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Copper-transporting P-Type Adenosine Triphosphatase (ATP7B) Is Expressed in Human Breast Carcinoma

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This is the first report to show that a copper-transporting P-type adenosine triphosphatase, ATP7B, is expressed in certain breast carcinomas, and *a priori* knowledge of its expression is important for the choice of therapy. We investigated the hypothesis that ATP7B, which was shown to be associated with cisplatin resistance *in vitro*, is expressed in certain breast carcinomas. To test this hypothesis, *ATP7B* expression and protein level were examined in 41 breast carcinomas using RT-PCR and immunohistochemistry. *ATP7B* gene/protein could be detected in 22.0% (9/41) of breast carcinomas and *ATP7B* gene expression was correlated well with the protein expression. In nine ATP7B-positive tumors, adjacent normal breast tissue was similarly analyzed, revealing that ATP7B is upregulated in breast carcinoma. *ATP7B* gene expression in poorly differentiated carcinoma was significantly higher than that in well-/moderately differentiated carcinoma (P=0.012). Furthermore, we found no association between the *ATP7B* gene/protein expression and that of *MDR1, MRP1, LRP* and *BCRP*. These findings suggested that *ATP7B* gene expression might be a chemoresistance marker for cisplatin in patients with poorly differentiated breast carcinoma.

Key words: MDR — ATP7B — Cisplatin — Breast carcinoma

Recently, it has been shown that a copper-transporting P-type adenosine triphosphatase (ATP7B) is associated with cisplatin resistance *in vitro*.[1)] The *ATP7B* gene was induced by exposure to cisplatin in human prostate cells and the *ATP7B*-transfected cells showed a dramatic decrease in cisplatin accumulation.[1)] Although an active efflux pump for cisplatin has yet to be identified, it is likely that ATP7B may function to efflux cisplatin from some carcinoma cells. Therefore, in addition to transporting copper, ATP7B can also transport cisplatin.

Cisplatin is one of the most effective chemotherapeutic agents for treating human cancer. Like many other agents in cancer chemotherapy, the problem of resistance remains a major obstacle to effective treatment. It is now well documented that multidrug resistance (MDR) plays a major role in drug resistance in tumor cells. Several genes involved in MDR, including *MDR1*, *MRP* (multidrug resistance protein) *1* and *LRP* (lung resistance associated protein), have been identified and analyzed.[2–4)] *MDR1* and *MRP1* function as a drug efflux pump and belong to the ABC transporter gene family,[3, 5)] expressed in both human solid tumors and hematological malignancies.[6, 7)] The 110-kd LRP, the major vault protein, is frequently overexpressed in multidrug-resistant cells, and has an important role in the transport of drugs from nuclei to cytoplasm, conferring MDR *in vitro*.[8)] Scheper and co-workers demonstrated that LRP expression in advanced ovarian carcinoma appears to be an indicator of poor response to cisplatin-containing chemotherapy.[9)] Expression of *BCRP* (breast cancer resistance protein) (*MXR*/*ABCP*) gene, a member of the ABC transporter family, has been described in breast, colon, gastric and fibrosarcoma cell lines.[10–13)] Overexpresssion of BCRP was induced by exposure of the cells to mitoxantrone or doxorubicin/verapamil and resulted in a different resistance profile from *MDR1* or *MRP* gene overexpression.[10–13)]

Copper is an essential trace element and is required for many enzymatic processes in both prokaryotes and eukaryotes.[14)] Excess copper is transported to the extracellular environment by an energy-dependent system.[15)] Wilson's disease (WND) is an autosomal recessive disease of copper transport, characterized by chronic liver and/or neurological disease, sometimes accompanied by kidney

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damage.[16] The *ATP7B* gene has been cloned and mapped to chromosome 13q14.3.[17] ATP7B is expressed in human liver, kidney, and placenta.[17] ATP7B protein includes the ATP-binding domain to the transmembrane segment[18] and is a member of a class of heavy metal-transporters that pump copper, cadmium, zinc, silver or lead.[19, 20] The fact that such transporters can also transport a small-molecular drug is intriguing and could potentially have a significant value in the clinic.

The aim of this study was to investigate the expression of *ATP7B* mRNA and protein in human breast carcinoma. We found that 22.0% (9/41 cases) of the breast carcinomas analyzed, expressed *ATP7B*. Furthermore, *ATP7B* mRNA expression is correlated with ATP7B protein expression. The role of ATP7B in other types of human tumor should be further explored.

MATERIALS AND METHODS

Patients and samples Surgical specimens from 41 patients with untreated breast carcinoma were provided for this study. The patients underwent surgery in Tokyo Metropolitan Hospital between 1983 and 1999. All the samples were immediately frozen at −80°C, embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo) and stored until use. The data on clinicopathologic variables, including age, TNM category and estrogen/progesterone receptor status are shown in Table I. The relapse and prognosis of the patients were followed up on June 30, 2000. All the patients were treated with doxorubicin-based chemotherapy. Informed consent was obtained from each patient, and Tokyo Metropolitan Komagome Hospital Committee approved this project prior to the study. After examining the histopathological features of sections stained with hematoxylin and eosin (H & E), the sections including more than 80% carcinoma cells were used for total RNA preparation.

RT-PCR Total RNA of human breast carcinoma was prepared by using Trizol (Gibco, Life Tech, Gaithersburg, MD). cDNA was synthesized with 3 μg of total RNA and random hexadeoxynucleotide primer (Gibco, Life Tech) in 20 μl of a solution containing reverse transcriptase. After synthesis, the cDNA was diluted 1:4 with water and stored at −20°C until use. PCR was performed with cDNA derived from 30 ng of RNA. PCR reactions were carried out in a total volume of 25 μl containing cDNA, dGTP,

Table I. Relationship of *ATP7B* Gene Expression and Clinicopathological Variables in Patients with Breast Carcinoma

Variables	All patients	*ATP7B*		Significance[a]
		Negative	Positive	
Total	41	32	9	
Age (yr.)				
Median		52	51	NS[b]
Range		(36–80)	(31–80)	
Histopathologic subtype				0.012
Differentiated	24	22 (91.7%)	2 (8.3%)	
Schirrous	17	10 (58.8%)	7 (41.2%)	
pT category				NS
T1+2	20	16 (80.0%)	4 (20.0%)	
T3+4	21	16 (66.2%)	5 (23.8%)	
pN category				NS
N0	12	10 (83.3%)	2 (16.7%)	
N1–4	29	22 (74.1%)	7 (25.9%)	
pM category				NS
M0	27	22 (71.5%)	5 (18.5%)	
M1	14	10 (71.4%)	4 (28.6%)	
Estrogen receptor				NS
Positive	25	18 (72.0%)	7 (28.0%)	
Negative	16	14 (77.5%)	2 (12.5%)	
Progestrone receptor				NS
Positive	26	22 (74.6%)	4 (15.4%)	
Negative	15	10 (66.7%)	5 (33.3%)	

a) Mann-Whitney *U* test or χ^2 test was used.
b) NS, not significant.

dATP, dTTP and [α-^{32}P]dCTP at a concentration of 200 *μM*, 4 *μM* of each primer and 0.25 unit of ExTaq polymerase (Takara Shuzo, Otsu, Shiga). The PCR cycling parameters were as follows: 10 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, and a final cycle at 72°C for 10 min. The PCR primer sequences of *ATP7B* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which was used as an internal control, were as follows: As for *ATP7B*, the primer sequences were: sense 5′-TCCTGGTGGCTATTGACG-GTGT-3′ and antisense 5′-CATTCAGGCGCAGAGAC-CACTT-3′ corresponding to 833 bp (fragment 3539 to 4349). With respect to *GAPDH*, the primer sequences were as follows: sense 5′-CCCCTGGCCAAGGTCATC-CATGACAACTTT-3′, and antisense 5′-GGCCATGAG-GTCCACCACCCTGTTGCTGTA-3′ corresponding to 513 bp (sequence 515 to 1027). Sequence analysis of a fraction of each PCR product was performed by Gene Scan version 2.1, in order to confirm the validity of the method. For correlation analysis with other known drug-resistance-associated transporters such as *MDR1*, *MRP1*, *LRP* and *BCRP*, we used the previously described data on the same sets of samples (see Ref. 21).

PCR and quantitative analysis of PCR products In order to evaluate the amplified PCR products semi-quantitatively, the optimal conditions for the detection of *ATP7B* and *GADPH* genes were determined using cDNA of placenta and human samples. Using 40 cycles of PCR, the relative yields of PCR products was similar, indicating that this number of cycles corresponded to the plateau. At 25 cycles or less, gene expression could not be clearly distinguished (data not shown). Thus, we used 35 PCR cycles as the optimal number for the detection of each target gene. The amplified cDNA fragments were electrophoresed on 6% polyacrylamide gels. Quantitative analysis of the amplified PCR products was performed using a BAS 2000 imaging plate (Fuji, Kanagawa).

Immunohistochemical analysis of *ATPB7* expression One 2.5-μm section of each submitted frozen block was first stained with H & E to verify the histopathologic diagnosis and quality of fixation for immunohistochemical analysis. Immunostaining was performed on cryostat sections using the standard immunoperoxidase procedure (Vectastain Elite ABC kit, Vector, Burlingame, CA). After recovery from O.C.T. compound, the sections were fixed in 10% neutral buffered formalin. Sections were then incubated in 0.03% H_2O_2 in absolute methanol for 30 min at room temperature, and blocked with 3% skimmed milk in phosphate-buffered saline (PBS) for 30 min at room temperature. The sections were incubated with 100-fold-diluted monoclonal antibody against the NH_2-terminal region of ATP7B, which includes the six copper-binding domains (amino acids 21 to 623; see Ref. 22) for 15 h at 4°C. After rinsing with PBS, the sections were incubated with biotinylated horse anti-mouse IgG at 1:200 with 1.5% normal horse serum for 30 min at room temperature. After rinsing with PBS, they were incubated for 30 min at room temperature with avidin-biotin-horseradish peroxidase macromolecular complex. After rinsing with PBS, the sections were incubated for 6 min in 0.05% diaminobenzidine in PBS with 0.03% H_2O_2. The slides were counterstained with hematoxylin, dehydrated and mounted. In this study, serial sections were routinely incubated with irrelevant mouse IgG as a negative control.

On representative samples, phenotyping of ATP7B-staining cells was performed by a double staining procedure, whereby the sections were stained sequentially with immunoalkaline phosphatase for CD68. Sections were examined for the presence of cells that were stained red alone (alkaline phosphatase staining of CD68), brown alone (peroxidase staining of ATP7B), or both red and brown (double staining). Serial sections were stained separately with either immunoperoxidase or immunoalkaline phosphatase as controls.

The slides were examined and scored independently by two observers (A. K. & Y. T.) without knowledge of clinical information of the patients. If more than 10% of the tumor cells were stained, the samples were considered to be ATP7B-positive carcinomas. The distribution of the percentage of carcinoma cells expressing ATP7B is shown in Fig. 1C. The 10% cutoff level was specified for the following reasons: 1) 10% positive cells was considered the lowest level of expression that could be consistently detected in cryostat sections, 2) Chan *et al.* demonstrated that a small percentage of cells positive for MDR-related proteins (i.e. P-glycoprotein) could have clinical significance.[23] The κ test of reliability between the two observers was 0.830, indicating fair to good agreement for the evaluation of *ATP7B* expression in breast carcinoma (data not shown). When the two observers differed from each other, they jointly re-evaluated the images of stained sections on a TV-capture station.

Statistical analysis Association of continuous variables was evaluated using the Mann-Whitney *U* test. The relationship between *ATP7B* gene expression and potential explanatory variables, including age, pT category, pN category, pM category and hormonal receptor status, was determined by χ^2 test. The correlation coefficient between different parameters was determined by simple regression analysis. The statistical analyses were performed using Statview J 5.0 software (Abacus, CA). Two-sided *P* values were calculated and a difference was considered significant if *P* was less than 0.05.

RESULTS

Expression of *ATP7B* gene in human breast carcinoma Forty-one primary breast carcinoma tissues were

used for the detection of *ATP7B*, *MDR1*, *MRP1*, *LRP* and *BCRP* genes. Expression of *MDR1*, *MRP1*, *LRP* and *BCRP* in these tissue samples was previously described (see Ref. 21). The PCR products of *ATP7B* and *GADPH* are shown in Fig. 1A. The expression level of the *ATP7B* gene is given as the ratio of *ATP7B* gene to *GADPH* gene. The expression levels of the *ATP7B* gene arranged in order of magnitude are shown in Fig. 1B. *ATP7B* gene expression was detected in 22% (9/41 cases) of human breast carcinomas. In addition, we confirmed the presence of the *ATP7B* mRNA by sequence analysis of PCR products in human breast carcinoma (data not shown). Moreover, we have examined *ATP7B* gene expression using 50 cycles of PCR and determined that the detectable cases were the same (data not shown). The expression level of the *ATP7B* gene varied from tumor to tumor. Among the cases of breast carcinoma expressing the *ATP7B* gene, the expression level was from 0.48 (mRNA of *ATP7B* gene/*GADPH* gene) to 11.5 (Fg. 1B).

ATP7B protein detection and its correlation with *ATP7B* gene expression To examine what kind of cells express *ATP7B* at the protein level in human breast carcinoma, we performed immunohistochemical analysis using a monoclonal antibody against ATP7B. This antibody specifically reacts with *ATP7B* in immunoblotting analysis (see Ref. 23). As shown in Fig. 2A, *ATP7B* expression was observed in the cytoplasm of breast carcinoma cells. A negative control did not reveal ATP7B protein (Fig. 2B). Furthermore, ATP7B immunoreactivity was detected as a characteristic granular cytoplamic staining in carcinoma cells (Fig. 2C). In contrast, ATP7B protein was not detectable in normal duct (Fig. 2D). In some cases, stromal cells appeared to express *ATP7B*, and these cells seemed to be macrophages. Therefore, we performed immunohistochemistry for ATP7B, CD-68, a marker for macrophages, and double staining in three serial sections. As shown in Fig. 2, E–G, many of the stromal cells stained for both ATP7B and CD68. However, some cells stained positive only for either ATP7B or CD68. This suggests that *ATP7B* is expressed in macrophages.

Next, we compared mRNA expression levels in carcinoma tissues and protein expression levels in carcinoma cells. As shown in Fig. 1D, there was a significant correlation (R=0.812, P<0.001). These data suggest that *ATP7B* gene expression level as well as protein level may be useful in clinical studies.

***ATP7B* gene expression in breast carcinoma is independent of *MDR1, MRP1, LRP* or *BCRP* expression** To observe the association of *ATP7B* gene expression with other transporters such as *MDR1*, *MRP1*, *LRP* and *BCRP*

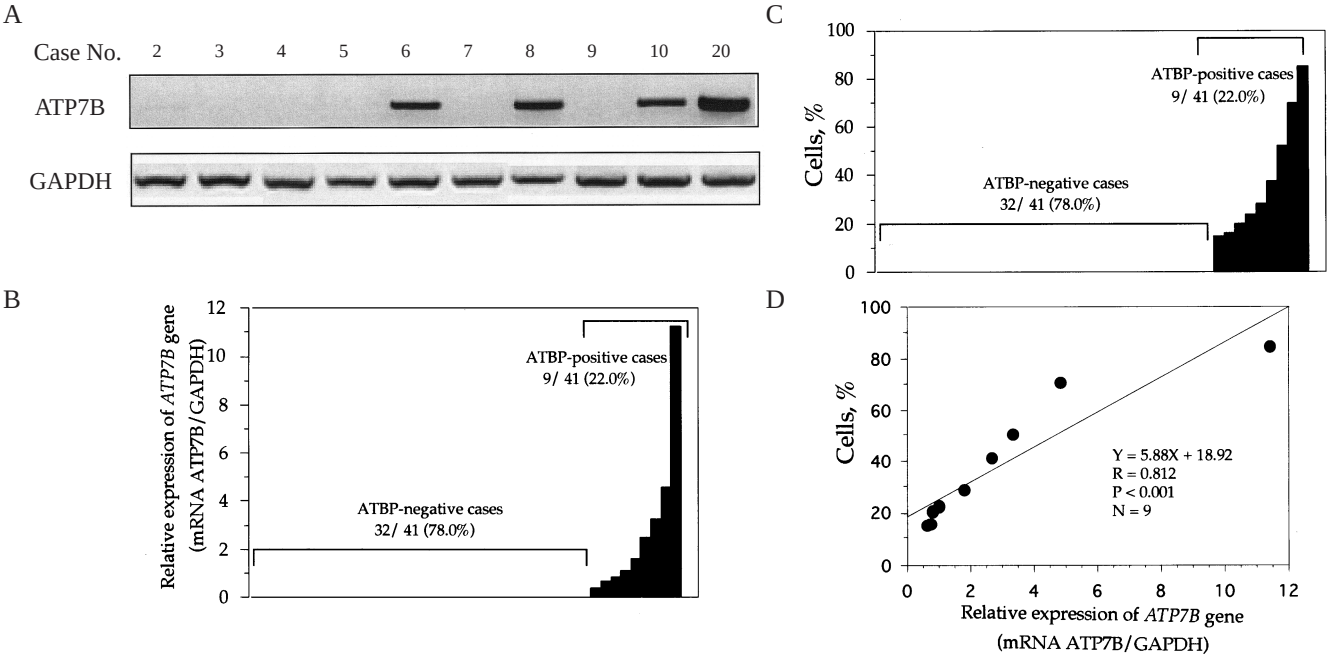

Fig. 1. *ATP7B* gene expression in human breast carcinoma. A. Expression of *ATP7B* and *GPDAH* gene in 41 patients with breast carcinoma, determined by RT-PCR, as described in "Materials and Methods." Data were expressed relative to the expression of the *GAPDH* gene in each breast carcinoma. B. Expression levels of *ATP7B* gene are arranged in the order of magnitude. C. Expression levels of ATP7B protein by immunohistochemistry are arranged in the order of magnitude. D. Correlation of expression levels of ATP7B protein and *ATP7B* gene.

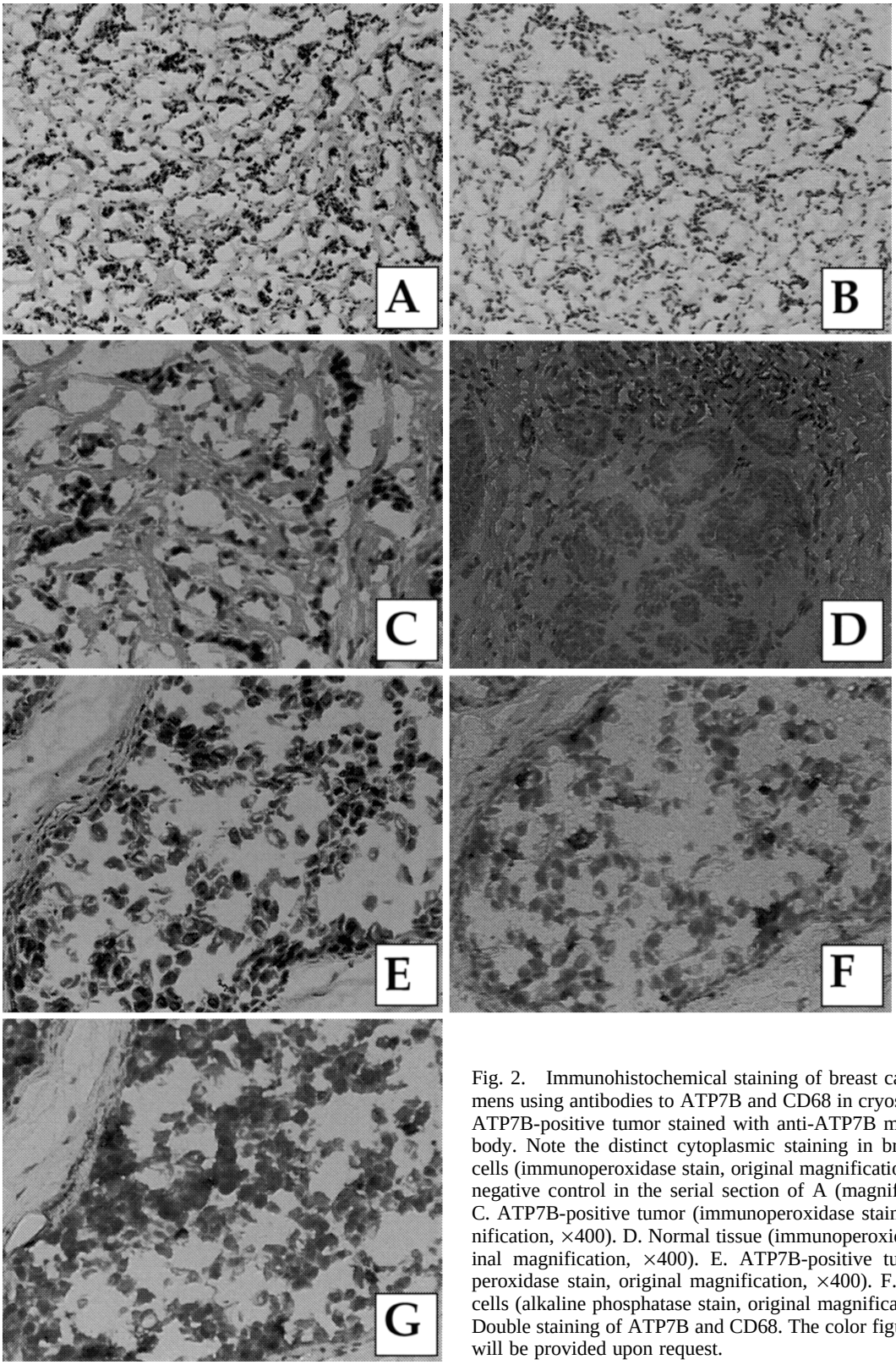

Fig. 2. Immunohistochemical staining of breast carcinoma specimens using antibodies to ATP7B and CD68 in cryostat sections. A. ATP7B-positive tumor stained with anti-ATP7B monoclonal antibody. Note the distinct cytoplasmic staining in breast carcinoma cells (immunoperoxidase stain, original magnification, ×200). B. A negative control in the serial section of A (magnification, ×200). C. ATP7B-positive tumor (immunoperoxidase stain, original magnification, ×400). D. Normal tissue (immunoperoxidase stain, original magnification, ×400). E. ATP7B-positive tumor (immunoperoxidase stain, original magnification, ×400). F. CD68-positive cells (alkaline phosphatase stain, original magnification, ×400). G. Double staining of ATP7B and CD68. The color figures (PDF files) will be provided upon request.

genes in human breast carcinoma, these gene expression levels were plotted in a graph (Fig. 3, A–E). The relationship between *MDR1* gene expression and *MRP1* gene expression (R=0.733, Fig. 3E) was significant. In contrast, no significant association between *ATP7B* gene expression and *MDR1* (R=0.473), *MRP1* (R=0.248), *LRP* (R=0.045) or *BCRP* genes (R=0.044) was observed.

Clinicopathologic significance of *ATP7B* gene expression in human breast carcinoma We examined the relationship between clinicopathologic variables and *ATP7B* gene expression. *ATP7B* gene expression level was independent of age, pT, pN and pM category and estrogen/progesterone receptor status (Table I). Concerning histopathologic type, *ATP7B* gene positivity in poorly differen-

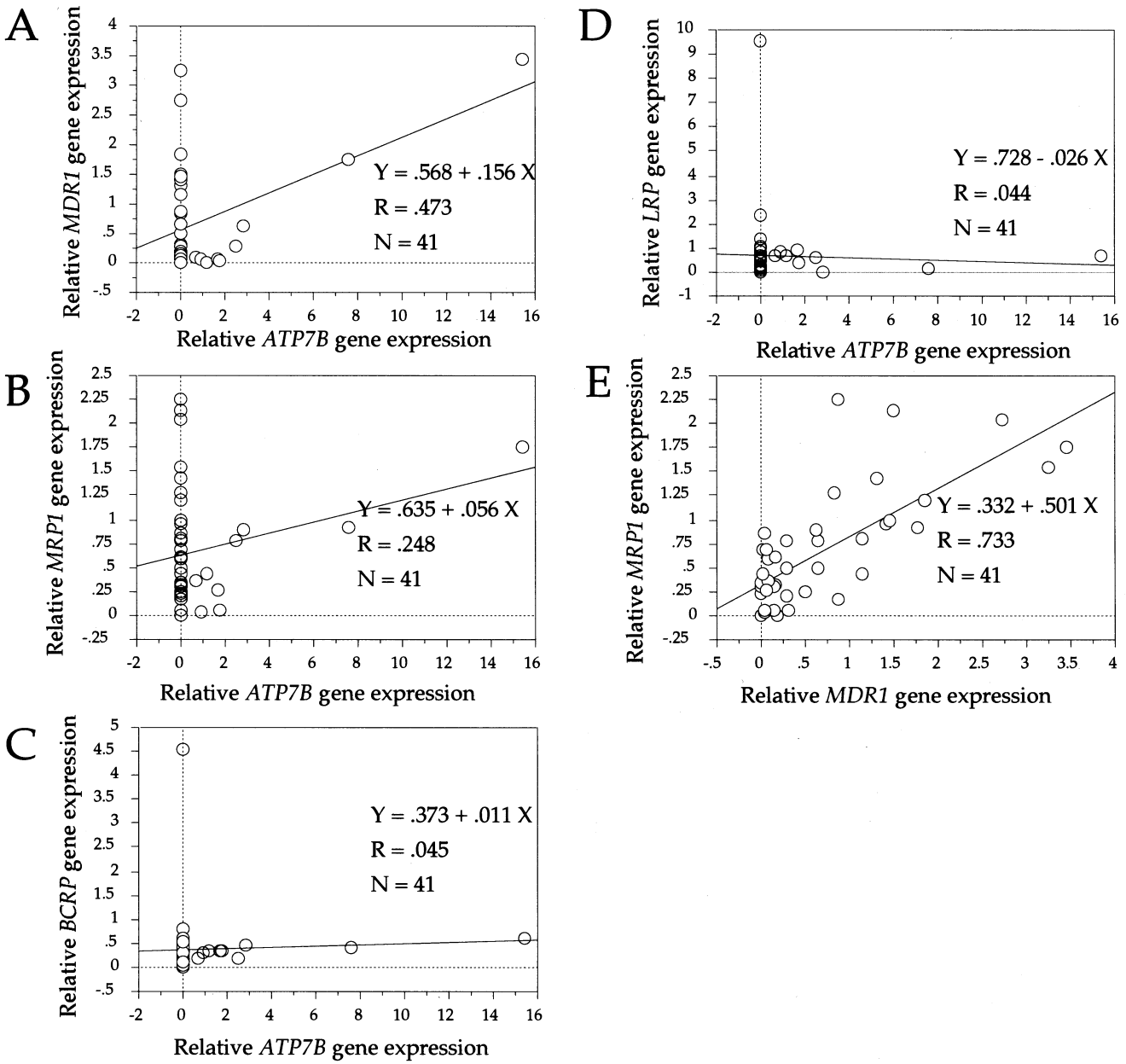

Fig. 3. Expression of the *ATP7B* gene independently of *MDR1*, *MRP1*, *LRP* or *BCRP* gene expression in breast carcinoma. Expression levels of *ATP7B*, *MDR1*, *MRP1*, *LRP* and *BCRP* genes in 43 patients with breast carcinoma were determined by RT-PCR, as described in "Materials and Methods." Each gene expression level is reported relative to the *GPADH* gene. Gene expression levels were plotted as follows. A. *ATP7B* vs. *MDR1* gene expression. B. *ATP7B* vs. *MRP1* gene expression. C. *ATP7B* vs. *BCRP* gene expression. D. *ATP7B* vs. *LRP* gene expression. E. *MDR1* vs. *MRP1* gene expression.

tiated carcinoma was significantly higher than that in well-/moderately differentiated carcinoma ($P=0.012$).

DISCUSSION

The present study provides the first evidence of *ATP7B* expression in human breast carcinoma (Figs. 1 and 2) by RT-PCR and immunohistochemical analysis. Further, *ATP7B* expression in poorly differentiated breast carcinoma is more frequent than in well-/moderately differentiated carcinoma (Table I).

The recent search for new marker(s) of chemoresistance has identified that ATP7B, an energy-dependent copper transporter, conferred resistance to cisplatin, and expression of the *ATP7B* gene was induced by exposure to cisplatin in human prostate cells.[1] Therefore, we examined the expression of *ATP7B* in human breast carcinomas, in order to elucidate its clinical significance. Interestingly, 22.0% (9/41 cases) of the breast carcinomas analyzed, expressed *ATP7B* (Fig. 1). Furthermore, *ATP7B* mRNA expression is correlated with ATP7B protein expression assessed by immunohistochemistry (Fig. 1D). ATP7B immunoreactivity was detected as a characteristic granular cytoplamic staining. In agreement with this observation, ATP7B has been reported to be abundant in the Golgi apparatus.[19] These findings are the first evidence(s) that ATP7B, which is involved in WND, is expressed in human breast carcinoma.

Interestingly, the expression of *ATP7B* in poorly differentiated carcinoma is significantly higher than that in well-/moderately differentiated breast carcinoma (Table I). Further, *ATP7B* gene and protein could not be detected in adjacent non-neoplastic tissues (Fig. 2). This suggests that the expression level of *ATP7B* is very low in normal differentiated breast tissue, but is upregulated in some breast carcinomas. The mechanism of this is not known, and further examination(s) is required.

MDR1 and *MRP1*, which function as drug efflux pumps and are classified as members of the ABC transporter gene family,[2, 3] are expressed in several types of tumor.[6, 7] The 110-kd LRP, the major vault protein, is frequently overexpressed in MDR cells, and has an important role in transportation of drugs from nuclei to cytoplasm and in conferring MDR *in vitro*.[4] *BCRP* (*MXR*/*ABCP*) gene, a member of the ABC transporter family, has been described in breast, colon, gastric and fibrosarcoma cell lines.[10–13] Recently, we reported the pattern of expression of these genes in the same sets of human breast carcinoma.[21] In the present study, we compared the expression levels of *ATP7B* and these genes in human breast carcinoma, in order to examine whether the expression patterns of these transporters are related to anti-cancer drug resistance (Fig. 3). Co-expression of *MDR1* and *MRP1* was observed, but *ATP7B* expression was independent of the expression of the other transporters. These data suggest that ATP7B may have an important role in human breast carcinomas that is different from that of the other transporters that confer MDR.

In conclusion, we have demonstrated that ATP7B, a transporter associated with chemoresistance, is expressed in a subset of breast carcinomas. Of especial interest is the finding that the expression is more frequent in undifferentiated carcinomas that are usually more refractory to therapy. Further examinations are required to find whether *ATP7B* expression is clinically relevant for the choice of therapy.

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