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Research Article

Expression in normals and in subjects with schizophrenia of a novel gene fragment originally isolated from monozygotic twins discordant for schizophrenia

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Abstract

Differentially expressed clones from subtracted cDNA libraries of a pair of monozygotic twins discordant for schizophrenia have been reported in the literature. The clones were expressed in lymphocytes from the healthy twin, but not from the schizophrenic twin. In the current study, we assessed the expression of one of these clones, oksc12b, in 10 normal controls and in 10 patients who met DSM-IV criteria for schizophrenia and had never received neuroleptic medication. We hypothesized that this clone would be differentially expressed in normal controls and in the schizophrenic patients, and that its expression could be a peripheral marker of the disease. Lymphocytes were isolated and total RNA was purified, reverse-transcribed, and quantified by two PCR methods. In the first PCR assay, oksc12b expression was measured relative to beta-actin gene expression. The second PCR assay consisted of a competitive procedure using a heterologous DNA internal standard. Neither method confirmed any difference in oksc12b expression between schizophrenic patients and normal controls. Subtypes of schizophrenia or the general heterogeneity of this syndrome may explain the discrepancy found. It is also possible that the differentially expressed clones are present in discordant monozygotic twins, but not in other patients.

Key words: monozygotic twins, mRNA, schizophrenia.

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Introduction

Subtractive hybridization can be used to identify differences in gene activity between discordant monozygotic twins (Travis *et al.*, 1987). Novel, differentially expressed clones have been isolated from subtracted lymphocyte cDNA libraries of a pair of twins discordant for schizophrenia (Friedhoff, *et al.* 1995). The clones were expressed in lymphocytes from the healthy twin, but not in lymphocytes from the twin with schizophrenia. The authors speculated that those mRNAs might code for some proteins which protected the healthy twin against schizophrenia. It is noteworthy that genes which have an important function in other tissues have been found to be lowly expressed in lymphocytes and without a known function. Such is the case of the dystrophin gene which plays a role in muscles (Chelly *et al.*, 1988), of dopaminergic receptors in the brain (Bondy *et al.* 1996; Nagai *et al.* 1993; Takahashi *et al.* 1992), and of glial protein RNAs, in the peripheral nervous system (Riol *et al.* 1997).

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There are several peripheral markers which, under controlled conditions, may represent what occurs in the brain in psychiatric diseases. We (Dávila *et al.*, 1996) and other authors have found that the variations in the plasma concentrations of homovanillic acid are a good predictor of the response to neuroleptics in schizophrenic patients, despite the fact that this dopamine metabolite has a central and peripheral origin. Several serotonin parameters in platelets are also good indicators of antidepressant efficacy in depressed patients (Bakish *et al.*, 1997). Morevoer, it has been reported (Nagai *et al.*, 1996) that the expression of the dopamine D3 receptor gene is diminished in lymphocytes from patients with Parkinson's disease, and that a decreased D3 expression correlates with the severity of the symptoms.

Coupled reverse transcription (RT) - polymerase chain reaction (PCR) is a highly sensitive method to assess gene expression, but, as it involves two enzymatic reactions, one of which with an exponential behavior, its reproducibility is low. Therefore, internal standards must be used to control the efficiencies of both the RT and the

PCR processes. The internal standard could be a synthetic homologous RNA added before or after total RNA purification, a synthetic DNA used to control only PCR efficiency, and/or an endogenous gene like beta-actin, whose expression is supposed to be conserved in each tissue.

We chose a double method of quantification, one involving beta-actin expression to control RNA extraction, RT and PCR, and the other using heterologous DNA to quantify the absolute expression of our gene in a competitive PCR assay.

In the original study, the oksc12b gene was expressed in the non-schizophrenic but not in the schizophrenic twin, so our working hypothesis was that the oksc12b gene would be differentially expressed in normal controls and in schizophrenics free of medication, and that the lack of expression of this gene could be a peripheral marker for the disease.

Material and Methods

A group of 10 normal controls was selected, 6 women and 4 men, mean age 32 years (s.d. = 6). They had no known illnesses and they were not receiving any medication at the time of the study. All gave 40 mL of blood for RNA analysis.

The group of 10 patients, 6 women and 4 men, mean age 24 years (s.d. = 5), met DSM-IV criteria for schizophrenia. They had never taken neuroleptic medication and were not receiving any medication at the time of the study. All were informed about the investigation and consented to participate. All gave 20 ml of blood for RNA analysis.

Isolation of total RNA

Blood was obtained in heparinized tubes, lymphocytes were isolated by separation on Ficoll-Paque (Pharmacia), and RNA was immediately isolated following the method of Chomczynski and Sacchi (1987). RNAs were incubated with RNAse-free DNAse (20 U/10 μg RNA) in the presence of 200 U of RNAsin Ribonuclease Inhibitor (Promega) for 30 min at 37 °C, to eliminate any possible DNA contamination. The amount of RNA was determined by optical absorption at 260nm, and its integrity was confirmed in a denaturing 1.2% agarose gel in MOPS stained with ethidium bromide.

Reverse transcription

2 μg of RNA were incubated with 1 μg of oligo-dT (15) primer for 5 min at 72 °C. After 5 min in an ice bath, samples were incubated with 100 U of M-MLV reverse transcriptase, 10 U of RNAsin, and 0.5 mM each of four dNTPs for 1 h at 37 °C. After inactivation at 95 °C for 3 min, cDNAs were stored at -20 °C until analysis.

PCR reaction

The cDNA studied consisted of a 201-bp fragment cloned in a cDNAII vector (Invitrogen); we selected a pair of primers using Primer Premier software (Biosoft International), which yielded a PCR fragment of 160 bp. The sense primer was 5'-GCTTTATCAGGCCAGGCACAGT, and the antisense primer was 5'-CCACCACCAATGGCTAA GTTTT. The beta-actin (ba) gene was amplified with the sense primer 5'-AAGAGAGGCATCCTCACCCT and the antisense primer 5'-TACATGGCTGGGGTGTTGAA, which rendered a PCR product of 218 bp.

A pair of composite primers was designed to generate a nonhomologous competitor DNA fragment. These primers contained the primers for the target sequence oksc12b pasted to the b-actin primers, and the PCR product was 262-bp long. The competitor standard was generated by two successive PCR amplifications of human lymphocyte cDNA, adding α^{32}P-dCTP to quantify the amount of final product. The first PCR product was generated with the composite primers, and the second one using the above product as a template with the oksc12b primers. The final competitor was purified on an agarose gel and quantified by measuring the α^{32}P-dCTP incorporated.

The PCR reaction was performed in a 50 μL volume containing 1xPCR buffer (1.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 °C), 0.01% Tween-20), 1.5 mM $MgCl_2$, 100 μM of each dNTP, 1.25 U of BioTaq DNA polymerase, ten million cpm of α^{32}P-dCTP, 0.3 μM of each oksc12b primer, and 0.75 μM of each beta-actin primer, in a Perkin Elmer/Cetus thermocycler.

The PCR conditions involved initial denaturing at 95 °C for 3 min, followed by 25 cycles of denaturing at 95 °C for 1 min, annealing at 62 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min.

PCR relative to beta-actin gene

A dilution series of each cDNA from lymphocytes, containing an equivalent of 5 to 60 ng of RNA and 10 million cpm of α^{32}P-d-CTP, were amplified in the presence of 0.3 μM primers for oksc12b plus 0.075 μM primers for the beta-actin gene. The beta-actin gene produced a 218-bp PCR fragment that was separated from the 160-bp oksc12b fragment on a 6% polyacrilamide gel or in a 2% agarose gel. The gel was stained in ethidium bromide and visualized under UV light. Bands were excised and the incorporated radionuclide was counted in a scintillation counter. For each sample, a regression line of the logarithm of radionuclide incorporation (cpm) against the logarithm of RNA amount was plotted. The relative response of oksc12b to beta-actin, measured in 25 ng of RNA, was used to compare expression in patient and control samples.

The measurement of beta-actin alone, expressed as the percentage of radionuclide incorporation per unit RNA

weight, was used to correct values obtained by competitive PCR.

Competitive PCR

Each sample was amplified in a series of tubes containing a fixed amount of cDNA from lymphocytes, equivalent to 300 ng of original RNA, and progressively larger amounts of competitor, from 0.002 to 4.63 attomoles. Products were separated on 2% agarose gel containing ethidium bromide. Bands were cut and radionuclide incorporation was measured; an aliquot of each reaction was also assessed by HPLC (Andía *et al.*, 2001). The amount of oksc12b in each sample was calculated by plotting the logarithm of the oksc12b/competitor ratio against the logarithm of attomoles of competitor. The point on the line where the ordinate value equaled the logarithm of 160/262 corresponded to the point where each had the same number of molecules. The amount of oksc12b in attomoles found with this method was normalized with respect to the amount of beta-actin obtained in experiments to correct the variability of the process of RNA purification and RT.

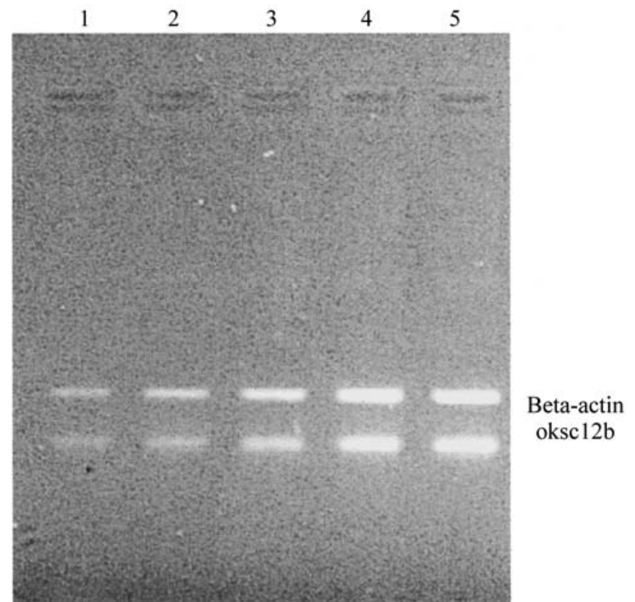

Figure 1 - 2% agarose gel separation of RT-PCR fragments corresponding to oksc12b gene (169 bp) and beta-actin gene (218 bp) amplified from cDNA of a normal control. Lanes 1 to 5 correspond to an equivalent of 5, 10, 20, 40, and 60 ng of RNA.

Results

We first attempted to quantify RT performance by adding a d-NTP marked with ^{32}P of highly specific activity (Pennetier *et al.*, 1992). After the reaction we tried to separate cDNA-incorporated radionuclide from free radionuclide using different methods: precipitation with 10% TCA followed by filtration, or separation in columns selective for the number of bases (Chromaspin 10, Clontech; Microcon 30, Millipore). These methods were unable to differentiate blank (for which water was used instead of template RNA) and samples. Therefore, we decided to correct performance by measuring relative beta-actin gene expression.

In 25 cycles, using RNA amounts from 5 ng to 60 ng, we found that PCR reactions in which beta-actin and oksc12b were measured simultaneously were always in an exponential phase. Figure 1 shows a 2% agarose gel separation of PCR products of the oksc12b gene (160 bp) and of the beta-actin gene (218 bp), after amplification of cDNA from a normal control. After trying beta-actin primer concentrations ranging from 0.025 μM to 0.1 μM under our conditions, we found that both products were easily measured at a concentration of 0.075 μM. Analysis of the same sample on 10 different days yielded a cv% of 20% for the oksc12b/ba ratio and of 18% for measurements of beta-actin alone.

Figure 2 shows a 2% agarose gel separation of PCR products corresponding to the oksc12b gene (160 bp) and the competitor product (262bp). The efficiency of the competitor in the PCR reaction was 59% (n = 6) and of oksc12b, 56%. Analysis of the same sample on 6 different days yielded a cv% of 17%.

The values of the measurements made in cDNA samples from normal controls and schizophrenic patients are shown in Table 1. We found no significant differences in the average values of any measurement of oksc12b gene expression between patients and controls. A comparison of the standard deviations of measurements of relative beta-actin gene expression showed a greater dispersion of values in schizophrenic patients ($F = 0.059$, $p < 0.001$).

The values obtained by plotting oksc12b expression in dilution samples with respect to beta-actin in the overall group of samples studied showed a weak correlation ($r = 0.577$, R-square = 33%) with absolute quantification measurements by competitive PCR. However, a correlation was found when we corrected the values obtained by quan-

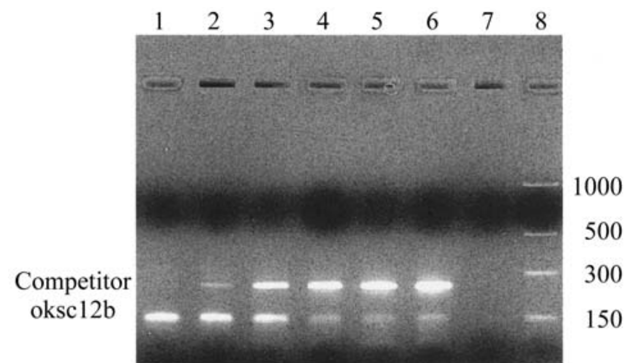

Figure 2 - 2% agarose gel separation of RT-PCR products corresponding to the oksc12b gene (160 bp) and the competitor DNA (262bp). Lanes 1 to 6 correspond to an equivalent of 300 ng of cDNA from a normal control amplified in the presence of 0.002, 0.023, 0.23, 1.16, 2.31, and 4.63 attmol of competitor. Lane 8 corresponds to a molecular weight marker containing 150, 300, 500 and 1000 bp.

Table 1 - Assessment of the okscl2b gene expression after RT-PCR assays of RNA from lymphocytes of control subjects and schizophrenic patients

	Controls	Schizophrenics
Competitive PCR (attmol okscl2b/μg RNA)	4.27 ± 3.90	4.89 ± 4.53
Titration assay (okscl2b/ba)	1.46 ± 1.00	1.96 ± 1.87
attmol okscl2b/%cpm ba incorporated	0.66 ± 0.44	1.45 ± 1.44*

*Significant difference between standard deviations, $F = 0.059$, $p < 0.001$.

titative PCR for beta-actin expression ($r = 0.864$, R-square = 75%). This may indicate variations in the efficiency of RT in different samples.

Discussion

An endogenous gene was chosen as the control for the efficiency of RT and the initial RNA measurement. RNA measurement by optical absorbance can give an approximate value; however, other products of purification, such as proteins, contaminating DNA, or partially degraded RNA, that also absorb at this wavelength may contribute significantly to producing inaccurate quantitation**.** Assay of a gene like beta-actin, which has been extensively used in the literature, is more useful for comparative studies. We adapted the measurement of beta-actin, which has a much higher level of expression than okscl2b, to the measurement of our gene by diluting the concentration of the beta-actin primers to one-fourth. This option, as opposed to the addition of beta-actin primers after a series of cycles, prevents cross-contamination and can be easily used for the study of genes with very different levels of expression.

Synthesis of a double-strand DNA competitor is simple, its quantification by radioactive incorporation is precise, and its conservation is stable. By establishing a relationship with the expression of another gene, more reliable, semi-quantitative information about the level of expression is provided than by using the titration technique. The combination of relative and competitive PCR will allow us to compare the expression of this gene with others that are also expressed in lymphocytes, thus establishing a true quantitative comparison between them**.** It should be pointed out that in this case, or in HPLC analysis, the amount of PCR products is not proportional to the number of strands, as occurs when using marked primers or hybridization. Consequently, competitor and template amounts are equal when the relation between their logarithms is equal to the relationship between the number of their bases, in this case 262 and 160.

In the sample populations studied, we detected no significant differences in the levels of expression of the okscl2b gene between controls and patients with schizophrenia. These results did not confirm the difference reported by the original study. During the development of our study, Friedhoff *et al.* (personal communication) measured again the expression of the 10 genes that were originally found to be differentially expressed. In lymphocytes obtained 2 years later from the same pair of twins, six of these genes were found differentially expressed in this new measurement, among them the okscl2b clone. The expression of okscl2b was measured in the Friedhoff studies by ribonuclease protection assay (RPA), a technique which is much less sensitive than RT-PCR. This may explain, in part, the lack of differential expression of okscl2b in the present study.

Familial versus non-familial schizophrenia (Dávila *et al.*, 1995), subtypes of schizophrenia (Dávila *et al.*, 1996) and the general heterogeneity of the syndrome may explain the discrepancy between the two studies. The fact that our gene-expression values were more dispersed in the patient sample than in controls may imply that there was more than one population in the study group. There are a number of findings suggesting that biological parameters vary more in schizophrenic patients than in healthy controls, especially parameters related to dopaminergic transmission (Syvälahti *et al.* 2000); these variations may reflect a dysregulated or labile system. Our original design did not allow us to address this issue, nor to establish a relation with parameters such as diagnosis, symptoms, and clinical evolution.

It also would be interesting to study a sample of monozygotic twins discordant for schizophrenia, as in the original study (Friedhoff *et al.*, 1995). In a study of DNA from twins discordant for schizophrenia, Tsujita *et al.* (1998) found differences in the presence of Notl restrictions sites with respect to control twins; these differences may be associated with different levels of methylation/demethylation of sites near the promoter of different genes, thus modifying the expression of these genes. It has been hypothesized that DNA-methylation mediated processes, among other epigenetic factors, may be of etiologic importance in schizophrenia (Petronis *et al.*, 1999). There may also be a detectable difference in okscl2b gene expression in patients with a longer clinical evolution. Such a modification would not be due to the disease itself, but arise as a consequence of the evolution of the disease. In our sample, in contrast with the twins, patients were young people with a short evolution of the illness. Neuroleptic treatment does not appear to be a significant factor, because neither the schizophrenic twin of the original study nor the patients in this study had received neuroleptic treatment.

Regardless of the results obtained, it seems that lymphocytes may be useful for the study of disturbances in the brain. Substances detected in human lymphocytes include, among others, mRNAs corresponding to the kappa opioid receptor (Takahashi *et al.*, 1992), an astroglial marker (Riol *et al.*, 1997), and the dopaminergic receptors D3 (Nagai *et al.*, 1993), D4 (Bondy *et al.*, 1996) and D5 (Takahashi *et al.*, 1992), which have a known function in the nervous sys-

tem, but no apparent function in lymphocytes. The assessment of mRNA in lymphocytes may serve as a peripheral marker of disease. In the brain and lymphocytes of schizophrenic patients, reduced expression of mitochondrial reductases has been found after flupentixol treatment (Whatley *et al.*, 1998). In patients with Parkinson's disease, a reduction in the expression of the dopaminergic receptor D3 has been found (Nagai *et al.*, 1996) in lymphocytes, which correlates with the severity of symptoms.

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