

Short communication

**DYSREGULATION OF GENE EXPRESSION IN *ABCC6* KNOCKDOWN
HepG2 CELLS**ROCCHINA MIGLIONICO, MARIA FRANCESCA ARMENTANO,
MONICA CARMOSINO, ANTONELLA MARIA SALVIA, FLAVIA
CUVIELLO, FAUSTINO BISACCIA* and ANGELA OSTUNIDepartment of Sciences, University of Basilicata, Viale dell'Ateneo Lucano 10,
85100 Potenza, Italy

Abstract: ABCC6 protein is an ATP-dependent transporter that is mainly found in the basolateral plasma membrane of hepatocytes. ABCC6 deficiency is the primary cause of several forms of ectopic mineralization syndrome. Mutations in the human *ABCC6* gene cause pseudoxanthoma elasticum (PXE), an autosomal recessive disease characterized by ectopic calcification of the elastic fibers in dermal, ocular and vascular tissues. Mutations in the mouse *ABCC6* gene were also associated with dystrophic cardiac calcification. Reduced levels of ABCC6 protein were found in a β -thalassemic mouse model. Moreover, some cases of generalized arterial calcification in infancy are due to *ABCC6* mutations. In order to study the role of ABCC6 in the pathogenesis of ectopic mineralization, the expressions of genes involved in this process were evaluated in HepG2 cells upon stable knockdown of *ABCC6* by small hairpin RNA (shRNA) technology. *ABCC6* knockdown in HepG2 cells causes a significant upregulation of the genes promoting mineralization, such as *TNAP*, and a parallel downregulation of genes with anti-mineralization activity, such as *NT5E*, *Fetuin A* and *Osteopontin*. Although the absence of ABCC6 has been already associated with ectopic mineralization syndromes, this study is the first to show a direct relationship between reduced ABCC6 levels and the expression of pro-mineralization genes in hepatocytes.

* Author for correspondence. Email: faustino.bisaccia@unibas.it; phone: +39 0971 205462; fax: +39 0971 205678

Abbreviations used: ABCC6 – ATP-binding cassette, sub-family C, member 6; ACDC – arterial calcifications due to deficiency in CD73; CD73 – cluster of differentiation 73; DCC – dystrophic cardiac calcification; Enpp1 – ecto-nucleotide pyrophosphatase/phosphodiesterase type I; GACI – generalized arterial calcification of infancy; MRP – multidrug resistance protein; NT5E – ecto-5'-nucleotidase; OPN – osteopontin; PXE – pseudoxanthoma elasticum; TNAP – tissue-nonspecific alkaline phosphatase

Keywords: ABCC6, Knockdown, HepG2 cells, Gene expression, Mineralization, TNAP, NT5E, OPN, Fetuin A

INTRODUCTION

Biom mineralization is the process by which minerals are deposited within or outside the cells in a variety of organisms. It is subject to complex regulatory networks. Physiological mineralization occurs in hard tissues, whereas pathological calcification occurs in soft tissues [1]. Various proteins and intracellular pathways are involved in these processes [2–4].

Several lines of evidence suggest that the ABCC6 transporter, encoded by the *ABCC6* gene and mainly expressed in the liver and kidneys, is implicated in several ectopic mineralization defects in humans and mice [5]. Mutations in the human *ABCC6* gene cause pseudoxanthoma elasticum (PXE), an autosomal recessive disease characterized by a progressive ectopic calcification of the elastic fibers in dermal, ocular and vascular tissues [6]. A PXE phenotype without mutations in *ABCC6* has frequently been associated with β -thalassemia patients [7]. A β -thalassemia mouse model showed a significant decrease in ABCC6 protein expression, even if it occurs too late in life and is thus insufficient to promote mineralization [8].

Generalized arterial calcification of infancy (GACI) is a hereditary disorder generally associated with mutations in the ecto-nucleotide pyrophosphatase/phosphodiesterase type I (*Enpp1*) gene [9]. It was recently reported that some cases of GACI are due to *ABCC6* mutations and show characteristic PXE clinical manifestations [10, 11]. In addition, deficiency in CD73 (encoded by the *NT5E* gene) causes a human disease called arterial calcifications due to deficiency in CD73 (ACDC), which is characterized by a spontaneous and premature onset of arterial calcification, closely related but phenotypically different from GACI and PXE [12]. Finally, mutations in the mouse *ABCC6* gene have been associated with dystrophic cardiac calcification (DCC), a disease characterized by hydroxyapatite deposition in necrotic myocytes [13, 14].

ABCC6 is expressed in the basolateral membrane of hepatocytes and in the proximal kidney tubules [15, 16]. The inability of this transporter to secrete its unknown substrate(s) in systemic circulation could be the primary cause of the ectopic calcification phenotype of PXE, β -thalassemia, GACI and DCC [17].

In this study, we analyzed the role of ABCC6 in the mineralization process using HepG2 cells, a suitable in vitro model system for the study of polarized human hepatocytes in which ABCC6 protein is endogenously expressed [18]. We observed a set of genes involved in hydroxyapatite deposition that are differentially expressed in *ABCC6* knockdown HepG2 cells, suggesting that the molecules transported by ABCC6 in the liver are involved in the regulation of genes directly engaged in the mineralization processes.

MATERIALS AND METHODS

HepG2 cell culture and isolation of HepG2 clones stably expressing *ABCC6*-shRNA

Human hepatocellular carcinoma cell line (HepG2) was maintained in Dulbecco's modified essential medium (DMEM) containing 4.5 g/l glucose, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C, in an atmosphere of 5% CO₂. Four short-hairpin RNAs (shRNAs) targeting different sequences in the coding regions of the *ABCC6* gene (*ABCC6*-shRNA) and a scrambled shRNA (scr-shRNA) cloned into the pRS vector were purchased from OriGene Technologies. After selection in a medium containing puromycin (2 µg/ml) for 2 weeks, individual resistant clones were expanded in medium without puromycin and evaluated via real-time PCR and western blot analysis. The *ABCC6*-shRNA targeting sequence 5'-AGCTTAGACGCGAGAGGTCCATCAAGTCA-3' from base pairs 2688 to 2716 yielded significantly effective *ABCC6* knockdown compared with HepG2 cells that had been stably transfected with scr-shRNA. The corresponding knockdown clone was used for further studies.

RT-PCR and real-time PCR

Total RNA was extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. The RNA was transcribed to cDNA using oligo (dT) primers and the GeneAmp RNA PCR Core Kit (Applied Biosystems). cDNA was amplified via real-time PCR using PowerSYBR Green PCR Master Mix (Promega) on the 7500 Fast Real-Time PCR System (Applied Biosystems). The *ABCC6* primers used in the real-time PCR experiments and the shRNA sequence used to knock down the *ABCC6* gene do not map to the *ABCC6* pseudogene sequence. All of the real-time PCR experiments were based on the use of primers spanning exon-exon junctions to eliminate any undesirable genomic DNA amplification. They were designed following these guidelines: GC content of 40–60%; product size of 80–150 bp; T_m of 60°C, no secondary structures, no primer dimers (Table 1).

Table 1. Primers used in the real-time RT-PCR assay.

Gene	Accession number	Forward primer	Reverse primer
<i>ABCC6</i>	NM_001171	AAGGAACCACCATCAGGAGGAG	ACCAGCGACACAGAGAAGAGG
<i>TNAP</i>	NM_000478	TACAAGCACTCCCACTTCATCTG	GCTCGAAGAGACCAATAGGTAGT
<i>Enpp1</i>	NM_006208	CCGTGGACAGAAATGACAGTTTC	ATGGACAGGACTAAGAGGAATTCTAAA
<i>NT5E</i>	NM_002526.3	GGGCGGAAGGTTCTCTGTAG	GAGGAGCCATCCAGATAGACA
<i>Fetuin A</i>	NM_001622	GCACGCCGCGAAAGC	TTCTCCAGCTGAAAATTGGA
<i>Osteopontin</i>	NM_001040058	AGGCAGAGCACAGCATCGT	TCGGCGTTTGCTGAGA
<i>β-actin</i>	NM_001101	CCTGGCACCCAGCACAAT	GCCGATCCACCGGAGTACT

To confirm PCR specificity, the PCR products were subjected to a melting-curve analysis. ABCC6 mRNA quantization was determined using the $\Delta\Delta C_t$ method, where $\Delta\Delta C_t = [C_{t_{\text{sample}}} - C_{t_{\beta\text{-actin}}}] - [C_{t_{\text{control}}} - C_{t_{\beta\text{-actin}}}]$. The results were expressed as percentages of the values for the scr-shRNA knockdown sample.

Western blot analysis

Cells (5×10^5) were lysed in RIPA buffer consisting of $1 \times$ PBS (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate supplemented with a protease and a phosphatase inhibitor cocktail. Proteins (100 μg) were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Bioscience). After blocking with 5% non fat dry milk in TBST, consisting of 100 mM NaCl, 50 mM Tris (pH 7.5) and 0.05% Tween-20, the membranes were incubated with 1:200 anti-ABCC6 H70 (Santa Cruz Biotechnology), 1:100 anti-tissue-nonspecific alkaline phosphatase (TNAP; Santa Cruz Biotechnology) or 1:1000 anti-NT5E primary antibodies (Antibody Verify) overnight at 4°C. This was followed by incubation with rabbit secondary horseradish-peroxidase-labeled antibody. Proteins were detected using Enhanced Chemiluminescence reagents (ECL, Promega).

Measurement of TNAP activity

Cells (1×10^6) were lysed with 0.1% Triton X-100 aqueous buffer, sonicated and centrifuged at 13,000 x rpm for 15 min at 4°C to pellet the cellular debris. A volume of 160 μl assay buffer consisting of 50 mM Tris-HCl, 100 mM glycine and 2 mM MgCl_2 (pH 10.3) was added into each well of a 96-well plate, followed by the addition of 20 μl of cell extract and 20 μl of p-nitrophenylphosphate solution (5 mM in assay buffer). The reaction mixture was incubated at 37°C for 30 min and stopped by adding 50 μl of 3 M NaOH. The $\text{OD}_{405\text{nm}}$ was measured using a Multiscan Go (Thermo) plate reader. Alkaline phosphatase activity was expressed as micromoles of p-nitrophenol produced per minute and was normalized to the protein content of each sample.

Statistical analysis

Data were expressed as the means \pm SD. The difference between any two groups was determined by ANOVA, using software R version 2.8.1 [19]. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

To investigate the possible mechanism by which ABCC6 could promote the ectopic mineralization process, several studies on *ABCC6*^{-/-} transgenic mice [20, 21] and on fibroblasts from PXE patients were previously performed [22]. However, these studies did not provide any insight into the pathways linking the ectopic mineralization processes to the absence of ABCC6 in the liver.

In this study, the *ABCC6* gene was stably knocked down in HepG2 cells and the effect on the RNA transcription level of a set of genes involved in tissue

calcification was evaluated. The knockdown occurred at both the transcriptional and translational levels, as confirmed via real-time PCR (Fig. 1A) and by western blot analysis (Fig. 1B), respectively.

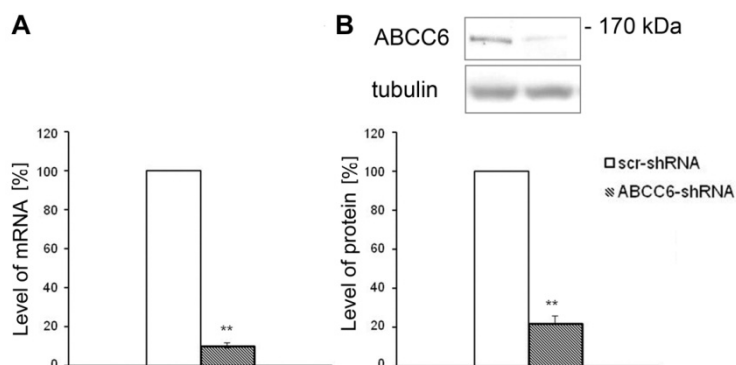


Fig. 1. The effect of shRNA-mediated knockdown on *ABCC6* gene expression. A – Fold change in *ABCC6* mRNA level compared to scr-shRNA transfected cells. Results are represented as the mean \pm S.D. (n = 3). **p < 0.01. B – Representative western blot and densitometric analyses of the *ABCC6* immunoreactive band in three independent experiments (means \pm SD). **p < 0.01. α -tubulin was used as a loading control.

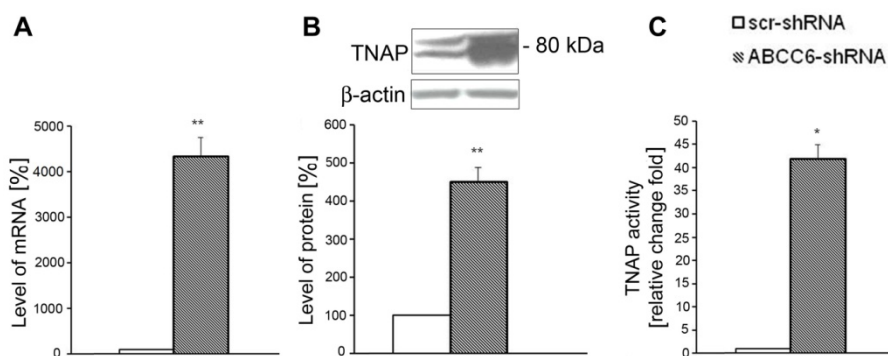


Fig. 2. Expression and enzymatic activity of TNAP in *ABCC6* knockdown cells. A – Fold change in the TNAP mRNA level compared to that for scr-shRNA transfected cells. The results are the means \pm SD (n = 3). **p < 0.01. B – Representative western blot and densitometric analyses of the TNAP immunoreactive band in three independent experiments (means \pm SD). **p < 0.01. α -tubulin was used as a loading control. C – TNAP activity in *ABCC6* knockdown cells expressed as the fold change relative to the control. The results are means \pm SD (n = 3). *p < 0.05.

ABCC6 knockdown affected the expression of TNAP, a membrane-bound enzyme facing the extracellular space that promotes the mineralization process, altering the extracellular inorganic phosphate/pyrophosphate ratio toward the

production of inorganic phosphate [23]. *TNAP* transcript levels in *ABCC6* knockdown cells were significantly higher than in scr-shRNA-transfected cells (Fig. 2A). The protein level increased accordingly (Fig. 2B). Finally, TNAP activity also increased in *ABCC6* knockdown cells by about 40-fold (Fig. 2C), indicating that the overexpressed protein is functionally active.

Interestingly, the expression of the ecto-5'-nucleotidase (NT5E), also known as CD73, decreased in *ABCC6* knockdown cells compared to the control at both the transcriptional and translational levels (Fig. 3). NT5E catalyzes the conversion of AMP to adenosine and inorganic phosphate [24] and is involved in ACDC [25]. This enzyme resides on the outer side of the plasma membrane of several cell types, supplying adenosine to receptors on the cell surface. Adenosine receptors trigger an intracellular signaling cascade that results in inhibition of TNAP activity. Thus, NT5E deficiency lowers pericellular adenosine concentrations, increasing alkaline phosphatase activity in the extracellular space and allowing calcification to occur [25, 26]. The synergic and opposite regulation of TNAP and NT5E, described in ACDC, is consistent with our findings showing that TNAP overexpression is associated with NT5E downregulation in *ABCC6* knockdown HepG2 cells. Indeed, we provided the first evidence that reduced levels of *ABCC6* activity in liver cells can upregulate TNAP and downregulate NT5E expression in the same cells, providing the possible molecular mechanism underlying the overlap between PXE and ACDC clinical phenotypes.

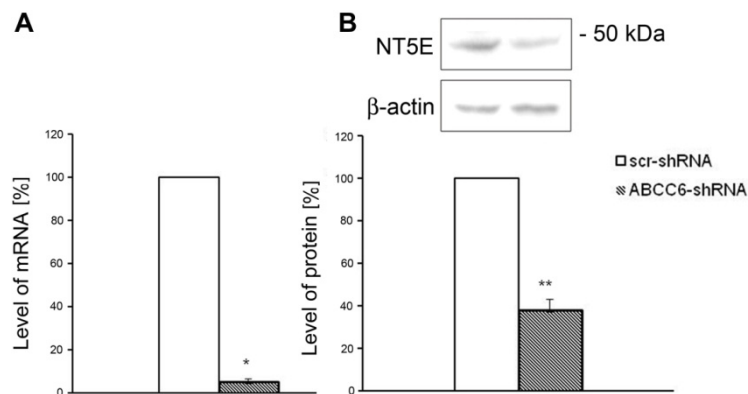


Fig. 3. Expression of NT5E in *ABCC6* knockdown cells. A – Fold change in *NT5E* gene expression compared to that for scr-shRNA transfected cells. The results are the means \pm SD ($n = 3$). * $p < 0.05$. B – Representative western blot and densitometric analyses of NT5E immunoreactive band in three independent experiments (means \pm SD). ** $p < 0.01$. β -actin was used as loading control.

Fig. 4 shows the mRNA quantification of other genes involved in the mineralization processes. The *Enpp1* gene encodes the enzyme hydrolyzing nucleoside 5' triphosphates to their corresponding monophosphates and

pyrophosphate, which in turn inhibit the mineralization processes. Interestingly, the *Enpp1* mRNA level did not significantly change in knockdown vs. control cells (data not shown). By contrast, the expression of *OPN* and *Fetuin A* genes, which encode two hepatic circulating proteins that are able to prevent deposition of hydroxyapatite, was downregulated in *ABCC6* knockdown cells. This finding concurs with the decreased levels of Fetuin A observed in *ABCC6*^{-/-} mouse serum [3] and PXE patients [27]. Moreover, it is evidence of a direct correlation between reduced levels of *ABCC6* and *OPN* gene downregulation in liver cells. A recent study has emphasized the correlation in expression between *ABCC6* and *OPN* in a different system [28].

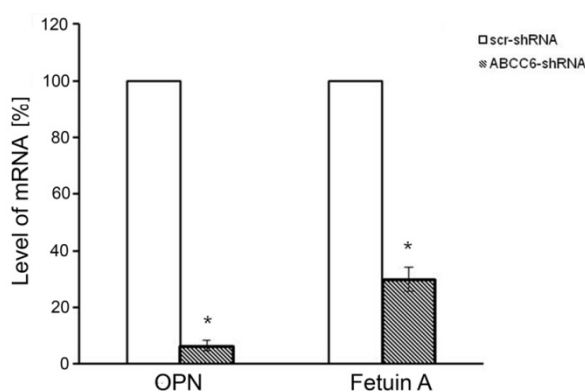


Fig. 4. *OPN* and *Fetuin A* transcripts levels in *ABCC6* knockdown cells. Fold change in gene expression in *ABCC6* knockdown cells compared to that for scr-shRNA transfected cells. The results are the means \pm SD (n = 3). *p < 0.05.

ABCC6 is localized at the basolateral surface of hepatocytes and, in analogy with other better-characterized members of the multidrug resistance protein (MRP) family, it is likely to serve as an efflux pump transporting substrates from hepatocytes to the circulation. It is conceivable that in the absence of functional *ABCC6*, serum becomes deficient in certain factors that inhibit ectopic mineralization [16].

The novelty of our data is that *ABCC6* substrates also act on the liver cells, themselves modulating the expression of genes involved in the mineralization pathways, suggesting the molecular basis of the observations made in *ABCC6*^{-/-} mice and PXE patients. It is indeed possible that, once secreted in the extracellular space, the *ABCC6* substrates may act as autocrine factors promoting the expression of anti-mineralization genes and downregulating the expression of pro-mineralization genes. In agreement with this hypothesis, it was recently reported that cells overexpressing *ABCC6* secrete an unidentified substrate that in turn induces a large efflux of nucleotide triphosphates providing the extracellular pyrophosphate, a potent inhibitor of mineralization [29]. Alternatively, it can be hypothesized that *ABCC6* deficiency leads to an

intracellular increase of substrates that might modulate the expression of genes involved in mineralization processes.

Importantly, the pro-mineralization genes dysregulated in ABCC6-lacking hepatocytes encode both the proteins expressed on the extracellular face of the hepatocyte plasma membrane (TNAP, NT5E) and those released into the blood stream (OPN, Fetuin A). The two types of protein can exert their actions in the extracellular space and in extra-liver tissues respectively in the absence of clinically evident modifications in hepatocyte metabolism or function.

CONCLUSION

Our findings represent a significant step forward in the understanding of the pathogenic mechanism of ectopic mineralization processes associated with ABCC6. We have clarified that reduced levels of ABCC6 activity have a direct effect on the cells that normally express it. Moreover, our experiments demonstrate that *ABCC6* knockdown HepG2 cells are a suitable model for recapitulating in vitro the ABCC6-lacking liver of PXE patients, thus shedding light on the complex pathogenesis of PXE.

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