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Evidence for interaction between 5-hydroxytryptamine (serotonin) receptor 2A and MHC type II molecules in development of rheumatoid arthritis.

Maria Seddighzadeh¹, Marina Korotkova¹, Henrik Källberg², Bo Ding², Nina Daha⁴, Fina A. S. Kurreeman⁴, Rene E. M. Toes⁴, Tom W. Huizinga⁴, Anca I Catrina¹, Lars Alfredsson^{2,3}, Lars Klareskog¹ and Leonid Padyukov^{1*}

¹Rheumatology Unit, Department of Medicine, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden, ²Institute of Environmental Medicine, Karolinska Institutet, ³Karolinska Institutet, and Stockholm Center for Public Health, Stockholm, Sweden, ⁴Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands

*Corresponding author: Leonid Padyukov, MD, PhD

Rheumatology Unit, Department of Medicine, Karolinska Institutet

CMM L8:O4, Karolinska Hospital, 17176, Stockholm, Sweden

Tel: +46 8 51776560; Fax: +46 8 51775562; E-mail: leonid.padyukov@ki.se

Running title: Interaction between HTR2A and MHC type 2 molecules

Abstract

It has repeatedly been suggested that the development of complex diseases can be elucidated by gene-gene interactions. Recently we found that *HTR2A*, the member of serotonin receptor family, is associated with RA. The current study was aimed to investigate the possibility of a gene-gene interaction between the *HTR2A* and the major genetic risk factor for RA, HLA-DRB1 shared epitope (SE) alleles. We studied 4095 RA cases and 3223 controls from three different populations; in Sweden, US and The Netherlands to test for interaction between the protective *HTR2A* haplotype and HLA-DRB1 SE alleles. Further, we analyzed mRNA and/or protein expression of *HTR2A* and HLA-DR in biopsies and in synovial fibroblasts from RA patients. The interaction was defined as departure from additivity of effects using attributable proportion due to interaction. First, we could demonstrate, and further replicate an interaction between a protective haplotype in the *HTR2A* and HLA-DRB1 SE alleles regarding risk of developing autoantibody positive RA. Second, we could show that both genes are constitutively expressed in fibroblasts from synovial tissue of RA patients and by double immunofluorescence staining we demonstrated that these two proteins are colocalized in these cells. In conclusion our data demonstrate a statistical interaction between *HTR2A* and HLA-DRB1 SE alleles as well as colocalization of the product of these two genes in inflamed synovial tissue, which suggest possible biological relationship between these two proteins. This finding may lead to the development of treatment based on enhancing protective features of 5-HT_{2A} in individuals with a certain HLA genotype.

Keywords:

Serotonin receptor; MHC class II; rheumatoid arthritis; gene-gene interaction

Introduction

Rheumatoid arthritis (RA [MIM 180300]) is a complex disease where much effort has been spent on finding risk genes for this disease. Up till today >10 replicated RA susceptibility genes/loci such as HLA-DRB1 shared epitope (SE) alleles,¹ *PTPN22*,² *STAT4*,³ *TRAF1-C5*^{4,5}, *CD40*⁶, etc. have been discovered. However, except for MHC II genes, the odds ratios (ORs) are modest and estimations of the total genetic contributions to the disease indicate that additional genetic factors remain to be identified. We suggest that some of the genetic risk that remains to be identified reside in yet unidentified gene-gene⁷ and gene environment interactions⁸, and we have previously identified an example of gene-gene interaction between the two major susceptibility genes, HLA-DRB1 and *PTPN22*.⁷

Serotonin (5-hydroxytryptamine-5-HT) is a neurotransmitter that plays an important role in the brain homeostasis. Surprisingly, only 1% of 5-HT in the human body is found in the CNS. The remaining 99% is found in other body compartments such as the gastro-intestinal tract and immune tissues, where serotonin acts as a hormone regulating various physiological functions. Moreover, it is well documented that 5-HT has stimulatory and inhibitory effects on immune cells including B, T, NK cells, monocytes and macrophages.^{9,10} The 5-HT_{2A} is one of the seven known serotonin receptors.¹¹ *HTR2A* (MIM 182135) is localized on the human chromosome 13q14–q21 and consists of 3 exons with 5 non-synonymous and 2 synonymous variations and two introns with more than 200 known variations. In addition to the neurons of the peripheral nervous system, 5-HT_{2A} is highly expressed on platelets and fibroblasts as well as in the peripheral blood cells and recently it was demonstrated that 5-HT_{2A} is expressed on dendritic cells.¹² There are several biological and clinical evidences for the connection between the function of 5-HT_{2A} and immune response.^{11,13} It has been demonstrated that the inhibition of production of TNF-alpha, an important pro-inflammatory

cytokine, by 5-HT is mediated by the 5-HT_{2A} in PBMCs¹⁴ and that the activation of 5-HT_{2A} suppresses TNF-alpha induced inflammation in primary aortic smooth muscle cells.¹⁵

Previously we have demonstrated that genetic variations in the *HTR2A* are in association with RA.¹⁶ However, since the strength of the association was moderate we hypothesized that the strongest genetic risk factor for RA, HLA-DRB1 SE alleles, may modulate it and that we can colocalize both products in rheumatoid tissue or in related cells.

The HLA-DRB1 SE alleles has consistently been shown to be associated with subtypes of RA characterized by presence of autoantibody production [e.g rheumatoid factor (RF) positive RA and/or anti-citrulline protein antibody (ACPA) positive RA].^{17,18} The ACPA positive subtype of RA represents a major clinically defined phenotype for the disease and accounts for ca 60% of all RA patients. There are multiple evidences that both genetic and environmental risk factors for ACPA positive and ACPA negative RA are different^{17,19} as well as the clinical course, the histology and the response to therapy.^{20,21}

The aim of this study was to use three different relatively large case-control studies to investigate potential interactions between *HTR2A* and HLA-DRB1 SE alleles in proving risk for RA, and to estimate where biological interaction may take place.

Materials and Methods

Study population

In this report the following three study populations have been included; Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA, 2158 cases and 1068 controls), North American Rheumatoid Arthritis Consortium (NARAC, 908 ACPA-positive cases and 1260 controls) and the Leiden Early Arthritis Clinic (EAC) (1029 cases and 895 controls) (Table 1). The EIRA study population is a population-based case-control study of incident cases of RA where all patients fulfilled the American College of Rheumatology (ACR) 1987 criteria.²² The controls were randomly selected from the Swedish national population registry,

taking into consideration the patient's age, sex and residential area. More details about the EIRA study population have been described elsewhere.¹⁹ Dutch Caucasian individuals with RA, all of whom fulfilled the ACR classification criteria for RA were studied and described elsewhere.²³ Controls were unrelated Dutch Caucasians with no history of RA. The cases in the NARAC study population consisted of RA patients of self-reported white ancestry that were randomly drawn from 4 different collections of patients and the controls were recruited from the New York Cancer Project.^{24, 25} All these studies were conducted according to the permissions from the Regional Ethics Committees and in accordance to the Declaration of Helsinki.

ACPA-status and genotyping

The levels of ACPA in samples from the EIRA and Leiden EAC study was determined using Immunoscan-RA Mark2 ELISA (Euro-diagnostica, Arnhem, The Netherlands) as described elsewhere.²⁶ Cases with antibody levels >25 U/ml were regarded as ACPA-positive. In the NARAC study ACPA levels were determined using the second generation commercial anti-CCP ELISA (INOVA Diagnostics Inc., CA, USA). Cases with serum antibody levels >20 U/ml were regarded as ACPA-positive. HLA-DRB1 type of samples of Swedish cohort was determined by sequence specific primer-polymerase chain reaction analysis using low resolution HLA-DR and DRB1*04 kits from Olerup SSP (Saltsjöbaden, Sweden). HLA-DRB1 alleles DRB1*01, DRB1*04, DRB1*10 were defined as SE. Subjects with SE allele were classified as having a single or double SE allele. The method used for genotyping in the Dutch cohort and the NARAC cohort has been reported elsewhere.²⁷ The method used for SNP genotyping of EIRA samples, which was performed previously,¹⁶ and the Leiden EAC samples was TaqMan allelic discrimination (Applied Biosystems). The call rates for rs6314 and rs1328674 in the all three cohorts were >97% and >98% respectively. For the NARAC

study genotyping data for rs6314 and rs1328674 were extracted from the genome-wide association study, which was performed on the Illumina HumanHap550 array.⁴

Real-time PCR

For mRNA expression analysis synovial fibroblasts from four individuals with RA were used (Dominion Pharmakine, S.L., Bizkaia, Spain). The cells were cultured without/with 400 ng/ml LPS (Sigma) and 200U/ml IFN-gamma (Nordic BioSite, Taby, Sweden) for 24h and harvested for RNA isolation. Cells were lysed and total RNA was extracted using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. Samples were treated with DNase (Qiagen RNase free DNase set) for 20 min at room temperature in order to avoid contamination with genomic DNA. 1µg total RNA for each sample was converted into cDNA using SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers. Real-time PCR was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and TaqMan Gene Expression Assays for *HTR2A*, *HLA-DRB1* and *GAPDH* (Hs00167241, Hs99999917 and 4310884E respectively) (Applied Biosystems) with a two-step protocol (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). The reactions were performed in duplicates in optical 384-well plates (Applied Biosystems) in a total volume 20 µl. Relative gene expression was calculated using the calibration curve method and normalized to the *GAPDH* expression.

Immunohistochemical analysis.

To perform detection of proteins in synovial tissue, we have used tissue biopsy samples from four RA patients. Staining of cryostat sections with mouse monoclonal anti-*HLA-DR* (BD Biosciences, San José, CeA, USA) and rabbit polyclonal anti-*5-HT2A* (Sigma Chemicals, St Louis, MO, USA) antibodies was performed using a standard protocol.²⁸ Isotype-matched irrelevant antibodies were used as negative controls. Tissue sections of mouse brain stem

were used as positive tissue control for 5-HT2A. Stained sections were examined using a Polyvar II microscope (Reichert-Jung, Vienna, Austria) and photographed with a digital Leica camera 300F (Leica, Cambridge, UK). Positive staining was indicated as brown deposits. Double immunofluorescence staining was performed using mouse monoclonal anti-HLA-DR (BD Biosciences, San José, CeA, USA) and rabbit polyclonal anti-5-HT2A antibodies (Sigma Chemicals, St Louis, MO, USA) as published previously.²⁹

Statistical analysis

Chi-square test was used to compare the genotype and allele frequencies between patients and controls in all the three studies. Haplotype analysis was performed using the HaploView 4.0 software. When a DNA sample genotyping failed for more than 50% of the SNPs it was excluded from the statistical evaluation. The distribution of the genotypes for the SNPs rs6314 and rs1328674 in all three studies were in agreement with Hardy-Weinberg equilibrium (HWE) ($p > 0.05$). The haplotypes for each individual was assigned using the PHASE software.³⁰ The interaction effects between *HTR2A* haplotype and HLA-DRB1 SE alleles from all three studies was evaluated using the departure from the additive effects as the interaction criteria³¹ and the attributable proportion due to interaction (AP) with the 95% CI was calculated. A more detailed description has been described elsewhere.³² For meta-analysis we used random effects model (excluding estimates that deviated from homogeneity of the odds ratios) to calculate pooled odds ratios.³³

Results

Interaction between *HTR2A* and HLA-DRB1 SE alleles

We performed an association study in the Swedish cohort as a follow up of our previous finding of an association between *HTR2A* and risk of developing RA.¹⁶ Our previous found haplotype consisted of four tagging SNPs. However, since not all four SNPs were genotyped in the NARAC study we decreased the number of SNPs to two. As could be seen from table

2, the TC-haplotype based on rs6314 and rs1328674, which is part of what was previously reported to be in association with RA¹⁶ demonstrates a protective effect, in the EIRA study ($p=0.005$ in Chi-2 test,). Further we addressed the hypothesis for association in two other study populations where we replicated it in the NARAC study ($p=0.006$ in Chi-2 test) but not in the Leiden EAC study ($p=0.8206$ in Chi-2 test), though control group had 2% higher frequency in comparison with patient's group (Table 2).

The interaction between *HTR2A* and HLA-DRB1 SE alleles was quantified by means of calculating the AP (Table 3). For EIRA and Leiden EAC interaction was investigated in relation to development of both ACPA positive and ACPA negative RA, respectively, but only a trend for a antagonistic interaction between these two genes regarding risk of developing ACPA-positive RA was observed in EIRA (AP 0.19 [95% CI -0.06-0.45]) as well as in the Leiden EAC study (AP 0.2 [95% CI -0.15–0.55]) (Table 3). No interaction was observed with regard to risk of ACPA negative RA in neither of these two studies.

Since EIRA and Leiden EAC studies demonstrated a trend towards interaction between *HTR2A* and HLA-DRB1 SE alleles in development of ACPA positive RA, we performed a meta-analysis for these two groups based on ORs. This analysis demonstrated a statistically significant interaction (AP 0.19 [95% CI 0.04-0.34]) (Table 4).

For the NARAC study which was restricted to ACPA positive patients only, the interaction between *HTR2A* and HLA-DRB1 SE alleles was statistically significant (AP 0.26 [95% CI 0.05-0.46]) (Table 3), which is a replication of our finding for two other studies (EIRA and EAC). We noticed that meta-analysis in all three groups demonstrated significant heterogeneity (not shown) and must be taken with caution.

5-HT2A and HLA-DR mRNA and protein expression

Using quantitative real-time PCR we observed that *HTR2A* and HLA-DRB1 mRNA are expressed in untreated synovial fibroblasts from RA patients. Induction with LPS and IFN-

gamma induced the expression of HLA-DRB1 mRNA while it did not change markedly the expression of *HTR2A* mRNA in RA synovial fibroblasts (Figure 1). Next, we investigated expression of HTR2A and HLA-DR proteins in synovial tissue from patients with RA and we could detect the cytoplasmic and membranous staining for 5-HT2A in synovial lining and sublining cells (Figures 2A-2C). As expected, HLA-DR was abundantly expressed in RA synovial tissue (data not shown).

To determine whether 5-HT2A and HLA-DR are co-localized in synovial fibroblasts, double staining of cells treated with IFN-gamma and LPS was performed (Figures 2D-2F). As it could be seen from figure 2, two proteins are colocalize in synovial fibroblasts although there were cells that expressed only 5-HT2A or HLA-DR.

Discussion

The major finding in our study is a discovery of interactions between one of the serotonin receptors and MHC class II molecules, which is important for the development of RA. Such relation has never been shown or indicated previously and may demonstrate an unknown pathway in the disease development, and it is also a potential tool to ameliorate influence of strongest genetic risk factor for RA, SE alleles. We found not only statistical evidence for such interaction, but also identified cells, which express both proteins, which is totally new finding.

Rheumatoid arthritis is a complex disease where the genetic and environmental factors contribute to the disease development. When investigating the association of single genes with risk of a complex disease the ORs are almost always low to moderate. This reflects that a specific phenotype is a result of a combination of different low-moderately contributing genes and environmental factors. One example is the *PTPN22* risk allele (R620W) which by it self is of a moderate or no risk factor for development of RA but when interacting with HLA-DRB1 SE alleles, a gene strongly connected to different autoimmune diseases, the ORs for

developing RA becomes very high.⁷ So taking in consideration the epistasis and interaction of genes with environmental factors is appropriate.

Polymorphisms in the *HTR2A* have frequently been reported to be in association with different psychiatric diseases such as schizophrenia and bipolar anxiety disorder.³⁴ However, there are more and more evidence for the involvement of the serotonin system in inflammation.¹³⁻¹⁵ Notably, RA is negatively correlated with schizophrenia.³⁵ Partition of RA into ACPA positive and ACPA negative subtypes is adequate since more evidence points toward the fact that these two subtypes differs in etiology and pathophysiology. In this paper we have shown that *HTR2A*, recently found to be in association with RA¹⁶, interacts with HLA-DRB1 SE alleles in the ACPA positive group of patients. HLA-DRB1 SE alleles has no impact on the development of ACPA negative RA and that could be the reason, why this interaction is not observed in this subgroup of the disease. The enhanced risk for development of RA due to interaction between *HTR2A* and HLA-DRB1 SE alleles was shown in meta-analysis of EIRA and Leiden EAC studies. Although we observed that the meta-analysis for three cohorts did not pass the test for heterogeneity, this may have an obvious reason which is that the estimates based on NARAC is significantly higher than the estimates from EIRA and the Leiden EAC studies. However, we could demonstrate a statistically significant interaction between *HTR2A* and HLA-DRB1-SE both in meta-analysis (EIRA and EAC) and in NARAC cohort. It is illustrative, as it was previously noticed for interaction between HLA-DRB1 SE alleles and *PTPN22*,⁷ that regardless substantial differences in design of the studies, data for all three populations consistently point to the gene-gene interaction. A possible biological background for the interaction between *HTR2A* and HLA-DRB1 SE alleles could be suggested at the cellular level by demonstrating that these two proteins are detected in inflamed synovial tissue simultaneously.

Based on the results from our study we can speculate about biological interaction between these two proteins. One possibility could be that there is a cross talk between signaling pathway related to *HTR2A* and MHC class II expression regulation. Furthermore, since we could detect these two proteins in the same cell in the synovial fibroblasts *in vitro*, a physical interaction between them should not be excluded. The protective association of the TC-haplotype in the *HTR2A* with the development RA and the interaction between *HTR2A* and HLA-DRB1 SE alleles makes it very tempting to speculate if this could be one of the reasons behind the inverse relation between schizophrenia and RA that has been reported frequently in the literature.³⁵ We can hypothesize that the protective haplotype in the *HTR2A* with regard to RA is a susceptible haplotype with regard to schizophrenia. Moreover, it has been shown that HLA-DRB1 SE alleles are associated with schizophrenia^{36,37} so perhaps in contrast to RA there is a synergistic interaction between this gene and the *HTR2A* TC-haplotype among schizophrenia patients. More functional studies have to be done before further details about the interaction between 5-HT_{2A} and HLA-DR proteins can be revealed, since our data is based on limited number of experiments. In conclusion, our data show strong evidence for an interaction between the 5-HT_{2A} and MHC type II molecules in development of RA.

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Table 1 Clinical Characteristics of EIRA, Leiden EAC and NARAC studies

Characteristic	EIRA	Leiden EAC	NARAC
No. of patients	2158	1029	908
No. of controls	1068	895	1260
Country of origin	Sweden	Netherlands	United States
Female sex (patients) %	71.5	66	73.6
Female sex (controls) %	74.8	46	71.3
Age of onset (mean \pm SD years)	50.7 \pm 12.3	56.4 \pm 15.4	(39.4 \pm 13.1) ^e
Disease duration (mean \pm SD years)	<1 year	<2 year	(16.2 \pm 11.8) ^e
ACPA ^a positive (%)	1145/1998 ^b (57.3)	375/625 ^b (60)	908/908 ^b (100)
HLA-DRB1 SE (patients) (%)	1422/1930 ^c (71.2)	590/842 ^c (70.1)	838/865 ^c (96.9)
HLA-DRB1 SE (controls) (%)	534/1056 ^d (50.6)	251/557 ^d (45.1)	543/1254 ^d (43.3)

NOTE. Details on the EIRA¹⁹, the Leiden EAC²³ and the NARAC^{24,25} studies have

been reported elsewhere.

^a ACPA= anti-citrullinated peptide antibody

^b The number of patients with defined ACPA status.

^c The number of patients with defined HLA-DRB1 SE status.

^d The number of controls with defined HLA-DRB1 SE status.

^e Refers to 840 patients reported previously.¹⁷

Table 2 The frequency of *HTR2A* TC-haplotype in EIRA^a, Leiden EAC^a and NARAC

Studies

Haplotype	Haplotype	Patient, Control	Patient, Control	p-value
	Frequencies	Ratio Counts	Frequencies	
EIRA				
CC	0.901	3829.7 : 414.3,		
		1903.4 : 214.6	0.902, 0.899	0.6422
TC	0.065	249.7 : 3994.3,		
		163.6 : 1954.4	0.059, 0.077	0.0050
CT	0.031	153.3 : 4090.7,		
		46.1 : 2071.9	0.036, 0.022	0.0020
Leiden EAC				
CC	0.859	1758.6 : 299.4,	0.855, 0.865	0.3682
		1547.7 : 242.3		
TC	0.094	191.6 : 1866.4,	0.093, 0.095	0.8206
		170.5 : 1619.5		
CT	0.044	103.2 : 1954.8,	0.050, 0.036	0.0322
		64.4 : 1725.6		
NARAC				
CC	0.863	1586.2 : 223.8,		
		2145.1 : 370.9	0.876, 0.853	0.0249
TC	0.096	147.3 : 1662.7,		
		267.7 : 2248.3	0.081, 0.106	0.0058
CT	0.037	69.5 : 1740.5,		
		90.3 : 2425.7	0.038, 0.036	0.6629

^a In the entire study

Table 3 Odds ratio together with 95% confidence interval for developing ACPA positive RA among subjects with different combinations of the *HTR2A* TC-haplotype and SE alleles, by study (EIRA, Leiden EAC and NARAC)

Study	TC-	SE	No. of	OR	95 % CI
	Haplotype		Patient/ Control		
EIRA					
	Yes	No	20/78	1.0 ^a
	No	No	146/444	1.3	0.7 – 2.2
	Yes	Yes	110/75	5.7	3.2 – 10.2
	No	Yes	858/459	7.4	4.4 – 12.3
	AP ^b : 0.19 (-0.06-0.45)				
Leiden EAC					
	Yes	No	10/62	1.0 ^a
	No	No	59/244	1.5	0.7 – 3.1
	Yes	Yes	44/49	5.6	2.5 – 12.2
	No	Yes	247/202	7.6	3.8 – 15.2
	AP ^b : 0.2 (-0.15 – 0.55)				
NARAC					
	Yes	No	4/140	1.0 ^a
	No	No	23/571	1.4	0.5 – 4.1
	Yes	Yes	131/109	42.1	15.1 – 117.3
	No	Yes	707/434	57.0	21.0 – 155.1
	AP ^b : 0.26 (0.05 – 0.46)				

^a Reference category, ^b Attributable proportion

Table 4 Odds ratios for different combinations of HTR2A and HLA-DRB1 SE alleles.

Meta-analysis based on the EIRA and Leiden EAC studies

Gene	OR ^a	95 % CI
No TC-haplotype	1.37	0.86 - 2.18
Yes SE-Yes TC haplotype	5.64	3.70 - 8.67
Yes SE-No TC Haplotype	7.47	4.96 - 11.25
AP ^b : 0.19 (0.04 – 0.34)		

^a Odds Ratio

^b Attributable proportion

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Figure 1. *HTR2A* and HLA-DRB1 mRNA are expressed in untreated fibroblasts from RA patients. Expression of *HTR2A* and HLA-DRB1 mRNA in RA synovial fibroblasts untreated (open bars) or stimulated (filled bars) with LPS and IFN-gamma for 24h. The data represent mean (\pm S.E.) of values obtained from three separate experiments. The results are expressed as fold changes, considering 1 as the value of untreated cells.

Figure 2. 5-HT_{2A} is expressed in synovial tissues of patients with RA and colocalize with HLA-DR in synovial fibroblasts. Photographs illustrating (A, B) brown immunoperoxidase staining of 5-HT_{2A} positive cells in representative synovial tissue sections from patients with RA counterstained with hematoxylin; (C) negative control (original magnification x250-500). Double fluorescence staining illustrating (D) 5-HT_{2A} staining, (E) HLA-DR staining and (F) merged images of rheumatoid arthritis synovial fibroblasts induced with IFN-gamma and LPS and (original magnification X500).

Figure 1

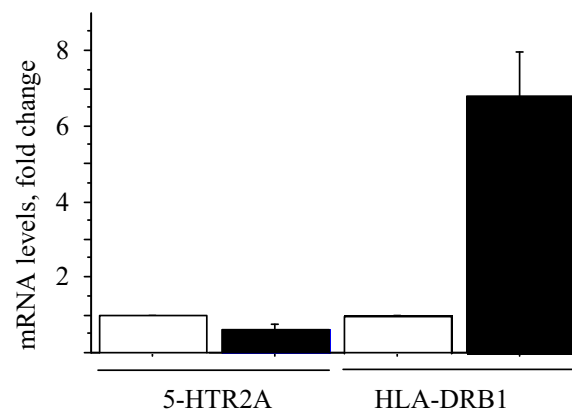


Figure 2

