Expression of Aggrus/podoplanin in bladder cancer and its role in pulmonary metastasis

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Platelet aggregation-inducing factor Aggrus, also known as podoplanin, is associated with tumor malignancy by promoting hematogenous metastasis. Aggrus overexpression has been reported in some tumor tissues including lung, esophagus, head and neck and brain. We here found the frequent upregulation of aggrus mRNA in urinary bladder cancers using cancer tissue panels from various organs. Immunohistochemical analysis confirmed Aggrus protein expression in urinary bladder cancers and suggested a positive correlation between Aggrus expression and metastatic tendency in bladder cancers. Endogenous expression of Aggrus protein on the cell surface was found in the mouse bladder cancer MBT-2 cell line and human bladder cancer SCaBER cell lines. Knockdown of Aggrus expression in MBT-2 cells decreased their ability to induce platelet aggregation and form pulmonary metastasis in syngeneic mouse models. Knockdown of Aggrus expression in the human bladder cancer SCaBER cells also attenuated their ability to induce platelet aggregation and form pulmonary metastasis in mice. Moreover, pulmonary metastasis of SCaBER cells was prevented by prior administration of our generated anti-Aggrus neutralizing monoclonal antibodies by attenuating their retention in lung. These results indicate that Aggrus plays an important role in bladder cancer metastasis. Thus, anti-Aggrus neutralizing antibodies would be useful for the prevention of hematogenous metastasis of Aggrus-positive bladder cancer.

Key words: Aggrus/podoplanin, platelet aggregation, bladder cancer

Abbreviations: ADC: adenocarcinomas; ADCC: antibody-dependent cellular cytotoxic; ATCC: American Type Culture Collection; CDC: complement-dependent cytotoxic; CLEC-2: C-type lectin-like receptor 2; ET-1: endothelin-1; FBS: fetal bovine serum; HBSS: Hanks' Balanced Salt Solutions; IHC: immunohistochemical; qRT-PCR: quantitative reverse transcription polymerase chain reaction; SCC: squamous cell carcinomas; TCC: transitional cell carcinomas. Additional Supporting Information may be found in the online version of this article.

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Grant sponsors: Advanced Research for Medical Products Mining Programme of the National Institute of Biomedical Innovation (NIBIO) (to N.F.), Grant-in-Aid for Scientific Research on Innovative Areas “Integrative Research on Cancer Microenvironment Network” from the Ministry of Education, Culture, Sports, Science and Technology of Japan. (to N.F.), by a Grant-in-Aid for Young Scientists (B) (to S.T.)

DOI: 10.1002/ijc.28602

History: Received 8 Aug 2013; Accepted 4 Nov 2013; Online 13 Nov 2013

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Bladder cancer is the sixth most common cancer in the western world; with an estimated 73,510 new cases of urinary bladder cancer being diagnosed in 2012 in the United States.¹² Bladder cancer is known to be a smoking-related disease that most frequently occurs in males living in industrialized countries.³ Approximately 90% of bladder cancers are transitional cell carcinomas (TCC), 5% are squamous cell carcinomas (SCC), and less than 2% are adenocarcinomas (ADC). Although 70% of patients with bladder cancer present with superficial tumors that are generally not life-threatening, 30% present with muscle-invasive disease associated with a high risk of death from distant metastasis.⁴ Despite of good prognosis for patients with non-muscle-invasive disease, recurrence is common and associated with the development of muscle invasion disease in up to 30% of the cases.⁵ The incidence of distant metastasis is 50% in recurrent TCC and 8–10% in SCC; distant metastasis is uncommon in ADC.⁶⁷ Lung and liver are two common sites of dissemination for bladder cancer. Some genes, including endothelin-1 (ET-1), CD24 and LAMC-2, are reportedly associated with lung and liver metastasis of bladder cancer.⁸–¹⁰

Aggrus, also known as podoplanin, T1-alpha, OTS-8, or D2–40 antigen, was previously identified as a platelet aggregation-inducing factor expressed in highly metastatic tumor cells.¹¹ Because Aggrus mutants lacking platelet aggregation-inducing abilities were unable to form hematogenous metastasis, Aggrus-induced platelet aggregation is directly associated with metastasis formation.¹¹ The metastasis-promoting effects of platelets have been revealed in several reports. For example, platelets have been reported to facilitate tumor cell survival in blood circulation by enhancing the formation of tumor cell clusters that increase...
Expression of the platelet-aggregation-inducing factor Aggrus (podoplanin) by tumor cells is associated with metastasis via the bloodstream. In this study, the authors found that Aggrus is over-expressed in highly metastatic human bladder cancers, and that knockdown of this factor decreased metastasis of both mouse and human bladder-cancer cells in mice. Anti-Aggrus antibodies were also able to prevent this metastasis. This type of antibody may therefore be a promising adjunct to bladder-cancer therapy, and Aggrus over-expression may be a useful biomarker for predicting metastasis.

**What’s new?**
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**Material and Methods**

**Quantitative and semi-quantitative reverse transcription polymerase chain reaction**

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a LightCycler 480 Probes Master (Roch, Basel, Switzerland) and the LightCycler 480 Real-time PCR System (Roch). TissueScan Cancer Survey Panel 4× 96-III (OriGene Technologies, Rockville, MD) was screened by qRT-PCR using primers for human aggrus and β-actin. Standard curves were generated from a dilution series of cDNA prepared from HT1080 cells that were reported to express endogenous Aggrus. The expression level of each aggrus mRNA was normalized by that of β-actin. Primer pairs used in qRT-PCR were as follows: human aggrus forward, 5′-AAATGTGGAGAAGTACTGTG-3′; human aggrus reverse, 5′-GCCAGCAATGTGTTCCAC-3′; human β-actin forward, 5′-CCAACCCGAGAAGATGTA-3′; and human β-actin reverse, 5′-CCAGAGGGTCAGAGGA TAG-3′. Semi-quantitative RT-PCR was performed using KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan) and the GeneAmp PCR System 9700. Complementary DNAs were prepared with SuperScript III RT according to the manufacturer’s protocols. Primer pairs used in semi-quantitative RT-PCR were as follows: human aggrus forward, 5′-ATG TGGAAGGTTGTACAGCTGC-3′; human aggrus reverse, 3′-GTGTGGTCTCATCCACAT CCTC-3′; human β-actin forward, 5′-ATCTGGCACCACACACTTCCAATG-3′; human β-actin reverse, 3′-CTGCAACTCCAACATCCACTGTAC-3′; mouse aggrus forward, 5′-TGTCTTTTCACTTCTTCC AAACTTC-3′; mouse aggrus reverse, 3′-AGCTTTAGGCGGAG AACCTTC-3′; mouse β-actin forward, 5′-GATATCGGTGAG CTGGAAGGCTGAC-3′; and mouse β-actin reverse, 3′-CAA GAAGGAAGGCTGGAAGA-3′.

**Immunohistochemistry**

Four human bladder cancer tissue arrays (BL801, BL804, BL806 and BL208) were obtained from US Biomax (Rockville, MD). Overlapped samples among the four arrays were used, and the remaining 135 samples were assessed. Tissue array sections were deparaffinized, rehydrated and treated with peroxidase-blocking solution (Dako, Glostrup, Denmark). Anti-human Aggrus/podoplanin mAb (clone: D2–40, Dako) was treated for 30 min at room temperature, then incubated with EnVision+ System-HRP labeled polymer anti-mouse (Dako). Color was developed with ImmPACT DAB (Vector Laboratories, Burlingame, CA). Mayer’s hematoxylin solution (Wako, Osaka, Japan) was used for nuclei counter staining. Evaluation of the stain score (defined as the sum of the proportion score and intensity score) was entrusted to Kyodo Byori (Hyogo, Japan). The proportion score (the percentage of positive staining) was defined as follows: 0: 0%; 1: <10%; 2: 11–49%; 3: 50–79%; 4: >80%.
intensity score (the average staining intensity) was defined as follows: 0: negative, 1: weakly positive, 2: moderately positive, 3: strongly positive. Scoring of immunohistochemical (IHC) analyzed slides was performed by two independent pathologists who were blind to diagnosis.

**Plasmid construction**
Human aggrus cDNA was cloned as described previously. MISSION shRNA targeting mouse aggrus (TRCN0000176005: shAgg), human aggrus (TRCN0000061924: shAgg1h and TRCN0000061926: shAgg2h) and empty vector (SHC001: shCont) were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell lines**
CHO cells were purchased from the American Type Culture Collection (ATCC) and MBT-2 cells were obtained from the RIKEN Cell Bank (Yokohama, Japan). Both cell lines were cultured in RPMI 1640 media containing 10% fetal bovine serum (FBS). HT1080 cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS. UM-UC-3 (ATCC) and T24 (RIKEN Cell Bank) cells were cultured in minimum essential medium (MEM) containing 10% FBS. UM-UC-5 cells (Health Protection Agency, Salisbury, UK) were cultured in MEM containing 1 mM nonessential amino acids (NEAA, Sigma-Aldrich) and 10% FBS. SCaBER (ATCC) and J82 (ATCC) cells were cultured in MEM containing 1 mM sodium pyruvate, 1 mM NEAA and 10% FBS. RT-4 cells (ATCC) were cultured in McCoy’s 5A media containing 10% FBS. CHO cells that had stably transfected with human aggrus gene (CHO/Aggrus) and NL-17 cells (a highly metastatic variant of colon 26 ADC) were established in our laboratory and cultured in RPMI 1640 media containing 10% FBS.

**Immunoblot analysis**
Sample preparation was performed as described previously. In brief, cells were lysed in lysis buffer [25 mM Tris–HCl (pH 7.4), 50 mM NaCl, 0.2% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 2% NP-40, 66 μM of aprotinin and 1 mM phenylmethylsulfonyl fluoride]. Cell lysates were electrophoresed in sodium dodecyl sulfate-polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes and immunoblotted with anti-human Aggrus/podoplanin mAb (clone: E-1, Santa Cruz Biotechnology), anti-mouse Aggrus mAb (clone: 8F11, established in our laboratory), or anti-β-actin mAb (clone: AC-15, Santa Cruz). Enhanced chemiluminescence reagent (GE Healthcare) and luminescence image analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan) were used in detection.

**Flow cytometric analysis**
Cells were harvested and treated with or without recombinant (His)_6-tagged human CLEC-2 (10 μg/ml) following incubation with the Alexa Fluor 488-conjugated anti-(His)_6 antibody (QIAGEN, Hilden, Germany). Flow cytometric analysis was performed using a Cytomics FC500 flow cytometry system (Beckman Coulter).

**Platelet-aggregation assay**
Murine whole blood was drawn by cardiac puncture from Jcl:ICR mice terminally anesthetized with chloroform and taken with heparin solution. Platelet aggregation was measured using the screen filtration pressure method and a WBA Carna aggregometer (IMI, Saitama, Japan). Whole blood samples (200 μl) were stirred in the reaction tubes at 1,000 rpm at 37°C and preincubated for 2 min, followed by cell addition (MBT-2 and its transfectants: 1.6 × 10^5 cells, SCaBER: 1.3 × 10^5 cells). Using a 3.7-mm diameter syringe containing screen microsieves made of nickel with 300 openings per 30 × 30 mm^2 in an area 1 mm in diameter, whole blood were sucked to detect aggregation pressure at 1–12 min later. The final platelet-aggregation pressure in each reaction tube was determined depending on the pressure rate (%) detected by the pressure sensor. In some experiments, SCaBER cells were preincubated for 30 min on ice with 300 μg/ml of anti-human Aggrus neutralizing mAbs, previously established in our laboratory (clone: P2-0 and MS-1), or control mouse IgG (Sigma-Aldrich).

**Animals**
Male C3H/HeNGr/Crlj and female CB17/ICr-Prkdc<sup>scid</sup>/CrlCrj mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Jcl:ICR mice were purchased from Clea Japan. (Tokyo, Japan). All animal procedures were performed using protocols approved by the Japanese Foundation for Cancer Research Animal Care and Use Committee.

**Experimental pulmonary metastasis**
MBT-2 cells were suspended in Hanks’ Balanced Salt Solutions (HBSS) and intravenously injected (6.0 × 10^5 cells/mouse) into the lateral tail vein of 8-week-old male C3H/HeNGr/Crlj mice. Control mouse IgG or anti-human Aggrus mAb (30 μg/mouse) was intravenously injected into the lateral tail vein of CB17/ICr-Prkdc<sup>scid</sup>/CrlCrj mice the day before intravenous injection of SCaBER cells suspended in HBSS (5.0 × 10^5 cells/mouse). After 17–20 days (MBT-2 cells) and 32–35 days (SCaBER cells), mice were euthanized; their lungs were harvested with saturated picric acid solution. Lung surface metastatic foci were then counted.

**In vitro and in vivo proliferation assays**
To assess in vitro cell proliferation, the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI) was used after 24, 48 or 72 h in cell culture. For the in vivo proliferation assay, SCaBER cells were harvested, washed and resuspended in HBSS. In total, 3.0 × 10^6 cells suspended in 50 μl HBSS were subcutaneously injected into the backs of 8-week-old male C3H/HeNGr/Crlj mice. Tumor volume was calculated using the following formula: volume = W^2 × L/2, where W is width (the shortest dimension) and L is length (the longest dimension).
Statistical analysis
The Mann–Whitney U test was performed to determine the statistical significance of the results of the metastasis assays. Some results were compared using Student t-test. Significant p values are shown as *p < 0.05, **p < 0.01. All statistical tests were two-sided.

Results
Aggrus expression in bladder cancers
To identify new tumors expressing Aggrus, qRT-PCR was initially performed using the TissueScan Cancer Survey Panel containing 381 human tissues from patients with 22 different forms of cancer. Upregulation of aggrus mRNA was detected in cancer tissues from esophagus, lung, testis and urinary bladder, which were then compared with normal tissues (Fig. 1). Because several groups have already reported Aggrus overexpression in esophageal, lung and testicular tumors, we focused on aggrus expression in urinary bladder cancers in this study. Although we compared the aggrus mRNA level among histological types or differentiation degrees of urinary bladder cancers, no correlation could be found (Supporting Information Table S1).

Correlation of Aggrus expression with metastatic tendency in bladder cancers
We next examined Aggrus protein expression in 135 different cases of human bladder cancer by IHC staining of tissue arrays with anti-human Aggrus/podoplanin mAb D2–40. Although most G1, G2 and G3 TCCs received scores of 0–3, approximately half of the metastatic TCC tissues received scores ≥4 (Fig. 2a and Supporting Information Table S2). Moreover, clear staining of SCC (but not ADC) of bladder was observed (Fig. 2b). Because distant metastasis is more common in TCC and SCC than in ADC, these results suggest that Aggrus would be useful as a biomarker for prediction of metastatic TCC and SCC of bladder.

Aggrus expression in mouse bladder cancer cell line MBT-2
Aggrus is known to enhance hematogenous metastasis of various cancers by interacting with and activating platelet receptor CLEC-2. To investigate the effects of Aggrus expression in bladder cancer metastasis, the chemically induced mouse bladder TCC cell line MBT-2 was initially utilized because of its applicability to syngeneic tumor models. Expression of mouse Aggrus protein has been reported in NL-17 cells, a highly metastatic subclone of the mouse colon ADC 26 cell line. Therefore, NL-17 cells were used as a positive control in this study. Aggrus protein and mRNA expression was detected in MBT-2 cells (Figs. 3a and 3b). Aggrus-knockdown MBT-2 cells were then established and designated as MBT-2/shAgg cells (Figs. 3c and 3d). These cells were examined for their binding capacity against the recombinant CLEC-2 protein. Although cell surface binding...
of CLEC-2 was detected by flow cytometry in a control cell line (MBT-2/shCont); the CLEC-2 binding was dramatically attenuated in MBT-2/shAgg cells (Fig. 3e). These results indicated an interaction of MBT-2 cells with the CLEC-2 protein through Aggrus expression on the cell surface.

Involvement of Aggrus in pulmonary metastasis of MBT-2 cells

Aggrus–CLEC-2 interaction is critical for Aggrus-induced platelet aggregation and hematogenous metastasis. Thus, platelet aggregation-inducing ability and lung metastasizing ability of Aggrus-knockdown cells were measured. It was observed that platelet-aggregation rate and the number of lung surface metastatic foci were attenuated in MBT-2/shAgg cells, which is consistent with the results shown in Figure 3e, but the attenuation was not observed in MBT-2/shCont cells (Figs. 4a–4c). The in vivo growth rate was similar among the established MBT-2 clones (Supporting Information Fig. S1). These results indicated that Aggrus plays an important role in pulmonary metastasis of MBT-2 cells.

Aggrus expression in human bladder cancer cells and its role in pulmonary metastasis

By collecting several human bladder cancer cell lines, we screened the expression of human Aggrus protein in these cells. Our previously established Aggrus-transfected CHO
(CHO/Aggrus) cells were used as a positive controls.\textsuperscript{15} Aggrus expression was detected in SCC cell lines such as UM-UC-5 and SCaBER, and a transitional cell papilloma cell line, RT4 (Figs. 5a and 5b). No Aggrus expression was detected in the collected TCC cell lines (J82, UM-UC-3 and T24). To investigate the role of Aggrus expression in hematogenous metastasis in human bladder cancer cells, Aggrus-knockdown SCaBER cells were established (SCaBER/shAgg1h and SCaBER/shAgg2h; Figs. 5c and 5d) and their role in pulmonary metastasis was examined. Metastatic ability was attenuated in SCaBER/shAgg1h and SCaBER/shAgg2h cells consistent with Aggrus expression levels (Figs. 5e and 5f). These results indicate that human Aggrus also plays an important role in pulmonary metastasis of human bladder cancer cells.

**Efficacy and mechanism of anti-Aggrus neutralizing antibodies on pulmonary metastasis of human bladder cancer cells**

We have previously established anti-human Aggrus neutralizing antibodies designated as P2-0 and MS-1, and clarified their efficacy as hematogenous metastasis inhibitors using Aggrus-overexpressing cell lines.\textsuperscript{31,32} Thus, we attempted to examine the efficacy of anti-human Aggrus neutralizing antibodies on SCaBER cell-mediated platelet aggregation. Platelet aggregation induced by SCaBER cells was significantly suppressed by the addition of P2-0 or MS-1 mAb (Fig. 6a). The effects of these antibodies on pulmonary metastasis of SCaBER cells were then investigated. Consistent with the earlier results, pulmonary metastasis of SCaBER cells was prevented by prior administration of P2-0 or MS-1 mAb into mice (Figs. 6b and 6c). To clarify how these mAbs suppress lung metastasis, calcein-AM-labeled SCaBER cells were intravenously injected into nude mice and fluorescent cells in lung microvessels were counted after 30 min. Fluorescent cells were detected in lungs of control IgG-administrated mice, indicating that several SCaBER cells were trapped in lung (Figs. 6d and 6e). In contrast, prior administration of P2-0 or MS-1 mAbs dramatically decreased the number of cells trapped in lung. These results indicated that P2-0 and MS-1 mAbs suppressed pulmonary metastasis of SCaBER cells by inhibiting cell retention in lung. Therefore, anti-Aggrus neutralizing antibodies would be useful for the prevention of pulmonary metastasis of Aggrus-positive bladder cancer cells.

**Discussion**

Although superficial tumors in patients with bladder cancer are generally not life-threatening, occult distant metastasis in recurrent cancer is associated with poor 5-year survival rates.\textsuperscript{5} Molecular markers to identify localized tumors with high metastatic potential are currently lacking.\textsuperscript{36} Thus, identification and inhibition of key molecules that induce distant metastasis is important for improving the 5-year survival rate of patients with bladder cancer.
In this study, upregulation of the platelet aggregation-inducing factor Aggrus was found in bladder TCC tissues from patients with distant metastasis (Fig. 2). Although Aggrus reportedly enhances hematogenous metastasis in experimental pulmonary metastasis models, the correlation between Aggrus expression and distant metastasis in cancer patients is not so clear. This is the first study to report the Aggrus upregulation in bladder cancer patients with distant metastasis and to indicate the possibility of Aggrus as a biomarker for predicting distant metastasis of bladder cancer.

Previous reports have examined ET-1, ET-1 receptor, and CD24 as possible diagnostic markers of distant metastasis in bladder cancer. ET-1 is known to promote tumor colonization through ET-1 receptor activation without affecting primary tumor growth. CD24 is reported to be involved in the progression of tumorigenesis and metastasis of male bladder cancer under androgen regulation. Using existing diagnostic markers such as ET-1 or CD24 in combination with Aggrus would improve accuracy in identification of distant metastasis in bladder cancer patients.

Moreover, Aggrus expression was found in clinical specimens of bladder SCC by IHC analysis (Fig. 2) and in bladder SCC cell lines (SCaBER and UM-UC-5, Fig. 5). Several groups have reported Aggrus upregulation in SCC of lung, esophagus and head and neck. Thus, our finding is consistent with those of the above mentioned reports.

An association of Aggrus with pulmonary metastasis of bladder cancer cell lines was also found in this study (Figs. 4 and 5). The results of this study indicate that pulmonary...
metastasis may be prevented by prior administration of anti-Aggrus neutralizing mAbs (Fig. 6). The two anti-Aggrus neutralizing mAbs that we established, P2-0 (mouse IgG1) and MS-1 (mouse IgG2a), possess similar affinity, epitope and platelet-neutralizing ability. However, only MS-1 exhibits Antibody-dependent cellular cytotoxic (ADCC) activity and complement-dependent cytotoxic (CDC) activity. Because P2-0 and MS-1 mAbs suppressed lung retention of bladder
cancer cells, the anti-metastatic effects of these anti-Aggrus neutralizing antibodies would be platelet-aggregation inhibitory activity rather than effector activity. Although inhibitory effects of P2-0 and MS-1 mAbs are similar in the platelet-aggregation assay (Fig. 6a), P2-0 mAb exhibited inhibitory activity higher than MS-1 mAb in the lung retention assay (Fig. 6d). We performed platelet-aggregation assay after mixing cancer cells with antibodies, while in the lung retention assay the cancer cells were intravenously inoculated on the day following administration of antibodies into mice. Because P2-0 and MS-1 mAbs are similar in the affinity (9.3 and 9.0 day following administration of antibodies into mice. Because antibody assay the cancer cells were intravenously inoculated on the lung retention (Fig. 6a) activity higher than MS-1 mAb in the lung retention assay with antibodies, while in the lung retention assay the cancer cells were intravenously inoculated on the day following administration of antibodies into mice. Because P2-0 and MS-1 mAbs are similar in the affinity (9.3 and 9.0 nM, respectively) but slightly different in the dissociation rate constant (8.5 × 10^{-7} and 1 × 10^{-2} sec^{-1}, respectively), it might be appeared as a difference of the capability to block Aggrus-CLEC-2 interaction in a short period until the retention of cancer cells in lung microvessels. We have also confirmed that the administration of either P2-0 or MS-1 mAb on the day following intravenous inoculation of tumor cells significantly suppressed the number of lung metastatic foci (data not shown). Thus, anti-Aggrus neutralizing antibodies may be appropriate as both neo-adjuvant and adjuvant therapy in patients with highly metastatic bladder cancer. However, further studies are required to evaluate the function of anti-Aggrus neutralizing mAbs on Aggrus-positive cancer cells in the bloodstream.

Because systemic chemotherapy regimens used to treat bladder TCC are generally ineffective for treatment of pure bladder SCC,38 effective molecular target drugs against bladder SCC are urgently needed. ADCC and CDC activities are effective modes of action for therapeutic antibodies. Drugs based on these activities have been approved for clinical usage (i.e., trastuzumab and rituximab). Abe et al. reported the efficacy of co-administration therapy of anti-Aggrus antibodies exhibiting ADCC activity with natural killer cells against Aggrus-positive malignant pleural mesothelioma,39 suggesting that anti-Aggrus antibody-based immunotherapy may be a promising strategy for the treatment of bladder SCC.

Acknowledgments

The authors thank Dr. R. Katayama and Dr. A. Takemoto for their valuable suggestions.

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