

Gene–disease relationship evidence: A clinical perspective focusing on ultra-rare diseases

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Abstract

The ACMG framework for variant interpretation is well-established and widely used. Although formal guidelines have been published on the establishment of the gene–disease relationships as well, these are not nearly as widely acknowledged or utilized, and implementation of these guidelines is lagging. In addition, for many genes so little information is available that the framework cannot be used in sufficient detail. In this manuscript, we highlight the importance of distinguishing between phenotype-first and genotype-first gene–disease relationships. We discuss the approaches currently available to establish gene–disease relationships and suggest a checklist to assist in evaluating gene–disease relationships for genes with very little available information. Several real-life examples from clinical practice are given to illustrate the importance of a thorough thought process on gene–disease relationships. We hope that these considerations and the checklist will provide help for clinicians and clinical scientists faced which variants in genes without robustly ascertained gene–disease relationships.

KEYWORDS

gene–disease relationships, HPO, in vitro, in vivo, rare diseases

1 | INTRODUCTION

Determining whether a DNA variant identified in a gene is causally related to the clinical features in a patient has many aspects. The two major considerations are: (1) whether mutations in the gene in question are indeed associated with well-described phenotypic features in humans. For many, often well-described conditions, such gene–disease relationships (GDRs) are usually obvious but not so for very rare or heterogeneous conditions; (2) whether the particular DNA variant identified, is (likely) pathogenic or not. The second part of this decision process is thoroughly covered by the DNA variant interpretation criteria designed by the American College of Medical Genetics and Genomics, which provide an excellent framework for

DNA variant interpretation in clinical practice and are widely used (Richards et al., 2015). The first part of this decision process, establishing the GDR in the first place, has been extensively discussed in many literature papers (Casanova et al., 2014; MacArthur et al., 2014; Strande et al., 2017), with a growing number of GDRs evaluated formally. However, the vast majority of GDRs for ultra-rare conditions (or ultra-rare potentially causative genes for less rare disorders such as intellectual disability) in the literature have not been formally evaluated, leaving clinicians and clinical scientists to assess these intuitively.

To establish a gene–disease relationship, historically, patients would be ascertained based on a specific clinical phenotype, followed by extensive genotyping using haplotype markers, single nucleotide

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polymorphism-arrays, or more recently by massive parallel DNA sequencing (phenotype-first GDR). Increasingly, however, the genotype is the *start* of a gene–disease relationship (genotype-first GDR), and as we will show in the following sections this distinction is important when assessing the strength of the evidence.

In this manuscript, we discuss the GDR for very rare genetic variants and propose a checklist for clinicians and clinical scientists to decide ad hoc if a GDR can be considered *bona fide*, or should be questioned in absence of further studies confirming the GDR. After the GDR has been established, clinical variant interpretation can follow using the DNA variant interpretation criteria by the American College of Medical Genetics and Genomics (Richards et al., 2015), or for the UK the modified version by the Association for Clinical Genome Science (<https://www.acgs.uk.com/media/11631/uk-practice-guidelines-for-variant-classification-v4-01-2020.pdf>). Determining the GDR has to be done *before* the ASHG DNA variant interpretation criteria can be applied, something which is acknowledged in the ASHG criteria who state “*These rules are intended to determine whether a variant in a gene with a definitive role in a Mendelian disorder may be pathogenic for that disorder*” (Richards et al., 2015). For many genes with a DNA variant identified in a single or just a few cases of an ultra-rare disorder, weighing the GDR before applying the ASHG criteria is, therefore, an essential step. Importantly, weighing the GDR is a key step for diagnostic genetic laboratories, to decide whether or not to even mention certain DNA variants in their diagnostic reports.

2 | GDR

For DNA variants in genes that are associated with common up to moderately rare conditions, establishing a GDR is usually not problematic as sufficient data of large cohorts of patients with the condition are available. Many of these GDRs have been evaluated by ClinGen's GDR framework (Strande et al., 2017), which we highlight as an important resource that may not be known to all, even amongst the clinical genetics community. In contrast, identifying a DNA variant in a single patient, or even a few patients, in a gene in which germline DNA variants are very rare does not prove that a variant in a “new” gene is truly causally related rather than just coincidentally identified in a patient with a particular disorder or clinical features. This problem is compounded when the disease itself is also ultra-rare. This holds true for so-called pathogenic DNA variants, as the ASHG DNA variant criteria make a clear distinction between two meanings of the term “pathogenic”: “... *it is important to consider the differences between implicating a variant as pathogenic (i.e. causative) for a disease, and a variant that may be predicted to be disruptive/damaging to the protein for which it codes, but is not necessarily implicated in a disease.*” (Richards et al., 2015).

The reason that problematic situations appear to occur more often in recent years is that whole-genome/exome sequencing has become widely applied across the globe, enabling identification of DNA variants in genes that are so far not, or have only been very

rarely, associated with human disease. Hence, increasingly, GDR is genotype-first, established after identifying DNA variants in a certain gene with retrospectively comparing the phenotypic features in these patients. In effect, the historic use of establishing GDRs phenotype-first has been replaced in more recent years by establishing GDRs in genotypically defined cohorts (genotype-first).

2.1 | Phenotype-first GDR

When a cohort consists of a specific phenotype, diagnosed a priori, as was common in the early days of exome sequencing and before, the specificity of the phenotype is more or less guaranteed as these patients were selected on shared phenotypical features that distinguished them from a larger group of patients with similar features. If the disorder defined by a specific constellation of phenotypic features is caused by *de novo* variants, that eases gene identification as they can be readily identified by trio exome sequencing. Since *de novo* variants in coding genes are sufficiently rare and considered damaging in the majority from an evolutionary perspective, the combination with a specific (very rare) phenotype provides evidence beyond reasonable doubt: or, in mathematic terms, the probabilities of multiple occurrences of the specific phenotype with a *de novo* DNA variant in the same gene, can be multiplied as they refer to independent probabilities. A typical example of this is the identification of one of the Coffin-Siris syndrome genes, by exome sequencing in 3 patients and identifying *de novo* mutations in *ARID1B* in all three (Santen, 2012).

2.2 | Genotype first GDR

The key difference between genotype-first and phenotype-first GDR is that the clinical features are assessed when the genotype is already known. This makes an important difference. When defining a cohort of patients with a clinically diagnosed syndromic condition, assessing features before genotyping, these features are compared with other patients and healthy individuals and deemed specific, because they occur exclusively (pathognomonic – the highest grade of specificity) or mostly only (specific) in patients of the selected cohort with an assumed common genetic cause. When assessed post-genotyping, many authors use “specific” differently, meaning that they observe that the feature in question occurs in most patients with the selected phenotype. This, however, is not true specificity in the statistical meaning of the word.

This is illustrated by the large number of research papers that report a cohort of patients with a certain diagnosis (e.g., a microchromosomal deletion), describe the various features, and then claim a “recognizable phenotype” on the basis of “specific features.” However, to many experienced clinicians, it is obvious that these “specific,” often facial dysmorphic, features cannot lead to a pre-genotype clinical diagnosis as these same features occur in many other patients with different conditions as well. The “recognizable

phenotype” and “specific features” mentioned in these papers point to postgenotyping qualifications. As the features in the selected cohort of patients with the same genotype are not being compared with patients with different genotypes, one cannot speak of true specificity of these features. In other words, the phenotype associated with the genotype can only be recognized with hindsight, after the genotypic diagnosis is known. Assessing a phenotype with knowledge of the genotype may easily lead to logical fallacies as “Texas Sharpshooter fallacy” or “cherry picking.” In mathematical terms, the phenotypic features assessed are not independently ascertained from the genotype and can therefore not be multiplied to achieve statistical significance.

Therefore, it follows that a more skeptical attitude towards genotype-first GDRs than towards phenotype-first GDRs is necessary.

This skeptical attitude towards genotype-first GDR has to be balanced with one of the advantages of genotype-first GDRs: it gives a clearer picture of the phenotypic variation in genetic conditions with wide variability in features, while the phenotype-first GDR will be restricted by having included patients with a certain defined phenotype in the first place. Conditions described by traditional phenotype-first GDRs have required re-evaluation in the increasingly genotype-first world, leading to a multitude of scientific papers in recent years, entitled “expanding the phenotype...”

2.3 | GDR: Premature and delayed assignment

In practice, when confronted with a single or very few patients with DNA variants in a gene hitherto not reported associated with a human phenotype there is a dilemma regarding the optimal timing of publishing a GDR: waiting too long or publishing prematurely can both have equally negative consequences in clinical practice.

The dangers of false-positive assumptions that DNA variants in a certain gene are causative is well-established in the medical literature (MacArthur et al., 2014; Piton et al., 2013). The negative consequences of delaying to publish or establish a GDR are less reported in the literature but do not seem less obvious. The clinical consequences of such delays are illustrated in the following examples coincidentally derived from the same paper.

Illustrative case 1: In 2020, DNA variants in four families with Osteogenesis Imperfecta KDELR2 were published, including functional studies and a plausible biological pathway for the KDELR2 protein to be causative for the condition (van Dijk et al., 2020). When proposing to add the KDELR2 gene to the UK gene panel for Osteogenesis Imperfecta, it was decided by the laboratory to postpone adding this gene to the panel until a second publication confirming the GDR would have been published (Personal Communication). This means that other patients with KDELR2-related Osteogenesis Imperfecta might remain unrecognized when applying this routinely used gene panel.

Illustrative case 2: in this same paper (van Dijk et al., 2020), Family 4 had exome sequencing in their local hospital, which was reported as negative, but one pathogenic variant in a known OI gene was detected. To look for the second variant, WGS was performed in a laboratory

specialized in OI. No second variant was detected, but two variants in KDELR2 were flagged as the laboratory was working on this gene. Prenatal diagnosis was performed in a second pregnancy even before the paper was published. Had the WGS not been performed in this specialized lab, the family would still have been without diagnosis.

2.4 | Establishing GDR for clinical purposes

From a mathematical viewpoint, the evidence of a GDR can be measured as the inverse of the likelihood that a given genotype and phenotype co-occur by chance. Thus, both the population frequency of the phenotype and the genotype play a role. Immediately one encounters an issue when realizing that each human phenotype (even that of identical twins) is unique and that the “uniqueness” of a certain phenotype is partly a matter of subjectivity or a matter of how many separate phenotypical features one wishes to take into account as part of the “unique” phenotype (“Gestalt Diagnosis”). Features like “upward-slanting palpebral fissures; hypotonia; sandal gap; flat facial profile; downward turned corners of the mouth; simple ear shape” are all rather nonspecific and happen in healthy subjects regularly. Taken together, they do form part of a well-established “Gestalt diagnosis,” and research has shown that this particular phenotype is specific in the sense that it almost exclusively only happens in Down Syndrome (Fried, 1980).

When looking for new GDRs in large cohorts, the combined use of several phenotypic features to arrive at a more specific combination of features to be used in determining the GDR can be formalized. As shown by Akawi et al. (2015). For each pair of patients, frequency of the common denominator of each pair of HPO-terms is established. If the common denominator is rare (such as e.g., “molar tooth sign”) then it will count strongly towards a specific phenotype, when it is frequent (such as “developmental delay”), it will count less. By combining this with a rigorous method to establish the probability of observing the overlapping genotype, a combined p value for the GDR is obtained for all identified DNA variants in the patient cohort. Although there are some downsides to this approach (it relies heavily on the precise specification of HPO terms which may not be available; there is no penalty for nonmatching phenotypes e.g.), the objective nature is laudable, and we strongly encourage the use of HPO in clinical practice and within clinical cohorts, as such large-scale clinical information will be key to establish GDR in the future. However, the situations where this approach can currently be used are limited to well-defined cohort of patients ascertained and analyzed in the same way, mainly because the baseline frequency of HPO defined phenotypic features and especially the combination of HPO features in single patients, is an unknown entity in most clinical situations. Another reason that such approaches are often not used is that, in our experience, both clinical scientists and reviewers, often assume that we are able to intuitively assess the significance of a GDR without such calculations.

As an example of how GDRs are routinely approached, in the Genomics England PanelApp (a web-based tool to make gene panels

[Stark et al., 2021]) inclusion of a gene in the “Green” category (the group with an established GDR) requires “There are plausible disease-causing mutations within, affecting or encompassing an interpretable functional region of this gene identified in multiple (3 or more) unrelated cases/families with the phenotype.” The crux in this criterion is “the phenotype,” for which Panelapp refers to the UK Genetic Testing Eligibility criteria (<https://www.england.nhs.uk/wp-content/uploads/2018/08/Rare-and-Inherited-Disease-Eligibility-Criteria-November-2020-21.pdf>) which contain clinical criteria for broad genetic disease categories and a few more common genetic syndromes, but not for rare genetic conditions. These eligibility criteria are therefore not helpful to compare phenotypes in new or scarcely described conditions.

Finally, we point again to the valuable ClinGen Gene-Disease Clinical Validity Curation portal (<https://clinicalgenome.org/curation-activities/gene-disease-validity/>) (Strande et al., 2017). Thus far (September 7, 2021) about 1500 GDR have been evaluated using a scoring system which includes aspects of segregation, in vitro, and in vivo observations in literature. Although this number is impressive, the vast majority of GDR have thus far not been scored. Whilst the number of evaluated GDR will undoubtedly grow, in our opinion there is a need for some guidance to help clinicians and clinical scientists make ad hoc assessments of genes which have thus far not been formally evaluated. In many cases the amount of available information is such that application of ClinGen framework is not feasible in a meaningful way. We acknowledge that the simple checklist we offer is no substitute for ClinGen's more rigorous framework, and if sufficient information is available to use ClinGen's framework we suggest that this should be used, instead of our checklist.

3 | GDR CHECKLIST FOR RARE GENETIC CONDITIONS

Having discussed the problems with establishing a GDR in very rare genetic conditions, we feel that it is important to suggest how to deal with this issue. Therefore, in Table 1, we have provided a checklist which can be used to assess GDR in a more structured manner.

Some of the criteria used in this GDR framework are highly similar to criteria used in the ACMG criteria since variant characteristics play an important role. For example, for rare diseases, variants in a gene should be rare to be potentially causal.

The phenotype of the patient and how that has been assessed plays an important role in the proposed checklist. The general rule for arriving at an established GDR is that there is an inverse relationship between the number of patients needed with a highly similar phenotype (harboring DNA variants in the same gene) and the specificity of their phenotypic features. In the medical literature, as in our proposed criteria, 3 or more independent observations of a specific phenotype with variants in the same gene are deemed sufficient for very strong evidence of a GDR. However, where there are exceptional gene function, molecular and disease-modeling data

for a rare variant then even a single patient may be sufficient to report a strong GDR (Casanova et al., 2014). That being said, we do feel that any variant reported on the basis of our checklist should not be designated a pathogenicity score above Class 4 (likely pathogenic). Only if the GDR is well-established using ClinGen's framework, should variants be labeled as Class 5 (pathogenic).

While a detailed discussion of functional validation is beyond the scope of this paper, we would highlight some key principles and future opportunities due to new and emerging technologies. First, the gene should be expressed in the correct tissue(s) and within a developmental time window that might feasibly lead to the phenotype. There are an increasing number of gene expression datasets obtained from human embryonic and fetal material that offers a rich resource to undertake this analysis, for instance, data derived from the Human Developmental Biology Resource (HDBR) or the Human Developmental Cell Atlas (Gerrelli et al., 2015; Haniffa et al., 2021). We would also highlight the recent findings that placental defects are a major contributor to abnormal development in mice, which could be a confounding factor for placenta-expressed genes in humans (Perez-Garcia et al., 2018). This study shows a value of model organisms, as have others (Hmeljak & Justice, 2019; Wangler et al., 2017). In particular, a demonstration that a similar (or ideally identical) genetic change in a model organism leads to a specific phenocopy remains a powerful approach to functional validation and will continue to play an important role in both diagnosis and downstream research, despite the advances in human model systems discussed below.

If gene expression data is compatible with the phenotype observed then functional testing is required. Simply demonstrating that a given variant disrupts intracellular protein characteristics (location, binding, etc.) or even disrupt gene function is insufficient evidence, as this still does not connect the variant to the disease in the patient(s). In human studies, the use of patient-derived cells is desirable, and easy-to-access cell types such as fibroblasts or peripheral blood mononuclear cells (PBMCs) are already routinely utilized (Tangye et al., 2020). This may allow direct assaying of patient cells to show that the specific function or pathway is defective—as has been shown for herpes simplex encephalitis and TLR3 pathway deficiencies (Zhang, 2020). Connecting the gene variant to the identified defect can be achieved by genetic rescue (editing the gene sequence back to wildtype sequence using gene-editing technologies) or by introducing the mutation to control cells to show the same deleterious effect.

However, fibroblasts or PBMCs may not be the ideal model for a number of reasons, most obviously if the relevant gene is not expressed in those cells. Therefore, this approach can be extended to essentially any cell type by using induced pluripotent stem (iPS) cell technology (Yamanaka, 2012). Patient-specific iPS cells are now facile to derive and correction of candidate gene variants to generate isogenic controls is a validated approach (Soldner et al., 2011). As iPS cells are pluripotent, this enables subsequent differentiation to the tissue(s) of interests using monolayer (“2D”) differentiation protocols or, increasingly, organoid (“3D”) approaches. In principle this

TABLE 1 Checklist when considering a newly identified gene–disease relationship (GDR)

Level	Description	Comments
Phenotype	Was the proposed GDR established phenotype-first or genotype-first?	For phenotype-first GDR, phenotype specificity can be taken for granted, and if the genetic data is convincing, a GDR is highly likely. For genotype-first GDR, look at how objectively the features have been assessed and how rare and specific the features are compared to individuals with phenotypically similar conditions and healthy controls.
Variant	Do all patients have the same variant type?	If only missense: do they cluster within gene regions? If there are mixed variants, be aware that some variants may not be pathogenic. A difference in disease severity or expression between variant types favors a GDR.
Population	Are variants as rare as you would expect based on the frequency of the disease?	For dominant conditions, if variants are in public databases (e.g. gnomAD, https://gnomad.broadinstitute.org/) with any measurable frequency, and the phenotype should not be present in these databases, this observation counts strongly against a GDR. Similarly, presence of truncating variants in public databases counts against a GDR if the presumed mechanism is haploinsufficiency. For very rare autosomal recessive diseases a majority of patients will harbor homozygous variants and if that is not the case, suspicion about a GDR is justified.
Gene	Does the gene product have an essential role in a pathway, which has been previously implicated in the disease?	If this is the case then this is a strong argument in favor of the GDR. The inverse is not true, unless the gene's function is well known, and cannot at all be matched to the phenotype. Be aware that this only holds if the function is strongly related to a group of diseases, for example, the RAS/MAPK pathway in Noonan syndrome. Therefore, this argument can only be used for well-established groups of genes.
Gene	Is the gene expressed in (developing) affected tissue?	If there is no expression in developing tissue, or precursors, then this is an argument against the GDR. Ideally, protein expression should be confirmed for protein-coding genes, in relevant tissues and developmental stages (i.e., https://www.humancellatlas.org/dca/ and https://www.hnbr.org).
In vitro model	Is the used in vitro model relevant for the gene's main function?	Especially for the interpretation of missense variants in vitro modeling may be crucial. Care should be taken to evaluate that the model is relevant with respect to the type of tissue and developmental stage of that tissue
Animal model	Is there overlap between animal model and human phenotypes?	A strong, specific overlap is a strong argument in favor of GDR, If there is overlap in multiple organ systems the evidence is stronger (http://www.informatics.jax.org). Absence of overlap may point against the GDR but is not a strong argument for rejection.

Note: Without giving a precise scoring system, by following these points, clinicians and molecular geneticists can discuss the strength of a GDR and if sufficiently strong, DNA variants in this gene can be further classified with the DNA variant interpretation criteria as published by the ACMG, with the caveat that variants should not be scored above Class 4 (likely pathogenic), taking the remaining uncertainty of the GDR into account.

approach allows the use of an assay-specific to the gene variant, in a relevant tissue, and with an isogenic control which allows an unambiguous connection between a gene variant and a pathogenic change, potentially providing strong evidence towards a GDR in one or a small number of patients. In practice, this approach can be challenging and arduous, is costly in terms of time and resources, and careful steps must be taken to ensure assays are robust and reproducible (Volpato & Webber, 2020). However, where only a single patient or family has been identified, we would argue a very high bar of functional evidence is necessary to make strong claims regarding a GDR.

The checklist does not provide a definitive answer about the certainty of a GDR: such a final decision can be obtained by

application of ClinGen's rigorous methodology. Rather, the checklist can function as a tool for clinicians and clinical scientists in ensuring that they have considered the most important aspects of the GDR, for instance in deciding if a GDR is strong enough to merit inclusion in a genetic result report or to highlight it as a promising avenue for future research.

One could argue that publication of weak relationships might still be worthwhile as a method to locate new patients. However, we feel that tools such as Genematcher (Sobreira et al., 2015) and DECIPHER (Firth et al., 2009) are a preferable approach, since a publication may in some cases lead to premature clinical uptake (see TOM1 example below). Genematcher is a web-based tool to link clinicians and laboratories who encounter very rare DNA variants in the same gene

(Sobreira et al., 2015), and has proven to be very successful, leading to many publications about new GDRs.

4 | CLINICAL EXAMPLES

- Example 1: In a girl with granulomatous inflammatory lesions in both her eyes, and a working diagnosis of “Phenomenon of Splenore Hoepfli,” a WES-based primary immunodeficiency gene panel was performed which flagged a heterozygous missense variant in *TOM1*, considered a VUS. *TOM1* has been linked to immunodeficiency and autoimmune disease in a single paper only, describing an inherited variant in a single-family (Keskitalo et al., 2019). Thus, even before classifying the variant itself according to ACMG criteria, the question should be asked: is there a GDR for *TOM1*? Many known immunodeficiency genes have first been described as single patients (Casanova et al., 2014), but in the current genomic era a single patient report should be treated with caution, since rather than sequencing genes because they were a priori thought to be implicated in a patient's disease, it is now routine to perform exome or genome sequencing. The GDR has not been formally evaluated by ClinGen. When we use our checklist, there is only one supporting criterion since the gene has a link to an immunological pathway. Importantly, there is no phenotype match between our and the published cases. In addition, the identified variant occurs in GnomAD with a frequency of 1/2000 which we consider too frequent. The absence of a clear GDR was discussed between the clinician and clinical scientist. Although it could be argued that reporting variants in *TOM1* is the only way to gain more evidence of its relevance, it was eventually decided to remove the *TOM1* gene from the immunodeficiency gene panel.
- Example 2: In a monozygotic twin pregnancy where both fetuses had microcephaly with suspected gyration delay at 20 weeks, prenatal exome sequencing was performed. Two rare missense variants in the *DMRTA2*-gene were identified in both. The *DMRTA2*-gene has previously been reported in a single-family with cortical brain malformation (Urquhart et al., 2016). The GDR has not been formally evaluated by ClinGen. It segregates in their family and preclinical models seem to support the involvement of the gene in brain development. Here again, even before classifying the variants, the question about GDR should be answered first. Arguments in favor of the GDR are that the gene is expressed in the developing brain, and that a knockout mouse has phenotypic similarities: A smaller telencephalon, midline brain defects, and no hippocampal structure. However, the variant was identified in a single-family only, and therefore we felt the evidence for the GDR was still limited. To gather more evidence, we asked the authors if more cases had come to their attention, or if an alternative diagnosis was reached in the family, which was not the case. We then decided to discuss the variants with the parents, emphasizing we could not be certain that they related to the phenotype of the twins, and if so, had little data on postnatal

consequences. The pregnancy was continued, and after birth we found microcephaly, but with normal gyration, making it less likely that the variants were causal.

Please note that, in both of the above cases, we do not mean to suggest that there is no evidence for a GDR of these genes. Rather, the evidence from a single-family is low, and taken together with our clinical cases we could still not establish a GDR.

5 | CONCLUSION

With the rapid identification of new genotype-first GDRs clinicians and clinical scientists are often faced with variants in genes lacking well-established GDRs. Therefore, it is important that clinicians and clinical scientists are able to judge the level of evidence for a GDR. We hope that our checklist provides some useful clues on how to assess GDR in a more structured manner. This checklist is to provide clinicians and clinical scientists with a quick estimate for a DNA variant's pathogenicity for a gene where very few putatively pathogenic variants have been reported and is by no means a replacement of ClinGen's framework. When sufficient data becomes available we would strongly advise subsequent use of ClinGen's framework to more formally establish the GDR, and we expect that this will become more and more feasible as ever-larger sequencing studies are being rolled out worldwide.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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