Biotechnology Reports 18 (2018) e00252

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Proseek single-plex protein assay kit system to detect sAxl and Gas6 in serological material of brain tumor patients

Heidi Jaksch-Bogensperger[a,b,*], Anna Hammerschmid[b], Ludwig Aigner[b], Eugen Trinka[d], Renate Gehwolf[e], Yvonne Ebner[f], Markus Hutterer[g], Sebastien Couillard-Despres[c]

[a] University Hospital for Obstetrics and Gynaecology, Paracelsus Medical University Salzburg, Müllner Hauptstrasse 48,A-5020, Salzburg, Austria
[b] Institute of Molecular Regenerative Medicine, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Paracelsus Medical University, Strubergasse 21, A-5020, Salzburg, Austria
[c] Institute of Experimental Neuroregeneration, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Paracelsus Medical University, Strubergasse 21, A-5020, Salzburg, Austria
[d] University Hospital of Neurology, Christian-Doppler-Klinik, Paracelsus Medical University Salzburg, Ignaz-Harrer-Straße 79, A-5020, Salzburg, Austria
[e] Institute of Tendon and Bone Regeneration, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Paracelsus Medical University, Strubergasse 21, A-5020, Salzburg, Austria
[f] University Hospital of Psychiatry, Psychotherapy and Psychosomatic, Paracelsus Medical University Salzburg, Ignaz-Harrer-Straße 79, A-5020, Salzburg, Austria
[g] Department of Neurology 1 – Neuromed Campus, Kepler University Hospital Linz, Wagner-Jauregg Weg 15, A-4020, Linz, Austria

ARTICLE INFO

Article history:
Received 4 December 2017
Received in revised form 1 March 2018
Accepted 4 April 2018

Keywords:
High-grade glioma (HGG)
sAxl
Gas6
Proximity extension assay (PEA)
Proseek
Serum

ABSTRACT

- The receptor tyrosine kinase (RTK) Axl and its ligand Gas6 are critically involved in the pathogenesis of high-grade glioma (HGG). Both proteins were found to be overexpressed e.g. in tumor cells, mediating cell proliferation and migration as well as tumor angiogenesis and neuroinflammation. The extracellular domain of Axl (sAxl) and Gas6 were found in the peri-tumoral edema and blood of animals as well as in human glioma tissue. Therefore, we monitored the level of sAxl and Gas6 in human blood samples. To increase the sensitivity of protein detection beyond commonly used standard methods we preliminary tested the innovative Proseek Single-Plex Protein Assay Kit System from Olink Bioscience together with new antibodies against the soluble RTK sAxl and its ligand Gas6. We conclude that the Proseek method is a highly sensitive and fast procedure that can be used as a possible powerful tool compared to routinely used ELISA-methods.

1. Introduction

High-grade glioma (HGG), WHO grade III-IV, are the most common primary brain tumors in adults with an incidence of 6–8/100.000 inhabitants per year. Within this group, glioblastome multiforme (GBM) WHO IV is the most aggressive subtype and represents the biggest group therein. Anaplastic glioma WHO grade III imply astrocytoma and oligodendroglioma [1,2]. The current standard treatment of GBM includes a maximal surgical tumor resection, followed by radio- and chemotherapy with temozolomide [3,4].

Since the receptor tyrosine kinase (RTK) Axl and its ligand growth arrest specific gene 6 (Gas6) are known to be co-expressed in HGG tissue correlating with a poor prognosis [5], we were interested on testing a new adapted open format reagent kit method system from Olink Bioscience called Proseek Single-Plex Protein Assay measuring the soluble (extracellular) portion of the Axl RTK (sAxl). The usual method of choice to measure levels of sAxl and Gas6 in human sera is a sandwich enzyme-linked immunosorbent assay (ELISA), which general protocol has been optimized recently based on challenging stability and storage conditions, but also masking effects of unknown components in serum [6]. Since ELISA measurement includes time-consuming washing procedures, the Proseek Single-Plex Assay provides results within 24 h without washing steps. Furthermore,

* Corresponding author.
E-mail addresses: h.jaksch-bogensperger@salk.at (H. Jaksch-Bogensperger), A.Hammerschmid@salk.at (A. Hammerschmid), Ludwig.Aigner@pmu.ac.at (L. Aigner), E.Trinka@salk.at (E. Trinka), Renate.Gehwolf@pmu.ac.at (R. Gehwolf), Y.Ebner@salk.at (Y. Ebner), markus.hutterer@kepleruniklinikum.at (M. Hutterer), Sebastien.Couillard-Despres@pmu.ac.at (S. Couillard-Despres).

https://doi.org/10.1016/j.btre.2018.e00252

only 1 μl of serum, plasma, or almost any other type of biological sample such as liquor, is sufficient for valide results. Another interesting part of the method is the combination of oligonucleotide-labeled antibodies (binding the target protein within the sample) and the formation of a new PCR target sequence, which can be quantified by standard real-time PCR. This powerful combination of protein detection and PCR amplification to quantify single proteins in solutions with a maximum of sensitivity and specificity is able to visualize low levels of proteins even before tumors can be detected by imaging technologies prior therapy and also should help for therapy monitoring. Therefore we were interested on the combination of this method with targeted human protein biomarkers for tumor vascularization, sAxl and Gas6.

The receptor tyrosine kinase Axl, beside Tyro3/Sky and Mer (syn. TAM family), is characterized by an extracellular domain (ECD) of two immunoglobuline-like domains abreast of two fibronection-type III domains [7,8].

After ligand binding (e.g. Gas6, Protein S), the tyrosine kinase domain of the receptors of the TAM family activates the intracellular signal transduction downstream by receptor dimerisation and initiation of autophosphorylation [9,10]. Axl/Gas6 signaling can induce apoptosis inhibition in a broad range of cells, [11–14] and is involved in cell migration, essential for tumor invasiveness, metastasis and neoangiongenesis. Therefore the Axl/Gas6-system is involved in various physiological processes, including angiogenesis and several types of human cancer [5,9,10,15,16].

2. Material and methods

2.1. Study population and sample collection

To generate the new method to measure the detection of sAxl and Gas6, 3 patients of ≥18 years and ≤80 years of age with the diagnosis of a first or second recurrent or progressed high-grade glioma measured by standard MRI were included. For comparison, the serum of a healthy 25-year old woman was added (control). Sera of patients with recurrent or progressive HGG receiving anti-angiogenic bevacizumab therapy (P02: female, age 47; P04: male, age 55; P05: male, age 42) were used. After baseline and treatment start with Avastin, clinical follow ups started with serum sample collections at week 1 ± 1 day, followed by clinical follow up visits at week 2. Afterwards every second week until clinical progression (e.g. P01 S1: patient 01 with serum sample S1 of the first week, S2 of the second week, and samples S3 to S7 for every second week), serum samples were collected and used for measurements. Ethical approval for this study was granted by the local research ethics committee (AM3752_LEK).

2.2. Sample preparation

For serum sample preparation, standard laboratory tests including chemistry (differential (%), coagulation, biochemistry) and haematology panels have been performed. At baseline evaluation a serum pregnancy test (female patient with reproductive potential) and a test for infections (Hepatitis B/C, HIV) have been carried out. All patients have been consented for the collection and storage of blood (University Hospital of Neurology, PMU, Salzburg), and markers have been evaluated using above-mentioned method. At baseline and during periodic follow-up visits in the course of an antiangiogenic treatment with Avastin (Bevacimzumab 10 mg/m2 body surface every two weeks), serum samples have been collected at baseline, in the first and second week and afterwards every 2 weeks (S1, S2, S3, . . .) until tumor progression for precise Proseek analysis. Therefore one serum tube derived from vein puncture for testing the combination of the new markers associated with tumor neovascularization (sAxl, Gas6) by Proseek-method have been used. Therefore blood samples were let for 10 min at room temperature and centrifuged for 10 min at 1408 × *g*. Aliquots of the supernatants were frozen to −80 °C. Serial dilutions for the proteins sAxl and Gas6 were prepared as a positive control and to establish a calibration curve. To this end, the recombinant and affinity purified Axl (DY154, R&D systems) and Gas6 (DY885, R&D systems) have been diluted in Calibrator Diluent as follows: Axl 139 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml, 0.1 pg/ml, 0.01 pg/ml, 0 (Calibrator Diluent); Gas6 230 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml, 0.1 pg/ml, 0.01 pg/ml, 1 fg/ml, 0 (Calibrator Diluent).

2.3. Proseek-proximity extension assay (PEA) technology

Proseek is a reagent Kit system from Olink Bioscience (Uppsala, Sweden) to detect and quantify proteins in sample material like serum and plasma, based on the Proximity Extension Assay (PEA) technology. The PEA is a method for single protein detection based on a Proximity-dependent DNA polymerization event. It can be performed using 2 Proximity probes. For this project, we took two polyclonal antibodies already established in ELISAs, but not for the Proseek reagent Kit system, to detect sAxl (AF154, R&D systems) and Gas6 (AF885, R&D systems). The Proseek Assay Development kit has been performed as described in the user manual version 3.0. First, the antibodies were conjugated either to the oligonucleotides (Oligonucleotide-labeled antibodies) A or B by using Proseek Probemaker A (Art.no. 93001-0010) or B (Art. No. 93002-0010) to create Proseek probes A and B. A dilution series of recombinant human sAxl (DY154, R&D systems) and recombinant human Gas6 (AF885, R&D systems) as antigen standard in Calibrator diluent (Proseek Assay Reagents, Art.no. 93003-1000) for standard curve was used. For negative control, Calibrator diluent without antigen was prepared for method application. The incubation of the dilution series and serum samples with Proseek probes A and B leads to the binding to the target protein. To dilute the Proseek probes and lower their real concentration, the Pre-Extension solution has been adducted before the Extension master mix has been added. Thereafter, the addition of a DNA polymerase will enable the extension of the hybridized oligonucleotides and the resulted sequence is detected and quantified by qPCR (real-time PCR amplicon, Proseek Assay Development Kit, Olink Bioscience).

2.4. Real-time based proseek technology

For this study, a two-step real-time PCR (qPCR) was performed by using the fluorophore FAM based on the TaqMan® Protein Assays Probe Development Protocol (LifeTechnologies, Applied Biosystems, Austria). Proseek reagents have been prepared according to the Proseek user manual (Olink, Bioscience, 2014). Samples, buffer (recommended background control by the TaqMan® Protein Expression Assay Protocol) or recombinant antibody for standard curve were incubated according to manual instruction. The PCR was performed using the TaqMan® Protein Expression Assay reagent kit according to the protocol (also Master Mix without Polymerase). Data analysis and sample calculation were carried out using Microsoft Excel 2007. For the calibration curve of each test of sAxl and Gas6, the calibrators were measured in duplicates. Afterwards the cycle of quantification (Cq) was calculated as the mean value of 3 independent experiments and the background values of the control were subtracted. To assess the concentration of the proteins the formula y = kx + d has been used.

2.5. ELISA

Calibration curves using human sAxl (DY154, R&D Systems) and Gas6 (DY885, R&D Systems) were also analyzed using the DuoSet