A Human-Specific α7-Nicotinic Acetylcholine Receptor Gene in Human Leukocytes: Identification, Regulation and the Consequences of CHRFAM7A Expression

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The human genome contains a variant form of the α7-nicotinic acetylcholine receptor (α7nAChR) gene that is uniquely human. This CHRFAM7A gene arose during human speciation and recent data suggests that its expression alters ligand tropism of the normally homopentameric human α7-AChR ligand-gated cell surface ion channel that is found on the surface of many different cell types. To understand its possible significance in regulating inflammation in humans, we investigated its expression in normal human leukocytes and leukocyte cell lines, compared CHRFAM7A expression to that of the CHRNA7 gene, mapped its promoter and characterized the effects of stable CHRFAM7A overexpression. We report here that CHRFAM7A is highly expressed in human leukocytes but that the levels of both CHRFAM7A and CHRNA7 mRNAs were independent and varied widely. To this end, mapping of the CHRFAM7A promoter in its 5′-untranslated region (UTR) identified a unique 1-kb sequence that independently regulates CHRFAM7A gene expression. Because overexpression of CHRFAM7A in THP1 cells altered the cell phenotype and modified the expression of genes associated with focal adhesion (for example, FAK, P13K, Akt, rho, GEE, Elk1, CyC), leukocyte transepithelial migration (Nox, ITG, MMPs, PKC) and cancer (kit, kItL, ras, cFos cyclinD1, Frizzled and GPCR), we conclude that CHRFAM7A is biologically active. Most surprisingly however, stable CHRFAM7A overexpression in THP1 cells upregulated CHRNA7, which, in turn, led to increased binding of the specific α7nAChR ligand, bungarotoxin, on the THP1 cell surface. Taken together, these data confirm the close association between CHRFAM7A and CHRNA7 expression, establish a biological consequence to CHRFAM7A expression in human leukocytes and support the possibility that this human-specific gene might contribute to, and/or gauge, a human-specific response to inflammation.

*TWC and XD contributed equally to the work presented in this study.

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CHRFAM7A gene that can gauge the function and ligand tropism of the normal α7nAChR ligand-gated channel (3,9).

Since its discovery in 1998, CHRFAM7A has largely been the focus of neuroscience and mental health research because historically the α7nAChR was viewed as a neuron-specific, ligand-gated ion channel. More recently, however, its detection in normal human leukocytes (10,11) has gained particular attention because several in vitro studies have shown that CHRFAM7A modifies α7nAChR channel activity and changes ligand tropism (12–14). Because α7nAChR activation is closely tied to the inflammatory responses of peripheral tissues, these observations raise the possibility that CHRFAM7A may be particularly relevant to gauging inflammation in humans. The Tracey laboratories, for example, established that efferent signaling of the vagus nerve acts exclusively via α7nAChR activation in the spleen to regulate systemic cytokine responses to infection in mice (15–20). Similarly, Costantini and colleagues demonstrated the existence of a similar α7nAChR-dependent regulation of the local inflammatory response in tissues (21–27). With α7nAChR activation clearly essential to inflammation, not to mention vagus nerve responsiveness and leukocyte function (28,29), we reasoned that it was therefore critical to understand how a human-specific α7nAChR in human leukocytes might influence human leukocyte function, the regulation of its expression and the biological consequences of its expression. Because newly evolved genes such as CHRFAM7A disproportionately segregate with complex human disease (30,31), the results point to the possible existence of human-specific responses to inflammation that are not present in other species. If so, CHRFAM7A may gauge the α7nAChR-mediated effects of the human vagus nerve.

MATERIALS AND METHODS

Materials

The plasmid encoding the full-length CHRFAM7A (variant 1: NM_139320.1) was from OriGene (Rockville, MD, USA) and differs from a shorter CHRFAM7A (variant 2: NM_148911) in that it encodes a 90-amino-acid amino terminus containing the FAMA7 sequence (NH2-MQKYCIYQHF QFOQFRLQHLW IAANCDI) and a α7nAChR amino terminus peptide (ADE RDFATFHTNV LYNSSGHCQY LPPGIFKSSC YIDVRWFPPFD VQHCCLKFGS WSYGGWSDL). The plasmid encoding full-length CHRNA7 (variant 2: NM_001190455) was from GeneCopoeia (Rockville, MD, USA) and differs from the shorter CHRNA7 (variant 1: NM_000746) by codons that encode a GKATAPPSTPPWDGHPASVRAPGP peptide introduced at His9 of the α7nAChR subunit. The pGL4 promoterless expression plasmid encoding firefly luciferase was purchased from Promega (Madison, WI, USA). All other chemicals and reagents were the products of Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise.

Human Peripheral Leukocytes

Informed consent was obtained from healthy volunteers for the collection of peripheral blood. Volunteers were recruited and enrolled by the University of California San Diego (UCSD) Clinical Translational Research Institute (San Diego, CA, USA). Venous blood was collected by peripheral venipuncture in BD Vacutainer blood collection tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Biosciences, San Jose, CA, USA) and placed on ice. Red blood cells were lysed using BD Pharm Lyse ammonium chloride solution (BD Biosciences) at room temperature for 15 min and leukocytes pelleted by centrifugation. Cell pellets were stored at −80°C until further analyses. The UCSD Institutional Review Board approved the enrollment of participants, consent forms and specimen collection protocols.

Cell Culture

All cell lines were originally purchased from ATCC (Manassas, VA, USA) and/or acquired through the UCSD Department of Surgery, Division of Trauma, Burns and Acute Care Surgery Cell Repository (San Diego, CA, USA). Thawed cells were washed in RPMI culture media containing 10% fetal calf serum (FCS), the pellet reconstituted in culture media and cells plated into six-well tissue culture plates. All cells were washed 48 h later and allowed to grow to 90% confluence and propagated with trypsin digestion as needed. For transduction studies, cells were seeded at 2 × 10^6 in 6-well tissue culture plates the day before the experiment. As indicated in each experiment, cells were harvested directly from the culture dishes for total RNA preparation, processed for stable or transient transfection or treated with 100 ng/mL LPS (L4391, Sigma-Aldrich) for 3 h. At the end of incubations, cells were harvested, total RNA isolated and the cDNA generated (see below) used for analyses of gene expression.

Lentivirus Constructs for CHRFAM7A Expression

The ORF of human CHRFAM7A (variant 1) was amplified by PCR from the CHRFAM7A-pCMV6-Entry plasmid (PS100001, OriGene, Rockville, MD, USA) using: forward primer: 5′-aGTcC TCGAGATGcaaaaatattgcatct-3′; reverse primer: 5′-attcGGATCTCCATTACCGAAAG TCTTGGACCGGC-3′.

The PCR products were purified and cloned into the bicistronic pLVX-IRE- ZsGreen1 lentivirus plasmid as recommended by the manufacturer. The identity of the plasmid was confirmed by DNA sequencing (Retrogen, San Diego, CA, USA). Lentivirus was packaged using the Lenti-X HTX Packaging System (631247, Clontech, Mountain View, CA, USA) following vendor instructions. After 48 h, the supernatant containing virus was collected and used to transduce THP1 cells. Stable transduced green fluorescent protein (GFP)-positive THP1 cells were detected by fluorescence microscopy and purified by cell sorting. Cells were expanded without further selection and the stability of transduction monitored by weekly flow cytometry.
Flow Cytometry and Cell Sorting of THP1 Cells

For flow cytometry analyses, cells were washed and fixed with Cytofix according to the manufacturer’s recommendations (BD Biosciences) for 10 min on ice. Cells were then incubated with labeled bungarotoxin (BD Biosciences) in FACS buffer (1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0.005% sodium azide) and washed in FACS buffer. Flow cytometry was performed with a Becton Dickinson FACSCalibur and data analysis performed with CellQuestPro software from Becton, Dickinson and Company (BD) (Franklin Lakes, NJ, USA), processed and analyzed using JFlow. To purify GFP-expressing THP1 cells, the transduced cells were sorted twice by FACS at the core facilities of the Center for AIDS Research at UCSD, selected for GFP expressing cells, the transduced and sorted THP1 cells using GFP as measured by flow cytometry. Stable expression was monitored weekly for retention of > 85% cells expressing GFP as measured by flow cytometry.

Cells were propagated in 10% RPMI1640.

RNAseq, Gene Expression and Pathway Analyses

Total RNA was prepared from transduced and sorted THP1 cells using RNeasy kit (Qiagen, San Diego, CA, USA) and was quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). One μg total RNA was used for RNAseq analyses and performed by contract with the Genomics Core, Cedars-Sinai Medical Center (Los Angeles, CA, USA). Bioinformatic analyses, differential gene expression and pathway analyses were performed by contract with Accura-Science (Johnston, IA, USA). For datasets and RNA-seq differential expression (DE) analysis, the BAM files for vector and stable CHRFAM7A-transduced cells were generated by RNA-seq at the genome core facilities at Cedars-Sinai Genomics core at Cedars Sinai Medical Center and were used for differential gene expression analyses. Comparisons were made between CHRFAM7A and vector using two methods to define differentially expression genes. DESeq (32) is one of the few methods suitable with limited replicates (33) and controls for false positive signals (34,35). The Python package HTSeq was used to produce the count table and a P value <0.05 was set as cutoff. In the second method, the results of DESeq were overlapped with Cuffdiff (36) and a P value <0.05 was chosen as cutoff. The differentially expressed gene groups defined by both analytical methods were used for functional enrichment analysis and GOseq in biocductor was used for gene ontology analysis (37) with up- and downregulated differentially expressed genes respectively. A P value cutoff of 0.05 was used to choose significant gene ontology (GO) terms. The Functional Class Scoring method implemented in GSEAbase was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (38) and a P value of <0.05 was used to define significant pathway categories.

Isolation of RNA from Cultured Cells and Preparation of cDNA for PCR and qPCR

Total RNA was prepared from cell lysates using the RNeasy kit and was quantified using a NanoDrop Spectrophotometer. One μg of the total RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, San Diego, CA, USA) in a 20 μL reaction as described by the manufacturer and 1 μL was used for RT-PCR or real-time qPCR analyses.

RT-PCR and Quantitative RT-PCR for CHRFAM7A and CHRNA7

RT-PCR was performed in a 50 μL reaction containing 45 μL PCR blue mix (Invitrogen [Thermo Fisher Scientific]), 1 μL of each primer (10 μmol/L), 1 μL cDNA and 2 μL water. The cycling conditions were 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 60 s and a final extension at 72°C for 5 min. Ten μL of each PCR product were resolved on a 2% agarose gel and images were acquired using an Alpha Innotech imaging system (Fisher Scientific [Thermo Fisher Scientific]). Real-time qPCR was performed in a 25 μL reaction containing 12.5 μL 2× SYBR Green PCR Master Mix (Bio-Rad), 0.5 μL of each primer (10 μmol/L), 1 μL cDNA and 10.5 μL water. PCR cycling conditions were: 95°C for 10 min followed by 45 cycles of 94°C for 25 s, 60°C for 25 s and 72°C for 40 s. Primer efficiency for CHRFAM7A and CHRNA7 were 100% and 94% respectively.

Primers for CHRFAM7A were: sense, 5′-ATAAGCTGCAAACTGCGATA-3′; antisense, 5′-cagcgtacatcgatgtagcag-3′. Primers for CHRNA7 were: sense, 5′-acATGctgtctgtcggagcctg-3′; antisense, 5′-gattgtagttcttgaccagct-3′. Primers for GAPDH were: sense, 5′-CATGGAAGTATGCAAACACCT-3′; antisense, 5′-AGTCCCTCCACGATAACGAACT-3′.

5′ RACE and Identification of CHRFAM7A Variant 1

5′ RACE was performed using SMARTer RACE cDNA Amplification Kit (Clontech) following vendor’s instructions. Briefly, total RNA was prepared from THP1 cell with RNeasy kit (Qiagen). Three μg total RNA was processed for mRNA using PolyA Spin mRNA Isolation Kit (New England Biolabs [NEB], Ipswich, MA, USA). One-fifth of the poly A mRNA was reverse transcribed and the resulting cDNA was amplified sequentially by PCR and nested PCR. Both gene-specific primers (GSP), GSP and nestGSP listed below, hybridize to the fifth exon of human CHRNA7/CHRFAM7A, with nestGSP 5′ to the GSP without overlapping. The nested PCR products were purified and cloned into pDrive (Qiagen). Colonies were sequenced to identify the 5′ initiation sites and the 5′ sequence upstream the fifth exon of CHRNA7/CHRFAM7A (GSP [5′-GCAGG TACTGCAGATGAAAAG-3′]; NestGSP [5′-TAGTGTTGAATGTGG CGTCAGCG-3′]).

Analyses of the CHRFAM7A Promoter

The putative CHRFAM7A promoter region spanning from -2363 to +22 relative to the open reading frame ATG start codon was amplified by PCR of genomic
DNA isolated from HEK293 cells. The longest fragment was cloned into pGL4 promotorless luciferase reporter plasmid (Promega) according to the manufacturer’s specifications and the resulting plasmid, pGL4-CHRFAM7A (~2400 bp), was confirmed by DNA sequencing and thereafter referred to as p2400 to reflect its size.

The primers were: sense, 5’-ATCAGCTGCTAGATAGAGCATTATTTA-3’ containing a NheI restriction site; antisense, 5’-GCATAGATCTGTAGATGCAATATTTCGCT-3’ containing an 8gIII restriction site.

Three serial 5’ deletion promoter constructs of 1800, 1000 and 500 bp were derived by PCR of the p2400 template using the same antisense primer described above, with one of three sense primers to obtain: f1800 (5’-ATCAGCTGCTAGAAGCC TTCATCAGTGGAAATGCTGATGACTCAAGTCGCATCTCAGTTGGAAG-3’); f1000 (5’-ATCAGCTGCTAGCTGATGCAATATTTTCGCT-3’); f500 (5’-ATCAGCTGCTGATGCAATATTTTCGCTGATGCAATATTTCGCT-3’). The fragments were generated and cloned into the pGL4 vector to create plasmids f1800, f1000 and f500, which were each sequenced to confirm their identity.

Three plasmids were then transiently transfected into THP1 cells as described above and luciferase activity was analyzed 30 h after transfection following the manufacturer’s instructions (Promega). Luciferase activity was normalized to protein concentration was expressed as fold changes over that of pGL4-transfected cells.

Statistical Analyses

CHRNA7 and CHRFAM7A gene expression were normalized to that of GAPDH and the fold changes calculated by ΔΔCt method using the relative expression software tool (REST) for group-wise comparisons and statistical analyses of relative expression results (39). The GraphPad (GraphPad Software Inc., La Jolla, CA, USA) program Prism 6 was used for other analyses including two-tailed unpaired t test, Wilcoxon signed rank test of unpaired t test and analysis of variance (ANOVA) as indicated in the text and in legends to the figures.

All supplementary materials are available online at www.molmed.org.

RESULTS

Detection of CHRFAM7A and CHRNA7 Expression in Human Leukocytes

During analysis of human-specific gene expression in leukocytes purified from the blood of normal volunteers, we examined whether we could specifically detect concomitantly expression of mRNAs encoding CHRFAM7A and CHRNA7 (Figure 1). As described in Materials and Methods and illustrated in Figure 1A, the full-length CHRFAM7A (variant 1, NM_139320.1) differs from the shorter CHRFAM7A (variant 2, NM_148911) in that it encodes a unique FAM7A peptide sequence (NH2-MQKYCIYQFQQLIQHFLWIAANCDI). Accordingly, PCR primers could be designed to specifically hybridize with CHRFAM7A variant 1. Because the CHRFAM7A variant 2 mRNA does not contain unique sequences that allow it to be differentiated from either CHRFAM7A (variant 1) or CHRNA7 (see Figure 1A), no specific RT-PCR primers could be designed to detect CHRFAM7A (variant 2) so we used 5’ RACE (see below).

In contrast, the full-length CHRNA7 mRNA (variant 2: NM_001190455) differs from the shorter CHRNA7 mRNA (variant 1: NM_000746) by encoding a GKATASPPSTPPWDPGHIPGASVRPAPG peptide that allows both transcripts differentiated by size when analyzed by gel electrophoresis (see Figure 1A). They are also measured simultaneously by quantitative RT-PCR. As shown in Figure 1B, PCR of leukocyte cDNA prepared from the mRNA of seven volunteers established the presence of CHRFAM7A mRNA in all samples (see Figure 1B). CHRNA7 was readily detected in four of seven samples (Figure 1C) and in all samples with longer exposures (not shown). Increased PCR cycle numbers showed ubiquitous expression of CHRNA7. Interestingly, leukocytes from different individuals appeared to express different combinations of CHRNA7 variants 1 and 2. For example, three samples (lanes 3, 4 and 7) had the longer CHRNA7 variant 1 transcript while a fourth (lane 4) only had variant 2 (see Figure 1C). Because no difference in the signal was obtained in the analyses of GAPDH (Figure 1D), the results suggest significant individual variability in expression of both leukocyte CHRNA7 and CHRFAM7A. On average, however, the differences detected between the levels of both genes are not statistically different in normal human leukocytes (Figure 1E). Both CHRNA7 and CHRFAM7A gene expression is markedly variable, ranging from 100 to 20,000 copies/μg of starting mRNA. Any differences in the
CHRFAM7A and CHRNA7 Gene Expression in Leukocyte Cell Lines

We used RT-PCR to survey the expression of both CHRFAM7A and CHRNA7 in human leukocyte lines (Figure 3). We found that HL60, RPMI-2286, U937, HEL92, Jurkat and ARH77 are like the premonocytic THP1 cell line and express both CHRFAM7A (Figure 3A) and CHRNA7 (Figure 3B) when compared with GAPDH (Figure 3C). As in normal leukocytes (see Figure 1), we detect both variant 1 and variant 2 mRNA transcripts of CHRNA7 (see Materials and Methods) depending on the cell evaluated. This raises the possibility that heteropentameric α7nAChRs exists on the leukocyte cell surface containing up to three distinct subunits: CHRNA7 variant 1; CHRNA7 variant 2; and CHRFAM7A. Three cell lines (HL60, HEL92 and Jurkat) express both transcripts 1 and 2 of CHRNA7 while U937 cells only express transcript 2 of CHRNA7. Three other cells (RPMI-2286, RH77 and THP1 cells) only express transcript 1 of CHRNA7. We conclude that significant heterogeneity of the α7nAChR subunits that compose the final cell surface pentameric channel may exist on the human leukocyte cell surface.

There are no antibodies that can distinguish the different molecular forms of CHRNA7 and CHRFAM7A. Accordingly, we used qRT-PCR to quantify the expression of both CHRNA7 (Figure 3D) and CHRFAM7A (Figure 3E) in the different leukocyte cell lines. Because HL60 cells appear to express equal amounts of variants 1 and 2 of CHRNA7 (see Figure 3B) and intermediate amounts of CHRFAM7A (see Figure 3A), the expression levels of both genes were normalized to the levels detected in HL60 cells. As shown in Figure 3F, no consistent pattern was observed in the ratio of CHRFAM7A to CHRNA7 which varied 10-fold to 10,000-fold higher in some cells (for example, HL-60, U937, HEL92 and THP1 cells) but were either near equal in others (RPMI-2286 cells) or 10 to 100 lower, for example in Jurkat and ARH77 cells. If mRNA ex-
expression correlates with protein expression, then the data suggest the existence of significant heterogeneity in the CHRFAM7A–CHRNA7 pentamer that forms the human cell surface α7nAChR on human leukocytes.

**CHRFAM7A Has a Unique and Distinct Promoter Regulating Gene Expression**

Expression levels of CHRFAM7A and CHRNA7 in leukocytes suggest their independent regulation. Furthermore the 5′ UTR sequence and the translation initiation site of the CHRFAM7A gene from the 5′ RACE analyses (see Figure 2) allowed us to infer the existence of the CHRFAM7A variant 1 protein in human leukocytes that others have detected by immunoblotting with anti-CHRNA7 antibodies (10,11). With these sequences, we used a bioinformatic approach to identify potential transcription factor binding sites (40) and experimental promoter mapping techniques to assess the regulation of CHRFAM7A gene expression. As illustrated in Figure 4A, five 5′ UTR plasmid constructs were prepared with DNA sequences that ranged from +22 to −2400 bp of the CHRFAM7A variant 1 transcript using the adenosine in the ATG ORF as +1 (see Materials and Methods). Each 5′ UTR–containing plasmid was then tested for its ability to activate luciferase gene expression in transiently transfected THP1 cells (Figure 4B). Promoter activity was observed within 500 bp of the CHRFAM7A ORF. Interestingly, 5′ extensions of this fragment encode dominantly negative inhibitory sequences that decrease the luciferase activity observed within the first 500 bp of the 5′ UTR. These data point to the existence of both stimulatory and inhibitory transcriptional elements regulating CHRFAM7A gene expression, per-
haps reflecting its reported downregulation by LPS treatment in leukocytes (10,11) and differential upregulation in human epithelial cells (41).

**Biological Consequence of CHRFAM7A Gene Expression in THP1 Cells**

We used the transduction of THP1 cells with lentivirus to stably overexpress CHRFAM7A in THP1 cells and determine whether CHRFAM7A altered THP1 cell phenotype and/or gene expression. As shown in Figure 5, GFP-transduced THP1 cells (Figure 5A) can be differentiated from CHRFAM7A-transduced THP1 cells (Figure 5B) in that the latter tend to proliferate as loosely associated cell clusters that are reminiscent of expanding, transformed and presumably clonal cells. This is a marked contrast to the even distribution of both parental (not shown) and GFP-transduced THP1 cells, suggesting that CHRFAM7A overexpression affects cell–cell adhesion.

To assess the functional consequence of stable CHRFAM7A overexpression, we used flow cytometry to first show that fluorescently labeled bungarotoxin (BT), a specific α7nAChR ligand, binds the leukocyte α7nAChR on THP1 cells (Figure 5C). BT is an irreversibly binding toxin that has been described extensively and shown to be a highly specific determinant of α7nAChR ligand binding. It also distinguishes the cell surface α7nAChR channel/receptor from other nicotinic receptors (42,43). As shown in Figure 5C, the addition of fluorescently labeled BT to THP1 cells produces a tenfold shift from baseline fluorescence labeling of THP1 cells that indicates the presence of the α7nAChR channel/receptor. This shift in BT binding was then compared between BT binding to GFP-vector and CHRFAM7A-transduced THP1 cells. As shown in Figure 5D, CHRFAM7A-transduced THP1 cells bind even more than labeled BT, suggesting that CHRFAM7A overexpression increases the α7nAChR channel and receptor at the THP1 cell surface. Quantitative analyses of the mean fluorescence intensity (MFI) in vector- and CHRFAM7A-transduced cells confirmed the significance of this shift (Figure 5E). These data suggest that stable CHRFAM7A overexpression either increased BT binding by altering ligand binding of a heteropentameric complex or by regulating CHRNA7 gene expression, thereby facilitating, not inhibiting, α7nAChR transport to the cell surface (3,9,12–14).

To further investigate the biological consequences of stable CHRFAM7A overexpression, its ability to increase α7nAChR dependent BT binding and induce a THP1 cell clumping phenotype, we isolated mRNA from both vector- and CHRFAM7A-transfected cells and analyzed their respective transcriptomes by RNA-seq. Clustering analyses of gene expression were performed using both DESeq (32) and CutDiff (36) analytical tools. In comparing the effects of CHRFAM7A and GFP-vector gene expression, DESeq identified 653 differentially expressed genes and 139 differentially expressed genes identified by Cutdiff. The top 30 up- and downregulated differentially expressed genes are presented in Table 1 and sorted on the basis of the statistical
The highest differentially expressed gene was **CHRFAM7A** (55.4-fold) in the CHRFAM7A-transduced cells. It is particularly noteworthy how- ever that an increase in **CHRNA7** expression (13.3-fold) was the second most significant difference in these stably transfected cells. This suggests that increased BT binding in CHRFAM7A cells (see Figure 5D) is the result of increased $\alpha_7$nAChR on the cell surface, rather then a reflection of an increase in a CHRFAM7A subunit in stably transfected cells. These data support the hypothesis that long-term and stable expression of CHRFAM7A modulates CHRNA7 availability to the surface. Alternatively, the CHRFAM7A protein may form a human-specific $\alpha_7$nAChR heteropentamer at the leukocyte cell surface with modified ligand specificity, tropism and binding kinetics, compared with the $\alpha_7$nAChR homopentamer found in all species (3,9,12–14).

**DISCUSSION**

Most genes are shared among all species but taxonomically restricted genes are only found in the genomes of specific taxa (for example, primates) and still others are species-specific, for example those uniquely localized to the human genome (29,44–52). While the emergence of human-specific genes is neither new nor unexpected, historically there has been the focus of evolu-
tionary biologists interested in the human-specific biology that relates to cognition, behavior, reproduction and mental health (30,53,54). Interestingly, the existence of human-specific genes is rarely invoked to explain unexpected human inflammatory responses to disease. Yet, human immunity is highly adaptive to the environment, the human inflammatory response is both highly plastic and highly significant to species survival, particularly after injury.

To date, there are more than 300 human-specific genes that have been identified, although their number, identity and significance remain contentious (30,31). Generally, these genes are thought to arise from segmental duplication of previously existing genes, human-specific alternative splicing that creates new open reading frames regulated by cognate promoters, mutational events during humanoid evolution or through the endogenization of integrated viral sequences (55–57). The evolutionary advantages that these changes confer drive their retention and presumably their activities outweigh “off-target” effects that might mediate disease. In hominid evolution, one such selection is likely tracked to burn injury and the control of fire, which is a uniquely human activity that dates back some 3 million years. To this end, it is interesting to note that it is controversial as to whether or not animal models mimic the programmed inflammatory response to burn injury (58,59).

In support of the existence of unexpected and off-target consequences to the emergence of new genes, newly evolved genes appear to be represented disproportionately in gene sets associated with complex diseases when they are compared with older and more evolutionarily conserved genes (31). Thus, it is thought that human-specific genes might confer subtle but differential responsiveness to human cells when compared with other species (60–62). Ironically, the absence of these human-specific genes in the genomes of animal models makes it impossible to study their contribution to human disease. CHRFAM7A is a case in point. It contributes human-specific activities that are tied to a widely distributed nicotinic ligand-gated ion channel called α7nAChR (CHRNA7) that plays a central role as a neurotransmitter receptor in the nervous system and in the regulation of inflammation.

The studies presented here are the first to establish a clear and unambiguous functional consequence to stable CHRFAM7A gene overexpression in a human leukocyte cell line. RNAseq of these stably transduced cells demonstrated increased CHRNA7 gene expression and increased bungarotoxin binding, which differs from the reported effects of transient overexpression (12–14). While the significance of this difference is not known, the studies estab-
lish a clear and unambiguous coregulatory link between CHRFAM7A and the α7nAChR neurotransmitter receptor encoded by CHRNA7. Furthermore, they may reflect the importance of the ratio of CHRFAM7A to CHRNA7 at the leukocyte cell surface in regulating the inflammation response (Figure 6). Pathway analyses of the changes in gene expression in these cells using both GO and KEGG algorithms show that CHRFAM7A has functional effects on leukocytes that tie the neurotransmitter receptor to leukocyte adhesion, trafficking and transformation. These sequelae may be direct effects of CHRFAM7A expression or indirect effects caused by downstream genes affected by CHRFAM7A.

In the experiments described here, we show that CHRFAM7A is normally expressed in human leukocytes (see Figures 1, 2) and at levels similar to those of CHRNA7. In view of its capacity to regulate α7nAChR activity after transient transfection in vitro (12–14), the data shown imply that CHRFAM7A has the potential to modulate human leukocyte function and by inference, α7nAChR-regulated inflammation (16). Interestingly, we also find that the stable transduction of CHRFAM7A increases basal expression of CHRNA7, thereby establishing the existence of a concomitant and compensatory response to high CHRFAM7A expression. These data point to the possibility that the ratio of CHRFAM7A and CHRNA7 expression may be important in defining the human α7nAChR response. To that end, we also established the identity of the THP1 CHRFAM7A variant, deduced the amino acid difference that distinguishes it from α7nAChR and mapped a unique promoter that differentially regulates CHRFAM7A from CHRNA7 in leukocytes (see Figure 4). Finally, we showed that stable CHRFAM7A expression affects several molecular pathways of cell function including cell

### Table 1. Top differentially up- and downregulated genes, sorted by significance of change.

**Upregulated genes**

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<th>Gene name or description</th>
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<td>9.50E-07</td>
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<td>ENSG00000105655</td>
<td>51477</td>
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<td>ENSG00000206337</td>
<td>10866</td>
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<td>ENSG00000183098</td>
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<td>ENSG00000179796</td>
<td>116135</td>
<td>LRRC3B</td>
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<td>ENSG00000165949</td>
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<td>ENSG00000081923</td>
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<td>ATPase, aminophospholipid transporter, type 8b member 1</td>
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**Downregulated genes**

<table>
<thead>
<tr>
<th>Ensembl</th>
<th>Entrez</th>
<th>Gene</th>
<th>Gene name or description</th>
<th>Δ</th>
<th>p</th>
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<tr>
<td>ENSG00000174099</td>
<td>253827</td>
<td>MSRB3</td>
<td>Methionine sulfoxide reductase B3</td>
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<tr>
<td>ENSG00000156515</td>
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<td>HK1</td>
<td>Hexokinase 1</td>
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<td>8.60E-07</td>
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<tr>
<td>ENSG00000100060</td>
<td>4242</td>
<td>MFNG</td>
<td>MFNG O-fucosyl 3-ß-N-acetylglucosaminyl-transferase</td>
<td>0.3</td>
<td>1.30E-06</td>
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<tr>
<td>ENSG00000100234</td>
<td>7078</td>
<td>TIMP3</td>
<td>TIMP metallopeptidase inhibitor 3</td>
<td>0.51</td>
<td>1.40E-06</td>
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<tr>
<td>ENSG00000182263</td>
<td>55137</td>
<td>FIGN</td>
<td>Fidgetin</td>
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</tr>
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<td>ENSG00000165659</td>
<td>1602</td>
<td>DACH1</td>
<td>Daechshund family transcription factor 1</td>
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<tr>
<td>ENSG00000134824</td>
<td>9415</td>
<td>FADS2</td>
<td>Fatty acid desaturase 2</td>
<td>0.58</td>
<td>0.0002</td>
</tr>
<tr>
<td>ENSG00000126767</td>
<td>2002</td>
<td>ELK1</td>
<td>ELK1, member of ETS oncogene family</td>
<td>0.42</td>
<td>0.0002</td>
</tr>
<tr>
<td>ENSG00000144712</td>
<td>23066</td>
<td>CAND2</td>
<td>Cullin-associated and neddylation-dissociated 2</td>
<td>0.31</td>
<td>0.0006</td>
</tr>
<tr>
<td>ENSG00000116100</td>
<td>7305</td>
<td>TYROBP</td>
<td>TYRO protein tyrosine kinase binding protein</td>
<td>0.59</td>
<td>0.0007</td>
</tr>
</tbody>
</table>
adhesion, cell growth and cell trafficking. Inasmuch as there are no analogous or independently regulated CHRFAM7A-like genes in the genomes of other species (3,9), these findings implicate the existence of a human-specific mechanism in human leukocytes to gauge the human inflammatory response.

**CONCLUSION**

Together these data provide compelling evidence supporting the hypothesis that CHRFAM7A has a significant role in human leukocyte cell biology. CHRFAM7A has been shown to have the capacity to form cell surface heteropolymers with the wild-type α7nAChR (see Figure 6) and is reported in some models either to exert a dominant negative effect on α7nAChR, regulating the appearance of α7nAChR on the cell surface, or to alter ligand tropism (12–14). As a role for α7nAChRs in leukocyte homeostasis is unequivocal (16), CHRFAM7A might then confer leukocytes with a human-specific responsiveness to trophic stimuli that remains to be defined. In one example, efferent signaling of the vagus nerve has a central role in regulating inflammation and α7nAChR activation is an obligatory intermediate, at least in mice (2,16–19,29,63,64). If the vagus nerve were presumed to have a similarly critical role in maintaining immune homeostasis in humans, then CHRFAM7A would likely gauge the immunomodulatory properties of human α7nAChR in a human-specific manner.

The human chromosome 15q13–14 locus contains the 10-exon human CHRNA7 gene that encodes the α7 subunit of the prototypic homopentameric α7nAChR ligand-gated ion channel. This locus, however, also underwent significant rearrangement during human evolution, leading to the emergence of the CHRFAM7A gene (reviewed in [3,9]). While the CHRFAM7A gene is structurally similar to CHRNA7 (4), it has lost five CHRNA7 exons and acquired five exons of a partially duplicated FAM7-UL kinase gene that was once on human chromosome 3. The in-frame fusion of these three partially duplicated genes led to the emergence of CHRFAM7A (3,9). Since its discovery in 1998 (4), numerous studies have shown that, like CHRNA7, CHRFAM7A is expressed in the central nervous system (CNS) and several genomic analyses have associated it and a

### Table 2. Top five significantly enriched GO terms in differentially expressed genes.

<table>
<thead>
<tr>
<th>Enriched GO terms</th>
<th>P value</th>
<th>False discovery rate</th>
</tr>
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<tbody>
<tr>
<td>Type I interferon signaling pathway</td>
<td>6.93E-019</td>
<td>5.23E-015</td>
</tr>
<tr>
<td>Cellular response to type I interferon</td>
<td>6.93E-019</td>
<td>5.23E-015</td>
</tr>
<tr>
<td>Response to type I interferon</td>
<td>8.79E-019</td>
<td>5.23E-015</td>
</tr>
<tr>
<td>Response to stimulus</td>
<td>7.83E-017</td>
<td>2.28E-013</td>
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<tr>
<td>Defense response to virus</td>
<td>8.82E-017</td>
<td>2.28E-013</td>
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</table>

### Table 3. Top five most significantly enriched KEGG pathways.

<table>
<thead>
<tr>
<th>Sample comparison</th>
<th>Enriched pathways</th>
<th>P value</th>
<th>False discovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHRFAM7A (DESeq)</td>
<td>Ribosome</td>
<td>3.35E-003</td>
<td>4.40E-001</td>
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<tr>
<td></td>
<td>Pathways in cancer</td>
<td>5.68E-003</td>
<td>4.40E-001</td>
</tr>
<tr>
<td></td>
<td>Hepatitis C</td>
<td>7.37E-003</td>
<td>4.40E-001</td>
</tr>
<tr>
<td></td>
<td>Colorectal cancer</td>
<td>1.16E-002</td>
<td>5.20E-001</td>
</tr>
<tr>
<td></td>
<td>Leukocyte transendothelial migration</td>
<td>1.78E-002</td>
<td>6.06E-001</td>
</tr>
<tr>
<td>CHRFAM7A (Cuffdiff)</td>
<td>Cytokine-receptor interactions</td>
<td>1.64E-002</td>
<td>4.05E-001</td>
</tr>
<tr>
<td></td>
<td>Osteoclast differentiation</td>
<td>1.74E-002</td>
<td>4.05E-001</td>
</tr>
<tr>
<td></td>
<td>RIG-I-like receptor signaling</td>
<td>1.78E-002</td>
<td>4.05E-001</td>
</tr>
<tr>
<td></td>
<td>Insulin signaling pathway</td>
<td>2.23E-002</td>
<td>4.05E-001</td>
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<td></td>
<td>TGF-β signaling pathway</td>
<td>2.76E-002</td>
<td>4.05E-001</td>
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</tbody>
</table>
2-bp mutation with neuropsychiatric disorders including schizophrenia (65), bipolar disorder (66) and autism (67). The natural ligand(s) responsible for CHRFAM7A activation is presumed to include acetylcholine, but its ligand selectivity is not known compared with other nAChRs, each of which have been described extensively (68). Normally, acetylcholine is thought to be the intrinsic ligand that activates the cell surface α7nAChR homopentameric channel, but there are numerous alternative ligands including choline, nicotine and even peptide hormone-like ligands (69–73) that can activate α7nAChR. The discovery of an alternative α7nAChR in human leukocytes raises the possibility that heteropentamers composed of CHRNA7 and CHRFAM7A proteins could modulate the cellular immune response to proinflammatory challenges (see Figure 6). In this way, the responsiveness of the nuclear factor kappa-B (NF-κB) and Jak2/STAT3 inflammatory signaling pathways (74,75) that has been normally ascribed to the homopentameric product of CHRNA7 may be altered by ligand binding to cells that express homopentameric CHRFAM7A or CHRNA7–CHRFAM7A heteropentamers on the cell surface. Interestingly, this subunit heterogeneity is well recognized for growth factors (for example, PDGF, activin and inhibin), receptors (for example, transforming growth factor [TGF]β, platelet-derived growth factor [PDGF] and fibroblast growth factor [FGF] receptors), integrins (for example, α/β/γ subunits) and other α/βAChR subunits from distinct genes. In the case of CHRFAM7A-encoded heteropentamers, they appear unique because CHRFAM7A only exists in the human genome and therefore, could define a currently unknown, human-specific response.

On a final note, it is worth noting that there is a mutagenized form of CHRFAM7A that has been more extensively studied in mental health research on the presumption that CHRFAM7A might have evolved as an alternative ligand-gated ion-channel-regulating neuronal function (76–78). There are no studies describing the possible effect of this mutant gene on human leukocyte function, let alone the evolutionary pressures that originally led to the emergence, selection and retention of CHRFAM7A in humans. The findings presented here add to a growing body of evidence that collectively underscores the need to dissect the functional link between genes that arose for human speciation and the off-target emergence of complex human disease. In light of genetic analyses that associate newly emerged genes with complex disease, it is incumbent on investigators to use emerging models of human disease that can serve to study the contribution of human-specific disease. Just as mouse models were adapted specifically to study viruses with human-specific tropism (for example, HIV), analogous models will be needed to help determine the contribution of human-specific and taxonomically restricted genes to human biology, physiology and the pathobiology of human diseases tied to inflammation.

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DISCLOSURE
The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.
REFERENCES


RESEARCH ARTICLE


