FACULTAD DE MEDICINA
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GRADO EN MEDICINA

TRABAJO FIN DE GRADO

Modelos animales de enfermedad humana

Animals models of human disease

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Santander, Septiembre 2019

INTRODUCTION

Humans have always wanted to fight the disease and live longer than we can, and we have certainly made great strides, moving from a life expectancy of 30 years to about 80 years throughout our history. And to make those advances, we certainly needed to investigate and test substances and processes in ourselves.

The use of model organisms in biological research is based on the concept of unity in biology, concept expressed by Jacques Monod and François Jacob: ´ ´ Anything that is found true in an E. coli must also be true for an elephant ´ ´
As people clearly are not going to submit to processes that may be harmful, and as there are also ethical implications for involving others, researchers had to develop another model of study, and decided to use animals.

95% of experiments are carried out on rodents

One of the most used, if not the most used, has been the house mouse or musculus musculus. Rattus norvegicus is also used, especially in psychological and neurological studies, since it was observed that their neural networks are more similar to ours.

This species has been chosen because of numerous similarities between the genetics and physiology of both species. We start from a common ancestor that existed 85 million years ago, so we share characteristics, such as the adaptive immune system, in which we can find the MHC genes and detect the T cell receptor. So, this allows us to study common physiological and genetic pathways very well. But few more similarities we will find, because for different reasons we differ.

For example, the metabolic rate, since mice have a rate 7 times higher than ours; or also the greater development of lymphoid tissue in the murine respiratory system. The reason for this may be that they live closer to the ground and are more exposed to soil pathogens. There are many other differences, such as the relationship between genotype and phenotype, or the resistance or sensitivity to oncological drugs, so when it comes to investigating diseases, we do not find them useful at all.

So, the use of these animals will have a number of disadvantages and benefits.

Benefits:

-The small size of the mouse, something that makes it less expensive in terms of care and storage.

-The great capacity of reproduction that they have, and that the humans have improved thanks to increase the fertile period and to increase the size of the litter.

-The common ancestor between both species, which provides some similarity in physiological pathways and genetics.

-The omnivorous diet of the mice, like us, which helps us to increase more the similarities.

-The similarity of the environment in which mouse and human live allow us to manipulate their genome in an inheritable way through Mendelian genetics

-We create mouse clones, so we eliminate inter-individual differences

A case that explains very well the usefulness of animals was the case of researchers from Harvard, Joseph and Charles Vacanti, who developed a mouse without a thymus gland (later these mice would be called Vacanti mouse), which allowed the transplantation of grafts from other individuals and species. What they did was to make the mouse grow an ear with human form, but made from cow cartilage. This experiment proved to be a success and in one case in 2018 we saw its usefulness, since a child suffering from microtia, a disease that produces a hypoplasia of the ears, was subjected to a transplant of this type, and saw how successfully the ear was not rejected by the body of the boy.

Fig 1. Mouse lacking an immune system with an engineered ear on the back.Labortory for tissue engineering and organ fabrication, Massachussetts General Hospital, Boston, MA, USA, Dr Joseph P. Vacanti

Disadvantages:

-The evolutionary development and lifestyle of both species is radically different. For example, there are many differences in the diet of both species (we for example need ascorbic acid and they are able to synthesize it) and its metabolic rate. Besides being much higher in the mouse, it also has a higher RO production and a higher sensitivity to O2. The cytochrome p450 of mice has different varieties than the human species, so toxic studies are not very useful.

-Differences between genotype and phenotype. Although we present similar genes, the difference lies in the way these genes connect and give rise to a different expression in each species.

-In diseases such as cancer, there are differences in epidemiology, since mice have more mesenchymal cancers and we epidermal, which added to the difference in metabolizing toxic, make mice of little use in studies of this disease.

They also have longer telomerases with much more expression than humans, causing murine cells to have higher rates of spontaneous transformation.

-Recently, animal rights support groups have sprung up, and people are increasingly being made aware of the bad life, to put it in a way, that these animals are suffering for our good.

-The use of genetically homogeneous mice, even if it helps us in the first place to discard variables that do not interest us in the experiment, gives rise to biases, since the findings discovered in the animal would not necessarily be the characteristic traits of a healthy animal.

An experiment that shows that this model is not perfect is that of thalidomide. Thalidomide was a drug that was marketed in order to prevent nausea during pregnancy, but over time it was observed that it was teratogenic and produced a large number of congenital malformations. And why was this not observed in the mouse? Because of the faster metabolism of these, which quickly undoes the drug, and also because of the greater number of antioxidants present in the murine embryo.

Conclusions

There are many differences between them and us so that the model has complete validity.

In certain aspects and studies, we could use them, as for example in genetic and metabolic diseases, although for example at the time of testing antineoplastic drugs, the majority of drugs do not leave to market, due to the difference of toxicity (as the thalidomide) and efficacy (the endostatin is more effective in mice) of the substances in both species. But we must be aware of all the limitations of the model, and not rely blindly on the results of the study.

And since it is inevitable for us to continue using them in order to improve our medicine, we must design and respect ethical and professional codes and protocols in order to improve the quality of life of these poor animals.

TYPES OF ANIMAL MODELS

We will use cancer as an example to explain the different types of animals obtained.

At first, when it came to studying cancer and drugs, scientists were limited to investigating human cancers implanted in mice, or some cancer specific to mice. But thanks to advances in technology, molecular biology and genetics, it has been possible to develop mice that naturally suffer a human neoplasm; or we can also promote the development of some relevant characteristic of any type of cancer.

Transgenic-The first transgenic mouse was developed at MIT in 1974, which was implanted with the Simian virus 40 within the embryonic blasts.

Their main use is to detect the toxicity of antineoplastic drugs and understand their mechanisms.

Production of transgenic mice

In the early 1980s, several teams began to develop techniques to introduce certain genes or sequences into the mouse genome.

They achieved this by introducing cDNA clones (which could be a sequence encoding a transcriptase, some interesting genes, and even DNA hybrids) through prokaryotic cell plasmids (mainly bacteria), using them as vectors and introducing DNA by injection into the embryo's pronucleus, and then implanting that embryo into a pseudo-pregnant female (female paired with a sterile male) This pseudo-pregnancy state is necessary because mice need sequential stimulation to prepare the reproductive tract for nesting.

The embryo is obtained from ovarian stimulation and subsequent mating of females other than those to be implanted.

The injection is performed approximately 24 hours after the male's service, which coincides with the ideal biological moment, which is between three and five hours before the pronuclei fusion occurs. This procedure leads to the stable integration of foreign DNA in 5-50% of the surviving embryos. In most cases, this integration occurs at the embryonic stage of a cell, so the resulting transgenic animal will carry the introduced DNA in all its cells, including the germline. However, 10-20% of transgenic animals may present integration at later stages of the embryo (embryos of more than one cell), producing "mosaic" animals for the transgene.

This technique has the limitation of not being able to be used to introduce genes in more advanced stages of the embryo's development and of only being able to add genetic material (never to subtract or replace).

Transgenes essentially consist of two components: the structural gene, which we want to study, and the so-called regulatory elements. The first will be the carrier of the genetic information necessary for the synthesis of the protein we want to analyse. Regulatory elements (promoters, activating sequences, etc.) are the DNA sequences responsible for our transgene expressing itself in a certain cell type and/or moment of development.

Transgenic models have been widely used for the identification and study of regulatory sequences of gene expression in transgenic constructions in which these sequences fuse to a molecule easy to detect by directing their expression.

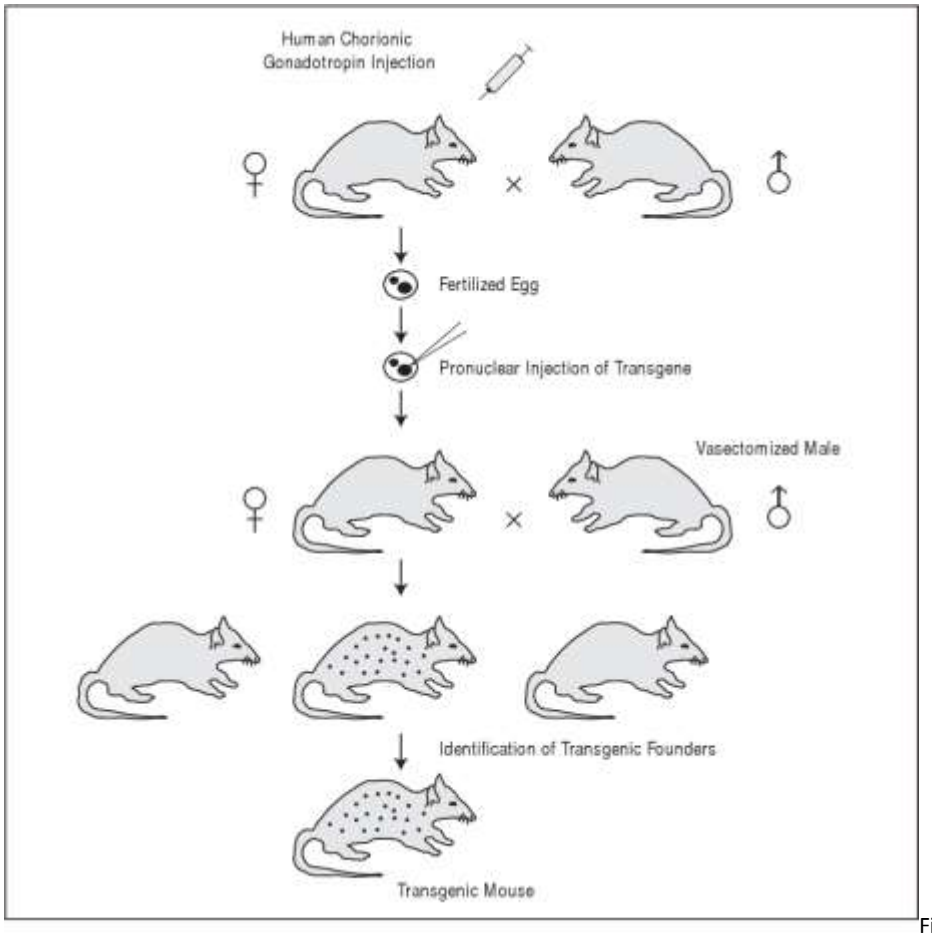


Fig 2. Principal steps of the creation of transgenic mouse

Xenografts: Immunosuppressed mice are transplanted with human oncological tissue to see the natural biological progression of the cancer, or for the selection of a certain cell lineage that under normal conditions or in vitro, would not grow. These implants have the advantage, or at least that is the belief we have, that they preserve characteristics of the original tumour, such as heterogeneous histology, biomolecular signature, malignant phenotypes and genotypes, as well as the architecture of the tumour and its vasculature. Most are implanted in the subcutaneous tissue, although it has recently begun to be implanted in some typical location of the tumour according to epidemiological data, which is believed to provide more information on its local growth and metastasis. The latter is called orthotopic xenotransplantation.

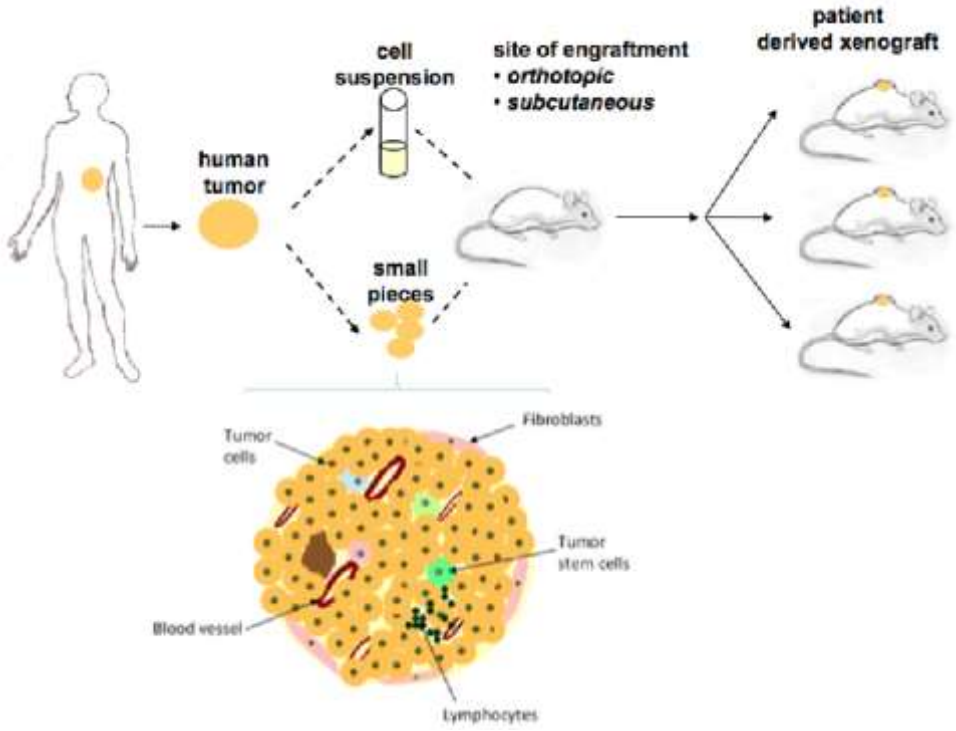


Fig 3 Xenograft of human tumour tissue in mice

Genetically Modified: As the genetics and histology of xenografts is not 100% similar to that of an original tumour, genetically modified animals were developed to perfect the model.

Modified animals are organisms that have alterations in specific genes to create disease models. It is defined as an animal carrying one or more foreign genes, deliberately introduced using recombinant DNA technology.

In modern models of modified animals, oncogenes are activated, and suppressor genes are inactivated. Because of this, animals will develop tumours in the tissues of interest, and then treated with the substance we want to test.

Some of the advantages are:

-safe and effective development and testing of products for human use production of recombinant products, such as anti-coagulants

-a means of studying the mechanisms of disease in complex organisms (diabetes) to understand the mechanisms and pathways that cause disease, thus allowing the optimal and safe development of treatments

But they also have their problems, and the most important is related to the patents that pharmaceutical companies are developing. Put more clearly, each genetically modified mouse has a copyright and cannot be used without the permission of the company that developed that mouse.

Knock out: A knockout mouse or KO mouse is a mouse genetically engineered so that one or more of its genes are inactivated by a technique called gene blocking. Its purpose is to understand the role of a gene that has been sequenced but whose function is unknown or incompletely known. By inactivating the gene and studying the differences in the affected mouse, researchers can infer the likely function of that gene.

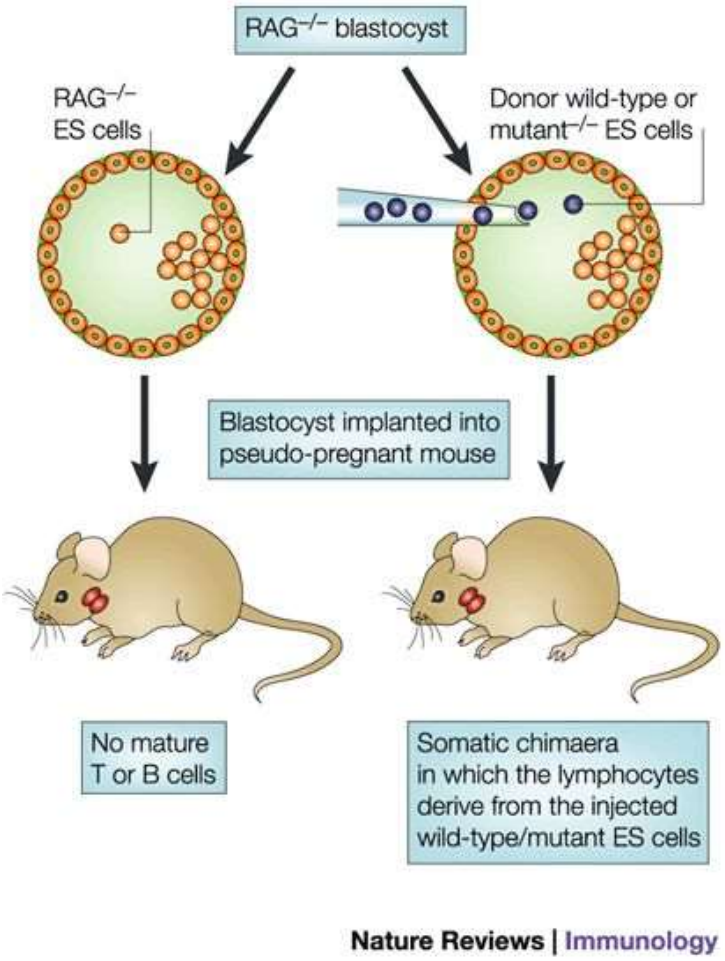


Fig 4 Production of Knockout mice

Knock in: introduction of a cDNA at a particular locus on the body's chromosome. In this way, a knock-in mouse is a mouse that has been replaced by a different or modified gene sequence.) It is a technique in which scientists can study the functioning of the regulatory machinery that governs the expression of the natural gene being replaced. This is achieved by observing the new phenotype of the organism in question.

Production of knockin and knock out mice (gene targeting)

These models differ from the previous ones in that the integration of the DNA that is introduced into the genome is directed to a specific position of the genome, with the aim of generating an intentional mutation and previously designed in that position. To date, this process is only available for the mouse. This limitation is due to the fact that the mouse is the only species in which pluripotent embryonic cells (ES cells) can be derived and inheritable mutations generated.

In general, there are two types of models generated by gene targeting.

It is a two-phase process. In the first, we start from a DNA sequence artificially constructed and homologous to the sequence containing the gene of interest. This artificial sequence is introduced into embryonic stem cells, where the researchers will try to create an environment (usually thanks to drugs) where the lines of cell clones of interest are selected. This homologous recombination, which happens in the nucleus, has a very low success rate, due to two things: the first is that these recombination events have a frequency of less than 0.1%, and the other is that the success rate when recombined is 1-5%, which gives a total possibility of 10-5.

For the selection there are also two ¨phases so to speak. One of positive selection. Normally the gene of neo-bacterial origin is used, which offers resistance against the antibiotic Neomycin (G418r) to the cells that express it. In addition, we use a second selection marker, in this case negative, flanking the gene sequence in order to enrich the reaction with targeted mutagenesis events. One of the most widely used negative selection markers is the herpes virus thymidine kinase gene (HSV-tk).

This whole process of positive/negative selection can increase up to 20 times the efficiency of the system and is in fact routinely employed by laboratories.

The second phase consists of selecting those cell clones that eliminated or promoted the function to be investigated, and transferring them to the blastocyte cavities of 3.5-day-old blastocyte embryos, and these embryos are transferred to other females in a pseudo-pregnancy state. The success rate for generating the desired mutation depends to a large extent on the care and good treatment with which embryonic stem cells were worked on.

A knockout mouse is the best model to study the function of a gene in the physiological context, analysing the phenotype resulting from the loss of its function.

Knockin models are mainly used to reproduce in the mouse genetic alterations such as point mutations or small deletions or insertions that are found in human pathologies or that are relevant to the study of gene function. For example, oncogenic mutations identified in human tumours. The possibility of generating conditional knockout or knockin mice allows the control of the cell type and the moment of development in which these mutations are expressed.

Thanks to new advances we can now create another type of mouse KO, the **conditioned ones**

The arrival of site-specific recombinase in the experimental manipulation of the mouse genome gives the possibility of creating KO mice conditioned to a tissue (conditional-tisular KO), or to a specific moment of development (conditional-temporal KO).

The enzymes Cre (from the bacteriophage virus P1) and Flp (obtained from a plasmid from the yeast Saccharomyces cerevisiae) are what allow us to do this. Both recognize a specific sequence of 34 bp,

known as loxP (in the case of Cre) and frt (in the case of the enzyme Flp). These recombinases perform "cut and paste" work whenever they encounter these specific sequences.

When both sequences are found in the same linear DNA molecule and with the same orientation, the DNA segment between the sites will be removed. If the specific cutting sites are on the same molecule but with inverted orientation, an inversion of the intermediate segment occurs. When sequences are found in separate DNA molecules the result is a translocation. All of these enzymatic reactions are reversible and recombinases work both in vitro and in vivo (and do not require any co-factor to activate). The strategy for creating conditional KO is based on spatial and/or temporal control of site-specific recombinases. This is possible thanks to the insertion of the specific sequence (generally loxP) in the flanking regions -or in the introns- of the gene we want to knock out. Thus, the function of the white gene is not altered, i.e. the gene is expressed normally despite the integration of the loxP sequence, as long as it does not interact with the recombinase Cre.

The axis of the technique lies in being able to manipulate the place (tissue) and time (time of development) for the expression of the Cre enzyme. In the first case, transgenic mice are produced (by the classical pronuclear microinjection technique) that express the Cre enzyme only in a predetermined tissue (through the use of specific tissue promoters). The final step is to cross these Cre transgenic mice (referred to as "deleters" mice) with the lines of mice carrying the loxP sites in the white gene. After this crossing, we will obtain a certain proportion of bigenic or double mutant mice (Cre plus floxeado gene), depending on the genotype of the parental lines (if they are both hemicigotes, Cre/+ and loxP/+, we will obtain 25% of conditional KO mice). The expression of the Cre enzyme in the specific tissue (determined by the chosen promoter) will make the loxP sites recombine leaving behind a deletion, and therefore a null allele (KO) of the gene in question.

In the case of conditional-temporal KO, the use of "inducible" promoters is used. Among the most prominent are promoters induced by antibiotics (e.g. tetracycline or its derivative doxycycline) and those induced by hormones.

In the second case, we work with fusion proteins between the Cre enzyme and an incomplete estrogen receptor (only the EBD portion, estrogen binding domain). This fusion protein is inactive, but administration of the drug tamoxifen (intraperitoneal) releases it and activates the Cre system. Therefore, it will result in recombinase action on specific sites and consequent recombination of DNA with target gene alteration in positive Cre/loxP mice.

The possibilities of creating models with the Cre/loxP system are enormous: modified sequences can be introduced and the expression of mutated alleles (knock-in) can originate. It is also possible to make "classical", i.e. unconditional KO mice with "clean" mutations, from which the selection markers have been removed. Another virtue of the system is to be able to produce deletions, inversions and translocations of large chromosomal segments.

BIOETHICS

Scientists who want to experiment with rodents should fill out a document explaining why the experiment requires animals. This can be summarized in the 3 R's: Reduce the number of animals used, replace the use of animals where possible, and improve experiments in order to improve animal welfare.

Royal Decree 53/2013, laying down the basic rules applicable to the protection of animals used in experiments and other scientific purposes says:

(a) the number of animals used in the procedures is reduced to a minimum, applying as far as possible alternative methods

(b) they are not unnecessarily caused pain, suffering, distress or lasting harm;

(c) any unnecessary duplication of procedures is avoided; and

(d) animals used, bred or supplied are given appropriate care.

Articles of interest:

Article 23. Wildlife and stray animals of domestic species.

Wild animals and stray animals of domestic species shall not be used in procedures. The competent organ may exceptionally authorize its use, provided that the following conditions are met:

a) There is an essential need to carry out studies related to the health and welfare of these animals or with serious threats to the environment or to human or animal health, and

(b) It has been scientifically justified that the purpose of the procedure can only be achieved by using stray or feral animals.

Article 22. Animals caught in the wild.

1. Animals caught in the wild shall not be used in procedures, unless expressly authorised by the competent body, which may grant such authorisation after scientific justification that the purpose of the procedure cannot be achieved by using animals bred for use in procedures.

2. The capture of animals in the wild shall only be carried out by a competent person using methods which do not cause avoidable pain, suffering, distress or lasting harm. Any animal which is injured or in poor health at the time of capture or after capture shall be examined by a veterinarian and measures shall be taken to minimise suffering. Only exceptionally and after scientific justification may the competent body authorise that such measures not be taken.

Which centres can investigate?

Generally, universities, pharmaceutical companies and public and private research centres.

Breeders, suppliers and users and their establishments must be authorised by the competent body before commencing their activities.

Breeders, suppliers and users must be registered in the General Register of Livestock Holdings.

Can anyone investigate with animals?

No, article 15 of Royal Decree 53/2013 says:

Persons performing the following functions must have adequate prior training:

a) Care of animals.

b) Euthanasia of animals.

c) Carrying out procedures.

d) Design of projects and procedures.

(e) Assume responsibility for on-site supervision of animal welfare and care.

(f) Assume the functions of designated veterinarian.

The competent body may authorise persons who have not yet demonstrated their full competence to perform these functions on a temporary basis and under responsible supervision.

What controls are in place?

RD 53/2013 describes several processes related to the evaluation and authorization of projects, as well as inspections or controls to the centres:

For the communication and request for authorization of a project, the user or person responsible for the project must submit to the competent body the project proposal, accompanied by the report of the ethical committee, a copy of the request for evaluation of the project. The competent body may suspend the authorisation of a project if it is not carried out in accordance with the authorisation, and withdraw it after a file has been processed and the interested party has been heard.

Project evaluation.

1. The evaluation of each project shall be carried out by the competent or empowered body at a level of detail appropriate to the type of project and shall consist in verifying that the project meets the following requirements:

(a) It is scientifically or educationally justified, or must be imposed by law or regulation;

(b) Its purpose justifies the use of animals; and

(c) It is designed in such a way that the procedures are carried out in the most humane and environmentally friendly way possible.

Article 40. Inspections or controls.

The competent or authorized bodies shall carry out regular controls or inspections of breeders, suppliers and users, including their establishments, to verify compliance with this royal decree. An adequate proportion of inspections shall be carried out without prior notice.

Article 44. Spanish Committee for the protection of animals used for scientific purposes.

It shall be the body in charge of advising the General State Administration, the autonomous communities and the cities of Ceuta and Melilla and the bodies in charge of animal welfare in matters related to the acquisition, breeding, accommodation, care and use of animals in procedures, as well as to guarantee that the best practices are shared, and for the due coordination.

The Committee will exchange information with the National Committees of the other Member States on the functioning of the bodies responsible for animal welfare and project evaluation, and will share best practices in the European Union.

Is there any ethical control over the use of animals in experiments?

If, in addition to the evaluation of the projects described above, there is a body in charge of animal welfare, RD 53/2013 establishes:

Creation of bodies in charge of animal welfare.

1. Each breeder, supplier and user shall establish a body in charge of animal welfare, hereinafter "ABO".

2. The ABO shall have an internal regulation that defines and develops, its composition and its basic functioning that guarantees impartiality in the decisions taken by it. Unipersonal ABOs shall be exempted from this requirement.

Article 38. Functions of the ABO. (body in charge of animal welfare)

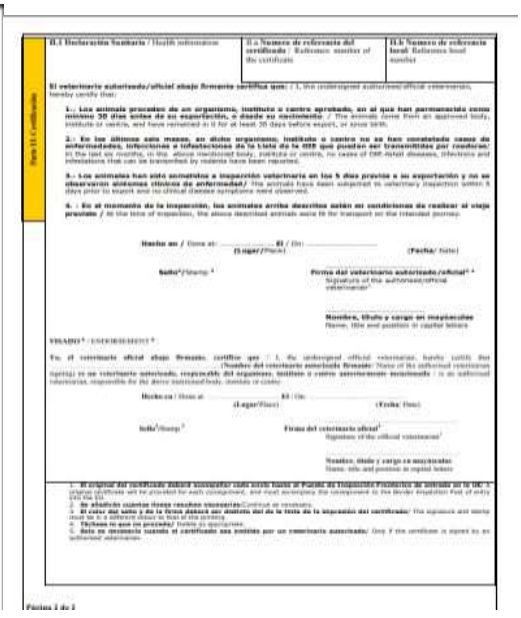
The ABLE shall perform at least the following functions:

a. To advise animal welfare personnel on animal welfare issues related to the acquisition, housing, care and use of animals.

b. To advise staff on the application of the replacement, reduction and refinement requirement, and to keep staff informed of technical and scientific developments in the application of that requirement.

c. Establish and review internal operational processes with respect to the control, communication and monitoring of animal welfare information.

d. Advise on rehousing or adoption regimes, including appropriate socialisation of animals to be rehoused or adopted.



Evaluation of medicinal products in animals before placing on the market

Law 29/2006, of 26 July, requires the authorization of medicines to prove that they are safe and effective.

To guarantee their safety, article 12 establishes: toxicological studies shall include acute and chronic toxicity tests, teratogenic tests, embryotoxicity, fertility, mutagenesis tests and, where appropriate, carcinogenesis tests and, in general, those others that are considered necessary for a correct evaluation of the safety and tolerance of a medicine under normal conditions of use and depending on the duration of the treatment. In any case, the regulations on the protection of animals used for scientific purposes shall be complied with. These studies shall be carried out in accordance with established good laboratory practice.

Are many animals slaughtered to test cosmetics?

The Regulation of the European Parliament and of the Council of 30 November 2009 on cosmetic products and Royal Decree 209/2005: Regulation of cosmetic products say:

Animal experiments must be replaced by alternative methods. The Regulation prohibits the performance of animal experiments in the European Union for:

Finished products;
Ingredients or combinations of ingredients.

The Regulation also prohibits the placing on the European Union market of:

Products the final formulation of which has been the subject of animal testing;
Products containing ingredients or combinations of ingredients which have been tested on animals.

From 11 March 2013, the use of experimental animals in all cosmetic safety tests is prohibited.

CRISPR/Cas9 TECHNOLOGY

It is a molecular tool for editing the genome, either by adding or removing DNA, or simply modifying the existing one. So, we are no longer limited to the mouse when it comes to using gene targeting techniques.

CRISPR/Cas9 stands for Clustered Regularly Interspaced Short Palindromic Repeats. The second is the name of a series of proteins, mainly nucleases, which were named after CRISPR associated system (i.e. "CRISPR associated system").

In 1987, an article was published which talked about how bacteria were able to differentiate their genetic material from that of the virus, destroying it; and in this way they defended themselves against their infection. And what is the use or interest of this?

There is a certain area of the genome of many microorganisms, especially archaeological ones, which is full of palindromic repetitions with no apparent function. These repetitions are separated from each other by sequences called "spacers" that resemble others of viruses and plasmids. Just in front of these repetitions and "spacers" there is a sequence called "leader". These sequences are what were called CRISPR. Very close to this grouping could be found some genes that coded for a type of nucleases: the cas.

The foundation of the technology is based on the fact that the virus normally enters the cell or bacteria and puts all the cellular machinery at its disposal. But there are bacteria that have Cas proteins attached to the RNA produced from the CRISPR sequences. If the genetic material of the virus were to interact with that complex, the virus would be inactivated by the degradation of its genetic material, and the bacteria would be safe. Although the system is even more efficient, since it takes this viral genetic material, and modifies it in order to integrate it into its genome, and that in subsequent contacts, the defence mechanism is much more effective.

In 2012, Dr. Emmanuelle Charpentier at the University of Umeå and Dr. Jennifer Doudna, at the University of California at Berkeley, published an article in the journal Science that demonstrated how to turn this natural machinery into a "programmable" editing tool that could cut any chain of DNA in vitro.

The process has two parts: the first, in which an artificial sequence of RNA is used, which is going to hybridize with the genetic material to be studied and arrive at Cas9, which acts by cutting that sequence.

The second phase consists of activating two DNA repair mechanisms present in the cell.

The first is called in-del makes a hole appear after the cutting site or inserts a piece of DNA, thus eliminating the original function of the cut DNA. The other mechanism allows us that incorporation of a specific sequence at the cutting site.

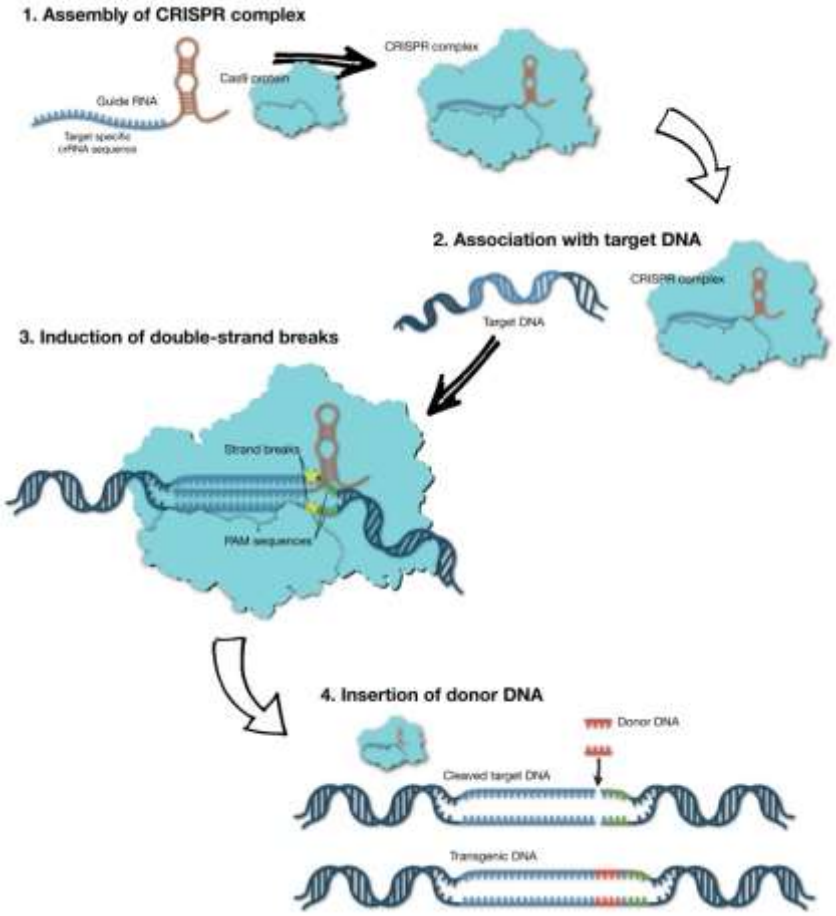


Fig 6 Process of CRISPR/Cas9 technology

This system has two disadvantages that are trying to be corrected: The first is derived from the fact that the specificity of the RNA guide is not total. In other words, this RNA can hybridize, join with more than one site in the genome, which would lead to the Cas9 enzyme cutting in a place that does not interest us. The second weak point of the technique is that Cas9 can cut without the RNA guide being present. This is solved with more precise enzymes.

Molecularly we can say that this tool can be used to regulate gene expression, label specific sites of the genome in living cells, identify and modify gene functions and correct defective genes. It is also already being used to create animal models to study complex diseases such as schizophrenia, for which previously there were no animal models.

And there is another very controversial point apart from its working mechanics:

Patent war

Shortly after the famous article by Doudna and Charpentier, in January 2013 the laboratories of George Church at Harvard and Feng Zhang at MIT's Broad Institute were the first to publish articles demonstrating that CRISPR/Cas9 served human cells. Doudna published his own independently just a few weeks later.

In April 2014 Zhang and the Broad Institute obtained the first of several general patents covering the use of CRISPR in eukaryotes. That gave them the rights to use CRISPR on mice, pigs, humans... on virtually any creature that was not a bacterium.

The speed at which the patent was obtained surprised some. And it was because the Broad Institute had paid discreetly to have it reviewed very quickly, in less than six months. In addition, the process was carried out in an almost "secret" manner. Along with the patent came more than a thousand pages of documents. Doudna had filed a patent application before Zhang. But, according to Zhang, Doudna's prediction in his application that his discovery would work on humans was a "mere conjecture" and that instead he was the first to prove it in a different and "surprising" act of invention. To prove that he was "the first to invent" the use of CRISPR-Cas on human cells, Zhang submitted photos of laboratory notebooks that he claimed showed the system was up and running in early 2012, even before Doudna and Charpentier published their results or applied for their own patent. That chronology would mean that he discovered the CRISPR-Cas system independently. In an interview, Zhang claimed that he had made the discoveries on his own. Asked what he had learned from Doudna and Charpentier's article, he said "not much.

Doudna and Charpentier's lawyers, on the other hand, did not stand idly by and are expected to mount an "interference procedure" in the United States, which is a legal process in which the winner takes everything and one inventor can take another's patent.

Three start-up companies are involved in the patent game, for which the control of these patents is key. These companies include Editas Medicine, Intellia Therapeutics, both from Cambridge, and CRISPR Therapeutics, a start-up from Basel (Switzerland) co-founded by Charpentier. Zhang co-founded Editas Medicine, which in December 2014 announced that it had purchased the license to use its patent from the Broad Institute. But Editas does not have a monopoly on CRISPR because Doudna was also a co-founder of the company. And since Zhang's patent came out, Doudna has broken with the company, and its intellectual property in the form of its own patent pending approval has been sold under license to Intellia. To further complicate matters, Charpentier sold its rights to the same patent application to CRISPR Therapeutics.

On the other hand, more and more voices are asking that, due to CRISPR-Cas9's great ability to cure diseases, the technology should not be protected by patent and should be left as public access. Charpentier herself assures that the technology has been made freely available to the research community, so she does not believe that the patent will pose any obstacle to scientific progress.

SPLIT HAND FOOT MALFORMATION

The SHFM is a highly variable malformation of the hand and/or foot characterized by the loss or deformity of the central rays that leads to a central cleft and the subsequent split appearance. SHFM is also termed ectrodactyly, cleft hand, and lobster claw hand. In humans, the non-syndromic SHFM is a genetically heterogeneous and rare congenital malformation with an incidence of 1 per 8,500-25,000 live born infants representing 8-17 % of all limb malformations.

It can range from a hypoplasia of a single phalanx, to the most severe form, which is an aplasia of the central and pre axial tracts of the limb. Because of this, it has great interindividual variety, and even in the same individual, there can be different malformations in each extremity.

The disease occurs sporadically, there are very few cases where family aggregation is seen, and its mode of transmission is autosomal dominant.

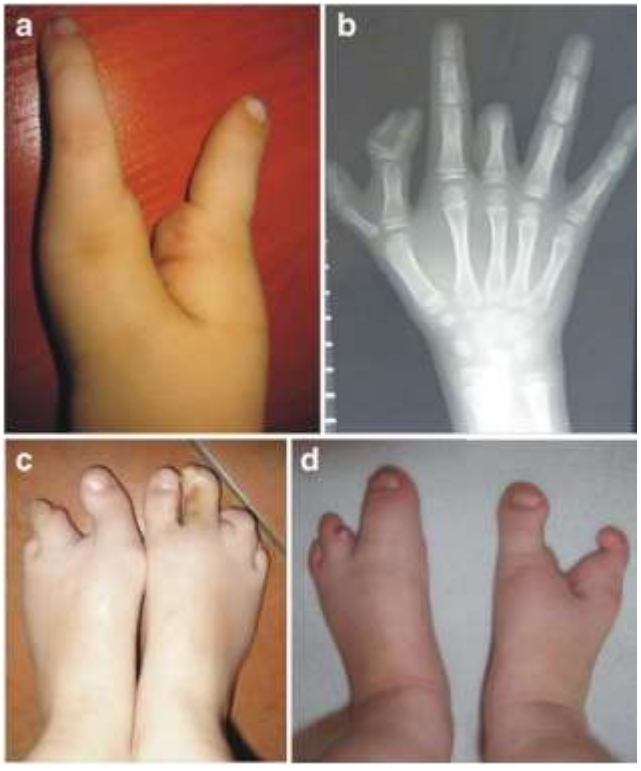


Fig 7. Limbs of the SHFM patients with different molecular origin of the defect (a) male proband with a classical cleft of the hands carrying a balanced chromosomal translocation t(7;12)(q21.2;q21.3) involving SHFM1 locus; (b, c) female proband carrying a typical 10q24 duplication of the SHFM3 locus showing ectrodactyly and preaxial polydactyly of the hands as well as ectrodactyly of the feet; (d) female proband with a classical cleft of the feet carrying a nonsense heterozygous point mutation c.G1974A(p.W658X) in the TP63 gene.

LIMB DEVELOPMENT

The developing limb bud consists of two cell layers: highly proliferating mesenchymal cells covered

by the ectodermal cells. The formation of limb bud is mediated by signalling molecules produced by three cell groups - the apical ectodermal ridge (AER), the progress zone (PZ) and the zone of polarizing activity (ZPA). The interaction between these regions determine the patterning of the limb in three spatial dimensions: proximo-distal, antero-posterior, and dorso-ventral. A number of signaling molecules and transcription factors such as fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs) or WNT and MSX proteins produced by AER keep the neighbouring mesenchymal cells in constant proliferation and undifferentiated state, giving rise to PZ,
Failure in maintaining the AER affects the formation of the autopod and leads to the development of SHFM phenotype.

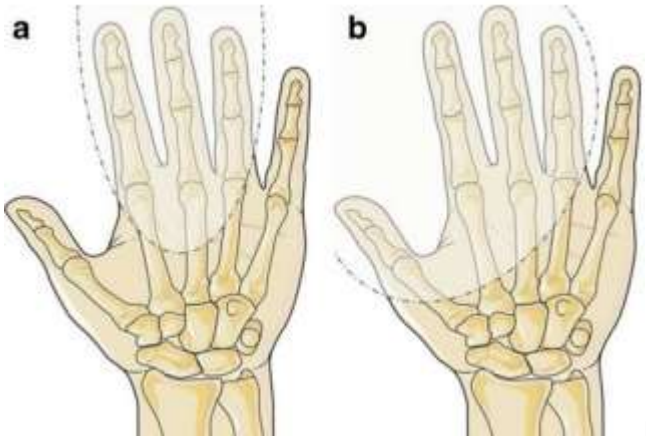


Fig 8. Schematic presentation of the different anatomical types of SHFM (a) classical cleft characterized by the aplasia of the central rays of the autopod; (b) monodactyly characterized by the aplasia of both central and preaxial rays of the autopod

Seven chromosomal loci involved in the syndrome have been identified: SHFM1 to 6 and SHFM/SHFLD, and 3 genes that can carry mutations related to this have been identified: TP63, WNT10B and DLX.

1. **SHFM type 1** is due to a mutation on chromosome 7q21, in a region that contains the two homeobox genes DLX5 and DLX6 are expressed in the AER. The involvement of DLX5 has been recently confirmed.
2. **SHFM type II** is very rare and maps to the Xq26 chromosome a region that includes FGF13 and TONDU.
3. **SHFM type III** maps to chromosome 10q24 a region that includes the FBXW4/Dactylin gene. The malformation is caused by duplications in this genomic region close to FGF8 and that includes many regulatory modules of FGF8, several of them embedded in the FBXW4 gene. Therefore, type III is probably the result of poor regulation of FGF8 resulting from the disruption of the normal architecture of the multiple enhancers present in this region.
4. **SHFM type IV** maps to chromosome 3q27 and TP63 is the gene involved.
5. **Type V SHFM** is located on chromosome 2q31 with DLX1 and DLX2, members of the DLX family also expressed in the AER, as candidate genes.
6. **SHFM type VI** is mapped to chromosome 12q13.12 and is caused by mutations in WNT10b although there is some doubt about whether these mutations are sufficient for the phenotype.

To date, we've only identified two genes that cause SHFM. No relationship has yet been found between SP6 and SP8 with SHFM in humans. There are corresponding mouse models for most types of SHFM, but phenotypes and inheritance patterns are not always identical because some of the mutations in humans appear to function in a dominant negative way.

Despite the genetic heterogeneity in SHFM, a convergence towards the Tp63 network can be clearly seen. TP63, responsible for type IV, is an upstream regulator of the Dlx genes, responsible for types I (DLX5/6) and probably type V (DLX1/2). Type III is due to the duplication of the normative landscape of Fgf8, a region where TP63 and Dlx junction sites abound.

Therefore, it seems likely that types I, III, IV and V of SHFM are due to defects in the TP63 network that direct the expression Fgf8. TP63 is a member of the p53 family of TFs that performs multiple functions including control of skin stratification and regulation of adult stem/progenitor cells. Tp63 also participates in the development of limbs that are necessary for the morphology and maintenance of ARE.

SHFM1 (7q21)

Hand/foot split type 1 malformation (SHFM1) is caused by chromosomal rearrangements of the region 7q21.3-q22.1. They occur mainly as de novo mutational events, but can also be inherited in an autosomal dominant manner, with reduced penetration and variable expression. Genomic alteration of the SHFM1 locus may be associated with isolated or syndromic malformations of the limb. Aberrations within this region involve translocations, inversions, duplications and, more frequently, eliminations. The minimum overlap region for SHFM1 encompasses several genes involved in limb development, such as DSS1, DLX5 and DLX6. Crackower found that the murine orthologist of DSS1 is expressed predominantly in the mesenchyme of the developing limb bud, as well as in the gill arches, genital tubercle and dermis. However, the other two genes located within the critical region of 7q21-22, DLX5 and DLX6, were found to be the most attractive candidate genes for SHFM. Both belong to the Wnt signalling pathway members, which is important in limb skeletal development and morphogenesis.

The expression of murine orthologists -Dlx5 and Dlx6- has been detected in the REA of the buds of the embryonic extremities, in the pharyngeal arches, in the osteoblasts of the developing bones and in the interneurons of the basal brain. Both gene products are critical for maintaining the proliferation of the medial REA cell population at the outbreak of developing limbs. The role of DLX5/DLX6 in limb morphogenesis has been demonstrated in mouse models, where the double knock-out of both genes (Dlx5/Dlx6-/-) resulted in a typical ectrodactyly, as well as craniofacial and inner ear anomalies. The suppression of either gene alone has resulted in ectrodactyly or another limb phenotype. It should be noted that SHFM in Dlx5/ Dlx6-/- null mice could only be rescued by overexpressing Dlx5 in the AER, suggesting redundancy of Dlx5 and Dlx6 in limb development. On the other hand, overexpression of Dlx5 in wild mice has no negative effect on limb formation. The hypothetical biological activity of DLX5 and/or DLX6 is that they function as repressors of the

downstream target genes, which in the absence of DLX5 and DLX6 are overexpressed, which results in the reduction of cell proliferation within the REA.

The mouse model for SHFM1 varies significantly from the clinical environment observed in human individuals. While the double mouse Dlx5/Dlx6-/- knock-out is recessive, human patients develop the condition if the aberration affects only a single copy of the SHFM1 locus. Consequently, human deletions of SHFM1 are inherited in an autosomal dominant manner. Therefore, based on the differences between species, the hypothesis can be made that the development of human limbs is more sensitive to reduced levels of DLX5/6 proteins. To make things more complex, the haploinsufficiency of the SHFM1 locus is not the only pathological mechanism leading to a limb defect in human individuals. Alternative mechanisms exist in the case of translocations or chromosomal inversions that affect the SHFM1 locus and include the "position effect" on the DLX5/DLX6 genes as a result of the interruption of the regulatory elements or the modification of the regulatory landscape in the 7q21. The transcriptional regulation of the DLX5 and DLX6 genes has been investigated by several authors. Iacono has shown that the DLX proteins and the transcription factor p63, encoded by the TP63 gene, are placed in the AER and that p63 acts as a regulator upstream of the transcription of Dlx5 and Dlx6 through binding to their cis action regulating elements at the promoter level. Heterozygous mutations in TP63 give rise to malformations of extremities in multiple congenital syndromes, as well as isolated SHFM (SHFM4). Genomic profiles of cis regulating elements and TP63 target genes, reported by the p63 binding site identified by Kouwenhoven - SHFM1-BS1, which can function as a potentiating element controlling the expression of DLX6, and possibly DLX5, by physical interaction with the Dlx6 promoter. The expression of Dlx5/Dlx6 is reduced by the inactivation of p63 (Lo Iacono) or the interruption of the regulatory elements controlled by p63 (Kouwenhoven). Recent studies on the transcriptional regulation of DLX5/6 resulted in the identification of additional enhancers located in protein coding sequences of the neighbouring genes. Birnbaum has described two new exonic limb enhancers, (eExons) DYNC1I1 eExon 15 and 17, which reside proximal to DLX5/6. With the use of transgenic enhancers in mice and zebrafish, the authors characterized the enhancer activity of DYNC1I1 eExon 15 in the REA and DYNC1I1 eExon 17 in the previous mesenchyme, thus demonstrating its regulatory influence on the expression of the extremities of Dlx5/6 (Birnbaum....). The previous findings gave a new vision of putative genes, regulatory elements and new mechanisms of disease in the etiopathogenesis of SHFM1. Recently, Shamseldi studied an autosomal recessive putative inbred family of autosomal SHFM and found that the homogeneous missense DLX5 mutation located in the homeostasis domain was causal to the limb phenotype. Therefore, the authors provided the first solid evidence on the direct involvement of DLX5 in the pathogenesis of human SHFM1 (Shamseldin).

DIAGNOSIS AND GENETIC COUNSELLING

Considering the reduction of penetration, variable expression or non-mendelian inheritance, as well as the distortion of segregation and sexual bias with over transmission of the genetic alteration from affected parents to children, it becomes evident that genetic counseling in cases of SHFM is quite difficult and challenging, not only in sporadic cases but also in relatives. In addition, many of the SHFM cases appear to come from a complex set of chromosomal mutations/aberrations and should be considered as disorders of two or more genes.

The largest cohort of MFHS patients described to date comprises 56 cases. The most frequent genetic abnormalities in this group were submicroscopic duplications of 10q24.3 (SHFM3) and 17p13.3 (SHFM/SHFLD) responsible for 20% and 16% of cases, respectively. Based on this finding and our own diagnostic experience, we propose that the SHFM tests begin with the selection of the number of copies for 10q24 followed by loci 17p13.3. The method of choice would be the Array CGH (aCGH), since it allows not only the detection of these changes, but also the identification of other unbalanced chromosomal aberrations, such as submicroscopic rearrangements affecting different sites.

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