Human clusterin gene polymorphisms associated with substance use disorders

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Abstract: Previous work revealed that the levels of clusterin in biological fluids are associated with loss of control over eating and with the duration and intensity of tobacco use in humans. In non-human primates, chronic cocaine upregulates clusterin gene expression in the nucleus accumbens, a key area in addiction. All these findings have led to suggest that clusterin could be associated with the presence of different kind of addictive behaviours. In this work possible associations between clusterin gene polymorphisms and drug use disorder were studied. Forty-four selected single nucleotide polymorphisms (SNPs) of the clusterin gene were studied in DNA samples from 499 subjects diagnosed of substance use disorder (addicted to alcohol, cocaine or both) and 500 control subjects. Genotyping was performed by using a multiplexing assay and data were analysed with logistic regression. Four SNPs (rs867231, rs867232, rs9331896 and rs11787077) were found significantly associated with the presence of substance use disorder. These associations further extend the hypothesis that clusterin could be a relevant protein in addiction.

Keywords: Clusterin; Substance Use Disorder; Addiction; Alcohol; Cocaine.

Resumen: ***Asociación entre polimorfismos del gen humano de clusterina y trastornos por consumo de sustancias***

Estudios previos han encontrado una asociación entre los niveles de clusterina en fluidos biológicos y la pérdida de control sobre la ingesta, así como entre dichos niveles y la duración e intensidad del consumo de tabaco. Por otra parte, la administración crónica de cocaína a primates no humanos incrementa la expresión del gen de clusterina en el Núcleo Accumbens, una región cerebral clave en las adicciones. Estos antecedentes sugieren que la clusterina podría estar implicada en distintos tipos de trastornos adictivos. En este trabajo se han estudiado posibles asociaciones entre polimorfismos de un solo nucleótido (*single nucleotide polymorphisms*, SNP) del gen de clusterina y trastornos por consumo de sustancias (TCS). Se genotiparon 44 SNP en muestras de ADN procedentes de 499 sujetos diagnosticados de TCS (adictos al alcohol, cocaína o ambas drogas) y 500 sujetos control. Para la secuenciación y el genotipado se utilizó la plataforma Sequenom y el análisis estadístico de los resultados se realizó mediante regresión logística. Se detectó una asociación significativa entre 4 SNP (rs867231, rs867232, rs9331896 y rs11787077) y la presencia de TCS. Estos resultados apoyan la hipótesis de que clusterina es una proteína relevante en las adicciones.

Palabras Clave: clusterina, trastornos por consume de sustancias, addicción, alcohol, cocaina.

Introduction

Recent proteomic studies performed in morbid obese patients revealed that the plasma concentration of clusterin positively correlated with loss of eating control, one of the main dimensions of food craving in these subjects (Rodríguez-Rivera et al., 2019). Clusterin is a multifunction protein that was already known to mediate the effects of both leptin and ghrelin on appetite (Gil et al., 2013), therefore the possibility exists that changes in the levels or function of this protein could be potentially associated to pathological conditions where problematic eating behaviours are expected to play a key role, i.e. the food addiction phenotype of obesity (Davis et al., 2011). This association could extend to substance use disorders (SUD), if we bear in mind the close relationship reported between the neurobiology of food and drug addiction (Volkow et al., 2013). According to this idea, previous studies in non-human primates revealed that chronic treatment with cocaine alters clusterin gene expression in the nucleus accumbens, a brain area deeply involved in different kind of addictions (Freeman et al., 2001). Recent reports of human studies are in agreement with these findings: thus, salivary levels of clusterin in nicotine addicts depended on the length of tobacco use and the number of daily cigarettes consumed, and decreased after smoking cessation (Pallardo-Fernández et al., 2018). Up to our knowledge the possible consequences of genetic variability in the clusterin gene on the risk of developing addictions have not been studied, despite the fact that this variability is known to be relevant in other diseases where clusterin is also involved, i.e. Alzheimer's disease (Foster et al., 2019). Accordingly, this work has explored possible associations between selected single nucleotide polymorphisms (SNPs) of the human clusterin gene and SUD.

Materials and Methods

Genetic variants of the clusterin gene were studied in 499 subjects diagnosed of SUD according to the Diagnostic and Statistical Manual of Mental Disorders (4th ed., text rev.; American Psychiatric Association, 2000) and 500 control subjects (table 1). The SUD group included patients that abused alcohol, cocaine or both drugs with predomination of males, which is in accordance with the reported sex-biased proportion of addicts that seek treatment (European Monitoring Centre for Drugs and Drug Addiction, 2019).

Table 1. *Main characteristics of the subjects included in the study. Numbers represent means ± SD.*

	Drug addicts	Controls
Sex (females : males)	108 : 391	250 : 250
Age (years)	44.4 ± 10.6	40.2 ± 3.9
Body mass index (kg/m^2)	25.4 ± 4.6	26.2 ± 4.4
Length of drug consumption (years)	21.8 ± 11.4	
Severity of drug dependence (SDSS/DSM-IV score)	7.5 ± 3.1	

DNA samples were collected and processed following standard operating procedures with the appropriate approval of the ethical and scientific committees of the biobanks involved and San Pablo CEU University (USP 191-17). Samples from drug addicts were provided by the Spanish Biobank of the Addiction Disease Network (Biobanco RTA), integrated in the Valencian Biobanking Network (Spain); these samples were prepared by extracting genomic DNA from peripheral blood using the Real Blood DNA kit (Durviz-Real Laboratory SL, Valencia, Spain) following manufacturer's instructions. Briefly, the protocol consists in a four-step process: lysis of red blood cells, lysis of white blood cells followed by RNA digestion, protein precipitation that leaves DNA in the supernatant, and DNA concentration and purification by isopropanol precipitation. The quantity and quality of the obtained DNA was measured by spectrophotometry using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington DE, USA). DNA samples were taken to a final concentration of 50 ng/µl

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and stored at -80 ºC. Samples from control subjects were provided by the National DNA Bank Carlos III (BNADN, Spain); genomic DNA was also obtained in this facility from peripheral blood, using the Gentra Puregene Blood kit (Qiagen, Vedbaek, Denmark). The steps were similar to those followed in the former procedure: red blood cell lysis followed by white cell lysis, protein precipitation and DNA precipitation with isopropanol. The DNA obtained was washed with 70% ethanol, resuspended in DNA Hydration Solution (10 mM Tris, 1 mM EDTA, pH = 7-8), normalized to 100 ng/µl and stored at -80 ºC. The concentration and purity of the DNA was determined with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington DE, USA). A ratio of 260nm/280nm absorbances higher than 1.8 was considered indicative of optimal quality, and integrity was confirmed by loading 1 µl of the samples in 0.7% agarose gel to check the presence of a single band after electrophoresis.

Genomic DNA samples were sent to the Spanish National Center for Genotyping (CEGEN-PRB3-ISCIII) to study 44 SNPs of the clusterin gene with a minor allele frequency greater than 5% that were selected from the dbSNP database (National Center for Biotechnology Information NCBI, USA) (table 2). Samples were normalized to 20 ng DNA/µl with Milli Q water in a Evo Freedom liquid handling robot (Tecan, Männedorf, Switzlerand), then analysed by using a multiplexing platform (Sequenom iPLEX Gold, Agena Bioscience, San Diego CA, USA) based on a simple single-base primer extension assay and Matrix-Assisted Laser Desorption/Ionisation, Time-of-Flight mass spectrometry (MALDI-TOF) for allelic discrimination. Briefly, polymerase chain reaction (PCR) primers were designed in a region of approximately 100 base pairs around each SNP of interest, and an extension primer was designed immediately adjacent to the SNP; PCR products were single-nucleotide-extended using dideoxynucleotide triphosphates (ddNTPs) with some of their atoms substituted in order to generate weight differences between alleles. Mass spectrometry was then used for allele differentiation by weight (Ragoussis et al., 2006).

The genotypes of drug addicts were compared to those of controls to investigate SNPs associated with SUD by using logistic regression. The model obtained fitted with data as shown by the Log Likelihood ratio test ($p = 4.39x10^{-17}$), Nagelkerke's R^2 (0.31) and the percentage of subjects properly classified by the adjusted logistic model (70%). The level of statistical significance was set at $p < 0.05$.

Results

Table 3 depicts four clusterin gene polymorphisms and the corresponding genotypes that were found to be significantly associated with the presence of SUD. As it is shown, three of these SNPs were located within the signal peptide exon, while the remaining one was intronic.

Discussion

The results obtained clearly show the existence of associations between several clusterin gene variants and the presence of SUD. Some Odds Ratios reached high values, which is expected when the corresponding genotype is highly predominant in the total sample (Cerda et al., 2013). These associations further extend the hypothesis that clusterin could be a relevant protein in addiction, as it was suggested by the antecedents previously commented (Freeman et al., 2001; Pallardo-Fernández et al., 2018; Rodríguez-Rivera et al., 2019). A biological interpretation of these results cannot be yet provided, bearing in mind that the functional consequences of the genetic variants detected are largely unknown and thus require additional, specific work to be fully understood. Despite of this, it is important to note that rs867232, rs867231 and rs9331896 could impact the signal peptide function, and hence could influence clusterin transportation and cellular location (Jarjanazi et al., 2008). By turn, the intronic SNP rs11787077 could affect splicing.

A neuroprotective role has been proposed for clusterin after being studied in models of neuronal damage induced by ischemia (May et al., 1992), β-amyloid deposition (Boggs et al., 1996), excitotoxicity (Park et al., 2007) and trauma (Huang et al., 2016). Accordingly, those genotypes linked to altered clusterin function could affect the vulnerability to suffer different diseases related with neuronal injury, such as dementia and drug addiction. In fact, the latter parallelism is observed in the case of rs9331896, since the T allele is associated with higher risk of both SUD and Alzheimer's disease (Lambert et

Table 2. *SNPs selected for this study. MAF, Minor Allele Frequency.*

SNP	Locus	MAF	Type	Wild allele
rs4732729	15319638	0.4726	A/C	C
rs9331936	15315658	0.0653	A/C	A
rs9331931	15316250	0.1478	C/G	C
rs9331930	15316440	0.2636	A/C	C
rs9331923	15319409	0.0581	C/T	C
rs7812347	15318331	0.2640	A/G	A
rs60056423	15317351	0.1480	A/C	C
rs66969288	15317786	0.1480	A/G	G
rs76604252	15319510	0.0597	C/T	T
rs9331908	15321764	0.4734	A/G	G
rs73679246	15321302	0.0715	A/G	G
rs9331888	15327008	0.3335	C/G	G
rs34109053	15328743	0.2198	A/G	G
rs72417182	15329066:15329067	0.2961	--/AG	-
rs17515931	15328342	0.2105	A/C	C
rs73231005	15328924	0.2109	C/G	C
rs867230	15326649	0.3247	A/C	C
rs545243	15328286	0.0603	G/T	G
rs546076	15328357	0.0529	C/T	C
rs867232	15325329	0.1290	C/T	T
rs9331892	15326151	0.0571	C/T	C
rs867231	15325538	0.1264	C/G	C
rs2070926	15325967	0.2352	C/G	C
rs9331896	15325832	0.3828	T/C	C
rs4732731	15334320	0.0673	C/T	C
rs9331950	15312828	0.1030	C/T	C
rs9331949	15312832	0.0966	A/G	A
rs9331942	15313260	0.1200	C/T	T
rs73679247	15323778	0.0731	A/C	A
rs1532277	15324327	0.2360	C/T	C
rs1532278	15324461	0.2636	C/T	C
rs1982229	15332745	0.2342	C/T	T
rs2279590	15314399	0.2406	A/G	G
rs3087554	15313588	0.2420	A/G	G
rs11787077	15323458	0.3786	C/T	T
rs10503814	15312721	0.0575	C/T	C
rs11136000	15322665	0.3782	C/T	T
rs1532276	15324303	0.3706	C/T	T
rs4236673	15323075	0.2346	A/G	G
rs7982	15320627	0.3355	C/T	C
rs9314349	15332348	0.2330	A/G	G
rs9331905	15322390	0.1348	A/G	A
rs9331902	15323947	0.0715	A/G	G
rs536332	15333913	0.3117	A/G	A

Table 3. *Clusterin gene variants significantly associated with drug use disorder. +, increased risk; -, decreased risk; OR = odds ratio; c.i. = 95% confidence interval.*

SNP	Locus	Association	Genotype	OR	c.i.
rs867232	Signal peptide exon	-	A_	0.24	0.06 – 1.00
			AA	0.12	0.03 – 0.50
rs867231	Signal peptide exon	+	G_	4.38	1.08 – 17.72
			GG	8.76	2.16 – 35.44
rs11787077	UTR / intron	+	T_	4.39	1.48 – 13.01
			TT	8.79	2.97 – 26.02
rs9331896	Signal peptide exon	-	C_	0.26	0.09 – 0.77
			CC	0.13	0.04 – 0.38

al., 2013), as well as other kinds of dementia (Nordestgaard et al., 2018). We did not find in the literature additional associations between the remaining three SNPs identified in our study and any other disease. All these findings strongly recommend further investigations on the role of clusterin gene variants and clusterin function in drug addiction.

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