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Genetics

The expression of the KAI1 gene, a tumor metastasis suppressor, is directly activated by p53

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ABSTRACT **KAI1 is a tumor metastasis suppressor gene that is capable of inhibiting the metastatic process in animals. The expression of the KAI1 gene also is found to be down-regulated during the tumor progression of prostate, breast, lung, bladder, and pancreatic cancers in humans, and this down-regulation appears to be at or posttranscription level. We have found that the tumor suppressor gene p53 can directly activate the KAI1 gene by interacting with the 5′ upstream region. The p53 responding region is located at approximately 860 bases upstream of the transcriptional initiation site, and it contains a typical tandem repeat of the p53 consensus-binding sequence. A gel-shift mobility analysis showed that this sequence indeed had the ability to bind to the purified p53 protein. Mutations of this sequence abolished the responsiveness to p53 and also the binding ability to the p53 protein. Furthermore, immunohistochemical analysis of 177 samples of human prostate tumors revealed that the expression of the KAI1 gene was correlated strongly to that of the p53 gene and that the loss of these two markers resulted in poor survivals of patients. Our data indicate a direct relationship between p53 and KAI1 genes and suggest that the loss of p53 function, which is commonly observed in many types of cancer, leads to the down-regulation of the KAI1 gene, which may result in the progression of metastasis.**

The development of metastasis is the main cause of death for most cancer patients and thus is a major obstacle to the successful treatment of those patients. However, the molecular aspect of metastatic development is as yet poorly understood, mainly because metastasis is a highly complex process and involves a variety of positive and negative factors (1). A possible breakthrough in our understanding of tumor metastasis has emerged with the hypothesis that metastasis is negatively controlled by suppressor genes. The KAI1 gene was isolated originally as a prostate-specific tumor metastasis suppressor gene (2, 3). It is located in the p11.2 region of human chromosome 11. When the KAI1 gene is transferred into a highly metastatic prostatic cancer cell, KAI1-expressing cancer cells are suppressed in their metastatic ability, whereas their primary tumor growth is not affected (2). DNA sequencing analysis of the KAI1 gene revealed that it is identical to CD82, a surface glycoprotein of leukocytes, which encodes 267 aa. The protein has four hydrophobic and presumably transmembrane domains and one large extracellular N-glycosylated domain (2). It appears to function in cell–cell and cell–extracellular matrix interaction, thereby potentially influencing the ability of cancer cells to invade tissues and to metastasize.

Consistent with the view that KAI1 is a metastasis suppressor gene, the immunohistochemical analysis of human tumor samples revealed that the expression of the gene in most cases is down-regulated during the tumor progression of not only prostate (4–6) but also lung (7), breast (8), bladder (9), and pancreatic (10) cancers. The down-regulation of the KAI1 gene expression is correlated with poor survival in patients with those cancers. Further studies of prostate tumors including 120 cases using the methods of PCR–single-strand conformational polymorphism and microsatellite analysis revealed that the KAI1 expression is down-regulated consistently during the progression of human prostatic cancer and that this down-regulation does not commonly involve either mutation or allelic loss of the KAI1 gene (4, 5). Therefore, the expression of this gene appears to be down-regulated in advanced tumor cells at or posttranscriptional level, presumably by the loss of an activator or gain of a suppressor. In search of such factors, we first dissected and analyzed the 5′ upstream region of the KAI1 gene. Here, we present evidence to show that the tumor suppressor gene p53 can directly activate the KAI1 gene.

MATERIALS AND METHODS

Cell Lines. Human prostatic carcinoma cell line ALVA41 and PPC-1 were kindly provided by W. Rosner (Columbia University, New York) and A. Brothman (Eastern Virginia Medical School, Norfolk), respectively. Human prostatic carcinoma cell line PC-3 and DU145 were purchased from American Type Culture Collection (Manassas, VA). All cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS and 250 nM dexamethasone.

Library Screening. A human placenta genomic library in the EMBL-3 Sp6/T7 lambda phage vector (CLONTECH) was screened by using a ^{32}P-labeled synthetic 58-mer oligonucleotide, which corresponds to nucleotide number 1–58 of the previously published KAI1 cDNA sequence (2).

CAT Reporter Gene Plasmids. A series of deletions for the KAI-CAT reporter plasmids was constructed by digesting KAI-2900 with *Hin*dIII followed by partial digestion with *Bam*HI or *Eco*RI or with limited digestion with *Exo*III. These plasmids were transfected to prostate cells by using Lipofectin (BRL), and cell extracts were prepared and assayed for CAT activities. For each transformation experiment, the luciferase expression plasmid, pGL3 (Promega), was added as an internal control and the CAT activity was normalized to the luciferase activity.

Site-Specific Mutagenesis. Site-specific mutations at the putative p53 binding site were generated by PCR using two

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primers: 5′-ggccagtccagg**AG**a**T**ctggggcagct**GC**agcctgtgactcagg (mutations in uppercase and bold) and 5′-cgggcgtgagcgggatccagc. The PCR was performed for 35 cycles by using KAI-2900 as a template with the following conditions: denaturation at 94°C for 30 sec, annealing at 58°C for 1 min, and extension at 68°C for 2 min. The PCR products were cloned into the pT7Blue T/A cloning vector (Novagen), and resultant plasmids were digested with *Hin*dIII and *Bam*HI. Gel-purified fragments then were subcloned into the KAI-2900 plasmid, which was digested with *Hin*dIII followed by a partial digestion with *Bam*HI, to produce KAI-902 (wild type) and KAI-902* (mutant). The mutation was confirmed by DNA sequencing.

Electrophoresis Mobility-Shift Assay. A double-stranded DNA with the sequence of 5′-gtccaggcaagctggggcagctcaagcctgtgac, which corresponds to the nucleotide number −896 to −863 of the KAI1 gene, or its mutant form as described above was synthesized and end-labeled with [γ-^{32}P]ATP. For the gel-shift assay, 25 ng of purified p53 protein (11) and 1 ng of probe were mixed and incubated at 0°C for 20 min. For the super-shift assay, anti-p53 (DO-1, Santa Cruz) was included in the reaction mixture. The competition experiment was done by adding 60–600 times excess of nonlabeled oligomers with wild-type or mutant sequences in the reaction mixture. The sample was run on 5% acrylamide gel, which was then dried and autoradiographed by PhosphorImager (Bio-Rad).

Reverse Transcription–PCR. The p53 expression plasmid, LNp53B (13), was transfected to ALVA cells. After 48 hr, the cells were harvested and total RNA was isolated by using the RNeasy Total RNA system (Qiagen). For reverse transcription–PCR, 5 μg of RNA was reverse-transcribed by using random primers and a Moloney leukemia virus reverse transcriptase (Perkin–Elmer). The cDNA then was amplified with a pair of 5′ and 3′ primers for the KAI1 gene (5′-ccgactgaggcacggagcgggtgacgctggg and 5′-gcttccttccacgaaaccagtgcagctgg) or human β-actin (Stratagene) in the reaction mixture containing [α-^{32}P]dATP. After the reaction, an aliquot of the product was subjected to 8% acrylamide gel electrophoresis. DNA products were visualized by staining them with ethidium bromide and photographed. The gel was dried and the radioactive bands were detected and quantified by PhosphorImager (Bio-Rad).

Immunohistochemical Analysis. Formaldehyde-fixed and paraffin-embedded human prostate tissue specimens from 177 patients (ranging from 53 to 91 years old) were studied. The specimens examined consisted of 79 cases of needle biopsy, 45 cases of transurethral resection, and 53 cases of radical prostatectomy. All samples were obtained from surgical pathology archives of the Akita Red Cross Hospital, Akita, Japan. The sectioned specimens were stained with hematoxylin/eosin (H&E), and immunological detection was performed by using a mouse monoclonal anti-human p53 antibody, DO-7 (DAKO), and a rabbit polyclonal anti-human KAI1 antibody, C-16 (Santa Cruz), respectively. Statistical significance was analyzed by χ^2 test.

RESULTS

We have cloned the 5′ upstream region of the KAI1 gene by screening a human genomic library in a lambda phage, using an oligonucleotide as a probe that corresponds to the sequence of the cDNA of the KAI1 gene. The cloned lambda phage DNA was analyzed by Southern blotting and PCR followed by a partial sequencing to determine the physical map (Fig. 1*A*). A 2.9-kb *Pst*I/*Xba*I fragment that contains the immediate upstream region of the KAI1 gene was subcloned into a reporter plasmid pBLCAT3 (12) so that the CAT (chloramphenicol acetyl transferase) gene is under the control of the KAI1 gene promoter. As a negative control, the *Pst*I/*Xba*I fragment was also cloned into pBLCAT3 in the reverse orientation. These plasmid constructs then were transfected to various human prostate cancer cell lines, and the resulting CAT activities were measured. As shown in Fig. 1*B*, when the reporter plasmid containing the 5′ upstream region of the KAI gene with ordered orientation (KAI-2900) was introduced into four different human prostatic tumor cells (ALVA, PC3, PPC-1, and DU145), all of these cells expressed the CAT gene. On the other hand, when the reporter plasmid containing the same upstream region with reverse orientation was introduced into these cells, no detectable level of the CAT activity was expressed, suggesting that 5′ promoter region is functioning in a specific manner in the prostate cells.

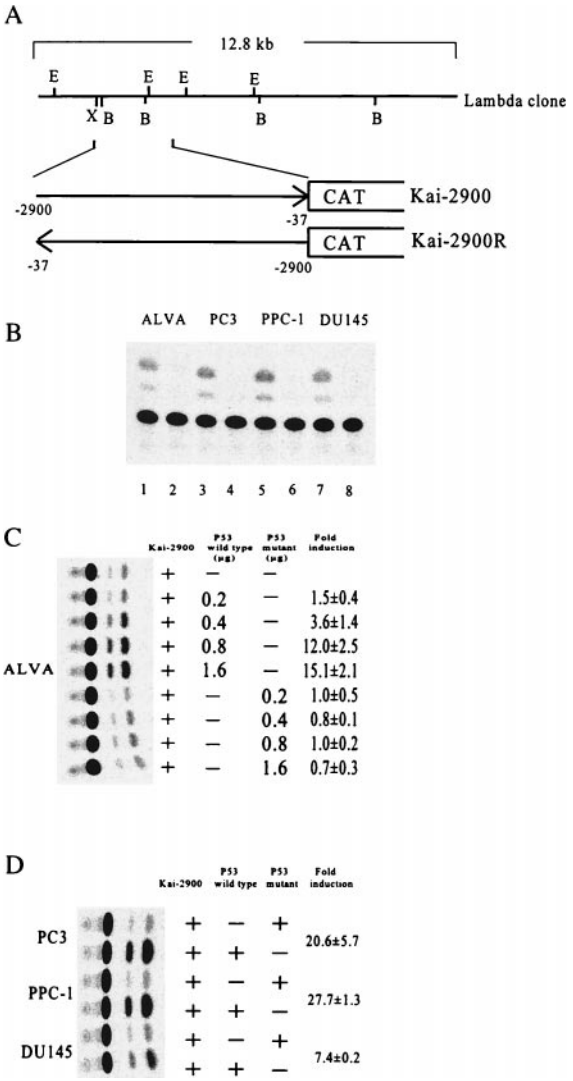

FIG. 1. p53 activates the KAI1 gene promoter. (*A*) A phage clone containing the 5′ upstream region of the KAI1 gene was obtained by screening a lambda phage human genomic library. The *Pst*I-*Xba*I fragment (2,900 bp) containing the immediate upstream region of the KAI1 gene was subcloned into the CAT reporter plasmid pBLCAT3 in both ordered (KAI-2900) and reverse orientations (KAI-2900R). (*B*) KAI-2900 (lanes 1, 3, 5, and 7) or KAI-2900R (lanes 2, 4, 6, and 8) were transfected to various human prostate cell lines (ALVA, PC3, PPC-1, and DU145) by using Lipofectin. After 48 hr, cell extracts were prepared and assayed for their CAT activities. (*C*) The dose-dependent response of the KAI1 promoter to p53 was examined. KAI-2900 was cotransfected with various amount of the p53 expression plasmid or its deletion mutant together with the control plasmid, pGL3, to human prostatic tissue cells, ALVA. After 48 hr, cell extracts were prepared and assayed for the CAT activities. The indicated numbers of the fold induction were calculated as means ± SE of at least three independent experiments. (*D*) The activation of the KAI1 promoter by p53 was examined in various human prostate cells including PC3, PPC-1, and DU145 as described in *C*.

Using this assay system, we have tested the effects of p53 on the promoter of the KAI1 gene. When ALVA cells were transfected with both KAI-2900 and an expression plasmid of p53 (LNp53B) (13), the expression of the CAT gene was augmented significantly by p53, up to 15 times, in a dose-dependent manner (Fig. 1*C*). Similar results also were obtained by using other human prostatic tumor cells, PC-3, PPC-1, and DU145, in which the KAI1 promoter was augmented, on average, 20-, 27-, and 7-fold, respectively (Fig. 1*D*). These results strongly suggest that the KAI1 gene is positively controlled by p53 at the transcriptional level in prostatic tumor cells.

To identify the responsive sequences to p53 in the 5′ upstream region of the KAI1 gene, we constructed a series of deletion mutants in this region. These CAT reporter plasmids were cotransfected with either wild type or mutant p53 expression plasmid to the ALVA cells, and the degree of augmentation of the CAT activity by p53 was measured. As shown in Fig. 2*A*, we found that the region of 272 bp that was approximately 860 bp upstream of the transcriptional initiation site was responsible for this p53 activation. DNA sequencing analysis of this region revealed that there was one region spanning from −892 to −868 that consisted of two tandem repeats of CAAG and showed strong homology to the dodecamer p53 consensus sequence (14) (Fig. 2*B*). This sequence is most similar to the p53-binding site of human Bax gene (15) (Fig. 2*C*). We then introduced site-specific mutations in this putative p53-binding domain as shown in Fig. 2*D*. When the CAT reporter plasmid with the mutation in the binding site was cotransfected with the p53 expression plasmid, the mutant almost completely lost the responsiveness to p53. Therefore, the p53 consensus sequence in the KAI1 promoter indeed is functionally responsible for the p53 activation.

To test the physical interaction between the putative p53−binding sequence and p53, we performed a gel-shift mobility assay by using a purified p53 protein. As shown in Fig. 3*A*, when the 34-bp DNA fragment corresponding to the −896 to −863 of the KAI1 gene was mixed with the p53 protein, the mobility of the DNA was shifted in an acrylamide gel, suggesting that the DNA binds to p53. This DNA-p53 complex was further shifted when the mAb against p53 also was added in the reaction mixture. Mutations in the p53-binding domain almost completely abolished the binding activity to p53 (Fig. 3*A*, lane 4). Furthermore, an addition of specific competitor DNA significantly reduced the complex formation (Fig. 3*B*), indicating that the binding of p53 is sequence-specific. Our results of the above experiments clearly indicate that p53 activates the KAI1 gene at the transcriptional level through its binding to the specific site of the 5′ upstream region.

We have also examined the effect of the p53 gene on the expression of the intracellular KAI1 gene. The p53 expression plasmid was transfected to ALVA cells, which are p53 negative (16), and mRNAs were prepared after 48 hr. The amount of expression of the KAI1 mRNA was measured by the reverse transcription–PCR as shown in Fig. 4. As we expected, the expression of intracellular KAI1 mRNA was augmented significantly by p53. Therefore, p53 is capable of effectively activating both endogenous and exogenous KAI1 genes. Our results of these experiments strongly support the notion that p53 activates the KAI1 gene and the loss of p53 function results in the down-regulation of the KAI1 gene.

We investigated to see whether the results of the above experiments indeed reflect the *in vivo* situation. Paraffin-blocked tumor tissue samples from 177 prostate cancer patients were examined immunohistochemically by using both KAI1 and p53 antibodies. Sliced samples were reacted with both antibodies followed by the DUB staining. Among 110

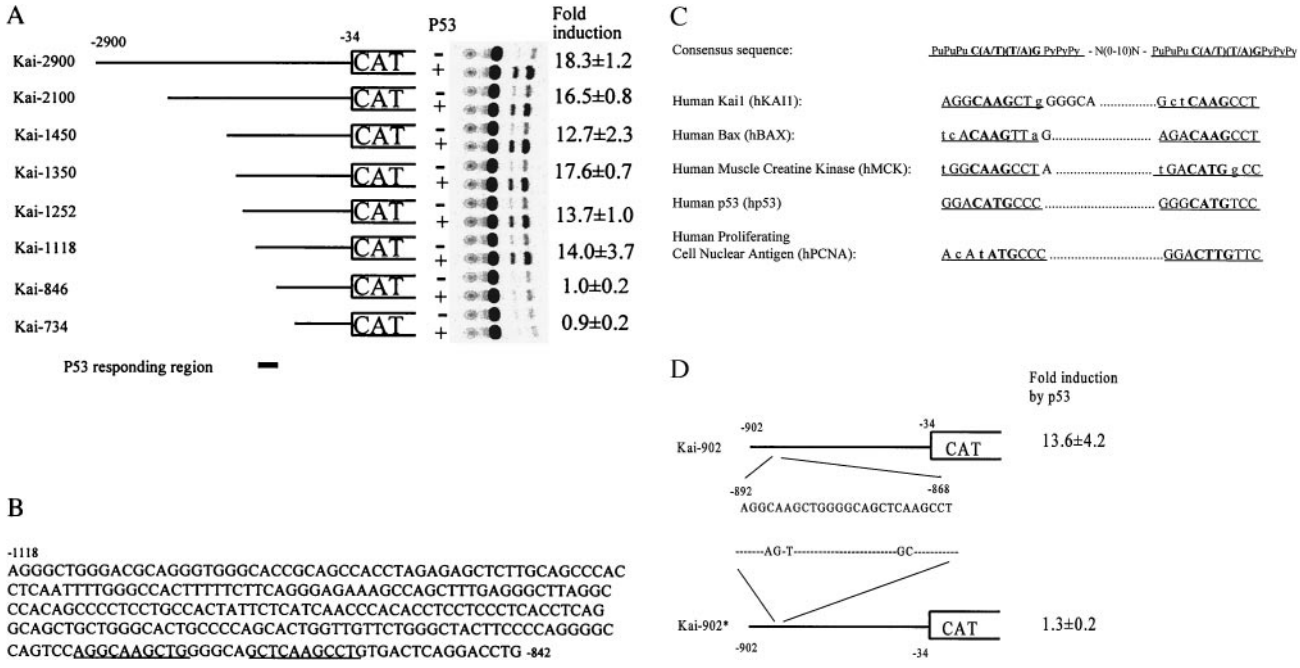

FIG. 2. The p53 responding sequence in the 5′ upstream region of the KAI1 gene. (*A*) Systematic deletions were introduced in the 5′ upstream region of the KAI1 gene, which was placed upstream of the CAT gene. These plasmids were cotransfected with the p53 expression plasmid (+) or its deletion mutant (−) together with pGL3 to ALVA cells, and the degree of the activation of the CAT expression by p53 was measured. The results were indicated as means ± SE of five independent experiments. (*B*) The p53 responding region of the KAI1 gene was sequenced. The underlined portion is the putative consensus sequence of the p53-binding site. (*C*) The putative p53-binding sequence of the KAI1 gene was aligned with p53-binding sequences of other genes and the general p53 consensus sequence. Uppercase letters indicate a perfect match to the consensus sequence. (*D*) Site-specific mutations were introduced in the dodecamer p53 consensus sequence by using a CAT reporter plasmid containing up to −902 bp upstream of the KAI1 gene. Either wild-type (KAI-902) or mutant (KAI-902*) plasmid was cotransfected with the p53 expression plasmid or its deletion mutant together with pGL3. After 48 hr, CAT activities were measured and the fold induction of the CAT activities by p53 was calculated. The numbers were indicated as means ± SE of at least three independent experiments.

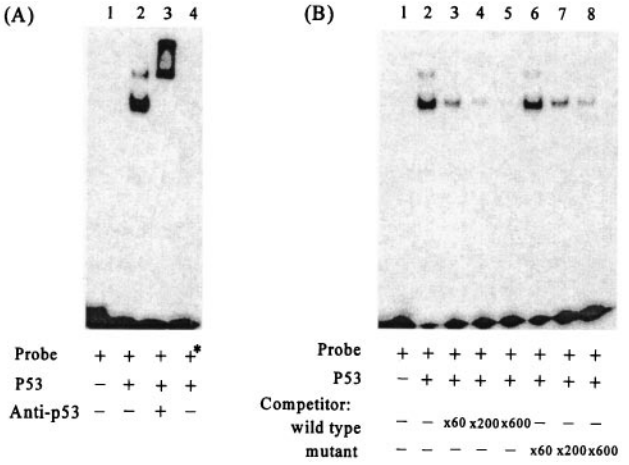

FIG. 3. Electrophoresis mobility-shift assay. (*A*) The gel-shift mobility assay was performed by using a 34-bp DNA fragment containing the putative p53-binding site of the KAI1 gene. The reaction contains DNA alone (lane 1), DNA plus purified p53 protein (lane 2), DNA, p53 protein, and mAb to p53 (lane 3). Lane 4 represents the reaction containing a probe DNA* with mutations in the binding site. After the reaction, samples were run on 5% native acrylamide gel and autoradiographed by PhosphorImager. (*B*) Competition experiment was performed by adding 60× (lanes 3 and 6), 200× (lanes 4 and 7), or 600× (lanes 5 and 8) excess of specific (lanes 3–5) or nonspecific (lanes 6–8) DNA fragment in the reaction mixture containing 34-bp DNA probe and p53 protein. Lane 2 represents the control reaction without competitor DNA.

cases of p53-positive samples, 85% were shown to be KAI1 positive (Table 1). On the other hand, among 67 cases of p53-negative tumor samples, 61% turned out to be KAI1 negative. These results suggest that the expression of the KAI1 gene has significant correlation with the expression of the p53 gene in these prostate tumor samples. Fig. 5 shows an example of the immunohistological staining of a tumor sample that was positive for both p53 and KAI1. In this poorly differentiated tumor specimen, most cells that expressed p53 were observed as strongly positive for the KAI1 expression. On the other hand, p53-negative cells stained with the KAI1 antibody but with much lesser degree, which is probably because of the basal level of the gene expression. Therefore, these results of the immunohistochemical examination is in good agreement with our *in vitro* results and strongly support our conclusion that the expression of the KAI1 gene is positively controlled by p53. Furthermore, as shown in Table 1, our follow-up data of 5 years' survival suggest that the loss of these two markers

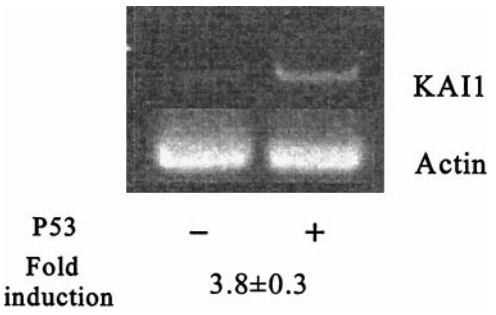

FIG. 4. Reverse transcription–PCR analysis of the expression of the endogenous KAI1 gene. The p53 expression plasmid was transfected to ALVA cells, and total RNA was isolated. mRNAs of both KAI1 and actin genes were converted to cDNA and amplified by PCR in the presence of [^{32}P]dATP. The amount of radioactivity was quantitated by PhosphorImager, and the relative amount of KAI1 mRNA was calculated by normalizing the radioactivity to that of actin. The result was indicated as mean ± SE of three independent experiments. The photograph shows an ethidium bromide-stained gel.

Table 1. Immunohistochemical analysis of prostate tumor tissues and patients' survival

	p53 + (n = 110)		p53 − (n = 67)	
	KAI1+	KAI1−	KAI1+	KAI1−
% of cases*	85% (93)	15% (17)	39% (26)	61% (41)
Average months of survival†				
Stage IV (n = 20)	42 mo (7)	20 mo (4)	21 mo (3)	9 mo (6)
Overall (n = 39)	44 mo (14)	21 mo (6)	20 mo (6)	12 mo (13)

Numbers in parentheses indicate actual numbers of patients.
*A total of 177 cases of tumor samples were immunohistochemically examined by using antibodies for p53 and KAI1. Percentage was calculated for each group of p53+ and p53−. The expression of the KAI1 gene has significant correlation with that of the p53 gene as determined by χ^2 test ($P < 0.001$).
†The average number of months of survived period was analyzed for 141 patients for up to 5 years. A total of 39 patients were decreased during the period.

predicted poor survival of patients. This is particularly clear among the patients at stage IV; p53+/KAI+ patients survived 42 months on average, while patients with p53−/KAI− survived only 9 months.

DISCUSSION

Our data presented here indicate the direct relationship between p53 and KAI1 genes. This line of evidence implies a possible direct link of a tumor suppressor to a metastasis suppressor gene. p53 is the gene most frequently found with mutations and deletions in a variety of tumors including prostate cancer (1). p53 alteration is considered to play a role in a subset of advanced stages of prostate cancer. However, studies on p53 protein expression in prostate cancer and the long-term clinical outcome in cancer patients have shown conflicting results (17). On the other hand, KAI1 expression has been shown to be well correlated with prostatic cancer progression (4, 5). Furthermore, the down-regulation of the KAI1 gene in advanced prostate cancer does not appear to involve mutations (4). These results and our present data further suggest that the loss of the p53 function, which is commonly observed in many types of cancer, leads to the down-regulation of the KAI1 gene, which in turn results in progression of metastasis. In fact, our data of the patient follow-up for the period of 5 years indicate that patients who were negative for both p53 and KAI1 had poor survival rates and that the combination of these two markers is considered to be a credible prognostic marker to predict the clinical outcome.

Biological significance of the KAI1 gene activation by p53 in a normal cell is as yet unknown. Based on its structure analysis, the KAI1 protein presumably is involved in cell adhesion or cell–cell interactions (2). On the other hand, the p53 gene is

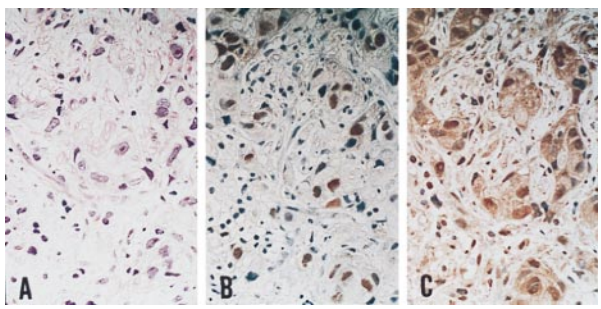

FIG. 5. Immunohistochemical analysis of a prostate tumor. A poorly differentiated prostatic tumor sample was sliced and stained with HE (*A*), p53 antibody (*B*), and KAI1 antibody (*C*).

believed to act as a gatekeeper for cellular transformation by playing a role in many key steps of cellular functions including cell cycle control and apoptosis (18). p53 accumulates in response to DNA damage by sensing single-stranded DNA fragments and arrests cell growth in part by induction of the p21 (19). During the event of cell-cycle arrest, a variety of genes are activated or suppressed by p53. Among these gene, GML (GPI-anchored molecule-like protein) was found recently to be up-regulated by p53 (20). GML belongs to a group of genes celled RIG-E; products of these genes are known to be involved in cell–cell adhesions and cell–matrix attachment. It is also found that the expression of E-cadherin, a key cell adhesion molecule, as well as P-cadherin, is down-regulated in p53-deficient mice (21). It is known that the loss of adhesion to specific components of the ECM can lead to apoptosis (22). Therefore, it is tempting to speculate that p53 activates cellular adhesion molecules such as KAI1 during the process of cell-cycle arrest so that the cell can be sustained from immediate death and allowed time to repair DNA damage. However, this hypothesis requires further investigation of the function of the KAI1 gene product.

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