Summary

Brain somatic variants are increasingly recognized as important causes of neurodevelopmental diseases, particularly in the pathogenesis of malformations of cortical development (MCDs), such as focal cortical dysplasia (FCD) and hemimegalencephaly. Therefore, the capability to detect such variants is critical to make genetic diagnosis in MCDs cases. Thanks to recent genomic technical improvements, multiple studies have implicated mTOR pathway brain somatic variants in various MCDs. The present review will show the main current approaches adopted to detect brain somatic variants, the role of mTOR signaling cascade in the pathogenesis of epilepsy with FCD and the therapeutic choices available at this time.

Key words: Somatic mosaicism, focal cortical dysplasia, mTOR pathway

Introduction

A postzygotic variant occurring during development can originate distinct populations of cells within an individual: this condition is referred to as mosaicism. If the variant occurs in the germline, it is indicated as “germline mosaicism”, and the new variant can be transmitted to the progeny. Otherwise, we talk about “somatic mosaicism” if the variant arises in a cell that will develop in the soma, and therefore will not be transmitted to the descendants. Somatic variants may occur in both dividing and non-dividing cells and can be
due to an error during DNA replication (only in dividing cells) or to environmental factors (the most remarkable are UV light and carcinogens) [1].

The frequency of such variants in a cell population depends on different factors: the timing during development when they occurred; whether they affect patterns or rates of cellular proliferation and if a selective pressure is applied to the cells carrying the variant.

This review will summarize the current methodologies and techniques to detect somatic variants, and their role in brain development, with a focus on focal cortical dysplasia (FCD) and epilepsy.

**Mosaicism discovery strategies**

Somatic variants can be characterized by alterations of the DNA sequence (single nucleotide variants and small insertions/deletions) or by genomic structural variations (mobile element insertions, copy number variants, loss of heterozygosity, inversions, translocations, chromosomal aneuploidies/multiploidies), both in nuclear and mitochondrial DNA [2]. Most of the applied techniques can detect only a subset of these variants.

Another important factor influencing the detection of a somatic variant is its frequency in the examined tissue. Rare variants (i.e. with a low alternative allele frequency, < 10%) are harder to detect, regardless of the strategy and experimental technique used. Bulk tissue analysis and single-cell analysis are two major strategies applied in the discovery of somatic variants. Bulk tissue analysis is applied when the genomic DNA is extracted directly from a primary tissue, where cells carrying the variant can be present together with cells without the variant (wild-type). The tissue can be subjected to the sorting of certain cell fractions (e.g. specific neuronal cells by fluorescence activated cell sort ing [FACS] of NeuN-positive cells), to analyze a more homogenous sample and to increase the percentage of cells carrying the variant. In the case of single-cell analysis, the genomic DNA of an individual cell is extracted, amplified and sequenced in a single experiment.

Experimental techniques usually applied to bulk tissue analyses during the variant discovery phase are whole exome sequencing (WES), whole genome sequencing (WGS), SNP (single nucleotide polymorphism) arrays, array-CGH (comparative genomic hybridization) and targeted high-coverage sequencing. Standard PCR (polymerase chain reaction), Sanger sequencing and digital droplet PCR are mostly applied as validation methods.

The bulk tissue approach is preferably chosen than the single-cell analysis because (i) it allows to obtain a greater amount of DNA, (ii) the preparation and handling of the sample is easier, (iii) it is less time consuming and less expensive and (iv) the technical validation of the identified variant in the original sample is equivalent to a biological duplicate. However, its main disadvantage consists in the fact that it can only detect variants at a relatively high allele frequency rate (~10%), unless a targeted high-depth sequencing is applied: in this case the detection has been proven to be as low as 0.1% [3]. The ability to detect these low frequency variants is important because evidence exists that these can lead to strong phenotypic effects. In the single-cell analysis, the genome of a single nucleus is sequenced in a unique experiment, and multiple cells are usually analyzed in parallel to obtain statistically significant findings. The main advantage of using the single-cell strategy is that it allows to discover variants present in the analyzed cell, regardless of its frequency in the tissue. However, for very rare variants, the analysis of a great number of individual cells would be necessary and the biological validation in the primary bulk tissue or in additional selected cells can be difficult. Therefore, the techniques applied to these studies must be robust. The first step in single-cell sequencing approaches consists in the whole genomic DNA amplification (WGA), during which cytosine deamination may occur resulting in common artifacts (artificial CG->TA transitions) [4]. Therefore, an error introduced during this phase will be propagated in the following sequencing steps, leading to a false positive call. An implementation of the currently used experimental procedures has been recently published, increasing the capability to detect somatic variants reducing false positive calls [5]. This will provide a greater insight into the pathogenic role of somatic variants in human disease. Lastly, the genomic sequence obtained by a single-cell analysis has always to be compared to the genome of a reference tissue to exclude germline variants: in single-cell sequencing, mosaic variants would appear at an alternative allele frequency of 50%, the same as germline heterozygous variants.

The technical and biological validation of an identified somatic variant is a crucial step. Depending on the discovery approach used, there are various recommended validation strategies, based on methods which differ for sensitivity, throughput and cost. If a bulk tissue analysis has been performed, the confirmation of the somatic call in the original sample is usually achieved using a technique more sensitive than the one used for the discovery phase, with a consequent biological and technical validation. For this reason, a targeted high-depth sequencing or digital droplet PCR are commonly adopted. If the mosaic variant has been identified through a single-cell sequencing approach, the biological confirmation, although very important, may not be achieved in the original bulk tissue, because the variant can be present at a frequency too low to be detected by any technique. Consequently, the technical validation is fundamental in these cases, though a variant artificially introduced during the DNA amplification phase of the single-cell sequencing approach will be validated anyway, leading to a false call. A comprehensive description of the cited techniques can be found in [6].
Brain somatic mosaicism and epilepsy with FCD

Mosaicism in the brain

The mutational burden in proliferating somatic cells is estimated to be very high: hypothetically, at each cell division a genetic variation can occur, with possible effects on cellular functions [15]. The role of somatic variants in the pathogenesis of most cancers is well known, but several studies have also demonstrated that somatic variants can lead to non-neoplastic diseases as well (e.g. Proteus syndrome, McCune-Albright syndrome and Sturge-Weber syndrome, which are skin disorders caused by somatic mutations in AKT1, GNAS1 and GNAQ genes respectively), and a small number of mutated cells can be sufficient to cause important structural/functional effects [15 - 19].

Most neurons do not face cell divisions during adult life; however, the cellular proliferation rate in the brain during the first half of the gestation period in animals is higher than in any other organ at any developmental phase. At the fourth week of gestation a limited number of neuronal progenitors are found in the developing brain, but at 24 gestation weeks 10^10 neurons will develop from these progenitors, with a 10^5 cell division rate per minute (higher than that of any cancerous or other somatic cell) [19]. The brain is therefore the organ with the highest risk of accumulating somatic variations during development, which could be linked to various neurodevelopmental disorders [19]. The development of the cerebral cortex involves different complex processes, including the proliferation of progenitor cells, migration and neuronal organization. Each of these steps may be affected by the occurrence of somatic variants in the DNA of subgroups of cells, causing phenotypes with different degrees of severity, from functional alteration of few neurons to malformations of cortical development (MCDs, including cortical layer disruption or enlarged brain) [20, 21].

Focal Cortical Dysplasia (FCD): definition and classification

MCDs are an important cause of pediatric and adult refractory epilepsy associated with developmental delay, in which seizures arise as a consequence of defective positioning of normal cortical neurons or due to abnormal cortical neurons leading to altered cortical circuitry [21 - 23]. A subgroup of MCDs include focal cortical dysplasia (FCD) and hemimegalencephaly (HME), cortical malformations limited to a portion or one entire brain hemisphere which can be identified by neuroimaging techniques (e.g. magnetic resonance imaging, MRI, and positron emission tomography, PET). The incidence and prevalence of FCD in the population is unknown; however, it is thought to account for most refractory epilepsy cases in childhood, and the proportion of FCD in surgical series is 9% [24]. Moreover, many cases of the so called non-lesional refractory focal epilepsy, undergoing surgical resection of the epileptogenic focus, result from small FCDs undetectable with standard MRI techniques, but which are confirmed by the histopathological analysis of the resected tissue.

Since its first description by Taylor and colleagues in 1971, several efforts for FCD classification have been made [25]. The last scheme was released by the International League Against Epilepsy (ILAE) in 2011, providing evidence for differences in morphology and protein expression among the different types and subtypes of FCD (Figure 1) [26].

It is to be noted that in the context of the same lesion, multiple FCD subtypes can coexist, as well as different severity grades are recognized among different tissue samples of the same FCD type, suggesting a common molecular mechanism that can have a graded effect with a spatial spectrum [27]. Nevertheless, the present classification is based on histopathological findings, without any correlation with genetic etiologies, which have been recently identified (see below). These findings may lead to another revision of the current classification [28].

FCDs share certain pathological phenotypes with HME: disorganized/absent cortical lamination, loss of radial neuronal orientation, and abnormal neuronal differentiation and maturation [13]. However, while HME leads to gross cortex malformation with the enlargement of an entire brain hemisphere, FCD is not always visible on MRI imaging, but may often be confirmed by histopathological examination of resected brain tissues from patients subjected to surgical removal of the epileptogenic focus. This finding together with the fact that most FCD and HME occur sporadically, suggests that somatic variants in genes involved in main brain developmental processes, as neuronal cell growth and migration, may be the leading cause.
FCD and mTOR signaling

The first suspicions about the pathogenic mechanisms leading to the development of FCD came in 2004, only 30 years after its first report, when Babys et al. and Miyata et al. described the hyperactivation of the mechanistic target of rapamycin (mTOR) pathway in human FCD and cortical tuber samples [29, 30]. Subsequently, this was also demonstrated in HME brain specimens [31, 32]. mTOR is a serine/threonine kinase expressed ubiquitously in mammalian tissues. Its signaling pathway has important roles in different cellular functions: protein synthesis and transcription regulation, cell growth and proliferation, metabolism, cell motility and death [33]. In the brain, mTOR signaling has been implicated in synaptic plasticity and learning, neurogenesis and dendritic/axonal morphology [34, 35]. Therefore, the involvement of mTOR signaling alteration was in line with the pathological findings of FCD and HME, in particular cytomegaly. However, the hyperphosphorylation of mTOR targets was only confirmed in a subset of cells in the brain lesions, suggesting that the molecular cause of this cellular phenotype would have been present in the same cells and not in the entire lesion. This led to hypothesize that a somatic variant could be the cause of FCD or HME. Thanks to technical sequencing advancements, brain somatic variants in mTOR pathway genes PIK3CA, AKT3 and MTOR itself have been firstly identified in HME patients [36] and more recently MTOR brain variants have been detected in about 15 - 46% of FCD patients [37, 38]. In these cases, the mosaic variant allele frequency rate can be as low as about 1%, further underlining that low level somatic variants can cause neurodevelopmental disorders (as also shown by in vivo mouse model of FCD) [37]. After these first reports, many others have involved mTOR pathway genes in the pathogenesis of FCD, further confirming that FCD belongs to the so-called "mTORopathies". Moreover, variants in MTOR are associated with a spectrum of brain malformations phenotypes that seem to be correlated with the levels of mosaicism [39, 40]. Not only somatic but also germline variants, and both gain of function as well as loss of function variants have been described. To date, FCD-associated gain of function variants in mTOR signaling have been reported in MTOR gene itself and PIK3CA, while variants with a loss of function effect (both null and missense variants) have been identified in inhibitors of the same signaling cascade (TSC1, TSC2, DEPDC5, NPRL2 and NPRL3), all leading to mTORC1 activation [41 - 43, 27, 44, 37, 45, 38, 46 - 49, 39] (Figure 2). The proteins encoded by DEPDC5, NPRL2 and NPRL3 genes constitute the GATOR1 complex, a negative regulator of mTORC1 complex, belonging to the amino acid sensing branch of the signaling. Several reports have underlined the role of loss of function GATOR1 variants in the pathogenesis of FCD.
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of focal epilepsy with MCDs, despite not all patients display MRI abnormalities. This has suggested that a second somatic hit (leading to the complete removal of inhibition of mTOR signaling) could be necessary for the MCD to occur: intriguingly, a second somatic nonsense variant was identified in the resected brain tissue of a patient with FCD carrying a germline nonsense variant of DEPDC5 [41]. This mechanism could also explain the differences seen among the brain lesions found in patients with FCD: the identification of the developmental time points at which the somatic variant occurs and of the progenitor cells involved may help to clarify how the same variant can lead to different histopathology features.

Figure 2. Schematic showing mTOR pathway main actors currently involved in the pathogenesis of epilepsy with FCD. Somatic and germline refer to the type of variant reported so far.
Epilepsy with FCD: Therapeutic strategies

A large spectrum of epileptic conditions characterizes the clinical manifestations of FCDs and depends on the age of seizures onset and on the extent and localization of the dysplasia. Despite the progresses made in the diagnosis of FCD-associated epilepsies, most cases are drug-resistant [50]. The standard treatment in cases of refractory epilepsy is the surgical resection of the lesion [22], with a fluctuating seizure freedom rate after the surgery. In fact, long term seizure outcome is influenced by diverse factors, such as the identification of the lesion on MRI and its complete removal, the localization and the extension of the lesion, histological findings, and the age at which the epilepsy surgery was conducted [51]. Recent publications have also highlighted that the seizure outcome in FCD patients is also related to the type of FCD diagnosed: in a long-term analysis of 211 FCD patients, Fauser and colleagues (2015) highlighted that FCD types I, II and IIIa have similar postoperative outcomes, with Engel class I at last follow-up (> 5 years) reported in 56% of FCD type I, 61% of FCD type II and 64% of FCD type III cases, and that a complete withdrawal of anticonvulsant drugs was significantly higher in FCD II patients [52]. However, in a more recent work, a significant difference among FCD Ila and I Ib subtypes was reported, with a better outcome in FCD I Ib (88% Engel Ia after 5 years) compared to FCD I Ia (57% Engel Ia after 5 years) [51]. Interestingly, FCD type I resulted to be the one with the lower surgery success rate, with seizure freedom achieved in 21% of the patients (at 5 years from the surgery, Engel Ia) [51]. This finding is consistent with the fact that FCD type I lesions are often difficult to see on MRI [53], which is one of the factors mostly impacting the surgery outcome, possibly due to incomplete resection of the abnormal tissue.

Due to the high percentage of refractory seizures in FCD patients, alternative therapeutic approaches (e.g. ketogenic diet and vagus nerve stimulation) could also be considered in combination with surgical resection of the lesion, or alone in those cases not suitable for surgery [54]. However, the identification of variants in mTOR pathway genes in surgically resected FCD tissues shows that mTOR inhibitors, such as rapamycin analogs or ATP-competitive mTOR inhibitors, could be considered as possible alternative antiepileptic drugs, as already done for TSC patients [55]. The development of novel molecules targeting specifically other mTOR pathway components such as GATOR1 complex could lead to a more specific anti-epileptogenic effect, in contrast to the systemic effect of rapamycin, with possible fewer side effects for the patients [55].

Our present inability to adequately treat many patients with refractory epilepsy caused by FCD is a significant clinical problem.

Conclusions

The present review has highlighted the established role of brain somatic variants in neurodevelopmental diseases, and FCD in particular. Advancements in DNA sequencing techniques have allowed to sequence DNA extracted from FCD tissues, leading to the discovery of brain mosaic variants in mTOR pathway genes, at allele frequencies as low as 1%. Moreover, as FCD is characterized by a mosaic pattern of abnormal cells, the single-cell sequencing approach seems to be very promising for FCD genetic diagnosis. The molecular mechanisms leading to the development of these malformations of cortical development are not yet well understood; further efforts will be needed to elucidate how an altered mTOR signaling drives the development of FCD and other MCDs, ultimately leading to epilepsy. The epileptic phenotypes associated with these malformations can be severe and are often drug-resistant. The current strategies to face these conditions include the combination of multiple antiepileptic drugs and surgical resection of the epileptogenic zone, but seizure outcome depends on multiple factors and the anticipation of a long-term outcome is still difficult to achieve.

References

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