Transcriptional regulation of the human *ABCC6* gene.

Booklet of Theses

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INTRODUCTION

Pseudoxanthoma elasticum (PXE, OMIM 264800) is a rare recessive genetic disorder with a prevalence of approximately 1 : 40.000. It is characterized by the calcification of elastic tissues of the body, including the skin, the arteries and the eyes. It has been shown that the development of the disease is due to loss of function mutations of an ATP binding cassette transporter called ABCC6. The gene encodes an organic anion transporter ABCC6/MRP6 that transports molecule(s) from the cytoplasm to the blood.

Intriguingly *ABCC6* is expressed in the liver and to a lower extent in the kidneys and in the intestines, while it is absent from tissues that are related to the PXE phenotype. This observation and other studies confirmed the theory of a metabolic disease due to the lack of export to the blood of a liver specific metabolite. To date the physiologic substrate of the transporter, and therefore the function of the protein are still unknown.

The transcriptional regulation of the *ABCC6* gene has been poorly studied. The proximal promoter of the gene has been characterized and a CpG island present in the promoter have been shown to be methylated in tissues that do not express

ABCC6. Moreover, an activator element has been identified in the proximal promoter of the gene. Finally, several transcription factors were suggested to modulate the expression of the gene. However, to date no cellular signaling pathway has been convincingly linked to the regulation of *ABCC6* expression, and the mechanism of regulation is poorly understood.

OBJECTIVES

1) My aim was to find a physiologic pathway regulating the human *ABCC6* expression. In that purpose I looked for different molecules that can increase or decrease the *ABCC6* expression. My strategy was to perform a screening on the liver HepG2 cell line, and to follow *ABCC6* expression variation by quantitative RT-PCR.

2) In parallel I aimed to map the different potential regulatory regions of the *ABCC6* gene. I used the DNAse I digestion technique to identify sequences with transcriptional regulatory abilities in the endogenous chromatin environment. This technique is used in a hypothesis free context, which is a major advantage since hypothesis could lead to over or underestimation of the importance of specific sequences. This

experiment was done on different cell lines to detect possible cell specific regulation profiles.

3) Finally I aimed to map and to analyzed the regulatory region(s), observed in the two firsts objectives, using the Luciferase reporter gene assay technique. Following the mapping on the *ABCC6* promoter of the drugs response elements (see aim 1) or of the DNaseI regulatory region (see aim 2), the regions were screened by bioinformatics tools to identify the factors potentially involved in the regulation of the gene.

METHODS

- **Cell culture**

I used different model cell lines in this study. The Hepato-carcinoma cell line HepG2, and the Caco2 cell lines were used as cell line expressing the *ABCC6* gene. HeLa, and HEK293 cell lines were used as cell lines non-expressing *ABCC6*.

- **Treatments**

HepG2 cells were cultured on 24 wells plates without serum for 24 hours. Selected molecules were added to the cells, and cells were harvested after 24hours treatment.

- **Quantitative PCR**

cDNA was synthesized from 500ng of total extracted RNA. qPCR was performed with specific primers for the *ABCC6* and the *ABL* genes (used as a control gene), using the SYBRgreen system with a LC480 roche qPCR machine.

- **DNase I hypersensitive asssay**

Whole cell genomic DNA was partially digested by different concentrations of DNaseI for 3 minutes on ice. The genomic DNA was extracted and digested by selected restriction enzymes. The presence of DNaseI Hypersensitive sites (HS) were revealed by Southern blot using a specific *ABCC6* probe.

- **Luciferase reporter gene assay**

Selected fragments were cloned into the Luciferase reporter plasmid pGL3. Cells were seeded in a 96 wells plate. After 24h, the plasmids were transfected by polyethyleneimine transfection reagent. 48 h after transfection the cells were harvested and lysed, and the activity of luciferase was measured.

RESULTS AND DISCUSSION

1. The ERK1/2 cascade down-regulates *ABCC6* expression.

By examining the gene modulation following treatment with various factors involved in major physiologic pathways in the cells, I found that the ERK1/2 cascade inhibits the expression of *ABCC6* at the transcriptional level. Indeed activation of the ERK1/2 cascade by growth factors leads to the inhibition of the gene expression, while co-treatment with U0126 a specific inhibitor of ERK1/2 totally prevented this negative effect.

2. The ERK1/2 response element is located in the proximal promoter of *ABCC6*.

Luciferase reporter gene assay with deletion mutant of the proximal promoter of the gene revealed that ERK1/2 modulate *ABCC6* in HepG2 cells. I demonstrated that this modulation was dependent on the proximal promoter between -209 and -145bp from the translation initiation site. Moreover, *ABCC6* expression was not affected by the ERK1/2 cascade in HeLa cells, showing that this modulation is cell type specific

3. HNF4alpha up-regulate *ABCC6* expression

In silico analysis of the region containing the ERK1/2 response element showed that a degenerate HNF4alpha

binding site is located between -166 and -154bp from the translation initiation site. It is known that the HeLa cells that do not express *ABCC6* neither HNF4alpha. Therefore I confirmed the role of HNF4alpha in the *ABCC6* gene regulation by co-expressing HNF4alpha and the *ABCC6* promoter construct in HeLa cells. I observed that HNF4alpha induces the expression of the gene. Interestingly the expression pattern of *ABCC6* and HNF4alpha are highly overlapping, and therefore I hypothesized that this factor is responsible for the tissue specific expression of *ABCC6*.

4. ERK1/2 cascade inhibits the HNF4alpha induction of *ABCC6*.

I wanted to observe if a relation exist between *ABCC6* expression, HNF4alpha and ERK1/2. In that purpose I expressed a mutated construct for the HNF4alpha binding site in HepG2 and I treated the cells with activators or inhibitor of the ERK1/2 cascade, and I observed that the ERK1/2 cascade didn't act anymore on ABCC6 expression with this mutation. Moreover co-expression of HNF4alpha and the mutated construct in HeLa cells showed similar results. Finally a last set of experiment confirmed these results since co-expression of HNF4alpha and MEKK1ca in HeLa cells lead to the inhibition of the *ABCC6* luciferase activity. Altogether these

results emphasized the crucial role of HNF4alpha in the regulation of *ABCC6* expression, but also highlighted the potentially important role of the ERK1/2 cascade in this modulation.

5. Three hypersensitive sites are located in the proximal promoter and the first intron of the human *ABCC6* gene.

To further examine the possible different elements involved in the regulation of *ABCC6*, I used the DNase I hypersensitive assay technique that reveals open chromatin region, known to be located in active promoter or enhancer region. I identified three hypersensitive sites in a 4.5kb region around the *ABCC6* translation initiation site and named from the 5' to 3' HS1, HS2 and HS3 and located in the proximal promoter for HS1 and in the first intron of *ABCC6* for HS2 and HS3. I assumed that HS1 marks the previously characterized proximal promoter of the gene, and I therefore focused on HS2 and HS3.

6. HS2 enhances the minimal promoter of the gene.

I observed that the presence of HS2 in addition to the minimal promoter enhances the activity of the -145/+72 region that marks the minimal basal promoter of the gene. Moreover, I demonstrated that the HS2 enhancer plays a role in the

constitutive expression of the gene since the construct containing the HS2 and the minimal promoter do not exhibit any tissue specific pattern.

7. HS3 enhances the effect of HNF4alpha.

Finally I demonstrated that the HS3 enhances dramatically the expression of the *ABCC6* gene, and that this enhancement is dependent of the -234/-209 region in the proximal promoter of *ABCC6*. I narrowed down the sequence containing the enhancer region to 60bp located in the first intron of *ABCC6* in the +629/+688 region, and I determined that it is possible that more than one factor is binding to this sequence.

8. Conclusions

In this study I have investigated the transcriptional regulation of the *ABCC6* gene, using different techniques. First, treatment with various molecules showed an interesting role of the ERK1/2 pathway in the *ABCC6* gene modulation. To understand the mechanism of this modulation, I showed that HNF4alpha is responsible of the tissue specific expression of the gene. Next I demonstrated that this transcription factor is under the negative modulation of the ERK1/2 cascade.

In Parallel, I used the DNaseI hypersensitive assay that allows the identification of regulatory elements in the chromatin

context. Using this technique I analyzed the mechanisms of the gene expression, and I identified two intronic enhancers that modulates the expression of the gene. I showed that HS3 enhances the expression of the gene in tissues expressing *ABCC6* while HS2 enhances the expression of the basal promoter in tissues that do not express *ABCC6*.

Altogether I identified crucial regions of the *ABCC6* gene that are involved in its regulation. I described a complex physiologic regulatory mechanism of this gene by demonstrating the interconnection between the different transcriptional regulatory elements present on the promoter and the first intron of *ABCC6*.

PUBLICATION LIST

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