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Expression of PiT1 and PiT2 retroviral receptors and transduction efficiency of tumor cells★✪

Piotr Grabarczyk[1, 2✉☼], Piotr J. Wysocki[1], Katarzyna Gryska[1] and Andrzej Mackiewicz[1]

[1]*Department of Cancer Immunology, Chair of Oncology, K. Marcinkowski University of Medical Sciences, GreatPoland Cancer Center, Poznań, Poland;* [2]*Postgraduate School of Molecular Medicine, Warszawa, Poland*

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Recombinant retroviral vectors are still the most common gene delivery vehicles for gene therapy purposes, especially for construction of genetically modified tumor vaccines (GMTV). However, these vehicles are characterized by relatively low titre and in the case of many tumor cell lines, low transduction efficiency. We constructed bicistronic retroviral vector pseudotypes of amphotropic murine leukemia virus (A-MuLV) and gibbon ape leukemia virus (GaLV), encoding enhanced green fluorescent protein (EGFP) as a rapid and easily detectable reporter gene. Transduction of five different human melanoma and four renal carcinoma cell lines by these two virus pseudotypes revealed differences in transduction efficiency, which wase markedly lower for the renal carcinoma cell lines. Stimulation of retroviral receptor expression (PiT1 and PiT2) by phosphate depletion induced a limited increase of receptor mRNA levels, but did not improve the gene transfer efficiency. In contrast, simultaneous transduction with both vector pseudotypes markedly increased the transduction efficiency, compared to GaLV or A-MuLV alone. The same effect could be achieved by several repeated exposures of target cells to fresh vector

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✉Correspondence: Piotr Grabarczyk, Chair of Oncology, Department of Cancer Immunology, K. Marcinkowski University of Medical Sciences, Greatpoland Cancer Center, Garbary 15, 61-866 Poznań, Poland, phone: (48 61) 854 0665, fax: (48 61) 852 8502, e-mail: pgrabarczyk@yahoo.com

Abbreviations: A-MuLV, amphotropic murine leukemia virus; EGFP, enhanced green fluorecent protein; GaLV, gibbon ape leukemia virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RCR, replication competent retroviral vector; scFvs, single-chain Fv antibody fragment.

preparation. Overexpression of GaLV receptor (PiT1) in target cells significantly increased the transduction rate and enabled retrovirus mediated gene transfer into the cells which normally are not transducible by GaLV pseudotypes. We demonstrated that, using different transduction strategies, the relatively inefficient, widely used retroviral vector systems could be significantly improved.

Efficiency and stability of gene transfer into target cells are the most important issues in gene therapy. Retroviral vectors are still widely used for that purpose. However, the utilization of amphotropic murine leukemia virus (A-MuLV) and gibbon ape leukemia virus (GaLV)-derived vectors, which have become the primary tool for gene delivery into mammalian cells, results in limited transduction efficiency in a number of human cell types. Many efforts have been undertaken to optimize gene transfer with retroviral vectors. Most of them rely on technical improvements, like temperature shift during transduction or low-speed centrifugation of virus particles and target cells. One of the novel strategies is the development of suspension packaging cell lines, which increases the vector titre up to 10^7 IU/ml and makes large scale vector production possible (Pizzato *et al.,* 2001). Another strategy that may prove to be promising for *in vivo* approaches is construction of recombinant vectors capable of replicating within the target cells. In a murine model, replication competent retroviral vector (RCR) has been shown to be highly effective in the transduction of tumor cells and surprisingly no viral particles were detected outside the tumor bed (Logg *et al.,* 2001). Other attempts to improve retroviral vectors focus on changing the vector host range by utilizing different envelope proteins. Examples include vesicular stomatitis virus Env, which does not demand a protein receptor for cell entry, or covalent modification of viral envelope with antibody fragments (scFvs) directed against human cell surface antigens (Engelstadter *et al.,* 2001). While VSV-pseudotyped vectors turned out to be very effective and have been adopted in lentiviral vectors, the second scFVs approach led to very limited success.

The aim of these studies was to determine if the commonly used vector pseudotypes A-MuLV and GaLV can be used to transduce human melanoma and renal carcinoma cell lines efficiently. We showed previously that these cell lines express receptors conferring susceptibility to both pseudotypes (PiT1 and PiT2), but the level of the receptors mRNAs varied in melanoma and renal carcinoma cells. Moreover, differences between cell lines derived from the same tumor were also observed. No direct relationship between the PiT1 or PiT2 mRNA level and transduction efficiency was revealed. Generally, human melanoma cells could be transduced with higher efficiencies than renal carcinoma cells (Grabarczyk *et al.*, 2001).

In these studies we have attempted to improve the retrovirus mediated gene transfer of human melanoma and renal carcinoma cell lines by several different strategies: (i) stimulation of PiT1 and PiT2 expression by phosphate depletion; (ii) simultaneous transduction with GaLV and A-MuLV pseudotyped vectors; (iii) repeated exposure of target cells to fresh infectious medium; (iv) overexpression of the PiT1 retroviral receptor in target cells.

MATERIALS AND METHODS

Cells and vector pseudotypes. PA317, TE-FLY-A, TE-FLY-GA, renal carcinoma (P54, P58, P66, P138) and human melanoma cell lines (A375, WM9, WM35, WM239, WM902b) were cultured in standard conditions.

The bicistronic retroviral vector, encoding enhanced green fluorescent protein (EGFP), was constructed basing on MSCV2.1 RV. The construct pMINV-EGFP was introduced into PA317 cells by electroporation, followed by se-

lection in G418 (800 μg/ml). The infection medium was used to transduce TE-FLY-A and TE-FLY-GA packaging cells. GaLV and A-MuLV pseudotypes carrying pMINV-EGFP vectors were harvested, filtered (0.45 μm pore size), titrated and used for infection assays. The functional titer of the vector was evaluated on 293 cell line. Relative virus concentration was measured by whole virion dot-blot hybridization, with radiolabeled EGFP-specific probe. Media of equal virus concentration were used for all transduction assays. The virus containing medium was supplemented with Polybrene (4 μg/ml). Transduction efficiency was evaluated by flow cytometry (FACS) analysis of EGFP expressing cells.

Quantitative competitive RT-PCR. Quantitative competitive RT-PCR involves the co-amplification of sample to be quantified with various known amounts of a competitive template, which competes for the same set of primers but the product of its amplification is different in size from the sample. The ratio of both PCR products reflects the initial amounts of the templates. Competitive templates were generated by amplification of PiT1, PiT2 and GAPDH fragments, ligation into pGEM T-easy vector and introduction of an 86 bp random stuffer sequence. Total RNA isolated from target cells was reverse transcribed and amplified with various amounts of competitive templates, using pairs of primers: GA: 5′-ATGAGCCCCAGGCTTCTCCA-T-3′, GS: 5′-GGTCGGAGTTCAACGGGAT-TT-3′, P1A: 5′-CAGCAACGGTGCTCCAG-3′, P1S: 5′-GTGTGGCAACTCGTGGCTTC-3′, P2A: 5′-GCTGGTCATGAGAGAGCCGTG-3′, P2S: 5′-CGGAACATCTTCGTGGCCTG-3′. PCR products were analyzed by agarose gel electrophoresis and a Vilber Lourmat (France) gel documentation system. The amounts of PiT1 and PiT2 mRNA were normalized to GAPDH as an internal standard.

Stimulation of PiT1 and PiT2 expression by inorganic phosphate depletion. Target cells (A275, WM9, WM35, WM239, WM902b and P54, P58, P66, P138) were cultured in phosphate depleted D-MEM medium (ICN Biomedicals, Aurora, OH, U.S.A.) for 72 h. Total mRNA was isolated from a part of each cell line, using RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.), reverse transcribed with SensiScript Reverse Transcription Kit (Qiagen, Valencia CA, U.S.A.) and PiT1, PiT2 and GAPDH mRNA levels were quantified by quatitative RT-PCR assay, as described above. The cells were transduced with the A-MuLV and GaLV MINV-EGFP vectors. The transduction efficiency was evaluated by FACS 72 h after transduction.

Determination of cell doubling time. For each cell line 4×10^5 cells were seeded into standard D-MEM (ICN Biomedicals, Aurora, OH, U.S.A.) and phosphate depleted D-MEM (ICN Biomedicals, Aurora, OH, U.S.A.) medium. Cells were trypsinized after 24, 48 and 72 h, counted, and the doubling time was calculated.

Simultaneous transduction with A-MuLV and GaLV pseudotypes. Target cells were seeded with the same density 24 h before tranduction. Media harvested from confluent TE-FLY A and TE-FLY-GA packaging cell cultures were filtered (0.45 μm). Equal volumes of each medium were combined, supplemented with Polybrene (4 μg/ml) and used for single transduction of target cells.

Repeated exposures of target cells to fresh virus. Target cells were exposed to GaLV-MINV-EGFP infection medium for 96 h. Every 24 h the fresh vector portion was added to the cell cultures. Target cells were harvested after 1, 2, 3 or 4 subsequent transductions and the percentage of EGFP expressing cells was evaluated by FACS.

Overexpression of PiT1 retroviral receptor. The PiT1 encoding sequence was amplified from total cDNA derived from P54 cell line, using PiT1 cds: 5′-ATGGCAACGCTG-ATTACCAG-3′ and PiT1 cda 5′-TCACATTC-TAGAGATGACATATCTG-3′ primers. The amplicon was cloned into the pGEM-Teasy vector (Promega, Madison, WI, U.S.A.) and sequenced (*f*mol, Promega). PiT1 cDNA was

digested with the *Not*I and subcloned into the *Not*I site of the pAdCMVLink expression plasmid. The construct was introduced into B78 murine melanoma and WM239 human melanoma cell line using the Effectene (Qiagen, Valencia, CA, U.S.A.) transfection reagent. Forty-eight hours post transfection the cells were transduced with the GaLV MINV-EGFP vector. Transduction efficiency was measured by FACS. PiT1 mRNA expression levels were evaluated as described above.

Statistics. All experiments were performed in triplicate. The data presented here represent the mean value and ±S.D. (standard deviation).

RESULTS AND DISCUSSION

Effect of phosphate (P_i) depletion on PiT1 and PiT2 expression and transduction efficiency

Since the role of PiT1 and PiT2 as sodium dependent phosphate symporters had been revealed, many groups reported increased expression of both receptors and improved transduction efficiency following incubation of target cells in P_i depleted media (Kavanaugh *et al.,* 1994; Kurre *et al.*, 1999). In fact, using this method, we were able to generate changes of PiT1 and PiT2 mRNA levels in human melanoma and renal carcinoma cell lines. In most cases, 72 h of culture in P_i-free medium resulted in a limited increase of both receptors' mRNAs. An interesting exception was P66 cell line, where the lack of P_i reduced the level of PiT1 mRNA. In one case (P138) no effect on receptors expression was observed (Fig. 1). The differences in PiT1 and PiT2 mRNA levels, if observed, were not significant.

Induction of retroviral receptors expression did not affect the transduction efficiency of stimulated cells. No improvement in gene transfer with the GaLV or A-MuLV pseudotyped vector could be demonstrated, compared with cells cultured in standard conditions, which can be explained by reduced proliferation of the target cells (data not shown). Inorganic phosphate is a critical nutrient for cells and is required for nucleic acids synthesis, hence its concentration can influence the

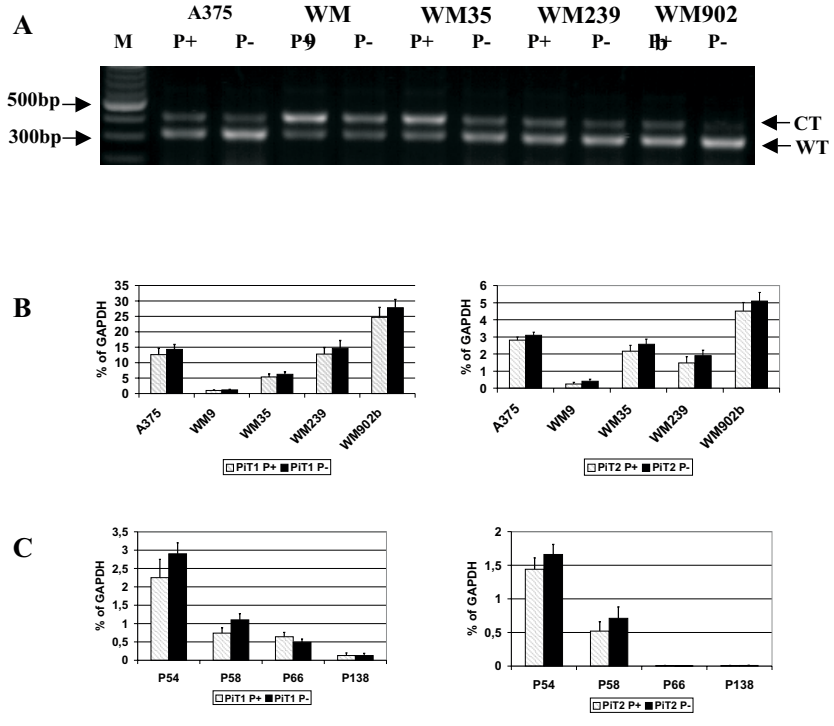

Figure 1 A. Quantitative competitive RT-PCR evaluation of PiT1 mRNA in P_i-depleted (P_i-) cultures of human melanoma cell lines.

M, 100 bp DNA ladder (Promega); CT, competitive template; WT, "wild" template. **B.** PiT1 and PiT2 mRNA levels in human melanoma cell lines, follow 72 h of incubation in P_i-depleted medium. **C.** PiT1 and PiT2 mRNA levels in renal carcinoma cell lines, following 72 h of incubation in P_i-depleted medium.

cell cycle, which in turn is critical for retroviral vector integration. We suggest that the lack of any improvement in transduction efficiency resulted from these two opposite effects.

Simultaneous targeting of PiT1 and PiT2 retroviral receptors improves transduction efficiency

We have reported previously that both PiT1 and PiT2 are expressed in all melanoma and renal carcinoma cell lines studied. However, the level of expression varied and did not reflect the susceptibility of the cells to the retroviral vector pseudotypes. It was of interest if a mixture of the reporter vector pseudotyped with GaLV and A-MuLV envelopes can transduce target cells with higher efficiencies than any of the pseudotypes alone. Equal volumes of vectors harvested from TE-FLY-A and TE-FLY-GA were combined and used for transduction of A275, WM9, WM35, WM239, WM902b and P54, P58, P66, P138 cells. The results were compared to cells transduced by single pseudotype. In all cell lines studied, this approach resulted in significant improvement of transduction rate. In most cases the effect was additive (Fig. 2).

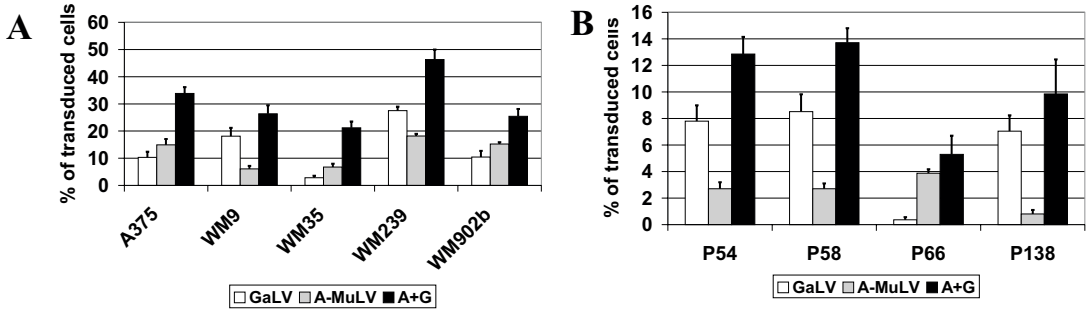

Figure 2. Effect of simultaneous transduction with GaLV and A-MuLV pseudotyped MINV- EGFP retroviral vector in human melanoma (A) and renal carcinoma (B) cell lines.

Another aspect of these studies was the predictive value of the multiplicity of infection (MOI) on transduction efficiency. Morgan *et al.* (1995) proved that the percentage of infected cells appeared to be governed predominantly by virus concentration but not by the absolute number of virus particles added. In our experiments, the mixture of the two vector pseudotypes contained two-fold dilution of each vector, but it seemed not to influence the transduction efficiency mediated by each vector. To further clarify the issue two different reporter genes should be applied.

Repeated exposures to GaLV pseudotyped MINV-EGFP vector significantly improve gene transfer

In a next series of experiments, we investigated the effect of several repeated exposures of target cells to fresh virus preparations. Due to the higher titres that could be obtained from the TE-FLY-GA packaging cell line only, our studies were limited to the GaLV enveloped vector. Target cells were exposed to infection medium for 96 h. Every 24 h the fresh vector preparation was added to the cell cultures. EGFP expression evaluation demonstrated a significant improvement of transduction in all melanoma cell lines. Starting from 20–30% of EGFP-positive cells after standard transduction (4–6 h), up to 80% transduced cells were obtained following four repeated infections. In renal carcinoma cell lines, which were generally less susceptible to retroviral vector transduction, the effectiveness of this procedure was reduced due to the

high sensitivity of these cells to Polybrene, which resulted in a significant loss of cell viability (Fig. 3).

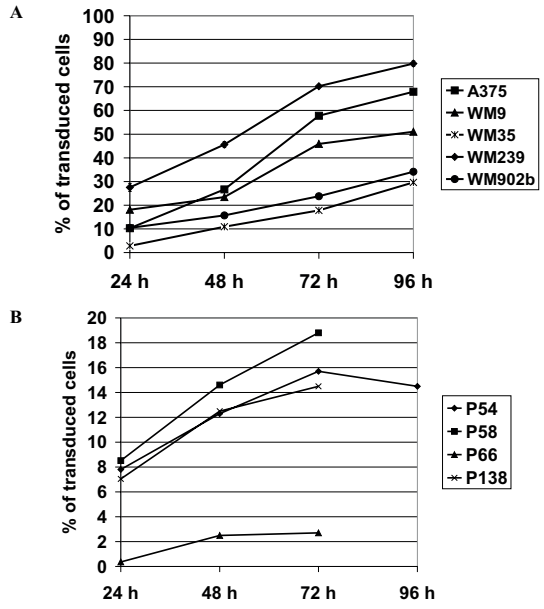

Figure 3. Efficiency of transduction after prolonged exposure of target cells to GaLV pseudotyped EGFP encoding vector.

A. Human melanoma cell lines. **B.** Renal carcinoma cell lines.

The superiority of this strategy can be partially explained by the limited stability of the vector. Results by Uckert *et al.* (2000) showed that GaLV pseudotyped vector half-live was 13 h, which means that after 24 h of transduction the vector concentration was about 25% of the initial value. Maintaining the high level of virus concentration over extended period of time enables cells to undergo division, which is indispensable for vector integration. Interestingly, in the case of human bone marrow cells, no increase in the transduction efficiency by repeated exposures to a new virus preparation was found. Those findings probably reflect a balance between the loss of cell viability associated with the repeated transduction protocol and gains achieved by the extended exposure to the virus (Hennemann *et al.,* 2000).

Overexpression of PiT1 in target cells

A promising alternative to the approaches described above was transient overexpression of retroviral receptor in the target cells. Limited success has been reported with an adenoviral vector encoding PiT2 (Yamaguchi *et al.*, 1995). High level, almost 100%, of transient expression of PiT2 in HeLa cells, which normally are resistant to A-MuLV pseudotyped retroviral vectors, allowed for about 30% retrovirus mediated gene transfer. For some reason, like higher stability and higher affinity of GaLV envelope to its receptor, PiT1 seemed a better choice for that purpose. We constructed an expression vector in which PiT1 was driven by the strong cytomegalovirus promoter (P_{CMV}). The functionality of the receptor was confirmed by introduction of the construct into the murine melanoma cell line B78, which normally does not express functional PiT1. Fourty-eight hours post infection, about 30% of transfected cells could be transduced by single exposure to GaLV enveloped reporter vector. This result is probably limited more by the transfection than the transduction efficiency, hence more effective vehicles for introduction of the receptor need to be used.

Transient overexpression of PiT1 in WM239 human melanoma cell line, normally expressing PiT1 and relatively susceptible to GaLV vectors, resulted in an over 80% increase of PiT1 mRNA level and almost 100% improvement of transduction (Fig. 4).

Preliminary results indicated that such approach not only increased the transduction rate, but also broadened the range of cells that could be a possible target for retroviral vectors. This strategy may be most effective in the cells in which the low level of receptor expression seems be the only limiting factor.

In conclusion, we presented several different approaches, based on typical, safe and widely used retroviral systems, which may markedly improve the transfer of genes into human cells *ex vivo*. Although our studies were focused on cell lines derived from human

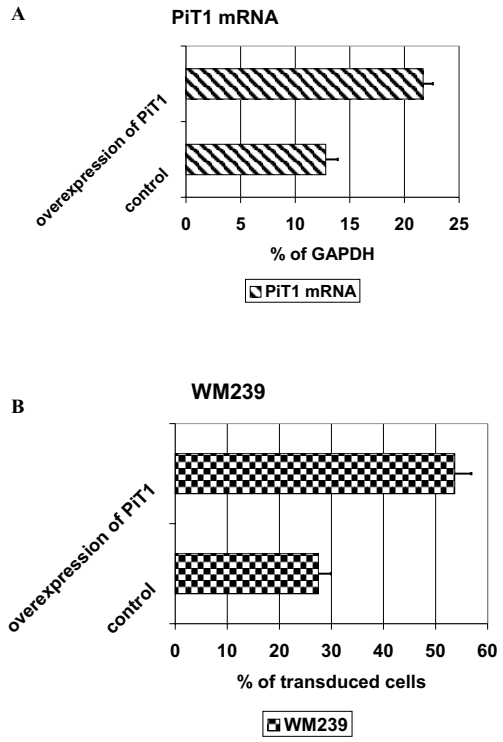

Figure 4. Effect of transient overexpression of PiT1 on receptor mRNA level (A) and transduction efficiency (B) of WM239 cell line.

melanoma and renal carcinoma, most of these findings can be utilized successfully for other cell types.

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