ultra pure water (Biosolve, Valkenswaard, The Netherlands). Protein extraction buffer contained 9 M Urea, 1% CHAPS and 1% Protease Inhibitor Cocktail. Binding buffers for ProteinChip retention chromatography were prepared as follows: 10% Acetonitrile/0.1% Trifluoro Acetic Acid for hydrophobic arrays, 0.1 M Ammonium Acetate pH 4 for Cation Exchange arrays, 50 mM Tris-HCl pH 9 for Anion Exchange arrays, and 0.1 M Sodium Phosphate/0.5 M Sodium Chloride for immobilized metal affinity arrays. 2.5% solutions of Sinnapinic Acid and a-Cyano Hydroxy Cinnaminic Acid (Ciphergen Biosystems, Fremont, CA) were prepared in 50% Acetonitrile/0.5% Trifluoro Acetic Acid.

Patient tumors

Four GISTs with *KIT* exon 9 insertion AY 502–503 and 3 GISTs with *KIT* exon 11 deletion WK557–558 were selected from the tumor bank (−80°C) of the University Hospital Gasthuisberg. All tissues were histopathologically classified as GISTs, positive for the KIT antigen (CD117 staining, DakoCytomation, Glostrup, Denmark).

Protein extraction

Protein extracts were prepared by crushing 50 mg of GIST tissue in liquid nitrogen with a mortar and pestle. The pulverised tissue was dissolved in 1 ml of protein extraction buffer by brief homogenisation with a tissue homogenisator. The extracts were centrifuged for 5 minutes at 10,000 g (4°C) and the supernatant was aliquoted and frozen at −80°C. Total protein concentrations were measured with a Bradford protein assay (Bio-Rad, Hercules, CA).

ProteinChip retention chromatography

All experiments were performed in duplicate. The protein extracts were normalized to a final concentration of 2 μg/μl in extraction buffer. These extracts were diluted 20 fold in the different ProteinChip binding buffers. Hydrophobic, cation exchange, anion exchange and copper-loaded metal affinity ProteinChip arrays were mounted in a bioprocessor and every spot was rinsed two times with 100 μl of the respective binding buffer for 5 minutes at room temperature. The arrays were than loaded with 100 μl of the diluted protein extract per spot, and incubated for 45 minutes at room temperature with vigorous shaking on a Micromix 5 (DPC, Los Angeles, CA). After incubation, the arrays were washed three times with 100 μl of binding buffer per spot for 5 minutes at room temperature. Cation exchange, anion exchange and metal affinity arrays were desalted prior to mass spectrometry (MS) by a bulk wash with ultra pure water.

Mass spectrometry

The energy absorbing matrices sinnapinic and cinnaminic acid were added twice (0.8 μl/spot), and the arrays were air-dried for 15 minutes in a dark room. The proteins bound to the retention chromatographic ProteinChip arrays were then analysed by SELDI TOF MS on a PBS II system (Ciphergen Biosystems, Fremont, CA). The instrument was externally calibrated with peptide and protein standards (Ciphergen Biosystems, Fremont, CA), according to the manufacturer's instructions, and was operated in an air conditioned room with a constant temperature of 22°C.

Data analysis

All spectra were normalised against the total ion current, and background was subtracted at 8 times the expected value of the peak width. Biomarker wizard software (Ciphergen Biosystems, Fremont, CA) was used to calculate statistically significant differences in protein expression between the two defined sample groups (GISTs with *KIT* exon 9 versus GISTs with *KIT* exon 11 mutation). Only peaks with a minimal signal to noise ratio of 5 were retained for the first pass cluster determination, and a minimal signal to noise ratio of 3 was applied for the second pass cluster determination.

Results

A total of 154 proteins displayed significant differential expression ($p < 0.05$) between GISTs with *KIT* mutation in exon 9 versus GISTs with *KIT* mutation in exon 11. The molecular weight of these proteins ranged from 2,150 to 150,178 Dalton. 61 proteins were upregulated in exon 9 mutants, and 93 proteins were upregulated in exon 11 mutants. Most differences were seen on metal affinity arrays (57), followed by hydrophobic arrays (45), cation

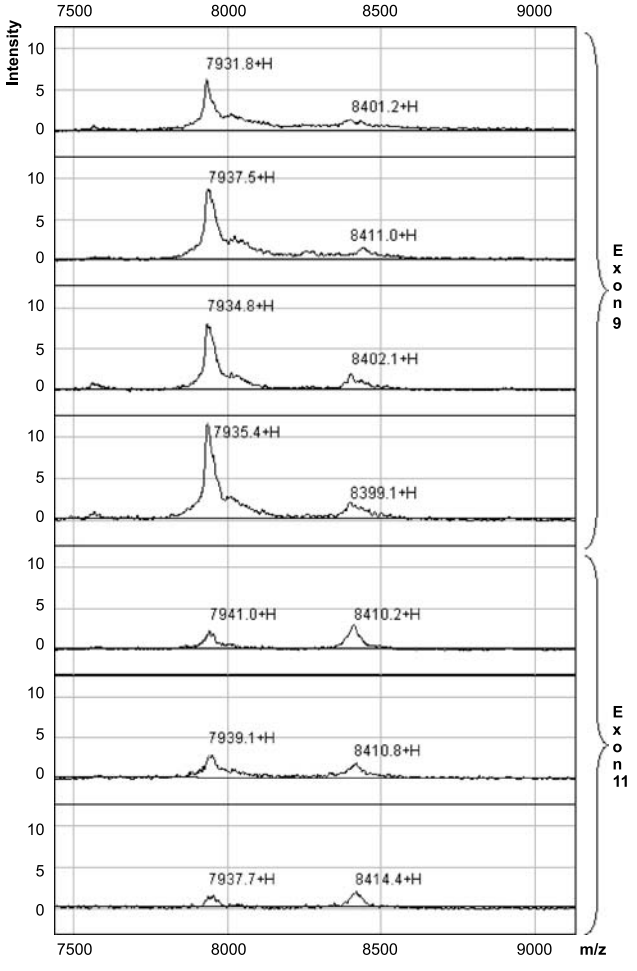

Fig. 1. Example of a significant difference in protein expression between GISTs with *KIT* mutation in exon 9 and exon 11, observed in the low molecular weight range on hydrophobic ProteinChip arrays

exchange arrays (28), and anion exchange arrays (24). An example of differential protein expression observed on the hydrophobic arrays in the low molecular weight range is presented in Fig. 1.

Discussion

These data clearly show that GISTs with *KIT* mutation in exon 11 express different proteins compared to GISTs with *KIT* mutation in exon 9. The observed ratio of differential expression is in line with the results obtained from gene expression profiling (Subramanian et al., 2004). GISTs with *KIT* mutation in exon 11 seem to express a more complex transcriptome and proteome compared to GISTs with *KIT* mutation in exon 9.

Most differences between the two groups of mutants were observed on metal affinity surfaces, which are known to have a high affinity for phosphorylated proteins. This observation allows us to hypothesize that *KIT* mutations in exon 9 and exon 11 initiate different signalling pathways, which might explain the different clinical response to the tyrosine kinase inhibitor Imatinib. These preliminary results require further validation in a larger group of patients, before the identification of significantly differentially expressed proteins. The future characterisation of these proteins could be very important in optimising the use of signal transduction inhibitors, perhaps aiding the development of combination regimens.

Acknowledgements

Bart Landuyt is a research fellow of the ''Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen'' (IWT-Vlaanderen). SELDI TOF MS data were generated by Ciphergen's Benelux demo facility at the University Hospital of Leuven. We are gratefull to Martin Highley of Ninewells Hospital (Dundee, Scotland, UK) for his suggestions to improve our English writing.

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