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メタデータ	言語: eng 出版者: Oxford University Press 公開日: 2013-08-27 キーワード (Ja): キーワード (En): 作成者: Yamada, Hidetaka メールアドレス: 所属:
URL	http://hdl.handle.net/10271/79

Identification and characterization of a novel germline *p53* mutation in familial gastric cancer in the Japanese population

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Abstract

Germline mutations of the *p53* gene are known to cause Li-Fraumeni syndrome, and a germline *p53* mutation has recently been reported in a small subset of familial gastric cancer (FGC) in Europe and Korea. Although the incidence of gastric cancer is very high in Japan and familial clustering is not uncommon, there has been little information on the genetic factors of FGC. Therefore, to determine the role of germline *p53* mutations in FGC in the Japanese population in this study we used sequencing analysis to examine 80 individuals from 35 Japanese FGC families without germline *CDH1* mutations for germline *p53* mutations. One missense (c.91G>A: p.Val31Ile) and two intronic germline mutations were found, and transcriptional activity of the Ile31 mutant on *p53* responsive genes was examined to determine the functional effect of the novel p.Val31Ile germline mutation. A luciferase reporter assay showed that the transcriptional activity of *p21* (*CDKN1A*) and *MDM2* promoters but not of the *BAX* promoter was significantly lower in the Ile31-type *p53* than in the wild-type *p53*. Next, doxycycline-regulated *p53*-inducible H1299 cell lines were established by applying a retrovirus-mediated gene transfer system to a *p53*-null human H1299 cell line. Under similar *p53* expression conditions shown by Western blot and immunofluorescence analyses, a cell proliferation assay showed that the Ile31-type *p53* had significantly lower cell proliferation suppressing activity than wild-type *p53*. These results suggest that Ile31-type *p53* may be partly involved in FGC because of its low transcriptional activity and low cell proliferation suppressing activity. (246 words)

Introduction

Gastric cancer is one of the most common cancers worldwide, and although its incidence has decreased in recent years, it is still high in East Asia, including Japan. Most gastric cancers are sporadic and develop in response to the cumulative effect of environmental risk factors and genetic susceptibility of individuals (1), but a small subset are aggregated in families. The risk of individuals in families with gastric cancer aggregation is higher than in individuals without familial aggregation (2, 3). Since the report of the existence of germline mutations that cause loss of function of the *CDHI* gene in New Zealand Maori families with diffuse type gastric cancer (4), germline mutations in the *CDHI* gene have been found in gastric cancer families in different ethnic groups (5-11). However, the frequency of germline *CDHI* mutations is low, even in families with aggregation of gastric cancer (8-10, 12, 13). Kim *et al.* recently reported finding a germline missense mutation in the *MET* receptor tyrosine kinase gene in familial gastric cancer (FGC) patients without a germline *CDHI* mutation (14). The above findings suggest that genetic factors are important to the pathogenesis of gastric cancer with familial aggregation and that germline mutations of genes other than *CDHI* may contribute to the development of FGC.

The tumor suppressor protein p53 mediates growth arrest or apoptosis in response to potentially oncogenic cellular stresses such as DNA damage (15, 16), and loss of p53 function plays a central role in the development of cancer, and is caused by mutations. Many somatic *p53* mutations lead to a loss of p53 function and are detected in gastric cancer (17, 18).

Germline *p53* mutations are known to cause Li-Fraumeni syndrome (LFS) (19-21), and interestingly, germline *p53* mutations have recently been found in European and Korean FGC patients (22-24). Since, to our knowledge only three FGC cases with a germline *p53* mutation have been reported (22-24) and none have been in Japanese FGC patients, in the present study we investigated whether germline *p53* mutations are responsible for FGC in a Japanese population by screening 35 Japanese FGC families for *p53* germline mutations. We identified a *p53*-p.Val31Ile germline mutation in one family and characterized the *p53* mutant.

Materials and Methods

Samples, cell lines, and ethical considerations

In a previous study 80 blood samples were collected from 35 Japanese FGC families, and DNA was extracted (10). All of the families had been shown to be negative for germline *CDH1* mutation (10). Cell lines H1299 and 293T were purchased from the American Type Culture Collection (Manassas, VA). DNA from specimens of sporadic gastric cancer were collected previously (25, 26). The research protocol was approved by the Institutional Review Board of Hamamatsu University School of Medicine (12-14).

Polymerase chain reaction (PCR) and sequencing analysis

Fragments covering the entire coding exons and boundary regions of the *p53* gene were

amplified by PCR with HotStarTaq DNA polymerase (QIAGEN, Valencia, CA). The primers used were: 5'-TTG GAA GTG TCT CAT GCT G-3' and 5'-TTG AAG TCT CAT GGA AGC CAG-3' for exons 2, 3, and 4, 5'-TTG TGC CCT GAC TTT CAA CTC-3' and 5'-GGA GGT CAA ATA AGC AGC AGG-3' for exons 5 and 6, 5'-CTC ATC TTG GGC CTG TGT TAT C-3' and 5'-ACT TTC CAC TTG ATA AGA GGT CCC-3' for exons 7, 8, and 9, and 5'-ATA CTT ACT TCT CCC CCT CCT CTG-3' and 5'-TTG CAA GCA AGG GTT CAA AG-3' for exons 10 and 11. The PCR products were purified with a PCR purification kit (QIAGEN) and directly sequenced with a BigDye Terminator Cycle Sequencing Reaction Kit and the ABI 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) as described previously (27).

Plasmid construction

Expression vectors for wild-type (wt) and mutant (Ala143) p53 were provided by Dr. B. Vogelstein (28, 29). Expression vector for Ile31-type p53 was generated by site-directed mutagenesis with a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) (30) and the primer set: 5'-CTT CCT GAA AAC AAC ATT CTG TCC CCC TTG C-3' and 5'-GCA AGG GGG ACA GAA TGT TGT TTT CAG GAA G-3'. Luciferase reporter plasmids for the *p21*, *Bax*, and *MDM2* promoters were constructed by inserting a DNA fragment containing each promoter amplified from genomic DNA derived from blood samples from a healthy individual into the *NheI*-*BglIII* site of the pGL4.10[*luc2*] vector (Promega, Madison, WI). The PCR primers used for amplification were: 5'-CTA GCT AGC

TAG CTT CCC AGG AAC ATG CTT G-3' and 5'-GAA GAT CTT CCT GAC TTC GGC AGC TGC TC-3' for the *p21* promoter, 5'-CTA GCT AGC TAG AAC AGA AAA GCA GGC CTG G-3' and 5'-GAA GAT CTT CAA TCG CAG CTC TAA TGC CTT C-3' for the *BAX* promoter, and 5'-TAG CTA GCT AGC TTT GCG GAG GTT TTG TTG-3' and 5'-AAG ATC TTC GGA AAT CAT GAA ACA TCT GTG G-3' for the *MDM2* promoter. To construct p53 expression vector with a retroviral-mediated tetracycline-regulation system, pRevTRE-Tight vector was first constructed by inserting the *P* tight promoter region amplified from pTRE-Tight vector (Clontech, Mountain View, CA) into the *Xho*I and *Bam*HI sites of the pRevTRE vector (Clontech). *p53* was then inserted into the pRevTRE-Tight vector at the *Bam*HI site, and the expression vector created was named pRevTRE-Tight p53 vector.

pQCXIN-Tet-On retroviral vector was constructed by inserting the Tet-On sequence amplified from the pRevTetOn vector (Clontech) into the *Bam*HI site of pQCXIN retroviral vector (Clontech). The following PCR primers were used: 5'-TCC GCG GAT CCA AGC TTG TTA ATT AAT ATG TC-3' and 5'-TCG ATG TGG ATC CTC GCG CCC CCT ACC CAC-3'. All the plasmid vectors were confirmed by sequencing.

Luciferase reporter assay

A *p53*-null H1299 human cancer cell line was cotransfected with 1 μ g of the p53 expression vector, 300 ng of firefly luciferase reporter vector pGL4.10 containing a fragment of the *p21* (*CDKN1A*), *BAX*, or *MDM2* promoter, and 3 ng of Renilla luciferase reporter vector pGL4.74[*hRluc*/TK] (Promega) by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad,

CA). The cells were harvested 24 h post-transfection. Luciferase activity was quantified by a Dual-Luciferase Reporter Assay System (Promega), and relative luciferase activity was calculated according to the manufacturer's instructions. The statistical comparisons were performed by Dunnett's multiple comparison.

Establishment of the doubly stable H1299 cell line expressing p53 under doxycycline (Dox) control

Lipofectamine 2000 reagent (Invitrogen) was used to cotransfect 293T cells with a combination of pQCXIN-Tet-On and pCL10A1 plasmid vectors (IMGENEX Corp., San Diego, CA) or pRevTRE-Tight p53 and pCL10A1 plasmid vectors. The resulting virus-containing supernatants were used to serially infect H1299 cells - first with pQCXIN-Tet-On virus to produce a 1 mg/ml G418 resistant stable H1299-Tet-On cell line, then with pRevTRE-Tight p53 virus to produce a 1 mg/ml G418- and 500 μ g/ml hygromycin B-resistant double stable H1299-Tet-On-p53 cell line. Positive clones were confirmed by Western blot and immunofluorescence analyses to express p53 protein in the presence of Dox (a derivative of tetracycline).

Western blot analysis

Cells were harvested in lysis buffer containing 10 mM HEPES (pH7.5), 1.0% Nonidet P-40, 1 mM EDTA, 1 mM DTT, and 0.1 mg/ml PMSF, and the whole-cell extracts were mixed with an equal volume of 2xSDS sample buffer, and boiled. A 25 μ g amount of extracts was

subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins obtained were electrophoretically transferred to a PVDF membrane (GE Healthcare Bio-Science Corp., Piscataway, NJ). The membrane was blocked with nonfat milk and incubated with anti-p53 monoclonal antibody (clone DO7; Novocastra Laboratories, Newcastle, UK). After washing with phosphate-buffered saline containing 0.1% Tween-20 (PBS-T), the membrane was incubated with anti-mouse HRP-conjugated secondary antibody (GE Healthcare Bio-Science Corp). After washing with PBS-T, immunoreactivity was visualized with an ECL chemiluminescence system (GE Healthcare Bio-Science Corp).

Indirect immunofluorescence analysis

Double-stable H1299-Tet-On-p53 cells were cultured on coverslips in the presence of Dox, and after 24 h the cells were washed with PBS and fixed for 20 min at room temperature with 10% formalin (WAKO, Tokyo, Japan). The cells were then permeabilized with 1% Nonidet P-40 in PBS for 5 min, and incubated with 10% normal goat serum blocking solution (DAKO, Kyoto, Japan) for 1 h. The cells were probed with anti-p53 monoclonal antibody DO7 (Novocastra Laboratories) at room temperature for 1 h, and indirect immunofluorescence labeling was performed at room temperature for 1 h with a Alexa Fluor 488-conjugated goat anti-mouse as the secondary antibody (Molecular Probes, Eugene, OR), and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (SIGMA, St. Louis, MO). The immunostained cells were examined under a fluorescence microscope (Olympus BX-51-FL; Olympus, Tokyo, Japan) equipped with epifluorescence filters and a photometric CCD camera

(Sensicam; PCO Company, Kelheim, Germany). The images captured were digitized and stored in the image analysis program (MetaMorph; Molecular Devices, CA).

Cell proliferation assay

Double-stable H1299-Tet-On-p53 cells were seeded at a density of 7,500 cells per well into 96-well plates in medium containing or not containing Dox. After 72 h, the number of viable cells was counted by using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The kit detects mitochondrial NADH-dehydrogenase activity in live cells by measuring reduction of the tetrazolium monosodium salt WST-8. The statistical comparisons between the results for wt p53 and Ile31-type p53 were performed by the two-tailed Student's *t* test.

Genotyping of the c.91G>A mutation

PCR-single-strand conformation polymorphism (SSCP) analysis was used to type the c.91G>A mutation. A 103-base nucleotide fragment containing the c.91G>A mutation was amplified by PCR with a primer set consisting of 5'-CCC CTA GCA GAG ACC TGT G-3' and 5'-CCA ACC CTT GTC CTT ACC AG-3', and SSCP was performed as described previously (27). In brief, PCR products were diluted with loading solution, and after applying them to 8% polyacrylamide gels, the products were electrophoresed at 4°C. The DNA bands obtained were detected by silver staining. Before typing the sporadic gastric cancer cases, bands derived from the DNA of a FGC20 patient that had shifted abnormally in

comparison with bands derived from the DNA of cases homozygous for the c.91G allele were confirmed to be the c.91A mutation by sequencing.

Results

Identification of the novel c.91G>A germline mutation of the p53 gene in a Japanese FGC family

We screened for *p53* germline mutations by PCR and subsequent sequencing analyses in 80 probands or relatives in 35 Japanese FGC families without germline *CDH1* mutations, and 11 variations were found in the *p53* gene locus. When we defined a germline mutation as a gene variation not registered on the dbSNP homepage of the NCBI web site (<http://www.ncbi.nlm.nih.gov/SNP/>), a database of Japanese single nucleotide polymorphisms (JSNP) (31), and the genetic polymorphism data of the IARC TP53 mutation database (<http://www-p53.iarc.fr/PolymorphismView.asp>) (32), three *p53* variations were classified as a germline mutation and each was found in unrelated families (Table I). One of them, the c.91G>A mutation located in exon 3, was detected only in the proband of the family with the identification (ID) number FGC20 who was a heterozygote, and the mutation leads to an amino acid substitution of isoleucine for valine in codon 31 (Figure 1A). Two members of the family FGC20 had gastric cancer, and the proband was diagnosed with five synchronous colon cancers at 42 years of age (Figure 1B). Interestingly, there were two members with hepatocellular carcinoma and two members with uterine cancer in the same family.

Unfortunately, no samples from family members other than the proband were available for genetic testing for the *p53* mutation. Detection of the c.91G>A mutation was previously reported as a somatic mutation in lung cancer (33), and has never been reported as a germline mutation. Nor has it ever been registered on the dbSNP or JSNP homepages as described above, implying that it is a novel germline mutation. The following eight *p53* gene variations besides the germline mutation were detected in our analysis: c.74+38G>C (dbSNP ID = rs1642785), c.96+16_96+31dup16 (rs17878362), c.97-29C>A (rs17883323), c.215G>C (rs1042522), c.672+62G>A (rs1625895), c.782+72C>T (rs12947788), c.782+92T>G (rs12951053), and c.1101-221G>A (rs6503048). One of them, the c.215G>C variation, was associated with an amino acid substitution (p.Arg72Pro), and the Arg72-type was shown to be more susceptible than the Pro72-type to degradation by the E6 following human papilloma virus infection (34). The c.96+16_96+31dup16 variant type was shown to be associated with a reduced level of *p53* mRNA (35). However, both c.215G>C and c.96+16_96+31dup16 are common genetic polymorphisms (35). The others were located in the intron, and as far as we know their functional effect has never been reported. These findings suggested that the *p53*-c.91G>A (p.Val31Ile) mutation specifically found in the Japanese FGC family is a novel germline mutation.

Low transcriptional activation function of the Ile31-type p53

To evaluate the functional effect of the p.Val31Ile mutation, we first examined the transcriptional activation potential of the Ile31-type, because the mutation is located in the

transactivation domain of p53 protein. *p53*-null human H1299 cells were transiently cotransfected with the p53 expression vector, luciferase reporter vector pGL4.10 containing a fragment of the *p21* (*CDKN1A*), *BAX*, or *MDM2* promoter, and a transfection control vector pGL4.74, and 24 hr after transfection, luciferase activity was measured by a Dual-Luciferase Reporter Assay System and compared in wt p53 and Ile31-type p53. The Ala143 mutation located in the DNA binding domain of p53 protein was used as a positive control in the analysis, because the mutant has been shown to have low transcriptional activity (36). Empty mammalian expression vector alone was also prepared as a negative control. The p53 responsiveness of the *p21*, *BAX*, and *MDM2* genes and the presence of the p53 binding sequence in the promoter of these genes has been shown previously (37). As expected, p21-, BAX-, and MDM2- luciferase activity was much lower level in the cells transfected with Ala143-type p53 expression vector or empty vector than in the cells transfected with wt p53 expression vector (Figure 2). The comparison between wt and Ile31-type p53 showed that p21-luciferase and MDM2-luciferase, but not BAX-luciferase, activity was significantly lower in the cells transfected with the Ile31-type p53 expression vector than in cells transfected with the wt p53 expression vector (Figure 2). These results suggested that Ile31-type p53 has low transcriptional activity in relation to a subset of p53-responsive promoters.

Establishment of p53-inducible cell line

To precisely characterize the p.Val31Ile mutation, we decided to establish cell lines

expressing various types of p53 under the control of Dox. First, *p53*-null human cell line H1299 was infected with virus-containing supernatants from 293T cells cotransfected with pQCXIN-Tet-On and pCL10A1 plasmids, and cells were selected with G418. Next, the stable H1299-Tet-On cell line produced was infected with virus-containing supernatants from 293T cells cotransfected with pRevTRE-Tight p53 and pCL10A1 plasmids, and cells were selected with hygromycin B. These steps resulted in the production of a G418- and hygromycin B-resistant stable H1299-Tet-On-p53 cell lines. p53 protein was expressed in the H1299-Tet-On-p53 cells in the presence of Dox. We adjusted the concentration of Dox in the medium so that the level of p53 protein expression by the wt, Ile31-type, and Ala143-type p53-inducible H1299 cells was almost the same according to the results of Western blot analysis with anti-p53 monoclonal antibody (Figure 3A). Nuclear expression of each type of p53 was confirmed by immunofluorescence analysis with anti-p53 monoclonal antibody (Figure 3B).

Low ability of the Ile31-type p53 protein to suppress cell proliferation

Cell proliferation suppressing activity is one of the major activities of p53 protein (15, 16), and we used the H1299-Tet-On-p53 cell lines to compare the cell proliferation suppressing activity of wt p53 and Ile31-type p53. Cells were plated in medium with or without Dox, and 72 hr later the number of viable cells was counted by measuring the reduction of the tetrazolium monosodium salt WST-8. In the absence of Dox, the number of viable H1299-Tet-On-wt p53 cells and H1299-Tet-On-Ile31-type p53 cells was similar (Figure 4A).

However, in the presence of Dox, there were significantly fewer viable H1299-Tet-On-wt p53 cells than H1299-Tet-On-Ile31-type p53 cells (Figure 4B). These results suggested that Ile31-type p53 has lower cell proliferation suppressing activity than wt p53.

Frequency of the c.91G>A (p.Val31Ile) germline mutation in sporadic gastric cancer

To more clearly demonstrate that the c.91G>A (p.Val31Ile) germline mutation associated with low p53 activity is specific to the FGC20 family, we genotyped DNA from the blood of 110 sporadic gastric cancer patients for the mutation by PCR-SSCP analysis. The c.91G>A mutation was not found in any of them (data not shown), suggesting that the c.91G>A germline mutation is specific to a family with gastric cancer clustering.

Discussion

Sequencing analysis for germline *p53* mutations was performed in 80 individuals from 35 Japanese FGC families without germline *CDH1* mutations, and one missense (c.91G>A: p.Val31Ile) and two intronic germline mutations were found. The missense mutation of p.Val31Ile was detected as a heterozygote in only the proband from one FGC family, and is a novel germline *p53* mutation. The functional effect of the p.Val31Ile mutation was then examined to investigate the role of the p.Val31Ile mutation in the familial clustering of cancers. A luciferase reporter assay showed that transcriptional activity on *p21* and *MDM2* promoters but not the *BAX* promoter was significantly lower in those with Ile31-type p53 than

wt p53. Next, by applying a retrovirus-mediated gene transfer system to *p53*-null human cell line H1299, we established a stable cell line H1299 expressing p53 under Dox control. Expression of p53 protein was regulated by addition of Dox at a similar level of the Western blot analysis, and nuclear p53 expression was confirmed by immunofluorescence analysis. A cell proliferation assay of Ile31-type p53 showed significantly lower cell proliferation suppressing activity than wt p53. These results suggest that Ile31-type p53 may be partly involved in FGC because of its low transcriptional activity and low cell proliferation suppressing activity in comparison with wt p53.

There have been three reports of FGC families in which a germline mutation of the *p53* gene has been identified in Europe and Korea (22-24). One of the families meets the clinical criteria for LFS (24), but, the other two do not (22, 23). LFS is a cancer predisposition syndrome associated with sarcoma, breast cancer, brain tumors, leukemia, adrenocortical carcinoma, melanoma, and other cancers, including gastric cancer (19-21). However, because of the relatively low frequency of gastric cancer in LFS families with the characteristics of both LFS and FGC are thought to be rare, and in fact, the case described above is the only one even reported (24). On the other hand, when our Japanese case is added to the previous two German and Portuguese cases (22, 23), the number of FGC families with a germline *p53* mutation that do not meet the clinical criteria for LFS rise to three. As regards frequency of the detection of FGC with a germline *p53* mutation, according to the previous papers (22, 23) and the present paper one FGC with a germline *p53* mutation was found in 35 Japanese families with FGC, one among 34 German families with FGC, and one

among 10 Portuguese families with FGC. The results of this and previous studies suggested that the *p53* gene should be included in genetic testing of FGC families.

p53 plays an important role in the prevention of cancer by functioning in a transcription dependent and independent manner. It functions in a transcription dependent manner as a transcription factor that induces expression of downstream target genes that negatively control cell growth and viability (15, 16). The luciferase assay in the present study showed that transcriptional activity by Ile31-type *p53* on *p21* and *MDM2* was significantly lower than by wt *p53*. However, interestingly, the *p21*- and *MDM2*-luciferase activity of Ile31-type *p53* was higher than that of Ala143-type *p53*, a well known transcription-deficient mutant. Moreover, BAX- luciferase activity did not differ between wt *p53* and Ile31-type *p53*, in contrast to the extremely low activity of Ala143-type *p53*. These results suggested that Ile31-type *p53* has reduced transcriptional activity in relation to a subset of *p53*-responsive promoters, and from a different point of view, this means that the Ile31-type *p53* retains partial transcriptional activity towards those promoters. The low as opposed to completely abolished activity of Ile31-type *p53* may be involved in FGC but not LFS.

When we turned our attention to the difference in activity between Ile31-type *p53* and Ala143-type *p53*, we noted that the transcriptional activity was lower in the Ala143-type than in the Ile31-type, but that they had similar cell proliferation suppressing activity. One possible explanation for this discordance is that the transcriptional activity and cell proliferation suppressing activity levels of *p53* mutants are not always parallel, as has been

shown previously (38). If in addition to the results of the current study we compared p53 activities of wt p53, Ile31-type p53, and Ala143-type p53 proteins other than their transcriptional activity and cell proliferation suppressing activity, we would be able to evaluate the level of the mutant p53 protein ability more precisely. That might enable us to better understand the role of Ile31-type p53 in the occurrence of FGC.

In this study we used a retrovirus-mediated gene transfer system to establish Dox-regulated cell lines with inducible, doubly stable expression of p53 from *p53*-null H1299 cells. The tetracycline- or Dox (a derivative of tetracycline)-regulated gene expression system enables tight quantitative and temporal control of gene expression and has been widely used for inducible gene expression in mammalian cell culture (39). To more tightly regulate p53 expression in the present study we newly constructed pRevTRE-Tight p53 vector by substituting the *P* tight promoter region from the pTRE-Tight vector for the promoter region of pRevTRE vector. Since an inappropriately high level of p53 expression is cytotoxic and similar levels of p53 expression are needed to compare the activity of the various types of p53, tight regulation of p53 expression is indispensable to the establishment of exogenous p53 expressing cell lines and comparison of differences in activity among various types of p53. The Dox-regulated inducible expression system was therefore adopted to evaluate the function of p53 mutant in our study.

Acknowledgements

We are grateful to Dr. B. Vogelstein for providing us with wt and Ala143-type p53 cDNA and to Dr. S. Tsugane for sporadic gastric cancer samples. This work was supported in part by a Grant-in-Aid from the Ministry of Health, Labour and Welfare for the Comprehensive 10-Year Strategy for Cancer Control (15-22 and 19-19), from the Japan Society for the Promotion of Science for Scientific Research, and from the Ministry of Education, Culture, Sports, Science and Technology for priority area (18014009) and the 21st century COE program “Medical Photonics”. H.Y. and M.G. are COE research assistants in Hamamatsu University School of Medicine. We acknowledge T. Kamo for technical assistance.

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Table I. Germline *p53* mutations detected in FGC families

Nucleotide change ^{a, b}	Location	Predicted effect	No. of individuals having the mutation	Homozygous / Heterozygous	Family ID
c.91G>A	exon 3	p.Val31Ile	1	heterozygous	FGC20
c.1100+410G>A	intron 10	unknown	1	heterozygous	FGC34
c.1101-160G>A	intron 10	unknown	1	heterozygous	FGC17

^aMutation nomenclature is according to den Dunnen and Antonarakis (40) and den Dunnen and Paalman (41). Nucleotide +1 is the A of the ATG-translation initiation codon.

^bIn this paper we defined a germline mutation as a gene variation not registered in the SNP databases (dbSNP or JSNP). The following variations were also found in our analysis, but are not listed in the table because they have been registered in the dbSNP or JSNP database: c.74+38G>C, c.96+16_96+31dup16, c.97-29C>A, c.215G>C, c.672+62G>A, c.782+72C>T, c.782+92T>G, and c.1101-221G>A.

Figure legends

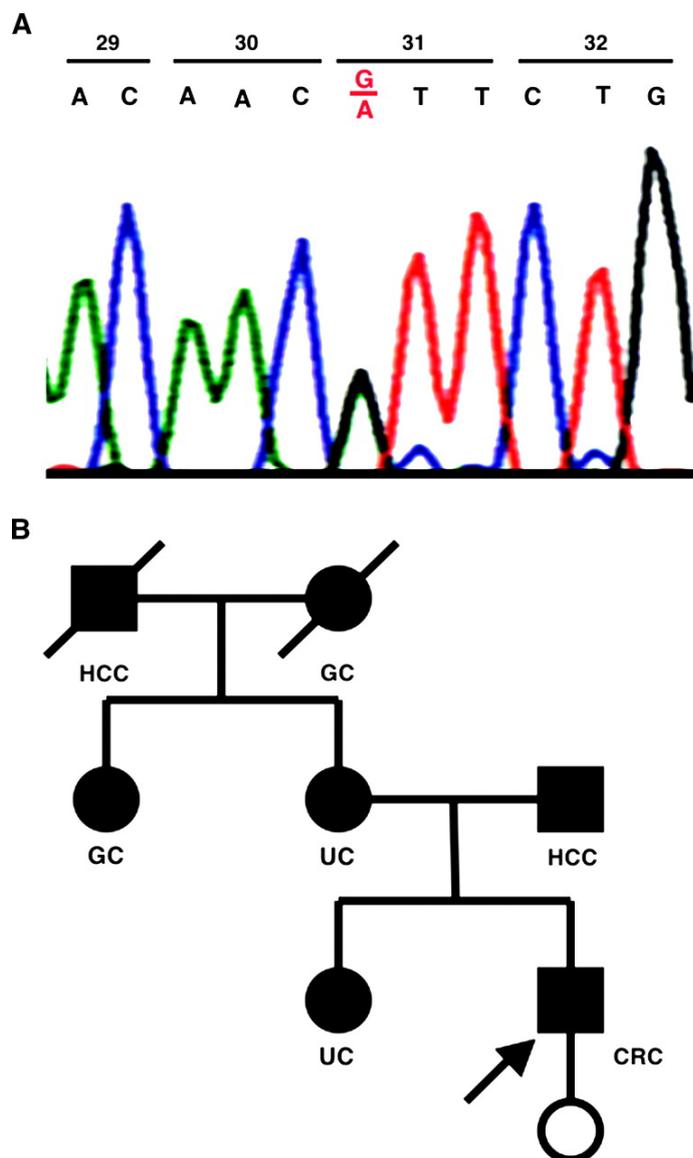


Fig. 1 Pedigree of the FGC family with the p.Val31Ile germline mutation of the *p53* gene.

(A) Detection of a germline *p53* mutation in DNA from the blood of a FGC family. Direct sequencing analysis of exon 3 of the *p53* gene revealed a heterozygote with a GTT allele and ATT allele at codon 31. (B) Pedigree of the FGC family. Square, male; circle, female.

Solid symbols represent cancer patients. Symbols with a slash indicate deceased individuals.

An arrow points to the proband. GC, gastric cancer; CRC, colorectal cancer; HCC,

hepatocellular carcinoma; UC, uterine cancer.

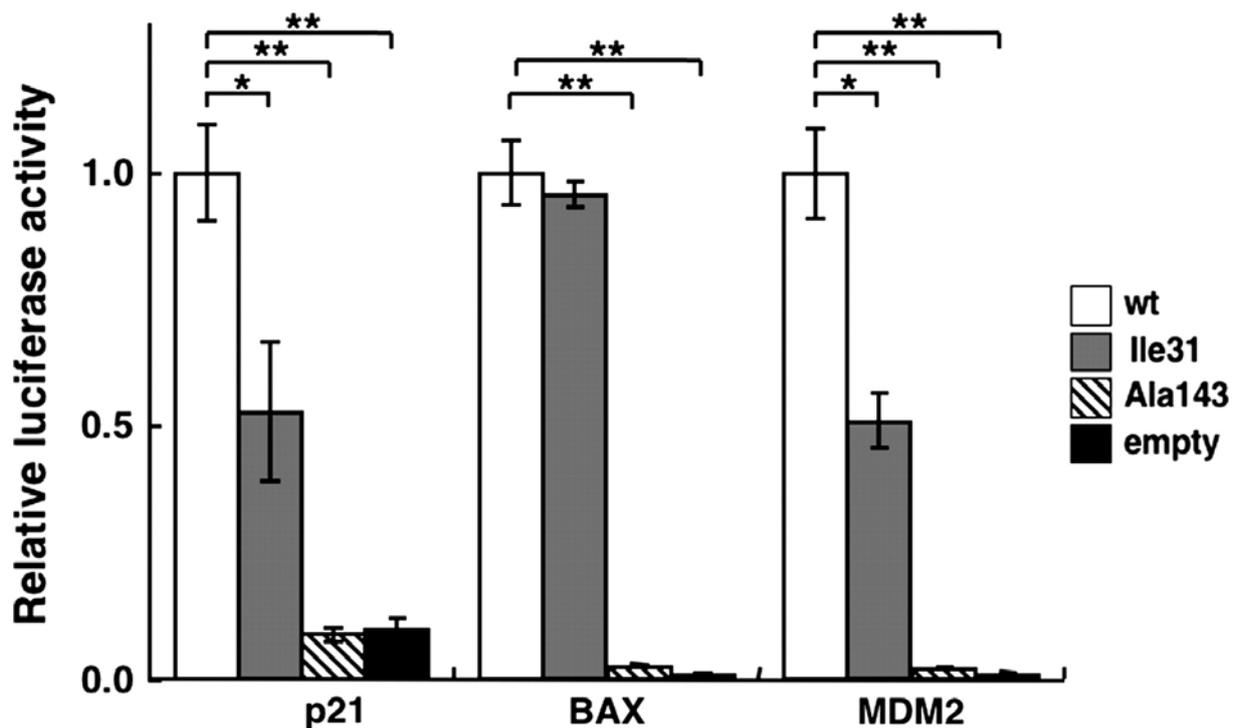


Fig. 2 Low transcriptional activation function of the Ile31-type p53 shown by luciferase assay. p21-, BAX-, and MDM2- luciferase activity was measured in p53-null H1299 cells transiently transfected with p53 expression vector, firefly luciferase reporter vector pGL4.10, and a transfection control vector pGL4.74. Luciferase activity was quantified with a Dual-Luciferase Reporter Assay System, and relative luciferase activity was calculated. Values are means \pm S.D. of three independent experiments. In each p53-responsive gene, i.e., p21, BAX, and MDM2 the luciferase activity of cells transfected with wt p53 was set equal to 1.0. P values were calculated by Dunnett's multiple comparison. *, $P < 0.001$; **, $P < 0.0001$.

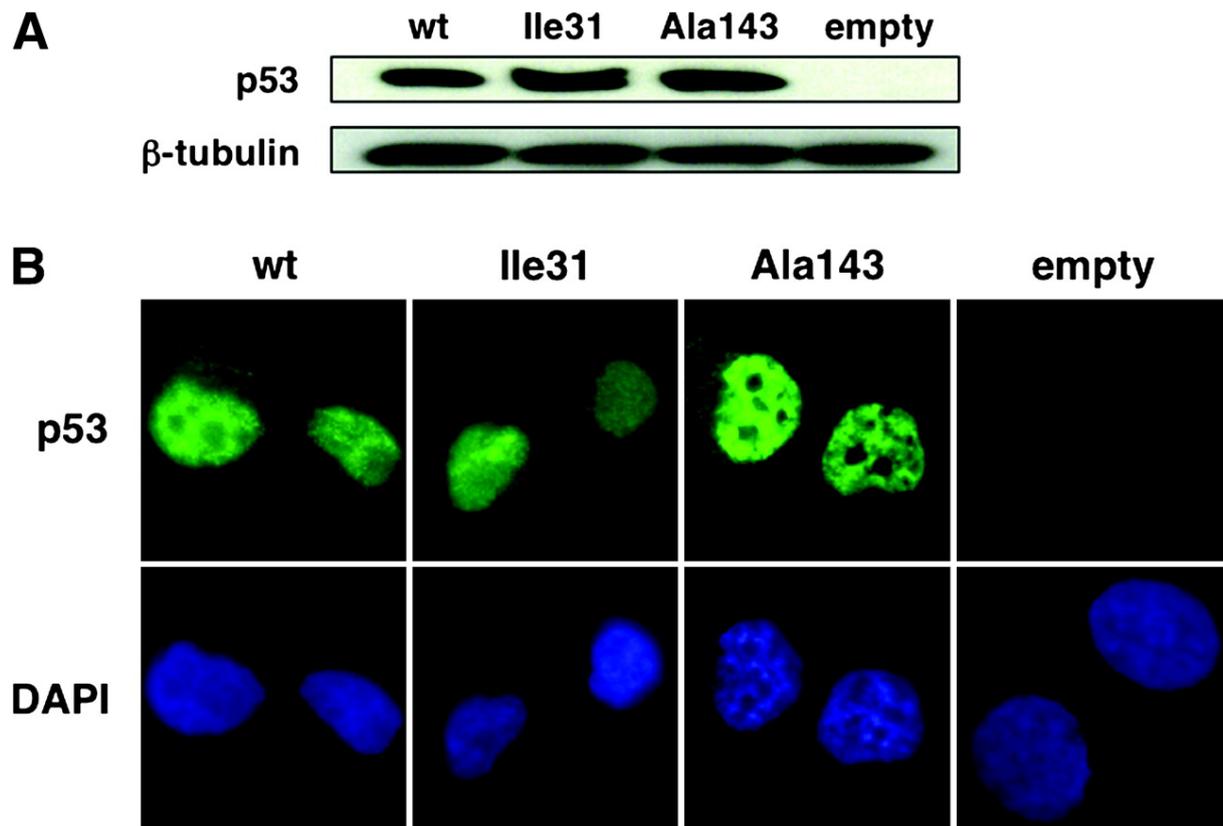


Fig. 3 Expression of p53 protein in p53-inducible H1299-Tet-On-p53 cell lines in the presence of Dox. (A) Detection of protein expression of wt, Ile31-type, and Ala143-type p53 by Western blot analysis with anti-p53 antibody DO7. (B) Detection of protein expression of wt, Ile31-type, and Ala143-type p53 by Immunofluorescence analysis. p53 protein (green) was stained with anti-p53 as the first antibody and with Alexa Fluor 488-conjugated goat anti-mouse IgG as the secondary antibody. The nuclei were counterstained with DAPI (blue).

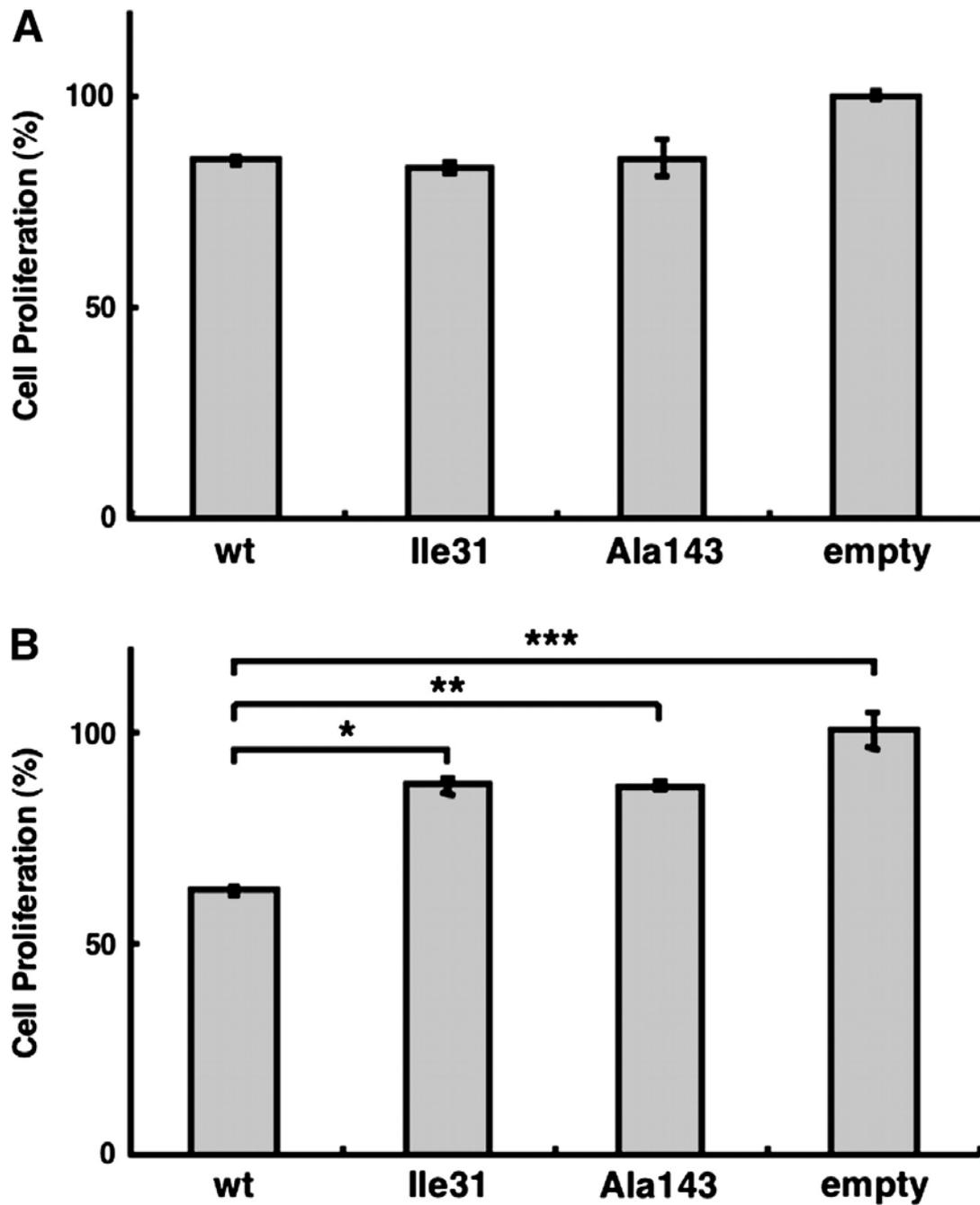


Fig. 4 Low ability of the Ile31-type p53 protein to suppress cell proliferation. The H1299-Tet-On-p53 cell lines were plated in medium without Dox (A) or with Dox (B), and 72 h later the number of viable cells was counted by measuring the reduction of the tetrazolium monosodium salt WST-8. Values are means \pm S.D. of three independent experiments. The number of H1299-Tet-On-empty vector cells was set equal to 100%. *P* values were calculated by the two-tailed Student's *t* test. *, $P < 0.0001$; **, $P < 0.001$; ***, $P < 0.01$.