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Anorexia nervosa and oligonucleotide microarray technique – pilot study

Małgorzata Janas-Kozik, Irena Krupka-Matuszczyk,
Małgorzata Stachowicz, Urszula Mazurek

SUMMARY

Aim: Evaluation of the transcript expression profile of selected genes encoding leptin and orexin A and B receptors with the oligonucleotide microarray technique (Affymetrix, HG-U133A).

Material and method: Peripheral blood mononuclear cells (PBMC) of 4 patients suffering from AN and fulfilling all criteria according to ICD–10 and DSM IV [14, 15] were analyzed. Two of these patients suffered from the restrictive form of AN (AN-R) and the remaining two suffered from the binge eating/purging form (AN-BP). The control group consisted of 4 patients not suffering from any eating disorders. The studied material consisted of RNA isolated from patients' PBMC. Obtained RNA was used to study the expression profile of selected genes at the transcript level with the oligonucleotide microarray technique (Affymetrix, HG-U133A). Hierarchical clustering (Cluster v 3.0) for six and then eight oligonucleotide microarrays was used to analyze the results.

Results and conclusions: Hierarchical clustering singled out two clusters for AN-R, AN-BP and control group patients. Based on the results of hierarchical clustering performed for six and then eight oligonucleotide microarrays showing different expression profiles for genes coding selected orexigenic (OXA and OXB) and anorexigenic (LEP) proteins, we may assume that this technique differentiates the two forms of AN: restrictive (AN-R) and binge eating/purging (AN-BP).

anorexia nervosa / oligonucleotide microarray

INTRODUCTION

In the past, the oligonucleotide microarray technique has mainly been used to study genes' expression profiles at the transcript level in tumor cells. However, this technique may also be used to study the expression profile of an isolated group of genes characteristic for a given type of cell or tissue. DNA microarrays, a.k.a. DNA chips, consist of an arranged number of probes in the form of single-strand DNA (cDNA microarray) or nucleotides (oligonucleotide microarrays) placed in a particular order on nylon membranes or glass or nylon slides. These cDNA microarrays may be used to study the expression of genes if one has the proper DNA clones multiplied through Reverse Transcriptase – Polymerase Chain Reaction and mRNA isolated from cells [1]. Oligonucleotide microarrays (DNA chips) were introduced by Affymetrix manufac-

Małgorzata Janas-Kozik*,, Irena Krupka-Matuszczyk*,**, Małgorzata Stachowicz***, Urszula Mazurek***:** *Psychiatry and Psychotherapy Department and Clinic, Silesian Medical University, Katowice, Poland; **Developmental Age Psychiatry and Psychotherapy Ward, Pediatric Center, Sosnowiec, Poland; ***Molecular Biology Department, Sosnowiec, Poland; Correspondence address: Małgorzata Janas-Kozik, Developmental Age Psychiatry and Psychotherapy Ward, John Paul II Pediatric Center, 3 G. Zapolskiej Str., 41–218 Sosnowiec, Poland; e-mail: mkozik@psychiatria.pl

turing through photolithography. Microarrays make it possible to study the expression of thousands of genes at the same time. The DNA microarray technique makes it possible to obtain incomparably more information on the transcriptive expression of genes than any other method. It evaluates the presence of gene transcripts in the studied material. Data obtained with the microarray technique provides information on expression of genes in the studied cells, therefore one can determine whether the transcription of a given gene was intensive, i.e. whether the gene was active or whether its expression was inhibited. Leptin (LEP) is one of the anorexigenic peptides produced by the fat tissue. LEP plays an important role in the regulation of energy expenditure, appetite and body mass [2]. The discovery of LEP found the missing link connecting functional energy storage (fat tissue) and the hypothalamus area regulating the amount of energy available to the organism. Leptin, apart from the role it plays in organism homeostasis, also interacts with the autonomic system [3]. Leptin is present in the human organism in two forms playing different functions: free and bound to plasma proteins [4]. Free circulating leptin controls the level of fat tissue, while when bound with a soluble form of the receptor, it controls energy expenditure [5].

The leptin receptor (OB-R) is one of the class I cytokine receptors. A few OB-R isoforms have been identified (created as a result of alternative transcript splicing [6]): Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re [7]. There is a relationship between a mutation in a leptin coding gene in mice and humans and obesity. Orexins, a.k.a. hypocretins, are orexigenic peptides. Orexins A and B (OXA and OXB) were first identified in a rat brain in 1998 by two independent scientists: de Lece and Sakurai [8,9]. Orexins are created when their common precursor – preproorexin – breaks down [10,11]. Its name derives from the Greek word *orexis* meaning "appetite". Orexins are mainly produced in two hypothalamic nuclei: the perifornical nucleus (PFN) and dorsomedial nucleus (DMN). Orexins are produced in these nuclei not only in amphibians, rodents and cattle, but also in humans. From these nuclei, the orexin fibers are connected to other areas of the brain: olfactory bulbs, cerebral cortex, thalamus, hypothalamus, brain stem and all levels of the spinal cord. Sakurai identified the orexin receptors OX-R1 and OX-R2, which are coded by two separate genes [9,12]. Studies performed mainly on the animal models showed that Orexin A (OXA) increases appetite [13,14,15,16] and plays a role in maintaining organism homeostasis [13,14,15,16]. OXA increases appetite 100 times more than OXB, this resulting from the fact that OXA acts through the OX-R1 receptor, stimulating orexigenic peptide activity in the hypothalamus [10]. OXA seems to play a more important role in the organism and therefore is better known than OXB, including its' molecular level activity.

Both peripheral and central mechanisms are active in maintaining organism homeostasis. Orexigenic peptides, i.e. OXA and OXB, and the anorexigenic peptide LEP play a role in maintaining homeostasis mainly by controlling appetite.

MATERIAL AND METHODS

The studied material consisted of RNA isolated from peripheral blood mononuclear cells (PBMC) (Total RNA Prep Plus kit, A&A Biotechnology) [17] of patients suffering from AN. PBMC of 4 patients suffering from AN and fulfilling all criteria according to ICD–10 and DSM IV [14, 15] were analyzed; two of these patients suffered from the restrictive form of AN (AN-R) and the remaining two suffered from the binge eating/purging form (AN-BP). The control group consisted of 4 patients not suffering from any eating disorders. The study was approved by the Bioethical Committee of the Silesian Medical University in Katowice, Poland. Written consent was obtained from all patients and/or their parents or legal guardians as well as the control group. Table 1 presents the patients' data.

The study evaluated the gene coding two isoforms of the leptin receptor (OB-Ra and Ob-Rb) as well as the genes coding the OXA and OXB receptors. Table 2 presents descriptions of particular transcripts evaluated with the oligonucleotide microarray technique (HGU–133A). Data comes from the Affymetrix database [20].

In order to evaluate the transcript expression profile for selected genes coding the anorexigenic (LEP) and orexigenic (OXA and OXB) proteins

Table 1. Patients' characteristics.

Patients' initials	Age	Weight upon admission to the Ward (kg)	Sex	Diagnosis
Characteristics of AN patients				
N.P.	17.5	42	female	AN-BP
N.M.	24	39	female	AN-R
T.S.	23	39	female	AN-R
H.P.	21	43	female	AN-BP
Characteristics of control group patients				
A.S.	19	60	female	Paranoid schizophrenia
B.K.	14	59	female	Adaptive disorders
K.W.	20	61	female	Mild mental impairment
M.P.	16	46	female	Adaptive disorders

Table 2. Characteristics of analyzed transcripts.

Probe Set ID	Gene Title	Gene Symbol
202378_s_at	LEPROT	leptin receptor overlapping transcript
202594_at	LEPROTL1	leptin receptor overlapping transcript-like 1
202595_s_at	LEPROTL1	leptin receptor overlapping transcript-like 1
207255_at	LEPR	leptin receptor
209894_at	LEPR	leptin receptor
211354_s_at	LEPR	leptin receptor
211355_x_at	LEPR	leptin receptor
211356_x_at	LEPR	leptin receptor
207642_at	HCRT	hypocretin (orexin) neuropeptide precursor
207619_at	HCRTR1	hypocretin (orexin) receptor 1
207393_at	HCRTR2	hypocretin (orexin) receptor 2

in patients suffering from AN, one 20 ml sample of blood was collected with Vacutainer tubes from each of the patients. RNA was isolated from PBMC (Total RNA Prep Plus kit, A&A Biotechnology) [17]. The material was purified with RNeasy Total RNA Mini Kit (Qiagen) and treated with DNasis I. Obtained RNA was used for the synthesis of double-stranded cDNA (Gibco BRL SuperScript Choice system) which became the array for the synthesis of biotinylated cRNA. Marked cRNA was purified with RNeasy Mini Kit (Qiagen), fragmentized and hybridized with a test array (Test 3), and then with Human Genome Arrays U133A (Affymetrix). Hybridized with arrays, cRNA was then marked with the streptavidin-phycoerythrin complex. Fluorescence intensity was evaluated with GeneArray Scanner G2500A. Obtained results were normalized with the RMAExpress software and then hierarchically clustered with Cluster v 3.0., which makes it possible to combine genes of a similar expression profile and create the so-called clusters.

RESULTS

Fig. 1 shows the results of hierarchical clustering for 6 oligonucleotide microarrays for the expression profile of transcripts of the gene coding the LEP receptor; Fig. 2 shows the results of hierarchical clustering for 8 oligonucleotide microarrays for the expression profile of transcripts of

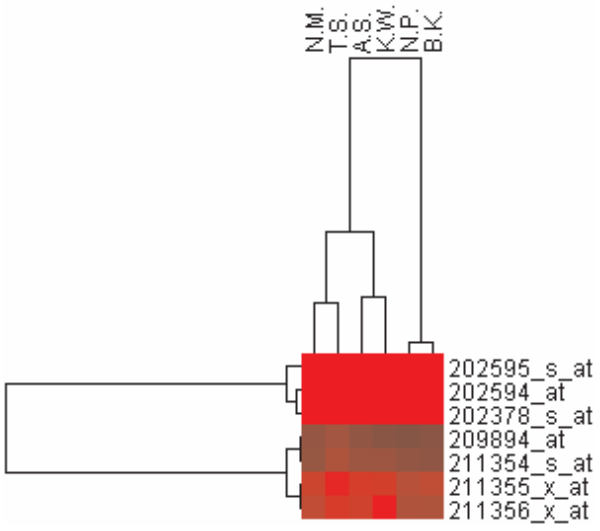

Fig. 1. Results of hierarchical clustering for 6 oligonucleotide microarrays – the expression profile of transcripts of the gene coding the LEP receptor.

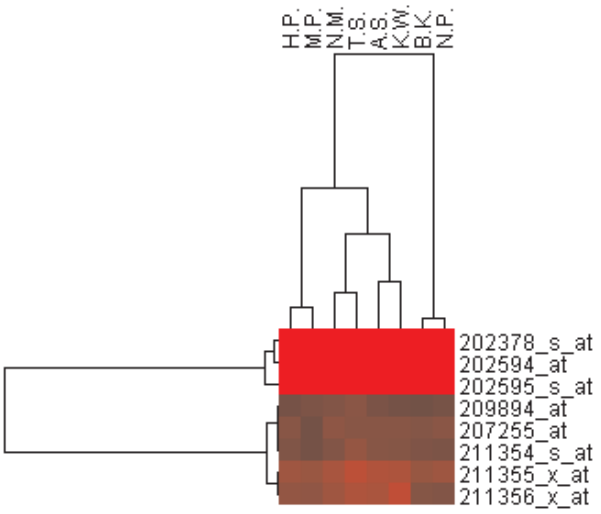

Fig. 2. Results of hierarchical clustering for 8 oligonucleotide microarrays – the expression profile of transcripts of the gene coding the LEP receptor

the gene coding the LEP receptor; Fig. 3 shows the results of hierarchical clustering for 8 oligonucleotide microarrays for the expression profile of transcripts of the genes coding the LEP and orexin receptors.

DISCUSSION

The present study used the oligonucleotide microarray technique (HG-U 133A, Affymetrix)

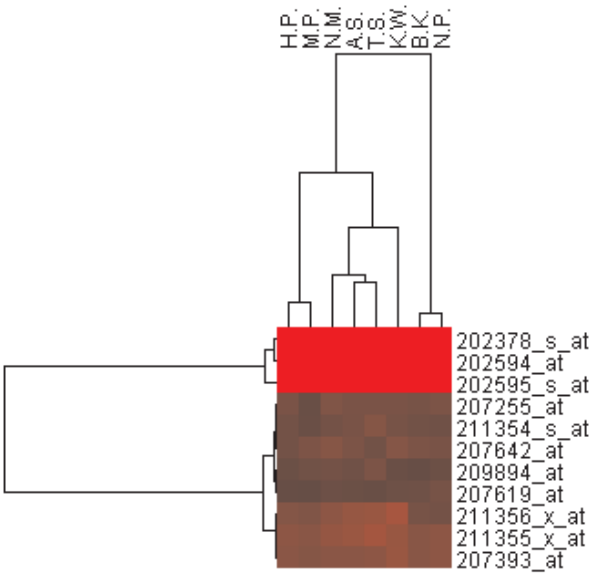

Fig. 3. Results of hierarchical clustering for 8 oligonucleotide microarrays – the expression profile of transcripts of the genes coding the LEP and orexin receptors

to analyze the expression profile of gene transcripts whose products are responsible for organism homeostasis by controlling hunger and satiety. Patients representing both the restrictive form of AN (AN-R) and the binge eating/purging form of AN (AN-BP) participated in the study. The oligonucleotide microarray technique (HG-U133A, Affymetrix) was used to evaluate the expression profiles of gene transcripts coding leptin and orexin receptors. In the initial tests, two isoforms of the LEP receptor were studied (Ob.-Ra and Ob.-Rb); their reference sequences were located in the National Center for Biotechnology Information (NCBI) database [21]. These sequences were used by Affymetrix to design probes for particular genes on the HG-U133A (Affymetrix) microarray, on which 7 transcripts of the gene coding LEP are present. Hierarchical clustering groups together genes' transcripts of a similar expression profile. All transcripts create the so-called "hierarchical tree" on which transcripts of a similar expression are located close to each other. The length of the "tree branches" determines the similarity of transcript expression profiles of studied genes – the shorter the branch the more similar the studied elements.

Initially, 3 AN patients (2–AN-R and 1 AN-BP) and 3 control group girls participated in the study and oligonucleotide microarrays were completed for all of them. The expression pro-

file for 7 transcripts of the gene coding LEP was analyzed. Hierarchical clustering resulted in 3 groups constituting separate clusters (Fig. 1). Patients belonging to cluster I (N.M. and T.S.) suffered from AN-R. Two control group patients not suffering from any eating disorders (A.S. and K.W.) belonged to cluster II, while the AN-BP patient (N.P.) and one control group patient not suffering from any eating disorders (B.K.) belonged to cluster III. Based on these results one could assume that the expression profile of transcripts of the gene coding leptin differentiates the two forms of AN: AN-R and AN-BP [22]. However, a question arose: why did B.K. – a control group patient not suffering from any eating disorders – belong to the same cluster as N.P. who suffered from AN-BP? Hierarchical clustering results allowed the assumption that the transcript expression profile of the gene coding the LEP receptor was similar for both patients. A few months after completion of therapy, B.K. was admitted to the Developmental Age Psychiatry and Psychotherapy Ward of the Pediatric Center in Sosnowiec, Poland for the second time – this time with a diagnosis of the binge eating/purging form of AN (AN-BP).

Additionally, two microarrays were completed for the gene coding different isoforms of LEP for additional patients: H.P. suffering from AN-BP and M.P., a control group patient not suffering from any eating disorders. Hierarchical clustering analysis for eight oligonucleotide microarrays resulted in four separate clusters (Fig. 2). H.P. suffering from AN-BP and M.P. from the control group belonged to cluster I. N.M. and T.S. – both suffering from AN-R – belonged to cluster II; A.S. and K.W. from the control group belonged to cluster III, while B.K. (control group) and N.P. (AN-BP) belonged to cluster IV. Hierarchical clustering for eight microarrays, similarly as in the case of six microarrays, grouped N.P. and B.K. in the same cluster, which allowed the assumption that molecular level changes may precede clinical symptoms of AN. However, it is important to bear in mind that there are other factors which may influence the clinical picture of AN, e.g. family, environment or personality.

The next study consisted of hierarchical clustering for eight oligonucleotide microarrays (HG-U133A, Affymetrix), evaluating the transcript expression profile of the gene coding the LEP receptor as well as genes coding OXA and OXB receptors. Hierarchical clustering results grouped the patients as follows:

- cluster I H.P. (AN-BP) and M.P (control group)
- cluster II A.S. (control group) and T.S. (AN-R)
- cluster III B.K. (control group, later diagnosed with AN-BP) and N.P. (AN-BP)

Patients N.M. (AN-R) and K.W. (control group) are grouped with cluster II.

The analysis of eight oligonucleotide microarrays (HG-U133A, Affymetrix) for genes coding selected receptors (LEP receptor and OXA and OXB receptors) showed that AN-R and AN-BP patients are grouped in different clusters.

CONCLUSIONS

Based on the results of hierarchical clustering performed for six and eight oligonucleotide microarrays presenting different expression profiles of genes coding selected orexigenic (OXA and OXB) and anorexigenic (LEP) peptides at the transcript level, we may assume that this method differentiates the two forms of anorexia nervosa: the restrictive form (AN-R) and the binge eating/purging form (AN-BP).

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