Brief Report

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Usefulness of salivary sampling for the molecular detection of a genetic variant associated with bipolar disorders

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Abstract

Under certain conditions, the hyperthymic temperament traits associated with an increased risk of developing bipolar disorders may in fact produce adaptive responses. The purpose of this study is to see if the type of biological material used for genetic analysis (saliva or blood) affects the detection of mutations in the CACNA1C (RS1006737) gene. The first experimental group consisted of Sardinian migrants ("volunteers") in South American and European megacities. The second experimental group consisted of older healthy subjects with hyperactivity and novelty-seeking characteristics from Cagliari, Italy. The genetic procedure included DNA extraction, real-time PCR, and the Sanger method. Nonetheless, the authors believe that saliva is the most appropriate biological material, given its many advantages. In contrast to blood, saliva can be collected by any type of healthcare provider after following a few simple instructions.

Keywords

Saliva, blood, CACNA1C gene, bipolar disorders, hyperthymic temperament

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The temperaments have generally been proven to belong to the domain of normality rather than the domain of pathology, in accordance with their putative adaptive role. The hyperthymic temperament can only be classified as abnormal in the presence of chronic hypomanic symptoms or advanced mood disorders.[1] Hyperthymic temperament is characterized by hyperactivity, goal achievement, novelty seeking, extroversion, a high energy level, emotional intensity, and a moderate need for sleep.[2] In fact, these features appear to provide well-defined advantages in leadership, competition, exploration, and territoriality.[3] Under certain circumstances, the traits of the hyperthymic temperament are linked with the increased probability of developing mental health pathologies, especially bipolar disorders, in fact could create adaptive responses.[4–6] This potentially adaptive effect may be more visible in megacities, where life is expected to occur 24 hours a day, 7 days a week, with complete changes in sleep-wake cycles.[7]

This paper is part of the evolutionary perspective, which objective is to confirm if a genetic component related to bipolar disorders can be identified in people without bipolar disorders pathology, but with hyperactivity/novelty seeking and exploration aspects. The paper's goal is to demonstrate whether the type of biological material used for genetic analysis affects the detection of mutations in the CACNA1C gene linked to bipolar disorder.

Until now, our approach was supported by two complementary studies in which the genetic variant RS1006737 (CACNA1C gene) was studied. The presence and the

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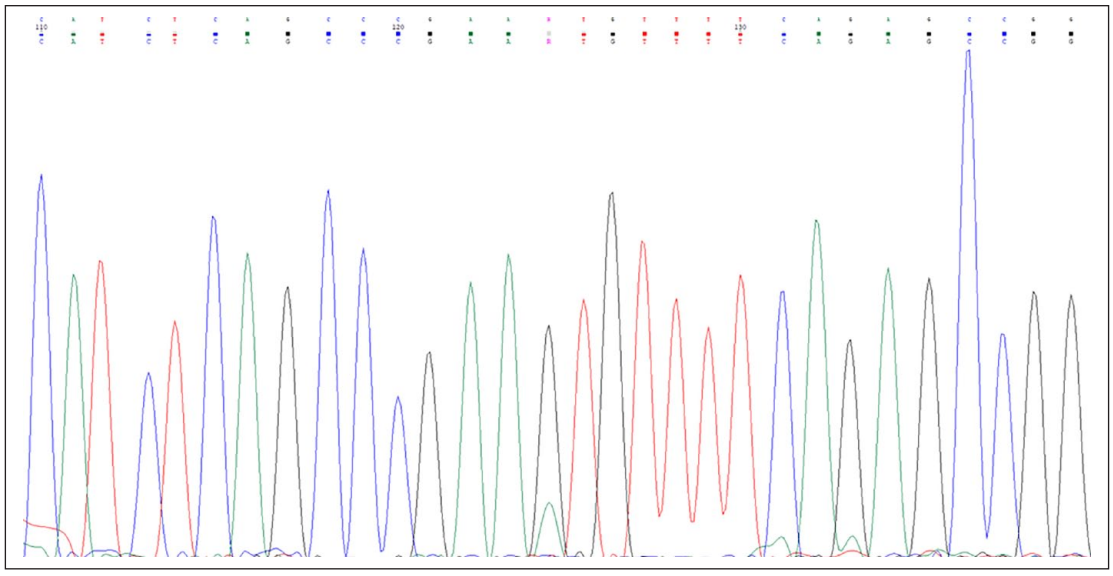

Figure 1. The presence of heterozygous mutation (G/A) in genetic variant RS1006737 (CACNA1C gene).

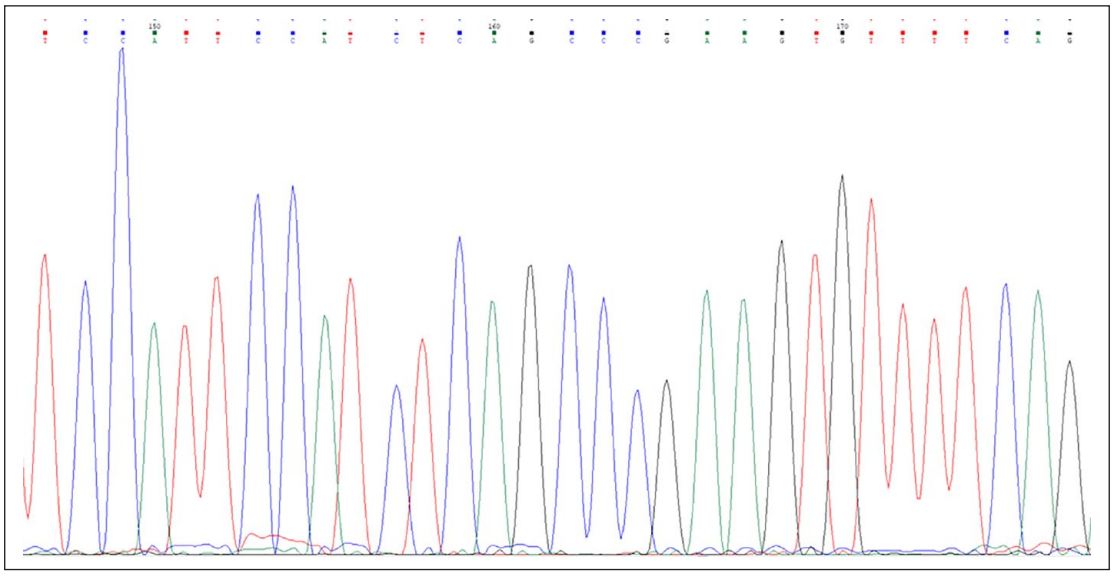

Figure 2. The wild type profile in genetic variant RS1006737 (CACNA1C gene).

absence of the mutation at this genetic variant are shown in Figures 1 and 2, respectively.

The first experimental group consisted of Sardinian migrants ("volunteers") in the megacities in South America and Europe.[8,9] In addition, we found that in the Sardinian migrants in the megacities of South America, the frequency of episodes of non-pathological hypomania was double that of Sardinians residing in Sardinia, which goes in favor of the adaptive and beneficial side of the hyperthymic temperament. Saliva was collected from them for genetic testing.[10]

The second and larger experimental group consisted of older healthy subjects with characteristics of hyperactivity and novelty seeking in the city area of Cagliari, Italy, previously involved in a clinical trial on the efficacy of middle/moderate exercise.[11,12] The inclusion criteria of the trial of exercise were: age 60 or over, both genders, living at home, and capacity of freely providing informed consent. Blood was collected from them for the genetic testing.

The control group in both studies included bipolar disorder (BD) patients attending the Centro di Psichiatria di Consultazione e Psicosomatica of the University Hospital of Cagliari.

Saliva is an extracellular fluid produced and secreted in the mouth by salivary glands. In humans contains

Table 1. Total number of positive and negative participants for the RS1006737 genetic variant, although the samples were recruited from different geographical areas and different subjects, the differences between the saliva and blood samples were not significant ($P > 0.01$).

	Positive participants (G; A)	Negative participants (G; G)	Total participants
Saliva	28 (59.57)	19 (40.43)	47
Blood	40 (65.57)	21 (34.43)	61

approximately 99% water, as well as electrolytes, mucus, white blood cells, epithelial cells (from which DNA can be extracted), enzymes (such as lipase and amylase), and antimicrobial agents (such as secretory IgA, and lysozymes).[13] Saliva sampling is a low-cost, non-invasive, and time-saving method. Another significant advantage is that the subject can take the material on their own after a brief explanation of the procedure. When collecting mucosal salivary samples, the buccal swab should be vigorously rubbed on the inside of the cheek, both right and left. One disadvantage of using saliva as a test material is that it is advised to avoid eating or drinking colored substances and smoking. It also necessary to consider also the possibility of ongoing oral infections that may result in bleeding or mucus production, so that the testing tool detects these as contaminants. Importantly, in some cases, the reason for re-sampling is a lack of DNA in the salivary sample. The blood is a body fluid in the circulatory system of humans that transports metabolic waste products away from cells while delivering necessary substances such as nutrients and oxygen to them. It is made up of blood cells suspended in plasma.[14] Blood sampling is more expensive than salivary sampling. It is a more invasive method that has the potential to coagulate and hemolyze the sample. As a result, certain temperature ranges associated with sample transport and storage must be observed. Taking a blood sample for genetic analysis, on the other hand, always provides a sufficient amount of DNA, and individuals receive no prior recommendations regarding food and drink restrictions. It is critical that no local or systemic infection poses a threat to providing a viable sample for genetic analysis.

Between March 2019 and September 2022, 108 samples were evaluated with the goal of detecting mutations of SNP variant 1006737 of the CACNAIC gene, which is strongly associated with bipolar disorder. This gene's mutations were discovered using real-time PCR and confirmed using Sanger sequencing.

A total of 47 salivary samples were collected with a brush and stored in 1 ml eppendorf tubes containing 400 µl of 500 µl EDTA and dimethyl sulfoxide. Sixty-one blood samples, on the other hand, were collected in tubes containing coagulation activators, which have a micronized silica particle coating that activates coagulation when the tube is gently inverted during the mixing process after collection. The samples were transported to the AOU of Cagliari's Molecular Biology laboratory and immediately centrifuged at 3000 rpm for 10 min. All samples were kept at −20°C.

The CTAB technique protocol was used to extract DNA from salivary swabs. Several variations were developed in order to adapt the technique to a wide range of organisms. These "homemade approach's" operational steps are outlined below. To 400 µl of biological material, 75 µl of SDS/Proteinase K was added. 100 µl of NaCl (5 M) and 100 µl of CTAB/NaCl preheated to 65°C were added after 1 h of incubation at 65°C. 750 µl of chloroform/isoamyl alcohol (24/1) was added after another hour of incubation. DNA was extracted from the upper liquid phase and transferred to a −20°C ice-cold tube. After being treated with 450 ml of cold isopropanol, the sample was incubated at −20°C for 2 h. A pellet wash with 100 µl of ethanol at 70° was performed, and the DNA was resuspended by adding TRIS/HCl at pH > 8. New biological approaches expanded the number of molecular diagnostic tests[15,16] and new molecular clinical tests are now avaible.[17,18]

Polymerase chain reaction (PCR) has been used to detect nucleotide polymorphisms (SNPs) associated with BD at the CACNA1C gene level.[19] The use of bioinformatics tools to design FRET probes was critical in detecting mutations at the gene of interest. For genotyping rs1006737 with greater specificity/selectivity, we proposed a molecular method based on FRET probes. This method has been shown to be faster, simpler, and more precise than older approaches such as RFLP or DNA sequencing technologies. FRET, in particular, is a probe that uses a dipole-dipole mechanism to transmit energy from an excited donor to an accepting group.[20] One of the two probes contains a fluorochrome that can transfer energy to the other probe's fluorochrome, causing it to emit light at a specific wavelength detectable by real-time PCR. As a result, the fluorescence is only detected when the PCR reaction results in the binding of both probes to the DNA. In FRET technologies, the upstream probe is labeled with fluorescein isothiocyanate (F1), while the downstream probe is labeled with Red 640 fluorophore (acceptor). Melting temperature is caused by nucleotide variation in the target acceptor DNA (Tm).[21] This result was observed in the PCR apparatus by observing the F2 fluorescence during sample heating and observing the melting peak. By evaluating melting temperatures, we predicted a high likelihood of allelic diversity in this gene region.[22]

Based on our previous findings (not shown), this method appears to be most sensitive to the saline concentration in saliva; in fact, the melting curves' Tm resolution grade is strictly dependent on the Na+ and Mg2++ concentrations. For

this reason, the novel techniques based on Sanger sequencing could represent an interesting molecular approach, especially for DNA mutation detection in salivary samples or for high-DNA GC-content secondary structure resolution. In this brief report, we describe the novel apparatus by using Sanger sequencing to confirm the detected mutations in SNP RS1006737. In this context, we used the SeqStudio Genetic Analyzer technology (Applied Biosystem, USA). It is a four-capillary, fluorescence-based capillary electrophoresis device made adaptable for genetic analysis research to high resolution. This protocol provided 2 PCR reactions, each of which contained a denatured DNA fragment to be sequenced, Primers, high processivity DNA polymerase and 4 triphosphate deoxyribonucleotides (dNTPs). The resulting mixture was divided into four fractions for each nucleotide base (A, T, G, C) to which the corresponding chain terminator was added (ddATP, ddTTP, ddGTP, ddCTP). These chain terminators were added by chance; they formed fragments of varying lengths with the labeled primer's initial sequence and terminated with dideoxyribonucleotide triphosphate. Following incubation, the four fractions were heat denatured to separate the paired nucleotide chains and electrophoretically run on a single polyacrylamide gel. Based on their size, the DNA sequence was then determined.[23] The electropherogram generated by the sequencer enabled us to analyze the data using software such as: Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi/) and Clustal W, (https://www.ebi.ac.uk/Tools/msa/clustalo).

Statistical analysis

The Cohen statistical procedure was used to estimate the level of agreement between the positive and negative rate for the G/A mutation in RS100673724. Although the samples were recruited from different geographical areas and different subjects. An adjunctive evaluation was performed by Chi-square test using the social statistics web program: https://www.socscistatistics.com/tests/

Results and Discussion

Two different mutations were identified in these analyzed samples relating to exonic position RS100673. The first was standard wild-type sequencing (G; G), which was linked to no risk of mood disorders. Following the SNPedia report, an increase in risk (especially for bipolar disorder) is reported for the homozygous mutation (A;A). While in this work, we have observed a good and interesting association with the heterozygous profile (A;G), Table 1. At the same time, this investigation demonstrated and suggested the use of capillary sequencing as a fast and reliable method to detect RS100673 mutations in the CACNA1C gene.

Conclusions

Although saliva and blood differ in composition and biological properties, we concluded that sample type has no effect on the detection of a mutation in the genetic variant of interest. Nonetheless, the authors believe that saliva is the most appropriate biological material, given its many advantages. Saliva, as opposed to blood, can be taken by any type of healthcare provider after following a few brief instructions.

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Authors contributions

AS and GK prepared the first draft, and SF, MP, GC, CIAG, MGC, GO, contributed to its improvement. The paper's final content was approved by all authors.

Declaration of conflicting interests

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Institutional review board statement

The studies were conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethical Committee of the Institutional Review Board of the University Hospital of Cagliari, Italy (First study—authorization signed on 27th February 2022, with a reference number NP/2019/1003; Second study—authorization signed on 11 July 2022, with a reference number NP/2022/2893).

Informed consent

Informed consent was obtained from all subjects involved in the study.

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