Tyrosine kinase activity profiling of metastatic malignant melanoma: Identification of possible therapeutic targets and markers predicting response to therapy

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Abstract

Metastatic melanoma is an aggressive form of cancer that responds poorly to conventional chemotherapy. The aim of this study was to obtain information about the tyrosine kinase activity in metastatic malignant melanoma in the presence and absence of two different kinase inhibitors. Tyrosine kinase activity was measured on 26 human metastatic stage IV melanoma samples and 4 normal skin samples using Tyrosine Kinase PamChip® arrays. The overall protein tyrosine kinase (PTK) activity was higher in melanoma compared to normal skin. Further, we attempted to evaluate the tyrosine kinase activity in response to the B-Raf inhibitor PLX4032, and the multi-targeted kinase inhibitor sunitinib, in order to receive important tumor-specific information on affected targets and pathways. Differential kinase activity was observed between *BRAF*^{V600E} and *BRAF*^{wt} in response to PLX4032. Based on the inhibition profiles, 41 peptide substrates were identified as significantly different between the two groups, with many of the peptides encoding for proteins involved in the MAPK pathway. Stronger inhibition was observed in tumors with *BRAF*^{V600E} compared to *BRAF*^{wt}. Treatment with sunitinib caused the expected pattern of kinase inhibition.

Introduction

Metastatic malignant melanoma is a life-threatening cancer type lacking optimal systematic treatment. Established standard chemotherapy with dacarbazine (DTIC) has shown poor response rates (10-20%) in patients with advanced melanoma, and more targeted therapies are urgently needed. Kinases are important key regulators of the cell, and the use of kinase inhibitors as cancer treatment has increased dramatically. In 2011, vemurafenib (PLX4032) was approved by the FDA for the treatment of advanced melanoma. Vemurafenib is a serine/threonine kinase inhibitor targeting B-Raf with *V600E* mutation. This type of mutation occurs in ~50% of all melanoma patients, and treatment with vemurafenib has shown positive results in patients harboring BRAF^{V600E} suffering from late stage melanoma (1). However, the occurrence of *de novo* and acquired resistance towards vemurafenib in patients with the *V600E* mutation needs further investigation on the molecular mechanisms involved. We studied the kinase activity profiles in metastatic malignant melanoma in the presence and absence of either PLX4032 or sunitinib. Our aim was to identify PTKs that are deregulated in metastatic malignant melanoma and possibly identify novel targets and pathways involved in resistance to the tested TKIs.

Materials and Methods

Tyrosine Kinase PamChip® array technology

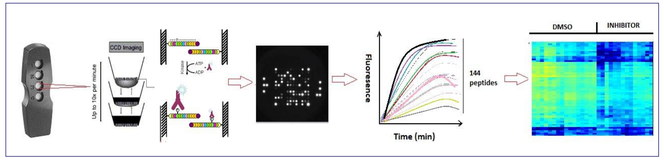

Fig.1 Tyrosine Kinase PamChip® Array for PamStation®12 consists of 144 peptide substrates with known phosphorylation sites, representing 100 different proteins. The technology allows automated, fast and easy identification of substrates for tyrosine kinases, and provides a tool for the multi-parallel detailed analysis of the kinetic mechanism of kinases (2).

Samples and data analysis

Tyrosine kinase activity profiling was performed on fresh frozen tumor tissue from 26 stage IV melanoma patients prior to DTIC treatment. All specimens have previously been described and screened for mutations in *BRAF, NRAS*, *CDKN2A* and *TP53* (3). In addition, 4 normal skin tissue samples were collected from the skin of individuals not affected by melanoma. The tissue was sliced in coupes and lysed in lysis buffer supplemented with phosphatase and protease inhibitors for determination of kinase activity profiles in the presence and absence of PLX4032 (40μM) and Sunitinib (7.5μM). 15μg of total protein lysate was used per array in the presence of 400μM ATP. Signal intensities were quantified and analyzed using BioNavigator. For the inhibition profiles log2 ratio's of inhibited vs. non-inhibited were calculated, and used for further data analysis.

26 metastatic malignant melanoma Prior to DTIC treatment + **4 normal skin tissue**

1. Tyrosine kinase activity profiling before treatment with inhibitor (control)
- **i)** Data was log2 transformed. All values <0.01 set to 0.01
- **ii)** Sample mean calculated for melanoma and normal skin
- **iii)** T-test was performed between cases and controls

26 metastatic malignant melanoma Prior to DTIC treatment

2. Measure the effect of inhibitor on tyrosine kinase activity (Inhibition)
- **i)** Data was log2 transformed
- **ii)** Inhibition profile = Effect of inhibitor on all 144 peptides (log2 (control) - log2 (inhibitor))

3. Tyrosine kinase activity profiles before and after inhibition were analyzed for correlation to different clinical parameters.

4. T-test was performed to identify significantly different peptides between groups. Only peptides with p.value >0.05 were selected for analyses.

Fig.2 Overview of samples involved in the study and the data analysis performed.

Results

Tyrosine kinase activity in metastatic malignant melanoma

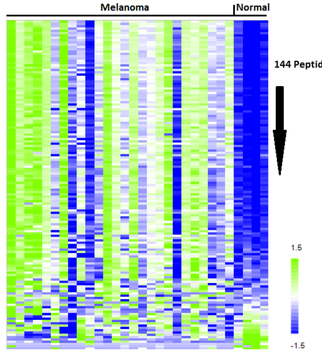

Fig.3 Heatmap of peptide phosphorylation intensity for 144 peptides in melanoma and normal samples. In general, most peptides (80-90%) had higher phosphorylation intensity in melanoma samples, and were identified as significantly different between the two groups.

BRAF^{V600E} and *BRAF*^{wt} inhibition profiles with PLX4032 in melanoma

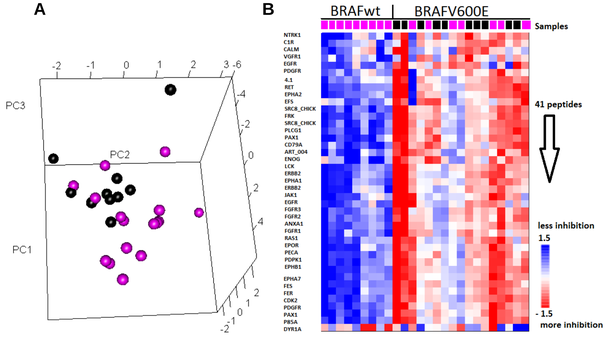

Fig.4 Inhibition profiles after treatment with PLX4032. A) PCA including all 144 peptides separated *BRAF*^{wt} (magenta) and *BRAF*^{V600E} (black) melanoma samples in two groups. B) Heatmap based on 41 peptides that were identified as significantly different (p.value <0.5) in phosphorylation intensity in *BRAF*^{wt} and *BRAF*^{V600E} in response to PLX4032. Stronger inhibition was observed in samples with mutant *BRAF* compared to wild type *BRAF*.

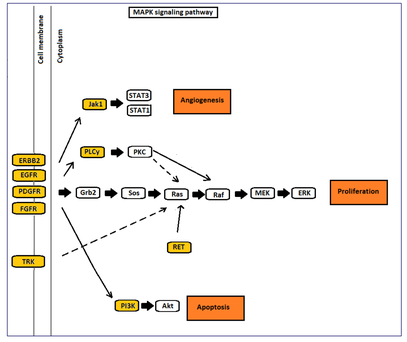

Fig. 5 Kinases involved in the MAPK pathway affected by PLX4032. In color are the kinases encoded by the peptide substrates identified as differentially affected by PLX4032 between *BRAF*^{V600E} and *BRAF*^{wt} samples. Angiogenesis, Proliferation and apoptosis were affected through the MAPK signaling pathway.

Sunitinib inhibition profiles in melanoma samples

For the analysis with sunitinib, inhibition of tyrosine kinase activity was observed as being expected. However, the inhibition profile obtained did not correlate to any known biological factors or clinical parameters.

Discussion

Our results show that the PTK activity is generally higher in metastatic malignant melanoma compared to normal skin. In the PLX4032 inhibition profiles, 41 peptides could differentiate between *BRAF*^{wt} and *BRAF*^{V600E} samples. Most of the peptides encoded for proteins involved in the MAPK pathway, previously identified to be important for the response and resistance to PLX4032 (4,5). Upregulated tumor expression of EGFR has been suggested to play a role in resistance towards PLX4032 in colon cancer patients with *BRAF*^{V600E} mutations (4). In melanoma, acquired resistance to PLX4032 is thought to develop through upregulated PDGFRB (5). Taken together, the results suggests that PTKs involved in the MAPK pathway may serve as important targets for treatment of metastatic malignant melanoma. The finding of human melanoma-samples with BRAF^{wt} behaving like BRAF^{V600E} after treatment with PLX4032 (fig.4) may indicate that some patients could potentially benefit from treatment with PLX4032, despite lacking the *V600E* mutation. This demands further clarification in the future.

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