

# *ALA-Porphyrin Science*

## **Screening of Transporters Involved in Porphyrin Accumulation after Administration of 5-Aminolevulinic Acid *in vitro***

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### **Summary**

In cancer patients, protoporphyrin IX (PpIX) and coproporphyrin III (CPIII) concentrations in blood and urine are reportedly elevated after administration of 5-aminolevulinic acid (ALA). Since porphyrins are fluorescent compounds, it is easy to detect them. Hitherto we have been examining whether porphyrins in blood and urine could be used as a potential tumor marker. We have recently found that PpIX is transported by human ABC transporter ABCG2, whereas the mechanism underlying tumor specific CPIII export is not understood. Therefore, in this study, we investigated CPIII transport from cancer cells *in vitro*. Biosynthesis of porphyrins in cancer cells were exerted by incubation of cells with ALA, and porphyrin levels were quantified by HPLC. Intracellular and extracellular PpIX levels were greatly changed by adding ABCG2 specific inhibitor fumitremogin C into the incubation medium, while CPIII level was not affected at all. These results suggest that ABCG2 is not responsible for the transport of CPIII from cancer cells. Thus, the mRNA levels of other ABC transporters were measured by RT-PCR. Several ABC transporters have been nominated as candidate for CPIII export transporters.

### **Keyword:**

5-aminolevulinic acid, porphyrin, ABC transporter

### **Introduction**

5-aminolevulinic acid (ALA) is one of the amino acid synthesized in our body and precursor of porphyrin and heme. It is well known that porphyrin is selectively accumulated in tumor cells after administration of ALA. Accumulated porphyrin is exported to extracellular. Therefore, porphyrin is detected in blood and urine. Since porphyrin is fluorescent compound, it is easy to detect them. Hitherto we have been examining whether porphyrins in blood and urine could be used as a potential tumor marker. In blood and urine, protoporphyrin IX (PpIX) and coproporphyrin III (CPIII) are detected (1-4). We have recently found that PpIX is transported by human ABC transporter ABCG2 (5,6), whereas the mechanism underlying tumor specific CPIII export is not understood (Fig. 1). In this study, CPIII export transporter is investigated.

## Experimentals

### Materials

5-Aminolevulinic acid hydrochloride was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Fumitremorgin C (FTC) was purchased from Enzo Life Sciences, Inc. All other reagents used were of the highest grade available.

### Cells and cell culture

The human gastric cancer cell lines KKLS and NKPS were provided by Dr. Mai of the Cancer Research Institute of Kanazawa University (Kanazawa, Japan), and TMK-1 cells were provided by Dr. Tahara (Hiroshima University, Hiroshima, Japan). MKN45 cells and MKN28 cells were provided by Dr. Suzuki (Fukushima Medical College, Fukushima, Japan). Cells were maintained under 5% CO<sub>2</sub> gas at 37°C in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin.

### Western blot analysis

Cell lysate was separated by 7.5% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). After incubating in 5% nonfat dry milk in TBST (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h, the membranes were incubated for 1 h with human primary antibody followed by incubation in a secondary antibody coupled to horseradish peroxidase. Target proteins were detected using an ECL kit (GE Healthcare, UK), and the image was captured using an LAS-4000 imager (GE Healthcare).

### Measurement of intracellular and extracellular porphyrin using HPLC

Cells were seeded in 6-well plates and allowed to attach overnight in culture medium. Then, they were incubated for 24 h in culture medium containing 1 mM ALA and/or 10 µM FTC. Cells were washed and lysed by 0.1 M NaOH. Then, lysates were extracted by adding 3-fold volume of DMF/2-propanol (100:1, v/v). The obtained mixture was centrifuged to remove protein, and supernatant was incubated at r.t. in dark palace for 1 day. 100 µL of the sample, thus prepared, was subjected to HPLC analysis. A chromatogram was obtained (excitation wavelength, 404 nm; detection wavelength, 624 nm). Porphyrin levels were normalized by intracellular protein level. Intracellular protein level was measured by Bradford protein assay.

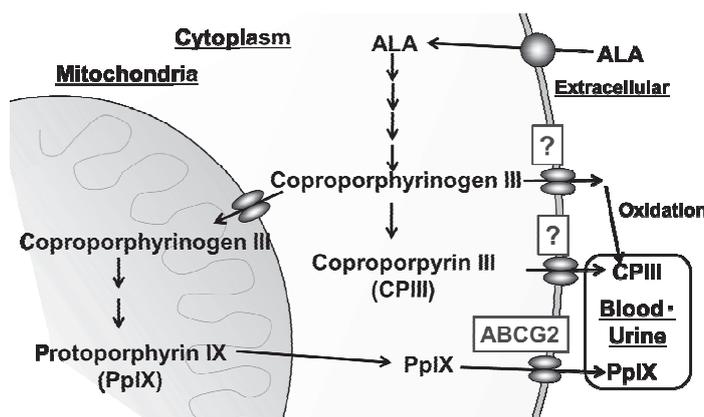


Fig. 1 Schematic illustration of porphyrin biosynthesis and export. *In vivo*, CPIII and PpIX are detected in blood and urine. PpIX is exported by ABC transporter ABCG2 (BCRP), whereas CPIII or coproporphyrinogen III export transporter is not clear.

## RT-PCR

Total RNA was isolated using High Pure Isolation kit (Roche). Concentration and quality of the RNA were analyzed on the UV-Vis (SHIMADZU). Total RNA was used for cDNA synthesis with the PrimeScript<sup>®</sup> RT reagent Kit (Perfect Real Time) (TaKaRa) according to the supplier's protocol. PCR reagents were obtained from TOYOBO (Quick Taq<sup>™</sup> HS DyeMix). Cycle parameters were 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, and 60°C for 30 sec. Amplified cDNA were separated by agarose gel electrophoresis. After ethidium bromide staining, bands were detected by ultraviolet light.

## qRT-PCR

cDNA was synthesized as described earlier. Quantitative real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa) using Thermal Cycler Dice<sup>®</sup> Real Time System Single (TaKaRa). The relative amount of the mRNA was normalized to the level of the internal control GAPDH mRNA.

## Results and discussion

### Porphyrin export

Since CP111 is very similar structure with PpIX, CP111 export by PpIX export transporter ABCG2 was investigated. Two gastric cancer cell lines which differed in ABCG2 expression level were used (Fig. 2). Intracellular and extracellular porphyrin levels were measured by HPLC after incubation of cells in medium containing ALA and/or ABCG2 specific inhibitor FTC for 24 hour (Fig. 3). It is expected that FTC is effective



Fig. 2 Western blot analysis of ABCG2.

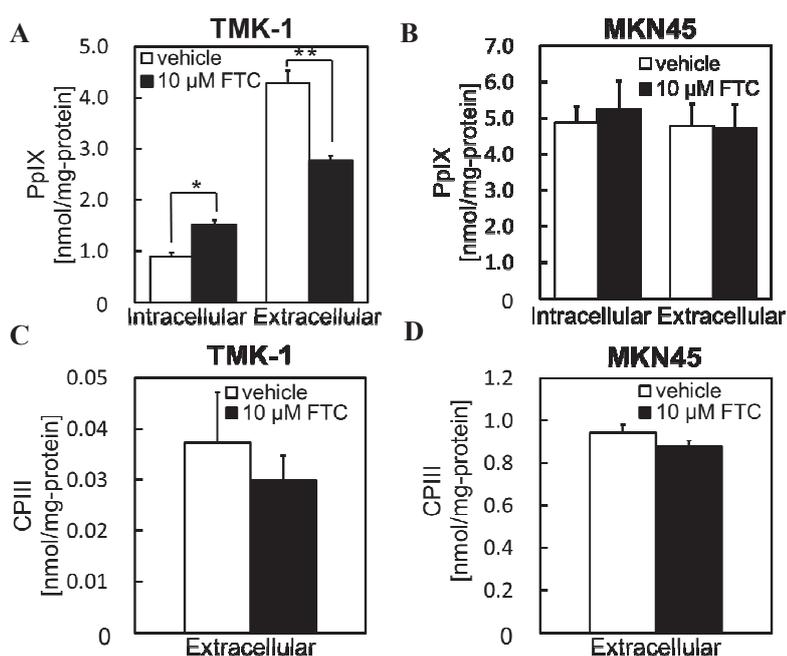


Fig. 3 Intracellular and extracellular porphyrin levels after incubation with 1 mM ALA for 24 h. Porphyrin levels were normalized by cell protein levels. White bar shows control and black bar shows adding FTC into medium. A, Intracellular and extracellular PpIX levels of TMK-1. B, Intracellular and extracellular PpIX levels of MKN45. C, Extracellular CP111 level of TMK-1. D, Extracellular CP111 level of MKN45. Bars represent SD of the means of three replicate experiments. \*;  $p < 0.001$ , \*\*;  $p < 0.005$  by Student's t-test.

for ABCG2 high expression cell line TMK-1, but is not effective for ABCG2 low expression cell line MKN45. Porphyrin levels were normalized by protein level of intracellular lysate. In TMK-1 (ABCG2 high expression cell line), intracellular PpIX level was increased and extracellular PpIX level was decreased by adding FTC into the incubation medium (Fig. 3A). On the other hand, intracellular and extracellular PpIX levels were not changed at all in MKN45 (ABCG2 low expression cell line) by FTC (Fig. 3B). If CPIII is exported by ABCG2, the change of extracellular CPIII level should be similar to that of extracellular PpIX level by adding FTC. However, extracellular CPIII levels of both cell lines were not changed at all (Fig. 3C, D). These results suggest that ABCG2 is not affect for the transport of CPIII in cancer cells and also suggest existence of other transporter involved in CPIII export.

### Screening of CPIII export transporter

Extracellular CPIII level of MKN45 was higher than that of TMK-1 (Fig. 3C, D). These results suggest that the expression level of CPIII export transporter of MKN45 was higher than that of TMK-1, therefore the mRNA levels of all 48 human ABC transporters were measured by RT-PCR. Table. 1 shows the status of mRNA expression levels of ABC transporters. 10 genes were not detected, and 28 genes were almost same expression levels. 3 transporters containing ABCG2 showed higher expression levels in TMK-1, and 7 transporters showed higher expression levels in MKN45. Therefore, 7 ABC transporters (ABCA3, ABCA12, ABCB4, ABCB11, ABCC8, ABCC12, ABCG4) have been nominated as candidate for CPIII export transporters.

Table. 1 The status of mRNA expression levels of ABC transporters. MKN45 high expression gene were candidates of CPIII export transporter.

Not detected	ABCA4, ABCA6, ABCA8, ABCA9, ABCB5, ABCC7, ABCC9, ABCD2, ABCG5, ABCG8
Not changed	ABCA1, ABCA2, ABCA5, ABCA7, ABCA10, ABCB2, ABCB3, ABCB6, ABCB7, ABCB8, ABCB9, ABCB10, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10, ABCC11, ABCD1, ABCD3, ABCD4, ABCE1, ABCF1, ABCF2, ABCF3, ABCG1
TMK-1 high expression	ABCA13, ABCB1, ABCG2
MKN45 high expression	ABCA3, ABCA12, ABCB4, ABCB11, ABCC8, ABCC12, ABCG4

In order to determine the candidate for CPIII export transporters from these 7 transporters, extracellular CPIII level and mRNA expression level were compared among 5 gastric cancer cell lines (TMK-1, NKPS, KKLS, MKN28, MKN45). Extracellular CPIII levels of 5 cell lines were measured by HPLC. Fig. 4A shows extracellular CPIII level of cell lines. CPIII export level of MKN45 was higher than that of other cell lines. Therefore, it is considered that mRNA level of CPIII export transporter in MKN45 was higher than that of other cell lines. mRNA levels of candidate for CPIII export transporters in cell lines were quantified by quantitative RT-PCR. mRNA levels were normalized by GAPDH. ABCC12 and ABCG4 were high expression in MKN45 (Fig. 4B, C). Other transporters were very low expression in TMK-1 and expressions of other cell lines

were very high levels (data not shown). Therefore ABCC12 and ABCG4 have been nominated as first candidate for CPIII export transporters.

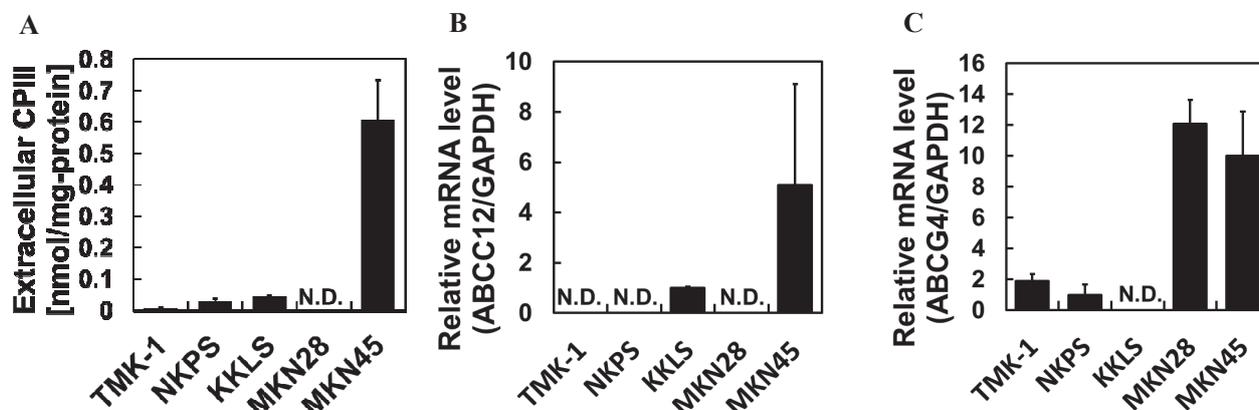


Fig. 4 Extracellular CPIII level and mRNA levels of 5 gastric cancer cell lines. A, Extracellular CPIII level measured by HPLC 24 h after administration of ALA. CPIII level were normalized by protein level of each cell lysates. B, relative mRNA level of ABCC12. C, relative mRNA level of ABCG4. mRNA levels were normalized by GAPDH.

## Conclusion

We demonstrated CPIII was not exported by PpIX export transporter ABCG2, suggesting that there are other transporters which export CPIII specifically. By measurement of extracellular CPIII and profiling of mRNA levels of 5 gastric cancer cell lines, ABCC12 and ABCG4 have been nominated as first candidate for CPIII export transporters.

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