Agència de Gestió d'Ajuts Universitaris i de Recerca

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Memòria justificativa de recerca de les beques predoctorals per a la formació de personal investigador (FI)

La memòria justificativa consta de les dues parts que venen a continuació:
1.- Dades bàsiques i resums
2.- Memòria del treball (informe científic)

Tots els camps són obligatoris

1.- Dades bàsiques i resums

Títol del projecte ha de sintetitzar la temàtica científica del vostre document.
I. Identification and characterisation of epigenetically alterated tumor supressor genes by proteomic and genomic approches.
II. Studies of genes regulated by MeCP2 union to its promoter regions by proteomic and genmomic approches

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Paraules clau: cal que esmenteu cinc conceptes que defineixin el contingut de la vostra memòria.
DNA methylation, cancer, proteomics, Rett syndrome, MeCP2

Data de presentació de la justificació

9/09/10

Resum en la llengua del projecte (màxim 300 paraules)

Resum en anglès(màxim 300 paraules)
DNA methylation has an important impact on normal cell physiology, thus any defects in this mechanism may be related to the development of various diseases. In this project we are interested in identifying epigenetically modified genes, in general controlled by processes related to the DNA methylation, by means of a new strategy: combining proteomic and genomic analyses. First, the two Dimensional-Difference Gel Electrophoresis (2-DIGE) protein analyses of extracts obtained from HCT-116 wt and double knockout for DNMT1 and DNMT3b (DKO) cells revealed 34 proteins overexpressed in the condition of DNMTs depletion. From five genes with higher transcript levels in DKO cells, comparing with HCT-116 wt, only AKR1B1, UCHL1 and VIM are methylated in HCT-116. As expected, the DNA methylation is lost in DKO cells. The methylation of VIM and UCHL1 promoters in some cancer samples has already been reported, thus further studies has been focused on AKR1B1. AKR1B1 repression due to DNA methylation of promoter region seems to occur specifically in the colon cancer cell lines, which was confirmed in the DNA methylation status and expression analyses, performed on 32 different cancer cell lines (including colon, breast, lymphoma, leukemia, neuroblastoma, glioma and lung cancer cell lines) as well as normal colon and normal lymphocytes samples. AKR1B1 expression after treatments with DNA demethylating agent (AZA) was rescued in 5 colon cancer cell lines confirming epigenetic regulation of the candidate gene. The methylation status of the rest of the genes identified in proteomic analysis was checked by methylation specific PCR (MSP) experiment and all appeared to be unmethylated. The similar research has been done also by means of Mecp2-null mouse model. For 14 selected candidate genes the analyses of expression levels, methylation status and MeCP2 interaction with promoters are currently being performed.

2.- Memòria del treball (informe científic sense limitació de paraules). Pot incloure altres fitxers de qualsevol mena, no més grans de 10 MB cadascun d'ells.

Introduction

Epigenetic can be defined as changes in gene expression that are not associated with any alteration in DNA sequence (Holliday 1987). Therefore, it seems obvious that proper epigenetic control is essential for development and cell functioning. Epigenetic regulation of gene expression is mediated by mechanisms such as methylation of DNA, modifications of histones and positioning of nucleosomes along the DNA. The interplay between epigenetic components guarantees proper balance between transcriptional activity and repression by changing chromatin architecture. Thus, regulation of packaging of DNA ensures maintenance of correct chromosome replication, gene expression and stable gene silencing (Esteller 2007). DNA methylation is one of the most intensely studied epigenetic modifications in mammals and it has an important impact on normal cell physiology. As this DNA modification seems to be a critical player in the transcriptional regulation, it is not surprising that defects in this mechanism may be related to the development of various diseases (Esteller 2008).

The methylation reaction of cytosines, within the CpG dinucleotides, is mediated by a class of enzymes called DNA methyltransferases (DNMTs) that catalyze the transfer of the methyl group from S-adenosyl methionine onto cytosine. Five members of the DNMT family have been identified in mammals: DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. However, as far as we know, only DNMT1, DNMT3a and DNMT3b interplay to produce the global cytosine methylation pattern. These independently encoded proteins are classified as *de novo* enzymes (DNMT3a and DNMT3b) or as a maintenance enzyme (DNMT1) (Bestor 2000; Robertson 2001). How DNA methylation contributes to the inhibition of expression still remains unclear and various hypotheses have been proposed. One of the models of gene inactivation mediated by DNA methylation is related to methyl-CpG binding domain proteins (MBDs). In general, DNA methylation is considered to be an initiation step for establishing the inactive chromatin state. It is followed by an MBDs association that, in turn, recruits histone deacetylases known as repressive epigenetic modification enzymes. The chromatin compacts and gene silencing is achieved.

It is commonly known that inactivation of certain tumor suppressor genes occurs as a consequence of hypermethylation of regulatory regions, consisting of CpG islands within the promoter region (Esteller 2007). Apart from cancer, alteration in epigenetic machinery may lead to the development of some other disease. For instance, mutation of the X-linked gene encoding MeCP2 protein (member of MBD proteins) results in progressive neurodevelopmental disorder, called Rett syndrome (RTT) (Bird 2008). As the epigenetic changes could be reversible, elucidating the epigenetic mechanisms such as DNA methylation phenomena and screening for new hypermethylated genes may provide possible targets for therapy or biomarkers (Mulero-Navarro et al., 2008).

So far, various approaches (gene-by-gene based as well as massive genomic screening procedures) have been employed to search for genes that undergo methylation-associated inactivation in development of different diseases. In this project the comparative proteomic and genomic analysis have been performed to search for epigenetically modified genes. We decided to use two different model systems with disrupted epigenetic machinery. First, we performed our analyses on the colorectal cancer cell line HCT-116 double knockout for DNMT1 and DNMT3b (DKO) that show a minimal DNA methyltransferase activity and a 95% reduction in 5-methylcytosine content (Rhee et al., 2002). Considering that CpG hypermethylation is one of the mechanisms responsible for silencing tumor suppressor genes, we compared the expression profiles of HCT-116 wt cell line vs. DKO cells. Thus, genes overexpressed in DKO cells which lack DNA methyltransferases could be silenced by hypermethylation in HCT-116 wt.

The similar research has been done also by means of *Mecp2*-null mouse model, presenting RTT phenotype (Guy et al., 2007). The detailed analysis of proteins differently expressed in specific parts of brain of wt and *Mecp2*-null mice will unmask the role of epigenetic silencing in the proper mental development and neuron functioning.

Main Objectives

The aim of this project is to identify new putative genes regulated by epigenetic mechanisms, in general related to the DNA methylation by means of a new strategy: combining proteomic and genomic analyses in the HCT-116 wt and DKO (knockout for DNMT1 and DNMT3b) model system and *Mecp2*-null mouse model system. Considering that CpG hypermethylation is one of the mechanisms responsible for silencing tumor suppressor genes, we will compare the expression profiles of HCT-116 wt cell line vs. DKO cells in terms to provide new hypermethylated markers for the putative translational use in patients.
It is also important to find genes regulated by MeCP2 binding into the promoter region. This might shed more light on the importance of epigenetic process in the brain physiology, as well as in development of neurological alterations resulting in Rett syndrome phenotype in mice.

Results

I. Studies of new putative tumor suppressor genes undergoing epigenetic alterations in human cancer by means of HCT-116 wt and DKO (knockout for DNMT1 and DNMT3b) model

1.Identification of candidate proteins differently expressed in HCT-116 wt and DKO cells

The two Dimensional-Difference Gel Electrophoresis (2-DIGE) protein analyses of extracts obtained from HCT-116 wt and DKO cells in our laboratory were performed in collaboration with the Danish Cancer Society (Department of Proteomics in Cancer) and CIC bioGUNE (Center for Cooperative Research in Biosciences, Technology Park of Bizkaia). The list of detected proteins with significantly higher expression levels in DKO, thus acting as possible tumor suppressors regulated by epigenetic mechanisms, was provided (Tab.1). Five of them, with the highest expression ratio between DKO and HCT-116 wt (AKR1B1, UCHL1, NAP1L1, CALR and NASP) were chosen to define the correlation between proteins and transcripts levels and exclude the hypothesis that their overexpression is due to post-transcriptional regulation. We included also seven other proteins which seemed interesting because of their probable implication in cancer formation (e.g. VIM). Real-time PCR results showed that only in the case of five genes: *AKR1B1, UCHL1, CALR, HSPB1* and *VIM* the transcript levels were higher in DKO cells (as a control three housekeeping genes *GAPDH, HPRT1* and *PSMD4* has been used). It should be noted that expression of *UCHL1* and *VIM* was not detected at all in HCT-116 cells, thus it seems that these genes are totally repressed in wt cells. *AKR1B1, CALR* and *HSPB1* are expressed in HCT-116 cells but their levels of expression in DKO cells are at least two times higher.

Table 1. Proteins overexpressed in DKO cells (= no differenced in expression between HCT-16 and DKO, ↑ - increase of transcript level in DKO, referring to HCT-116 wt cells, ↑↑ - transcript not detected in HCT-116 wt and expressed in DKO cell line)

		RT-PCR (HCT116 vs DKO)	Promoter methylation in HCT-116	
UniGene	*Protein name*		*BSP analysis*	*MSP analysis*
AKR1B1	Aldose reductase	↑	Methylated	
UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	↑↑	Methylated	
NAP1L1	Nucleosome assembly protein 1-like 1	=		
CALR	Calreticulin	↑	UNmethylated	
NASP	Nuclear autoantigenic sperm protein	=	UNmethylated	
EIF5A	Eukaryotic translation initiation factor 5A-1			UNmethylated
LGALS1	Galectin-1	=		
TXN	Thioredoxin peroxidase 1			UNmethylated
MT-CO2 (COX2)	Cytochrome c oxidase subunit 2			UNmethylated
HSPB1	Heat shock protein beta-1	↑	UNmethylated	
TOMM40	Mitochondrial import receptor subunit TOM40			UNmethylated
ACTB	Actin, cytoplasmic 1			UNmethylated
EIF4A2	Eukaryotic initiation factor 4A-II		UNmethylated	
HNRNPL	Heterogeneous nuclear ribonucleoprotein L			UNmethylated
GMPS	GMP synthase			UNmethylated
VIM	Vimentin	↑↑	Methylated	
NME1	Nucleoside diphosphate kinase A	=	UNmethylated	
TPI1	Triosephosphate isomerase	=	UNmethylated	
DHRS2	Dehydrogenase/reductase SDR family member 2		NO CpG island	
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1			UNmethylated
RPLP0	60S acidic ribosomal protein P0			
FUS	RNA-binding protein FUS	=		UNmethylated
SERPINH1	Serpin H1			UNmethylated
HSP60	Heat Shock protein 60kDa		UNmethylated	
1433E	14-3-3 protein epsilon	=	UNmethylated	
TPM4	Tropomyosin alpha-4			UNmethylated
NDUS3	NADH dehydrogenase subunit 3		NO CpG island	
EEF2	Translation elongation factor 2			UNmethylated
ANXA5	Annexin 5			UNmethylated
DNAJB11	DnaJ homolog			UNmethylated
TPM3	Tropomyosin 3			UNmethylated
EIF3I	Translation initation factor 3, subunit 2			UNmethylated
ENO1	Enolase 1			UNmethylated
VIL2(EZR)	Ezrin			UNmethylated

2. Validation of epigenetic regulation of candidate genes

In order to evaluate if the overexpression of genes of concern in DKO could be directly associated to the lack of DNA methyltransferases function, their methylation pattern, focusing on promoter regions, was analyzed by means of bisulphite genomic sequencing (BSP). First, the CpG island (CGI) location in the promoter region for each of the candidate genes was determined (Methyl Primer Express Software v 1.0).Next, specific primers were designed for sequencing possibly the biggest fragment of CGI (containing at least 6-8 CpGs) in the proximity of transcription start site. The BSP analysis revealed that CGI located in the promoter region of three of the candidate genes *AKR1B1, UCHL1* and *VIM* are methylated in HCT-116. As expected, the DNA methylation is lost in DKO cells.

In order to confirm epigenetic regulation of candidate genes, it was checked if the treatments with DNA demethylating agent, 5-aza-2'-deoxycytidine (AZA), and/or histone deacetylases (HDAC) inhibitor (TSA) will rescue their expression in HCT-116 wt. The AZA treatment was performed during 72 hours (final concentration 5 μM), replenishing the drug every 24 hours as it has a short half life. TSA was added for the final 24 hours of treatment (final concentration

300 nM). According to the real-time PCR analyses, AZA as well as AZA/TSA indeed result in reexpression of *UCHL1*, *VIM* and higher expression level of *AKR1B1*. TSA alone have not effect on gene expression. BSP of promoter regions confirmed the partial loss of DNA methylation in HCT-116 cells after AZA treatment.
For the rest of the genes encoding the proteins significantly overexpressed in DKO cells, according to the proteomic analysis, the methylation specific PCR (MSP) was performed to quickly assess their DNA methylation status (Tab.1). None of them was methylated, indicating that most probably they are not directly regulated by DNMTs activity. However there might be some additional, upstream factors controlling their expression in HCT-116/DKO cell lines, regulated by epigenetic processes.

3. Studies of *AKR1B1*, putative new tumor suppressor gene

As the methylation of *VIM* and *UCHL1* promoters in some cancer samples has already been reported, further studies has been focused on *AKR1B1* which possible regulation by DNA methylation has not been described. To establish if the DNA methylation in *AKR1B1* promoter region is also present in other cancer cell lines, the MSP analysis has been performed. 32 different cancer cell lines have been checked (including colon, breast, lymphoma, leukemia, neuroblastoma, glioma and lung cancer cell lines) (Fig.1.B). The DNA methylation of *AKR1B1* occurred only in the colon cancer cell lines. After this first screening, BSP analysis proved the hypermethylation of *AKR1B1* in 9 colon cancer cell lines checked. Moreover, we did not find the methylation of *AKR1B1* promoter neither in normal colon (one sample checked) nor in normal lymphocytes (2 samples checked). Semi-quantitative PCR analysis showed that methylation in colon cancer cell lines correlates with loss of *AKR1B1* expression. On the other hand, in normal colon and normal lymphocytes samples as well as in unmethylated cell lines, used as a control, *AKR1B1* is expressed. This finding could confirm that *AKR1B1* becomes methylated and as a consequence repressed in colon cancer. However, it is indispensable to validate its methylation status in tumor samples as well as in a larger number of normal colon samples.

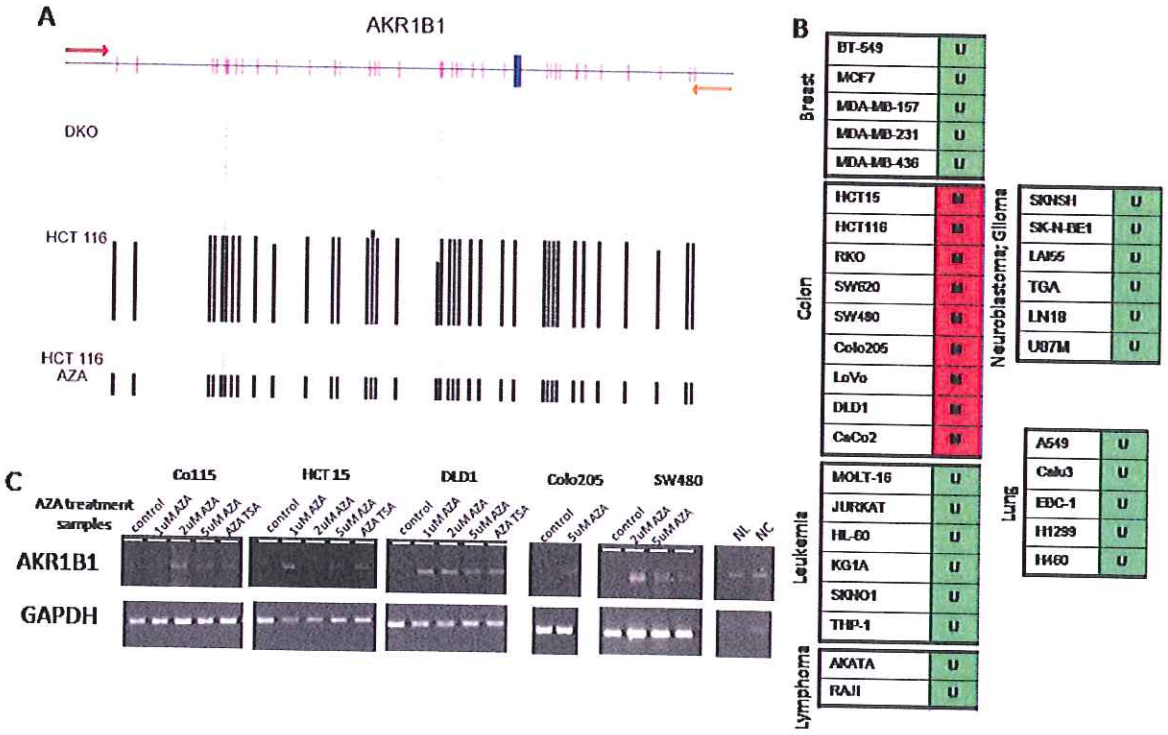

Fig 1.
A. BSP of *AKR1B1* promoter region in HCT-116, DKO and HCT-116 treated with 5μM of AZA
B. MSP for *AKR1B1* in different cancer cell lines
C. Semi-quantitative RT-PCR of *AKR1B1* as well as *GAPDH* (positive control) in colon cancer cell lines samples, treated with AZA in different concentrations. (NL=Normal Lymphocytes, NC=Normal Colon)

As a following step, the AZA treatments of 5 methylated colon cancer cell lines: Co115, DLD1, HCT-15, SW480 and Colo205 were performed. In all of the cases, reexpression of AKR1B1 after AZA treatment was observed, even when low drug concentration was applied (final concentration 1 μM) (Fig.1.C).

II. Studies of genes regulated by MeCP2 union to its promoter regions by means of Rett syndrome Mecp2-null and wt mice model

1. Identification of candidate proteins differently expressed in *Mecp2*-null vs. wt mice

In collaboration with CIC bioGUNE (Center for Cooperative Research in Biosciences, Technology Park of Bizkaia) the protein expression profiles of wild type (WT) and *Mecp2*-null (KO) mice of three different brain regions (cortex, midbrain, and cerebellum) were compared by 2-DIGE approach. Proteins which expression in this mouse model differs significantly were identified and subjected to the further analyses (Tab.1.).

Proteins downregulated in Mecp2-null mice vs wt					
Cortex		Mesencephalon		Cerebellum	
GPDA	Glycerol-3-phosphate dehydrogenase [NAD+]	PYC	Pyruvate carboxylase, mitochondrial	**SGTA**	**Small glutamine-rich tetratricopeptide repeat-containing protein alpha**
PDIA3	Protein disulfide-isomerase A	DHE3	Glutamate dehydrogenase 1, mitochondrial	**APOA1**	**Apolipoprotein A-I**
GFAP	**Glial fibrillary acidic protein**	ACLY	ATP-citrate synthase	ATPB	ATP synthase subunit beta, mitochondrial
		EF2	Elongation factor 2	ARPC4	Actin-related protein 2/3 complex subunit 4
				CHM4B	Charged multivesicular body protein 4b
Proteins upregulated in Mecp2-null mice vs wt					
Cortex		Mesencephalon		Cerebellum	
MYL12B	**Myosin regulatory light chain 12B**	**SNCA**	**Alpha-synuclein**	**MYL12B**	**Myosin regulatory light chain 12B**
GSTP1	**Glutathione S-transferase P 1**	**ARI5B**	**AT-rich interactive domain-containing protein 5B**	EF2	Elongation factor 2
VDAC1	**Voltage-dependent anion-selective channel protein 1**	**SEPT3**	**Neuronal-specific septin-3**	**LMNB1**	**Lamin-B**
VDAC2	**Voltage-dependent anion-selective channel protein 2**			GPDM	Glycerol-3-phosphate dehydrogenase, mitochondrial
SOD2	**Superoxide dismutase [Mn], mitochondrial**			**NEFL**	**Neurofilament light polypeptide**
				GLNA	Glutamine synthetase
				PPIA	Peptidyl-prolyl cis-trans isomerase A
				HNRPK	Heterogeneous nuclear ribonucleoprotein K

Tab 1. Proteins differentially expressed in Mecp2-null and wt mice. Genes selected for further analyses marked in bold.

2. Validation of methylation status of candidate genes

For further analyses, we selected 14 genes with the highest expression ratio between mutant and wt mice or representing interesting biological functions (marked in bold in the tab1). Their methylation patterns, focusing on promoter regions, were analyzed by means of bisulphite genomic sequencing (BSP). First, the CpG island (CGI) location in the promoter region for each of the candidate genes was determined (Methyl Primer Express Software v 1.0). Next, specific primers were designed for sequencing possibly the biggest fragment of CGI (containing at least 6-8 CpGs) in the proximity of transcription start site, in the plausible binding site of MeCP2. The BSP analysis, performed on two pairs of Rett and wt mice samples, revealed that the promoter region of only two genes *Gfap* and *Apoa1* are methylated both in Rett and wt samples. Moreover, there was no difference in methylation pattern detected in the tree different part of the brain: cerebellum, cortex and mesencephalon.

3. Expression analysis of candidate genes

To define the correlation between selected proteins and transcripts levels and exclude the hypothesis that their overexpression is due to post-transcriptional regulation, the real-time PCR experiment was performed. By now, we analyzed expression profiles in 3 pairs of Rett and wt mice samples. According to some preliminary studies, the changes in the protein level of MYL12B and LMNB1 seems to be due to some post-translational modification, as there was no difference in the expression at the transcript level (checked by real-time PCR analysis, with two different pairs of Rett model mice). However in the case of NEFL, the overexpression in Mecp2-null mouse was observed uniquely in cerebellum sample, confirming the proteomic analysis. That lead us to the conclusion that this gene may be indeed regulated by MeCP2 binding to its promoter region.

Apparently, there are no significant differences in the transcript levels of two genes that we showed to be methylated, *Gfap* and *Apoa1*, in the samples checked. However, more trials with a larger number of samples should be performed, as it might be possible that only a slight difference in the mRNA level provokes changes in protein expression.

4. Optimization of Chromatin Immunoprecipitation Analysis (ChIP)

To confirm the possibility that MeCP2 binds to the promoter regions of detected genes and regulate the protein expression, the ChIP analysis is necessary. We are currently working on optimization of that method with mouse brain tissue samples, searching for efficient conditions of chromatin extraction and fragmentation as well as checking different MeCP2 antibodies.

Conclusions

In search of the genes regulated by epigenetic mechanisms (focusing on the role of DNA methylation phenomena), proteomic analysis was performed in two well established model systems with disrupted epigenetic machinery: 1) HCT-116wt and DKO, knockout for DNMT1 and DNMT3b colon cancer cell line model and 2) *Mecp2*-null and wt mice model.

In the condition of DNMTs depletion in HCT-116 colon cancer cell line, several proteins significantly overexpressed were identified but so far it seems the expression of only three of them (UCHL1, VIM and AKR1B1) is controlled by DNA methylation. UCHL1 (ubiquitin carboxyl-terminal esterase L1) plays role in cytoplasmic protein degradation and recycling of free ubiquitin and it was shown to be methylated in many cancer cell lines (e.g. head and neck, prostate, breast, esophageal squamous cell carcinoma etc) (Seliger et al., 2009). VIM (vimentin) is responsible for maintaining cell shape, stabilizing cytoskeletal interactions and is one of the major markers of epithelial-mesenchymal transition (EMT). The gene encoded vimentin has been shown to be frequently methylated in gastric cancer samples (Chen et al., 2005). Thus, we focused our studies on *AKR1B1*, gene encoding one of the members of aldo-keto reductase family 1, which has never been reported to be controlled by epigenetic mechanisms. AKR1B1 catalyzes the reduction of a number of aldehydes, including the aldehyde form of glucose and it has been shown to be implicated in the development of diabetic complications because it facilitates the reduction of glucose to sorbitol (Demaine 2003). The putative role of AKR1B1 in cancer remains unclear. According to Lefrançois-Martinez *et al.* expression of AKR1B1 is decreased in adrenocortical carcinoma compared with adenomas (Lefrancois-Martinez et al., 2004). As AKR1B1 is proposed to be involved in neutralization of toxic aldehydes derived from lipid peroxidation and steroidogenesis, it might be a part of the cellular defense against oxidative stress. However it still remains to be elucidated whether the lower level of AKR1B1 in malignant tumors is a cause or consequence of the tumor progression.

In our studies we showed that the expression of *AKR1B1* is decreased particularly in colon cancer cell lines and that it seems to be a direct consequence of DNA methylation in the promoter region of that gene. It has been also proved that upon AZA treatment, gene expression is recovered. Currently we are working on establishing the DNA methylation status in tumor samples as well as on explaining the role of methylation of AKR1B1 in tumor formation.

Proteomic analysis was also performed in Rett syndrome *Mecp2*-null and wt mice model. 14 candidate genes were selected and analyses of their expression, methylation pattern as well as MeCP2 binding to their promoter region should be carried out in a large number of mice samples.

The exact role of MeCP2 in the expression of the genes determined in 2-DIGE analysis of *Mecp2*-null and wt mouse brain protein extracts still need to be determined. Nevertheless, at that stage of studies, the analysis of already published data concerning the identified candidate genes lead us to the conclusion that epigenetic mechanisms might be involved in neuron formation as well as defense against oxidative stress. The detailed studies of expression levels, methylation status and MeCP2 interaction with promoters of candidate genes will be performed.

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