DOCTORAL THESIS

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HUMAN ACYL-COA SYNTHETASE 5 (ACSL5) VARIANTS: FROM GENE TO PATHOLOGY

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HUMAN ACYL-COA SYNTHETASE 5 (ACSL5) VARIANTS: FROM GENE TO PATHOLOGY

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Presented by;

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Don ANTONIO ALCINA MADUEÑO y Doña FUENCISLA MATESANZ DEL BARRIO, Doctores en Ciencias Biológicas,

CERTIFICAN:

Que el trabajo que presenta Dña. Dorothy Ndagire con el titulo **"Human Acyl-Coenzyme A synthetase 5 (ACSL5) variants: From Gene to Pathology"** ha sido realizado en el Departamento de Biología Celular y Inmunología del Instituto de Parasitología y Biomedicina "Lopez Neyra" (IPBLN-CSIC) bajo nuestra dirección, para la obtención del título de Doctora en Biología Molecular e Inmunología**.**

Y para que conste y surta sus efectos en el expedienté correspondiente, expido la presente certificación en Granada, a 13 de Abril de 2012.

Fdo. Antonio Alcina Madueño   Fuencisla Matesanz del Barrio

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Esta Tesis Doctoral ha sido realizada en el Instituto de Parasitología y Biomedicina "López Neyra" (CSIC) y ha podido ser realizada gracias a unas becas de la Agencia Española de Cooperación Internacional (A.E.C.I) y la Fundación Española de Esclerosis Múltiple (FEDEM).

Los resultados de esta Tesis han sido publicados y otros envidos para su publicación con los títulos:

1. Differential expression of RNA isoforms from long-chain acyl CoA synthetase 5 (ACSL5) gene in peripheral blood and leukemic cell lines

2. The ACSL5 gene polymorphisms and potential relevance of its alternative promoters in multiple sclerosis

3. High ACSL5 transcript levels associate with systemic lupus erythematosus and apoptosis in jurkat T lymphocytes and peripheral blood cells

Publicaciones adicionales son resultado de mi participación en diferentes temáticas del grupo de investigación en el que he realizado mi tesis doctoral y indicadas al final de esta memoria.

The single most significant decision one can make on a day today basis is the choice of attitude. It is more important than the past, education, bankroll, successes or failures, fame or pain, what other people may think or say about one's, circumstances, or position. Attitude keeps one going or cripples one's progress. It alone fuels the fire or assaults our hope. When attitudes are right, there is no barrier too high, no valley too deep, no dream too extreme and no challenge too great for anybody.

To my family
In memory of the late, Mr. Blasio Waswa Kyamumi.

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ABBREVIATIONS

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ACR	American college of rheumatology
ACSL	Long-chain acyl-CoA synthetase
AICD	Activated induced cell death
AMP	Adenine monophosphate
AP2	Activator protein
AS	Alternative splicing
ATP	Adenine triphosphate
AUG	Start codon
Bcl2	B cell leukemia/lymphoma 2 *genes*
CASP3	Cysteine-aspartic acid protease
CNS	Central nervous system
CoA	Co-enzyme A
DNA	Deoxyribonucleic acid
EMSA	Electrophoretic mobility shift assay
FA	Fatty acid
IL	Interleukin
Inr	Initiator elements
Io	Ionomycin
LD	Linkage disequilibrium
mRNA	Messenger ribonucleic Acid
MS	Multiple sclerosis
PBMC	Peripheral blood mononuclear cells
PCD	Programmed cell death
PCR	Polymerase chain reaction
PKc	Protein kinase -c
PMA	Phorbol 12-myristate 13-acetate
PPARs	Peroxisome proliferators-activated receptors
PP$_i$	Inorganic pyrophosphatase
qRT-PCR	Quantitative real time polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNA	Ribonuclelic acid
RR	Relapsing-remitting

SI	Stimulation index
SiRNA	Small interfering ribonucleotides
SLE	Systemic lupus erythrematosus
SNP	Single nucleotide polymorphism
SP	Secondary progressive
Sp1	Specificity protein 1
TCR	T-cell receptor signalling
TNF	Tumour necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TSS	Transcription start sites
UTR	Untranslated region

RESUMEN

I.   INTRODUCCIÓN

Las Acil-CoA sintetasas de cadena larga humanas (ACSL, EC6.2.1.3) activan ácidos grasos de cadenas de 12 a 20 átomos de carbono mediante esterificación con coenzima A. Esta activación requiere una reacción de dos pasos: en el primero se forma un compuesto intermedio acil-AMP (usando ATP como precursor), mientras que en el segundo el AMP es intercambiado por coenzima A para producir el acilo activado. De este modo las ACSL se clasifican dentro de la superfamilia de enzimas productoras de AMP (Bar-Tana et al., 1973).

<u>Reacción química</u>

(i)   Acido graso + ATP→ Acyl−AMP −AMP + PPi

(ii)   Acyl−AMP + CoA→ Acyl−CoA + AMP

Las ACSL forman una familia de enzimas que consiste de 5 isoformas si nos basamos en las propiedades de la cadena de los ácidos grasos preferidos, ACSL1, ACSL3, ACSL4, ACSL5 y ACSL6. Así, estas enzimas se diferencian en la especificidad de sustrato, localización subcelular, cinética enzimática, distribución tisular y respuesta a regulación nutricional y hormonal (Van, 2005; Kim, 2001; Lewin, 2001; Coleman, 2000). Hoy en día, estas enzimas reciben considerable atención debido a que sus funciones individuales parecen no ser redundantes. Los acil-CoA producidos son esenciales para la síntesis de lípidos complejos, modificación de macromoléculas (proteínas y ADN), beta-oxidación y regulación de procesos fisiológicos y remodelación de membranas (Soupene and Kuypers, 2008) (figura 1).
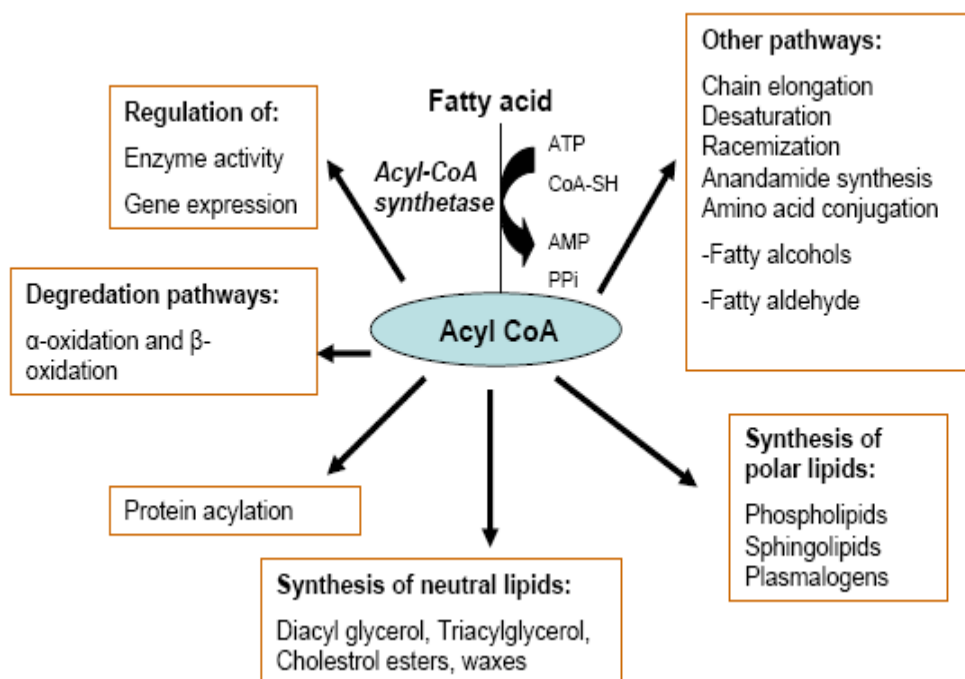
Figura 1. Destinos metabólicos de acidos grasos (AG) activados. La mayoría de las vías del metabolismo celular de AG requieren su activación previa por tioesterificación a la CoA. La isoforma ACS que participa en una vía específica depende con frecuencia del tejido, tipo celular, localización subcelular, y la longitud de la cadena de AG. Además de las vías ilustradas, acil-CoAs pueden ser degradados por tioesterasas que cortan la union del AG: con CoA, y por hidrolasas NUDIX que cortan el enlace de pirofosfato con el resto de CoA.

Varios estudios *in vitro* han asociado los acil-CoA con apoptosis o muerte celular programada y crecimiento tumoral, principalmente debido al efecto que tienen sobre la estabilidad de membranas, señalización celular y porque son precursores de ceramida, diacilglicerol y cardiolipina (Yamashita et al., 2000; Hardy et al., 2003; Heimli et al., 2003). De hecho, la inhibición de la actividad de ACSL se ha propuesto como posible alternativa para inducir apoptosis de células tumorales defectivas para el p53 (Mashima et al., 2005).

ACSL5 es especial por ser la única que se localiza en la membrana externa de mitocondria y presenta varias variantes transcripcionales. La expresión diferencial de las variantes de ACSL5 en diferentes líneas celulares hematopoyéticas podría implicar la producción de numerosas proteínas, la mayoría de las cuales con función

desconocida. En adición a lo expuesto, se cree que variaciones anormales en *splicing* se implican en enfermedad ya que una gran proporción de desordenes genéticos en humanos resultan de variantes de *splicing* (Matlin et al., 2005). Del mismo modo se cree que estas variantes de *splicing* contribuyen al desarrollo del cáncer (Skotheim and Nees, 2007), aunque estos productos aberrantes de *splicing* son, bajo condiciones normales, eliminados por un mecanismo de control de calidad posttranscripcional (Danckwardt et al., 2002). Además, a través del análisis de exones en todo el genoma, el ACSL5 es uno de los 9 genes que muestran el uso específico de tumor TSS alternativo, tanto en el adenoma colorrectal como en las muestras de cáncer (Thorsen et al., 2011)

Varios estudios recientes han mostrado un papel de ACSL5 involucrado en apoptosis; por ejemplo la expresión de ACSL5 se ha correlacionado con el estado de la arquitectura de la villi y la homeostasis epitelial en intestino delgado humano, pudiendo ejercer su función sensibilizando a las células epiteliales a seguir la vía apoptótica inducida específicamente por el ligando TRAIL, y también se ha relacionado con supervivencia tumoral y respuesta a dieta (Reinartz et al., 2010; Mashima et al., 2009; Gassler et al., 2007; Marshek et al., 2006). Por lo tanto, esta enzima podría funcionar como una diana terapéutica en cáncer.

Variaciones en la transcripción génica son importantes en mediar susceptibilidad genética a enfermedades. Polimorfismos del ADN (SNP) identificados en regiones activas de promotores pueden alterar la expresión génica de dicho gen al que afectan y causar una desregulación fisiológica o una enfermedad compleja (Cheung and Spielman, 2009). El SNP rs2419621, localizado en uno de los promotores alternativos de ACSL5 afecta su expresión en células musculares y se asocia a respuesta a la dieta (Adamo et al., 2007). Por lo tanto, la información sobre las distintas isoformas transcripcionales y el uso de promotores alternativos debería ser considerado en todos

los análisis del genoma en numerosas enfermedades genéticas (Pan et al., 2008). Alteraciones de los procesos de *splicing* que afectan a ACSL5 en células intestinales, por otro lado, se han asociado con desarrollo de cáncer colorrectal (Gassler et al., 2007). Sin embargo, aunque se han realizado numerosos estudios sobre ACSL5, todavía no existe información sobre la regulación transcripcional de este gen en diferentes tipos celulares, y con mayor importancia para enfermedades autoinmunes, en linfocitos.

Nuestro trabajo ha consistido en el estudio de la trascripción génica de ACSL5 humana en diferentes líneas celulares hematopoyéticas, así como en PBMC (células sanguíneas periféricas). Encontramos 6 exones iniciales alternativos y sitios de inicio de la transcripción (TSS) móviles. Sin embargo, no analizamos la región 3'-UTR. Observamos diferentes variantes expresadas entre la línea eritroblástica K562 y las demás líneas celulares y PBMC. Localizamos el promotor más importante en la regulación de la expresión de ACSL5 en estas líneas celulares y PBMC. Entre los datos de ACSL5 en la base de datos UCSC se indica a un sitio en el promotor del gen con el que se une el factor de transcripción SP1 como se estudió en las células linfoblastoides GM12878. En nuestro estudio, hemos demostrado mediante el ensayo de EMSA la interacción de esta proteína nuclear con el ADN en la misma región. Además, aunque la contribución de las rutas metabólicas lipídicas a la autoinmunidad están poco esclarecidas, estudios in vivo e *in vitro* indican el involucramiento de ACSL en algunas de las complicaciones asociadas con mecanismos que al final conducen a enfermedades metabólicas y autoinmunes. Dentro de estas se encuentran tanto la esclerosis múltiple (EM) como el lupus eritematoso sistémico (LES), la primera una compleja enfermedad crónica inflamatoria que cursa con desmielinizacion y neurodegeneración en el sistema nervioso central (SNC) (Munger, 2009; Ramgolam et al., 2011), y la segunda una

enfermedad autoinmune sistémica caracterizada por una severa desregulación del sistema inmune.

En EM, los oligodendrocitos, las células productoras de mielina del SNC, son células diana, por lo que la causa que provoca la apoptosis de oligodendrocitos y posterior activación de células gliales, linfocitos y macrófagos y infiltración a través de la barrera hematoencefálica – son eventos críticos en la etiología de la enfermedad (Veto et al., 2010; Watzlawik et al., 2010). Desde que diferentes estudios han mostrado la relación entre obesidad y distintos ácidos grasos con la EM, nosotros evaluamos si determinados SNP en ACSL5 podrían modificar su función de activación de ácidos grasos y por tanto del control de rutas metabólicas y apoptosis en las células. En búsqueda de la asociación del gen de ACSL5 a un riesgo de padecer EM, realizamos un mapeo fino del locus mediante un estudio Tag-SNP.

Una anormal activación de células T y apoptosis marca la patología del LES (Kyttaris et al., 2005). Células T y B potencialmente autorreactivas son eliminadas de la circulación durante la selección negativa en la hematopoyesis, pero en el caso del LES esta regulación esta desnivelada. Varios estudios *in vivo* e *in vitro* indican que ACSL pueden jugar un importante papel en la disfunción inmunologica en modelos de ratones de lupus (Ando et al., 1993; de Calvalho et al., 2008; Posadas et al., 2004). Nosotros hemos investigado la asociación del metabolismo lipídico con LES mediante cuantificación de la expresión de las distintas isoformas ACSL en PBMC de pacientes de LES y controles sanos, junto a otras variables de interés como la importancia del tratamiento, sexo y otras.

II.   Objetivo General

La expresión de los diferentes isoformas de transcritos de la ACSL5 en distintas líneas celulares hematopoyéticas y PBMCs, los niveles de transcripción y la presencia de relevantes polimorfismos puede significar la producción de una multiplicidad de proteínas de diferente calidad y la variabilidad en su cantidad. Objetivo general de este trabajo fue estudio de la función de estos isoformas, desconocida prácticamente en este momento en inmunopatologías, como el lupus eritematoso sistémico (LES) y la esclerosis múltiple (EM), en cuales se supone una desregulación del metabolismo lipídico.

III.   Objetivos especificos

1.   Determinar los sitios de inicio de la transcripción (TSS), las variantes de *splicing* alternativos y polimorfismos de secuencia del gen ACSL5.

2   Determinar los niveles de expresión de cada isoforma de RNA en diferentes tipos celulares, que son: células T Jurkat, monocitos U937, células B Raji, eritroleucemia K562, glioma U87, oligodendroglioma KG-1C y células de sangre periférica (PBMC).

3.   Localizar y definir el promotor funcional de ACSL5 en celulas Jurkat limfoblastoma estimulados con phorbol 12-myristate 13-acetate and Ionomycin (PMA+Io).

4.   Medir los niveles de transcripción de las diferentes ACSLs en PBMCs de pacientes LES y controles sanos y determinar si los niveles de transcripción se asocian con el lupus eritematoso sistémico.

5. Determinar el papel de ACSL5 en la apoptosis inducida durante el proceso de activación in vitro de linfocitos (PBMCs y cellulás Jurkat).

6. Estudio genético Tag-SNP de la asociación de ACSL5 con la susceptibilidad a la esclerosis múltiple (EM).

7. Implicación de los promotores anternativos específicos de tipo célular y polimorfismos de ACSL5 en esclerosis múltiple (EM).

IV. Metodos

IV.1. Recogida y tratamiento de las muestras de Pacientes y controles:

Durante el estudio, se obtuvieron muestras sanguíneas de los pacientes, con esclerosis múltiple o lupus eritematoso sistémico, de acuerdo con los criterios de *Poser* (Poser et al., 1983) y de la American College of Rheumatology (ACR) (Costenbader et al., 2002), respectivamente. Todos los pacientes y controles confirmaron su consentimiento antes de inscribirse en el estudio y los pacientes de EM fueron clasificados como RR (remitente-recurrente) o SP (secundaria progresiva) EM.

Las muestras de ADN utilizadas en el estudio fueron obtenidos de un cohorte de pacientes con esclerosis múltiple de cuatro hospitales ubicados en Andalucía, España (hospitales Clínico San Cecilio y Virgen de las Nieves de Granada, hospital Carlos Haya de Málaga y hospital Virgen de la Macarena de Sevilla). Por su parte, las muestras de ARN fueron obtenidas a partir de PBMC's aisladas de pacientes con lupus del hospital Virgen de las Nieves de Granada. Los controles utilizados en el estudio fueron obtenidos de los bancos de sangre de cada una de las cuatro provincias (Granada-Almería, Málaga y Sevilla).

Para determinar si la ACSL5 está involucrada en la patogenia de la esclerosis múltiple fue realizado un estudio caso-control del gen-candidato, compuesto por 830 pacientes y 974 controles. Una región de 60Kb del cromosoma 10 fue seleccionada en la posición comprendida desde 114,115,874 hasta 114,175,873 (NCBI 36 *Assembly*) y de esta región - 6 Tag-SNPs de la base de datos del proyecto HapMap.

Tag-SNPs en la población caucásica de pacientes y controles fueron genotipados utilizando tecnología Taqman, basada en la actividad 5'-3' exonucleasa de la Taq-polimerasa sobre una sonda de doble marcaje durante la hibridación con la secuencia

diana complementaria y la detección basada en fluoróforo. Las dos sondas discriminan entre los dos alelos del SNP.

IV.2 Cultivo celular, transfección celular, análisis de ADN y ARN, y citometría de flujo

Con el fin de llevar a cabo los estudios funcionales, tanto linfocitos primarios como líneas celulares fueron utilizados, y se cultivaron con métodos estándar. El ADN extraído de muestras de sangre se amplificó mediante la reacción en cadena de la polimerasa (PCR) y los ensayos cuantitativos de expresión de diferentes genes fueron realizados por transcripción reversa del ARN celular y posterior PCR a tiempo realutilizando SYBR Green como fluoróforo.

Para determinar la ubicación del promotor de la ACSL5, diferentes secuencias 5' *upstream* del gen fueron clonados en el plásmido de *Luciferasa* y co-transfectadas con el plásmido reportero *Renilla* en las células T Jurkat. Actividades promotoras fueron obtenidas mediante la medición de expresión de luciferasa normalizada con la expresión de renilla en los extractos celulares.

Ensayo EMSA se utilizó para determinar la cantidad de proteína nuclear específica (factor de transcripción) que se une a secuencias del promotor de la ACSL5. Este sistema utiliza secuencias consenso para llevar a cabo ensayos de competencia y así determinar la unión específica de las proteínas al ADN. El análisis citometría de flujo fue usado a determinar apoptosis tanto en células Jurkat como en PBMCS. La secuenciación del ADN se realizó utilizando un método de secuenciación, el BigDye Terminator.

V.   Resultados y Discusión

V.1.   Articulo 1: Differential expression of RNA isoforms from long-chain acyl CoA synthetase 5 (ACSL5) gene in peripheral blood and leukemic cell lines

Los principales hallazgos de este estudio fueron siguientes. La estimulación con PMA + Io sobre-reguló la expresión de ACSL5 en células T Jurkat, monocitos U937 y eritroblastos K562, lo que sugiere que este gen puede ser modulado por factores que afectan la señalización por el receptor de células T (TCR), causando una mayor absorción de calcio y de activación de las proteínas quinasa C celulares (Huang et al., 2004). También se observó que la estimulación provoca un aumento rápido de la expresión del gen interleuquina 2, mucho antes que la ACSL5, en la activación de las células T Jurkat. Las células B Raji, sin embargo, expresaron ACSL5 sin necesidad de estimulación con PMA + Io.

La caracterización de los sitios del inicio de transcripción (TSS) de la ACSL5 utilizando la técnica 5'RACE reveló la presencia de seis TSS principales, que hemos denominado como 1A-1A', 1A' 1B, 1C, 1D y 2, que significa la presencia de promotores alternativos para este gen. Sin embargo, otro TSS 1A' se identificó desde el navegador UCSC para la ACSL5 y su existencia se determinó mediante QRT-PCR en células eritroblástoides K562.

Varios clones del experimento 5'-RACE que fueron elegidos para representar a los sitios de inicio de las regiones 1B, 1C y 2 representan fluctuaciones en los sitios de inicio de transcripción. Este hallazgo está en línea con los recientes estudios de genoma completo en TSS que demuestran que la mayoría de los promotores no tienen un único TSS sino un conjunto de TSS relacionados con diferentes iniciaciones transcripcionales (Frith et al., 2008). Ninguno de los fluctuantes TSS reveló una secuencia de iniciación

específica consenso como se muestra en algunos genes (Anish et al., 2009).

Este estudio demostró que la cuantificación del ARN de la ACSL5 que parte de diferentes TSS resultó en una clara evidencia de expresión especifica de las distintas isoformas de la ACSL5 dependiente del tipo celular debido al uso de promotores alternativos, como se ha mostrado en la línea celular K562 en diferencia de la de Jurkat, U939, Raji o PBMCs.

La proporción de transcripción alternativa es la principal fuente en la diversidad del transcriptoma y del proteoma en eucariotas superiores, y pueden contribuir al desarrollo de complejas actividades funcionales y a la implicación en diferentes enfermedades (Jacox et al., 2010). La presencia de múltiples perfiles en variantes de expresión de la ACSL5 que son específicas de tipo celular, en diferentes líneas leucémicas y PBMCs, sugiere que sus funciones no pueden ser redundantes; de hecho, muestran una regulación en la expresión de tipo diferencial. Teniendo en cuenta la participación de la actividad de la proteína ACSL5 en apoptosis en tejidos de cáncer diferentes, estos resultados pueden ser útiles para encontrar las funciones específicas de cada isoforma en los diferentes tipos de la leucemia y en células de sangre periférica.

Hemos identificado el promotor de la ACSL5 más abundantemente utilizado en los linfocitos y monocitos. El análisis de esta secuencia promotora en Jurkat reveló que carecía de elementos TATA e INR, lo que lo coloca como *TATA/Inr-null*, elementos típicamente encontrados regulando la expresión de los genes de referencia (Novina y Roy 1996). Una búsqueda posterior de secuencias TATA o CAAT en promotores de otros TSS no mostró ninguna. La presencia de una caja TATA o CAAT a menudo se relaciona con la existencia de TSS y sitios de unión del factor de transcripción SP1, y lo hemos confirmado en este estudio. A pesar de que la presencia de islas CpG es una característica de promotores sin caja TATA (*TATA-less*), nuestro análisis de la

secuencia de la región *upstream* del promotor no mostró islas CpG. El porcentaje de G+C fue inferior al 50%.

Otros estudios han demostrado que la unión de SP1 a los motivos GC estimula promotores tipo *TATA-less* como se muestra en el promotor de la terminal desoxinucleotidil transferasa específica de linfocitos (Smale y Baltimore, 1989).La presencia de sitios de unión de SP1 en el promotor principal de ACSL5 Jurkat (1C) demostrada en nuestro estudio sugiere que estos factores de transcripción pueden modular la expresión de este gen después de la activación con PMA + Io. Los experimentos de luciferasa y de cambio de movilidad electroforética (EMSA), revelaron que el factor de transcripción SP1 se une a la secuencia promotor de la ACSL5 que confiere actividad promotora en Jurkat, por lo que puede estar jugando un papel importante en su transcripción.

V.2. Articulo 2: The ACSL5 Gene Polymorphisms and Potential Relevance of its Alternative Promoters in Multiple Sclerosis

Siguiendo el hecho de que la esclerosis múltiple (EM) es una enfermedad inflamatoria crónica y multifactorial del sistema nervioso central (SNC) que se cree es causada por factores genéticos y ambientales (Tan y Waschek, 2011), este estudio tuvo como objetivo determinar si existe alguna evidencia de que el proceso del metabolismo de los ácidos grasos juegue algún papel en la fisiopatología de esta enfermedad. Con este fin, analizamos la asociación genética de ACSL5 con la susceptibilidad a MS basándonos en varios hechos anteriores, tales como: la asociación de ACSL5 con la apoptosis en células hepáticas (Reinartz et al, 2010.) y diferentes tumores, y la participación de ACSL5 en la constante renovación celular en el intestino delgado humano (Gassler et al., 2007).

Los oligodendrocitos son los principales dianas en las enfermedades autoinmunes como la esclerosis múltiple y en enfermedades desmielinizantes genéticas como la adrenoleucodistrofia ligada al cromosoma X (ALD-X) (Cimini et al., 2003). Estas células producen la mielina cuyas membranas son altamente ricas en lípidos. Por lo tanto, cualquier desregulación de lípidos o del metabolismo de ácidos grasos puede conducir a daño de estas células y por lo tanto al progreso de la enfermedad. Es interesante mencionar que isotipos del factor de transcripción PPAR-delta pueden ser activados por ácidos grasos (Vanden, 1999) en oligodendrocitos. Debido a esto, las enzimas ACSL's tienen un papel crucial en el control de la expresión génica mediante la activación de varios factores de transcripción.

Además, mutaciones en el gen PPAR-gamma, que responde a ACSL5, están asociadas con la respuesta a la dieta (Adamo et al., 2007) y, adicionalmente un estudio realizado por Munger et al., 2009 ha demostrado una relación entre la obesidad y el riesgo de desarrollar esclerosis múltiple.

Los resultados de este estudio caso-control del gen-candidato fueron obtenidos por genotipado de 6 Tag-SNP's en la población caucasica de 824 pacientes con EM y 960 controles del sur de España. Entre los SNP's genotipados sólo dos SNP's (rs2419621 y rs2419629) se asociaron con MS bajo el modelo recesivo. El modelo que se suma rs2419629 a rs2419621 fue estadísticamente significativo y proporcionaba un mayor efecto protector.

Por lo tanto, la asociación dentro de este gen podría explicarse mejor por locus de la enfermedad dos marcados por rs2419621 y rs2419629. Hemos observado, sin embargo, que hasta que se identifique un conjunto más completo de polimorfismos y se genotipe en una mayor colección de casos y controles, no podemos excluir una variante en LD con estos SNP's que determine el/los polimorfismo/s causal/es.Un estudio ha demostrado que

el polimorfismo rs2419621 (C> T) en el promotor de ACSL5 se asocia con la pérdida rápida de peso en mujeres caucásicas obesas y elevados niveles de mRNA de ACSL5 en biopsias de músculo esquelético de los portadores del alelo (T). Este SNP se encuentra a 12 nucleótidos del segundo TSS del gen ACSL5, que es un gen de respuesta a PPAR-γ (Adamo et al, 2007; Teng et al, 2009).

El SNP rs2419621 juega un papel diferencial en la expresión de las isoformas ACSL5 en diferentes líneas celulares como las células que forman la mielina (U-87) y los linfocitos, por lo tanto la asociación de este SNP con EM es importante. Este estudio presentó nuevas evidencias de la relación entre los polimorfismos del gen ACSL5 y susceptibilidad a EM en una población española.

V.3. Articulo 3: High ACSL5 Transcript Levels Associate with Systemic Lupus Erythematosus and Apoptosis in Jurkat T lymphocytes and Peripheral Blood Cells

El objetivo del estudio fue determinar la participación de la ACSL5 en la patogénesis del LES. La contribución de la vía metabólica de lípidos a la patogénesis autoinmune ha sido establecida. Previamente. Se ha demostrado en estudios con ratones autoinmunes (MRL-lpr/lpr) que las células T de esos ratones muestran complejos defectos en la biosíntesis y la rotación de los fosfolípidos de membrana lo que sugiere que estas aberraciones metabólicas de células T contribuyen en las enfermedades autoinmunes (Ando et al, 1993; Tomita et al, 1990).

Para estudiar este tema, se midieron los niveles de transcripción de ACSL5 en PBMC de pacientes con LES y controles mediante qRT-PCR antes y después de la activación con PMA+Io. El análisis de los datos demostró unos niveles de transcripción de ACSL5 significativamente mayor en PBMC's de pacientes de LES en comparación con controles, y esta asociación no fue influenciada por la diferencia en la proporción de

hombres / mujeres. El índice de estimulación de ACSL5 entre PBMC's de controles fue mayor en comparación con los pacientes, posiblemente debido al hecho de que los pacientes con LES presentan un estado persistente de activación de los linfocitos con subsecuente secreción de citoquinas proinflamatorias (Aurămescu et al., 2010). Usando PBMC's de controles y células Jurkat como un modelo para las células T, investigamos el efecto de la activación de células T con PMA+Io y también su efecto sobre la expresión del ARNm de ACSL5 en estas células. Nuestros resultados mostraron que la activación PMA+Io induce muerte celular (AICD) en estos linfocitos, descrito también por Yahata et al., 1999, entre otros.

Basándonos en este hecho, determinamos la función de ACSL5 en la AICD que ocurre en células T Jurkat con el uso de la tecnología siRNA, y encontramos que la expresión de ACSL5 después de la activación con PMA+Io correlaciona con la apoptosis inducida por PMA+Io en PBMC's y en las células Jurkat. Además, el alto grado de apoptosis en los linfocitos T en pacientes con LES correlaciona con la actividad de la enfermedad, ya que el tratamiento con corticosteroides reduce la actividad de la enfermedad y la inflamación en LES, y que de forma interesante correlaciona con una disminución de los niveles de transcripción de ACSL5, lo que sugiere una asociación entre ACSL5, apoptosis e inflamación.

Una característica importante en el LES es la incapacidad del cuerpo para eliminar el material celular apoptótico por fagocitosis lo que conduce a la producción de autoanticuerpos y, por tanto a inflamación crónica. Otros estudios han demostrado que la ACSL5 está involucrada en procesos de escape de apoptosis y supervivencia de células tumorales (Mashima et al., 2009). En nuestro estudio se demuestra sin embargo que juega un papel pro-apoptótico en el proceso de AICD de PBMC's y células Jurkat. Mismo efecto se detectó en células hepáticas en las que se incremento la apoptosis

mediada por el TNF y TRAIL (Reinartz et al., 2010), en referido estudio estos genes se han encontrado sobre-expresados junto a otros pro-apoptóticos como Fas, FasL o Caspasa 3.

VI. Conclusiones

1. Las diferentes líneas celulares exhiben expresión específica de isoforma de ACSL5 según el tipo celular, debido a la presencia de promotores alternativos y numerosos TSS en este gen.

2. Factor de transcripción: Sp1 puede estar involucrado en la regulación de ACSL5 inducida por phorbol 12- myristate 13-acetate y Ionomycin (PMA+Io).

3. Múltiples perfiles de expresión de los tránscritos de ACSL5 específicos de tipo celular sugieren una función que puede no ser redundante, debido a esta regulación específica en cada tipo celular.

4. Los SNPs rs2419621 y rs2419629 están asociados a esclerosis múltiple, el primero ha sido previamente involucrado en regulación de la expresión de ACSL5 en músculo esquelético y en la respuesta a la dieta.

5. Altos niveles de mRNA de ACSL5 están asociados a lupus eritematoso sistémico debido a que provoca el apoptosis espontánea de los linfocitos que ocurre en esta enfermedad.

6. Altos niveles de ACSL5 están asociados a apoptosis en linfocitos y la interferencia de ACSL5 neutraliza la apoptosis inducida por activación en linfocitos.

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DISSERTATION RESULTS

ARTICLE 1

Differential expression of RNA isoforms from long-chain acyl CoA synthetase 5 (ACSL5) gene in peripheral blood and leukemic cell lines

Differential expression of RNA isoforms from long-chain acyl CoA synthetase 5 (*ACSL5*) gene in peripheral blood and leukemic cell lines

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ABSTRACT

BACKGROUND: Diverse type of polymorphisms and differential expression levels of RNA are being determined as potential genetic factors associated with complex diseases. Since long-Chain Acyl-Coenzyme A synthetase 5 (ACSL5) has been implicated in apoptosis, differentiation, cancer cell survival, diet response and systemic lupus erythematosus, we sought to study its transcript variants and their cell-type expression preference in peripheral blood mononuclear cells and different human leukemic cell lines.

RESULTS: By using rapid amplification of 5' cDNA ends technique, quantitative PCR and sequencing of transcripts, we found that the *ACSL5* gene was transcribed from 6 alternative promoters and corresponding initial exons (1A-1A', 1A', 1B, 1C, 1D and 2). There were fluctuations of transcription start sites (TSS) in different exons. No consensus initiator (Inr) or TATA box elements were revealed. The erythroleukemia K562 expressed a very different TSS profile compared to other lymphocyte-monocyte cell lines and peripheral blood mononuclear cells. No sequence changes from exon 3 to 21 were found comparing transcript variants from Jurkat T cells and K562. Luciferase reporter analysis of promoter regions (p1A-1A', p1A', p1B and p1C) uncover a region with maximal promoter activity of 100 nucleotides in p1C as the most active in Jurkat T cells and containing a specific protein 1 (SP1) binding sequence that was predicted by electrophoresis mobility shift assay (EMSA) and by the ENCODE database of UCSC browser.

CONCLUSIONS: These results demonstrate the existence of a broad repertoire of transcription start sites and a cell-type specific expression profile derived from the use of 6 alternative promoters. This suggests a cell-type-dependent expression regulation and the potentiality of producing N-truncated polymorphisms that could play a role in different tissues, developmental stages or diseases.

Background

Human long-chain acyl-CoA synthetases (ACSL, EC6.2.1.3) activate fatty acids with chain lengths of 12 to 20 carbon atoms by esterification with coenzyme A (CoA). Activation of fatty acids requires a two-step reaction catalyzed by these enzymes. In the first step, an acyl-AMP intermediate is originated by using ATP as precursor. The AMP is then exchanged with CoA to produce the activated acyl-CoA. Release of AMP in this reaction defines the super family of AMP-forming enzymes. The acyl-CoAs formed are essential for complex lipid synthesis, protein modification, beta-oxidation, and regulation of various physiological processes and remodelling of membranes [1].

Although differing in fatty acid types, ACSL5 has a substrate preference for C16-C18 unsaturated fatty acids. Confocal microscopy revealed that adenoviral-mediated overexpression of rat ACSL5 in hepatoma cells localized the protein in mitochondria and endoplasmic reticulum [2]. According to database annotations reviewed by Soupene and Kuypers [1], human ACSL5 has two *in-frame* AUG-translational initiators that produce isoforms of different length. A longer protein is initiated at the first AUG codon, AUG1, and a shorter one is initiated at the downstream AUG2. As predicted by signal peptide analysis, these variations could result in different cellular localization.

Several *in vitro* studies have associated acyl-CoAs with apoptosis or "programmed cell death" (PCD) mostly due to their effect on membrane stability, signaling pathways and because these complexes are precursors of ceramide, diacylglycerol and cardiolipin [3-7]. In fact, inhibition of acyl-CoA synthetase activity has been suggested as a novel strategy to induce the death of p53-defective tumor cells [8].

ACSL5 is up-regulated in hepatocyte steatosis causing increased susceptibility to hepatic cell death [9]. Its expression has been shown to define the state of villus architecture and epithelial homeostasis in human small intestines and it may sensitize

epithelial cells to undergo apoptosis specifically triggered by the death ligand TRAIL [10]. It has been shown that ACSL5 can promote cellular surviving under proapoptotic or acidosis (tumoral ambient) conditions [11]. Furthermore ACSL5 activity has also been suggested to have a role in cancer growth [4, 7, 10, 12, 13].

Variation in gene transcription is important in mediating disease susceptibility. DNA polymorphisms identified in the active promoter regions may alter gene expression causing physiological deregulation or complex diseases [14-16]. The *ACSL5* polymorphism rs2419621, located in one of the alternative promoters, affects its expression in muscle cells and is associated with diet response [17]. Isoform information and the use of alternative promoters should be considered in all genomic analyses since they are observed to occur in many genetic diseases. For instance, alterations of the splicing process affecting ACSL5 in intestine cells have been associated with colorectal cancer [10, 13].

The process of T cell activation through the TCR/CD3 pathway has been broadly described. It has been demonstrated an increase in cytoplasmic free calcium and activation of PKC (protein kinase C) as essentials for correct activation and maturation of T cells. Phorbol-myristate-acetate (PMA) is an activator of PKC while ionomycin is an ionofore for calcium and the combination of both drugs is sufficient for stimulation and proliferation of T cells [18].

In the present work, we have studied the human *ACSL5* gene transcription in different leukemic cell lines and peripheral blood mononuclear cells (PBMCs) before and after stimulation with PMA plus ionomycin (Io). We found 6 alternative initial exons of the *ACSL5* gene and fluctuating transcription start sites at alternative exon 1B, 1C and 2 but no other sequence or splicing differences between RNA isoforms. A different profile of transcript isoform expression was observed in erythroleukemia

K562, Jurkat T lymphoma, Raji B lymphoma and U937 monocyte precursor leukemic cells and PBMCs. We localized the major proximal promoter controlling the expression of *ACSL5* in PMA+Io activated Jurkat T cells and showed specific binding of Specific protein 1 (SP1) transcription factor which is also detected in the same region by Chip analysis in GM12878 lymphoblastoid cells (ENCODE transcript factor Chip-Seq, UCSC browser). Studies analyzing all these cell-type specific isoform expression profiles regarding autoimmune diseases are underway.

Results

ACSL5 transcription in the Jurkat T-cells

Since the cells analyzed in this study belonged to the immune system we wanted to study the relationship of ACSL5 expression with the process of cellular activation. To this end, we used PMA+Io to stimulate Jurkat T cells which are activated and differentiated to produce IL2. As shown in Figure 1, the IL2 transcription was produced long before (4 to 8 h) than ACSL5 (24 h). In addition, IL2 expression was much more repressed before stimulus than *ACSL5*. As it is shown in Figure 3 and 4, peripheral blood mononuclear cells (PBMCs) and other leukemic cell lines like U937 (monocytes) and the erythroleukemia K562 cells, produced ACSL5 mRNA at relatively low levels but were highly up-regulated after stimulation with PMA+Io for 24 h. On the contrary, Raji B-cells did not show an inducible pattern of ACSL5 transcription after PMA+Io treatment.

Identification of *ACSL5* transcription start sites in Jurkat T cells

In order to determine the *ACSL5* transcript variants, RNA from PMA+Io activated Jurkat T cells was used for 5' RACE experiments. The PCR products were cloned, sequenced and compared with the GenBank genomic *ACSL5* sequence for homology. We used the UCSC genome browser, assembly NCBI Build 36/hg18 for all coordinates, as depicted in Figure 2A and 2B. Analysis of the cloned sequences showed the starting 5'-end nucleotide designated (+1), which defined the TSS, and the extension of the transcribed segment, which defined the length of the corresponding exon. Figure 2B and 2C represent in red the information from our results and in blue the one already annotated in the UCSC. We observed the presence of different ACSL5 transcripts sequences starting at exons 1A-1A', 1A' Genebank: NM_203379, 1B GeneBank:

NM_203380 , 1C GeneBank: NM_016234 , 1D and 2 GeneBank: NM_016234 (exact position is indicated in additional file 1). The presence of exon 1A', with a promoter region between 1A and 1A', was deduced from the qPCR experiments in the erytroleukemia K562 cell line since it was not expressed in Jurkat (Figure 4D). Exon 1D was obtained from 5'-RACE in Jurkat T cells but it was not detected in any of the cell-types subjected to qPCR experiments. Exon 1A, always detected in combination of 1A', was the most transcribed in K562 cells. Intronic regions between each of these exons and 2nd exon were spliced and therefore, the exonic segments were considered alternative first exons. Sequencing of the transcript variants starting from each TSS up to exon 21 from Jurkat cells, PBMCs and the K562 erythroleukemia cells showed no differences apart from those found in the region of alternative first and 2nd exon as shown in Figure 2.

Exon/ intron junction in the new exonic sequences found

Isoforms 1A and 1D were not described in UCSC browser, so they were analyzed for the presence of splicing-junction sites (Additional file 2). Intronic space between 1A and 1A' was 1259 nucleotide long. Exon-intron and intron-exon junction sequences between exons 1A-1A' (A/GT---AG/A), 1A'- exon 2 (G/GT---AG/G), 1D- exón 2 (G/GT---AG/G) conformed the standard splice site consensus sequences for U2 (major class) introns in pre-mRNA.

Fluctuating transcription start sites

5'-RACE experiments gave us information on the +1 nucleotide position in the RNA transcript of the different TSS. In the genome, this coordinate supplies information regarding the position of the core promoter, which lies adjacent to and overlapping the TSS [19]. Several TSS were found in the exons 1B, 1C and exon 2 for which we

obtained several RACE clones, as shown in Figure 2C and additional file 1. The analysis of sequences from -9 to the +2 nucleotide position in the different TSS did not reveal a specific initiator nor a consensus sequence (not shown). However, the analysis of TSS of exon 1C revealed a partial homology with the consensus sequence XCEP2, initially found in mRNA of HBV and then in many other genes [20]. These data are indicated in additional file 3. Fluctuating TSSs have been found in many genes and are being annotated in RNA-seq databases as "http://dbtss.hgc.jp/".

Quantification of *ACSL5* transcript variants in different cell types

We went ahead to quantify the *ACSL5* transcript variants in different cell-types using RNA from activated and non-activated cells. This was performed by means of qRT-PCR relative to the gene expression of the ubiquitin-conjugating enzyme UBcH5B [21] with primers that hybridized to the corresponding exons (Figure 3A).

As shown in Figures 3 and 4, alternative exon 1C was the principal initial exon from which transcription occurred in leukemic lymphocytes (Jurkat and Raji) and monocytes (U937) after activation with PMA+Io as well as in PBMCs. Alternative exon 1B was expressed at a much lower level than 1C and 2nd in all of these cell-types. Traces of exon 1A-1A', 1A' and 1D must exist in Jurkat cells since they were cloned and sequenced in the 5'-RACE experiment though were undetected in quantification experiments. On the contrary, the erythroleukemia K562 showed a completely different *ACSL5* variant expression profile. In this case, the most abundant transcript variant started at exon 1A' compared with transcripts from exon 1C followed by 1A-1A', 1C and 1D. Variants that started in exon 1D were not detected by qRT-PCR in any of these cells.

Determination of the active promoter in lymphocytes

In order to determine the alternative promoter region driving the expression of the different *ACSL5* isoforms in lymphocytes, luciferase reporter plasmid constructs containing the 5' upstream region of the alternative exons (1A, 1A', 1B and 1C) were generated (Figure 5). The constructs, together with renilla luciferase vector as normalizing transfection control, were transfected into Jurkat T cells. Results showed that apart from the -354/-45 plasmid construct with promoter activity lower than 1 fold, all other constructs (766/-45, -679 /-45 and -454/-45) in the upstream region of exon 1C, demonstrated promoter activity approximately 4-5-fold higher than the empty plasmid control (PGL). The 100-bp fragment in the construct P1C (-454/-45) which was absent in the P1C (-354/-45) produced the highest fold increase of luciferase activity. Therefore this region was considered the binding site of transcription factors (TFs) responsible for the expression of the gene. The rest of constructs containing the 5' upstream regions of the alternative exons 1A, 1A' and 1B showed expression activity at the background level (PGL), and therefore, we considered all these promoters not active in Jurkat T cell under stimulation with PMA+Io.

ACSL5 promoter region analysis

We used the information on the *ACSL5* gene in the UCSC database (http://genome.ucsc.edu/) as reference for our study (Figure 6). From Ref-seq, three transcript isoforms 1A', 1B, and 1C (in blue) and corresponding promoter regions are annotated. Exon 1A' is shorter than the one we described in this work and no information was found on exon 1A and 1D (Figure 6). From ENCODE, DNA regions corresponding with the promoters of the different *ACSL5* variants showed different epigenetic marks but nothing in the upstream region of the exon 1A for which we found promoter activity in the erythroleukemia K562. The promoter 1C lacked a TATA box.

Generally, TATA-less promoters may be regulated by the involvement of SP1 (Specificity protein 1)-like factors [22] and CpG islands. SP1 binding was reported in the ENCODE transcription factor ChIP-seq as is observed in Figure 6. However, we searched the presence of CpG islands upstream from the TSS 1C using the EMBOSS-CpG plot program and did not find any, the %G + %C was less than 50%.

Identification of transcription factors

Using computer based tools such as Genomatix Matinspector (http://www.genomatix.de), TFSEARCH (http://www.cbrc.jp) and UCSC, we selected 6 potential transcription factor (TF) binding sites for SP1, IK3, CREB, ETS, SRY, AMA-1A upstream of exon 1C that could be potential transcription enhancers lying within the 100-bp fragment with maximum promoter activity.

SP1 binding is reported in the UCSC, ENCODE transcription factor ChIP-seq using GM12878 lymphoblastoid cells in the same region (Figure 6D). Therefore, we went ahead and determined if the predicted SP1 binding site in this study had the potential to interact with nuclear proteins in this region.

Firstly, qRT-PCR was used to determine the expression of SP1 gene in Jurkat before and after PMA+Io stimulation (Figure 7B). Electrophoretic mobility shift assay (EMSA) with radiolabeled probes were used to assess the interaction of the mentioned transcription factor with the specific promoter sequences. The oligonucleotide with SP1 binding site from -454 to -426 was retarded indicating DNA-protein interactions (Figure 7A). PMA+Io activation did not appear to alter SP1 binding.

To evaluate the sequence specificity of the binding to these oligonucleotides, we performed competition EMSA using 50-fold molar excess of unlabeled probes with consensus sequences. The specificity of the protein binding was demonstrated as their

formation was inhibited by competition with molar excesses of the unlabeled

homologous oligonucleotides for SP1 wild (Wt) type probes). Mutant probes (Mut

probe) did not compete. Taken together, these results demonstrated that this

transcription factor interacted with its corresponding binding sequence located in the

promoter 1C of *ACSL5* gene.

Discussion

Recently, high level ACSL5 mRNA has been associated with systemic lupus erythematosus (SLE), a systemic autoimmune disease producing a wide repertoire of complications, as well as with in vitro apoptosis of lymphocytes [23]. On the other hand, by means of genome-wide exon array analysis ACSL5 is among the 9 genes showing tumor-specific alternative TSS usage in both colorectal adenoma and cancer samples [13]. With this precedents, we determine the variety of ACSL5 RNAs and their level of expression in peripheral blood cells and in different types of leukemia cell lines which can be of great interest as base for studying its pathological activity in the immunological system.

We found the existence of 6 alternative initial exons (we called 1A-1A', 1A', 1B, 1C, 1D and 2) and their corresponding promoters that showed a cell-type specific expression profile. TSS at exon 1A' was found in the qPCR experiments in K562 and from UCSC, though the size we report and the one annotated in UCSC browser database are different. Alternative initial exon 1D, though isolated by 5'-RACE from Jurkat T cell was not practically detected by qPCR experiments in the cells used in this study. Intronic sequences at exon-intron junction sites between 1A-1A', 1A' - exon 2 and 1D-exon 2, conformed to the standard splice site consensus sequences for U2 (major class) introns in pre-mRNA which supports the possibility not to be artefacts, especially exon 1D which was not detected by qPCR. The analysis of the different TSSs (from -9 to +2) did not reveal a specific sequence, however some of the sequences in exon 1C showed partial similarity with a new core promoter element named XCPE2 (X core promoter element 2) which has been found initially in hepatitis B virus X gene mRNA and later in many other TSS-containing TATA-less promoters [20] (see Additional file 3).

We did not find differences in the nucleotide sequence from the exon 3 up to the 21 in the different isoforms either from one cell or distinct cell-types (Jurkat vs K562). The splice variant ACSL5Δ20 that lacks 72bp corresponding to exon 20, assumed to be of functional relevance for death ligand-induced apoptosis of enterocytes [10] was not found in Jurkat and K562 cells. We did not study the 3'-UTR (untranslated region). So, the only apparent consequence of this 5' variability is the potential generation of mRNA isoforms bearing different 5'-UTR and the location of the first *in-frame* translation initiation codon (AUG) in the corresponding mRNAs. Some of the ACSL5 transcripts starts downstream of the annotated translation start codons (AUG) located in exon 1C and 2 [1], so these RNAs have to initiate translation at the next in-frame AUG start codon with optimal context of nucleotides [24]. These possible N-truncated ACSL5 variants might be functionally important since they would affect signal sequences and be targeted to other cellular compartments [1]. Similarly, the sequence analysis of the alternative exons did not find N-terminally extended variants. In most cases, the significance of such "translational protein polymorphism" for cellular and organellar proteomes is unclear [24, 25].

The quantification of the RNA levels serves as an initial proxy for the activity of the corresponding promoters [26]. Thus, we observed two completely different TSS profiles: one for Jurkat, Raji, U937 and PBMCs (lymphocyte-monocyte group) and the other for the K562 erythroleukemia cell line. This differential activity of the alternative promoters between K562 and the rest of cells indicates an RNA transcription regulation specific of different cellular types. The lymphocyte-monocyte group transcribed mainly the isoforms from promoter 1C and much less from promoter 1B and exon 2, whereas in K562 it was mainly from 1A and 1A', and much less from 1B, 1C and exon 2. Therefore, the expression profiles of different isoforms were specific of cellular type

even though all cells were treated with the same stimulant (PMA+Io), suggesting that the transcription regulation of this locus was determined by an active feature of the cells, probably specific transcription factors. These results are supported by a recent study in which the ACSL5 has been identified as one of the genes showing tumor-specific TSS usage in adenoma and different cancer samples [13].

Polymorphisms identified in these promoter regions may represent candidates for altering gene expression and causing complex diseases [15, 16, 26, 27]. Exon 1B contains a functional polymorphism (rs2419621) that regulates *ACSL5* expression in skeletal muscle and associates with diet-induced weight loss [17, 28]. This promoter was active in all cells analysed in this work, although at much lower level than the promoter 1C in lymphocyte and monocytes as well as lower than 1A-1A' in K562. The T allele of rs2419621 polymorphism creates a functional cis-regulatory E-box element (CANNTG) that is recognized by the myogenic regulatory factor MyoD promoting *ACSL5* transcription in skeletal muscle cells. In addition, the T allele, in conjunction with a PPARgamma2 polymorphism (SNP Pro(12Ala)), has been associated with more rapid diet-induced weight loss whereas the G allele showed a significant decline [17, 28]. Therefore, the rs2419621 polymorphism is functionally relevant in the cell-types that transcribe *ACSL5* from alternative exon 1B.

The analysis of the promoter region upstream of exon 1C, the most active in the lymphocyte-manocyte group, neither revealed a TATA-box nor a specific TSS initiator sequence, rather mRNAs started in several positions along the exon 1C sequence. Our results are in agreement with a recent genome-wide study of TSSs revealing that most core promoters do not have a single TSS, but an array of closely located TSSs with different rates of initiation [29]. Thus, we can consider *ACSL5* gene in the category of TATA-/Inr-`null´ elements.

TATA-less promoters are characterised by the presence of multiple transcripts, SP1 transcription factor binding sites and CpG islands. However, *ACSL5* did not show CpG islands. TATA box and CpG-island are two of the most-characterized promoter features related with tissue specificity. The majority of tissue-specific genes possess neither CpG-island nor TATA-boxes in their core promoters (60.2%) to whose group *ACSL5* belongs. TATA- / CpG+ promoters constitute more than 78% of mammalian housekeeping genes [30]. TATA-less promoters are regulated by the involvement of SP like factors which act as tethering agents to recruit specific transcription factors and bind to CpG rich motifs. The analysis of the *ACSL5* promoter sequence 1C and EMSA demonstrated the physical presence of SP1 response elements. Annotated data for the promoter region of exon 1C in the UCSC database from ENCODE indicate that it binds SP1 as studied in GM12878 lymphoblastoid cells, in the same region. SP1 transcription factor is constitutively and ubiquitously expressed in housekeeping and tissue-specific genes and distributed in the promoters, enhancers and locus control regions [31]. *ACSL5* is regulated by PPAR-gamma transcription factor but we did not find any DNA binding-site for it (a PPAR-gamma response element), it is possible that there is either an indirect PPAR gamma regulation or an interaction between PPARgamma and SP1 as described for the human resistin gene [32].

The human *ACSL3* and *ACSL4* promoters have binding sites for AP2, NF-Y, c-Myb and SP1, AP2, GCF, CREB, respectively [33, 34]. The presence of SP1 binding site in the luciferase construct with the highest promoter activity might qualify it as a potential regulator of the *ACSL5* gene, along with other transcription factors detected by the computer based search tools. SP1 plays an important role in promoter function during the activation process of T cells and it has been described as involved in inducible expression of several genes. Binding of SP1 to the GC motif sites stimulates TATA-less

promoters [22]. SP1 transcripts were not only highly expressed in activated and non activated Jurkat T cells but also demonstrated their capacity to bind to specific DNA sequences in the promoter with maximum luciferase activity. Thus, these results suggest that SP1 transcription factor may be playing a role in the *ACSL5* transcription in Jurkat cells. However, we have not determined the functional activity of SP1 site with a direct test because under PMA+Io stimulation, there was no difference in expression of the SP1 gene and protein.

ACSL5 has been involved in cell development and maturation, physiopathological processes, apoptosis and tumorigenesis [4, 7, 9, 10, 12, 13, 23, 35-37] what highlights the importance of this study and suggests the need to address the complete profile of ACSL5 expression in different cell types and pathological conditions.

Conclusions

ACSL5 has been involved in cell development and maturation, physiopathological processes, apoptosis and tumorigenesis. Recently, high level ACSL5 mRNA has been associated with systemic lupus erythematosus (SLE) as well as with in vitro apoptosis of lymphocytes. On the other hand, by means of genome-wide exon array analysis, alternative TSS usage in colorectal adenoma and cancer samples has been shown for nine genes including the ACSL5. We have found that ACSL5 gene transcribes a broad repertoire of different RNAs derived from the use of 6 alternative promoters and many fluctuating transcription start sites. This RNA variability shows a cell-type specific expression profile suggesting a precise regulation and the potentiality of producing many N-truncated protein variants that could play a role in different tissues, developmental stages or diseases. In spite of these suggestive precedents, this study is the first to approach ACSL5 diversity in leukemic cell lines and peripheral blood cells at

the transcriptional level as base to clarify the complex panel of effects of ACSL5, which might be a good example of a gene whose final function could depend on the proportion of each isoform or variant.

Methods

Cell Cultures

Jurkat cells (T cell lymphoma), Raji B cells (human B lymphocyte Burkitts lymphoma), U-937 monocytic cells (human leukemic promonocyte lymphoma cell line) and K-562 eryhtroleukemia cell line, all of them can be found in American Type Culture Collection (ATCC). PBMCs (peripheral blood mononuclear cells) came from anonymous blood donors at the Blood Bank of Granada, Spain, from unprocessed blood for-transfusion. Thus in this case ethics committee approval and confirmation or informed consent was not a requirement.

Jurkat cells, Raji cells, U937 cells and PBMC were cultured in RPMI 1640 medium supplemented with gentamycin. K562 cells were grown in Dulbecco's Modified Eagle medium (DMEM) (PAA laboratories (GmbH) Austria) that was supplemented with 50U/ml penicillin/50μg/ml streptomycin. All culture medium contained 10% foetal bovine serum. Incubation was done at 37oC in a 5% humidified CO_2 incubator. Stimulation of cells was done using phorbol-12-myristate-13-acetate (PMA) (50 ng/ml) and ionomycin (Io) (10 ng/ml) as previously described [15].

Purification of RNA and quantitative real-time PCR (qRT-PCR)

Total RNA was purified from each cell-type using RNeasy kit (QIAGEN Cat.no. 74134). cDNA synthesis was carried out with superscript III reverse transcription reagents and random primer according to the manufacturer's instructions (Invitrogen). qRT-PCR measurements were performed in triplicates each using the Biorad 2x Syber green Master Mix in a Biorad Mini Opticon cycler with the primers for ACSL5 indicated in each case. Relative RNA levels were calculated by the comparative cycle threshold method with respect to the house keeping gene ubiquitin-conjugating enzyme

UBcH5B (also called UBE2D2) [21] (Ensemble transcript ID, ENST00000398733) using the following primers: sence (5'- AAGAGAATCCACAAGGAATTG -3') and antisence (5'-TGAAGAGAATCCACAAGGAATTGA-3'). All primers for quanitification of genes are indicated in Additional file 4.

Determination of ACSL5 transcription start sites (TSS) and quantification

Rapid Amplification of the 5'-cDNA ends (5'RACE) was performed using the SMART RACE cDNA Amplification Kit according to the manufacturer's instructions (Clontech). Briefly, to synthesize 5'-full length cDNA from Jurkat cells, a total of 600 ng of mRNA, was combined with SMART II A oligonucleotide, 5'-CDS primer and MMLV reverse transcription reagents. The 5'-end DNA fragments were amplified using Advantage 2 PCR kit using universal primer mix and a gene specific primer: 5'-GCCATGTGTAGGGCTGG TTTGGTTTTCT-3'). The resultant PCR products were cloned into the PGEM-T Easy vector (Promega) and clones analyzed for homology with the ACSL5 gene sequence available in the GenBank database. The primers indicated in additional file 4 were used to quantify the amount of ACSL5 transcripts from each of the transcription start sites (TSS) in the different cell types analysed.

Sequencing of ACSL5 transcripts from Jurkat and K562

cDNA was synthesized from Jurkat and K562 RNA. A forward primer from the start of the alternative exon 1B, 1C and exon 2 and a reverse primer in exon 21 of the ACSL5 gene were synthesized (Primer sequences in additional file 6). cDNA was amplified polymerase chain reaction (PCR) using each primer set. The bands were visualized on agarose gel. The PCR products were cloned into a pCR II-TOPO vector. Sequencing was done using the BigDye Terminator v3.1 Cycle Sequencing method.

Construction of plasmids for promoter analysis in Jurkat cells

All ACSL5 promoter fragments were cloned into pGL4.10 luciferase reporter vector (Promega) and sequenced. Construct naming is based on the positions of the promoter fragments relative to translation initiation site in exon 1C. DNA constructs with progressive deletions from the 5' region of the *ACSL5* promoter 1C were engineered by PCR from human genomic DNA and plasmid constructs of promoters for 1A, 1A' and 1B were made. The PCR products were sub-cloned into pCR-blunt II-TOPO vector. The fragments were restricted out with Xho I and Kpn I and sub-cloned into pGL4.10.basic vector (Promega). The constructs included; 1A-418bp (-22942/-22525), 1A'- 524bp (-21307/-20784), 1B- 495bp (-20161-19667), 1C- 722bp (766/-45), 635bp (-679/-45), 410bp (-454/-45), 310bp (-354/-45). Primers for cloning are indicated in additional file 5.

Transient transfections and analysis of luciferase activity

Transfections were performed with Nucleofector kit V (Amaxa Bosystems) according to the manufactures instructions. Quantification of luciferase activity was performed as already described [15]. Briefly, 1×10^6 cells were transfected with 1 μg of the various ACSL5 promoter constructs or pGL4.10 basic vector and 0.2 μg of renilla luciferase reporter gene plasmid pRL-TK (Promega) for normalizing transfection efficiency. Twenty hours after transfection cells were stimulated for 24h. Cells were harvested and washed twice with phosphate-buffered saline (PBS), lysed with passive lysis buffer (Promega) and 30 μg of protein was assayed for both firefly and renilla luciferase activities using the FB12 luminometer (Berthold Detection Systems).

Bioinformatic analysis of ACSL5 promoter region and data sources

The putative binding sites of transcription factors within the 5' regulatory region of the *ACSL5* gene were analyzed with the following software packages: MatInspector 8.0 (http://www.genomatix.de) and TFSEARCH (http://www.cbrc.jp). Genomic annotations (RefSeq annotations, mRNAs, ENCODE and ChIP) were obtained from the UCSC database (http://genome.ucsc.edu). TSS database was consulted for complement our data at (http://dbtss.hgc.jp/).

Extraction of the cytosolic and nuclear proteins

The extraction was done using the ProteoJet Cytoplasmic and nuclear protein extraction kit K0311 (Fermentas) at $4\,^{o}$C. Briefly, 5×10^{6} Jurkat cells were washed in PBS and lysed in buffer containing 0.1 M DTT and protease inhibitor cocktail. The cytoplasmic fraction was separated by centrifugation. The nuclei pellet was then washed twice with nuclei washing buffer containing 0.1M DTT and protease cocktail inhibitor, nuclei lysis reagent was added to the nuclei suspension. The mixture was vortexed and incubated on a shaker for 15 min, the nuclear lysate was cleared by centrifugation. The supernatant was stored in aliquots at $-80\,^{o}$C.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using five pmol of the following double stranded oligonucleotide that was labelled as probe with 25 μCi of $(\gamma\text{-}^{32}\text{P})$ ATP (PerkinElmer) and T4 polynucleotide kinase (New England Biolabs) for 40 min at 37 ^{o}C (only sense chain is indicated): SP1, 5'-AAGGCAGGCAGCTCTGGCGACTGGAGAGAG GCGGGGTA-3'. The 35000 cpm of labelled and purified probes were incubated with 3 μg of Jurkat nuclear protein extract from untreated or 24 h PMA+Io activated cells, binding buffer (7.5 mM HEPES pH 8; 35 mM Nacl; 1 mM $MgCl_2$; 0.05 mM EDTA;

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7.5% glycerol; 0.5 mM DTT), 1 µg of poly(dI-dC) and 1 µg bovine serum albumin (BSA). For the competition assays, following unlabelled consensus oligonucleodite and its correspondent mutated form was added for 30 min prior to addition of labelled probe: SP1 consensus, 5'-ATTCGATCGGGGCGGGGCGAGC-3'; SP1 mutant, 5'-ATTCGATCGGttCGGGGCGAGC-3'. Reactions were carried out for 30 min on ice and analyzed by 4% polyacrylamide gel electrophoresis in 0.5 x TBE buffer and followed by autoradiography.

Statistics

Paired Student T test was performed for comparisons between control and PMA+Io activated cells. Errors bars are presented as mean (standard deviation) from three independent experiments. All analyses used a 2-sided level of significance of 5% (P< 0.05).

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

DN performed the qPCRs, luciferase constructs, statistical analysis and participated in drafting the manuscript. MF performed the RACE experiments and quantification of luciferase activity. ACR participated in qPCR experiments, cell growth and participated in drafting the manuscript. RBM maintained the leukemic cells, extracted RNA from cell lines and PBMCs. JAL conceived of the study and participated in its design and drafting the manuscript. FM conceived and designed of the study, sequence analysis with databases and performed original preliminary qPCR experiments. AA conceived and designed of the study and wrote the paper and all authors contributed to revising it. All authors read and approved the final manuscript.

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Figures

Figure 1 -Time course of ACSL5 and interleukin 2 (IL2) RNA expressions in activated Jurkat T cells

Cells were cultured with an optimal concentration of PMA+Io for 4 h, 8 h and 24 h. RNA expression was quantified relative to the *UBcH5B* RNA determined by qRT-PCR. Results are given by means of three independent experiments and bars show the standard deviation. *P-value* has been calculated with paired Student *t* test.

Figure 2 - ACSL5 transcription start sites (TSS) expressed in Jurkat T cells as determined by 5'-RACE

(**A**) Location of the *ACSL5* TSS, in red are those identified in this study and in blue are TSS from the UCSC database. (**B**) Scheme of ACSL5 genomic organization in which boxes in blue stand for exons whose position have been obtained from UCSC database (Chr 10, NCBI36/hg18). Boxes (red) and numbers in black stand for exons found in this work. Intronic regions are represented by black thin lines between boxes. Translation initiation codons in DNA (ATG) and their positions within the corresponding exons, as well as one functional polymorphism (rs2419621) [17] (GenBank sequence GI: 224589801) are indicated**. (C)** Results from 5'-RACE experiments using RNA from PMA+Io activated Jurkat cells are indicated. Transcripts in blue (thick line) and their size in nucleotides represent the ACSL5 exons annotated in UCSC data base. Dotted lines represent spliced sequences. Transcripts in black (thick lines) represent the number of clones and their position respect to the ones from UCSC database. Only one cDNA clone containing 1A-1A'-2 exons and one clone containing 1D-2 exons were found. The existence of a TSS at exon 1A' was deduced from quantification experiments in K562

(Figure 4D) and confirmed from UCSC annotations. Exact position of the different

TSS, ATG and rs are indicated in additional file 1. Sequence for intron-exon junctions

1A-1A'-2 and 1D-2 is shown in additional file 2.

Figure 3 - Quantification of ACSL5 RNA and alternative isoforms from PBMCs

(**A**) Schematic representation of *ACSL5* gene and primer design for amplifying total

RNA and alternative isoforms. Exact position of primers hybridization is indicated in

additional file 4. (**B**) Quantification by qRT-PCR of purified RNA isolated from

nonactivated cells (NA, green) and PMA+Io activated (ACT, red) PBMCs, using

primers that hybridized within each alternative exon, indicated as 1A, 1A', 1B, 1C, 1D

and at exon 2; and exon 7 and 8 for quantifying total RNA. Results were from three

independent experiments and bars show the standard deviation.

Figure 4 -Quantification of ACSL5 RNA and alternative isoforms in leukemic cell lines

Quantification by qRT-PCR of purified RNA isolated from nonactivated control (NA)

and PMA+Io activated (ACT) Jurkat T cells (**A**), Raji B cells (**B**), U937 monocyte cells

(**C**) and K562 erythroleukemia cells (**D**), using primers that hybridized within each

alternative exon indicated as 1A, 1A', 1B, 1C, 1D and exon 2; and exon 7 and 8 for total

RNA. Results were from three independent experiments and bars show the standard

deviation. *P-value* has been calculated with paired Student *t* test. Primer design is

represented in Figure 3A and exact position is indicated in additional file 4.

Figure 5 - Determination of active promoter for ACSL5 transcription in lymphocytes

Schematic illustration of the *ACSL5* promoter region subjected to 5′to 3′ deletion constructs and cloned in the luciferease (LUC) reporter pGL4.10 vector (red lines). The size and exact position of the DNA constructs are referred to the translation initiation codon (ATG) of the exon 1C (position +1). Exact position of the fragments upstream of exon 1C for amplification and cloning is indicated in additional file 5. Relative position of potential transcription factor SP1 located in the relevant clones is indicated in the genomic scheme. Luciferase activity of *ACSL5* deletion constructs in activated Jurkat T cells is also indicated. The four promoter constructs for promoter 1C plus the promoter constructs for 1A-1A', 1A' and 1B, including the pGL basic vector, and the pRL-TK (renilla) plasmid were cotransfected into Jurkat cells. Firefly luciferase activities were measured 24 hours after cell stimulation with PMA+Io. Values were normalized by renilla luciferase activities and represented as the mean ± SD from at least three separate experiments, each done in triplicates.*** $P < 0.001$ versus control group by *t*-test, ** $P < 0.01$ and * $P < 0.05$ between groups.

Figure 6 - UCSC annotations for human ACSL5 gene (NCBI, Bild 36)

(A) Exon/Intron structure of *ACSL5* promoter region according with the data obtained in this work in red. **(B)** mRNA transcripts from Ref-Seq. **(C)** Schematic representation of regions with histone methylation and acetylation in different cell lines from UCSC ENCODE. **(D)** Schematic representation of regions with transcription factors binding by ChIP-seq from UCSC ENCODE Chip annotations.

Figure 7 - SP1 transcription factor binding to promoter DNA sequence and expression in Jurkat cells

(A) Gel shift binding assays with [32P]-labelled *ACSL5* promoter sequences from upstream of exon 1C: -454 to -426 (probe SP1) in the presence of nuclear extract from activated lanes (2, 4 and 6) or nonactivated 1, 3 and 5) Jurkat T cells. Competition for binding was carried out with 50-fold molar excess of the unlabelled consensus oligonucleotides (Wt probe: lanes 3 and 4) or with 50-fold molar excess of mutant consensus oligonucleotides (Mut probe: lanes 5, 6). Arrows indicate specific transcription factor bound proteins. **(B)** Relative transcript levels of transcription of SP1 in PMA+Io activated (+PMA/Io) and nonactivated control (CTL) Jurkat T cells, quantified by qRT-PCR using primers that amplify SP1 (Additional file 4).

Additional files

Additional file 1 - Tables with exact position of TSS, ATG and a SNP

Includes the names of the different alternative initial exons (1A, 1A', 1B, 1C and exon 2) and a list of the different transcription start sites (TSS) obtained from the 5'-RACE experiments for each alternative transcript variant. The numerations were obtained from chromosome 10 (ncbi36/hg18).

Sequences from -9 to +2 are included for each TSS. The single nucleotide Polymorphism (SNP) with its sequence and ATG positions on chromosome 10 (ncbi36/hg18) are indicated.

Additional file 2 - Exon-intron sequences of 1A-1A' and 1D-2

This file includes the exon-intron and intron-exon junction sequences for 1A-1A', 1D-2 and sequences for 1A, 1A', 1D and exon 2.

Additional file 3 - TSS homology with XCPE2 sequences

The TSS homology of the different fluctuating TSS of transcripts that start in 1C with the XCEP2 consensus sequence is shown. Sequences from position -9 to the +2 nucleotide of each clone were aligned and analysed for similarity with the XCEP2 Inr element shown to occur in many genes. Nucleotides matching the XCEP2 consensus sequence are labelled in red.

Additional file 4 - Primers for qPCR and their chromosomal location

Shows a list of primers used in quantitative real time PCR for the different transcript variants labelled as 1A, 1A', 1B, 1C and exon 2, SP1 transcription factor and Interleukin 2 (IL2).

Additional file 5 - Primers for cloning in luciferase reporter vector

The table includes the primers used for PCR amplification of the promoters for 1A, 1A', 1B and 1C. The chromosomal location of each primer on chromosome 10 (ncbi36/hg18) is shown.

Additional file 6 - Primers for amplification and sequencing ACSL5 variants

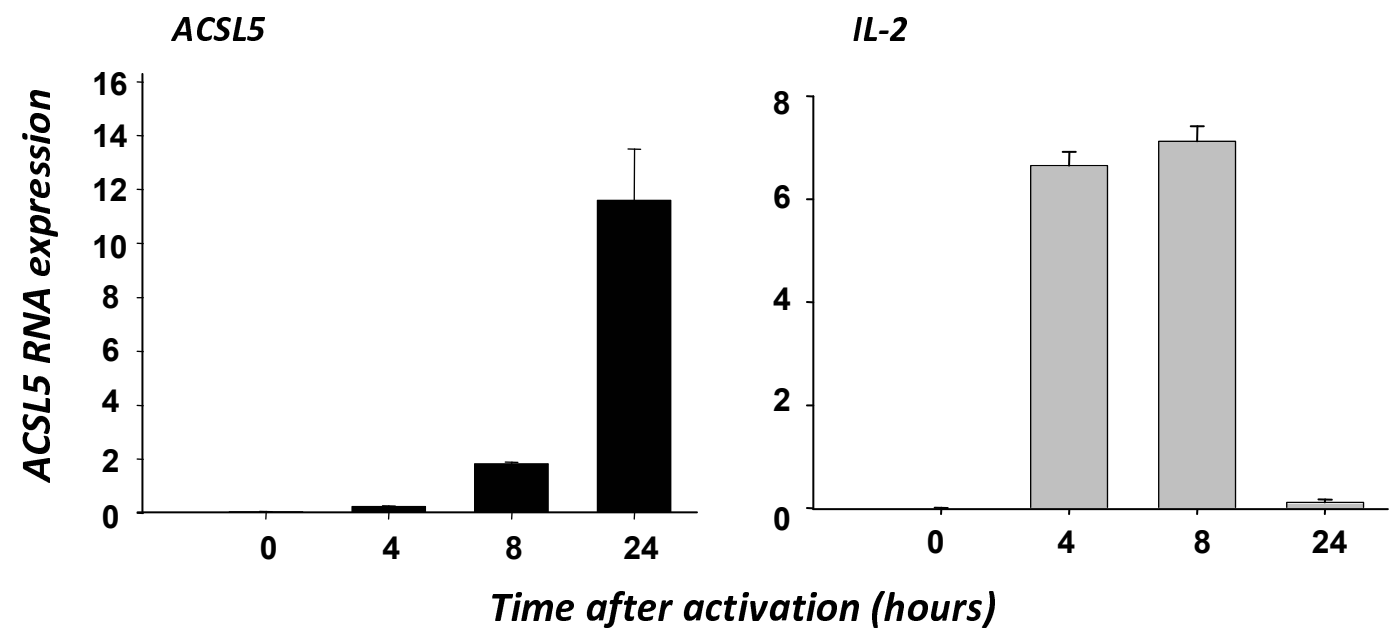A list of primers used for PCR amplification of each transcript variant 1A, 1A', 1B, 1C and exon 2 is shown.
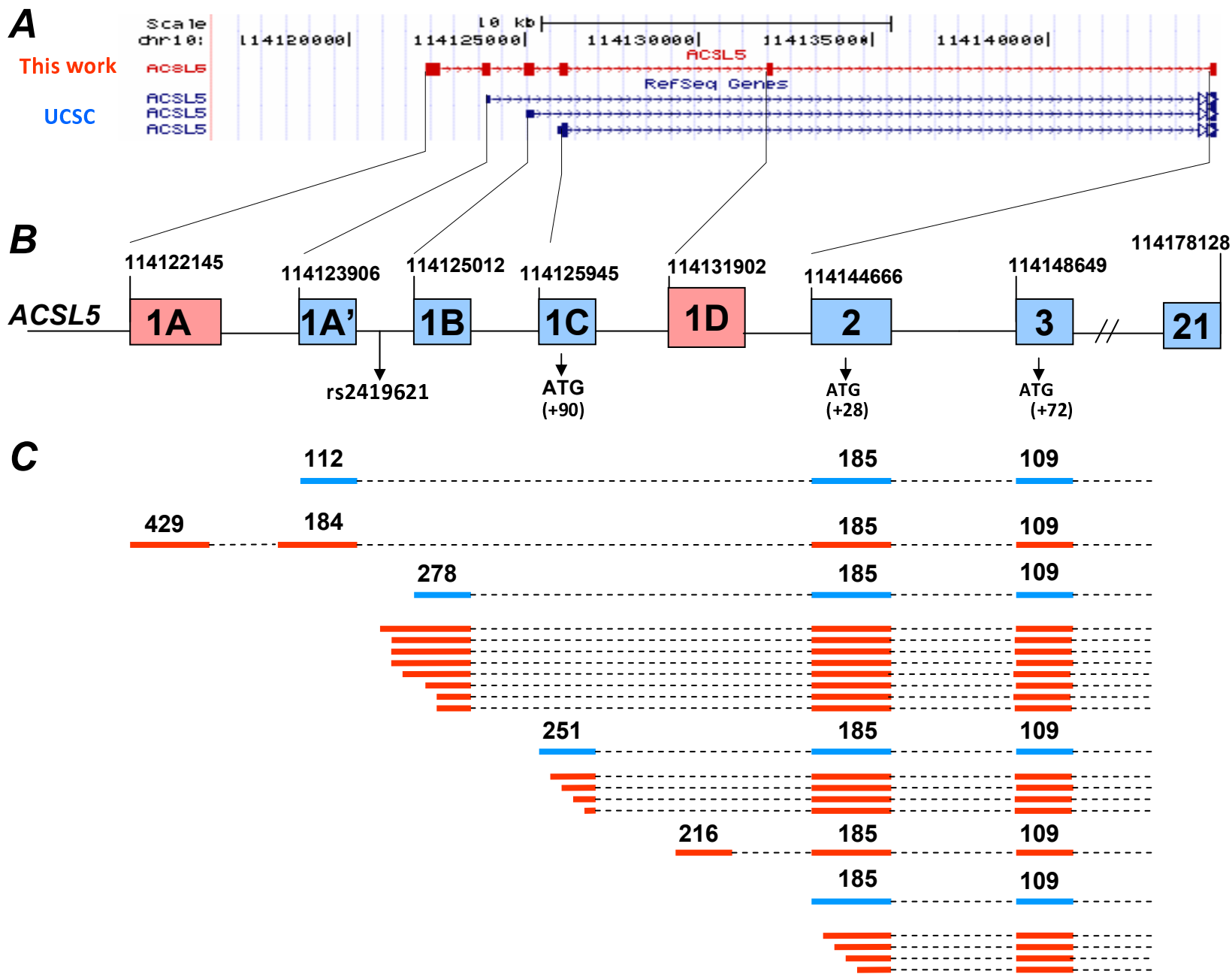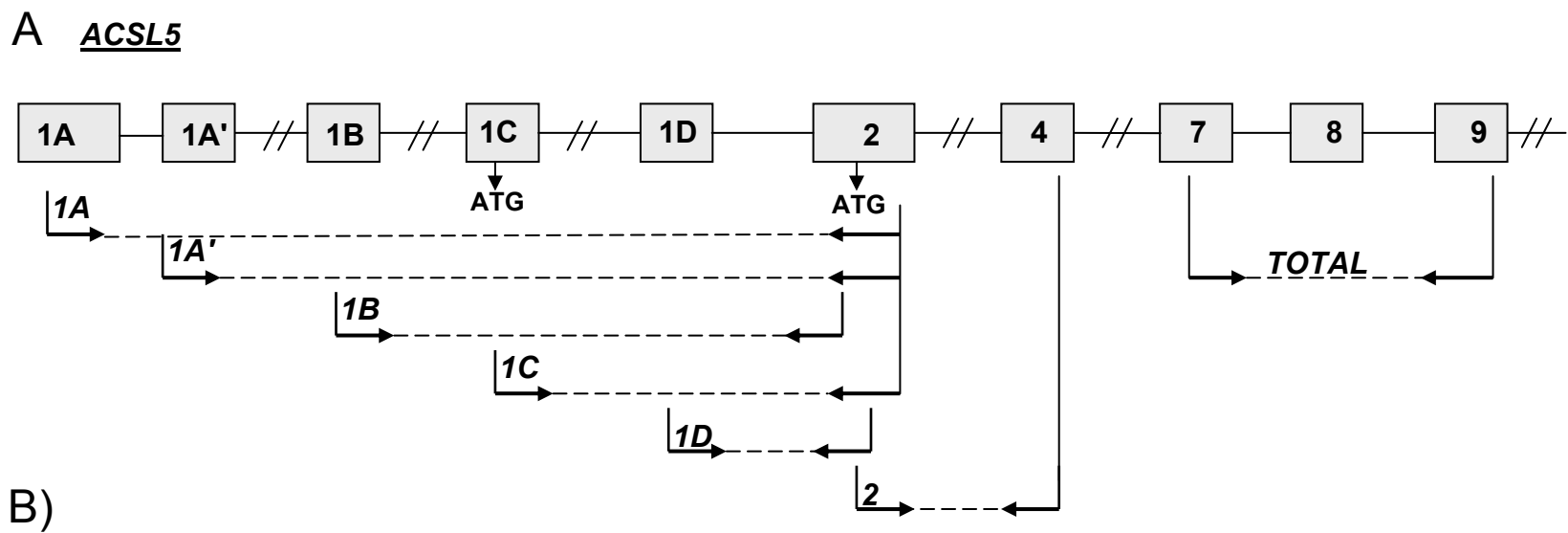
Figure 1

Figure 1

Figure 2

Figure 3
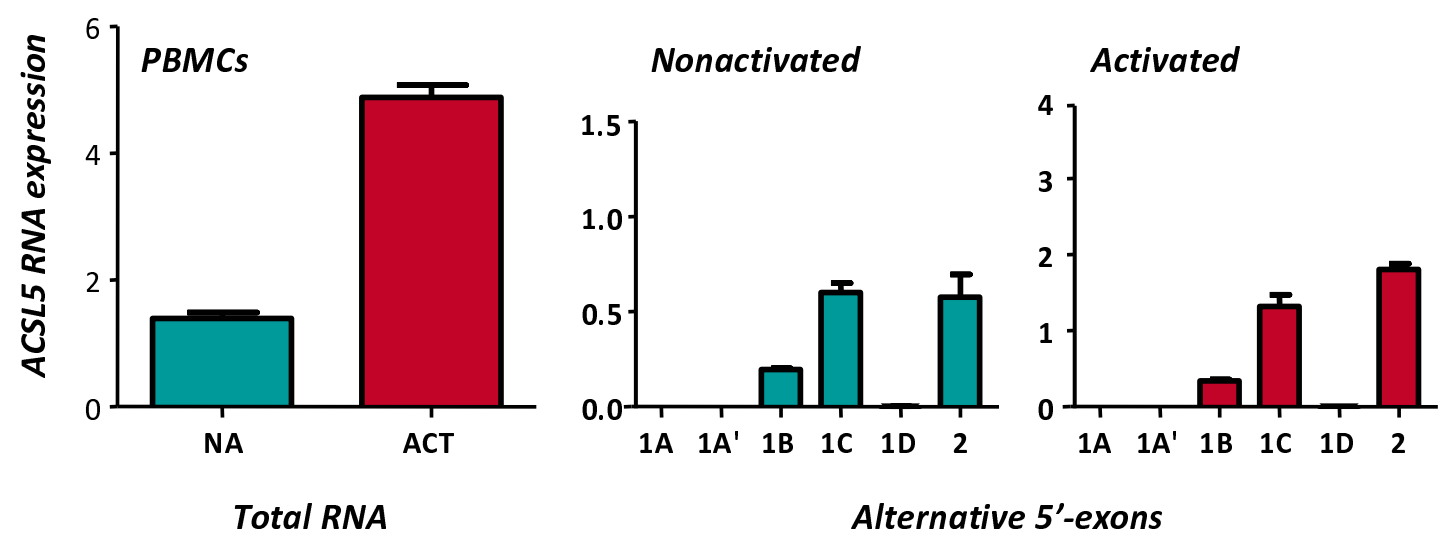
Figure 3
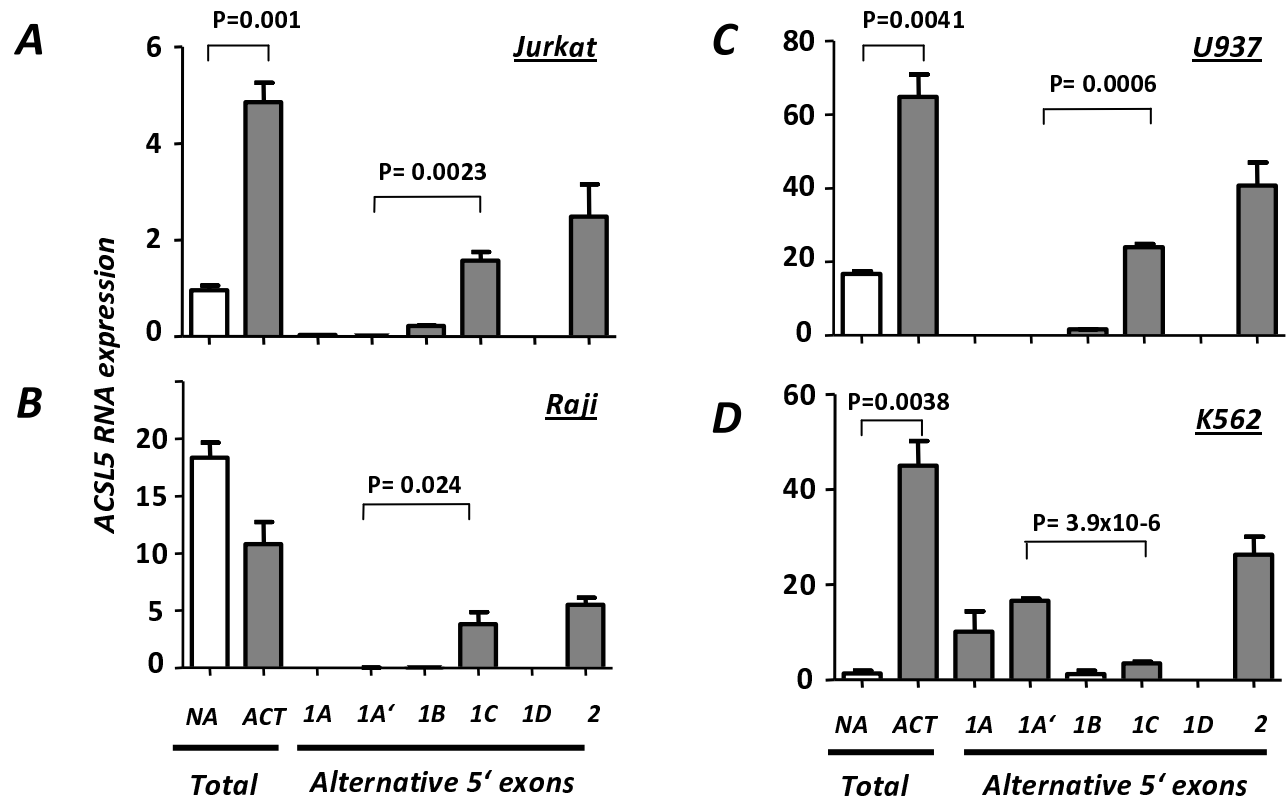
Figure 4

Figure 4

Figure 5

ACSL5 Alternative promoters

Figure 5

Figure 6

Figure 7

A

SP1

PMA / Io	-	+	-	+	-	+
Wt probe	-	-	+	+	-	-
Mut probe	-	-	-	-	+	+

1 2 3 4 5 6

B

SP1 RNA expression

NA   ACT
(PMA+Io)
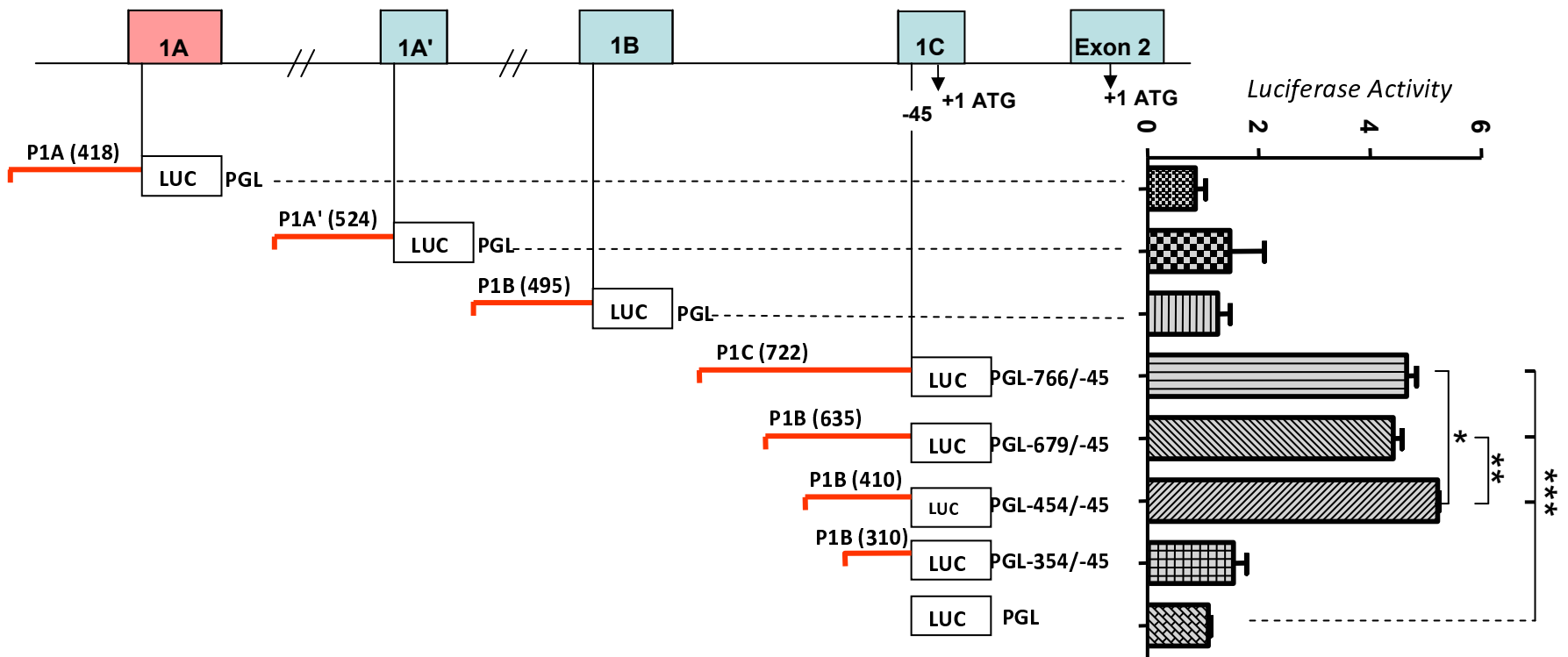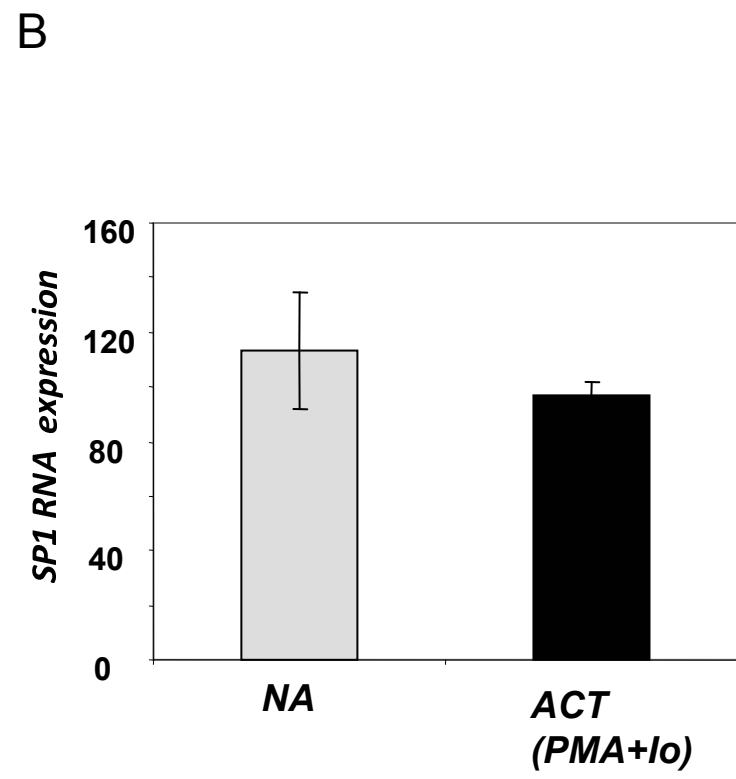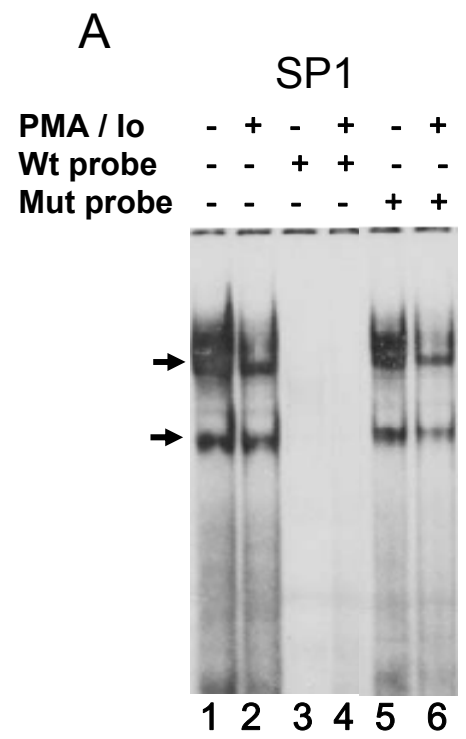
Figure 7

Additional files provided with this submission:

Additional file 1: Additional file 1 6 3 12.doc, 35K
http://www.biomedcentral.com/imedia/2820830516894169/supp1.doc
Additional file 2: Additional file 2 6 3 12.doc, 41K
http://www.biomedcentral.com/imedia/3372750546894169/supp2.doc
Additional file 3: Additional file 3 6 3 12.doc, 39K
http://www.biomedcentral.com/imedia/1757767856689416/supp3.doc
Additional file 4: Additional file 4 6 3 12.doc, 38K
http://www.biomedcentral.com/imedia/4546994886894169/supp4.doc
Additional file 5: Additional file 5 6 3 12.doc, 36K
http://www.biomedcentral.com/imedia/1433010366689416/supp5.doc
Additional file 6: Additional file 6 6 3 12.doc, 28K
http://www.biomedcentral.com/imedia/1997699324689416/supp6.doc

Additional file 1. Tables with exact position of TSS, ATG and a SNP

TSS LOCATION	CHROMOSOME 10 LOCATION OF TSS (NCBI36/HG18)	Position -9 to +2 (green is start position)
1A	114122145	gcccggagtgg
1A'	114123905 (UCSC) 114123834 (clone)	tgtcccagtca ttgtgacagat
1B	114125012 (UCSC) 114124955 (1 clone) 114124963 (3 clones) 114124971 (1 clone) 114125023 (1 clone) 114125133 (2 clones)	cgtgagatgag ttgcctcttgg tggggagctga tgagttcctga ccagaggatgg gactgtggcac
1C	114125945 (UCSC) 114125969 (1 clone) 114125995 (1 clone) 114126012 (1 clone) 114126158 (1 clone)	aaaaaaaaatt gaagtccccga tgacatggcct ggacagctcag gaagcccccat
1D	114131902 (1 clone)	aatggatacat
2	114144665 (UCSC) 114144739 (2 clones) 114144764 (1 clone) 114144767 (1 clone)	cctgctgctgt tccgaccccgg ctgacatttgg acatttggagc

NAME	LOCATION ON CHROMOSOME 10
rs2419621 (C/T)	114125003
ATG in Exon 1C	114126058 which is +113bp
ATG in Exon 2	114144695 which is + 30bp
ATG in Exon 3	114148721 which is + 72bp

Rs2419621 SNP
AAGCAAATGACAAGTGCTCCTCCAGC[C/T]GTGAGATGAGCCAGAGGATGG
AATG

Additional file 2 - Exon-intron sequences of 1A-1A' and 1D-2

Splicing 1A-1A'-2

Exon 1A start-end (light blue)

```
GGCAACCACC TGGGGTTCTT TTCACACTGT GGAAGGTTTG TTCTTTAGCT   114122194
CTTTGCAATA AATCTTGCTA CTGCTCACTC TTTGGGTCTA CGCTGCTTTT   114122244
ATGAGCTGTA ATGCTCACTG CAAAGGTCTG TAGCTTTACT CCTGAGCCAG   114122294
CAAGACCACA AACCCACCAG AAGGAAGAAA CTCTGAACAC ATCTGAACAT   114122344
CAGAAGGAAC AAACTCTAGA CACACCGCCT TTAAGAACTG TAACACTCAC   114122394
TGCGAGGGTC CGCGGCTTCA TTCTGGTAGC TAAACATACA CAGATCAATG   114122444
GGGCAGCATA GGGGCCAAGG CACTGCAGGG GTTCTCATGA CTCTGAGTCA   114122494
GGTGCTGTCT TGACCGACTG GTTGTGTGAT TCCAGAAGCC TTCTTTCACC   114122544
TGAAAAGGCC TCACTGGCCT CACCTATACA
```

intron 1A-1A' start-end (red)

```
                              GTAAGTTTGC ACATCATCTC   114122594
CAGGGTTTCT TTCATTTCTA CCCACTGTTT TTGTTTAGTA ATGATGCCTT   114122644
GCAGTCCTAA TGATCTTGGA ATCAAGAATG GTGACTAGAG AACTTCAAAG   114122694
TGGAAAGAGA GGCTGGGCAT GGTGGCTCAT GCCTATAATC CCAGCACTTC   114122744
GGGAGGCCGA GGCAGGCAGA TCACTTGAAG TCAGGAGTTC GAGACCAGCC   114122794
TGGCCAACAT GGTGAAACCC TGTCTCTGCT AAAAATACAA AAAAAAAAAA   114122844
AAAAATTAGC CAGGCATTGT GGTGCATGCC TGTAATCCCA GCCACTCCAG   114122894
AGGCTGAGGC AGGAGAATTG CTTGAACTCA GGAAGTGGAG GTTGCGGTGA   114122944
GACTCCGTCT CAAAAAAAAA AAAAAAAAAA AAAAGGAGA GAGTAGGATG   114122994
GCTGAGCCAT GACGCAGTAG CAATGCTTCT TTTTTTTTTT TTTTTTTTGA   114123044
GACAGAGTCT CGCTGTGTCG CCCAGGCTGG AGTGCAGTGG CGCGATCTTG   114123094
GCTCACTGCA AGCTCCACCT CCCGGGTTCA CACCATTCTC CTGCCTCAGC   114123144
CTCCCAAGTA GCTGGGACTA CAGGGGCCCA CCACCACGCC CGGCTAATTT   114123194
TTTGTGTATT CAGTAGAGAC GGGGTTTCAC CGTGTTAGCC AGGAAGCAAT   114123244
GCTTCCTATT CTAGCGCCTT GCTAAACTAT GTGTGCTGCA GACTGTGTTC   114123294
GTGATTCCGA ATCCTAAAAA CAACCGTCCT AGGCTTGTCT CTCAGCCTAG   114123344
ACTCTAGACT GATTTGGCTC ATTTGGTTGT CTTGGAGACG TTCTAATTCT   114123394
GAAGGGGGCA GTGGTCCCTT CTAGAAGATA GCTGGTCTGC AAATGCACAC   114123444
CTCTGCTTAG TCTGAATCCT AAAGTTATAG CTTGAGCCAA TTAAAGTGGC   114123494
TTCTCATTTT TCTCTCACTC TGATCTCAAG GAGCCCTTTT TCTTCTAGTA   114123544
ATCTAGACCT TCCTCTTCCT ATCACAGTCT CAAGGATTGT GCCCCCAAAG   114123594
CAGATGCAAA GCCCTGTCTA CCCCTTCTCC TTCCCCTGAG GTAGAGCTTT   114123644
ATCGAAGGTT TCTCTTGGTA AACGTTAGCT TTCTGAACTA AGGGCCGTCT   114123694
TTTTTTTTTT TCCCGCTTAA GCCCATTGCA TAACAATCTC TTGTTTTAAT   114123744
CTTTTGCCCA ACACTGCTTT TCTTACTGTC AGAGGTTACT ACAAGTGTTT   114123794
GAACAGGTAA CTTCTACCTT TGAACTTCTG TTGTGACAG
```

Exon 1A' start-end (light blue)

```
                                      A TTCAAAGGTT   114123844
CCAGGGGAGT GTGTTGGCCA CTCTCAGGAC AGTACACAGT AGCTTCGGGT   114123894
GTGTCCCAGT CAGTCCTAGG AGCTGTGGAA AGAGTAGAAG TGCCTGAATG   114123944
TGGTGCTGAA TCAATACAGC CAGCTGTGAG GGGAGCACTT CCTGGACCCA   114123994
GGAAGGGAGA GTCTTCTTCC AAG
```

Exon 2 start-end (light blue)

```
             GTCTGA ATTTCCTGCT GCTGTTCACA AAGATGCTTT   114144701
TTATCTTTAA CTTTTTGTTT TCCCCACTTC CGACCCGGC GTTGATCTGC   114144751
ATCCTGACAT TTGGAGCTGC CATCTTCTTG TGGCTGATCA CCAGACCTCA   114144801
ACCCGTCTTA CCTCTTCTTG ACCTGAACAA TCAGTCTGTG GGAATTGAG   114144851
```

69

Splicing 1D-2

Exon 1D start-end (light blue)

```
ATTATGAGGG AACCCGCCCT CACTTAGGTC GGACTGCTTA GCGTTGCCCT  114131951
TCTCATGGCA TGAAGCCCCT GGCTGTGGCA TTCCTGAGGA AGACTCCGCC  114132001
GACCCTGTCC TGCTGTGTGC ATAGAAGCAT GTTCTGTTCC TTGTGGGATT  114132051
GAACACTGCA AACTTTCCTT CAGCAGACTC TCCTCGCTAG TTGCCTTTCT  114132101
CTCAAATTGT GCCAATG
```

intron start-end (red)

```
                  GTG AGTAAAAGGT GAAGGTGGCT TGGTAACAGC  114132151
AGCTTCCCAC TGTGTCTCAG GTGCCATGCT AGGTGCAGTG GAATCCACAG  114132201
GTGAGGATGG TCACTATGTG CATGAGTGCT CACAGCACTG GGAGGCCTAT  114132251
TGACACACAG ATCCATGAGT CCCAGGAAGC CTCACTGGGA CCCAAACCCG  114132301
AGGAAAGGGC TTATTCTGCT CCACCTCTTC TATCTCTTCT GAGCTCTGGC  114132351
TCCTGTAGCT TTTCCTGTGC AATTCAGCAG CAAAGATTTT ACCAGTGGAG  114132401
CTGCATCGAA CGCCCTAATG ATTTATTTAT GGTATGCTTG CCTGTGGCCC  114132451
AGGACACCTG TCTGAAGCCT AGACTCTTAA CCCTTTCTCT GCCAGCTGTC  114132501
TTACACATTG GGACTGTTAG ATGCTGTCTA GCAATTGGGC TTGGAAGAGA  114132551
......................................................................
......................................................................
......................................................................
....................................GCCCAG CCCTGTGCCT  114143701
GGCATACAGT GAGTGCTCAT GTGTTTGCTG CTATTATATT CTCCTAATCC  114143751
AGTTTGAAAG CTGTGAGGCA GCAACTTTCC AGGAAAGAT GGCTTCAGCC  114143801
AAGGGTATGT TCCTAGGACC TACCCTAAAG TTAGTCTCTC CTATTAGAGT  114143851
AGTTTGAAGC TGAACTTGGC TGAAAACTAT AAGAAAGGCT GGTTTAGGGG  114143901
AAGCCTTTGT TACTTTTAGT TTCTTGAGAT CTCTTCTTGA CCTAACTCTT  114143951
CAATGCTCAC CACGTGTCAT CCATTCTCCA GCACTGTTTC TGTGTACCTG  114144001
ACTGACAGTT TGCCTCACAG TAAAATGATT CCTCTGGGCT CCCTAGGTGA  114144051
AAGGATGTTG GGCATAGCAA GTTGTAATGC TGTTGATCTC AGAAATCCTT  114144101
CCGATGCTCC AAAAAATAAA GCAATGAGCA AGACATGTCT AGCCAGCAGG  114144151
AAATATCCTT CATACAAAAT GGTTAATTTC TAAAACCTGT GGCATCACCT  114144201
TGACAGATCT CTTCTGTTTG GTTTATACGG GGGAATCCAA CTGGAAGAGT  114144251
CCTTCCTGAC CCTTGGTCTG CCTGGGCAGA GGAGGTACTG TCCTCTGACC  114144301
ATCAGATTAT GTTGGGTCTA GCAGGCTGCC TTCTTCCAGG CTTTGTGCAC  114144351
CAACATTAAA AAGGCAGTCA TTTCTCAAAA TTGTTTGGGA AATGATTAAC  114144401
TTGTTTCCTT TGATCCTCCC TGCTCTGTGT GAGGACAGTC AAGTAGCAGA  114144451
GGTGGAGAAT GTGTTCTATT ATTTTCAACA GTGCTAAGTA AACACTGGGG  114144501
GAAATTCTGC CACTAGAGAG GTACAACTGT GTTTGAGGGT TTGAAGGCGT  114144551
GCGCGCGTGT GTGTGTGTAT GTGTGTGTGT GTGTCCTTCT GAAAACATAG  114144601
AGCTATTGAG TACAAAAATA TGGCCATTTC CTCTAAATTT TCTTTCCCCT  114144651
GTTTTTTTTT TAAG
```

Exon 2 start-end (light blue)

```
               GTCTGA ATTTCCTGCT GCTGTTCACA AAGATGCTTT  114144701
TTATCTTTAA CTTTTTGTTT TCCCCACTTC CGACCCGGC GTTGATCTGC  114144751
ATCCTGACAT TTGGAGCTGC CATCTTCTTG TGGCTGATCA CCAGACCTCA  114144801
ACCCGTCTTA CCTCTTCTTG ACCTGAACAA TCAGTCTGTG GGAATTGAG  114144851
```

Additional file 3 - TSS homology with XCPE2 sequences

TSS homology with other gene's TSS. Exon 1C TSS sequences from the -9 position, at the genomic upstream region, to the +2 nucleotide in the sequence of the clones from the 5' RACE experiments are indicated. TSS 1C showed certain level of similarity with the XCEP2 Inr element present in many genes, whose consensus sequence is published by Anish et al. [20]. Nucleotides matching the XCEP2 consensus sequence are labelled in red.

Additional file 4 - Primers for qPCR and their chromosomal location

Exon	Direction	Sequence (5'-3')	Location in Chr 10 (NCBI36/hg18)
1A	Forward Reverse	TCACCTGAAAAGGCCTCACT CCCACAGACTGATTGTTCAGG	114122540-114122559 114144823-114144843
1A'	Forward Reverse	AGCTGTGAGGGGAGCACTT CCCACAGACTGATTGTTCAGG	114123966-114123984 114144823-114144843
1B	Forward Reverse	CACCCTGGCTTCCTTAGTAGG CTTTGTGAACAGCAGCAGGA	114125139-114125159 114144675-114144694
1C	Forward Reverse	TCACTAGAAGCACTGAGAGATGC CCCACAGACTGATTGTTCAGG	114126160-114126182 114144823-114144843
1D	Forward Reverse	GTGGGATTGAACACTGCAAA AGCTCCAAATGTCAGGATGC	114132044-114132063 114144750-114144769
2	Forward Reverse	TTTTTGTTTTCCCCACTTCC TAGCCATCTGTAGGGCTGG	114144713-114144732 114154292-114154311
Total ACSL5	Forward Reverse	AAGGCATTGGTGCTGATAGG TCAGGTCTTCTGGGCTAGGA	114159290-114159309 114160334-114160353

Primers for quantification of SP1 transcription factor

Transcription factor	Direction	Sequence (5'-3')
SP1	Forward Reverse	GGCCTCCAGACCATTAACCT GGGCTGTTTTCTCCTTCCTC

Cytokine gene	Direction	Sequence (5'-3')
IL2	Forward Reverse	CAAGAAGCAGTTCTGTGGCCTTCTTG GTAGAACGCACCTACTTCAAGTTCTAC

Additional file 5 - Primers for cloning in luciferase reporter vector.

Cloned Segment (bp)	*Direction*	*Primer Sequence (5'-3')*	*Location in Chr 10 (NCBI36/hg18)*
1A promoter	Forward Reverse	TGGTAGGGACTTGGGTAACCT TGTGAAAAGAACCCCAGGTG	114121753- 114121773 114122151- 114122170
1A' promoter	Forward Reverse	TAATTCTGAAGGGGGCAGTG AGGACTGACTGGGACACACC	114123388- 114123407 114123892- 114123911
1B promoter	Forward Reverse	ATAGTGCGTCTCCAGGCTGT CATTCCATCCTCTGGCTCAT	114124534-114124553 114125009- 114125028
1C, 310bp Fragment	Forward Reverse	TCTGCCTCTGTGAGGCTTCT CTGAGCTGTCCCGAGTCAG	114125704-114125723 114125994-114126012
1C, 410bp Fragment	Forward Reverse	GAGAGAGGCGGGGTAGAAGG CTGAGCTGTCCCGAGTCAG	114125604-114125623 114125994-114126012
1C, 635bp Fragment	Forward Reverse	CAGCCTTAGGCGGATCAAAA CTGAGCTGTCCCGAGTCAG	114125379-114125398 114125994-114126012
1C, 722bp Fragment	Forward Reverse	TATCTGTGAGGTGGGGAAGG CTGAGCTGTCCCGAGTCAG	114125292- 114125311 114125994-114126012

Additional file 6 - Primers for amplification and sequencing ACSL5 variants

Transcript	Direction	Primer Sequence (5'-3')
1A	Forward	GCCTCACTGGCCTCACCTAT
	Reverse	TCGAAGATGGGAAAGACAGG
1A'	Forward	ACCCAGGAAGGGAGAGTCTT
	Reverse	TCGAAGATGGGAAAGACAGG
1B	Forward	GAACTTCCTGTGAGCCTTCG
	Reverse	TCGAAGATGGGAAAGACAGG
1C	Forward	GGGAAGAAGGACAGGGACTC
	Reverse	TCGAAGATGGGAAAGACAGG
Exon 2	Forward	CCTGCTGCTGTTCACAAAGA
	Reverse	TCGAAGATGGGAAAGACAGG

ARTICLE 2

The ACSL5 Gene Polymorphisms and Potential Relevance of its Alternative Promoters in Multiple Sclerosis

The ACSL5 Gene Polymorphisms and Potential Relevance of its Alternative Promoters in Multiple Sclerosis

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Keywords: Multiple sclerosis (MS); Tag-SNP analysis; polymorphisms; genetics; ACSL5 gene; association.

Running Title: The *ACSL5* gene locus in multiple sclerosis

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ABSTRACT

BACKGROUND: The aetiology of multiple sclerosis (MS) is poorly known. Long-chain Acyl-CoA synthetase-5 (ACSL5) has an important role in fatty acid (FA) metabolism and apoptosis, two processes that are involved in MS, a central nervous system (CNS) inflammatory and neurodegenerative disease. Therefore, we wanted to test whether this gene was involved in this disease.

FINDINGS: A case-control study was performed with 830 Caucasian Spanish MS patients and 974 healthy subjects. Firstly, we genotyped the functional *ACSL5*-promoter polymorphism (rs2419621) involved in diet response predisposition and affecting gene expression. Since promising data were obtained [genotype GG vs GT+TT, P= 0.04; OR, 95% CI=0.68 (0.46-0.99)], we performed a Tag-SNP analysis of the locus with 6 additional SNPs covering 42 polymorphisms. From these, the rs2419629 was associated with MS under a recessive model [genotype GG vs GT+TT, P= 0.0176; OR (95% CI) = 0.60 (0.46-0.93)] without withstanding correction for multiple testing. Cell-type quantification of ACSL5 transcripts starting at different alternative promoters revealed that neither peripheral blood mononuclear cells nor different lymphocyte cell lines used the promoter containing rs2419621 for ACSL5 transcription under phorbol myristate acetate and ionomycin stimulation, however it was considerably functional in the human U-87 glioma cell line contributing to about 50% of the total full length transcripts.

CONCLUSIONS: Altogether, these data suggests a modest protective role of ACSL5 for MS and, at least the rs2419621 may have disease relevance in CNS glia cells. This is the first genetics evidence of the association of the metabolism of long chain fatty acids with MS.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). Oligodendrocyte damage and subsequent axonal demyelination is a hallmark of this disease. The aetiology is unknown though different pathomechanisms may be involved in MS [1] for instance immune-mediated inflammation, oxidative stress and excitotoxicity. Extensive epidemiological data confirm that genetic variation is an important determinant of susceptibility to MS, and suggest that such variation also influences the timing of symptom onset, the course of the disease, and the treatment response [2, 3]. In addition, current evidence suggests that it involves complex interactions of both genetic and environmental factors being childhood or adolescence thought to be a critical period [4].

This candidate-gene case-control study was set up based on several evidences involving the metabolism of fatty acids and lipids in the pathophysiology of MS. To end this, we have made a relevant compilation of recent findings that justify the analysis of the long-chain acyl-Coenzyme A (CoA) synthetase 5 gene (*ACSL5*) and analyzed its genetic association with diseases susceptibility. The ACSL5 is one of the five members of the ACSL family whose isoenzymes activate fatty acids of length C12 to C20, by esterification with Coenzyme A (CoA), to produce activated acyl-CoAs [5]. This is the first essential step in fatty acid utilization for beta-oxidation or membrane biosynthesis amongst other functions. The protein encoded by the ACSL5 gene is mainly located in mitochondria [6] and though this could suggests a main role in beta-oxidation other works have shown that ACSL5 is also involved in the anabolic fate of fatty acids [7]. In several *in vitro* and in vivo studies, ACSL5 has been involved

in apoptosis of hepatic cells [8], glioma cells, other tumor cells [9, 10], and enterocytes [11]. Oligodendrocytes, the myelin-forming cells of the central nervous system (CNS), are target cells in MS and new insights suggest apoptosis as one of the critical events followed by glial activation and infiltration of lymphocytes and macrophages [12, 13].

The T allele of the SNP rs2419621, located in one of the alternative promoters of *ACSL5*, increases the mRNA and has been associated with rapid weight loss in obese Caucasian females [14]. This SNP sinergizes with peroxisome proliferator-activated receptor-gamma (PPAR-g) [15].

On the other hand, several studies have given evidences of the implication of obesity and different types of fatty acids in MS. Obesity at age 18 (body mass index $\geq$ 30 kg/m(2)) was associated with a greater than twofold increased risk of MS (multivariate relative risk(pooled) = 2.25, 95% CI: 1.50-3.37, p trend <0.001) in a large study of women in the Nurses' Health Study (n = 121,700) and Nurses' Health Study II (n = 116,671) by which it has been suggested that prevention of adolescent obesity may contribute to reduced MS risk [16].

Ecologic correlations suggest that higher intake of saturated fat and lower intake of polyunsaturated fat might increase the risk of multiple sclerosis (MS) [17]. However, case-control studies on fatty acids and MS have given inconsistent results [18].

Polyunsaturated fatty acid (PUFA) and antioxidant deficiencies along with decreased cellular antioxidant defence mechanisms have been observed in MS patients. Antioxidant and PUFA treatment in experimental allergic encephalomyelitis, an animal model of MS, decreased the clinical signs of disease. Both dietary

antioxidants and PUFAs have the potential to diminish disease symptoms by targeting specific pathomechanisms and supporting recovery in MS [19, 20].

Studies of fatty acid composition within the different phospholipid fractions of red blood and peripheral blood mononuclear cell (PBMC) membranes have been correlated with the severity of neurological outcome of MS [21].

On this base, we hypothesized that the ACSL5 could be involved in the susceptibility to MS and started to analyze the 5' promoter SNP rs2419621 and as promising results were obtained, we performed a fine mapping by a tag-SNP study of the whole ACSL5 gene locus.

Material and Methods

Study subjects

Case samples comprised 830 patients with clinically defined MS according to Poser´s criteria [22]. They were obtained from four public hospitals: the Hospital Clínico in Granada (n=126), the Hospital Virgen de las Nieves de Granada (n=164), the Hospital Carlos Haya in Málaga (n=380) and the Hospital Virgen de la Macarena in Seville (n=160) all three cities within a 200 km radius in the South of Spain. The mean age at the sample collection of the cases was 36 years and mean age of controls at interview was 38 years. The percentage of females was 68% for cases and 68 % for controls. All of them were classified as RR (relapsing-remitting) or SP (secondary progressive) MS cases. Controls were 974 blood donors with no history of inflammatory disease attending the blood banks of Granada (n=823), Seville (n=71) and Málaga (n=80). The study was approved by the Ethics Committees of each of the hospitals participating in the study and written informed consent was obtained from all participants.

Genotyping

High-molecular-weight DNA was isolated from whole blood using the Flexigene Kit (Qiagen. Hildren. Gemany) according to the manufacturer´s protocol. The 7 SNPs were genotyped by TaqMan technology under conditions recommended by the manufacturer (Applied Biosystems, Foster City, CA. USA).

Sample Power Calculation

This combined sample set of 830 patients and 974 controls is powered at 80% to find effect sizes as low as 1.33 with a minor allele frequency (MAF) of 0.1, assuming a multiplicative model, at $\alpha = 0.05$ (conditions assumed for the Tag-SNP). Since the

recessive model is the one that adjust best of all to our result, we calculated the power for this model which was 67% (OR= 0.66, MAF=0.3, α = 0.05).

Statistical Analysis

Departure from Hardy-Weinberg equilibrium (HWE) for all the biallelic SNP markers was tested using an exact test [23]. For individual SNP association analyses, genotype frequencies were assessed by recessive a model which fits the best for the first analysis performed for the rs2419621. Logistic regression models were used to estimate crude odds ratios (ORs) and 95% confidence intervals (95% CI). In order to determine which SNP is more associated with MS, multiple logistic regression models were computed by using likelihood ratio test. We compared the effect of each SNP adjusted by the most promising SNP found in the crude analysis. These analyses were performed using the SNPassoc R package [24].

Cell Cultures

Jurkat cells, Raji cells, U937 cells and PBMC were cultured in RPMI 1640 medium supplemented with gentamycin. All culture medium contained 10% foetal bovine serum. Incubation was done at 37oC in a 5% humidified CO_2 incubator. Stimulation of cells was done using phorbol-12-myristate-13-acetate (PMA) (50 ng/ml) and ionomycin (Io) (10 ng/ml)

Purification of Total RNA and mRNA

Total RNA was purified from each cell type using RNeasy kit (QIAGEN Cat.no. 74134) mRNA was purified from the total RNA by using the GenElute mRNA Miniprep kit from Sigma.

Reverse transcriptase and Real time PCR

A total of 200ng mRNA was reverse transcribed using 1μl random hexamer(50ng), 1μl superscript III reverse transcriptase (200U/μl) with 1μl 10mM dNTP mix, 4 μl 5x First strand buffer, 0.1M DTT and RnaseOUT. The mixture was heated to 50°C for 60min then inactivated by heating at 70 for 15min. Amplifications were done in triplicates of 30μl each using the biorad 2x sybr green mastermix for RT-PCR (Biorad) under the following conditions; 1 cycle at 95°C (3min); 40 cycles at 95°C (2 s),64-60 ^{o}C(40s), and 72 ^{o}C (20s). As a reference gene to calculate the relative expression we used the UbcH5B, which is relative constant during the entire differentiation period and differ less than 4-fold in several human tissues [25]

Quantification of ACSL5 transcripts from different alternative exons in different cell lines

The following primers were used to quantify the amount of ACSL5 transcripts from each of the transcription start sites of the alternative exons as following indicated: Exon 1A forward (gcctcactggcctcacctat) and reverse (cccacagactgattgttcagg), Exon 1A' forward (agctgtgaggggagcactt) and reverse (cccacagactgattgttcagg) Exon 1B forward (caccctggcttccttagtagg) and reverse (ctttgtgaacagcagcagga); Exon 1C forward (tcactagaagcactgagagatgc) and reverse (cccacagactgattgttcagg); Exon 1D forward

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(gtgggattgaacactgcaaa) and reverse (agctccaaatgtcaggatgc); Exon 2I (initial site)

forward (tttttgttttccccacttcc) and reverse (tagccatctgtagggctggt); Exon 2F (final site)

forward (gacctcaacccgtcttacctc) and reverse (tagccatctgtagggctggt).

RESULTS AND DISCUSSION

This study is the first to present novel evidences for the relationship between polymorphisms of the *ACSL5* gene locus and multiple sclerosis. Table 1 summarizes the genotypic frequency of the functional *ACSL5* polymorphism rs2419621 in Spanish patients suffering from MS and compared with ethnically-matched healthy controls.

Results conformed to Hardy-Weinberg expectations. As shown, the frequency of the homozygous minor allele carrier was lower in MS patients than in controls (P=0.04, odds ratio (OR) = 0.68 (0.46-0.99)). To clarify if the association observed was due to the rs2419621 variant or to other polymorphism of the region, we performed a fine mapping of the locus by a Tag-SNP analysis.

A region of 60Kb of the chromosome 10 at position 114115874-114175873 (NCBI 36 assembly) was selected (Fig.1). To analyze this region we chose 6 Tag-SNPs by pairwise from the HapMap CEU population data release 24/phaseII which captured 42 markers with a minor-allele frequency (MAF) $\geq$ 0.1 and $r^{2} >$ 0.7 (mean $r^2 = 0.922$). As observed in Fig. 1, the LD pattern among markers with the Spanish population is very similar to that obtained for the CEU population of the HapMap database.

We genotyped the Tag-SNPs in a Caucasian population of 824 MS patients and 960 controls from the South of Spain. The results of genotypic distribution between cases and controls are shown in Table 2. The frequency of the homozygous minor allele carrier was lower in MS patients than in controls for the rs2419629 variant (P=0.017, OR=0.66 (0.46-0.93), however, it did not survive Bonferroni correction.

Since in this Tag-SNP study two out of seven polymorphisms showed to be significantly associated with MS under a recessive model as shown in Table 1 for rs2419621 and Table 2 for rs2419629, we performed a logistic regression analysis to test the addition of each SNP to both SNPs (independently) and determined the primarily associated marker (Table 3). The model that adds rs2419629 to rs2419621 was statistically significant and increased the protective effect by 10% respect to the simple model (P from likelihood ratio test equal to 0.007; 0R= 0.59). The rs2419629, when it was adjusted by the rs2419621, remains associated without any change in the effect ($P = 0.028$, OR= 0.68). This data indicated that both SNPs conferred protection and contributed as independent factors that modelled the association with MS but the rs2419629 represented a confounding factor that seems to add protection against MS. Therefore, the association within this gene could best be explained by two disease locus tagged by rs2419621 and rs2419629.

The T allele of rs2419621 creates a functional cis-regulatory E-box element (CANNTG) that is recognized by the myogenic regulatory factor MyoD which promotes MyoD-dependent ACSL5 transcription activation in skeletal muscle and in non muscle CV1 cells. Furthermore, the T allele, in conjunction with PPARgamma2 (SNP Pro(12Ala)), has been associated with more rapid diet-induced weight loss whereas the G allele showed a significant decline [14, 15]. The Pro12Ala SNP of PPARgamma may influence the onset of MS in 116 patients and 211 age-matched healthy controls [26].

As the polymorphism rs2419621 is located in one of the several alternative promoters of *ACSL5* gene and has a functional role in transcription regulation [14, 15], we wanted to quantify the production of ACSL5 RNA in different cell types including;

KG-1C (oligodendroglial), Peripheral blood mononuclear cells and the human glioma U-87 (Fig. 2B and 2C). Using the primer design shown in figure 2A ,we found that U-87 cells transcribed *ACSL5* gene mainly from 3 promoters, from one containing the functional SNP rs2419621 (P1B) and from the next one (P1C and also promoter (P1A) and similarly KG-1C cells, however, its expression profile was similar to that of PBMCs which contain T-cells, B-cells and monocytes.

ACSL5 transcription was much less from promoter 1B for PBMCs and KG-1C in comparision with U-87 cells and much more from prmoter 1C (90%). This implies potential differential roles of rs2419621 depending on the cell type. Therefore, the association of this SNP with MS would be relevant in cells like the glioma U-87 but minimal in lymphocytes. In this sense, it is interesting to note that lipidosin, an 80-kDa protein with long-chain acyl-CoA synthetase activity, was greatly increased in astrocytes in the area of remyelination, following experimental demyelination induced by the administration of cuprizone to mice, suggesting that this protein is involved in fatty acid metabolism during reconstruction of the myelin sheath [27].

ACSL5 gene expression is regulated at transcriptional level, amongst other factors [7], by PPARgamma. This transcription factor regulates cell growth, differentiation and homeostasis. Since PPARgamma agonists have a crucial role in preventing CNS inflammation and demyelination in experimental allergic encephalomyelitis (EAE) [28, 29], the animal model of MS, our results of *ACSL5* association and cell-type specific transcription suggest a potential role for the rs2419621 in glia cells of the brain in disease.

In this study we revealed a role for *ACSL5* gene as a protective factor for MS under the condition of a recessive model, therefore, having GG in both associated genotypes

for rs2419621 and rs2419629. We noted, however, that until a more complete set of polymorphisms is identified and genotyped in a large collection of cases and control subjects, we cannot exclude another variant in LD with these SNPs to determine the causal polymorphisms. Future resequencing of the locus may provide as-yet-undiscovered variants that will need to be assessed for diseases susceptibility.
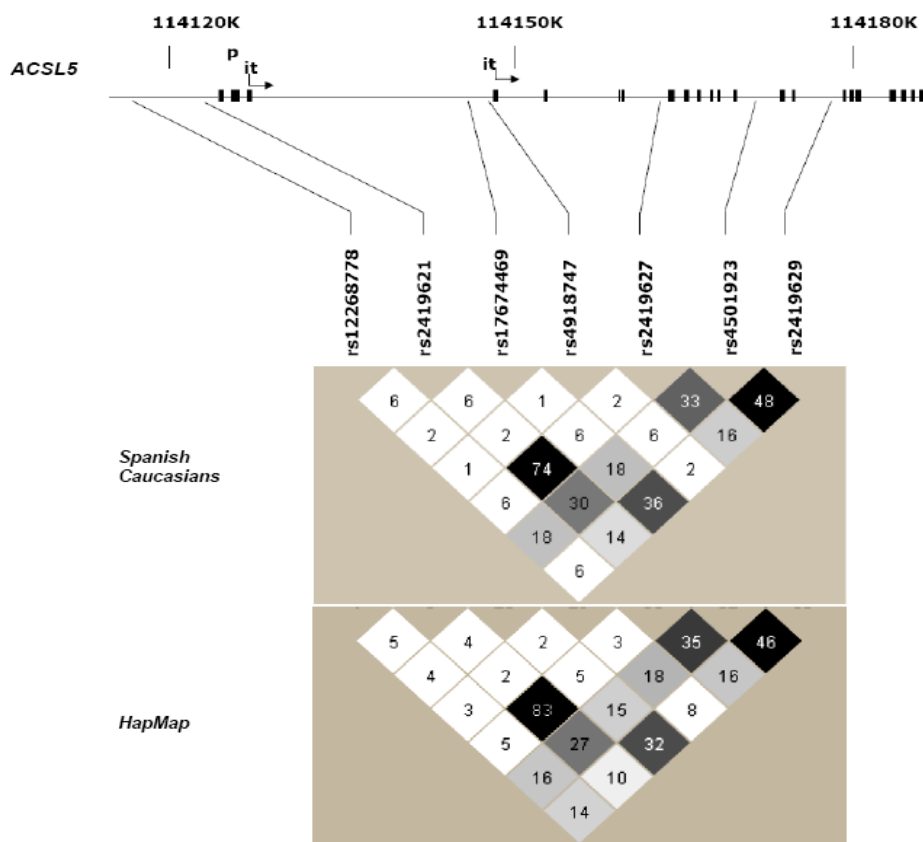
Figure 1. Haplotype block representation of the 7 investigated SNPs in the *ACSL5* gene showing the positions of the polymorphisms based on the NCBI Bild 35 and the linkage disequilibrium (LD) structure as pairwise r^2 – values. The calculations of LDs were performed by the software Haploview from our data (Spanish Caucasians) or from HapMap Data Rel 24/phase II (HapMap). In the scheme of the ACSL5 gene it is shown the exons (black boxes) with untranslated regions (horizontal line), the different alternative promoters (P) and the initiation of translation (it).
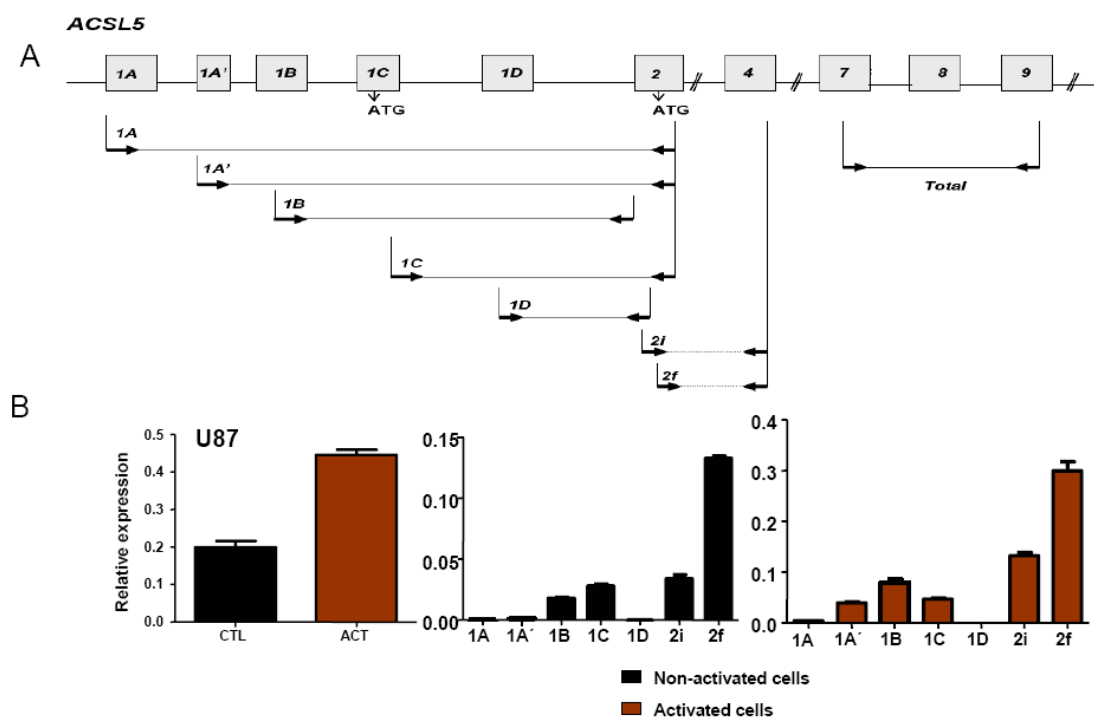
Figure. 2. Quantification of *ACSL5* transcript variants in U89 glioma cells

A) Schematic representation of ACSL5 gene including the first alternative exons and arrows show primer design for amplifiying the alternative exons. B) U87 alternative TSS expression indicated as (1A, 1A´, 1B, 1D and 1C). The quantity of ACSL5 transcript in exon 2 (2i and 2f) was also determined. Data represent means ± SEM (n= 3). Black and brown stand for non activated and activated cells respectively.
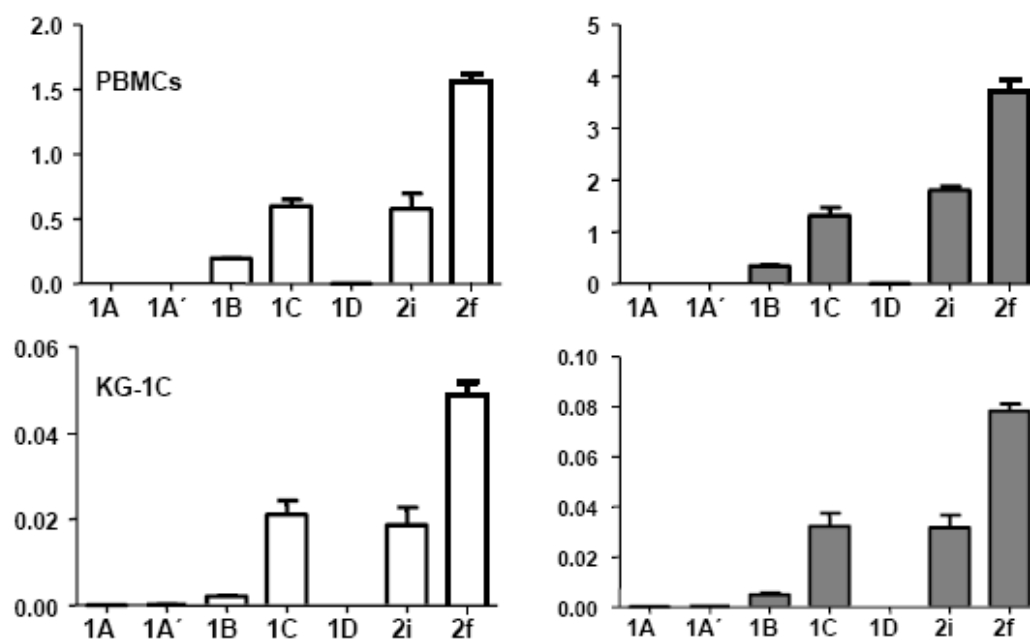
Figure. 2C. Quantification of *ACSL5* transcript variants in PBMCs and Kg-1c cells

The quantity of ACSL5 transcript in each of the alternative first exons 1A, 1A', 1B, 1C,1D and exon 2 (2i and 2f) was determined. Data represent means ± SD (n= 3). White and black stand for non activated and activated cells respectively.

Table 1. Genotype distribution of rs2419621 in multiple sclerosis patients and controls.

Genotypes	CTL n (%)	MS n (%)	P	OR (96% CI)
Codominant model				
AA	416 (47.5)	344 (48.6)	0.104	
AG	378 (43.2)	317 (44.8)		
GG	81 (9.2)	46 (6.5)		
Recessive model				
AA+AG	794 (90.7)	661 (93.4)	**0.04**	1
GG	81 (9.3)	46 (6.6)		0.68 (0.46-0.99)

OR, odds ratio

Table 2. Tag-SNP mapping of the *ACSL5* locus

SNP	Genotypes	Recessive Model	MULTIPLE SCLEROSIS N (frequency)	HEALTHY CONTROLS	P	OR odds ratio	[95% CI]
rs12268778	AA		596 (0.73)	688 (0.73)			
	AG		209(0.25)	235 (0.25)			
	GG		17 (0.02)	20 (0.02)			
		AA+AG	805 (0.98)	923 (0.98)	0.8931		
		GG	17 (0.02)	20 (0.02)			
rs17674469	AA		592 (0.72)	684 (0.73)			
	AG		212(0.26)	226 (0.24)			
	GG		13(O.02)	24 (0.03)			
		AA+AG	804 (0.98)	910 (0.97)	0.105		
		GG	13 (0.02)	24 (0.03)			
rs4918747	GG		697 (0.86)	803 (0.86)			
	AG		109 (0.14)	120(0.13)			
	AA		1 (0.001)	5 (0.005)			
		GG+AG	738 (0.99)	849 (0.99)	0.088		
		AA	60 (0.001)	92 (0.005)			
rs2419627	GG		402 (0.5)	465 (0.49)			
	AG		336 (0.42)	384 (0.41)			
	AA		60 (0.08)	92 (0.1)			
		GG+AG	738 (0.92)	849 (0.9)	0.061		
		AA	60 (0.08)	92 (0.1)			
rs4501923	CC		172 (0.21)	211 (0.23)			
	AC		414 (0.5)	460(0.49)			
	AA		238(0.29)	267(0.28)			
		CC+AC	586 (0.71)	671 (0.72)	**0.047**		
		AA	238 (0.29)	267 (0.28)			
rs2419629	AA		386 (0.48)	453 (0.47)			
	AG		366 (0.45)	413 (0.43)			
	GG		54 (0.07)	94 (0.1)			
		AA+AG	752(0.93)	866 (0.9)	**0.017**	1	
		GG	54 (0.07)	94 (0.1)		0.66	(0.46-0.93)

Table 3. Regression analysis adding **rs2419621** to **rs2419629** and reverse regression analysis for 830 MS cases and 974.

Locus	Add locus to rs2419621		Add rs2419621 to locus	
	P	OR (95% c.i.)	*P*	OR (95% c.i.)
rs2419629	0.007	0.59 (0.41-0.87)	0.028	0.66 (0.96-0.46)

Results assuming recessive effect model. OR, odds ratio.

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DISCLOSURE

The authors declare that they have no competing interests.

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ARTICLE 3

High ACSL5 Transcript Levels Associate with Systemic Lupus Erythematosus and Apoptosis in Jurkat T Lymphocytes and Peripheral Blood Cells

High ACSL5 Transcript Levels Associate with Systemic Lupus Erythematosus and Apoptosis in Jurkat T lymphocytes and Peripheral Blood Cells

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Running Title: ACSL5 in SLE and apoptosis in Jurkat T cell*s*

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ABSTRACT

BACKGROUND: Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease in which increased apoptosis and decreased apoptotic cells removal has been described as most relevant in the pathogenesis. Long-chain acyl-coenzyme A synthetases (ACSLs) have been involved in the immunological dysfunction of mouse models of lupus-like autoimmunity and apoptosis in different *in vitro* cell systems. The aim of this work was to assess amongst the ACSL isoforms, the involvement of ACSL4, ACSL5 and ACSL6 in SLE pathogenesis.

FINDINGS: With this end, we determined the ACSL2, ACSL4 and ACSL5 transcript levels in peripheral blood mononuclear cells (PBMCs) of 45 SLE patients and 49 healthy controls by quantitative real time-PCR (q-PCR). We found that patients with SLE had higher ACSL5 transcript levels than healthy controls [median (range), healthy controls= 16.5 (12.3-18.0) vs SLE= 26.5 (17.8-41.7), P=3.9 x 10 E-5)] but no differences were found for ACSL4 and ACSL6. In *in vitro* experiments, ACSL5 mRNA expression was greatly increased when inducing apoptosis on Jurkat T cells and PBMCs by Phorbol-Myristate-Acetate plus Ionomycin (PMA+Io). On the other hand, short interference RNA (siRNA)-mediated silencing of ACSL5 decreased induced apoptosis in Jurkat T cells up to the control levels as well as decreased mRNA expression of FAS, FASLG and TNF.

CONCLUSIONS: These findings indicated that the ACSL5 may have a role in the apoptosis that takes place in SLE. Our results point to ACSL5 as a potential novel functional marker of pathogenesis and a possible therapeutic target in SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex genetic autoimmune disorder which predominantly affects women and leads to the production of antibodies against an individual's own healthy tissues, aberrant formation of immune complexes (IC), and inflammation of multiple organs. As a consequence skin rashes, joint pain, anaemia, cardiovascular-atherosclerosis and renal diseases are the principal clinical manifestations. While no known cure for SLE exists, current treatments that range from antimalarials to corticosteroids and immunosuppressive agents have markedly reduced short-term mortality rates. Long-term mortality rates, on the other hand, are increasingly influenced by cardiovascular complications [1].

Accelerated apoptosis of circulating lymphocytes and/or impaired clearance of apoptotic cells are already known to be a hallmark of SLE [2]. Impaired engulfment of early apoptotic cells may cause secondary necrosis and release of intracellular autoantigens, and then trigger autoimmune reactions in SLE [3, 4]. The dysfunction of apoptosis may be a direct consequence of alterations in proteins/genes such as Fas, FasL, Bcl-2 and C1q among others [5,6].

Human long-chain acyl-CoA synthetases (ACSL, EC6.2.1.3) activate fatty acids with chain lengths from 12 to 20 carbon atoms by esterification with coenzyme A (CoA). The acyl-CoAs formed are essential for complex lipid synthesis, protein modification, beta-oxidation, regulation of various physiological processes and remodelling of membranes [7]. ACSLs differ in fatty acid types and tissue expression preference. ACSL5 has a substrate preference for C16-C18 unsaturated fatty acids and is expressed in small intestine, as well as in lungs, liver and other tissues, localizing at the outer mitochondrial membrane and microsomes [8, 9].

Acyl-coAs are lipid metabolic intermediates that have been associated to metabolism regulation systems and gene expression [10]. Besides, acyl-coAs have long been associated to apoptosis, mostly because of their effect on membrane stability, signaling pathways and secondary metabolite activity [11-13].

Although the contribution of lipid metabolic pathways to autoimmunity, and specifically to SLE, is poorly understood, several *in vivo* and *in vitro* evidences indicate that ACSLs may play an important role in the immune dysfunction of lupus-like mouse models (14,15). In addition, autoimmunity and inflammation are associated with marked changes in lipid and lipoprotein metabolism in SLE [16, 17]. ACSL5, on the contrary, has been associated to cell development and maturation, physiopathological processes, apoptosis and tumorigenesis [18-21].

In spite of these suggestive precedents, this study is the first to approach a possible involvement of ACSL5 in autoimmune diseases and specifically in SLE. We hypothesized an implication of ACSL5 in the pathogenesis of SLE, which is associated with the increased apoptosis seen in the disease, since this gene has already been associated to apoptosis in other tissues and diseases. For that, we investigated the ACSL5 transcripts levels in peripheral blood mononuclear cells (PBMCs) from SLE patients and healthy donors to seek for significant differences. Moreover, we focused on whether ACSL5 transcript levels were related to activation-induced cell death (AICD) in Jurkat T cells and PBMCs as models already described [22], and discuss its potential association with apoptosis found in SLE. Our results indicated that ACSL5 transcript levels were higher in SLE than in controls and that silencing ACSL5 mRNA by short interference RNA (siRNA) decreased the apoptosis induced by phorbol-myristateacetate plus ionomycin (PMA+Io)-activation of Jurkat T cells, thus implicating ACSL5 in AICD.

MATERIALS AND METODS

Participants

Forty five Caucasian patients (39 female, 6 male), fulfilling the American College of Rheumatology criteria for SLE [33], who attended the out-patient clinic of the University Hospital Virgen de las Nieves of Granada, Spain, were included (Table 1). Exclusion criteria were less than 1 year of follow-up in our unit. Current clinical assessment were made during a routinely visit and other demographic and clinical data were obtained from the medical records in a computer database. Disease activity and accumulated organ damage were measured with the SLE Disease Activity Index (SLEDAI) and the Systemic Lupus International Collaborating Clinics/ACR Damage Index (SDI) respectively as indicated in Table 1. Controls were healthy individuals attended to the Blood Bank of Granada. Mean age of this group was similar to the SLE group (P>.05) but the % of females (49%) was rather different to the SLE group (88%). So, we tested the effect on the ACSL5 transcript levels of males and females as indicated in Table 1.

Ethics Statment

All participants provided a written informed consent to participate in this study, which was approved by the Institutional Review Board of Hospital Virgen de las Nieves of Granada, Spain.

Cell cultures

Jurkat T cells (Clone E6-1, LGC/ATCC[R]) and peripheral blood mononuclear cells (PBMCs) from anonymous donors in the Blood Bank of Granada, Spain, were cultured in RPMI 1640 complete medium (PAA Laboratories, GmbH), supplemented with 10%

(v/v) heat-inactivated fetal bovine serum, 2mM (w/v) glutamine, and 100U/mL penicillin and 100ug/mL streptomycin (all from Gibco, Invitrogen. Carlsbad, CA), at 37 ºC / 5% CO_2 atmosphere. Cells were maintained in an exponential growth phase for all experiments. To study effect of Activating-Induced Cell Death (AICD), 50ng/mL Phorbol 12-Myristate 13-Acetate (PMA) and 10ng/mL Ionomycin (Io) (Sigma-Aldrich Inc.) were added to the cultured cells for different-time experiments. Viable-cell counting was carried out by Trypan Blue solution (Sigma-Aldrich Inc.) staining.

ACSL5 transcript measurement

PBMCs were obtained from whole blood samples by Ficoll-Hystopaque (Sigma-Aldrich Inc.) density centrifugation. Total RNAs were isolated by using the RNeasy mini-kit (Qiagen, Valencia, CA) and the mRNA purified by using the GenElute mRNA Miniprep kit (Sigma-Aldrich Inc.) and stored at -80°C until used.

cDNA was synthesized using a total of 200-500ng mRNA of each sample, reverse transcribed using the Superscript III reverse transcription reagents (Invitrogen S.A., Invitrogene Ltd., UK) and then subjected to RT-PCR. As a reference gene for normalization to calculate the ACSL5 relative expression, the ubiquitin-conjugating enzyme UBcH5B was used [34]. Amplifications of cDNAs were done in triplicates using the sybr green[R] Mastermix for real time-PCR (Biorad Laboratories, Inc.) and 50nmol of the following primer sets: *ACSL2* Forward 5'-ttcgaagaagccctgaaaga-3' and Reverse 5'-agaaatcagccaccacgttc-3' (renamed as ACSL6, ENST00000354273); ACSL4 Forward 5'- tccaagtttgggaagaagga-3' and Reverse 5'-ggcaatggtgttctttggtt-3' (EnsembleID, ENST00000354273), _ACSL5_: Forward: 5' – AAGGCATTGGTGC TGATAGG – 3' and Reverse: 5' – TCAGGTCTTCTGGGCTAGGA – 3' (Ensemble transcript ID ENST00000357430); and _UbcH5_ as reference gene: Forward: 5' –

CAATTCCGAAGAGAATCCACAAGGAATTG – 3' and Reverse: 5' – GTGTTCCAA

CAGGACCTGCTGAACAC–3'(Ensemble transcript ID, ENST00000398733).

PCR conditions were as follows: 1 cycle of 95 ^{o}C for 3 min, followed by 40 cycles of 95 ^{o}C for 20 s, 62 ^{o}C for 20s, and 72 ^{o}C for 20 s. All assays were validated for linearity of amplification efficiency. PCR efficiencies were calculated using a relative standard curve derived from mixed cDNA of different samples (a twofold dilution series with four measuring points). To ensure the absence of amplification artefacts and primer dimmer formation, end point PCR products were initially assessed on ethidium bromide stained agarose gels that gave a single band of the expected size for each assay. Negative controls containing no template cDNA were run in each condition and gave no results. The reactions were quantified when the PCR product of interest was first detected (cycle threshold, CT). Calculations for relative mRNA transcript levels were performed using the comparative CT method_2_ΔΔCT_ between cycle thresholds of different reactions using Image Quant (Bio-Rad Laboratories Inc).

ACSL5 from non-activated (N) and activated (A) PBMCs: stimulation index (SI)

In a first step, we quantified *ACSL5* mRNA levels from freshly extracted PBMCs. The second type of measurement was done after 24 h of *in vitro* stimulation by phorbol-myristate-acetate (PMA) and ionomycin (Io). Finally, a parameter that we set up to equilibrate differences due to undetermined factors between measurements, was the stimulation index (SI), that is, the ratio between activated and non-activated expression levels [23].

Apoptosis related mRNA expression

We quantified some relevant genes in the apoptotic pathway: BCL-2 (ENSG00000171791), FAS (ENSG00000026103), FASLG (ENSG00000117560), TRAIL (TNFSF10; ENSG00000121858), TNF (ENSG00000232810) and CASP3 (ENSG00000164305). Primers sequences can be seen in supporting material table S1. cDNA was synthesized using a total of 200-500ng mRNA of each sample and amplifications of cDNAs were done in triplicates. PCR conditions were as follows: 1 cycle of 95 ^{o}C for 3 min, followed by 45 cycles of 95 ^{o}C for 20 s, 63 ^{o}C for 20s, and 72 ^{o}C for 20 s.

Short interference RNA (siRNA)

To silence *ACSL5* mRNA expression we electroporated Jurkat cells with specific siRNA [35]. 300 nM of siACSL5 or the transfection control (siRNA) (Dharmacon, Thermo Fisher Scientific. Lafayette, CO) were introduced into Jurkat T cells with Cell Line Nucleofector^R Kit V Solution Box (Amaxa, Lonza Cologne GmbH), following supplier instructions, and using the Nucleofector^R II (Amaxa, Lonza Cologne GmbH) electroporator. After transfection Jurkat T cells were cultured in complete medium for 18 h prior to further experiment.

Flow cytometry

We used Propidium Iodide (PI) (Sigma-Aldrich Inc.) staining to detect hipodiploid cell fragments produced in PMA+Io-induced apoptosis [36]. Cultured PBMCs or Jurkat T cells (activated and non activated by PMA+Io for 24 h) were collected at 2, 6 and 24 h, washed twice with PBS-glucose at 2mg/mL (VWR International, LLC. Amresco Inc., OH) and fixed with cold 70% ethanol for 15 minutes at 4 ºC. Then we washed cells with PBS-glucose and stained the activated and non activated cells with final concentration of 100 ug/mL Propidium Iodide (PI) prior to analysis with BD FACSCalibur^{TM} flow cytometer (Becton, Dickinson and Company).

Annexin V-FITC kit for apoptosis (Sigma-Aldrich Inc.) was used for detection of phosphatidylserine in the outer plasma membrane as a specific apoptotic marker [37], following manufacturer's instructions. Cells were cultured for 24 h in presence or absence of PMA and ionomycin and collected at 2 and 24 h. Analyses of samples were carried out with the BD FACSCalibur^{TM} flow cytometer.

Statistics

Statistical analysis was performed using SPSS 15.0 for the Windows software package (SPSS, Chicago, USA). We used the nonparametric Mann-Whitney rank-sum test to test the significance of the difference in the transcript levels of ACSL5(N), ACSL5 (A) and ACSL5 (SI) between the healthy controls and SLE patients; females and males from the control group; and prednisone treated SLE patient group versus the untreated SLE group. Relative ACSL5 mRNA levels are presented as medians (range). To determine the effect on risk, we used simple logistic regression analysis and results are expressed as logistic coefficient (B), and odds ratios with the 95% confidence intervals. Receiver-operating characteristic (ROC) curves (not shown) and the area under the ROC curve (AUC) were used to assess the feasibility of using peripheral blood mononuclear cell ACSL mRNA concentration as diagnostic tools for detecting SLE. Paired Student T test was performed for comparisons between control and PMA+Io-activated and/or transfected Jurkat T cells. Errors bars are presented as mean (standard deviation) from three independent experiments. All analyses used a 2-sided level of significance of 5% ($P<0.05$).

RESULTS

ACSLs measurements in patients and controls

The aim of this study was to investigate the mRNA expression levels of ACSL5 in PBMCs from SLE patients and control subjects by means of qPCR. The main characteristics of the SLE patients and controls relevant for this study are listed in Table 1. We have quantified mRNA levels in two conditions. Firstly, from freshly extracted and non-activated PBMCs named as ACSL5 (N), potentially indicative of physiological status of each group, either patients or controls. The second type of measurement for ACSL5 was done after 24 h of *in vitro* stimulation with PMA+Io named ACSL5 (A). This kind of activation may resemble to a natural polyclonal activation conditioned by the pathological status of the individuals from whom PBMCs were extracted and may be indicative of the maximum response potential of PBMCs. Finally, a parameter that we set up to equilibrate differences owning to undetermined factors between measurements (supposing to affect similarly the untreated and activated cultures), was the stimulation index (SI), that is, the ratio between ACSL5 (A) and ACSL5 (N) expression levels [23].

Table 1. Main characteristics of study subjects with systemic lupus erythematosus.

General Characterisitics	Patients (n =45)
Age (yrs)[1]	42 ± 13
Female (%)[2]	88
SLE characteristics	
Disease duration (yrs)	13.3 ± 8.6
Age at SLE diagnosis (yrs)	31.6 ± 13.1
SLE complications	
Arthritis (%)	90
Renal involvement (%)	28
Serositis (%)	23
Haematological manifestations (%)	33
Neurological manifestations (%)	23
Positive anti-dsDNA (ever) (%)	83
Antiphospholipid antibody (ever) (%)	28
Activity	4.4 ± 3.6
SLEDAI score	1.23 ± 1.46
SDI score	
Treatments	68
Current use of prednisone (%)[3]	74
Current use of HCQ (%)	3.6 ± 3.1
Mean current prednisone dose (mg/day)	91 ± 59
	33
Mean current HCQ dose (mg/day)	
Immunosuppressant agents (%)	

(1) Age of Control group: n=49, mean ± SD = 36.91 ±14 (19-61). No significant difference with SLE group (P-value> 0.05).

(2) Female percentage of control group: 50%. No difference of ACSL5 levels between male and females of control group (see Figure 1B).

(3) Prednisone treatment to SLE patients affected ACSL5 transcript levels (see Figure 1C).

ACSL5 in SLE patients vs. healthy controls

As shown in Figure 1A, comparing with healthy subjects, freshly collected PBMCs from SLE patients had higher expression levels of mRNA for ACSL5 than controls [median (range); CTL= 16.5 (12.3-18) vs. SLE= 26.5 (17.8-41.7), P=3.9 x 10^{-5}]. No significant difference was found for ACSL2 and ACSL4 between healthy controls and SLE patients (unpublished data). In activated PBMCs, there were no differences between expression levels from SLE patients and controls. ACSL5 (SI) was significantly lower in SLE patients than in controls (median (range); CTL= 5.1 (2.5-6.8) vs. SLE= 1.9 (1.2-3.4), P= 1.8 x 10^{-5}), suggesting an enhanced transcription of ACSL5 in SLE patients that was close to maximum state of provoked activation.

ACSL5 difference between male and female healthy controls

While SLE group had similar age to the healthy control group, the male to female ratio was clearly different, so we took this last characteristic into account and presented experiments to determine gender influence into ACSL5 transcript levels. As shown in Figure 1B, ACSL5 (N), (A) and (SI) values were similar in male and female groups of healthy controls, therefore it was deduced that gender was not associated with differences of ACSL5 transcript levels and hence we could establish that there were no differences between SLE patients and controls
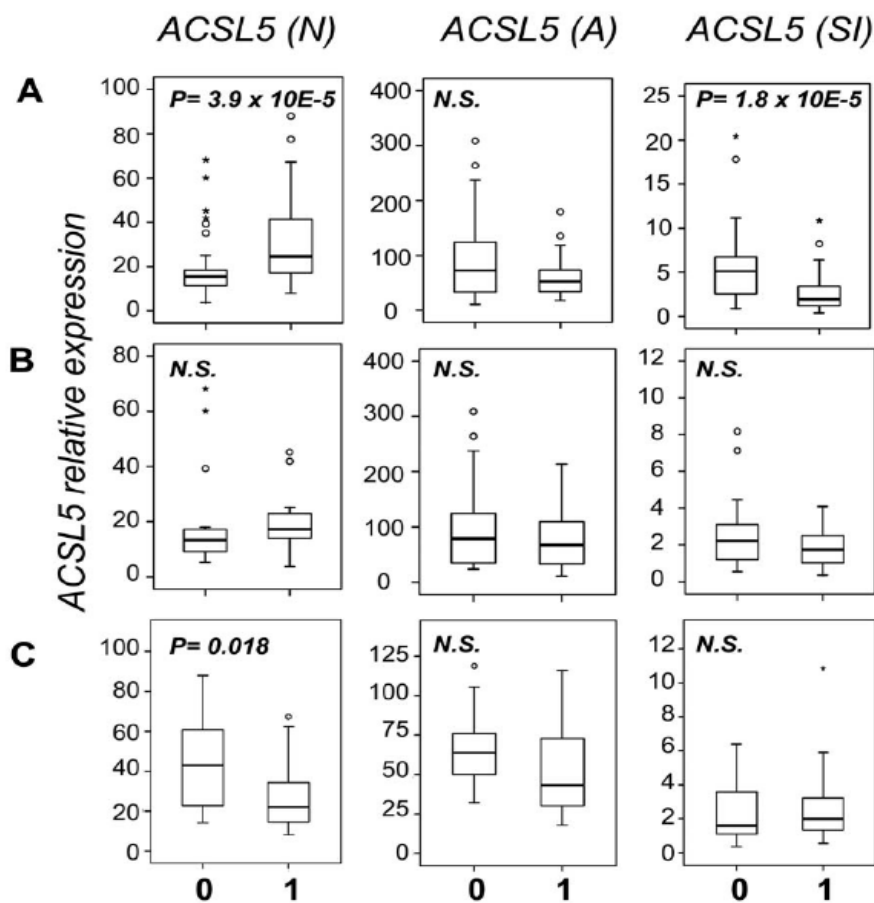
Figure 1. Association of ACSL5 mRNA expression levels with SLE. A) ACSL5 transcript levels in PBMCs from healthy controls (0) and SLE patients (1) for ACSL5(N) (number of samples n: 0= 49, 1= 45), activated ACSL5(A) (n, 0=34, 1=40) and the ratio A/N (ACSL5(SI) (n, 0= 34, 1= 40). B) ACSL5 transcript levels in PBMCs from males (0) (n= 24) and females (1) (n= 25), all of them from the control group. C) ACSL5 transcript levels in PBMCs from prednisone treated (1) (n= 28) and untreated (0) (n= 12) SLE patients. Results are represented in box plots given medians, quartiles, outsiders (circle points) and extremes (asterisk points). *P-values* of differences between the groups (0) and (1) are defined by Mann-Whitney Statistics. *N.S.* stands for non significant.

ACSL5 differences between prednisone treated and untreated SLE patients

Another important consideration to discard confounder factors was the effect of drug treatments in the ACSL5 mRNA. Amongst the several drug-treatments of SLE patients indicated in Table 1, only the corticosteroid prednisone affected ACSL5 mRNA. Comparing transcript level differences between these two groups of SLE patients, we found a lower ACSL5 (N) transcript levels in treated than untreated group as shown in Figure 1C. These results point to an even higher ACSL5 transcript levels associated with pathology if patients were not treated with prednisone which would follow our previous results referring to higher ACSL5 expression in SLE patients than in healthy controls.

ACSL effect in SLE and as diagnostic marker

To determine the effect on SLE (odds ratio, OR), logistic regression analysis was performed showing the beta coefficient and odds ratio with 95% confidence intervals (Table 2). ACSL5 (SI) had an OR of 0.68 (95% CI= 0.544-0.854) for each increasing unit variation. Analysis of the ROC curves for ACSL5 from PBMCs showed that the AUC was highest for ACSL5 (SI) (AUC =0.776) as indicated in Table 2, which reflected a potential utility in diagnosis

ACSL5	Coefficient	Wald	P-value	OR, 95% CI	AUC
ACSL5(N)	0.047	7.26	0.007	1.048 (1.013-1.084)	0.732
ACSL5(A)	-0.011	5.316	0.021	0.989 (0.980-0.998)	0.598
ACSL5(SI)	0.384	11.138	0.001	0.681 (0.544-0.854)	0.776

Table 2. Logistic regression analysis to determine the effect on diseases and the area under curve (AUC) of ACSL5 values in PBMCs. Results are represented with the beta coefficient and the odds ratios *(OR)* with 95% confidence intervals *(CI).*

Effect of PMA+Io activation in PBMCs

We obtained PBMCs from two controls and cultured them with or without PMA+Io up to 24 h followed by PI alone or Annexin V/PI double staining as described in material and methods (Figure 2 A-B). Results showed more than 50% of PI incorporation after 24 h of activation with PMA+Io compared to controls (P= 0.0289). Annexin V/PI double staining showed no diferences compared to controls due to an abnormally elevated apoptosis in controls. We analyzed then mRNA expression of several relevant genes associated with apoptosis to partly determine the effect of PMA+Io in the apoptotic pathway (Figure 2 D-I). BCL-2 showed a 4-fold decrease, while FAS, FASLG, TRAIL (TNFSF10), TNF and CASP3 increased by 3-, 5-, 2-, 3- and 2-fold respectively, thus corroborating and induction of AICD in PBMCs by PMA+Io activation.

On the other hand, ACSL5 mRNA levels increased in a time-dependent course when activated with PMA+Io (mean ± SD; 24 h= 5.51 ± 0.41 vs. CTL= 1.02 ± 0.13; P=

0.0049) (Figure 2C), thus correlating with the expression of pro-apoptosis genes and the induction of apoptosis in PBMCs.
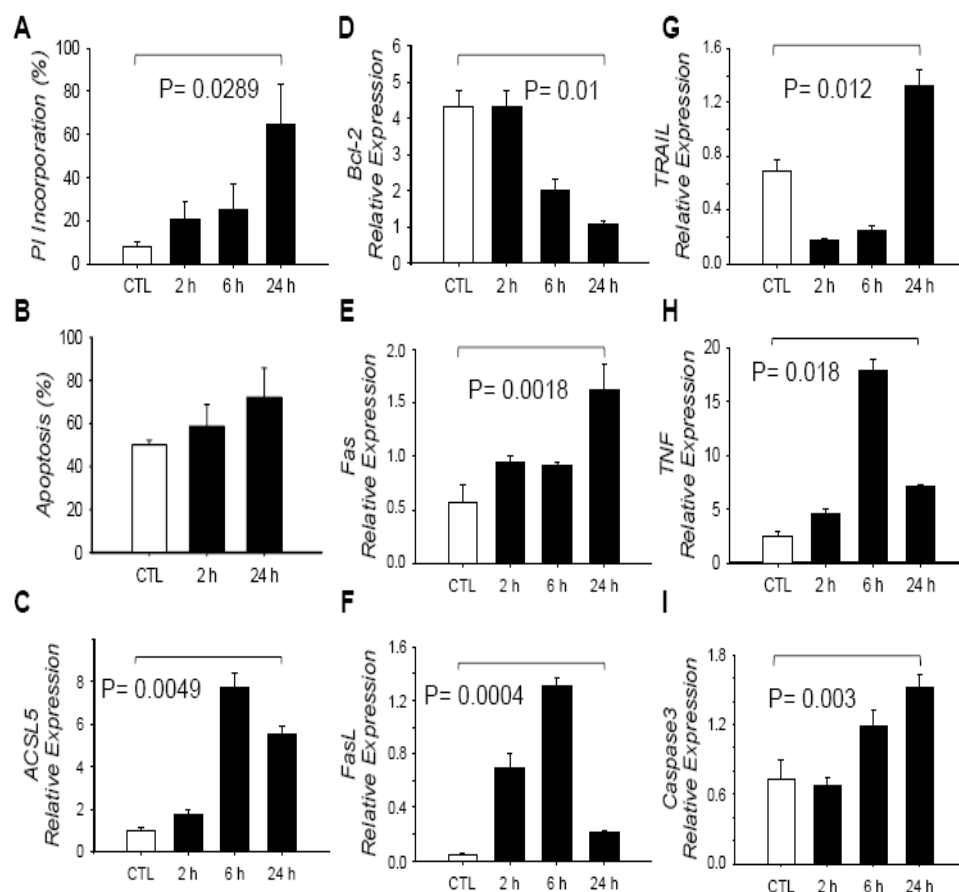
Figure 2. Effect of PMA+Io activation in PBMCs. PBMCs from healthy controls were obtained and cultured in the presence or absence of PMA+Io for up to 24 h. A) Cells were collected at defined times, washed with PBS, fixed with 70% Ethanol, stained with Propidium Iodide (PI) and analyzed in a FACSCaliburTM flow cytometer to determine the apoptotic hipodiploid cell fragments (defined as percentage of PI incorporation). B) Cells were washed twice with PBS and double stained with Annexin V and PI, then analyzed by FACS to determine the percentage of apoptotic cells (Annexin V positive and double positive cells). C-I) Total RNA was extracted, cDNA synthesized and qRT-PCR implemented to determine mRNA expression. Results are given by means of three independent experiments and the bars show the standard deviation. *P-value* has been calculated with the paired Student *t* test.

Effect of PMA+Io-activation on AICD in Jurkat T cells

Jurkat T cells were activated with PMA+Io for up to 24 h, followed by PI alone or Annexin V/PI double staining (Figure 3A-B). PI positive cells in PMA+Io-activated Jurkat cells increased to 20, 27 and 40% at 2, 6 and 24 h respectively, whereas it was 8% in untreated control cells. On the other hand ,Annexin V positive cells increased 2 and 23% at 2 and 24h, respectively compared with controls (P=0.0256)- These results agree with several studies describing PMA+Io-induced AICD in lymphocytes [22]. On the other hand, Annexin V positive cells increased, compared to controls, 2 and 23% at 2 and 24 h, respectively. We analyzed, as with PBMCs, mRNA expression of several relevant genes associated to apoptosis to partly determine the effect of PMA+Io in the apoptotic pathway (Figure 3 D-I). FASLG, TNF and TRAIL (TNFSF10) were induced 14, 8 and 3 times respectively, thus agreeing with cytometry results.

Effect of PMA+Io-activation in ACSL5 mRNA expression

Jurkat T cells activated with PMA+Io showed an increased level of ACSL5 mRNA – time dependent – by approximately 5-fold at 24 h compared to untreated control cells (mean ± SD; PMA+Io= 0.36 ± 0.04 vs. CTL= 0.079 ± 0.002; P= 0.018) (Figure 3C), so indicating that ACSL5 is inducible by PMA+Io in Jurkat T cells. Here we concluded that the increased ACSL5 transcript levels correlated with PMA+Io-induced apoptosis in Jurkat cells.
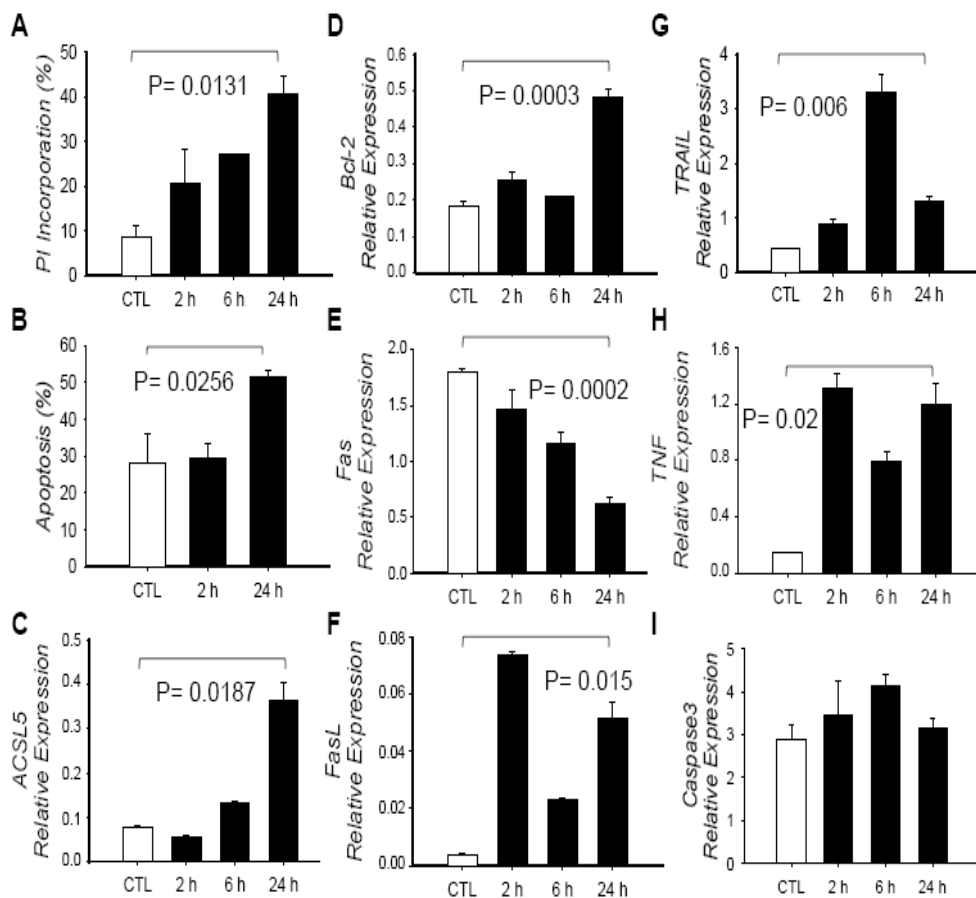
Figure 3. Effect of PMA+Io activation in Jurkat T cells. Jurkat Tcells were cultured in the presence or absence of PMA+Io for up to 24 h. A) Cells were collected at defined times, washed with PBS, fixed with 70% Ethanol, stained with Propidium Iodide (PI) and analyzed in a FACSCaliburTM flow cytometer to determine the apoptotic hipodiploid cell fragments (defined as percentage of PI incorporation). B) Cells were washed twice with PBS and double stained with Annexin V and PI, then analyzed by FACS to determine the percentage of apoptotic cells (Annexin V positive and double positive cells). C-I) Total RNA was extracted, cDNA synthesized and qRT-PCR implemented to determine mRNA expression. Results are given by means of three independent experiments and the bars show the standard deviation. *P-value* has been calculated with the paired Student *t* test.

Effect of ACSL5 transcript levels in PMA+Io-inducing apoptosis

We used short interference RNA (siRNA) technology to silence ACSL5 gene expression to test whether it was implicated in AICD in Jurkat T cells. For that purpose siACSL5 and a transfection control (siRNA) were introduced by electroporation into Jurkat T cells, obtaining 80% silencing approximately after 18 h of transfection (Figure 4A). Silencing was particularly important in PMA+Io-activated cells (mean ± SD; siACSL5= 0.034 ± 0.0005 vs. siRNA= 0.19 ± 0.08; P= 0.038) due to a low expression level of ACSL5 in untreated cells but high expression following PMA+Io activation.

We then proceeded to test ACSL5 implication in PMA+Io-induced apoptosis in Jurkat T cells by FACS (Figure 4 B-C). Following PI alone or Annexin V/PI double staining, analysis of transfected Jurkat samples showed a 35% decrease approximately in both experiments − lowering to control levels in siACSL5 compared to siRNA transfected Jurkat T cells when they were activated with PMA+Io (mean ± SD; PI Incorporation: siACSL5= 47 ± 7 vs. siRNA= 75 ± 6; P= 0.0246. Annexin V positive cells: siACSL5= 47 ± 9 vs. siRNA= 72 ± 2; P= 0.0242). It is important to note that there was an elevated apoptosis in controls and possibly in stimulated too due to the necessity to stimulate cells with PMA+Io right after transfection because of short life of siRNAs, and so the impossibility to eliminate dead cells from the nucleofection process.

Following prior experiments we analyzed mRNA expression of several apoptotic genes, finding that FAS, FASLG and TNF decreased 1.3-, 2.6- and 1.6-fold in siACSL5-transfected cells compared to controls after stimulation with PMA+Io. These findings imply a direct role of ACSL5 in PMA+Io-induced apoptosis in Jurkat cells.
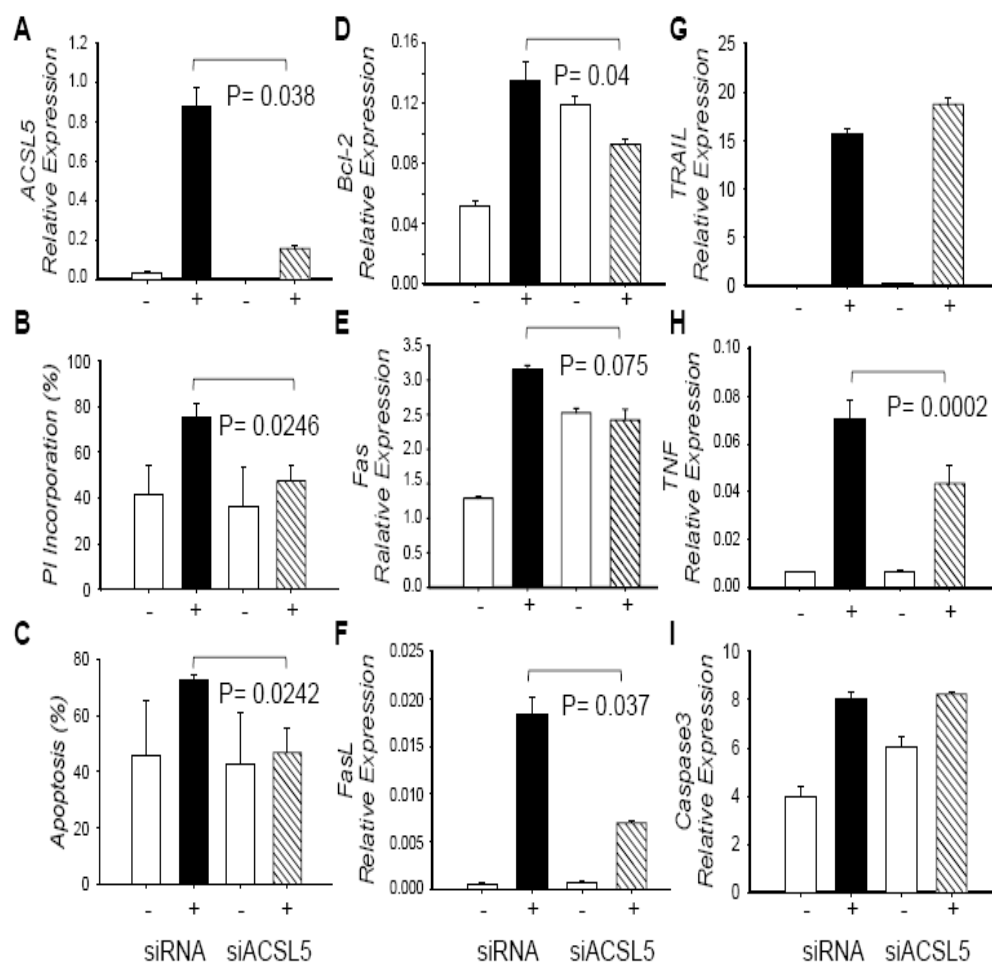
Figure 4. Effect of ACSL5 silencing in the apoptosis induced in Jurkat T cells by PMA+Io activation. Jurkat T cells were electropororated with either siRNA for ACSL5 (siACSL5) or unspecific control of siRNA (siRNA), and left for 18 h in culture media. Then both siACSL5 and siRNA Jurkat T cells were cultured with or without PMA+Io for 24 h. A) ACSL5 expression at 24 h was determined in each type of culture as described in material and methods. B) Percentage of apoptosis in siACSL5 or siRNA Jurkat T cells measured by cytometry as Annexin V positive and double Annexin V and PI positive cells. C-I) Total RNA was extracted, cDNA synthesized and qRT-PCR implemented to determine mRNA expression. Results are given by means of three independent experiments and the bars show the standard deviation. *P-value* has been calculated with the paired Student *t* test.

DISCUSSION

The aim of this study was to investigate the role of ACSL5 in SLE pathogenesis. We found that ACSL5 transcript level was significantly increased in PBMCs from SLE patients compared to controls. This association was not influenced by the different female/male ratio of the cohorts since they did not show ACSL5 differences. The stimulation index (SI), which equilibrates undetermined individual differences, showed a significantly greater stimulation increase in PBMCs from controls compared to SLE patients. Taken that SLE patients present a persistent state of activation of lymphocytes with oversecretion of pro-inflammatory cytokines [24], we could suggest that ACSL5 mRNA was overexpressed in SLE patients as a consequence of this chronic activated state of cells and thus, exert a role in lymphocyte activation. Unfortunately, we could not establish a relationship between ACSL5 and cytokine expression in hematopoietic cell lines (unpublished data). In this way, a higher degree of apoptosis in T-lymphocytes in SLE patients has been found to be directly correlated with disease activity [4]. In addition, we observed that corticosteroids, used to reduce disease activity and systemic inflammation in SLE, decreased ACSL5 transcript levels, suggesting a strength link between inflammation (disease activity), apoptosis and ACSL5 levels.

There is little information about ACSL expression in PBMCs and diseases. One interesting evidence come from a microarray gene expression study of about 10,000 genes in PBMCs from 7 rheumatoid arthritis (RA) patients with rheumatoid factor (RF), 6 without RF and 7 healthy individuals. Though it showed no significant differences between RF-positive and RF-negative patients, comparisons of gene expression patterns from all RA patients and healthy controls identified a subset of discriminative genes, amongst others, a significantly higher expression in RA patients of the fatty-acid-Coenzyme A ligase (corresponding with an ACSL, although specific isoform is

undetermined) together with others involved in immunoinflammatory responses, especially those related to altered phagocytic functions [25].

As shown in fas-deficient mice and humans, autoimmunity can be caused by the inability of the immune system to eliminate self-reactive lymphocytes and hence maintaining autoreactive cells that will recognise autoantibodies [5]. However, as shown in complement deficiencies, increased apoptotic material and altered clearance of apoptotic cells is found in patients with SLE [2-4]. This suggest that what is found in rare individuals with genetic deficiencies that develop SLE or SLE-like disease may be found in the larger population of SLE patients as a common end point pattern of unbalanced process of both apoptosis and clearance of apoptotic material. The dysfunction of apoptosis may be a direct consequence of alterations in proteins/genes such as Fas, FasL, Bcl-2 and C1q [6]. On the other hand, ACSL5 has previously been associated to both apoptosis and surviving processes of tumors from different tissues [20, 26], thereby implying a role of ACSL5 in these pathways. In addition, it has been described in SLE patients an augmented spontaneous apoptosis of lymphocytes [5], which, in the same line with our findings in patients, points to ACSL5 as a possible key molecule regulating apoptosis in SLE, and hence exerting a role in SLE pathogenesis.

Our results from experiments *in vitro* confirmed a pro-apoptotic role of ACSL5 in AICD occurring in lymphocyte cells, as this had been previously demonstrated in hepatocytic cells, increasing TNF- and TRAIL-mediated apoptosis [20]. Unlike this hypothesis, other works have shown enhanced ACSL5 expression associated to the development of colorectal cancer [21] and involved in surviving of glioma cells under acidosis conditions [26], pointing to an anti-apoptotic role. Taken together, ACSL5 may be able to play both pro- and anti-apoptotic roles depending on tissue specificity,

physiopathology conditions and other factors, thus contributing to different clinical manifestations appearing in complex diseases as SLE.

Apoptosis is a genetically controlled process initiated by two principal pathways. The extrinsic pathway is activated by the ligation of death receptors, and the intrinsic pathway emerges from the mitochondria [5]. Lipids have long been associated to apoptosis through lipid-peroxidation and mitochondrial permeability transition [12], and, even though poorly understood, there have been described marked changes in lipoproteins and triglycerides in SLE patients [17]. Oxidized fatty acids found augmented in sera from SLE patients include hydroxyls and aldehydes that are linked to modification of lipids, proteins and DNA; moreover, immunization with oxidatively modified autoantigens accelerate disease progress in MLR/lpr mice [27, 28]. ACSLs are essential for lipid metabolism (synthesis and degradation) and acyl-modification of cellular components, mediated by its enzymatic activation of fatty acids [7]. This, consequently, points to these enzymes as possible regulators of these processes.

ACSL5 localizes in the mitochondria and microsomes [8,9], from where it may execute its role in apoptosis, probably by supplying fatty acids into the mitochondria, where they can participate in oxidative reactions, increase ceramide synthesis and affect the mitochondrial membrane potential [11, 12, 27-29]. Lipid-peroxidation is augmented in SLE and correlates with disease activity [30, 31], and has also been associated to exposition of oxidized phosphatidylserine in the plasma membrane, associated with immunity and apoptosis due to recognition by macrophages [32]. Overexpression of ACSL5 in SLE patients may be an important key in predisposition and progression of autoimmunity; pointing to the involvement of this enzyme in supplying the material necessary for oxidative modification of cellular and extracellular components in early stages of SLE and SLE-like diseases, being ACSL5 directly associated with apoptosis

and thus leading to an augmented apoptosis and recognition of these modified antigens by macrophages, which in the end could activate lymphocytes and promote autoantibody production.

In conclusion, our findings point to ACSL5 as a key regulator of AICD in lymphocytes, playing a pro-apoptotic role. We have found that silencing ACSL5 decreases FAS, FASLG and TNF expression. This supports the hypothesis that this enzyme is implicated in SLE pathology not only by directly mediating the spontaneous apoptosis occurring in SLE but also indirectly involved in presenting self-antigens to immune cells and hence, promoting the pro-inflammatory state for the predisposition and progression of autoimmune diseases such as SLE. And probably by inducing TNF expression among other pro-inflammatory genes. Future perspectives should take into account the knowledge of molecular contribution of ACSL5 in the apoptotic pathway. We therefore propose ACSL5 as a diagnostic marker and potential therapeutic target for SLE.

FUNDING

DISCLOSURE

The authors declare that they have no competing interests.

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Supporting information Table S1. Primers used for quantification of different apoptosis associated genes.

Gene	Primer	Sequence (5'-3')
BCL2	Forward	GTG AAC TGG GGG AGG ATT GT
	Reverse	CCA GCC TCC GTT ATC CTG
FAS	Forward	CAA GGG ATT GGA ATT GAG GA
	Reverse	TGG AAG AAA AAT GGG CTT TG
FASL	Forward	TGG GGA TGT TTC AGC TCT TC
	Reverse	CAG AGG CAT GGA CCT TGA GT
TRAIL	Forward	TTC ACA GTG CTC CTG CAG TC
	Reverse	CAG CAG GGG CTG TTC ATA CT
TNF	Forward	TGC TTG TTC CTC AGC CTC TT
	Reverse	CAG CTT GAG GGT TTG CTA CA
CASP3	Forward	ATG GAA GCG AAT CAA TGG AC
	Reverse	GCT GCA TCG ACA TCT GTA CC

PATENT PAPER

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

Número de solicitud:	P201030630
Fecha de recepción:	28 abril 2010, 17:53 (CEST)
Oficina receptora:	OEPM Madrid
Su referencia:	ES1641.515
Solicitante:	CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC) (80%)
Número de solicitantes:	2
País:	ES
Título:	ACIL COENZIMA-A SINTETASAS DE CADENA LARGA (ACSLs) COMO BIOMARCADORES CITO-SEROLÓGICOS EN ENFERMEDADES INFLAMATORIAS O AUTOINMUNES
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(1) MODALIDAD:		
	PATENTE DE INVENCIÓN	[✓]
	MODELO DE UTILIDAD	[ ]

(2) TIPO DE SOLICITUD:		
	PRIMERA PRESENTACIÓN	[✓]
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	ADICIÓN A LA PATENTE ESPAÑOLA	[ ]
	SOLICITUD DIVISIONAL	[ ]
	CAMBIO DE MODALIDAD	[ ]
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	PCT: ENTRADA FASE NACIONAL	[ ]

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	N.º SOLICITUD:	
	FECHA SOLICITUD:	

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	SUCESIÓN:	[ ]

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CÓDIGO PAÍS:	ES
DNI/PASAPORTE:	

(7) TÍTULO DE LA INVENCIÓN:	ACIL COENZIMA-A SINTETASAS DE CADENA LARGA (ACSLs) COMO BIOMARCADORES CITO-SEROLÓGICOS EN ENFERMEDADES INFLAMATORIAS O AUTOINMUNES

(8) PETICIÓN DE INFORME SOBRE EL ESTADO DE LA TÉCNICA: SI NO	[ ] [✓]

(9) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN SI NO	[ ] [✓]

(10) EFECTUADO DEPÓSITO DE MATERIA BIOLÓGICA: SI NO	[ ] [✓]

(11) DEPÓSITO: REFERENCIA DE IDENTIFICACIÓN: INSTITUCIÓN DE DEPÓSITO: NÚMERO DE DEPÓSITO: ACCESIBILIDAD RESTRINGIDA A UN EXPERTO (ART. 45.1. B):	

(12) DECLARACIONES RELATIVAS A LA LISTA DE SECUENCIAS: LA LISTA DE SECUENCIAS NO VA MÁS ALLÁ DEL CONTENIDO DE LA SOLICITUD LA LISTA DE SECUENCIAS EN FORMATO PDF Y ASCII SON IDENTICOS	[✓] [✓]

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ADDITIONAL PUBLICATIONS

PLoS one

Multiple Sclerosis Risk Variant *HLA-DRB1*1501* Associates with High Expression of *DRB1* Gene in Different Human Populations

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Abstract

The human leukocyte antigen (HLA) *DRB1*1501* has been consistently associated with multiple sclerosis (MS) in nearly all populations tested. This points to a specific antigen presentation as the pathogenic mechanism though this does not fully explain the disease association. The identification of expression quantitative trait loci (eQTL) for genes in the HLA locus poses the question of the role of gene expression in MS susceptibility. We analyzed the eQTLs in the HLA region with respect to MS-associated HLA-variants obtained from genome-wide association studies (GWAS). We found that the Tag of *DRB1*1501*, rs3135388 A allele, correlated with high expression of *DRB1*, *DRB5* and *DQB1* genes in a Caucasian population. In quantitative terms, the MS-risk AA genotype carriers of rs3135388 were associated with 15.7-, 5.2- and 8.3-fold higher expression of *DQB1*, *DRB5* and *DRB1*, respectively, than the non-risk GG carriers. The haplotype analysis of expression-associated variants in a Spanish MS cohort revealed that high expression of *DRB1* and *DQB1* alone did not contribute to the disease. However, in Caucasian, Asian and African American populations, the *DRB1*1501* allele was always highly expressed. In other immune related diseases such as type 1 diabetes, inflammatory bowel disease, ulcerative colitis, asthma and IgA deficiency, the best GWAS-associated HLA SNPs were also eQTLs for different HLA Class II genes. Our data suggest that the DR/DQ expression levels, together with specific structural properties of alleles, seem to be the causal effect in MS and in other immunopathologies rather than specific antigen presentation alone.

Competing Interests: As a declaration of competing financial interest, authors JG and AR are employees and/or shareholders in Neocodex. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Multiple sclerosis (MS) is a common inflammatory disorder of the central nervous system characterized by demyelination with axonal and neuronal degeneration [1]. The cause of MS is unknown; however, susceptibility to MS is thought to be conferred by a combination of genetic and environmental factors [2].

The human leukocyte antigen (HLA) exerts the largest genetic contribution to MS susceptibility but exactly how it alters the risk of developing MS is not yet fully understood [3,4]. Association studies based first on serological typing and more recently on genome-wide association studies (GWAS) have been conducted for MS and other autoimmune diseases, and have identified specific HLA-DR/DQ genes. However, the remarkably strong linkage disequilibrium (LD) across the HLA region has hampered the

unequivocal ascertainment of the primary disease-risk HLA gene. This Class II association has been mapped to the *DRB5*0101-DRB1*1501-DQA1*0102-DQB1*0602* haplotype in the North European population [5]. These alleles are almost always present together in this population, making it impossible to distinguish the primary association. The mechanism for the strong LD in these HLA haplotypes has been shown to be consistent with a functional epistatic interaction between *DRB1*1501* and *DRB5*0101* alleles. This functional epistasis is associated with a milder form of experimental autoimmune encephalomyelitis (EAE) in mice [6].

On the other hand, association studies in African-American populations have suggested that the *DRB1*1501* allele itself determines MS-associated susceptibility [7]. However, in other populations, the risk allele or haplotype is different or does not contain *DRB1*1501* as in Sardinians where MS is associated with

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ARTICLE

Tag-SNP analysis of the *GFI1-EVI5-RPL5-FAM69* risk locus for multiple sclerosis

Antonio Alcina[1], Óscar Fernández[2], Juan Ramón Gonzalez[3], Antonio Catalá-Rabasa[1], María Fedetz[1], Dorothy Ndagire[1], Laura Leyva[2], Miguel Guerrero[2], Carmen Arnal[4], Concepción Delgado[5], Miguel Lucas[6], Guillermo Izquierdo[7] and Fuencisla Matesanz[*,1]

A recent genome-wide association study conducted by the International Multiple Sclerosis Genetic Consortium (IMSGC) identified, among others, a number of putative multiple sclerosis (MS) susceptibility variants at position 1p22. Twenty-one SNPs positively associated with MS were located at the *GFI-EVI5-RPL5-FAM69A* locus. In this study, we performed an analysis and fine mapping of this locus, genotyping eight Tag-SNPs in 732 MS patients and 974 controls from Spain. We observed an association with MS in three of eight Tag-SNPs: rs11804321 ($P = 0.008$, OR = 1.29; 95% CI = 1.08–1.54), rs11808092 ($P = 0.048$, OR = 1.19; 95% CI = 1.03–1.39) and rs6680578 ($P = 0.0082$, OR = 1.23; 95% CI = 1.07–1.41). After correcting for multiple comparisons and using logistic regression analysis to test the addition of each SNP to the most associated SNPs, we observed that rs11804321 alone was sufficient to model the association. This Tag-SNP captures two SNPs in complete linkage disequilibrium ($r^2 = 1$), both located within the 17th intron of the EVI5 gene. Our findings agree with the corresponding data of the recent IMSGC study and present new genetic evidence that points to *EVI5* as a factor of susceptibility to MS.
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INTRODUCTION

Multiple sclerosis (MS) is a complex disease presumed to be auto-immune and characterized by inflammation and demyelination with axonal and neuronal degeneration. MS is the most common central nervous system disease in young adults.[1] The prevalence and incidence rates in Spain are around 77/1 00000 habitants and 5.3/1 00000 habitants per year, respectively, similar to that found in Britain.[2,3] Susceptibility to MS is thought to be conferred by the combination of genetic and environmental factors.[1,4]

The best-established region implicated in predisposition to MS is the major histocompatibility complex on chromosome 6p21, specifically the HLA-DRB1* 1501 class II allele, but this accounts for less than 50% of MS genetics.[1,5] Recently, other regions have been implicated in MS susceptibility and replicated in different independent populations such as the interleukin 7 receptor alpha (*IL7RA*),[6–8] the interferon regulatory factor 5 (*IRF5*) gene[9] and the interleukin-2 receptor alpha (*IL2RA*).[8,10] However, except for *IL7RA*, the causal SNP of the new determined risk loci and the functional effect in the encoded proteins are unknown.

A recent genome-wide association study conducted by the International Multiple Sclerosis Genetics Consortium (IMSGC) has identified a number of putative MS susceptibility genes,[8] among them, 21 SNPs that are located in the locus containing the growth factor-independent 1 (*GFI1*), ecotropic viral integration site 5 (*EVI5*), ribosomal protein L5 (*RPL5*) and family with sequence similarity 69 (*FAM69*). The association of two of these SNPs has been replicated in a posterior study with 1318 MS Canadian patients.[11] In this study, our aim was to validate the association of the *GFI1-EVI5-RPL5-FAM69A* locus in a Caucasian Spanish population and to try to identify the gene responsible for the association with MS by a Tag-SNP strategy that captures 279 common variants at a pairwise $r^2 \geq 0.6$.

MATERIALS AND METHODS

Study subjects

Case samples comprised 732 patients with clinically defined MS according to Poser's criteria.[12] They were obtained from four public hospitals: Hospital Clínico of Granada ($n=126$), Hospital Virgen de las Nieves Of Granada ($n=165$), Hospital Carlos Haya of Málaga ($n=365$) and Hospital Virgen de la Macarena of Seville ($n=76$); all three cities located within a 200km radius in the South of Spain. The mean age of cases at the moment of sample collection was 36 years and the mean age of controls at interview was 38 years. The percentage of females was 68% for cases and 68% for controls. All of them were classified as relapsing remitting (RR) or secondary progressive (SP) MS cases. Controls were 974 blood donors with no history of inflammatory disease visiting the blood banks of Granada ($n=823$), Seville ($n=71$) and Málaga ($n=80$). The study was approved by the ethics committees of each of the hospitals participating in the study and written informed consent was obtained from all participants.

Genotyping

High-molecular-weight DNA was isolated from whole blood using the Flexigene Kit (Qiagen, Hilden, Gemany) according to the manufacturer's protocol. The eight SNPs were genotyped by TaqMan technology under conditions recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA).

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SHORT REPORT

Hexose-6-phosphate dehydrogenase: a new risk gene for multiple sclerosis

Antonio Alcina[1], Sreeram V Ramagopalan[2], Óscar Fernández[3], Antonio Catalá-Rabasa[1], María Fedetz[1], Dorothy Ndagire[1], Laura Leyva[3], Carmen Arnal[4], Concepción Delgado[5], Miguel Lucas[6], Guillermo Izquierdo[7], George C Ebers[2] and Fuencisla Matesanz*,[1]

A recent genome-wide association study (GWAS) performed by the The Wellcome Trust Case–Control Consortium based on 12 374 nonsynonymous single-nucleotide polymorphisms (SNPs) provided evidence for several genes involved in multiple sclerosis (MS) susceptibility. In this study, we aimed at verifying the association of 19 SNPs with MS, with P-values ≤ 0.005, in an independent cohort of 732 patients and 974 controls, all Caucasian from the South of Spain. We observed an association of the rs17368528 polymorphism with MS ($P=0.04$, odds ratio (OR)$=0.801$, 95% confidence interval (CI)$=0.648$–0.990). The association of this polymorphism with MS was further validated in an independent set of 1318 patients from the Canadian Collaborative Project ($P=0.04$, OR$=0.838$, 95% CI$=0.716$–0.964). This marker is located on chromosome 1p36.22, which is 1 Mb away from the MS-associated kinesin motor protein $KIF1B$, although linkage disequilibrium was not observed between these two markers. The rs17368528 SNP results in an amino-acid substitution (proline to leucine) in the fifth exon of the hexose-6-phosphate dehydrogenase ($H6PD$) gene, in which some variants have been reported to attenuate or abolish H6PD activity, in individuals with cortisone reductase deficiency. This study corroborates the association of one locus determined by GWAS and points to $H6PD$ as a new candidate gene for MS.

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease with a complex pathogenesis, in which demyelination and neurodegeneration are the main contributors to disability.[1] Susceptibility to MS is thought to be conferred by a combination of genetic and environmental factors.[2] The most established region in predisposition to MS is the major histocompatibility complex on chromosome 6p21, specifically, the HLA-DRB1*1501 class II allele.[3] Currently, other loci have been identified that have convincing evidence for an association with MS, such as the IL2RA,[4,5] IL7RA,[6,7] KIF1B,[8] IRF5,[9] EVI5,[4,10] CD226[11,12] and CLEC16A[12] genes. Some of these genes were identified through a classical candidate gene approach; however, many new candidate risk factors arise from the hypothesis-free approach provided by genome-wide association studies (GWAS). The Wellcome Trust Case–Control Consortium (WTCCC) carried out a GWAS based on 12 374 nonsynonymous single-nucleotide polymorphisms (SNPs) typed in 975 patients and 1466 controls.[13] No SNP outside the HLA region provided a significant association with MS at the genome-wide level in this study. However, the initial screen yielded promising signals. One of the signals located at the tyrosine kinase 2 (TYK2) gene has since been confirmed with an independent cohort.[14]

In this study, we assessed the risk contribution of 19 SNPs that showed some degree of association in the GWAS performed by the WTCCC using MS patients and controls from the

south of Spain. We identified hexose-6-phosphate dehydrogenase (H6PD, glucose-1-dehydrogenase) as a novel risk gene for MS and validated this finding in an independent cohort of Canadian MS patients.

MATERIALS AND METHODS

Subjects

Case samples comprised 732 patients with clinically definite MS, according to Poser's criteria.[15] They were obtained from four public hospitals: the Hospital Clínico in Granada ($n=126$), the Hospital Virgen de las Nieves in Granada ($n=165$), the Hospital Carlos Haya in Málaga ($n=365$) and the Hospital Virgen de la Macarena in Seville ($n=76$). All three cities are within a 200 km radius in the South of Spain. The mean age ($\pm$ SD) of cases at the time of sample collection was 29.84 ± 10.66 years and that of controls was 33.43 ± 12.19 years. The percentage of females was 68% for cases and 68% for controls. All patients were classified as RR (relapsing remitting) or SP (secondary progressive) MS cases. Controls were 974 blood donors with no history of inflammatory disease attending the blood banks of Granada ($n=823$), Seville ($n=71$) and Málaga ($n=80$). Both cases and controls were Caucasians. The study was approved by the Ethics Committees of each of the hospitals participating in the study and a written informed consent was obtained from all participants.

Genotyping of individuals from the Spanish population

In this analysis, we included the 19 SNPs identified in the WTCCC GWAS with a P-value ≤ 0.005 from all chromosomes, except chromosome 6. We typed all 19 SNPs in 732 patients and 974 controls. Genotyping was performed using

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IL2RA/CD25 Gene Polymorphisms: Uneven Association with Multiple Sclerosis (MS) and Type 1 Diabetes (T1D)

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Abstract

Background: IL-2 receptor (IL2R) alpha is the specific component of the high affinity IL2R system involved in the immune response and in the control of autoimmunity.

Methods and Results: Here we perform a replication and fine mapping of the *IL2RA* gene region analyzing 3 SNPs previously associated with multiple sclerosis (MS) and 5 SNPs associated with type 1 diabetes (T1D) in a collection of 798 MS patients and 927 matched Caucasian controls from the south of Spain. We observed association with MS in 6 of 8 SNPs. The rs1570538, at the 3'-UTR extreme of the gene, previously reported to have a weak association with MS, is replicated here (P = 0.032). The most associated T1D SNP (rs41295061) was not associated with MS in the present study. However, the rs35285258, belonging to another independent group of SNPs associated with T1D, showed the maximal association in this study but different risk allele. We replicated the association of only one (rs2104286) of the two *IL2RA* SNPs identified in the recently performed genome-wide association study of MS.

Conclusions: These findings confirm and extend the association of this gene with MS and reveal a genetic heterogeneity of the associated polymorphisms and risk alleles between MS and T1D suggesting different immunopathological roles of IL2RA in these two diseases.

Introduction

Multiple sclerosis (MS) is the most common central nervous system disease in young adults, and one of the leading causes of disability in this age group affecting over 2.5 million individuals world-wide [1] The prevalence and incidence rates in Spain are around 77/100 000 habitants and 5.3/100 000 habitants per year respectively similar to what has been found in Britain [2,3] The disorder, which is presumed to be autoimmune in nature, is characterized by inflammation and demyelination, with axonal and neuronal degeneration. Susceptibility to MS is thought to be conferred by the combination of many common gene variants (not aberrant gene products) and environmental factors, which are mostly unknown [1,4].

The most strongly associated region implicated in predisposition to MS is the major histocompatibility complex (MHC) on chromosome 6p21, specifically the HLA-DRB1* 1501 class II allele; but, this account for less than 50% of MS genetics [1,5]. Recently, other regions have been implicated in MS susceptibility

and replicated in different independent populations as the interleukin 7 receptor alpha (*IL7RA*) [6–8], the interferon regulatory factor 5 (*IRF5*) gene [9] and the interleukin-2 receptor alpha (*IL2RA*) [8,10,11]. The *IL2RA* gene has also been associated with type 1 diabetes (T1D) [12–14] and localized the association region in two independent groups of SNPs, spanning overlapping regions of 14 and 40 Kb encompassing *IL2RA* intron 1 and the 5' regions of *IL2RA* and the RNA binding motif protein 17 (*RBM17*) genes.

Diverse autoimmune diseases may coexist in the same individual and in families, suggesting they might share common susceptibility gene variants implying a common etiology [15,16]. For example, in families with systemic lupus erythematosus (SLE), other autoimmune mediated diseases, such as MS and rheumatoid arthritis (RA) [17], or families with T1D and MS in Sardinian population [18], occur more frequently than in the general population. Such observations and others suggest the existence of shared genes or involvement of common biochemical pathways in these diseases. This hypothesis is supported by numbers of reports

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BRIEF COMMUNICATION

The T244I variant of the interleukin-7 receptor-alpha gene and multiple sclerosis

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Abstract

Several but not all studies have provided evidence for the association between multiple sclerosis (MS) and the T244I variant of the interleukin-7 receptor-alpha gene ($IL7RA$), rs6897932. We performed a new replication case–control study in 599 MS patients and 594 healthy controls, all Caucasians from the south of Spain. The genotype and allele frequencies differed between MS cases and controls. The $IL7RA$ rs6897932 C allele and the CC genotype were found to be factors for disease susceptibility [per allele odds ratio (OR) 1.32, 95% CI 1.1–1.6, $P = 0.0031$; per CC genotype vs TT + TC genotypes, OR 1.5, 95% CI 1.18–1.87, $P = 0.0007$]. The combined data analysis included 3324 cases and 5032 controls of Europeans and Americans of European origin resulting in stronger association with similar OR ($P = 1.9 \times 10E-9$). These findings in our sample support previous reported association studies between $IL7RA$ rs6897932 and MS.

Several studies have provided evidence for the association between multiple sclerosis (MS) and the T244I variant of the interleukin-7 receptor-alpha gene ($IL7RA$), rs6897932, but some others have been negative. As consistent replication of an effect for $IL7RA$ in various populations would provide stronger evidence of causality (1, 2), we investigated in this study whether the $IL7RA$ rs6897932 is associated with MS in an independent set of Caucasian population from the south of Spain.

MS is chronic inflammatory and demyelinating disease of the central nervous system estimated to affect over 2 million individuals worldwide. The disease manifests itself by immune-mediated demyelination and damage to axons resulting in progressive neurological disability in the absence of apparent ongoing infection (3). Genes influence susceptibility to MS; however, results of linkage and association studies are inconsistent, aside from the identification of human leukocyte antigen (HLA) class II haplotypes where the HLA-DRB1*1501 allele is a well-established genetic risk factor for MS (4–8). This locus, however, does not account for the whole genetic component of MS, although former and recently performed large linkage and association studies suggest that genes other than those in the HLA locus have small effects (4). Numerous candidate gene studies have also been performed in MS, but findings from one population have been difficult to replicate in other populations (9). The protein kinase C alpha ($PRKCA1$) gene (10, 11), the interleukin-2 receptor-alpha

BRIEF COMMUNICATION

Multiple sclerosis association study with the *TENR-IL2-IL21* region in a Spanish population

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Abstract

Polymorphisms from the *TENR-IL2-IL21* block in the 4q27 chromosome were recently associated with type 1 diabetes, celiac disease, rheumatoid arthritis and psoriasis. We undertook this study to investigate the potential role of polymorphisms rs3136534, rs6822844 and rs2069762 (-330 T/G *IL2*) in multiple sclerosis (MS) (805 patients of Spanish Caucasian origin and 952 health controls). We did not find evidence for association with any single nucleotide polymorphisms (SNPs) tested. Allele and genotype frequencies of the SNPs, which were studied, were similar in *DRB1*15*-positive or *DRB1*15*-negative patients. After stratification of MS patients by clinical course, a weak association was observed with rs2069762 G allele and haplotype bearing this allele with secondary progressive MS, although these cases represent 22% of the MS cases. Our results did not show major influence of *TENR-IL2-IL21* locus on susceptibility or disease progression in MS. However, we could not exclude completely the effect in MS for this region. Additional studies, using much larger sample sizes and analysis of additional polymorphisms in the gene and its flanking region, will be required to ascertain their contributions to MS susceptibility.

As a result of genome-wide association studies, several polymorphisms in the chromosome 4q27 region have been recently associated with susceptibility to type 1 diabetes (T1D) and Graves' disease (1), celiac disease (CD) and rheumatoid arthritis (RA) (2) and psoriasis (3). The Wellcome Trust Case Control Consortium study reports that rs17388568 and rs3136534 from 4q27 *TENR-IL2-IL21* region are associated with T1D and Graves' disease (1). van Heel et al. (2) indicate that rs6822844 is significantly associated with CD in three ethnic cohorts. This SNP also mapped to the *TENR-IL2-IL21* high-degree linkage dis-

equilibrium (LD) block. The functional promoter polymorphism rs2069762 (-330 T/G *IL2*), which affects the gene expression, has been found associated with the secondary progressive (SP) course of multiple sclerosis (MS) in Caucasians by our group (4). The aim of this work was to assess the possible association of the rs3136534, rs6822844 and rs2069762 polymorphisms from *TENR-IL2-IL21* region with MS.

The pathogenesis of MS remains to be discovered, although it is widely regarded as an autoimmune disease directed against myelin proteins and/or other oligodendrocyte