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ORIGINAL ARTICLE

DNA polymorphisms as tools for spinal cord injury research

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Study Design: Data mining of single nucleotide polymorphisms (SNPs) in gene pathways related to spinal cord injury (SCI).
Objectives: To identify gene polymorphisms putatively implicated with neuronal damage evolution pathways, potentially useful to SCI study.
Setting: Departments of Psychiatry and Orthopedics, Faculdade de Medicina, Universidade de São Paulo, Brazil.
Methods: Genes involved with processes related to SCI, such as apoptosis, inflammatory response, axonogenesis, peripheral nervous system development and axon ensheathment, were determined by evaluating the 'Biological Process' annotation of Gene Ontology (GO). Each gene of these pathways was mapped using MapViewer, and gene coordinates were used to identify their polymorphisms in the SNP database. As a proof of concept, the frequency of subset of SNPs, located in four genes (ALOX12, APOE, BDNF and NINJ1) was evaluated in the DNA of a group of 28 SCI patients and 38 individuals with no SC lesions.
Results: We could identify a total of 95 276 SNPs in a set of 588 genes associated with the selected GO terms, including 3912 nucleotide alterations located in coding regions of genes. The five non-synonymous SNPs genotyped in our small group of patients, showed a significant frequency, reinforcing their potential use for the investigation of SCI evolution.
Conclusion: Despite the importance of SNPs in many aspects of gene expression and protein activity, these gene alterations have not been explored in SCI research. Here we describe a set of potentially useful SNPs, some of which could underlie the genetic mechanisms involved in the post trauma spinal cord damage.
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Keywords: single nucleotide polymorphism; spinal cord injury; NINJ1; APOE; ALOX12; BDNF

Introduction

Immediately after nerve injury, intrinsic and extrinsic factors, involving inhibition and induction of myelination, axonal repair, apoptosis and inflammatory pathways, will participate in the lesion evolution process in progressive waves of secondary injuries.[1] A delicate balance between the modulations of all these pathways will generate substantial secondary damage within the cord, culminating in different degrees of injury ranging from partial restoration of lesion to a complete neurological damage at the injury level.[2]

Spinal cord injury (SCI) leads to considerable loss of neurological function below the level of injury and functionally affects multiple body systems. SCI most often occurs in people in their mid-20s and seriously diminishes patients' quality of life. Data from 2005 indicate that worldwide over 2.5 million trauma survivors live with paralysis induced by SCI. The economic impact of the long-term cost of care and social welfare support reaches in excess of tens of billions of dollars each year (http://rickhansenregistry.org/page192.htm). The therapeutic action range has focused on three lines of approach: acute neuroprotection, enhanced regeneration and treatment of demyelination.[3] At present, the scientific

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community is receiving studies reporting optimistic advances in spine treatment with enthusiasm. Controversially, most of these studies are not reproducible or are followed by contradictory findings, which provide insights on individual patterns of gene expression with trauma and modulation of neurodegenerative and neuronal repair process. In this scenario, individual genomic variations may play a very important role.

One of these genomic variations are single nucleotide polymorphisms, defined as 'single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals in some population(s), wherein the least frequent allele has an abundance of 1% or greater'.[4] Single nucleotide polymorphisms (SNPs) are among the most abundant variation class in human genome being responsible for approximately 83.6% of the total detected genetic variation in gene expression.[5]

SNPs have a potential to impact phenotypic variation in different and independent ways: directly modulating gene expression,[5] creating or abolishing binding sites for microRNAs,[6] modifying splicing patterns,[7] or spatial conformations and/or functions of proteins due to amino-acid alterations in the protein sequence[8] or by altering protein translation kinetics.[9]

Two reasons make SNPs particularly useful when compared to other polymorphic markers. At first, more than 6.2 million SNPs covering the entire human genome have been validated (http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi), with exact genomic locations determined and allelic frequencies assessed in several ethnic groups. Second, diverse and flexible SNP investigation methodologies are available, allowing from single genotyping by PCR to arrays that can examine 500 000 SNPs at a time. These methodologies make it possible to accurately obtain genotypes and allele frequencies to fulfill the needs and capacities of different research protocols.

The impact of SNPs on the development and progression of a vast number of human pathologies has been studied during the past few years. However, this important tool remains to be explored in SCI research. To foster SNP analysis in SCI we used a bioinformatics approach to define a list of SNPs, putatively related to inflammatory, axonal, myelination and apoptosis pathways creating a dataset of candidate targets for the study of spinal cord injury. To prove the potential of this strategy, we conducted an exploratory analysis of five SNPs within a cohort of patients with spinal cord injury as well as non-SCI individuals, to determine the frequency of these SNPs and their potential for the investigation of SCI evolution.

Materials and methods

Dataset of candidate targets

We used the Gene Ontology annotation (http://www.geneontology.org) on Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene) to identify the human genes associated with some of the most relevant pathways related to SCI, including apoptosis (GO:0006915), inflammatory response (GO:0006954), axonogenesis (GO:0007409), peripheral nervous system development (GO:0007422) and axon ensheathment (GO:0008366). Human genes retrieved by these GOs terms were mapped with data available from Map Viewer repository (human genome build 36.2, ftp://ftp.ncbi.nih.gov/genomes/MapView/Homo_sapiens/sequence/BUILD.36.2/initial_release/seq_gene.md.gz), and mapping coordinates were used to identify the polymorphisms present in each gene, according to dbSNP (http://www.ncbi.nlm.nih.gov/SNP; build 128). The list obtained was curated limiting the SNP class by only true SNPs excluding others non-SNPs polymorphisms, like insertions/deletions and microsatellites. Information concerning function class (untranslated region/intron, synonymous or non-synonymous) and type of substitution (transition or transversion) were obtained in the description of each SNP.

Genotyping

Polymorphisms of arachidonate 12-lipoxygenase (ALOX12), apolipoprotein E (APOE), brain-derived neurotrophic factor (BDNF) and nerve-induced injury protein 1 (NINJ1) were genotyped in samples derived from patients with severe SCI and individuals with no SC lesions.

Genotypes of APOE C112R and R158C, and NINJ1 D110A were determined following the protocols previously described by Hisxon and Vernier[10] and Cardoso *et al*.[11] respectively, based on PCR amplification followed by restriction enzyme digestion. Genotypes of ALOX12 R261Q where determined as described by Fridman *et al*.[12] The BDNF V66M genotypes were obtained with a single base extension kit (SNaPshot, Applied Biosystems). Initially, the genomic segment was amplified using the primers BDNF F-5′ AAACATCCGAGGACAAGGTG 3′ and BDNF R-5′ AGAAGAG GAGGCTCCAAAGG 3′. PCR was performed in a total volume of 10 μl consisting of 50 ng of genomic DNA, 0.15 μM of each primer, 0.65 U of Taq DNA polymerase (Invitrogen), 1 × PCR buffer (including 1.5 mM $MgCl_2$) and 0.15 mM of dNTPs. Thermal cycling was performed under the following conditions: 5 min at 95 °C, 35 × (30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C). Using the PCR products as templates we followed with the single base extension using the primer BDNFSNAP-5′ $A_{(22)}$GCTGACACTTTCGAACAC 3′, according to the manufacturer.

Samples

Samples used in this study were obtained from the Department of Orthopedics, Faculdade de Medicina da Universidade de São Paulo. A total of 28 patients with complete neurological damage after SCI and 38 individuals without any neurological pathology or SCI were analyzed. Approximately 10 ml of blood was drawn from patients and non-SCI individuals and genomic DNA was extracted.

Subjects with complete neurological damage after SCI complied with the following inclusion criteria: (i) to have complete neurological damage caused by spinal cord injury at least for 2 years; (ii) subjects with no vertebral canal stenosis at the time of the SCI; (iii) subjects who had burst vertebral fracture, in the absence of facet dislocation, at the level of the

spinal cord in the vertebral canal, and never at the level of the spinal cord cone or the nervous roots; (iv) subjects who were younger than 35 years at the time of the injury; (v) and who did not experience neurological alterations as a result of surgical intervention; (vi) subjects treated either clinically or surgically by the same medical team (Spinal Cord Injury Unit at Departmento de Ortopedia e Traumatologia, Universidade de São Paulo), following the same protocols for all cases. All of them were informed about the risks in participating and agreed to sign up the informed consent paper.

Results

Dataset of candidate targets

Using selected GO terms we identified a total of 588 genes encoding proteins potentially associated with second damage pathways, distributed as follows: 322 genes in apoptosis, 206 genes in inflammatory response, 33 genes in axonogenesis, 20 genes in peripheral nervous system development and seven genes in axon ensheathment pathways. Using the mapping limits of each of these genes we scanned a total of 28.4 million bases of human genome, selecting a set of 95 276 SNPs, a mean of one SNP for each 298.6 bases. Most of the SNPs, 91 364 (95.9%), were located in untranslated regions/introns and 3912 SNPs (4.1%) were located in coding regions of genes being 1773 (1.9%) synonymous alterations and 2139 (2.2%) non-synonymous ones. (Table 1). The complete list of genes and their SNPs are given in the supplementary table that accompanies this paper.

Determination of SNP frequencies

Five SNP frequencies were determined in four genes. SNPs in two of these genes, APOE and BDNF, have been studied by many groups, and were included here as controls to determine if the frequencies found in our population resemble those previously described by other groups. The other two SNPs (ALOX12 and NINJ1) have been published by some of us[11,12] and, up to now, were evaluated in a specific set of samples. Allelic frequencies for all these evaluated SNPs are presented in (Table 2). Our results show that the selected SNPs have variable frequencies (ranging from 5.3% in APOE gene to 42.8% in ALOX12 gene) in the Brazilian samples studied, suggesting the applicability of some of them in the study of SCI lesions with different progressions.

Discussion

Complex pathologies, such as SCI, involve individual and environmental factors that contribute to their development, evolution and treatment response. Modulation of post-traumatic process involves different pathways with multiple genes evocated. Even more, data from a huge number of studies have shown that intrinsic gene variants are responsible for modulating all genes/pathways involved with pathological history (for example, Stranger *et al*.[5]). One of the most abundant genetic variants in the human genome is the SNP, a powerful tool to understand the behavior of circuits involved in different pathways.

Almost 7 years ago, together with human genome publication, the first systematic map of human genome SNPs, containing 1.42 million SNPs, was described.[13] Today, the human SNP database (build 128) describes 11 883 685 SNPs, corresponding to approximately one SNP at every 270 bases of our genome. Many of these variations can be involved with phenotypic outcomes, many with important clinical implications.

Table 1 Characterization of SNPs identified by GO term

GO term; accession	*Genes identified*	*SNPs identified*	*Function class*			*Substitution type*	
			Untranslated region/intron	*Synonymous*	*Non-synonymous*	*Transition*	*Transversion*
Apoptosis; GO6915	322	56371	54367	904	1100	37576	18795
Inflammatory response; GO6954	206	26551	25018	680	853	18018	8533
Axonogenesis; GO7409	33	7562	7364	93	105	5061	2501
Peripheral nervous system development; GO7422	20	3451	3318	69	64	2265	1186
Axon ensheathment; GO8366	7	1341	1297	27	17	925	416
Total	588	95276	91364	1773	2139	63845	31431

Abbreviations: GO, Gene Ontology; SNP, single nucleotide polymorphism.

Table 2 Distribution of alleles in SCI patients and health individuals

	ALOX12		*APOE*			*BDNF*		*NINJ1*	
	A	*G*	*ε2*	*ε3*	*ε4*	*A*	*G*	*A*	*C*
SCI patients	24 (42.8%)	32 (57.2%)	3 (5.4%)	47 (83.9%)	6 (10.7%)	8 (14.3%)	48 (85.7%)	50 (89.3%)	6 (10.7%)
Non-SCI	30 (39.5%)	46 (60.5%)	5 (6.6%)	67 (88.1%)	4 (5.3%)	11 (14.5%)	65 (85.5%)	56 (73.7%)	20 (26.3%)

Abbreviations: ALOX12, arachidonate 12-lipoxygenase; APOE, apolipoprotein E; BDNF, brain-derived neurotrophic factor; NINJ1, nerve-induced injury protein 1; SCI, spinal cord injury.

An example of the clinical application of SNPs is the recent description of an SNP associated with coronary heart disease (CHD).[14,15] Using appropriate sample sizes, these groups found that homozygous individuals for one specific allele have an ~20–40% increased risk of developing CHD and that ~20–25% of individuals analyzed are homozygous for this variant. This kind of data can identify subjects with a higher risk for specific diseases, helping to design better strategies for the management of the patients and contributing to decipher the molecular basis of this disease.

Using cross searches in PubMed we can mine more than 3.5 thousand articles that have already been published involving the study of SNPs in cancer, more than 1.4 thousand articles published involving SNPs in diabetes, close to 500 articles involving SNPs in arthrosis but only six papers using SNPs as tools to study SCI, all from the same research group (Supplementary information), showing that the human genome is still a wild landscape to be explored in SCI research.

Our analysis helps foster the use of genetic variants in the study of SCI. Using a robust bioinformatics routine we were able to identify more than 95 000 SNPs in 588 genes associated with second damage pathways, creating a focused dataset for SCI research. Non-synonymous alterations that alter the sequence of protein by amino-acid substitution constitute the more obvious SNPs with the direct potential to impact the phenotype. According to the Human Gene Mutation Database, alterations involving modifications in the protein sequence account for almost half of all the DNA variations that are known to cause genetic disease (http://www.hgmd.cf.ac.uk). Our dataset contains 2139 non-synonymous SNPs distributed around all pathways analyzed.

In many cases, the potential clinical value of an SNP depend not only on its consequences over the mRNA or the encoded protein, but also on its frequency on the population under study. The statistically significant association of a specific SNP with a relative risk of an event (pathology development, drug response, etc) requires adequate sample sizes that is dependent on the frequency of the minor allele on the SNP. The impact of inadequate sample sizes is more relevant when modest effects are expected or on reports of positive associations identified in small samples. Most of functional genetic variants presents subtle effects on disease risk but may have a large population impact owing the prevalence of SNP. Sample sizes required for association studies of polymorphisms with high prevalence in population are smaller than those present at a lower prevalence. Rare SNPs (with frequencies $<5\%$) require larger sample sets,[16] which are difficult to obtain for some analysis, including the comparison of patient groups with different SCI outcomes. To evaluate in our population, the frequency of some SNPs identified here, we genotyped a set of samples derived from SCI and non-SCI subjects (Table 2). These SNPs are located in genes related with key aspects of the physiological pathways involved in the concepts already described.

The genes selected for this frequency analysis were: APOE (involved with cholesterol metabolism, which is essential for myelin membrane growth[17]), ALOX12 (involved in the modulation of inflammatory response,[18] neurite regeneration[19] and nerve cell death by oxidative stress under glutamate depletion[20]), BDNF (a neurotrophic factor that promotes the development, regeneration, survival and maintenance of function of neurons,[21] overexpressed in fibroblasts used to promote regeneration of SCI[22,23]) and NINJ1 (a membrane protein that is upregulated after nerve injury[24]).

SNPs with prevalence of 50% and 20% will require sample sizes of 387 and 535 individuals, respectively, to be statistically significantly associated ($P<0.05$) to a particular event with a 1.5 odds ratio value. In contrast, for rare SNPs (with frequency of the minor allele $<5\%$) much larger sample sets (>1700 individuals) are required to produce statistically significant findings, with a 1.5 odds ratio value.[16]

The allelic frequencies observed here shows that all polymorphic alleles evaluated, with the exception of ApoE2 and ApoE4, have relatively high frequencies, consequently demanding smaller sample size to be appropriately evaluated. The polymorphism of ALOX12 is highly prevalent in both SCI (42.8%) and non-SCI samples (39.5%) being an excellent candidate for an initial study (Table 2).

Here we provide relevant data that can be used to support and stimulate additional genetic studies in SCI, concerning the use of SNPs as tools, to better understand the participation of individual variations in the evolution in spinal cord injury.

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Supplementary Information accompanies the paper on the Spinal Cord website (http://www.nature.com/sc)