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Research paper

LOCAL ALLELE FREQUENCIES OF THE 5-HTTLPR SEROTONIN TRANSPORTER PROMOTER POLYMORPHISM

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Abstract. The Serotonin Transporter protein (5-Hydroxytryptamine transporter; 5-HTT) is an important reuptake receptor of serotonin from the synaptic cleft. The protein is encoded by the *SLC6A4* gene. A size polymorphism, the 5-HTT Linked Polymorphic Region (5-HTTLPR; *SLC6A4*, 44-BP INS/DEL), exists within the promoter of this gene. The presence of this polymorphism has been associated with an increased susceptibility for a variety of neurological conditions including Parkinson disease, chronic pain, anxiety and depression related phenotypes. This 5' regulatory promoter polymorphism consists of a 44–base pair insertion resulting in a long or short allele. The short allele is linked to a pronounced reduction in transcriptional efficiency producing lower numbers of transporter protein and a reduced rate of serotonin reuptake. Allele frequencies for this polymorphism show substantial variation in different populations. The frequency of the 5-HTTLPR in the population of Malta was determined in 608 cord blood DNA samples. Allele size difference of the 5-HTTLPR was detected using Polymerase Chain Reaction (PCR) and agarose gel electrophoresis. In total, 288 samples were found to be heterozygous (L/S) carrying 1 copy of the short allele and 1 copy of the long allele, while 129 samples were homozygous for the short allele (S/S) and 189 samples were homozygous for the long allele (L/L). Unexpectedly, 2 samples were found to carry a copy of the extra-long allele (XL) which is reportedly only found in African and Asian populations. Allele frequencies for L, S and XL alleles were 54.86%, 44.98% and 0.16% respectively. These local frequencies are similar to those of other European populations with the exception of the occurrence of the XL allele. These findings highlight the changing dynamics of population gene pools, the importance of selecting suitably matched controls for case-control studies and the importance of ethnicity information in the design, execution and interpretation of genetic diagnostic tests.

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1 Introduction

Serotonin (5-HT) is an inhibitory neurotransmitter with pleiotropic functions. It is required in adequate concentrations for mood stability and for balancing any excessive excitatory brain neurotransmitter secretions. Serotonin is also a regulator of several other processes which include carbohydrate cravings, sleep cycle and sleep (Jacobs, 1985), pain control and appropriate digestion (Yeo et al., 2004). Serotonin levels have also been associated with variations in function of the immune system (Mossner and Lesch, 1998). It has also been suggested that 5-HT is involved in the feeling of fear as well as pathological anxiety (Hariri et al., 2002). Furthermore, serotonin is an indolamine with vasoconstrictive and aggregating properties. It is considered a weak soluble platelet agonist, but with the ability to potentiate the aggregation induced by other agonists such as adenosine diphosphate (Baumgartner and Born, 1968; Gomez-Gil et al., 2002; Rand et al., 2003; Vanags et al., 1992). Thus, serotonin increases the procoagulant activity of activated platelets (Lopez-Vilchez et al., 2009).

In the central nervous system (CNS), serotonin is involved in the regulation of a variety of behavioural and visceral functions, including: mood, anxiety, aggression, gastrointestinal motility and vascular resistance (Karwautz et al., 2007). Its activity is implicated in many physiological functions (sleep, appetite and pain) as well as in pathological conditions such as depression and schizophrenia (Lesch et al., 1996; Lesch and Gutknecht, 2005). Furthermore, genetic polymorphisms within the serotonin reuptake transporter have been linked to many conditions such as pain perception (Hampf, 1989), psychiatric disorders such as mood, anxiety and depression (Kenna et al., 2012), Parkinson's Disease (Zhang et al., 2014) and cardiovascular disease (Lopez-Vilchez et al., 2009).

The reuptake of 5-HT from cells and neurones is mediated by a specific transporter protein, the 5-Hydroxytryptamine Transporter (5-HTT), a sodium-dependent transporter protein that contains 12 trans-membrane spanning regions

and enables the specific transport of 5-HT from the extracellular space of the synaptic cleft to the neuronal cytoplasm (Amara and Kuhar, 1993). It is the principal regulator of serotonin neurotransmission (Lesch and Mossner, 1998). Through its function, 5-HTT determines the level and duration of postsynaptic receptor-mediated signalling and acts as a prevention mechanism to hinder overstimulation or desensitisation of the sensorimotor responses. Thus, serotonin can be reutilised and is readily available to be transmitted to other receptors when stimulated (Hooten et al., 2013). The serotonin transporter protein is the main target of selective serotonin reuptake inhibitors (SSRIs) such as sertraline and paroxetine.

The gene encoding 5-HTT, *Solute Carrier Family 6 Member 4,* is located on chromosome 17q11.2 (Ramamoorthy et al., 1993). The promoter of 5-HTT contains a size polymorphism (the 5-HTT Linked Polymorphic Region; 5-HTTLPR) which gives rise to two common alleles differing by 44bp in size (Heils et al., 1996): a long allele (L) and a short allele (S) which have been shown *in vitro* to differentially regulate 5-HTT transcription and expression (Heils et al., 1996). The S allele has been shown to impair transcriptional efficiency resulting in decreased production of the transporter protein leading to a reduced re-uptake rate of serotonin from the synaptic cleft (Nakamura et al., 2000; Haberstick et al., 2015).

Allele frequencies for this polymorphism show substantial variation in different populations worldwide (Gelernter et al., 1999; Nakamura et al., 2000). Population studies of *SLC6A4* suggest that the 5-HTTLPR genotype frequencies also show considerable variation across different ethnic groups (Willeit et al., 2003). It has also been suggested that the existence of at least two functionally different alleles in the promoter of *SLC6A4* might reflect balancing selection, with certain beneficial traits balancing the negative outcomes of the anxiety-related phenotype (Gelernter et al., 1999). Since random genetic drift can also be used to explain the global variation in allele frequencies, any associations with the polymorphism can also be interpreted through an evolutionary perspective (Haberstick et al., 2015; Gelernter et al., 1999).

The allele frequency of the 5-HTTLPR serotonin transporter gene polymorphism in the Maltese population has never been determined. However, there is a general interest in this gene and its polymorphisms especially in relation to Parkinson's disease, chronic facial pain and cardiovascular disease. Therefore, the current study was designed to determine the allele frequencies of the 5-HTTLPR in Maltese Population.

2 Methods

Study Population

Cord blood samples had been successively collected by the Malta BioBank over a 2-month period in 2010 to reflect the current population of Malta and Gozo. Cord blood was collected in Ethylenediaminetetraacetic acid (EDTA) vacutainers and frozen until required for DNA extraction. Extracted DNA was stored in the biobank for use in population studies (University Research Ethics Committee approval 48/2002). The cord blood collection was fully anonymised, however, the samples were coded into 5 sub-groups: those from babies born to two Maltese parents (MT), 2 foreign parents (OO), 1 foreign and 1 Maltese parent (O) and samples from babies born in Gozo (G). Samples with incomplete parental details were denoted by ND. In this study, these different sub-groups are referred to as nation-based subgroups.

The study population included samples from 323 male and 307 female newborns. Of these, 305 samples were from infants with 2 Maltese parents (MT), 65 samples from infants with 2 foreign parents (OO), 75 samples from infants with 1 Maltese and 1 foreign parent (O), and 155 samples from infants whose parental details were incomplete (ND). The remaining 30 samples were from infants born at Gozo General hospital (G) and no parental details were available for these samples.

Genotyping

DNA had been previously extracted using the salting out technique (Miller et al., 1988). The primer sequences (Table 1) used to amplify the region surrounding the 5-HTTLPR 44-BP INS/DEL in the promoter of *SLC6A4* were obtained from literature (Hooten et al., 2013).

Table 1: Primers used to amplify the 5-HTT Linked Polymorphic Region

Forward Primer	5' – TGGGGGTTGCAGGGGAGATCCTG – 3'
Reverse Primer	5' – TCCGCTTTGGCGCCTCTTCC – 3'

A master mix with 5μM primers (Bioneer, Korea), water and One *Taq* 2x PCR buffer (New England Biolabs, USA), was prepared for each set of samples to be tested and 9 μL master mix was mixed with 1 μL DNA for each assay. Samples were tested in sets of 95 and a negative control was included with each set. PCR was carried out over 29 cycles with an annealing temperature of 60ºC. Sizing of PCR products was carried out on a 1.5% agarose gel.

DNA Sequencing

Two samples of each different genotype were selected for dideoxy sequencing. Prior to sequencing, PCR products were cleaned using a PCR purification kit (AccuPrep® Bioneer, Korea). Sequencing was carried out at LGC Genomics (Germany). Sequencing data was viewed using Chromas v.2.4.4 (Technelysium Pty Ltd.) and aligned using Sequencher v.5.4.1. PolyPeakParser (http://yosttools.genetics.utah.edu/PolyPeakParser/) was used to de-convolute the sequences of the different alleles in heterozygous samples (Hill, 2014).

Data Analysis

The population was tested for Hardy Weinberg Equilibrium (HWE) using the HWTriExact function in R v3.6.0 (R Core Team, 2013). The HWTriExact function is an exact test for HWE for triallelic variants and is part of the HardyWeinberg R package (Graffelman 2008; Graffelman 2015). Allele and genotype frequencies for the whole collection of samples and for different nation-based subgroups were calculated. These frequencies were compared to European American, African American and Asian cohort data. The Mann Whitney two-tailed test was used to compare genotype frequencies between males and females.

3 Results

Genotyping results were obtained for 608 samples (Figure 1, Table 2). From the data collected during this study, 47.37% of the Maltese population was found to be heterozygous (L/S), 31.09% were homozygous L/L and 21.22% were homozygous S/S. Unexpectedly, two individuals (0.32%) were found to be heterozygous for the 20-repeat XL allele as confirmed by Sanger sequencing (Appendix A). Allele frequency for S, L and XL alleles were 44.98%, 54.86% and 0.16% respectively. The 2 samples carrying the XL allele were both offspring of 2 foreign parents (OO).

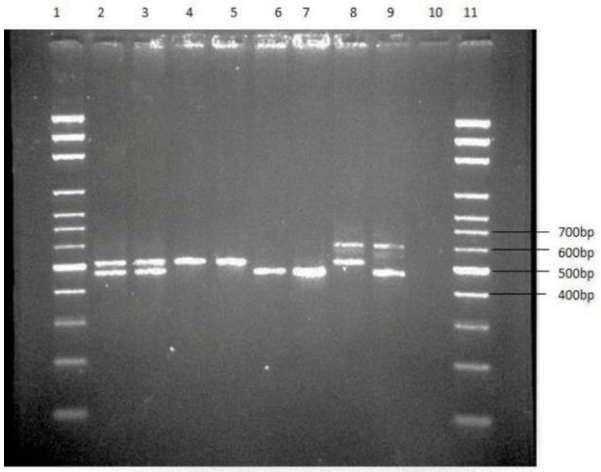

Figure 1. Genotyping results of representative samples from the study population. Lanes 1 and 11 show the 100bp ladder. Lanes 2 and 3 show L/S heterozygotes, lanes 4 and 5 show L/L homozygotes, lanes 6 and 7 show S/S homozygotes and lanes 8 and 9 show the XL/L and XL/S genotype respectively. Lane 10 shows the negative control. The PCR products observed are of expected size of 466bp for the S allele and 509bp for the L allele. Lanes 8 and 9 both show the XL band which is larger in size.

Table 2: Allele and genotype frequencies for 5-HTTLPR in the different subgroups

Genotype	Overall N (%)	MT N (%)	OO N (%)
L/L	189 (31.09)	81 (26.53)	19 (29.03)
L/S	288 (47.37)	166 (54.42)	33 (51.61)
S/S	129 (21.22)	58 (19.05)	10 (16.13)
L/XL	1 (0.16)	0 (0)	1 (1.61)
S/XL	1 (0.16)	0 (0)	1 (1.61)
Allele frequency L	0.549	0.538	0.563
Allele frequency S	0.450	0.462	0.422
Allele frequency XL	0.002	0.000	0.016

Notwithstanding the unexpected XL alleles, the study population was found to be in HWE. The allele frequencies for both genders indicate no gender bias and HWE testing of the MT and the OO subgroups showed that the subgroups representing the local gene pool (MT) and the immigrant gene pool (OO) are both in HWE.

Allele frequencies for the Maltese population were found to be comparable to the allele frequencies of neighbouring European populations (Figure 2), that is, a higher proportion of heterozygous individuals with homozygous L/L observed more than homozygous S/S.

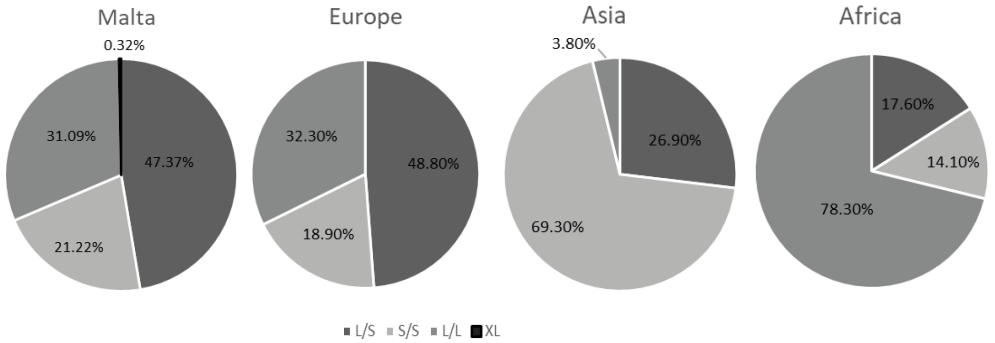

Figure 2. 5-HTTLPR genotype frequencies in Maltese, European, Asian and African populations. Results from the Maltese population are closest to he European populations with the exception of the presence of the XL allele which is typically found, albeit rarely, in African, Asian and non-white Hispanic populations. (European, Asian and African population data obtained from: Gonzales-Vigilar, 2015).

4 Discussion

Genetic demographic data shows a greater population frequency of the 5-HTTLPR S-allele within certain geographical regions of the world such as East Asia. In a typical East Asian population, 70–80% of individuals carry the S allele compared to a typical European population where only 40–45% of individuals carry the S allele (Gelernter et al., 1997 ; Nakamura et al., 2000). Conversely, African populations have a higher frequency of the L-allele when compared to European and Asian populations (Haberstick et al., 2015). One possible explanation for the differences in allele and genotype frequencies between different geographical populations is that geographical variability and environmental pressures have influenced genetic selection (Chiao and Blizinsky, 2010).

The genetic diversity between human populations shows clear variation with respect to geographical distance and geographical barriers, because individuals from more distant populations are less likely to interbreed with each other than individuals from less distant populations due to isolation by distance. This separation hence affects the rate of interbreeding with other populations which can be overcome through freedom of movement. European and Asian genetics have until recently been characterized by distinctive isolation by distance due to geographical barriers. Europeans are on average, more closely genetically related to the Middle-East rather than to Central and East Asians (Eisenberg and Hayes, 2011). This may imply that even with no natural selection, the historical relationship between populations has created a variation in allele frequencies moving from Western Europe into East Asia (Eisenberg and Hayes, 2011).

In the representative sample of the Maltese population studied, 2 individuals showed the possession of an XL allele consisting of 20 repeats with a genotype and allele frequency of 0.32% and 0.16% respectively. Other uncommon alleles longer than the L allele have been previously reported in other populations (Delbruck et al., 1997; Gelernter et al., 1997 ; Kunugi et al., 1997; Nakamura et al., 2000; Narita et al., 2001). Murdoch et al., (2013), studied 2500 individuals from 47 populations distributed globally for 5-HTTLPR frequencies that may reflect the migration patterns from Africa and Asia into Europe and America. The vast majority of the tested individuals carried the 14 and/or 16 repeats; the S and L alleles respectively. Additionally, two different 20-repeat alleles which had been previously observed in the Japanese (Nakamura et al., 2000; Frisch et al., 2000) and in individuals of African ancestry (Delbruck et al., 1997) were observed in East Asian populations. Alleles showing the 20-repeat pattern were also seen in two West African populations (Yoruba and Hausa), in the Durze population of Southwest Asia and in the Chagga from East Africa (Murdoch et al., 2013).

Hence, the presence of the XL allele in the Maltese population can be reasonably attributed to migration from African and/or Asian populations which has led to the introduction of this allele into the local gene pool. This is further supported by reports that no XL allele was identified in European populations (Noskova et al., 2008; Nonnis Marzano et al., 2008; Fumeron et al., 2002; Willeit et al., 2003; Gutierrez et al., 1998; Surtees et al., 2006; Szekely et al., 2004; Lang et al., 2004) and in adult Maltese populations (Muscat, 2017; Muscat, 2018).

The introduction of new alleles in the local gene pool is not a novel occurrence. A number of studies report potential historical founder effects (Koziell et al., 2002; Farrugia et al., 2007; Vidal et al., 2009) through which specific gene

variants were introduce into the gene pool of the Maltese population and subsequently spread through the population during a phase of population growth. These findings highlight the changing dynamics of population gene pools, the importance of selecting suitably matched controls for case-control studies and the importance of ethnicity information in the design, execution and interpretation of genetic diagnostic tests.

5 Conclusion

In conclusion, the allele frequency distributions of the 5-HTTLPR size polymorphism in the Maltese population are similar to the frequencies reported in other Europe populations, with the addition of the XL allele. This highlights the need for clinician and healthcare service providers to be prepared for the occurrence of conditions not previously encountered in the local population.

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8 Conflicts of Interest

The authors report no conflicts of interest

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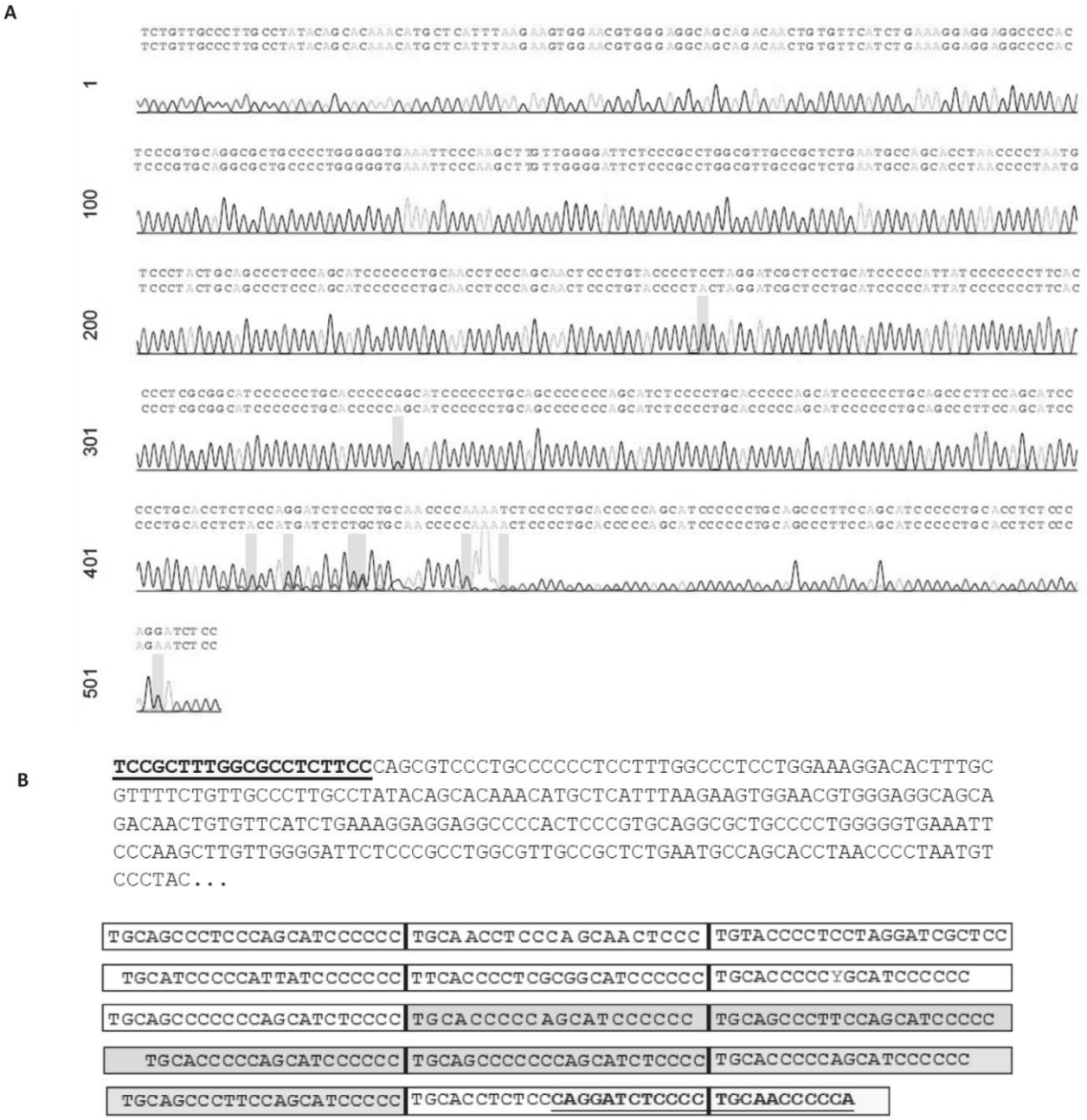
APPENDIX A



Appendix A. (A) Sequence of the 20-repeat XL allele extracted from sanger sequencing data using PolyPeakParser (Hill et al., 2014) showing the sequence of the XL allele. (B) Sequence of the amplified XL fragment. The yellow highlighted nucleotides show primer regions, blue nucleotides show the 44 base pair insertion of the L allele and the green highlighted nucleotides show the longer sequence found in the XL allele.