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Next-generation sequencing of human opioid receptor genes based on a custom AmpliSeq™ library and ion torrent personal genome machine

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ABSTRACT

Background: The opioid system is involved in the control of pain, reward, addictive behaviors and vegetative effects. Opioids exert their pharmacological actions through the agonistic binding at opioid receptors and variation in the coding genes has been found to modulate opioid receptor expression or signaling. However, a limited selection of functional opioid receptor variants is perceived as insufficient in providing a genetic diagnosis of clinical phenotypes and therefore, unrestricted access to opioid receptor genetics is required.
Methods: Next-generation sequencing (NGS) workflow was based on a custom AmpliSeq™ panel and designed for sequencing of human genes related to the opioid receptor group (*OPRM1*, *OPRD1*, *OPRK1*, *SIGMA1*, *OPRL1*) on an Ion PGM™ Sequencer. A cohort of 79 previously studied chronic pain patients was screened to evaluate and validate the detection of exomic sequences of the coding genes with 25 base pair exon padding. *In-silico* analysis was performed using SNP and Variation Suite® software.
Results: The amplicons covered approximately 90% of the target sequence. A median of 2.54×10^6 reads per run was obtained generating a total of 35,447 nucleotide reads from each DNA sample. This identified approximately 100 chromosome loci where nucleotides deviated from the reference sequence GRCh37 hg19, including functional variants such as the *OPRM1* rs1799971 SNP (118 A > G) as the most scientifically regarded variant or rs563649 SNP coding for μ-opioid receptor splice variants. Correspondence between NGS and Sanger derived nucleotide sequences was 100%.
Conclusion: Results suggested that the NGS approach based on AmpliSeq™ libraries and Ion PGM sequencing is a highly efficient mutation detection method. It is suitable for large-scale sequencing of opioid receptor genes. The method includes the variants studied so far for functional associations and adds a large amount of genetic information as a basis for complete analysis of human opioid receptor genetics and its functional consequences.

1. Introduction

Opioid signaling is triggered by endogenous opioid peptides comprising endorphins, enkephalins and dynorphins with preferential selectivities at the μ-, κ- and δ-opioid receptors, respectively [1]. The existence of opioid binding sites in the brain was established in 1973 [2] and subsequently located in various different physiological systems. In peripheral [3,4] and central [5–7] parts of the nociceptive system, activation confers an effective nocifensive mechanism. Activation at the *Area postrema* causes respiratory depression [8], activation in brain areas belonging to the reward system [9] is involved in addiction [10–12], activation in the gastrointestinal tract causes constipation [13], and activation at the hypothalamic–pituitary–adrenal (HPA) axis is involved in stress responses [14].

Genetic variation of human opioid receptors is an active research topic that has identified several possible modulators of the individual response to endogenous and exogenous opioids [15]. Variants were associated with the modulation of opioid receptor expression [16,17] or signaling [17–19] up to an almost complete functional loss [18,20]. However, the collection of certain opioid receptor variants has provided only modest explanations of clinical phenotypes [21,22]. Therefore, a limited selection of functional opioid receptor variants for which specific genetic assays had been established [23] is perceived as insufficient in providing a genetic diagnosis of the clinical phenotype [24].

Since the early 70th of the last century when the first processes of Sanger sequencing were introduced [25,26], the access to the whole genomic information has been widely accepted as a valuable method in clinical research [27]. With the recent broader availability of next generation sequencing (NGS) [28] the limitation to known functional variants has therefore fallen in favor of unrestricted access to opioid receptor genetics, which has already been shown to provide a working genetic marker for opioid-related clinical phenotypes [29]. In this report, the evaluation of a new NGS method based on a custom AmpliSeq™ library and Ion Torrent sequencing for the fast detection of genetic variations in human opioid receptor genes is described.

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2. Methods

2.1. Gene selection

Exomic genotyping was performed for the human *OPRM1*, *OPRK1* and *OPRD1* genes (NCBI IDs 4985, 4988 and 4986), located on chromosomes 6, 8, and 1, and encoding for the three major (μ, κ, δ) opioid receptors. Their endogenous ligands are opioid neuropeptides comprising endorphins/endomorphines, dynorphins, enkephalins, respectively, and they are the main targets of opioid analgesics as listed in the DrugBank database (version 4.1; http://www.drugbank.ca [30]). In particular *OPRM1* is addressed by most exogenous opioids. In addition, the *SIGMAR1* gene (NCBI ID 10280) located on chromosome 9, was included that codes for σ_1-opioid receptors. These are not activated or blocked by opioid peptides or naloxone, respectively, however, historically belong to the opioid receptors [31], are targets of some drugs classified as opioids such as pentazocine [32] or dextromethorphan [33] and their activation modulates peripheral μ-opioid analgesia [34]. Furthermore, the gene panel was extended to the opiate receptor-like 1 gene (*OPRL1*; NCBI ID 4987), located on chromosome 20 and coding for the nociceptin receptor that shows a high degree of structural homology to the classical opioid receptors although opioid peptides or morphine-like compounds have little or no affinity for it [35]. Its endogenous ligand is nociceptin, however, exogenous opioids comprising buprenorphine [36] and its glucuronide metabolites [37] have been shown to exert agonist activity that seems to contribute to their clinical effects, and the nonselective opioid agonist etorphine also binds at the *OPRL1* gene product [38], which supports the inclusion of this gene in the present panel.

2.2. DNA template preparation and amplification

The investigation followed the Declaration of Helsinki on Biomedical Research Involving Human Subjects and was approved by the Ethics Committee of the Medical Faculty of the Goethe-University, Frankfurt, Germany. All subjects had provided informed written consent covering the genotyping. Genomic DNA was available from venous blood samples drawn from 79 pain patients in a previously reported [29] context of the pharmacogenomics of opioid dosing requirements.

DNA was extracted from 200 μl blood on a BioRobot EZ1 workstation applying the blood and body fluid spin protocol provided in the EZ1 DNA Blood 200 μl Kit (Qiagen, Hilden, Germany). A multiplex PCR amplification strategy for the coding DNA sequences was accomplished online (Ion Ampliseq™ Designer; http://www.ampliseq.com) to amplify the target region specified above (for primer sequences, see Supplementary Table 1) with 25 base pair exon padding. After a comparison of several primer design options, the design providing the maximum target sequence coverage was chosen. The ordered amplicons covered approximately 90% of the target sequence (Table 1). A total of 10 ng DNA per sample were used for the target enrichment by a multiplex PCR and each DNA pool was amplified with the Ion Ampliseq™ Library Kit in conjunction with the Ion Ampliseq™ "custom Primer Pool" - protocols according to the manufacturer procedures (Life Technologies, Darmstadt, Germany).

After each pool had undergone 17 PCR cycles, the PCR primers were removed with FuPa Reagent and the amplicons were ligated to the sequencing adaptors with short stretches of index sequences (barcodes) that enabled sample multiplexing for subsequent steps (Ion Xpress™ Barcode Adapters Kit; Life Technologies). After purification with AMPure XP beads (Beckman Coulter, Krefeld, Germany), the barcoded libraries were quantified with a Qubit® 2.0 Fluorimeter (Life Technologies, Darmstadt, Germany) and normalized for DNA concentration to a final concentration of 20 pmol/l using the Ion Library Equalizer™ Kit (Life Technologies, Darmstadt, Germany). Equalized barcoded libraries from 11 to 12 samples at a time were pooled. To clonally amplify the library DNA onto the Ion Sphere Particles (ISPs; Life Technologies, Darmstadt, Germany), the library pool was subjected to emulsion PCR by using an IT OneTouch template kit on an IT OneTouch system (Life Technologies, Darmstadt, Germany) following the manufacturer's protocol.

2.3. Sequencing

Enriched ISPs which carried many copies of the same DNA fragment were subjected to sequencing on an Ion 316 Chip to sequence pooled libraries with eleven to twelve samples. The 316 chip was chosen (instead of the low-capacity 314 or the high-capacity 318 chip) to obtain a mean sequencing depth of coverage of 50× which means that, on average, each base has been sequenced 50×, when eleven samples were loaded. A larger number of samples could be analyzed simultaneously using the 318 chip, but this would increase the turnaround time for each sample, depending on the number of samples that are received by the laboratory. Sequencing was performed using the sequencing kit (Ion PGM 200 Sequencing Kit; Life Technologies, Darmstadt, Germany) as per the manufacturer's instructions with the 200-bp single-end run configuration.

2.4. Bioinformatics generation of sequence information

The raw data (unmapped BAM-files) from the sequencing runs were processed using Torrent Suite Software (Version 4.4.2, Life Technologies, Darmstadt, Germany) to generate read alignments which are filtered by the software into mapped BAM-files using the reference genomic sequence (hg19) of target genes. Variant calling was performed with the Torrent Variant Caller Plugin using as key parameters: minimum allele frequency = 0.015, minimum quality = 10, minimum coverage = 20 and minimum coverage on either strand = 3.

The annotation of called variants was done using the Ion Reporter Software (Version 4.4; Life Technologies, Darmstadt, Germany) for the VCF files that contained the nucleotide reads. The GenomeBrowse® software (Version 2.0.4, Golden Helix, Bozeman, MT, USA) was used to map the observed sequences to the reference sequences GRCh37 hg19 (dated February 2009). The SNP and Variation Suite software (Version 8.4.4; Golden Helix, Bozeman, MT, USA) was used for the analysis of sequence quality, coverage and also for variant identification.

2.5. Method validation

For method validation, two genomic regions were chosen at random from the whole analyzed region for validation by Sanger sequencing [25,26] in an independent external laboratory (AGOWA, Berlin, Germany), which was performed in ten DNA samples randomly chosen from the n = 79 samples in the present cohort. Amplification of the respective DNA segments was done using PCR primer pairs (forward, reverse) of (i) 5´-ATGAAGACAGCAACCAACATTTAC-3´ and 5´-CCAGATGCAGATATTGATGATCTT-3´ and (ii) 5´-TTTAACCTG GCTTTGGCAGATG-3´ and 5´-ACATCGACGTCTTCCCTGACT-3´. The results of Sanger sequencing were aligned with the genomic sequence and analyzed using Chromas Lite® (Version 2.1.1, Technelysium Pty Ltd., South Brisbane, Australia) and the GenomeBrowse® (Version 2.0.4, Golden Helix, Bozeman, MT, USA) was used to compare the sequences obtained with NGS or Sanger techniques.

3. Results

The NGS assay of human opioid receptors was established on 79 genomic DNA samples [29]. As proposed previously [39], only exons and their boundary sequences for which read-depths >20 for each nucleotide could be obtained were considered as successfully analyzed. Applying this criterion, complete or nearly complete coverage of the relevant sequences was obtained (Table 1; for details on missing variants, see Supplementary Table 2).