Gene regulatory biomarker identification for skin toxicities induced by EGFR inhibitor treatment

Vivien Hichert¹, Michael Steffens¹, Tanusree Paul², Catharina Scholl³,⁴, Sumit Parmar², Stefan Rüdiger³, Christian Schumann⁵, Thomas Seufferlein⁵, and Julia C. Stingl¹,²

¹Federal Institute for Drugs and Medical Devices (BfArM), Research Division, Bonn, ²Institute of Pharmacology of Natural Products and Clinical Pharmacology, ³Department of Internal Medicine II, University of Ulm, Ulm, ⁴Pneumology, Thoracic Oncology, Sleep- and Respiratory Critical Care Medicine, Clinics Kempten-Oberallgäu, Kempten, and ⁵Department of Internal Medicine I, University of Ulm, Ulm, and ⁶Department of Internal Medicine I, Martin Luther University Halle-Wittenberg, Germany

Introduction

The epidermal growth factor receptor (EGFR) is a tyrosine kinase belonging to the ErbB family, which is (over-) expressed in numerous solid (epithelial) tumors such as non-small cell lung carcinoma, adenocarcinoma of the pancreas, and colorectal carcinoma [1]. EGFR influences essential cellular processes such as proliferation, differentiation, and apoptosis by means of various signaling cascades involving mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT, and the signal transducer and activator of transcription 3 (STAT3) [2].

EGFR inhibition by monoclonal antibodies (cetuximab, panitumumab) and tyrosine kinase inhibitors (erlotinib, gefitinib) is frequently used in cancer therapy. Since EGFR is expressed not only in cancer cells but also in normal epithelial cells, especially in skin, a typical adverse effect of EGFR inhibitors (EGFRIs) is specific skin toxicity, including folliculitis and acneiform skin rash. This adverse effect is seen for all 4 described inhibitors and regardless of the tumor entity. Occurrence and severity of this skin rash are positively correlated with patient outcomes and survival.

The pathophysiology of EGFR-induced skin toxicities is not yet fully elucidated. However, gene regulatory elements, e.g., microRNAs (miRNAs), might play a role. MiRNAs are noncoding RNAs with a chain length of between 21 and 25 nucleotides. They are partially complementary to specific messenger RNA (mRNA) molecules to which they can bind to inhibit translation. So far, ~ 900 miRNAs have been identified within the human genome, but for most of these, the targets and exact functions are still under investigation. However, certain miRNAs have already been found to be involved in the regulation of genes of EGFR-signaling cascades, including miR-21 and let-7 [2].

The aims of this study were: 1) To identify gene regulatory determinants of skin toxicities associated with EGFRI treatment such as the miRNAs. 2) To elucidate the pathophysiology of these toxicities. Clinical data and genetic information for this purpose were obtained during a prospective pharmacogenetic study involving cancer patients undergoing first-time treatment with an EGFRI. An additional aim was to investigate the use of human adult dermal fibroblasts as a cell-culture model for further studies on the skin toxicities described above. An initial step in this work was to compare miRNA profiles in EGFRI-treated and untreated fibroblasts and to identify those miRNAs that are important in the response to EGFRIs. It was hoped that this approach might reveal which miRNAs can be used as biomarkers for assessing the likelihood of patients developing skin toxicities and for predicting the effectiveness of EGFRI cancer therapy.
Material and methods

Prospective pharmacogenetic study

Beginning in September 2008, a multi-center, prospective pharmacogenetic study was conducted, as described in a previous publication [3]. So far, the study has included more than 150 cancer patients undergoing first-time treatment with EGFRIs (cetuximab, panitumumab, erlotinib, or gefitinib). Survival time, skin toxicity, and other side effects were monitored for 1 year after initiation of EGFR therapy, and clinical variables (e.g., type of tumor entity, tumor status, age, drug treatment) were also determined. Blood samples for future genetic analyses were obtained 4 weeks after initiation of EGFR therapy.

Cell culture

Primary human dermal fibroblasts were obtained from healthy tissue removed by dermal excision from patients at the Clinical Pharmacology Department of the University Medicine Göttingen (kindly provided by Dr. med. Markus Schirmer). They were cultured in Basal Fibroblast Growth Medium 2 (PromoCell, Heidelberg, Germany) supplemented with 0.02 mL/mL fetal calf serum (FCS, PromoCell, Heidelberg, Germany) and antibiotics (100 U/mL penicillin; 100 µg/mL streptomycin; BioWest, Nuaillé, France) and grown at 37 °C and 5% CO₂. The medium was changed every 2 – 3 days, and cells were passaged every 7 – 10 days. miRNA precursor profiling was carried out after incubation of cells with 5 µM erlotinib-HCl (Santa Cruz Biotechnology, Dallas, TX, USA) for 24 hours and isolation of total RNA.

Next generation sequencing

Total RNA was isolated from erlotinib-treated and untreated human dermal fibroblasts using peqGOLD TriFast (peqlab, Erlangen, Germany). The total RNA was used to generate a barcoded cDNA library with aid of the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, Frankfurt, Germany) according to the manufacturer’s instructions. miRNA precursor profiles were determined by next generation sequencing of the cDNA libraries using the Illumina MiSeq platform and the miRBase database (containing predicted hairpin portions of miRNA transcripts). Data were analyzed using the Illumina MiSeq Reporter Software and the DESeq2 package [4]. The differential expression of miRNA precursors with an adjusted p-value < 0.05 was considered to be significant.

Results

Patients who experienced skin toxicity within the first 4 weeks of EGFR therapy showed a significantly longer overall survival than patients with no skin toxicities (log rank p = 0.0003; data not shown). The various types of tumor entities were distributed equally across the four groups of skin rash (grade 0 to 3). Diarrhea, as a nonspecific side effect, was not associated with a prolonged survival (data not shown).

Complex computational analysis, including thorough normalization of miRNA precursor counts, showed that several miRNA precursors were differentially expressed in erlotinib-treated as compared to untreated fibroblasts. The 10 miRNA precursors for which the differential expression was most significant (adjusted p-value ≤ 0.02) are listed in Table 1.

<table>
<thead>
<tr>
<th>miRNA precursor</th>
<th>log2fold-change</th>
<th>p-value</th>
<th>adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-mir-214</td>
<td>-0.91</td>
<td>9.1 × 10⁻⁶</td>
<td>1.9 × 10⁻³</td>
</tr>
<tr>
<td>hsa-mir-1273a</td>
<td>-1.16</td>
<td>1.8 × 10⁻⁴</td>
<td>1.2 × 10⁻²</td>
</tr>
<tr>
<td>hsa-mir-999a</td>
<td>-0.64</td>
<td>2.5 × 10⁻⁴</td>
<td>1.2 × 10⁻²</td>
</tr>
<tr>
<td>hsa-mir-484</td>
<td>0.81</td>
<td>2.8 × 10⁻⁴</td>
<td>1.2 × 10⁻²</td>
</tr>
<tr>
<td>hsa-mir-660</td>
<td>0.84</td>
<td>2.9 × 10⁻⁴</td>
<td>1.2 × 10⁻²</td>
</tr>
<tr>
<td>hsa-mir-129-1</td>
<td>-1.60</td>
<td>6.2 × 10⁻⁴</td>
<td>1.6 × 10⁻²</td>
</tr>
<tr>
<td>hsa-mir-99b</td>
<td>-0.56</td>
<td>6.8 × 10⁻⁴</td>
<td>1.6 × 10⁻²</td>
</tr>
<tr>
<td>hsa-mir-151a</td>
<td>-1.04</td>
<td>7.1 × 10⁻⁴</td>
<td>1.6 × 10⁻²</td>
</tr>
<tr>
<td>hsa-mir-425</td>
<td>-0.50</td>
<td>7.1 × 10⁻⁴</td>
<td>2.0 × 10⁻²</td>
</tr>
<tr>
<td>hsa-mir-186</td>
<td>0.79</td>
<td>9.5 × 10⁻⁴</td>
<td>2.0 × 10⁻²</td>
</tr>
</tbody>
</table>
Discussion and conclusions

This prospective pharmacogenetic study with more than 150 cancer patients confirms results obtained in several previous studies showing that the occurrence of skin toxicity induced by EGFRIs significantly correlates with overall survival.

The determination of miRNA precursor profiles in human dermal fibroblasts revealed a number of miRNA precursors that are differentially expressed in erlotinib-treated as compared to untreated fibroblasts. Although the calculated fold-changes, after averaging the miRNA counts in all cell samples and thorough normalization, seem rather low (fold-changes 1.4 – 3; log2-fold-change 0.5 – 1.6), they might still provide hints about which miRNAs are involved in the response to EGFRIs in skin cells. Several studies have already been published suggesting functional roles in EGFR signaling pathways for some of the differentially expressed miRNA precursors found in our study (or their corresponding mature miRNA molecules). MiRNA hsa-mir-214 for instance has been found to target the 3’ untranslated region (UTR) of the phosphatase and tensin homolog (PTEN) gene resulting in the down-regulation of PTEN protein, which is a phosphatase gene resulting in the down-regulation of PTEN and the phosphatase and tensin homolog (PTEN) targeting the 3’ untranslated region (UTR) of NShsa-mir-214 for instance has been found responding mature miRNA molecules). MiR-cursors found in our study (or their corresponding mature miRNA molecules). MiRNA hsa-mir-214 for instance has been found to target the 3’ untranslated region (UTR) of the phosphatase and tensin homolog (PTEN) gene resulting in the down-regulation of PTEN protein, which is a phosphatase gene resulting in the down-regulation of PTEN and the phosphatase and tensin homolog (PTEN) targeting the 3’ untranslated region (UTR) of NA

The impact of the miRNAs listed in Table 1 on the occurrence of EGFRI-induced skin toxicities has already been found in tumor cell lines [7]. Our results might help to identify an miRNA signature predictive for the development of EGFRI-induced skin toxicities, which would also be predictive for the effectiveness of the therapy. This could serve as an alternative to analyses in tumor cells and could be determined even before surgical removal of the tumor and also independently of tumor entity, stage, etc. Such an miRNA signature, or even single specific miRNAs, as biomarkers for EGFRI-induced skin toxicity, might help the early identification of patients in whom an EGFRI therapy would be less effective. This would facilitate therapeutic management and make cancer therapy more efficient and safer for patients.

Acknowledgment

This work was supported by a grant from the Wilhelm Sander Foundation (Grant No. 2008.017.1).

References


