Next Generation Sequencing Based Comprehensive Molecular Analysis in Iranian Patients with Restless Legs Syndrome (RLS)

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Research

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Abstract

Restless legs syndrome (RLS) is a common sensory-motor neurodegenerative disorder. It usually occurs at night and every 10 to 60 seconds with unpleasant sensations in the legs, burning and itching. Need to move and feel Uncomfortable legs improve with movement.In 20% of patients, symptoms can also include the arm and other parts of the body, which often occur in a more severe form of the disease. But generally, according to research, this syndrome is a multifactorial syndrome. These factors are divided into genetic and environmental or medical factors.

In this study, this disorder was investigated genetically and molecularly and the genes affecting the disease in Iranian population were identified by NGS method.

Materials and Methods: In this study, NGS and SEQUENCING methods were used to investigate the relationship between genes that influence disease and patients.

Conclusion: In this study, we found that ATXN7, MEIS1 and TANC1 genes are effective in restless legs syndrome. In this study, In this study, the effect of TANC gene on the development of restless legs syndrome in Iranian families was confirmed.

Discussion: The results of molecular analysis of restless legs syndrome using NGS method led to the identification of new gene loci on ATXN7 and TANC1 genes in Iranian population. Also, gene loci on MEIS1 gene, previous studies on its involvement in restless legs syndrome Confirmed.

Introduction

Restless Legs Syndrome (RLS), is a common sensorimotor of sleep disorders. Restless Legs Syndrome, or RLS, is often referred to as periodic leg movements (PLM) with involuntary movements and muscle spasms in the lower limbs. PLM may occur in either waking state (PLMW) or during sleep (PLMS). Pathological incidence of PLMS during sleep is found in about 80% of patients with RLS. The prevalence of RLS in the general population is about 3-5% and about 2-3% of the general population suffers from observable clinical symptoms (which occur twice a week with moderate to severe severity). It has been observed that with increasing age, the prevalence of the disease has increased and is higher in women than men. Studies strongly support the role of genetics in the development of this disease. Pharmacology and brain imaging also confirm the involvement of iron and opioid dopaminergic pathways in the brain and spinal cord in the incidence and exacerbation of the disease. Dopamine is required for smooth and purposeful muscle activity and movements, and as a result, disruption of these pathways usually leads to unwanted movements. Because of this, people with Parkinson's syndrome, which disrupts the dopamine pathways, are more likely to develop Restless Legs Syndrome. The RLS phenotype depends on the age of onset of the disorder. RLS or Restless Legs Syndrome is a multifactorial disease or polymorphism in which, in addition to genetics, the influence of environmental factors such as iron deficiency or other diseases such as diabetes and kidney disease has been proven. This disorder causes restlessness and severe concussions, mainly in the legs, due to decreased dopamine secretion in the brain neurons, which

is why it is known as Restless Legs Syndrome. However, the symptoms of this disease also appear in different parts of the body, including the arms. This disease disrupts the sleep of the affected people at night (figure 1) .

The development of molecular diagnosis in the genetics of hereditary diseases is done to determine genetic defects along with the development of new technologies in this field. New technologies often lead to the rapid discovery of new genes and diseases that lead to molecular diagnosis, management, and disease care. Numerous studies have shown that 60% of patients have a positive family history. Phenocypes and possibly non-pantrans (non-pantrans) are a distinct genomic feature that does not occur in the individual's own phenotype and in contrast to pantrats. It sometimes happens that people carrying the same genes show different phenotypes. This problem, which depends on how the environment relates to and affects genes, is called "gene penetration" (PTR), making it difficult to identify a common separating haplotype in families.

Accurate identification of the candidate region is difficult due to non-familial, allelic and inorganic heterogeneous reasons. An important prerequisite for future successful genetic studies on Restless Legs Syndrome is the full availability of the phenotype of a large community of patients and their families. Studies also show that restless legs syndrome (RLS), autosomal dominant, is highly pervasive and is passed from parent to child. Numerous studies have been performed on the human genome to determine the genetic status of this disease and important results have been obtained. Despite numerous reports suggesting a genetic contribution to idiopathic RLS Ethiopia, numerous molecular genetic studies have attempted to identify genes that may be susceptible to the disorder. In particular, the encoded genes for GABA A receptor subunits, the glycine receptor alpha1 subunit gene, and the genes involved in dopaminergic transmission and metabolism have been analyzed and significant findings reported.

Types of diseases

Restless legs syndrome comes in two forms:

1. Primary type: Starting at an early age (less than 15 years) and with a set of common symptoms

Genetics are associated in a family. Primary RLS (which accounts for 20% of all RLS cases) has an earlier onset, slower development, and a stronger family link, and is associated with dopaminergic (associated with dopamine transporters in the brain), iron deficiency, and sleep-wake cycles. Is.

2. Secondary type: usually starts at older ages and is related to environmental factors. Secondary causes of RLS include iron deficiency, kidney failure, peripheral neuropathy, diabetes, MS, Parkinson's disease, pregnancy, and drugs that stimulate RLS, such as neuroleptics, selective serotonin reuptake inhibitors, lithium (and neuropathy), neuropathy, radiculopathy Metabolic disorders such as iron deficiency, diabetes, B vitamin deficiency, thyroid dysfunction (and rheumatic diseases) are rheumatoid arthritis.(Alibabaei, 2018)

Evaluation of people with genetically restless legs syndrome using NGS method to determine the genes involved in the development of the disease:

Using the NGS method, or the study of exons, can help in the path of molecular diagnosis based on the disease panel. By examining the sequence of the exome or genome, a set of disease-causing genes can be reached. Exon examination, in addition to determining disease-causing mutations in index genes, can also be a good way to diagnose diseases and related new genes.

In this study, we examined the exon profile of infected individuals and examined the possibility of undetermined genetic mutations and new disease-related determinants in the Iranian population.

The main hypotheses of the research

Family studies and possible genetic predictions

According to genetic studies, restless legs syndrome is a disease of genetic origin and the role of genetics in the occurrence of this disease has been proven. In this study, blood samples were taken from 30 members of three Iranian families who were involved in severe to mild degrees of the disease, and by extracting DNA from the blood samples of the subjects, the possible genes involved in the development of the disease were examined.

Inheritance pattern studies

Although previous studies have shown that the disease follows an autosomal dominant pattern, but considering that this study was conducted for the first time in Iran, the study of hereditary patterns and how the disease is transmitted from parents to children was examined.

Materials And Methods

In order to conduct this study, 30 blood samples were collected at the rate of 5 cc from three different genealogies from Fars, Tehran and Mazandaran provinces. A sample from each family was selected as a control and examined in the study. Patients with Restless Legs Syndrome were identified based on their symptoms and family history with the approval of a neurologist. Before sampling, patients were examined for non-involvement of non-hereditary factors such as iron deficiency, which is the most important non-genetic cause of the disease. Patients were justified in terms of the type of research and its stages, and special research questionnaires were completed with the help of the research community. Questionnaire questions were selected based on the research design. The questionnaire included questions from patients about symptoms, time and severity of symptoms, family history, medications, history of certain diseases such as diabetes, hemodialysis, Parkinson's and mental disorders. Also, patients with restless legs syndrome who were receiving drug treatment were identified and the type of drug used was determined. Age, sex, geographical location and family relationship of patients with each other were determined and genealogy was drawn. Based on the interpretation of the questionnaire and the study of previous research in other countries, the role of heredity in the disease was confirmed and

blood samples were collected from patients and transferred to Tehran for further treatment. Prior to blood sampling, patients with restless legs syndrome in these families were identified through a questionnaire. Patients were evaluated for other environmental factors affecting the disease such as iron deficiency and diabetes and other environmental factors and according to the genealogy and ensuring the role of heredity in these patients, sampling was performed. Samples were placed in tubes for collecting human k3 blood samples containing EDETEA and marked for ease of use of samples 1 to 30.

Preparation of samples

At this stage, the blood samples were taken out of the freezer at room temperature to return from freezing and return to normal. The specimens were placed vertically under the hood. In the laboratory, the study site was a laminar hood.

Extraction of genomic DNA from blood

After taking blood samples from individuals, DNA extraction from the samples began. At this stage, according to the protocol in the kit, first RBC was lysed in several stages and the samples were centrifuged and after complete lysis, the obtained sample was used for extraction.

Mapping the genome and conducting studies on it to obtain a specific genetic link

In this study, we have investigated the molecular causes of Restless Legs Syndrome using the NGS method using the experimental method. For this purpose, the DNA sample of a person with severe restless legs syndrome was selected and NGS was performed. Then, by analyzing the obtained data, 3 genes:TANC1,ATXN1 and MEIS1 were selected as the final genes and a primer was designed for these genes. Of these three genes, only the MEIS1 gene is a common gene known in Restless Legs Syndrome, and the other two genes were tested for the first time and new variants were identified.

The final product of PCR, after electrophoresis, samples with suitable bands were selected for sequencing or sequencing and sent for sequencing. Finally, sequence analysis was performed and new variants effective in the incidence of disease were identified.

Study on candidate genes

After selecting the final genes, primers were designed and PCR was applied to all samples and the PCR product was sent for sequencing. Then, by analyzing the obtained sequences, a study was performed on the candidate genes and the effect of these genes on the incidence of the disease.

NGS

Exom sequencing data, due to the high volume of information they need, requires special software to study and study. The primary data obtained from sequencing devices is often in FASTQ format, in which there are components of each sequence that is a hack of four types of openings, along with other information that indicates the reading quality of each base. FASTQ data are readings of a certain length,

the number of which varies depending on the size of the cover (for example, with a coverage of 150, about 100 million readings are obtained). The stages of analyzing exome data are generally divided into 5 stages: quality control and correction of readings, alignment, calling variants, screening (filtering), writing reports of each variant.

Exom data were analyzed using CLC genomics workbench and Biomedical genomic workbench software, which generally includes the following 6 steps:

A) Quality control and correction of readings

At this stage, the quality of each reading as well as each of the bases was evaluated. After determining the qualities, the correction was performed, so that if the game had a score of less than 20, it was removed. A person's score is calculated according to the following equation:

Q = 101og p / 1-p

At this stage, other information that showed the accuracy of the sequencer was checked, such as G-C content, reading length and number of repetitions. Adapters that were added to each of the readings in the sequencing step were also removed in this step.

B) Alignment

After the corrections made in the previous step, the readings that have passed that step are aligned with the reference human genome, which uses different algorithms.

Some examples of common algorithms for aligning exom data are:

1. Burroes-Wheeler Transfermation (BWT)
2. Smith-Waterman (SW)
3. Dynamic programming, Hash-based index
4. Seed-and-extend approach.

The human reference genome in this study was hg19 (Human reference genome).

C) Post-alignment processes

These processes included the following 2 steps:

1-Elimination of duplicates: When readings are attached to the reference genome to match it, there are too many connections in some parts of the genome, and this causes problems in later stages due to the high volume of these sequences, so with Given the quality and degree of similarity of duplicate sequences, some of them are omitted.

2- Re-alignment: In the alignment stage, some bases are placed awkwardly and others are placed correctly. The mismatch indicates that our sequence is similar to the reference genome sequence, and that mismatch may indicate a SNP. However, in some cases, this is due to a poorly aligned process. Rearranging is an attempt to eliminate these inconsistencies and empty spaces created during alignment by using algorithms. The alignment performed at this stage is called the local alignment.

D) Calling variants

At this point, all the bases that are awkwardly aligned with the reference genome sequence are identified.

They turn. These base changes can take the form of single nucleotide polymorphisms, addition, deletion, variation in the number of replications, and structural variation.

E) Screening

In humans, there can be variations of the game, and this variation does not cause disease. At this stage, non-pathogenic variants can be isolated using common variants obtained from the genomic projects of different individuals. These projects include the Thousand Genomes Project, the EXAC Project, the NHLBI Exome Project, the SNP Database, the DiscoverEHR Project, as well as local projects involving people in a specific region (such as Iranum). Another concept in the screening phase is the minimum allelic frequency. This means that the frequency of the desired alleles that are studied in different projects, if it is less than a minimum value, that allele is considered rare. Screening is also performed at this stage, based on the type of inheritance pattern, the elimination of large recurrences, and the isolation of areas that are likely to be unrelated to the disease.

C) Write reports of each variant

The last step in analyzing Exome data is to write reports for each variant. At this stage, the number of variants has been greatly reduced due to their screening compared to the first case. Using different databases with information on different genes and alleles, we begin to examine the variants. These databases contain information on biological, molecular and cellular pathways and through them the relationship between the desired variant and the disease can be investigated. These databases include the SNP RSID database,

The DDD (Deciphering Developmental) study cited articles in various databases such as pubMed, the OMIM database, and COSMIC cancer information.

In addition to examining the above databases, predicting the pathogenicity of a variant is done by different algorithms. The most famous of them are SIFT, GWAVA, POLYPHEN and CADD. Each of these patterns scores the variants, which indicates the pathogenicity of that variant.

The WES test was performed with a 150X cover with the Illumina Hiseq 4000 system platform with a read length of 30 million and 8.5 GB of data was generated.

data analysis:

In order to align the sequences, custom software such as NextGenMap was used to create SAM / BAM files.

Variant calling was performed using the GENOME ANALYSIS TOOLKIT (GATK) tool and databases including NCBI OMIM, HGMD and 1000 Genomes were used.

VCF files were annotated using ClinVar and EmVar.

PolyPhen and SIFT software were used to predict whether the displaced amino acid was likely to be detrimental to the function of the protein produced, based on the degree of retention of the displaced evolution during evolution.

The quality of the sequenced data was first checked by FastQC software version 0.11.3 (Andrews, 2010). Versatile software version 0.36 was used to filter the raw data credit and sort the low open quality data at both ends, and readings less than 36 were not considered.

Low-reading readings (Phred Score, Q≥30) were used for bioinformatics studies.

SANGER SEQUENCING

After analyzing the data and identifying the candidate genes, 30 samples were examined, sequenced and using websites ensembl,ncbi,mutation tester,varsome and finchtv,clc,dnstar and igv softwars,final results were extracted.

Results

This study was conducted on a community with Restless Legs Syndrome in Iran. According to previous research and proving the role of geographical environment in the occurrence of genetic mutations in infected people, samples were selected from three geographical areas of Fars, Tehran and Mazandaran provinces. All participants in the study completed the questionnaire according to the standards of the World Association of Restless Legs Syndrome. All patients had a strong family history or family history. No mutation or disease-related genetic disorder was observed in the control samples. The age of the patients was between 45-10 years and according to the research done from childhood, they had symptoms of the disease. Patients show varying degrees of disease from mild to severe. In the study population, in a 12-year-old girl, mild symptoms of the disease were observed, which in molecular studies also carries the identified variant. The results showed that 10% of patients were women and 6.6% were men. These data show that, as previously demonstrated, women are more likely to develop Restless Legs Syndrome than men. The role of heredity in the development of this syndrome was also proven in this study.

Based on the sequencing analysis of genes TANC1, the following results were obtained:

1-TANC1:

The TANC1 gene was identified as the main cause of the disease, because this mutation was observed in all three generations F1, F2, F3. This mutation was observed in infected women.

chr2:159954270G>A: The gene locus identified for this gene is located on the long arm of chromosome 2, region 2, band 4 and subband 2 and on exon 27.

The mutation is of the synonymous single nucleotide type. In this sequence, G is replaced by A. Heterozygous and variant was identified as a pathogen.

This variant was common in 5 patients from 2 pedigree. four patients were of the one pedigree and mutations were observed in three generations of F1, F2 and F3.

Pedigree of a family with Restless Legs Syndrome with mutations in the TANC1 gene

2-ATXN7

Based on the results of this study, 8 variants were observed in the study population. the following variant was identified as a pathogen in three patients. variant chr3:63898457:G>A was observed in three patients with the same pedigree. Although due to the abundance of this variant in a pedigree, it can be introduced as a pathogen, but more research is needed. All three carriers of this variant had mild to severe restless legs syndrome. One patient was male and the other two were female.

3-MEIS1

This gene is known to be the main cause of Restless Legs Syndrome. In our study population, this woman showed the highest rate of mutation in the Iranian family. This confirms previous studies on the pathogenicity of this gene. In this study, 12 variants were identified for the MEIS1 gene. This site is located in the protein encoding region.

chr2:66664990T>G :According to studies, this mutation is one of the very rare mutations in the copper gene that before this study, only one case was identified in North Asia, and this is the second case of this mutation in the Iranian population studied in two The genealogy was observed. This mutation is on the 2p14 locus of chromosome 3.The effectiveness of this variant in the development of restless legs syndrome requires further studies and research.

Discussion

Based on the results of this study, the effect of all three genes on the occurrence of restless legs syndrome in the studied families was confirmed. The TANC1 gene was identified as the main cause of the disease, because this mutation was observed in all three generations F1, F2, F3, and most importantly, this mutation is present in infected people in all three generations.

This study shows that mutations in the TANC1 gene are mainly seen in women with restless syndrome. While known mutations in the ATXN7 gene are more common in infected men. But in the case of the MEIS1 gene, no significant gender differences are observed, and most mutations occur almost equally in men and women. The highlight of this study was the observation of a variant in the TANC1 gene that the female patient, who inherited the disease from a homozygous father, had a pure homozygous mutation. The table below shows the frequency of genes in people with mutations.(table1)

According to the evidence, this disease is autosomal dominant type with high penetration power. The important point is that This variant was observed in both homozygous and heterozygous forms. The frequency of variants is higher in women than men.

This confirms previous observations that restless legs syndrome is more common in women than men. This mutation was observed in the age group of 30-50 years. However, the identification of an infected 14-year-old girl needs further investigation.(table 2)

In this study, the disease was observed autosomally in the Iranian population. Examination of the data confirms the role of polymorphism in this disease.

According to the results of this study, it can be said that Restless Legs Syndrome is an autosomal dominant disease in the Iranian population and the discovery of new gene loci in the Iranian population proves the effect of environmental and geographical conditions on the incidence of the disease. This has already been confirmed in research on different families of other races in other countries. This study showed that restless legs syndrome has a genetic origin in the studied patients and confirms the previous data about restless legs syndrome.(table3)

Conclusion

The aim of this study was to investigate the molecular restlessness syndrome in Iranian society. This study was performed on families from Fars, Tehran and Mazandaran provinces. Sequence analysis showed that in the Iranian population studied, all three genes obtained from the patient's NGS analysis had an effect on the incidence of the disease(table4).

TANC1 has been confirmed as a new gene. On this gene, 1 variant was identified at a locus on the long arm of chromosome 3, in four samples that were common in patients with close kinship (sister, brother, father and daughter). This position was higher in female patients, which confirms previous data on the prevalence of this disease in women.

ATXN7 In this gene, 8 variants were identified on the short arm of chromosome 2, in the Iranian family.

MEIS1 This gene has long been studied and confirmed as an effective candidate gene for restless legs syndrome worldwide. The presence of gene variants in this gene in the Iranian family also confirms the role of MEIS1 in the development of restless legs syndrome in the Iranian population. 11 variants were identified on this gene.

It is hoped that in the future, with further studies and studies on more patients with restless legs syndrome, these new findings can be further examined and confirmed. Also, the study of the protein structure of pathogenic mutations can help how these mutations work. Slowly It is hoped that in the near future, genetic diagnosis kits will be designed using mutations identified in the Iranian population. These kits will play an effective role in faster identification of patients.

Gene therapy, which has gained its place in genetics today as a new and effective method in the treatment of genetic diseases, could be the next step in further studies.

Declarations

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Tables

Table 1- The frequency distribution of genes: TANC1,MEIS1 AND ATXN7

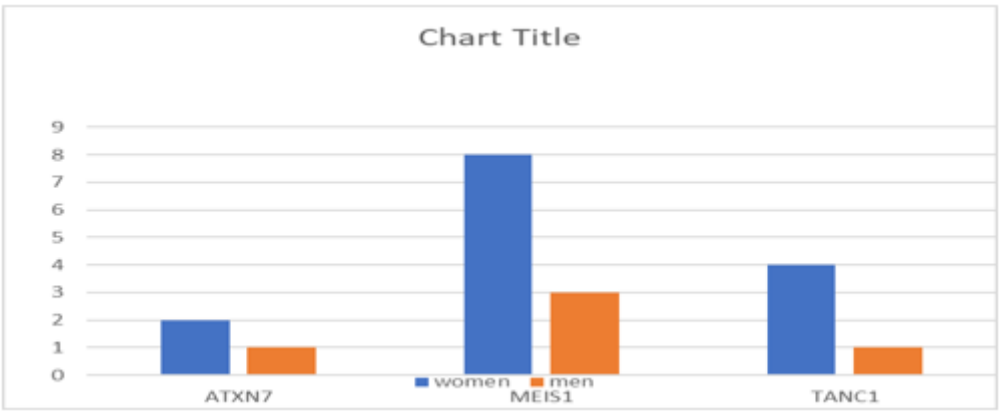

Table 2.Table of age frequency in the subjects

Valid	10-20	6	12.8	12.8	12.8
	20-30	2	4.3	4.3	17.0
	30-40	15	31.9	31.9	48.9
	40-50	14	29.8	29.8	78.7
	50-60	6	12.8	12.8	91.5
	60-70	4	8.5	8.5	100.0
	Total	47	100.0	100.0	

Table3-Comparison of studied genes

Gene	Variant	DANN	classification	Mutation tester	Publication ferequency
ATXN7	G183A	Pathogenic	Uncertain Significance	disease causing	$f = 0.00000634$
	G191A	Damaging	Likely Benign	Polymorphism	$f = 0.0000057$
	196G>T	Tolerated	Likely Benign	Polymorphism	44.58%
	215C>T	Pathogenic	Unknown	Polymorphism	$f = 0.00000997$
	240G>A	Pathogenic	Uncertain Significance	disease causing	$f = 0.00000421$
	242G>A	Disease causing	Benign	disease causing	$f = 0.000021$
	211T>C	Pathogenic	Likely Benign	Polymorphism	$f = 0.00001$
TANC1	183G>A	Disease causing	Uncertain Significance	disease causing	$f = 0.000016$
MEIS1	18C>A	disease causing	Uncertain Significance	disease causing	$f = 0.00000403$
	33C >T	Pathogenic	Uncertain Significance	disease causing	$f = 0.0000121$
	45T>C	Pathogenic	Uncertain Significance	disease causing	$f = 0.0000121$
	56T>G	Pathogenic	Uncertain Significance	disease causing	$f = 0.00000804$
	69G>A	Damaging	Uncertain Significance	disease causing	$f = 0.00000803$
	78C>A	Damaging	Likely Benign	disease causing	$f = 0.000189$
	114C>T	Pathogenic	Uncertain Significance	disease causing	$f = 0.00000402$
	134T>G	Damaging	Uncertain Significance	disease causing	$f = 0.00000404$
	141G>C	Pathogenic	Uncertain Significance	disease causing	$f = 0.0000081$
	149A>G	Disease causing	Uncertain Significance	disease causing	$f = 0.00000407$
	188T>A	Disease causing	Uncertain Significance	disease causing	$f = 0.00000436$
	207C>T	Pathogenic	Likely Benign	disease causing	$f = 0.000335$

Table4.Table of frequency of pathogens in the subjects

	Frequency	percent	valid	Cumulative percent
	percent			
patient	21	44.7	44.7	44.7
Healthy	11	23.4	23.4	68.1
Carrier of pathogenic genes	15	31.9	31.9	100.0
total	47	100.0	100.0	

Figures

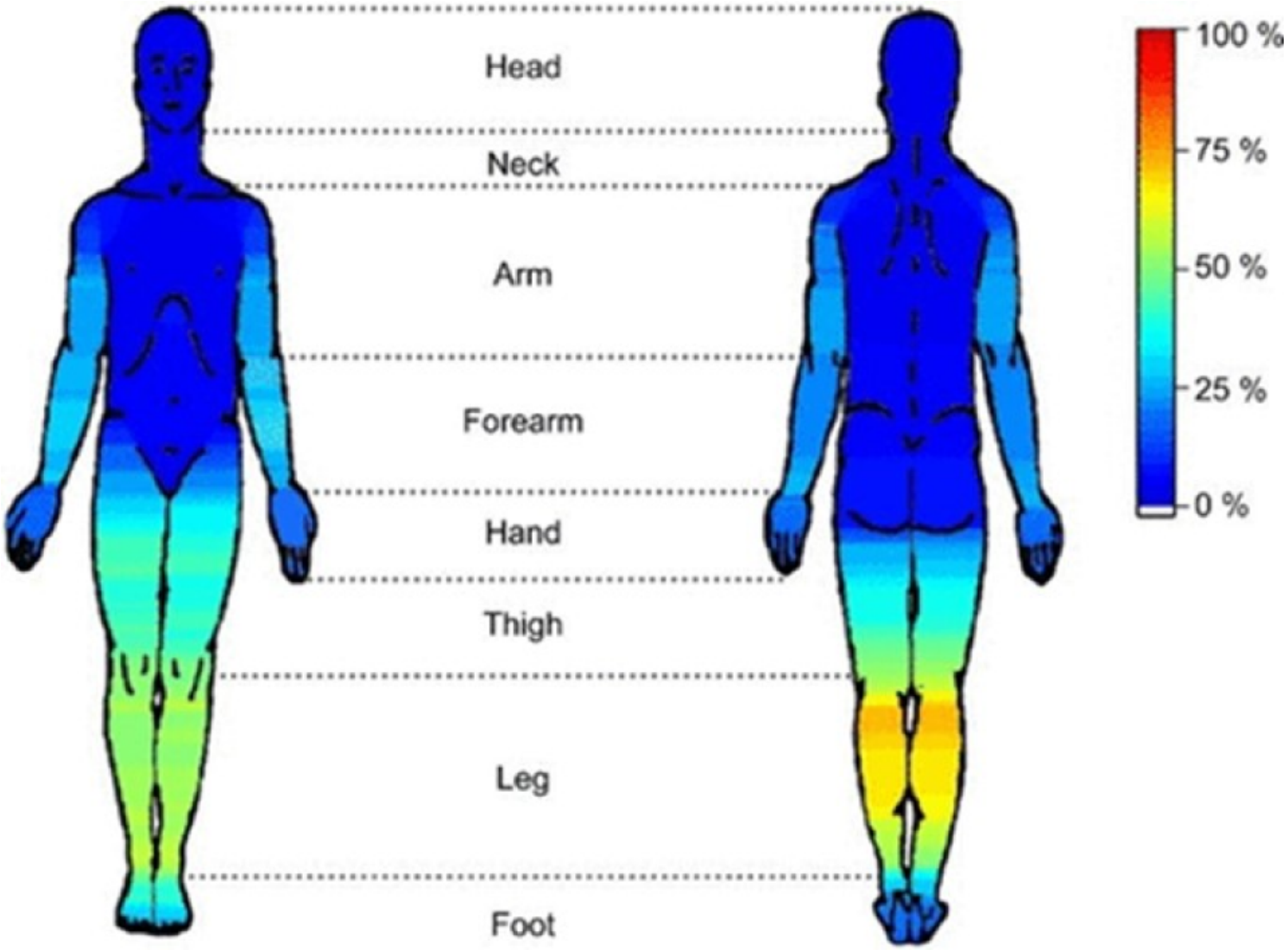

Figure 1

Diagrams of parts of the body that show signs of restless legs syndrome