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Targeted Resequencing of Epilepsy Genes: A Pharmaco-Therapeutic Perspective

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Abstract

More than half of all epilepsies have some genetic basis and single gene defects in ion channels or neurotransmitter receptors are associated with some inherited forms of epilepsy. Genetic research even in the field of epilepsy disorders is increasing in term of testing platform for the investigation of sequence and structural variation. Next generation sequencing (NGS), i.e., high-throughput sequencing technologies now allow analyses that were previously prohibitive. Targeted resequencing methods by diagnostic panels enable to sequence all the genes associated with a certain disease simultaneously within a few weeks. In this review, we will discuss the overall helpfulness and convenience of simultaneous genotyping of multiple epilepsy genes by NGS in a pharmacogenetics perspective.

Keywords: Epilepsy; Genetics, Next-generation sequencing; Candidate genes

Introduction

It has been estimated that more than half of all epilepsies have some genetic basis and over the pastdecade important progresses have been made in the understanding of the genetic causes of many ofthese epilepsies. Single gene defects in ion channels or neurotransmitter receptors are associated withsome inherited forms of epilepsy [1-6]. In addition, genetic discoveries in the field of infantile epilepsy syndromes have highlighted the possible different aetiologies other than channelopathies can result in epilepsy [7]. The importance of genetic testing has been highlighted inclinical contexts where the identification of a specific cause may clarify the prognosis, identify the bestavailable treatment, and define the risk of recurrence for current and future family members and avoidinvasive and or prolonged diagnostic processes [8]. Moreover, genetic testing allows understanding therole of the mutation and its relationship with the phenotype, thus helping in the definition of points of action for future therapies. The lack of substantial and long-lasting relief of unprovoked seizures, regardless the introduction in the past 15 years of a wide number of second-generation antiepileptic drugs (AEDs), epitomizes the necessity of a paradigm shift including a massive effort in the clarification of certain genetic determinants of this chronic neurological disorder [9,10]. As far aspharmacological research is assimilatedin the domain of the "system biology" [11], the idea of designing high selective ligands for complex multi-factorial syndromes is losing its attractiveness and clinical usability [12]. In its place, the "network pharmacology" approach (Hopkins [13]) may permit to abandon the genetic reductionism and the *magic bullets* philosophy, shifting our attention towards *magic shotguns* aimed at discovering unexpected multiple drug targets and customized pharmacotherapies [14,15].

Nowadays, the genomic era is entering in the most challenging phase, in which the identification of multiple genes involved in the pathophysiology of heterogeneous diseases requires to be intertwinedwith the mechanisms of action of existing as well as novel therapeutic entities. Genetic research even inthe field of epilepsy disorders is increasing in term of testing platform for the investigation of sequence and structural variation. Next generation sequencing (NGS) refers to high-throughput sequencing technologies that have developed during the past years. Thanks to these technologies clonally amplifiedDNA templates, or single DNA molecules, are sequenced in a massively parallel way in a flow cell.

Thus, each clonal template or single molecule is "individually" sequenced and can be counted amongthe total sequences generated. The high-throughput combination of qualitative and quantitative sequence information generated has allowed analyses that were previously either not technically feasible or cost prohibitive. Targeted resequencing methods by diagnostic panels enable to sequence all the genes associated with a certain disease simultaneously within a few weeks. It is considerably less expensive than sequencing genes individually and also increases the probability to detect the genetic cause of the illness. Variant detection is based on careful and conservative mapping of the reads to the genome reference and extraction of the alignments corresponding to the targeted genes. Variants called by the primary analysis software are annotated including data on heterozigosity/homozigosity ratio when existing; localization with respect to the gene structure; function (missense, nonsense etc) and possible involvement in disease of all the variants overlapping with a dbSNP or 1000. Moreover, the discovery of novel variants can generate new evidences to add to the panel catalog. The systematic review of all genes associated with seizure disorders is beyond the scope of this paper whereas we will discuss the overall helpfulness and convenience of simultaneous genotyping of multiple epilepsy genes by NGS in a pharmacogenetics perspective.

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Epilepsy: A Complex Disease with Several Potential Therapeutic Targets

Epilepsy and pharmacogenetics

The progression in the pharmacogenetic of epilepsies opens new perspectives for the understanding of epileptogenesis and early diagnosis. In the field of drug discovery for safer, effective and tailored antiepileptic prescriptions, the term pharmacogenetic is generally applied to the investigation of the relationship between individuals' genetic makeup and the response to a certain class of medications including toxicity and the incidence and magnitude of unwanted adverse effects [16]. Pharmacogenetics or pharmacogenomics focuses on the problem of variation in DNA and RNA, at genomic level, and impact of these changes (e.g., expression, activity, specificity of gene product) in individual variability in drug response.In this view, pharmacogenetics should help to detect patients that may benefit from a selected therapy ("magic shotguns") characterized by lower risk of adverse reactions and possible pharmacoresistance.

Emblematically, pharmacoresistance and refractoriness to AEDs is a recurrent problem in epilepsy pharmacotherapy. Pharmacoresistance to AED treatment occurs in 25-30% of patients [17] and current mechanistic hypotheses point towards the involvement of drug transporters in the blood brain barrier such as P-glycoprotein (P-gp), whose expression is increased in brain tissue from epileptic patients, in models of brain epileptogenic and inversely associated to many AEDs brain concentration [18, 19].Moreover, many AEDs (e.g., phenytoin, levetiracetam, lamotrigine, topiramate) are recognized high- low-grade affinity substrates of P-gp [20-22]. In humans MDR1 (*ABCB1*) and MDR2 (*ABCB4*) genes [23] encode P-gp and exploring the genetic of P-gp overexpression in epileptic brain may assist to explore the dilemma about constitutive or developed pharmacoresistance as result of recurrent seizures or chronic AEDs treatment. Despite controversialdata and poor confirmation [24, 25], a polymorphism of *ABCB1* expression (C to T) at position 3435(CC genotype) has been describedin association with pharmacoresistance in epilepsy [26]. Nevertheless, analysisand further examination of different polymorphisms in other transporters,as for multidrug resistance-associated protein MRP2 (*ABCC2*),may provide great advancement for the improvement of efficacy ofsomeAEDs [27, 28].On the other hand, possible morphological alterations of the molecular site of action of AEDs may underlie the occurrence of pharmacoresistant seizures. A genetic form underlying the alterations of the target of phenytoin and carbamazepine has been described in case of polymorphism of the *SCN1A* gene encoding an isoform of the voltage-activated sodium channels [29, 30].

The study of individuals' drug resistance to antiepileptic therapy or refractory seizures significantly challenges the clinical management of epilepsies. Thus, the first fundamental benefit of the pharmacogenetic approach is that it may offer a tool to recognize the consistency between epilepsy-associated genes and molecular targets of current AEDs. In this view, the assessment of eligible AEDs is "gene-driven hypothesis" and do not consider the genomic action of AEDs as major method of analysis.

Voltage- and ligand-gated ion channels

In lack of clearly detectable "structural" originof epileptogenesis, the "idiopathic generalized epilepsy" (IGE) represents the most common disease entity.Probably the most investigated epilepsy genes are those related to the syndromic entity known as channelopathies [31] in which mutations of single gene encoding voltage-gated sodium, potassium,calcium, potassium and chloride channels are increasingly recognized [32]. Thus, uncovering the genetic basis of channelopathies has strengthens the notion of epilepsy as ion channel disorder and led to develop genetic testing as clinical tool for diagnosis and treatment. Voltage-gated sodium channels (VGSCs) are critical to evoke action potential in excitable cells such as neurons. Not surprisingly, considering the key function of VGSCs in cell depolarization and propagation of action potential to another axon segment, the majority of genetic epilepsy syndromes (e.g., benign familial neonatal infantile seizures or severe myoclonic epilepsy of infancy) are described in association with sodium channels mutations [33,34].

Nevertheless, equally important is the role attributed to mutations of ligand-gated ion channel and pathogenesis of idiopathic epilepsy and in particular to those identified in nicotinic acetylcholine receptor (AChR) and GABAA receptor subunits [35,36]. For example, the role of polymorphisms and mutations in genes encoding extrasynaptic GABAA receptor and their association with human epilepsies is mirrored by the tonic inhibition provided by GABAA receptors and its importance for the efficacy of AEDs such as barbiturates, topiramate and retigabine [36-39] but also for benzodiazepine-resistant seizures [40]. The importance of ligand-gated ion channels for seizure disorders is epitomized by theclinically relevant consequences caused by mutations occurring in single subunits ofnicotinic AChRs. As formerly noted [41], the association between mutation in *CHRNA4*, the gene encoding for the alpha 4 subunit of the nicotinic AChR, and the autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) became paradigmatic of the importance ofnicotinic AChRs for the understanding of epileptogenesis.

Gene mutations, voltage- and ligand-gated ion channels and epilepsy syndromes

Prototypicals targets, in this regard, are the frequent mutations recognized in genes known to encode distinct isoforms of alfa-1 (Nav1.1), alfa-2 (Nav1.2) and beta-1 sodium channels subunits (Table 1).

Missense mutations, and therefore alterations of codon nucleotide sequences and incorporation of different amino acids into channel subunits, have been identified for SCN1A (Nav1.1), SCN2A (Nav1.2) and SCN1B (subunit beta-1). These missense mutations are recognized responsible of different dominant idiopathic epilepsies and seizures termed "generalized epilepsy with febrile seizure plus" (GEFS+) [42-45]. Besides GEFS+, a high percentage of patients diagnosed for severe myoclonic epilepsy of infancy (SMEI or Dravet syndrome) show concomitant mutations in the SCN1A gene [46,47]. Less frequently Dravet syndrome has been described in association with SCN2A [48], SCN9A [49], SCN3A and SCN1B gene mutations [50,51]. Moreover, a contribution of the SCN2A gene is also recognized in the benign familial neonatal-infantile (BFNIS) and benign familial infantile seizures (BFIS) [52,53]. Mutations underlying the GEFS+ phenotype show the contribution of another key target of epileptic channelopathies. Fast synaptic inhibition is mainly mediated in the brain by the 5-subunits pentameric channels gamma-aminobutyric acid (GABA) A receptor (GABAA-R) and aberrant GABAAR-mediated signaling is largely responsible for facilitation of seizures occurrence. Indeed, the inhibitory GABAAR signaling allows chloride influx through the channel pore, thus leading to cell hyperpolarization.

Excessive inhibition may be a cause of seizures (e.g., via dishinibition of epileptogenic network) and enhanced GABAA-R tonic inhibition of

Human Gene	VL-Gio	Identified Epilepsy syndromes	Mostly Prescribed AEDs
SCN1A	$Na_v1.1$	GEFS+/SMEI	CLB (BDZ), LEV, STP, TPM, VA
SCN2A	$Na_v1.2$	GEFS+/SMEI/BFNIS/Ecps	CLB (BDZ), LEV, STP, TPM, VA
SCN3A	$Na_v1.3$	SMEI	STP, TPM, VA
SCN1B	sodium channel β-1 subunit	GEFS+/SMEI	CLB (BDZ), LEV, STP, TPM, VA
SCN9A	$Na_v1.7$	SMEI	STP, TPM, VA
CACNA1A	HVA - Cav2.1	progressive ataxia with AS	VA, ETX, LMT+VA, AZA, LEV (JME)/
			TPM (GTCS), ZNS (t/aAE; GTCS; LG)
CACNA1G	LVA - Cav3.1	IGE (CAE, JME)	ETX, LMT, VA (CAE)/ LMT, VA, TPM (JME)
CACNA1H	LVA - Cav3.2	IAE (CAE)	AZA, ETX, LEV (JME), LMT+VA, VA
			TPM (GTCS), ZNS (t/aAE; GTCS; LG)
CACNG3	calcium channel γ-3 subunit	AS (CAE)	ETX, LMT, VA
KCNQ2	Kv7.2	BFNS	PHE, VA, RTG
KCNQ3	Kv7.3	BFNS	PHE, VA, RTG
KCNA1	Shaker-related subfamily, member 1	EA1m	AZA
KCNAB2	Shaker-related subfamily	1p36DS	BDZ?
KCND2	Shal-related subfamily	TLE	VNS, TL
CACNA1H	T-type, alfa-1 subunit	IGEs/CAE	AZA, ETX, LEV, LMT, VA
CACNA1A	P/Q type, alfa-1 subunit	IGEs/EA2/FHM/CAE	AZA, ETX, LEV, LMT, VA
CACNG3	gamma-3 subunit	CAE	ETX, LMT, VA
GABRA1	alfa-1 subunit	JME/CAE	RTG, ZNS
GABRD	delta subunit	IGEs	ETX, VA, LMT, VA, TPM
GABRB3	beta-3 subunit	CAE	ETX, LMT, VA
GABRG2	$GABA_A$-R γ-2 subunit	GEFS+/SMEI/CAE/JME	CLB (BDZ), ETX, LEV, RTG, STP, TPM, VA

Table 1 Main Genes, Epilepsies and AEDs

1p36DS, seizure-associated 1p36 deletion syndrome; ADNFLE, autosomal dominant nocturnal frontal lobe epilepsy; AS, absence seizures; AZA, acetazolamide; BDZ, benzodiazepines; BFNS, benign familial neonatal seizures; BFNIS, benign familial neonatal-infantile seizures; CAE, childhood absence epilepsy; CBZ, carbamazepine; CLB, clobazam; EA1m, episodic ataxia with myokymia; EA2,episodic ataxia type 2; Ecps, encephalopathies; ESL, eslicarbazepine; ETX, ethosuximide; FHM, familial hemiplegic migraine; GEFS+, generalized epilepsy with febrile seizure plus; GTCS, generalized tonic–clonic seizures; JME, juvenile myoclonic epilepsy; IAE, idiopathic absence epilepsy; IGE, idiophatic generalized epilepsy; HVA, high-voltage activated; LEV, levetiracetam; LG, Lennox-Gastaux syndrome; LCM, lacosamide; LMT, lamotrigine; LVA, low-voltage activated; OCBZ, oxcarbazepine; PHE, phenytoin; RFM, rufinamide; RTG, retigabine; SMEI, OR Dravet syndrome (severe myoclonic epilepsy of infancy); STP, stiripentol; t/aAE, typical/atypical absence epilepsy; TL, temporal lobectomy; TPM, topiramate; VA, valproic acid; VL-Gio, Voltage and ligand-gated ion channels; VNS, vagus nerve stimulation; ZNS, zonisamide

thalamocortical neurons has a critical importance in the pathogenesis of absence epilepsy and autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [54]. However, autosomal-dominant epilepsy such as GEFS+ may be also associated to mutations in GABAA-R, and in particular to the gene encoding the γ-2 subunit (GABRG2) that have been reported in febrile seizures, SMEI and GEFS+ families [35,55-57]. Notably, it has been recently shown that increasing temperature in neurons expressing recombined K289M (missens mutation) mutant γ-2 subunit can change the membrane dynamics and the postsynaptic clustering of GABAARs, thus reducing the frequency of GABA-mediated miniature inhibitory postsynaptic currents and efficacy of synaptic inhibition [58]. Considering further that mutations in the gene encoding the Nav1.1 (SCN1A) are a common cause of dominantly inherited and sporadic epilepsy, several knockout, knockin or transgenic mouse models with null or missense mutations of this gene have been generated to produce distinct seizure phenotypes [59,60]. Prototypic AEDs such as carbamazepine and phenytoin bind to the inactive state of sodium channels to block depolarized membrane potentials, thus inducing a decrease in conduction that is both voltage- and frequency-dependent [61,62]. Some newer second- and third generation AEDs also show a similar primary mechanism of action, and eslicarbazepine, oxcarbazepine, lamotrigine and rufinamide are all drug of choice for partial-onset seizures and generalized tonic-clonic seizures (GTCS) but also for the control (e.g., lamotrigine, rufinamide) of Lennox-Gastaut syndrome [63,64]. Interestingly, in spite of their mechanism of action (i.e, voltage-dependent sodium channels inhibition), AEDs such as carbamazepine, phenytoin and lamotrigine can exacerbate the occurrence of myoclonic seizures in patients diagnosed for GEFS+ and SMEI syndromes [65].

The heterogeneous group of monogenic and polygenic epilepsies known as idiopathic absence epilepsies (IAEs) includes all the forms represented by the idiopathic generalized epilepsies (IGEs). In the IGEgroup are further recognized the childhood absence epilepsy (CAE), the juvenile absence epilepsy (JAE) as well as the juvenile myoclonic epilepsy (JME) [66]. Hence, since IGEs include a variety ofsyndromes and families often display more than one single phenotype, it is possible to hypothesize the presence of genes in common to different IGE subtypes. Typically, absence epilepsies are nonconvulsive epileptic seizures and genotypization (e.g., candidate gene approach, association analysis) has allowed to detect important susceptible genes of GABAA-R subunits involved in these complex idiopathic epilepsies, such as: GABRA1 (α-1 subunit), GABRD (δ subunit), GABRB3 (β-3 subunit), GABRG2 (γ-2 subunit) [67,68]. Mutations of GABRG2 gene hasbeen associated with an autosomal dominant form of CAE with febrile seizures [69,70] and frameshift mutations (i.e., 1-2 nucleotides deletion or insertion with changes of downstream codons) of GABRA1 gene have been revealed in autosomal dominant JME as well as in CAE patients [71,72]. GABRB3 missense mutations in coding sequences were found associated with CAE [73] while decrease of GABAA-R current amplitude and δ subunit-mediated tonic inhibition associated with different GABRD variants and autosomal dominant IGE [74,75].

IAEs have been also diagnosed in association with mutations of voltage-dependent calcium and potassium channels, such as: CACNA1H (T-type calcium channel, α-1H subunit), CACNA1A (P/Q type calcium channel, α-1A subunit), CACNG3 (calcium channel, γ-3 subunit), KCND2 (potassium channel Shal-related subfamily, member 2), KCNAB2 (potassium, shaker-related subfamily, beta

member 2), KCNA1 (potassium, shaker-related subfamily, member 1), KCNQ2 and KCNQ3 (potassium channels KQT-like subfamily, member 2 and 3, respectively) [67,68,75,76]. In contrast to high voltage-activated (HVA) calcium channels, low voltage-activated (LVA) T-type calcium channels are activated by small, near resting membrane potential, depolarizations. T-channels modulate calcium influx, membrane depolarization, generation of low threshold spikes and sodium-dependent action potentials and the effects of T-type channels upregulation in IGE has been supported by several animal models of absence epilepsy [77,78]. For instance, absence epilepsy is accompanied by 3-4 Hz spike wave discharge and the thalamocortical circuit is believed to reinforce the synchronized oscillatory activity in this circuit and maintain this pattern of discharge. Hence, LVA calcium T-channels are considered essential players in the regulation of neural pacemaker activity and synchronized neuronal oscillations of thalamocortical networks and responsible of spike wave discharge in absence epilepsy [79,80]. Indeed, variants in the CACNA1H gene are considered risk factors for susceptibility to IGEs pathogenesis [81] and different single nucleotide polymorphisms of this gene have been found associated to CAE [82].

Moreover, introducing these polymorphisms into human Cav3.2 cDNA was demonstrated to increase calcium influx and neural firing propensity (e.g., increased T-type channel activity) in transfected HEK-293 cells, thus supporting the idea that susceptibility of CACNA1H gene may increases liability to seizures and CAE-like polygenic disorders [83].

The CACNA1A gene encodes the alfa-1A subunit of HVA calcium channels, which belongs to Cav2 family that includes the splice isoform to Cav2.1 subfamily (P/Q type calcium channel) [84]. The alfa- 1A subunit is mostly expressed in the neuronal tissue at presynaptic nerve terminals and the functional activity of P/Q type channels appears linked to activity-evoked excitatory neurotransmitter release and signaling integration [85,86]. As noted above, synchronic and bilateralspike wave discharges involving the thalamocortical network are a hallmark for the diagnosis of IGEs. In this regard, different mouse lines and spontaneously occurring homozygous mutants (e.g., tottering and rocker mice) harbouring mutations in the genes coding for brain Cav2.1 channels have been exploited as suitable models for human absence epilepsy reproducing both neural hyperexcitability and bilateral spike wave discharges [87, 88]. Notably, mutations in P/Q-type calcium channels and in particular CACNA1A missense mutations are recognized causes of a wide spectrum of neurological diseases, such as episodic ataxia type 2 (EA2) and familial hemiplegic migraine (FHM) [89-92] with co-occurrence of childhood epilepsy [93]. Interestingly, a CAE-like syndrome has been hypothesized to be associated with a stop mutation in the gene (CACNA1A) encoding P/Q-type calcium channel α-1A subunit, with a loss of Cav2.1 function and decrease of calcium current in a complex case of mixed cerebellar ataxia and absence seizuresphenotype [94].

A benign familial neonatal seizure (BFNS) is an autosomal dominant epilepsy syndrome characterized by focal and unprovoked tonic-clonic seizures. It is now recognized that mutations in the genes encoding the potassium channel KQT-like subfamily member 2 (Kv7.2) and member 3 (Kv7.3) are accountable for the BFNS pathophysiology. Indeed, the co-segregation of both chromosome 20q13.3 and 8q24 deletions and BNFS phenotype, has allowed the characterization of missense mutations of KCNQ2 (Kv7.2) and KCNQ3 (Kv7.3) as candidate genes of primary importance in BNFS etiology [95-102].

KCNQ2 and KCNQ3 mutations or duplications have been described in association to BNFS clinical features [103,104]. As members of the potassium channel of KCNQ gene family, KCNQ2 and KCNQ3 subunits can co-assemble and form heteromultimeric or heterotretamers channels that mediate neuronal muscarinic-regulated current (M-current or M-type K+ channel) [105,106]. The M-current is active near the threshold for action potential initiation and slowly originate when neurons depolarizes, thus enabling membrane repolarization and re-establishing of resting potential and firing ceasing. The M-current is therefore a low-threshold noninactivating potassium conductance that plays a key role in neuronal excitability and responsiveness to synaptic input and both KCNQ2 and KCNQ3 subunits have been shown to contribute to this current [107,108]. From a functional point of view, KCNQ2 and KCNQ3 mutations and consequent loss of heteromeric KCNQ2/KCNQ3-channel function are translated in a significant decrease of the K+-mediated currents of Kv7.2 and Kv7.3 channels that produce facilitation of membrane depolarization, neuronal hyperexcitability and seizure susceptibility in BFNC [109]. Hence, since the suppression of M-current evokes membrane depolarization and increase of neural input resistance, muscarinic cholinergic agonists possess an epileptogenic potential that is conveniently exploited in epilepsy models [109,110]. Interestingly, a recent study has described eight novel KCNQ2 mutations in families with BFNS that include missense start codon mutations, frameshift mutation andtruncation, and putative splice sites and missense mutations in the C-terminal region of KCNQ2 [110]. In particular, this study has provided evidence that C-terminal missense mutation can change the conformation of KCNQ2 C-terminal domain, thus suppressing the ability to bind to calcium protein calmodulin (CaM) with a possible decrease of channel opening and impairment of M-currents. Hence, according to these results, CaM is potentially involved in the BFNS pathogenesis. KCNA1 (Kv1.1) is another interesting epilepsy-susceptibility gene, a member (1) of shaker-related subfamily that appears mutated in episodic ataxia type 1 (EA1 with myokymia) [111], a rare autosomal dominant disorder frequently associated with an increased incidence of epilepsy [112].

Novel Therapeutic Options for Epilepsy Management and Gene Targets

Drug discovery for novel anticonvulsants (AEDs) may potentially greatly benefit from the identification of susceptible genes involved in epilepsy pathophysiology. Recent progress in the identification of human genetic mutations has been exploited to develop animal models able to recapitulate many phenotypic characteristics of channel dysfunction in different epilepsy syndromes. For instance, mice carrying mutations in the gene encoding voltage-gated sodium channel Nav1.1 (SCN1A) show reduced sodium currents in GABAergic inhibitory interneurons and replicate hyperexcitability and spontaneous seizures observed in human SMEI [113]. A Scn1b knockin mouse for the C121W mutation of the beta-1 sodium channel subunit reproduced the heterozygous SCN1B mutation found in patients with GEFS+, and identified in beta-1 subunit modulation of excitability of the membrane of axon initial segments of pyramidal neurons a possible determinant of heat-sensitive seizures [114]. A different model of GEFS+ was engineered to harbour the GABRG2 (R43Q) mutation and mimic the phenotype observed in patients suffering from GEFS+ or CAE [115]. Mouse models carrying the missense point mutations in Kcnq2 and Kcnq3 described in human BFNC exhibit spontaneous tonic-clonic seizures or reduced threshold to experimentally evoked seizures as well as enhanced liability to seizures later in life [116].

Up to now, the availability of a variety of mouse mutant mimicking human epilepsy has been inadequately implemented in antiepileptic drug discovery. It is, however, widely accepted that someolder and newer AEDs such as carbamazepine, eslicarbazepine, lacosamide, gabapentin, phenytoin, topiramate and valproate block or slowly inactivate (e.g., eslicarbazepine) voltage-gated sodium or calcium channels, thus inhibiting repetitive and sustained neuronal firing. Nevertheless, AEDs share multiple mechanisms of action and there are novel recognized molecular targets both for traditional and emerging AEDs that encourage the identification of gene mutations in epilepsies. Despite the initial underestimation of the role of voltage-gated potassium channels in the mechanism of action of several AEDs, it is now accepted that this large family of ion channels is a target of different AEDs. Phenytoin can also inhibit A-type potassium channel [117], topiramate facilitates membrane hyperpolarization by acting on potassium conductance [118] and lamotrigine can block hippocampal A-type potassium channels [119]. In parallel, lamotrigine has also been shown to reduce hippocampal glutamate-mediated transmission by inhibiting postsynaptic AMPA receptors [120].

Other second-generation antiepileptics, such as the piracetam-related levetiracetam and the carbamazepine-related oxcarbazepine, decrease delayed rectifier potassium currents and block sustained repetitive firing [121,122]. Notably, retigabine is a newly discovered and recently marketed AED that show an interesting mechanism of action. Indeed, retigabine may be considered a prototype of a new class of antiepileptic compounds targeting Kv7.x potassium channels (KCNQ) [123]. Although retigabine can enhance GABAergic transmission and GABA-induced chloride currents, it mainly acts as enhancer of the neuronal M-type potassium current mediated by Kv7.2-Kv7.3 channels [124]. Probably by destabilization of channels closed conformation and enhancement of open channels probability, retigabine reduces and accelerates the threshold for activation of M-current, thus increasing the current amplitude at negative potentials [124-126].

A different target for different novel AEDs is represented by the synaptic vesicle protein 2 isoform A (SV2A), a regulatory membrane glycoprotein found on synaptic vesicles of neurons and endocrine cells that contributes to membrane fusion processes underlying calcium-regulated neurotransmitter secretion. In the brain, SV2A is the binding site for the newly developed AED levetiracetam and its high-affinity structural analogue brivaracetam that also inhibits neuronal voltage-gated sodium channels [127].

Although the mechanism by which levetiracetam prevents seizures is not yet clarified, it is known that Sv2a/2b double knockout mice exhibit sustained increase in calcium-dependent synaptic transmission and spontaneous seizures compatible with a state of heightened epileptogenicity [128]. Sv2a dysfunction may therefore lead to calcium accumulation, excitatory transmission facilitation and attenuation of inhibition and levetiracetam treatment has been shown effective in patients with refractory epilepsy [129] as well as in management of human pain [130,131]. Hence, theidentification of SV2A as binding site for levetiracetam and brivaracetam [127,132] opens new avenues in the discovery of novel molecular targets for seizures treatment.

Similarly, human genetic analysis has helped to disclose another not obvious potential novel molecular target for AEDs in the gene (SLC2A1) encoding the glucose transporter GLUT1, which is the main transporter for D-glucose across the blood brain barrier. De novo or inherited heterozygous mutations in the GLUT1/SLC2A1 gene give rise to cerebral energy failure and a clinical condition known as GLUT1-deficiency syndrome (GLUT1-DS). Within the different phenotypes described to occur in GLUT1-DS, the familial paroxysmal exertion-induced dyskinesia (PED) can result associated to epileptic seizures.

Indeed, the hypothesis of shared genetic determinants for paroxysmal dyskinesia (PD) and epilepsy appears corroborated by the co-occurrence of PED and epileptic seizures (i.e., generalized tonic-clonic or absences) in patients carrying mutations in SLC2A1 gene [105]. This study identified a region on chromosome 1p35–p31 including SLC2A1, with heterozygous missense and frameshift mutations segregating in families with a similar phenotype in which some patients positively responded to ketogenic diet (KD) treatment [133]. Hence, according to this report, the concomitant manifestation of PED and epilepsy appears attributable to autosomal dominant heterozygous SLC2A1 mutations, whichhave also been found in a sub-cohort of patients with early-onset absence epilepsy diagnosis with andwithout PED [134]. In addition, a large European study on revealed that GLUT1 defects are a rare cause of familial IGEs. In particular, SLC2A1 screening should be considered in patients featuring absence epilepsies with onset from early childhood to adult life. This diagnosis may have important implications for treatment and genetic counseling [135]. In fact, in many cases, these individuals can be responsive to KD treatment that is the object of renewed attention for the management of intractable epilepsies in child as well as in adult patients [136,137]. When glucose transport is defective, as in GLUT1 deficiency, the KD allows easily deliveringof ketone bodies and energy supplement to the brain. However, although ketone bodies possess anticonvulsant properties, the mechanisms underlying the clinical efficacy of sustained ketosis remain elusive [138]. KD is based on the ingestion of very low-carbohydrate diet and one hypothesis of KD efficacy predicts that KD-induced seizure control might depend on the inhibition of glycolysis and carbohydrate metabolism. In support of this view, the administration of the glycolysis inhibitor 2-deoxy-D-glucose (2DG) is reported to induce both in vitro and in vivo anticonvulsant actions by reducing pharmacologically-induced interictal epileptiform bursting in hippocampal slices as well as 6Hz stimulation-evoked seizures in mice and the progression of epileptogenesis in kindling model of TLE in rats [139,140]. Despite the large number of availableAEDs, several epilepsy syndromes are medically intractable and TLE shows one of the worst prognoses. In this context, the further investigation of 2DG inhibitors is one more promising path worth pursuing. Recently confirmed in a high number of familial cases examined [135,141], the phenotypic spectrum (e.g., IGEs) associated to SLC2A1 mutations highlights the importance of GLUT1 deficiency as monogenic cause of absence epilepsies and suggests new potential strategies of intervention.

Targeted Resequencing of Epilepsy Genes: From Genomes to Diagnostic Panels of Genes

Using sequencing machines available today, high-throughput next generation sequencing permits the simultaneous sequencing of several billion basepairs within one week, i.e. the complete haploid human genome (3 billion basepairs in total). Because of higher accuracy and data quality and for reasons of better understanding of variation there is an advantage to focus on the coding regions of the human genome. The human exome, the totality of all coding regions in the genome reduces the amount of basepairs to be analyzed to 30 million. Sequencing an exome is useful if there are strong indications for a genetic disease, all known genes associated with epilepsy have been ruled out and this approach is applied and evaluated by experts in the particular field within the scope of a research project. In contrast, a diagnostic panel for epilepsy is the targeted simultaneous screening of a list of known genes that have already been described as the cause of

seizures. A diagnostic panel in general clearly differs from the purely scientific and explorative approach of exome sequencing. A diagnostic panel analyses only the genes whose connection with the disease have been established (Table 1). As new genes are described almost every week the list has to be updated on a regular basis. Based on the gene identifier and the respective genomic sequences complementary RNA baits are designed to capture the regions of interest.

On array and in solution hybridization technologies are used in these cases allowing massive parallel hybridization and capture of relevant sequences. Target amplification is another option and can be used as standalone method or in combination with hybridization based methods in order to cover problematic repetitive or GC rich regions. After enrichment for the defined target that can be up to several 10 megabases in size, the DNA fragments are amplified on beads and subsequently massively sequenced. Before a diagnostic panel can be offered for diagnostic purposes it must be validated. Within the framework of such validation patients whose variations are already known are "post"-sequenced on the panel. This requires that 100% of the known variations are found by the panel sequencing approach. Furthermore, the enrichment of the genomic regions must be efficient, specific and reproducible. Epilepsies are very suitable for a targeted next generation sequencing approach as several large genes and gene families have been described as cause and genetic diagnostics in cases was not only very time-consuming, but was either inefficient or not performed because of high costs. In comparison to whole exome sequencing, the decreased number of genes within the panel allows a significant increase in coverage on target sequences. This helps to reliably detect mutations, to screen tens of patients in parallel and most important to prevent detection, validation and interpretation of variants in genes not known to account for a patient's phenotype.

Lemke and co-authors [142] put together a panel of genes comprising the most relevant epilepsy genes and epilepsy phenotypes (265 genes in total). A pilot cohort of 33 index patients representing both isolated and familial cases with either concise epilepsy phenotypes was considered. The panel covers exonic regions as well as exon-intron boundaries of the vast majority of all currently well-known epilepsy genes. To facilitate further analysis the 265 genes were subdivided into subpanels according to clinically relevant groups of epilepsy phenotypes (Table 1). Using a customized target in solution enrichment followed by next generation sequencing a screening for mutations in the 265 candidate genes (875.678 base pairs in total) was performed. The detected variants were then evaluated with respect to the individual phenotype and all putatively causative mutations were validated by conventional Sanger sequencing. To determine the impact of novel variants segregation analysis in families was applied if possible. In a preliminary study [115], this diagnostic epilepsy panel identified gene mutations in 15 out of 33 (45%) patients. A known KCNQ3 mutation was detected in a patient with rolandic epilepsy and a SCN1A de novo mutation (R393H) was identified in a patient with Dravet syndrome.

Notably, in a handful of cases mutations were found in genes that are not routinely sequenced. For instance, a homozygous missense mutation was identified in the KCTD7 gene in some patients with progressive myoclonic epilepsy [143]. Additionally, pathogenic mutations were found in STXBP1, ARHGEF9, TPP1, MFSD8, KCNJ10, FLNA, SCN1A, and SCN2A [142]. Most interestingly and most intriguing, known pathogenic SCN1A mutations were detected by next generation sequencing in two patients with classic Dravet syndrome that have been tested negative by conventional Sanger sequencing in accredited laboratories. After knowledge of these mutations thevariants were confirmed with subsequent Sanger sequencing in both cases. A precise delineation of a patient's epilepsy phenotype can often be difficult due to a significant phenotypic overlap shared by many seizure disorders. Using massive parallel sequencing the detection rate of genetic aberrations does no longer depend on the prerequisite of a precise clinical diagnosis and consecutive prediction of putatively casual genes. It seems rather sufficient to categorize the patient's epilepsy in a rough group of phenotypes (e.g., according to a subpanel) and primarily screen the respective genes. Compared to the classic time, effort and cost consuming stepwise Sanger sequencing screening of disease specific panels seems more than just a straightforward and efficient alternative. The significantly higher coverage of targeted genes compared to whole exome sequencing within the epilepsy panel considerably increases the probability to detect relevant variations within the genes of interest.

Conclusions

The ultimate goal of personalized medicine is to develop individualized treatments tailored to every patient. Variants in the genome have a demonstrated impact in different aspects of drug therapy: drug effect on receptors and signaling pathways, and drug dose-related adverse reactions drug transportation, drug metabolism. Disease specific NGS panel of genes represents a powerful tool for uncovering underlying genetic defects in patients suffering from various seizure disorders and contributes to genotype-phenotype correlations of rare epilepsy syndromes and the choice of the best therapy.

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