Abstract. We hypothesize that dysregulated expression levels of the developmental regulatory genes in the adult body result in tumor development and malignant progression. PAX genes discovered as human orthologous genes of Drosophila 'paired' encode transcription factors, which control the expression of target genes to go on along the program of development. In this study, we first quantified expression of 9 PAX genes in human nevus pigmentosus tissues, melanoma tissues and melanoma cell lines by the real-time reverse transcription-PCR method. As a result, we found that the expression levels of PAX4 and PAX9 were extremely low in melanoma tissues and cell lines compared to nevus pigmentosus tissues. We then established melanoma cells overexpressing PAX4 and examined roles of PAX4 in cell growth. PAX4-overexpression reduced in vitro cell growth of human melanoma C8161 and MeWo cells. BrdU-uptake assay and cell cycle analysis by flow cytometry indicated that the retardation of cell proliferation by PAX4-overexpression was due to decreased DNA synthesis and cell cycle arrest at the G0/G1 phase. Furthermore, treatment of C8161 and MeWo cells with 5-azacytidine, a DNA demethylating agent, induced the expression of PAX4, suggesting that DNA methylation repressed the PAX4 gene expression in human melanoma. These results suggest that PAX4 functions as a potent tumor suppressor.

Introduction

The development of cancer and its malignant progression can be considered as a phenomenon where cancer cells go on along a part of the program of embryogenesis. The genes regulating embryonic development spatio-temporally express themselves in an exquisitely controlled manner and execute programs such as cell growth, migration, differentiation and death. It is thought that abnormal cell behaviour of tumor cells result from the mis-execution of such a program.

PAX genes, one of the developmental regulatory genes, were discovered as a human orthologous gene of Drosophila 'paired' (1). In human, 9 PAX genes have been identified (2,3). All PAX genes commonly possess a paired box, which consists of 381 bp. The paired box encodes paired domain through which PAX proteins bind to DNA in a sequence-specific manner in order to function as transcription factors.

It is known that abnormal expression of PAX genes causes various types of congenital abnormality. For example, mutations of Pax1 and Pax3 show the developmental defect of centrum and intervertebral disc and Splotch in mice, respectively (4,5). The mutation of PAX2 and PAX6 causes optic nerve colobomas, renal anomalies and congenital eye abnormalities including aniridia, respectively (6,7).

There are also some studies indicating that abnormal expression of PAX genes is associated with cancer development and behaviour. Abnormal expression levels of PAX genes through chromosomal translocations are found in rhabdomyosarcoma, thyroid cancer and acute lymphoblastic leukemia (8-11). Constitutive expression of PAX2, PAX3, PAX5 or PAX6 was detected in Ewing sarcoma, Wilms' tumor, medulloblastoma or thyroid cancer (12-15). Forced expression of Pax1, Pax2, Pax3, Pax6 or Pax8 transforms mouse fibroblasts (16). Some studies show that human PAX2 or PAX3 plays an essential role in survival of bladder and ovarian cancer cells or melanoma cells (17,18). PAX proteins are also known to alter the expression of tumor suppressor genes: PAX2 and PAX3 regulate the expression of WT1 and PAX2, PAX5 and PAX8 repress the expression of p53 (19-21).
dysregulated expression of a particular PAX gene(s) is likely to be involved in carcinogenesis and the malignant progression of a variety of cancers.

To explore malignant behaviour-associated PAX genes, we established an analysis system to quantify the expression of 9 human PAX genes at the level of mRNA. Using this system, we first compared the expression levels of 9 PAX genes between malignant melanoma specimens and nevus pigmentedous specimens. Furthermore, we addressed the tumor-suppressive role of PAX4 of which expression was lost or extremely low in melanoma compared to nevus tissues.

Materials and methods

Clinical specimens. We studied 16 cutaneous melanoma tissues and 5 nevus tissues from patients who had undergone surgery at the Department of Plastic Surgery, Hokkaido University Medical Hospital between 1996 and 2003. The age of the patients ranged from 29 to 87 years and gender breakdown was 6 males and 10 females. The tumor status according to the TNM classification (UICC, 6th edition) was pT4aN0M0 in 2 cases, pT4bN0M0 in 3 cases, pT4bN1aM0 in 1 case, pT2aN0M0 in 3 cases, pT3aN1aM0 in 1 case, pT1aN0M0 in 3 cases, pT1bN0M0 in 1 case, pT1bN1aM0 in 1 case and pT4bN2aM0 in 1 case. Complete written informed consent was obtained from all the patients.

Cells and culture condition. Human melanoma C8161 cells were kindly donated by Dr Motowo Nakajima (Johnson and Johnson, Tokyo, Japan). Origin and institution by which other cell lines (MeWo, G361, 9711, GAK, A375M and MMIV) were provided were described in our previous report (22). All the cell lines were grown on tissue culture dishes in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F12 medium (DME/F12), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Cambrex Bio Science, Wolkersville, MD). The cells were cultured at 37˚C in a CO2 incubator (5% CO2 and 95% air).

Transfection and cell cloning. The human PAX4 expression vector, pCDNA3.1HisA-PAX4, was kindly donated by Dr Takahide Miyamoto (Shinshu University School of Medicine) (23). The transfection of pCDNA3.1HisA-PAX4 or pCDNA3.1HisA (Invitrogen, Carlsbad, CA) into MeWo and C8161 cells was performed with Lipofectamine (Invitrogen) according to the manufacturer's instructions. Total RNA was extracted from monolayer cultures of each cell line and clinical specimens by using Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was subjected to cDNA synthesis in 100 μl of reaction mixture containing Taq Man RT buffer (Applied Biosystems, Foster City, CA), 5.5 mM MgCl2, 500 μM dNTP, 2.5 μM random hexamers, 0.4 U/μl RNAase inhibitor, 1.25 U/μl MultiScribe reverse transcriptase. The reverse transcription reaction was performed sequentially for 10 min at 25˚C, for 30 min at 48˚C and for 5 min at 95˚C.

Quantitative real-time PCR. The PCR primers were designed with the use of Primer Express 1.5 (Applied Biosystems). The primer sequences for amplification of 9 PAX genes and β-actin are listed in Table I. Real-time PCR was carried out with a 15 min hot start at 95˚C followed by a denaturation step...
at 94˚C for 15 sec, an annealing step at 60˚C for 30 sec and an extention step at 72˚C for 1 min for 40 cycles. Primer concentrations of reactions of each PAX gene were 0.6 μM except 0.3 μM for PAX7. Data were analysed with Sequence Detector Systems version 2.0 software (Applied Biosystems). The copy number of PCR products (cDNA) was quantified by using the standard curve method. Standard curves were drawn in the same method described in our previous study (24). Expression levels of PAX genes were shown as the ratio of the target PAX gene to the internal reference gene (ß-actin) expression based on the initial copy number calibrated along the standard curve.

Cell growth assay. The number of viable cells was estimated by colorimetric WST-8 assay using the tetrazolium salt/formazan system (Dojindo, Kumamoto, Japan). Cell suspension (100 μl) (1x10⁴/ml, DME/F12-10% FBS) was placed in each well of a 96-well tissue culture plate. At intervals of 24 h after the cell seeding, 10 μl of WST-8 solution was added to the wells in a quadruplicate manner and incubated in a CO₂ incubator. After 1 h of incubation, absorbance of soluble formazan was measured at 450 nm wavelength (655 nm reference wave length) by using a Microplate reader (Bio-Rad Laboratory, Hercules, CA). The data are presented as the mean ± SD of quadruplicate wells.

Cell cycle analysis by flow cytometry. After cells were detached by the treatment with PBS (-) containing 0.2% trypsin and 2 mM EDTA, they were washed twice with PBS (-). Cells (1x10⁶) were fixed in 70% EtOH at room temperature for 1 h. After washing the cells with PBS, 100 μl of ribonuclease solution (100 μg/ml) was added to the cell pellet. After being kept at room temperature for 5 min, the cells were stained with 400 μl of propidium iodide (PI) solution (50 μg/ml) for evaluation of DNA content. Fluorescent intensity of PI was analysed by using FACS Calibur (Becton-Dickinson, San Jose, CA).

Assay for DNA synthesis. DNA synthesis was measured by means of 5-Bromo-2-deoxyuridine (BrdU) incorporation into cellular DNA with the use of colorimetric Cell Proliferation ELISA (BrdU) assay kit (Roche Diagnostics, Indianapolis, IN). Cells (1x10³/100 μl/well, DME/F12-10% FBS) seeded on 96-well tissue culture plate were incubated in CO₂ incubator for 24 h. The medium was replaced with DME/F12-10% FBS containing 10 μM BrdU. After BrdU-pulsing for 2 h, the cells were fixed and incubated with peroxidase-labelled anti-BrdU antibody. Then, tetramethyl-benzidine, a peroxidase substrate, was added to each well and the reaction was then stopped with 0.2 M H₂SO₄. Absorbance was measured at 450 nm wavelength (655 nm reference wave length) by using a Microplate reader. The data are presented as the mean ±SD of quadruplicate wells.
Treatment of melanoma cells with 5-azacytidine, a DNA demethylating agent or trichostatin A, a histone deacetylase inhibitor. Cells were plated in 100-mm tissue culture dishes at a cell density of 1x10⁶/dish. After 24 h, the cells were treated with 5 μM 5-azacytidine (Sigma, St. Louis, MO) for 2 days, or with 1 to 100 nM trichostatin A (Sigma) for 2 to 5 days.

Results

Expression levels of 9 PAX genes in human melanoma specimens, melanoma cell lines and nevus specimens. We first analysed the expression levels of 9 types of PAX genes in 16 melanoma specimens, 7 melanoma cell lines and 5 nevus specimens by a real-time RT-PCR method. Of the 9 PAX genes, 3 PAX genes such as PAX3, PAX4 and PAX9 were expressed in nevus tissues. As shown in Fig. 1, the expression levels of PAX4 and PAX9 in the melanoma specimens and melanoma cell lines were significantly low compared to those in the nevus specimens (p<0.01, Mann-Whitney U test). None of the melanoma cell lines showed expression of the two PAX genes.

Overexpression of PAX4 in human melanoma C8161 and MeWo cells transfected with the PAX4 expression vector. As
the expression of PAX4 was lost or low in melanoma tissues and melanoma cell lines compared to nevus tissues, we speculated that PAX4 functioned as a tumor suppressor. To examine whether the expression of PAX4 influences the proliferative behaviour of melanoma cells, we stably transfected C8161 and MeWo cells with a PAX4 expression vector, pCDNA3.1HisA-PAX4. The cell lines were transfected with empty pCDNA3.1HisA (mock transfectants) as a control. RNA was extracted from each parent line and transfected sublines and subjected to the analysis of expression of PAX4 by a real-time RT-PCR method. In the C8161 and MeWo cell lines, all the cloned cell lines transfected with PAX4 and PAX4-transfected cell lines before cell cloning showed higher expression of PAX4 than the parent and mock-transfected cells (Fig. 2A and B). Immunoblot analysis also revealed that PAX4-transfected cells expressed the PAX4 protein (Fig. 2C).

Decreased cell growth of PAX4-overexpressing melanoma cells. We examined the influence of PAX4-overexpression on cell growth of melanoma cells by WST-8 assay. As shown in Fig. 3A, the growth of all the C8161 cell lines overexpressing PAX4 was significantly lower than the control cell lines (parent and Mock-transfectants) after incubation for 93.5 h (p<0.01). In MeWo cells, the growth of all the cell lines overexpressing PAX4 was significantly lower than any of the control cell lines after incubation for 89 h (p<0.01). The inhibitory effect of PAX4-overexpression on the cell growth was more prominent in MeWo cells compared to C8161 cells.

Decrease in DNA synthesis in melanoma cells by PAX4-overexpression. To examine whether the growth inhibition by PAX4-overexpression was due to a decrease in DNA synthesis, we performed a BrdU-uptake assay. As shown in Fig. 4A and B, the activity of DNA synthesis diminished by PAX4-overexpression in C8161 and MeWo cells.

Increase in G0/G1 phase fraction of cell cycle in melanoma cells by PAX4-overexpression. We then analysed cell cycle...
distribution by flow cytometry in order to clarify whether decreased DNA synthesis by PAX4-overexpression was related to cell cycle arrest. In C8161 and MeWo cells, PAX4-overexpression extended the G0/G1 phase fraction and reduced S or G2/M phase fraction (Fig. 5), while no essential changes were detected in the Mock transfectants as compared to the parent cells.

**Induction of PAX4 expression by treatment with 5-azacytidine.** We tested whether the silencing of the PAX4 gene in C8161 and MeWo cells was due to the DNA methylation or histone deacetylation. When the two cell lines were treated with 5 μM of the demethylating agent 5-azacytidine for 2 days, they expressed the PAX4 gene (Fig. 6). However, treatment with histone deacetylase inhibitor trichostatin A did not induce the expression of the PAX4 gene in any of the cell lines (data not shown).

**Discussion**

Nine members have been identified in the human PAX gene family. They are classified into 4 groups based on the structural domains other than the paired domain (25). The first group (group III: PAX3 and PAX7) contains an octapeptide motif and a homeodomain; second one (group II: PAX2, PAX5 and PAX8) contains an octapeptide motif and a truncated homeodomain; third one (group IV: PAX4 and PAX6) or fourth one (group I: PAX1 and PAX9) contains only a homeodomain or an octapeptide motif. So far, there are many studies indicating that PAX genes of group II and III contribute to oncogenesis. These PAX genes are often highly expressed in a variety of tumors: PAX2 in prostate cancer (26), PAX5 in medulloblastoma (14), PAX8 in ovarian cancer (17) and PAX3 and PAX7 in Ewing sarcoma (12,27). Furthermore, experimental analyses also showed oncogenic functions of these PAX genes: Silencing of PAX2 by antisense oligonucleotides or siRNA suppressed the growth of renal cancer cells (28) or induced apoptosis in ovarian and bladder cancer cells (17); mouse PAX1, 2, 3, 6 and 8 in vitro transformed NIH 3T3 cells and the transformants formed tumors in mice (16); PAX2, 5 and 8 inhibited transactivation of a p53-responsive reporter in culture cells (21).

However, there are few studies on anti-oncogenic functions of PAX genes; for example, PAX2 and PAX8 are able to transactivate WT1, one of the tumor suppressor genes and thus may be considered to work tumor-suppressively (19,29). A low level of PAX6 expression in malignant astrocytic gliomas correlates with unfavourable outcomes (30) and overexpression of PAX6 suppresses cell growth due to G1 arrest of cell cycle in human glioma cells (31).

Here, we showed for the first time that PAX4 potentially functioned as a tumor suppressor in human melanoma cells. Namely, the expression levels of PAX4 were significantly low in melanoma tissues compared to nevus tissues. Furthermore, forced expression of PAX4 suppressed the growth of melanoma cells, mainly due to a decrease in DNA synthesis through cell cycle arrest at the G1 phase. This phenomenon is similar to the growth inhibition of glioma cells by PAX6, as mentioned above (31). It is notable that PAX4 has common features with PAX6. Both belong to the same group (group IV) of the PAX family and are required for the normal development of pancreas, especially hormone-producing endocrine cells (32). Additionally, they are capable of binding a common element in the glucagon, insulin and somatostatin promoters (33,34).

It is unlikely that regulatory functions of the transcription of these genes related to the pancreas development work as tumor suppressors. However, it may be reasonable to think that PAX4 as well as PAX6 affects the expression levels of or interactions with molecules associated with the control system of the cell cycle, not as yet characterized. Further studies should help to identify the molecules targeted by PAX4.

**How is the expression of PAX4 suppressed in melanoma?** The expression levels of PAX4 in C8161 and MeWo were induced by treatment with 5-azacytidine, a DNA-demethylating agent but not TSA, a histone deacetylase inhibitor. No deletion of the PAX4 gene in these cell lines was detected by genomic DNA analysis (unpublished data). Furthermore, the deletion of chromosome 7q on which the PAX4 gene was localized was not detected although its amplification was reported (35,36). Thus, the suppression of PAX4 in melanoma is likely to be due to DNA-methylation although we need to identify the methylated sites.

This is the first study suggesting that PAX4 potentially functions as a tumor suppressor. Although it is necessary that further studies reveal detailed molecular mechanisms by which PAX4 works as a tumor suppressor, PAX4 may provide an important avenue for a therapeutic strategy to melanoma.

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**References**


