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ONLINE SUPPLEMENT

TARGETING CHEMOKINE RECEPTOR CXCR4 FOR MOLECULAR IMAGING OF ALDOSTERONE-PRODUCING ADENOMA

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Short title: CXCR4 expression in aldosterone-producing adenoma

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MATERIALS AND METHODS

Immunohistochemical analysis

As a screening test for primary aldosteronism the aldosterone / renin concentration ratio (ARR) after withdrawal of interfering drugs had been used and diagnosis of PA had subsequently been confirmed by saline infusion test. After confirmation of PA, 103 out of 117 patients underwent adrenal vein sampling. In the remaining 14 patients the decision for adrenalectomy had been based on detection of an adrenal lesion in conventional adrenal imaging combined with younger age (below 45 years) and/or positive postural test. After adrenalectomy, surgical specimens underwent histopathological evaluation by a local pathologist. Unilateral disease was confirmed by normalization of ARR and potassium levels as well as cure or amelioration of hypertension at follow-up (available for 113/117 patients).
All patients with cortisol producing adenoma had an abnormal dexamethasone suppression test defined as serum-cortisol level after 1 mg dexamethasone above 1.8 µg/dl. Mean serum cortisol levels after dexamethasone were 15.9 µg/dl (2.2 – 30.6 µg/dl). In addition, at least one further pathological test of the HPA axis was required for inclusion as CPA: 24 hour urine cortisol levels above the normal range and increased salivary or serum cortisol levels at midnight.
Patients with non-functioning adrenocortical adenoma had no clinical evidence of hormone excess and furthermore normal tests results for aldosterone to renin ratio and cortisol levels.
The respective local specialists of the departments of pathology confirmed histological diagnosis of adrenocortical adenoma.
Tissue sections were deparaffinized in xylene for 2x12 min and dehydrated in ethanol (100%, 90%, 80%, 70%, each concentration for 5 min). Immunohistochemical detection was performed using an indirect immunoperoxidase technique after high temperature antigen retrieval in 10 mM citric acid monohydrate buffer (pH 6.5) in a pressure cooker for 13 min. Blocking of unspecific protein-antibody interactions was performed with 20% human AB-serum in PBS for 1 hour at room temperature. The primary CXCR4 antibody was used at a dilution of 1:100 at RT for 1 h together with the N-Universal Negative Control Anti-Rabbit (Dako, Glostrup, Denmark). CXCR4 and CXCL12 antibodies were from abcam (Cambridge, United Kingdom (12824, 9797)) Specific antibodies against human CYP11B1 (dilution 1:100) and CYP11B2 (dilution 1:200) were kindly provided by Celso Gomez-Sanchez, University of Mississippi, USA. Signal amplification was achieved by En-Vision System Labeled Polymer-Horseradish peroxidase (Dako, Santa Clara, USA) for 40 min and developed for 10 min with Diaminobenzidine Substrate Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Nuclei were counterstained with Mayer's hematoxylin for 2 min. Negative controls were carried out by treating the slides with a nonimmune serum instead of the primary antibody, yielding a nearly complete loss of staining with only some faint background.
The anti-CXCR4, anti-CYP11B2 and anti-CYP11B1 staining intensity was evaluated with a grading score of 0, 1, 2, or 3, which corresponded to negative, weak, moderate, or strong staining intensity, respectively. The percentage of positive tumor cells was calculated for each specimen and scored 0 if 0% were positive, 0.1 if 1–9%, 0.5 if 10–49%, and 1 if >50%. A semi-quantitative h-score was then calculated by multiplying the staining intensity grading score with the proportion score as previously described [1]. Calculation of h-score was separately performed for membrane and cytoplasmatic staining in tumor tissue as well as the adjacent adrenal gland. For membrane and cytoplasmatic staining an h-score ≤ 1 was rated as

low, whereas and h-score > 1 was rated as high. Investigators were blinded to the patient data.

Analysis of chemokine receptor expression in adrenocortical adenomas by quantitative PCR

40 ng cDNA was used for each PCR reaction and each sample was performed in duplicate. Transcript levels were determined using the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, USA), the CFX96 real-time thermocycler (Bio-Rad, Hercules, CA, USA) and Bio-Rad CFX Manager 2.0 software. Cycling conditions were 95°C for three min followed by 50 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. Using the ΔCT method [2], the gene expression levels were normalized to those of b-actin.

Autoradiographic experiments with 68Ga-Pentixafor

Human tissues were snap-frozen in liquid nitrogen to block further biological processes including protein degradation and tissue hardening and stored at –80 °C. Experiments were repeated thrice. Before cutting, tissues were transferred to a –20 °C freezer. Frozen samples were sectioned in a micro-cryotome into 20 µm tissue slices for all tissues and thaw-mounted onto superfrost slides, dried and then stored at –80 °C until processing. A barrier pen was used (Invitrogen Mini PAP PEN, Zymed® Laboratories, Carlsbad, CA, USA). For the experimental set-up the tissue slices were fixated for 10 min with 0.4%PFA and then pre-incubated for 5 min at room temperature with buffer solution (pH 7.4, 50 mM Tris). Afterwards the slices were incubated with 20% human AB-serum in PBS for 60 min. After blocking for 1 hour, one slide was treated with 2 MBq 68Ga-Pentixafor and another slide was incubated with 2 MBq 68Ga-Pentixafor plus 10 µM of the non-radioactive compound to monitor the specific binding. Slides were subsequently washed twice in 0.5% Tween 50 mM Tris HCl buffer solution (pH 7.4) and dried. Thereafter, samples were placed for 30 min on Phosphor Imager plates in dedicated lead shielded cassettes. Autoradiographic images were analyzed with a Phosphor Imager (CR35BIO Imageplate scanner) and data analysis was performed with Amide software Version 0.9.0 and Microsoft Excel® 2007.

Imaging of patients by 68Ga-Pentixafor-PET

In the four patients receiving PET/CT, corresponding CT low dose scans for attenuation correction were acquired using a low-dose protocol (20 mAs, 120keV, a 512x512 matrix, 5mm slice thickness, increment of 30mm/s, rotation time of 0.5s, and pitch index of 0.8) including the base of the skull to the proximal thighs for the reference group and including one bed position over the abdomen for the patients with primary aldosteronism. Consecutively, PET emission data were acquired in three-dimensional mode with a 200x200 matrix with 2-3min emission time per bed position. After decay and scatter correction, PET data were reconstructed iteratively with attenuation correction using a dedicated software (Siemens Esoft).
In the remaining five patients, PET and MR data were simultaneously acquired for 20 min on the integrated PET/MR system 50 to 69 min after injection of 68Ga-Pentixafor. The PET scan was acquired in 3D-list-mode including PET-based respiratory gating, attenuation correction was accomplished using 2-point Dixon MR sequences. For anatomical correlation T1w and T2 fatsat axial sequences, as well as T2w coronal sequences were acquired.

In all cases 68Ga-Pentixafor scans were performed on a dedicated PET/CT (Siemens Biograph mCT 64 and Biograph mCT 128; Siemens Medical Solutions, Erlangen, Germany) or PET/MRI System (Siemens Biograph mMR; Siemens Medical Solutions, Erlangen, Germany). Injected activity ranged from 115 to 207 MBq and image acquisition started after 50 to 86 min.
Semi-quantitative PET scan analysis comprised calculation of the highest metabolic activity within each adrenal gland (maximum standardized uptake values, SUVmax) by 2D regions of interest (ROI) with a diameter of 1 cm around the hottest pixel. CT and MRI scans were read qualitatively by reporting the location and size of adrenal lesions.
68Ga-Pentixafor was administered under the conditions of pharmaceutical law (The German Medicinal Products Act, AMG §13 2b) according to the German law and in accordance with the responsible regulatory body (Regierung von Unterfranken, Regierung von Oberbayern, Germany) [3]. All patients gave written informed consent prior to 68Ga-Pentixafor-PET imaging.

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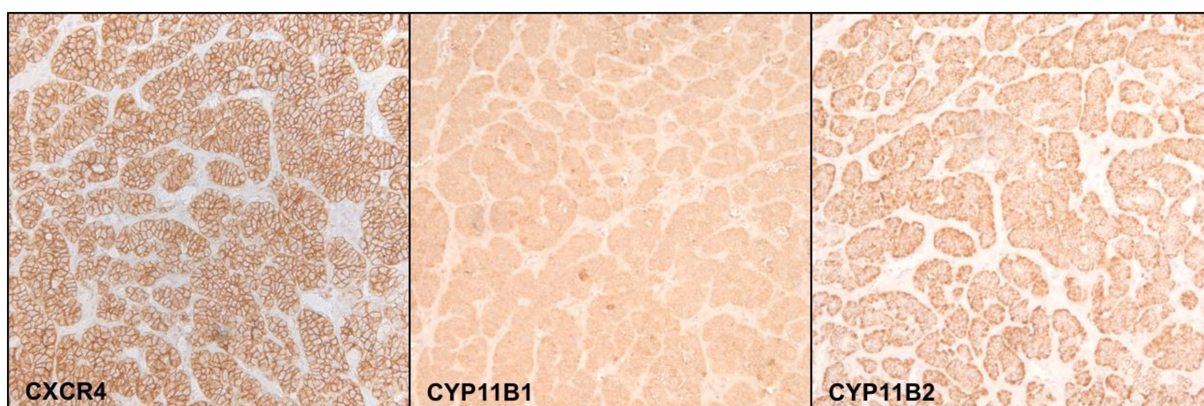
Results

Chemokine Receptor	Taqman Gene Expression assay
CCR1	Hs00928897_s1
CCR2	Hs00704702_s1
CCR3	Hs01847760_s1
CCR4	Hs00747615_s1
CCR5	Hs99999149_s1
CCR6	Hs10890706_s1
CCR7	Hs01013469_m1
CCR8	Hs00174764_m1
CCR9	Hs01890924_s1
CCR10	Hs00706455_s1
CCR11	Hs00664347_s1
CXCR1	Hs01921207_s1
CXCR2	Hs01891184_s1
CXCR3	Hs01847760_s1
CXCR4	Hs00607978_s1
CXCR5	Hs00540548_s1
CXCR6	Hs01890898_s1
CXCR7	Hs00664172_s1
b-actin	Hs9999903_m1

S1. Specific Taqman Gene Expression assays by Applied Biosystems (Darmstadt, Germany).



S2. Immunohistochemical staining of CXCR4, CYP11B1 and CYP11B2 in aldosterone-producing adenoma, Magnification 10x.