Author's response to reviews

Title: Gene expression profiles in Finnish twins with multiple sclerosis

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Author's response to reviews: see over
Re: MS: 1846958146628954 –Gene expression profiles in Finnish twins with multiple sclerosis

Dear The Biomed Central Editorial Team

Thank you very much for your letter and for comments by reviewers. We have done our best in order to refine our manuscript according to suggestions of reviewers. Our point-by-point response is the following

Reviewer Bernadette Kalman

Minor revision

Point 1. Four patients in our study were treated with betainterferon. One of them had received this treatment for 1 year and the rest for two to three years. This is now indicated in the revised manuscript (please see page 5, sections subjects, line 9). In addition, most of our twins with MS had various symptomatic medications including treatment for neuropathic pain, spasticity, fatigue and urine incontinence (page 5).

Point 2. We agree with the reviewer that the increase in the expression of GIP3 (Interferon-induced protein 6-16) and MX2 may be related to treatment since both of these genes are interferon-responsive. This is now mentioned in the manuscript (see page 11). However, even if up-regulation of these genes is resulted by betainterferon, there still remain 4 other genes of interest. The next step in our project is to confirm differential expression of these genes in larger MS patient cohort.

Point 3. The study using MS twin approach published in Ann Neurol 1996; 40: 108-112 is included in the text and references (please see page 4, ref. 16).

Discretionary revision:

Point 1. Sp3 was not included in the BD Atlas microarray.

Point 2. We agree with the reviewer that it is unlikely that 40% of patients would differentially express the same genes due to random events. Likewise, we agree that the inclusion of a few unaffected MZ twin pairs would have been informative, but unfortunately, currently such twin pairs are not available. Since inclusion of housekeeping gene to figure 1 requires new analysis of the whole data that would be technically very complicated and cumbersome, we ask for the allowance to keep figure 1 unchanged.
**Point 3.** The decrease of actin expression was seen in 2/8 affected patients. According to our understanding it is difficult to give any clear relevance to this observation.

Reviewer Anat Achiron

**Point 1.** In the revised manuscript we have indicated that four of eight patients were treated by betainterferon (table 1 and page 5, subjects). On pages 10 and 11 we have indicated that over-expression of 2 genes (GIP3 and MX2) may be related to interferon. However, even if up-regulation of these genes is resulted by betainterfeton, there still remain 4 other genes of interest. The next step in our project is to confirm differential expression of these genes in larger MS patient cohort.

**Point 2.** We agree with the reviewer that confounders may influence the data in twins with long duration of MS. However, in spite of that, as mentioned by other reviewer our study may be the largest molecular study in MZ twins discordant for MS, and therefore we believe that the obtained observations represent significant interest to the MS community.

**Points 3-7.** Since the questions 3 to 7 are related to microarray methodology and statistical evaluation that links them to each other, we give an answer to these points together below:

**Briefly about the methodology,** in our experiments, RNA sample obtained from healthy monozygotic twin (tissue No 1) was compared to tissue No 2 obtained from monozygotic twin with MS by using cDNA subtraction method. Please see the principle of the cDNA array and following cDNA subtraction procedure: www. clontech.com. Atlas cDNA Expression Arrays User Manual, Protokol #PT3140-1, Version #PROX591). In practice, after hybridization we performed the normalization of the arrays to the same level using the BD AtlasImageTM 2.7 Beta software (BD Biosciences Clontech, Palo Alto, CA, USA) and data was globally normalized by using the widely used and accepted sum method. Based on this methodology, the comparison was done for all 8 discordant monozygotic twin pairs (together for 16 persons) and the cDNA subtraction values were calculated for all 8300 genes.

The definition of significantly expressed genes in our array experiments was based on the instructions of the cDNA array manufacturer (Clontech, Palo Alto, CA, USA) and commonly used and accepted criteria (please see e.g., BMC Genomics. 2004 Apr 27;5(1):26; Adv Biochem Eng Biotechnol. 2004;86:191-213; Mol Carcinog. 2001 Feb;30(2):79-87). In our study cDNA subtraction ratios of gene expression greater than two-fold were considered significant, based on a 99 % confidence interval. As requested by reviewer, we have now clarified the definition of significantly expressed gene in the text of revised manuscript (page 8, Statistical analysis, Table 2). According to manufacturer from those genes with at least two-fold subtraction expression difference when compared to controls – about 90 % of the results should be reproducible by quantitative QRT-PCR method, used also in our study.

**Points 3-6.** It is important to note that using this analysis it is possible to obtain only one value, i.e. difference (cDNA subtraction) between control and patients for all studied 8300 genes. This is the reason why separate values for controls and MS patients are not generated by this methodology, and consequently, the group comparison performed by T-test is not possible. The analysis of categoric variables like those in our study requires statistical test like McNemar or $\chi^2$ test (categorical tests) which are generally used for the testing of the frequencies between two groups. In our case we tested result by both McNemar and more conventional $\chi^2$ test with similar results. This is indicated in Statistics (please see page 8 and Table 2.)
In order to clarify the used methodology, the description of our array (cDNA subtracation) is now given in the revised manuscript on page 6, section cDNA microarray.

We have also added few sentences to the discussion (please see page 9 and 10, lines 1-2) in order to answer to question raised by the reviewer (Point 5) “What does it mean to evaluate the differences of up- and down-regulated genes”.

**Point 6 continued.** Please also see answer to point 5. As shown in table 2, we found that from 8300 genes studied 305 genes were differentially expressed in our cDNA subtracation procedure at least in one sibpair and the rest were not found to be differentially expressed or were not expressed at all in studied tissues, i.e., no cDNA subtracation value could be calculated or the value was less than two-fold expression, i.e., near 1. Thus, since the array gives just categorical data, i.e., no or yes significant cDNA subtraction value difference between control and patients according to manufactures recommended criteria. Due to this fact, there is no significant expression value for controls and patients separately (only differences) that makes the required statistical comparison between groups impossible. (please see e.g., BMC Genomics. 2004 Apr 27;5(1):26; Adv Biochem Eng Biotechnol. 2004;86:191-213; Mol Carcinog. 2001 Feb;30(2):79-87). This is the reason why the table 2 do not include any statistical test. On pages 8 and 9, results, we however showed that the proportion of up-regulated genes was significantly higher compared to the proportion of down-regulated genes i.e., p=0.023 for the difference, McNemar or χ² test, within 1/8 group and within 2/8 group of table 2, p=0.01.

We have now added these p-values also to the footnote of the Table 2.

**Point 7.** We have now clarified both in the text and table 2 of the revised manuscript how the statistical testing was done and what the differences and obtained p-values mean. Please also see answer to point 3,5,6 above.

**Point 7. continued.** Reviewer: “Moreover .... Interferon alpha inducible protein --....However , this gene does not appear in the list in the list 3. Actually this gene is mentioned in the table 3. The other name of this gene is “G1P3, and in the table 3 we used this name for that gene in the description part of the same table “interferon-induced protein 6-16”. The abbreviation “G1P3” is also given in the parenthesis in the text after interferon alpha inducible protein (page 9, line 4 and in the abstract).

**Point 8.** The MRI sequences are given on page 5, section subjects.

**Point 9.** Patients and healthy twins are indicated in the legend for Figure 2.

**Point 10.** Please see answers to points 3-7 above.

**Point 11.** Please see also answer to the point 5 above. Figure 1. One box in Figure 1 already describes the difference in gene expression between patient with MS (i.e., cDNA subtracation value) and his healthy sibling.
**Point 12.** The origin of number 415 indicated by reviewer is not clear for us (we cannot find it in the text). The correct number of genes that showed over two-fold difference in gene expression in one twin pair was 305.

We thank once again The BioMed Central team and reviewers for the valuable comments that helped us to refine our manuscript. We hope that after this revision it is acceptable for publication in The BioMed Central.

With kind regards
Irina Elovaara, professor