Genetic alterations and reduced expression of tumor suppressor p33ING1b in human exocrine pancreatic carcinoma

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AIM: To detect the expression of p33ING1b protein and the change of p33ING1b gene in pancreatic carcinoma and to evaluate the significance of p33ING1b in pancreatic cell carcinogenesis.

METHODS: Pathological specimens from pancreatic carcinoma and matched non-tumor pancreatic tissues were examined for p33ING1b expression and mutation by immunohistochemistry, polymerase chain reaction single-strand conformation polymorphisms (PCR-SSCP) and loss of heterozygosity (LOH).

RESULTS: The rate of p33ING1b protein expression was 85% (34/40). A single germline missense mutation was detected in 1 of 40 tumors located at codon 215:TGC-TCC (Cys-Ser). Fourteen (60.9%) of 23 tumor samples showed LOH in all of the informative markers tested, but no mutation was detected in these tumors and only two of the informative tumors lacked expressions of p33ING1b protein.

CONCLUSION: Mutation and loss of expression are not the main reasons for the disfunction of p33ING1b in pancreatic carcinoma, an abnormality at the level of chromosome and/or transcription may inhibit their normal functions, potentially contributing to pancreatic cell carcinogenesis.

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INTRODUCTION

A candidate tumor suppressor gene, ING1, has recently been cloned and mapped on human chromosome 13q34, encoding p33ING1b mainly, a nuclear protein which physically interacts with p53[3,4] and cooperates with it in many ways[5,6]. Forced overexpression of the ING1 gene could lead to cell arrest in the G1 phase of the cell cycle and induce apoptosis in several cell types[5,6]. Conversely, inhibition of ING1 expression by antisense constructs could promote the transformation of mouse breast epithelial cells and increase the frequency of focus formation with NIH3T3 cells and protect cells from apoptosis. Recently, p33ING1b protein has been found to contain many specific structures, such as plant homeodomain (PHD)-like finger, nuclear localization sequence (NLS), nucleolar targeting sequence (NTS), proliferating cell nuclear antigen (PCNA) interacting protein (PIP) domain, Sin3-associated polypeptide (SAP30) interaction region and bromodomain which implicate that the p33ING1b protein might play a critical role with other genes in oncogenesis, apoptosis, DNA repair and cell cycle regulation[7-9].

High rates of 13q loss of heterozygosity (LOH) have been detected in cancer of the esophagus, ovary, breast and head and neck[10]. And several gene mutations and reduced protein expression of p33ING1b were found in head and neck, esophageal, lung, bladder, ovary, kidney, breast, and liver cell cancers[7]. Major sequence features and biological effects and genetic alterations of p33ING1b indicate that p33ING1b may be a tumor suppressor and that functional loss of p33ING1b might contribute to tumorigenesis. To the author’s knowledge, only one study showed that p33ING1b expression was increased in pancreatic adenocarcinoma cell line Mia PaCa-2 after incubation with COX-2 inhibitors for specific times[11]. But little is known about its role and changes in pancreatic cancer. Accordingly, we examined whether genetic alterations, such as allelic imbalance or mutations of p33ING1b gene, as well as altered protein expression of p33ING1b, might be responsible for the emergence and progression of human pancreatic carcinoma using immunohistochemical study, LOH analysis, and PCR-SSCP.

MATERIALS AND METHODS

Materials

Fourty tumor and 23 matched normal tissues were obtained from 40 patients with pancreatic carcinoma undergoing surgical resection as primary therapy for their disease after informed consent was obtained. All patients were from the Department of Pathology, Changhai Hospital. Another four pancreatitis and 5 normal samples were also from the department. Histological studies were performed and all tumors were confirmed as exocrine pancreatic carcinoma. Among these patients whose age ranged from 31 to 86 years, 24 were males and 16 were females, and the average age was 60.5 years. Four types of tumor were involved namely, adenocarcinoma, adeno-squamous cell carcinoma, cystadenocarcinoma, and acinar cell carcinoma. The number of cases was 36, 2, 1, and 1, respectively. Thirteen cases of adenocarcinoma were well differentiated, and the other 23 cases were moderately or poorly differentiated.

DNA Extraction

Genomic DNAs were isolated from formalin-fixed paraffin-embedded sections and frozen by proteinase K treatment (method D)[12], phenol-chloroform extraction, and ethanol precipitation following the manufacturer’s instructions.

Immunohistochemistry

The expression of p33ING1b protein in paraffin-embedded
histological sections was determined using the EnVision method. Histological sections (4 µm thick) on 0.2 g/L poly-L-lysine-coated slides were deparaffinized and rehydrated, and the endogenous peroxidase activity was blocked by incubation with 20 mL/L H2O2 in phosphate buffer, followed by pre-treatment with proteinase K. Non-specific binding was blocked with normal goat serum, and sections were incubated with p33ING1b antibody (supplied by Dr. Riabowol, Canada)\[1\]. After washed with phosphate buffer, the sections were incubated with secondary antibody and enhanced labelled polymers for 3 h and washed with phosphate buffer, followed by incubation with DAB. Then the sections were washed and counterstained with methyl green. Sections of normal pancreas tissue were used as positive control for the expression of p33ING1b, and the sections incubated with PBS instead of the corresponding primary antibody were used as negative controls. To quantitate the p33ING1b expression in various samples, a scoring method was adopted\[13\]. A mean percentage of positive tumor cells was determined in at least five areas at ×400 magnification and assigned to one of the five following categories: 0, <5%; 1, 5-25%; 2, 25-50%; 3, 50-70%; and 4, >75%. The intensities of immunostaining were scored as follows: weak, 1+; moderate, 2+; and intense, 3+. For tumors showing heterogeneous staining, the predominant pattern was taken into account for scoring. The percentage of positive tumor cells and the staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores of less than 1 were defined as negative, otherwise as positive.

SSCP Analysis

The coding region of exon1 was amplified by PCR with primers on the flanking regions, primers S1 (5′-ATCTTGAGCTCTGCCAACGGGGA) and AS1 (5′-CTCCTGTTATTTGCGTGCA), 138 bp; exon2 was amplified as four overlapping fragments with four primer sets: (a) S2 (5′-ATCTTGAGCTCTGCCAACGGGGA) and AGTGCTAGCTGACCGATGCAGCTGACGGGGA) and AS2 (5′-CTCCTGTTATTTGCGTGCA), 246 bp; (b) S3 (5′-TTCCAGGCGAGCTGACGGGGA) and AS3 (5′-CTCCTGTTATTTGCGTGCA), 210 bp; (c) S4 (5′-CAAAGGCAAGCCAGCCAGCAAGCGGCGAAGCGGGGGA) and AS4 (5′-TCAAGGCAAGCCAGCAAGCGGCGAAGCGGGGGA) and AS5 (5′-CTCCTGTTATTTGCGTGCA), 235 bp. The PCR mixture contained 400 ng of DNA, 5 µL of 25 mmol/L MgCl2, 5 µL of 10xPCR buffer, 5 µL of 2.5 mmol/L deoxynucleotide triphosphate, 25 µL of each primer, and 1.25 unit of Taq DNA polymerase (Takara, Kyoto, Japan) in a 50-µL volume. Initial denaturation at 94 °C for 4 min was followed by 35 amplification cycles, each consisting of a denaturation at 94 °C for 1 min, an annealing at 55 °C for 1 min, and an extension at 72 °C for 1 min. A final extension step at 72 °C for 10 min was carried out. Fifteen µL of PCR products as mixed with 8 µL of 3xloading dye (95% formamide, 0.5 g/L EDTA, 0.5 g/L bromphenol blue, and 0.5 g/L xylene cyanol), heat denatured, chilled on ice, applied onto an 80 g/L polyacrylamide gel containing 8 mol/L urea. DNA bands were visualized by silver staining. LOH was scored if one of the heterozygous alleles showed at least 50% reduced intensity in tumor DNA as compared with the corresponding normal DNA.

Statistical analysis

The SPSS 11.0 software package for Macintosh (SPSS Inc., Chicago, IL) was used for all statistical analyses. Variables associated with p33ING1b expression were analyzed by χ² test. Differences in expression of p33ING1b mRNA between groups were checked by independent t test. P<0.05 was considered statistically significant.

RESULTS

Detection of p33ING1b gene mutation

To investigate whether the p33ING1b gene was the target of functional loss in tumors, we searched for mutations in the coding regions of the gene in all the 40 samples of pancreatic carcinoma. Only one possible mutation was identified by SSCP and DNA sequencing (Figure 2). The sample showed a substitution of cysteine (TGC) with serine (TCC) at codon 215, which located in plant homeodomain (PHD) finger. This change might affect the PHD finger and break the three-dimensional structure of ING1 protein, leading to the loss of function. But this sample still showed p33ING1b protein expression, implicating that the protein expressed in tumor could not exert its normal function.

LOH analysis

We examined DNA from 23 pairs of matched pancreatic carcinomas and normal tissues for losses at four microsatellite markers (D13S261, D13S1047, D13S1315 and DS42490) on the chromosome 13q33-34 region. Figure 3 shows the summary and representative examples. Fourteen of 23 informative tumors (60.9%) showed LOH of at least one marker. The rates of LOH at D13S261, D13S1047, D13S1315 and DS42490 loci were 8.7%, 21.5%, 13.0% and 26.1%, respectively. The most frequent loss was seen at marker DS42490, which was located in the ING1 region. These markers were less than 3 Cm. Only two of these pancreatic carcinomas showed no p33ING1b protein expression and none had a single mutation.
Figure 1  Representative examples of IHC staining for p33ING1b (×200) on normal pancreatic tissues and cancer tissues. A: Weak positive pancreatic cells for p33ING1b; B: No p33ING1b; C and D: Extremely strong staining in pancreatic cancer samples.

Figure 2  SSCP analysis of p33ING1b gene in genomic DNA from pancreatic carcinomas. N: normal DNA; T: tumor DNA. Arrow point indicates the altered band in tumor DNA as compared with the corresponding normal DNA. The amino acid substitution of p33ING1b are shown to the right.

Figure 3  LOH analysis on chromosome 13q33-34 in PCs. A: Submatic representation of LOH distribution; ● LOH; ○ without LOH; a: cancer; b: matched cancer; B: Analysis of D13S1315; C: LOH analysis of D13S261; D: LOH analysis of DS42490; E: LOH analysis of D13S1047.
DISCUSSION

Several studies showed that reduced $p33^{ING1}$ expression was found in esophageal squamous cell cancer (100%)\[12\] and hepatocellular carcinoma (66.3%)\[17\]. In our study, positive human diploid fibroblast\[30\]. More detailed studies are needed pancreatic carcinomas and no missense mutation of p33ING1 gene loss of p33ING1b protein expression (8.7%) was very low in D13S1047 located on the upstream of ING1, D13S1315 on the rate of LOH in esophageal/head and neck squamous cell cancers was 48% (21/44)\[10\]. But the range between every two markers was more than 1 cm, far from ING1 locus, and these markers could not reflect the particular information about ING1. So we selected four markers, D13S261, D13S1047, D13S1315, and D5S2490, to detect LOH in 23 cancer tissues. D13S261 and D13S1047 located on the upstream of ING1, D13S1315 on the downstream, while D5S2490 in the ING1 locus. The results were described as above. As compared with the LOH (60.9%), the loss of $p33^{ING1}$ protein expression (8.7%) was very low in pancreatic carcinomas and no missense mutation of $p33^{ING1}$ gene was found. The lack of mutations suggests that the ING1 gene is not the suppressor gene target of tumors in which frequent LOH was observed at 13q. The high frequency of LOH at ING1 locus and the vicinity may affect the function of $p33^{ING1}$ but not the expression of $p33^{ING1}$ protein. The reason is very complex. An alternative target of inactivation may be involved in this tumor type which can affect the function of $p33^{ING1}$ protein. In addition, alterations at the transcriptional or posttranscriptional level may be another reason for the abnormal expression of $p33^{ING1}$ protein, for several experiments also detected reduced expression of $p33^{ING1}$ mRNA in primary breast cancers and cell lines\[23,28\], human gastric cancer\[22\], myeloid leukaemia\[29\], head and neck squamous cell carcinoma\[20\] and human diploid fibroblast\[50\]. More detailed studies are needed to elucidate this possibility.

REFERENCES

1. Garkavtsev I, Kazarov A, Gudkov A, Riabowol K. Suppression of the novel growth inhibitor $p33^{ING1}$ promotes neoplastic transformation. Nat Genet 1996; 14: 415-420
2. Cheung KJ Jr, Li G. The tumor suppressor ING1 structure and function. Exp Cell Res 2001; 268: 1-6
3. Garkavtsev I, Grigorian IA, Ossovskaya VS, Chernov MV, Chumakov PM, Gudkov AV. The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control. Nature 1998; 391: 295-298
4. Leung KM, Po LS, Tsang FC, Siu WY, Lau A, Ho HT, Poon RY. The candidate tumor suppressor ING1b can stabilize p53 by disrupting the regulation of p53 by MDM2. Cancer Res 2002; 62: 4890-4893
5. Helbing CC, Veillette C, Riabowol K, Johnston RN, Garkavtsev I. A novel candidate tumor suppressor, ING1, is involved in the regulation of apoptosis. Cancer Res 1997; 57: 1255-1258
6. Shimada H, Liu TL, Ochiai T, Shimizu T, Haupyt Y, Hamada H, Abe T, Oka M, Takiguchi M, Hiwasaki T. Facilitated adenoviral wild-type p53-induced apoptotic cell death by overexpression of $p33^{ING1}$ in T. Th human esophageal carcinoma cells. Oncogene 2002; 21: 1208-1216
7. Feng X, Hara Y, Riabowol K. Different HATS of the ING1 gene family. Trends Cell Biol 2002; 12: 532-538
8. Scott M, Bonnefin P, Vieyra D, Boisvert FM, Young D, Bazett-Jones DP, Riabowol K. UV-induced binding of ING1 to PCNA regulates the induction of apoptosis. J Cell Sci 2001; 114(Pt 19): 3453-3462
9. Aasland R, Gibson TJ, Stewart AF. The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem Sci 1995; 20: 56-59
10. Sanchez-Cespedes M, Okami K, Cairns P, Sidransky D. Molecular analysis of the candidate tumor suppressor gene ING1 in human head and neck tumors with 13q deletions. Genes Chromosomes Cancer 2000; 27: 319-322
11. Tseng WW, Deganiutti A, Chen MN, Saxton RE, Liu CJ. Selective cyclooxygenase-2 inhibitor rofecoxib (Vioxx) induces expression of cell cycle arrest genes and slows tumor growth in human pancreatic cancer. J Gastrointest Surg 2002; 6: 838-844
12. Frank TS, Svoboda-Newman SM, Hsi ED. Comparison of methods for extracting DNA from formalin-fixed paraffin sections for nonisotopic PCR. Diagn Mol Pathol 1996; 5: 220-224
13. Lu CD, Altieri DC, Tanigawa N. Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. Cancer Res 1998; 58: 1808-1812
14. Bassam BJ, Caetano-Anolles G, Greshoff PM. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 1991; 196: 80-83
15. Borodina TA, Ivanov DV, Khusnutdinova EK, Spitsyn VA, Baranov A, Iankovskii NK. A new pentanucleotide STR-marker, located in the intron of the ING1 tumor suppressor gene and its allelic polymorphism. Genetika 2001; 37: 117-119
16. Chen L, Matsbara N, Yoshino T, Nagasaka T, Itsuzu of adenoviral wild-type p53-induced apoptotic cell death by overexpression of $p33^{ING1}$ in T. Th human esophageal carcinoma cells. Oncogene 2002; 21: 1208-1216
17. Ohgi T, Masaki T, Nakai S, Morishita A, Yamakata S, Nagai M, Miyauchi Y, Funaki T, Kurokohchi K, Watanabe S, Kuriyama S. Expression of $p33^{ING1}$ in hematopoietic cell carcinoma: relationships to tumour differentiation and cyclin E kinase activity. Scand J Gastroenterol 2002; 37: 1440-1448
18. Campos EI, Cheung KJ Jr, Murray A, Li S, Li G. The novel tumour suppressor gene ING1 is overexpressed in human melanoma cell lines. Br J Dermatol 2002; 146: 574-580
19. Chen B, Campos EI, Crawford R, Martinka M, Li G. Analyses of the tumour suppressor ING1 expression and gene mutation in human basal cell carcinoma. Int J Oncol 2003; 22: 927-931
20. Gunduz M, Ochida M, Fukushima K, Hanafusa H, Etani T, Nishioka S, Nishizaki K, Shimizu K. Genomic structure of the human ING1 gene and tumor-specific mutations detected in head and neck squamous cell carcinomas. Cancer Res 2000; 60: 3143-3146
21. Hara Y, Zheng Z, Evans SC, Malatajian D, Riddell DC, Guernsey DL, Wang LD, Riabowol K, Casson AG. ING1 and p53
tumor suppressor gene alterations in adenocarcinomas of the esophagogastric junction. Cancer Lett 2003; 192: 109-116

22 Oki E, Maehara Y, Tokunaga E, Kakeji Y, Sugimachi K. Reduced expression of p33 (ING1) and the relationship with p53 expression in human gastric cancer. Cancer Lett 1999; 147: 157-162

23 Toyama T, Iwase H, Watson P, Muzik H, Saettler E, Magliocco A, DiFrancesco L, Forsyth P, Garkavtsev I, Kobayashi S, Riabowol K. Suppression of ING1 expression in sporadic breast cancer. Oncogene 1999; 18: 5187-5193

24 Krishnamurthy J, Kannan K, Feng J, Mohanprasad BK, Tsuchida N, Shanmugam G. Mutational analysis of the candidate tumor suppressor gene ING1 in Indian oral squamous cell carcinoma. Oral Oncol 2001; 37: 222-224

25 Ohmori M, Nagai M, Tasaka T, Koeffler HP, Toyama T, Riabowol K, Takahara J. Decreased expression of p33ING1 mRNA in lymphoid malignancies. Am J Hematol 1999; 62: 118-119

26 Sarela AI, Farmery SM, Markham AF, Guillou PJ. The candidate tumour suppressor gene, ING1, is retained in colorectal carcinomas. Eur J Cancer 1999; 35: 1264-1267

27 Bromidge T, Lynas C. Relative levels of alternative transcripts of the ING1 gene and lack of mutations of p33/ING1 in haematological malignancies. Leuk Res 2002; 26: 631-635

28 Tokunaga E, Maehara Y, Oki E, Kitamura K, Kakeji Y, Ohno S, Sugimachi K. Diminished expression of ING1 mRNA and the correlation with P53 expression in breast cancers. Cancer Lett 2000; 52: 15-22

29 Ito K, Kinjo K, Nakazato T, Ikeda Y, Kizaki M. Expression and sequence analyses of p33 (ING1) gene in myeloid leukemia. Am J Hematol 2002; 69: 141-143

30 Garkavtsev I, Riabowol K. Extension of the replicative life span of human diploid fibroblast by inhibition of the P33ING1, candidate tumor suppressor. Mol Cell Biol 1997; 17: 2014-2019

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