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Received May 2, 2007; revised September 5, 2007; accepted September 12, 2007

Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 251 (2007) 211–219
CANCER Letters
www.elsevier.com/locate/canlet

Increased expression of DNA methyltransferase 1 (DNMT1) protein in uterine cervix squamous cell carcinoma and its precursor lesion

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Received 1 September 2006; received in revised form 17 November 2006; accepted 21 November 2006

Abstract

Aberrant DNA methylation has been shown to play important roles during multistage carcinogenesis in various human organs. The aim of this study was to evaluate the significance of DNA methyltransferase 1 (DNMT1) protein expression during cervical carcinogenesis. We carried out an immunohistochemical examination for DNMT1 in 34 samples of histologically normal squamous epithelium, 36 samples of low-grade cervical intraepithelial neoplasia (CIN), 61 samples of higher-grade CIN and 30 samples of squamous cell carcinoma of the uterine cervix. The DNMT1 protein expression score, reflecting the intensity and incidence of DNMT1 nuclear immunoreactivity, was increased even in low-grade CIN ($P < 0.0001$) in comparison with histologically normal squamous epithelium and was further increased in higher-grade CIN ($P < 0.0001$ compared to low-grade CIN). The DNMT1 protein expression score remained at a plateau in microinvasive carcinoma (Stage IA, $P = 0.0690$ compared to higher-grade CIN) and then decreased with cancer invasion (Stage IB or more, $P = 0.0176$ compared to Stage IA), whereas the proliferating cell nuclear antigen (PCNA) labeling index did not decrease with cancer invasion ($P = 0.8259$ between Stage IA and Stage IB or more). Thus, the DNMT1 protein expression score and the PCNA labeling index were not mutually correlated in squamous cell carcinoma of the uterine cervix ($P = 0.2304$). These data suggest that progressively increasing expression of DNMT1 protein is not entirely a secondary result of increased cell proliferative activity, but is associated with an early step of multistage cervical carcinogenesis.

Keywords: DNA methylation; DNA methyltransferase 1 (DNMT1); Proliferating cell nuclear antigen (PCNA); Cervical intraepithelial neoplasia (CIN); Squamous cell carcinoma; Uterine cervix

1. Introduction

DNA methylation plays important roles in transcriptional regulation, chromatin remodeling and genetic stability. Overall DNA hypomethylation and regional DNA hypermethylation are commonly

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doi:10.1016/j.canlet.2006.11.023

observed in human cancers of various organs [1–3]. Aberrant DNA methylation may play roles in carcinogenesis as a result of (a) increased gene mutagenicity due to deamination of 5-methylcytosine to thymine; (b) possible association of aberrant DNA methylation with genetic instability; and (c) silencing of tumor-related genes through DNA methylation on CpG islands in cooperation with histone modification [1]. Furthermore, accumulating evidence suggests that aberrant DNA methylation is involved even in the early and precancerous stages of human carcinogenesis [4,5]. Precancerous conditions showing aberrant DNA methylation may rapidly progress and generate more malignant cancers [6,7].

DNA methyltransferase 1 (DNMT1) is the major human DNMT [1]. As DNMT1 shows a preference for hemimethylated rather than unmethylated substrates *in vitro* [8], and targets replication foci by binding to proliferating cell nuclear antigen (PCNA) [9], it has been considered to be a maintenance form of DNMT that copies methylation patterns after DNA replication. However, some workers have proposed that DNMT1 possesses both maintenance and *de novo* DNA methylation activity *in vivo* [10,11], regardless of its *in vitro* substrate preference. We have reported that DNMT1 protein overexpression precedes an increase of the PCNA labeling index in precancerous conditions in the urinary bladder [12], and is significantly correlated with poorer differentiation of liver [13], stomach [14] and pancreatic [15] cancers and a poorer prognosis of patients with liver [13] and pancreatic [15] cancers. Moreover, in stomach [14], colorectal [16], urinary bladder [17] and pancreatic [18] cancers, DNMT1 overexpression is significantly correlated with the CpG island methylator phenotype (CIMP) [19], which is defined as frequent DNA hypermethylation on C-type CpG islands that are usually methylated in a cancer-specific (not age-dependent) manner, or accumulation of DNA hypermethylation of multiple tumor-related genes.

With respect to cervical carcinogenesis, an early study demonstrated global DNA hypomethylation in squamous cell carcinoma and its precursor lesion [20], and some subsequent studies have revealed accumulation of DNA hypermethylation of tumor-related genes in squamous cell carcinoma [21–24]. However, to our knowledge, expression of DNMT1 has never been reported during multistage cervical carcinogenesis to date. In this study we carried out an immunohistochemical examination of DNMT1 in a series of tissue samples of uterine cervix squamous cell carcinoma and its precursor lesion.

2. Materials and methods

2.1. Patients and samples

Thirty-four samples of histologically normal squamous epithelium, 36 samples of low-grade CIN (CIN1) [25], 61 samples of higher-grade CIN (CIN2 and CIN3) [25] and 30 samples of squamous cell carcinoma (10 samples of microinvasive caricinoma [Stage IA] [26] and 20 samples of invasive carcinoma [Stage IB or more] [26]) of the uterine cervix were used for immunohistochemistry. Histopathological diagnosis and clinical staging were performed on the basis of previously described criteria [25,26]. All 161 tissue samples were obtained from 49 patients (mean age ± SD, 46.88 ± 11.72 years [range, 26–74 years]) who underwent conization or hysterectomy because of higher-grade CIN or squamous cell carcinoma of the uterine cervix at the National Cancer Center Hospital, Tokyo. With respect to 10 samples of microinvasive carcinoma, their microinvasive components, not non-invasive components, were examined immunohistochemically. There were no patients from whom multiple tissue samples were obtained metachronously. This study was approved by the Ethics Committee of the National Cancer Center, Tokyo.

2.2. Immunohistochemistry

Five-micrometer-thick sections of formalin-fixed, paraffin-embedded tissue specimens were deparaffinized and dehydrated. For antigen retrieval, the sections were heated for 10 min at 120 °C in an autoclave. Non-specific reactions were blocked with 2% normal swine serum. All sections were incubated with specific primary antibodies directed against DNMT1 (goat polyclonal antibody, sc-10219; Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:1000) and PCNA (mouse monoclonal antibody, p56720; Transduction Laboratories, Lexington, KY; dilution 1:200). We had previously confirmed the specificity of the goat anti-human DNMT1 polyclonal antibody by Western blotting analysis: an immunoreactive band of about 193.5 kDa, corresponding to the molecular mass of DNMT1, was detected in human cancer cells, but no non-specific bands were detected [13]. Both primary antibody incubations were conducted at 4 °C overnight, and were followed by incubation with biotinylated secondary antibodies (anti-goat IgG or anti-mouse IgG, Vector Laboratories, Burlingame, CA; dilution 1:200) at room temperature for 30 min. The sections were then treated with Vectastain Elite ABC reagent (Vector Laboratories).

3.3′-Diaminobenzidine tetrahydrochloride was used as the chromogen, and all sections were counterstained with hematoxylin. As a negative control, the primary antibodies were omitted from the reaction sequence.

In all examined patients without exception, uniform intensity of DNMT1 immunoreactivity was detected in the nucleus of parabasal cells of the histologically normal squamous epithelium of the uterine cervix. Therefore, parabasal cells were used as internal positive controls in all samples. Without exception, DNMT1 immunoreactivity was detected only in the nucleus, and never in the cytoplasm or cell membrane. The intensity of DNMT1 nuclear immunoreactivity in each sample was graded as 0 (less than the internal positive control), 1 (equal to the internal positive control) and 2 (more than the internal positive control). For each sample, at least 500 cells were randomly counted. If the lesion was small with less than 500 cells, all the cells were counted. The incidence of DNMT1 nuclear immunoreactivity in each sample was graded as 0 (less than 20% of the counted cells), 1 (20% or more, and less than 50%) and 2 (50% or more). The DNMT1 protein expression score (0, 1, 2 or 4) was determined as the product of the intensity grade (0, 1 or 2) and the incidence grade (0, 1 or 2). The PCNA labeling index in each sample was expressed as a percentage of all the cells counted.

2.3. Statistics

Comparisons of the DNMT1 protein expression score and the PCNA labeling index between sample groups were analyzed by the chi-squared test and the Mann–Whitney *U*-test, respectively. Comparisons of mean patient age among sample groups were analyzed by the Kruskal–Wallis test. Correlations of the DNMT1 protein expression score on the one hand and patient age or the PCNA labeling index on the other were analyzed by the chi-squared test and the Mann–Whitney *U*-test, respectively. For all tests, $P < 0.05$ was considered to be the level of significance.

3. Results

3.1. DNMT1 protein expression score in uterine cervix squamous cell carcinoma and its precursor lesion

Fig. 1 shows examples of immunohistochemical staining for DNMT1 in tissue samples. The distribution of the DNMT1 protein expression score in each sample group is summarized in Table 1. The DNMT1 protein expression score increased progressively from histologically normal squamous epithelium, to low-grade CIN ($P < 0.0001$ compared to histologically normal squamous epithelium), and to higher-grade CIN ($P < 0.0001$ compared to low-grade CIN). DNMT1 protein expression peaked in higher-grade CIN, and there was no significant increase of the DNMT1

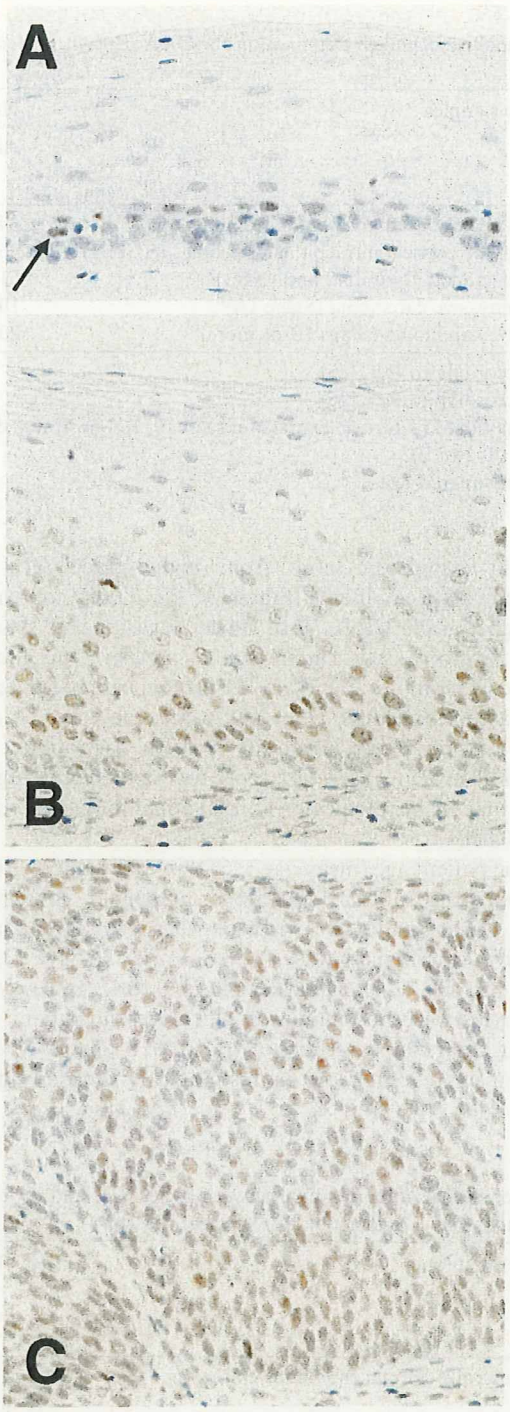

Fig. 1. Immunohistochemical examination for DNMT1 in histologically normal squamous epithelium (A) and CINs (B and C) of the uterine cervix. (A) Parabasal cells (arrow) of histologically normal squamous epithelium served as positive controls. The DNMT1 protein expression score was 0 in this sample. (B) DNMT1-positive cells were observed in low-grade CIN (DNMT1 protein expression score: 1). (C) The incidence of DNMT1 nuclear immunoreactivity was further increased in higher-grade CIN (DNMT1 protein expression score: 2) 280×.

Table 1
Immunohistochemical examination for DNA methyltransferase (DNMT) 1 in uterine cervix squamous cell carcinoma and its precursor lesion

Tissue samples	Number of specimens (%)					P^d
	Analyzed	DNMT1 protein expression score[c]				
		0	1	2	4	
Histologically normal squamous epithelium	34	34 (100)	0 (0)	0 (0)	0 (0)	<0.0001
Low-grade cervical intraepithelial neoplasia (CIN1)[a]	36	16 (44.4)	18 (50)	2 (5.6)	0 (0)	<0.0001
Higher-grade CIN (CIN2 and CIN3)[a]	61	3 (4.9)	29 (47.5)	29 (47.5)	0 (0)	0.0690
Microinvasive carcinoma (Stage IA)[b]	10	1 (10)	5 (50)	3 (30)	1 (10)	0.0176
Invasive carcinoma (Stage IB or more)[b]	20	8 (40)	12 (60)	0 (0)	0 (0)	

[a] According to Ref. [25].
[b] According to Ref. [26].
[c] The DNMT1 protein expression score (0, 1, 2 or 4) was determined as the product of the intensity grade (0, 1 or 2) and the incidence grade (0, 1 or 2).
[d] Chi-squared test.

protein expression score from higher-grade CIN to microinvasive carcinoma (Stage IA, $P = 0.0690$ compared to higher-grade CIN). With cancer invasion and extent, from microinvasive carcinoma to invasive carcinoma (Stage IB or more), the DNMT1 protein expression score decreased significantly ($P = 0.0176$ between Stage IA and Stage IB or more). Even in invasive carcinoma, the DNMT1 protein expression score was significantly higher than that in histologically normal squamous epithelium ($P < 0.0001$).

The patient age (mean age ± SD) in each sample group (histologically normal squamous epithelium, low-grade CIN, higher-grade CIN, microinvasive carcinoma and invasive carcinoma) was 45.53 ± 11.70, 45.89 ± 11.97, 44.21 ± 11.69, 42.40 ± 8.86 and 48.25 ± 13.47, respectively, and there were no significant differences in patient age among the sample groups ($P = 0.7682$). The DNMT1 protein expression score in histologically normal squamous epithelium, low-grade CIN, higher-grade CIN and squamous cell carcinoma each did not correlate with patient age (Table 2). The DNMT1 protein expression score in squamous cell carcinoma ($n = 30$) did not correlate with the clinicopathological parameters (presence or absence of lymphatic vessel involvement [$P = 0.2954$], venous involvement [$P = 0.7370$] and lymph node metastasis [$P = 0.6163$], data not shown).

As mentioned above, the average DNMT1 protein expression score in invasive carcinoma (Stage IB or more) was lower than that in microinvasive carcinoma or non-invasive higher-grade CIN. In fact, the incidence of DNMT1 nuclear immunoreactivity was lower in the central portion of large nests of invading cancer cells than that in non-invasive lesions. However, even in such invasive carcinoma, cancer cells often showed particularly strong DNMT1 nuclear immunoreactivity at the leading front of deep invasion (Fig. 2).

Table 2
Lack of significant correlation between patient age and expression level of DNA methyltransferase (DNMT) 1 protein in uterine cervix squamous cell carcinoma and its precursor lesion

Tissue samples	Age (y)	Number of patients (%)					P^c
		Analyzed	DNMT1 protein expression score[b]				
			0	1	2	4	
Histologically normal squamous epithelium	Less than 47	18	18 (100)	0 (0)	0 (0)	0 (0)	
	47 or more	16	16 (100)	0 (0)	0 (0)	0 (0)	
Low-grade cervical intraepithelial neoplasia (CIN1)[a]	Less than 47	19	10 (52.6)	7 (36.8)	2 (10.5)	0 (0)	0.1504
	47 or more	17	6 (35.3)	11 (64.7)	0 (0)	0 (0)	
Higher-grade CIN (CIN2 and CIN3)[a]	Less than 47	35	1 (2.9)	19 (54.3)	15 (42.9)	0 (0)	0.3918
	47 or more	26	2 (7.7)	10 (38.5)	14 (53.8)	0 (0)	
Squamous cell carcinoma	Less than 47	15	4 (26.7)	9 (60)	2 (13.3)	0 (0)	0.6815
	47 or more	15	5 (33.3)	8 (53.3)	1 (6.7)	1 (6.7)	

[a] According to Ref. [25].
[b] The DNMT1 protein expression score (0, 1, 2 or 4) was determined as the product of the intensity grade (0, 1 or 2) and the incidence grade (0, 1 or 2).
[c] Chi-squared test.

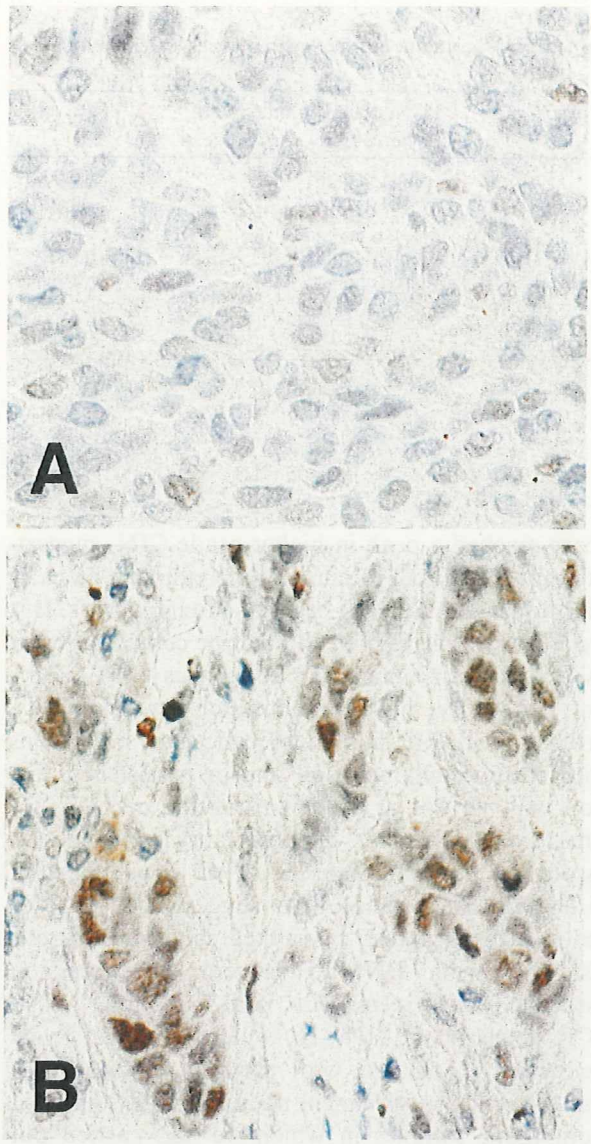

Fig. 2. Immunohistochemical examination for DNMT1 in invasive squamous cell carcinoma of the uterine cervix. Although only weak DNMT1 nuclear immunoreactivity (DNMT1 protein expression score: 1) was detected in the center of a large nest of invasive carcinoma (A), cancer cells showed particularly strong nuclear DNMT1 immunoreactivity at the invading front (B) 560×.

3.2. PCNA lebeling index in uterine cervix squamous cell carcinoma and its precursor lesion

Fig. 3 shows examples of immunohistochemical staining for PCNA in tissue samples. The PCNA labeling index of each sample group (a mean ± SD) is summarized in Table 3. Unlike the DNMT1 protein expression score, the PCNA labeling index increased progressively from histologically normal squamous epithelium, to low-grade CIN ($P < 0.0001$ compared to histologically normal squamous epithelium), higher-grade CIN ($P < 0.0001$ compared to low-grade CIN) and microinvasive carcinoma (Stage IA, $P = 0.0112$ compared to higher-grade CIN). The PCNA labeling index did not decrease with cancer invasion and remained at a plateau in invasive carcinoma (Stage IB or more, $P = 0.8259$ to Stage IA). Thus, the DNMT1 protein expression score and the PCNA labeling index did not show mutual correlation in squamous cell carcinoma of the uterine cervix ($n = 30$, $P = 0.2304$, Fig. 4).

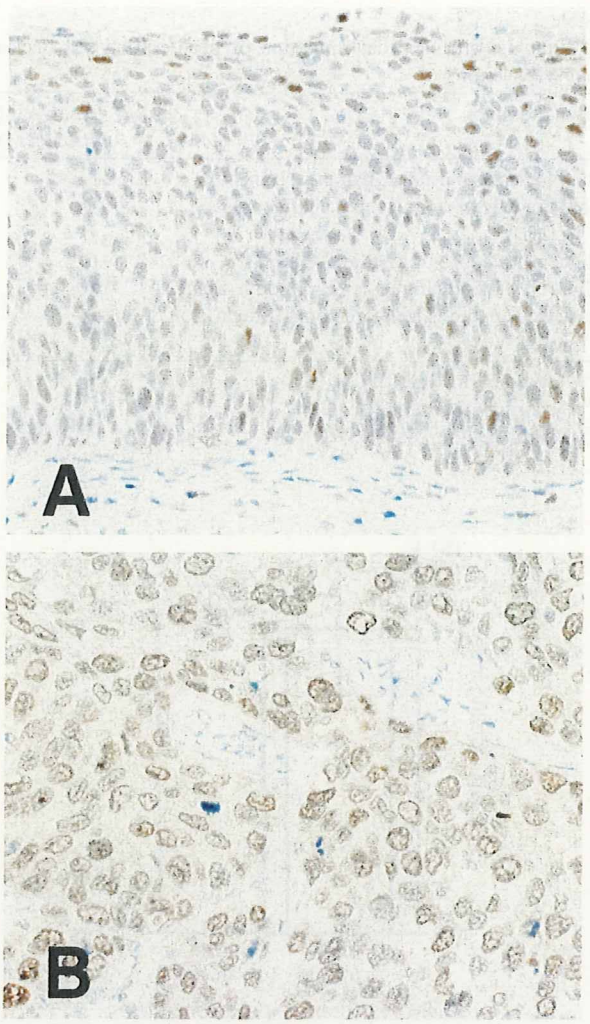

Fig. 3. Immunohistochemical examination for PCNA in higher-grade CIN (A) and invasive squamous cell carcinoma (B) of the uterine cervix. The PCNA labeling index did not decrease with cancer invasion. 280×.

4. Discussion

We believe that this is the first report to describe DNMT1 protein expression in uterine cervix squamous cell carcinoma and its precursor lesion.

Table 3
Immunohistochemical determination of proliferating cell nuclear antigen (PCNA) in uterine cervix squamous cell carcinoma and its precursor lesion

Tissue samples	Analyzed (number of specimens)	PCNA labeling index (mean ± SD)	P^c
Histologically normal squamous epithelium	34	4.03 ± 8.581	<0.0001
Low-grade cervical intraepithelial neoplasia (CIN1)[a]	36	37.419 ± 13.595	<0.0001
Higher-grade CIN (CIN2 and CIN3)[a]	61	74.711 ± 20.716	0.0112
Microinvasive squamous cell carcinoma (Stage IA)[b]	10	89.190 ± 9.563	0.8259
Invasive squamous cell carcinoma (StageIB or more)[b]	20	87.310 ± 13.601	

[a] According to Ref. [25].
[b] According to Ref. [26].
[c] Mann–Whitney *U*-test.

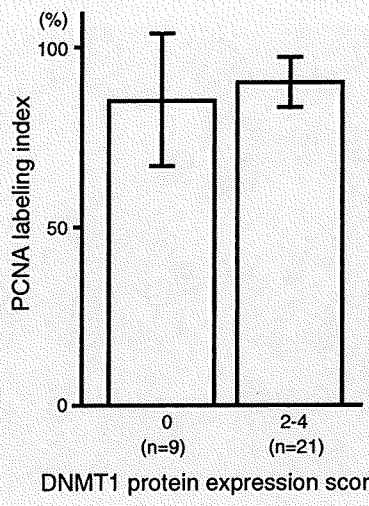

Fig. 4. DNMT1 protein expression score and PCNA labeling index in squamous cell carcinom of the uterine cervix. The PCNA labeling index is expressed as mean ± SD. DNMT1 protein expression level and cell proliferative activity were not mutually correlated ($P = 0.2304$).

DNMT1 protein expression was found to be significantly increased in squamous cell carcinoma in comparison with histologically normal squamous epithelium. There were no significant differences in patient age between the sample groups. Therefore, DNMT1 protein overexpression was not attributable to aging, but showed a possible association with cervical carcinogenesis.

Histopathological and clinical observations have revealed that CIN is a precursor lesion for squamous cell carcinoma of the uterine cervix [25]. Even in low-grade CIN, significant elevation of the DNMT1 protein expression score was detected in comparison with histologically normal squamous epithelium, and the DNMT1 protein expression score reached a peak in higher-grade CIN, suggesting that DNMT1 protein overexpression is an early event during multistage cervical carcinogenesis. It is well known that CIN of the uterine cervix is closely associated with human papillomavirus (HPV) infection [27,28]. We have frequently observed DNMT1 protein overexpression in precancerous conditions with chronic inflammation and/or persistent infection with viruses or other pathogenic microorganisms, such as chronic hepatitis and liver cirrhosis associated with hepatitis B or C virus infection [6,29–35]. The present data suggest that CIN of the uterine cervix can be ranked as a precancerous lesion showing DNMT1 protein overexpression associated with persistent viral infection. When PCR detection of HPV-16, which is known to be frequently associated with cervical carcinogensis, was performed in specimens microscopically dissected from tissue samples in which we had carried out immunohistochemical examination for DNMT1, the DNMT1 protein expression score did not correlate with the presence or absence of HPV-16 infection in low-grade CIN ($P = 0.1715$), higher-grade CIN ($P = 0.4569$) and squamous cell carcinoma ($P = 0.5836$, data not shown). Although HPV-16 infection may not directly affect the DNMT1 expression level, HPV-16 E7 protein has been reported to directly associate with DNMT1 and stimulate the methyltransferase activity of DNMT1 *in vitro* [36]. Overexpressed DNMT1 may be activated by HPV-16 E7 protein during cervical carcinogenesis. Since DNMT1 protein overexpression is a very early event during multistage cervical carcinogenesis, it is not surprising that the DNMT1 protein expression score did not correlate with clinicopathological parameters reflecting tumor aggressiveness

or progression, such as lymphatic and venous involvement, and lymph node metastasis.

DNMT1 mRNA is expressed mainly during S-phase in normal cells, and because tumor tissue presumably contains a greater proportion of dividing cells than normal tissue, there has been some debate as to whether increased DNMT1 mRNA expression is due to an increase in the proportion of dividing cells or to an acute increase in DNMT1 expression per individual cell [37]. This continuing discussion prompted us to compare the DNMT1 protein expression score and the PCNA labeling index in tissue samples. DNMT1 protein overexpression reached a peak in higher-grade CIN and microinvasive carcinoma, and the peak of the DNMT1 protein expression score preceded that of the PCNA labeling index. The DNMT1 protein expression score was decreased in invasive carcinoma, whereas the PCNA labeling index was retained at the same level. Thus, the DNMT1 protein expression level did not correlate with cell proliferative activity in tissue specimens, suggesting that DNMT1 protein overexpression does not result entirely from increased numbers of dividing cells during cervical carcinogenesis. We have previously observed a similar discrepancy between DNMT1 protein expression level and cell proliferative activity in precancerous conditions in the urinary bladder [12] and in certain subgroups of stomach [14] and renal [7] cancers. Several mechanisms for regulation of DNMT1 expression have been proposed in human cancers. Dysfunction of p53 tumor suppressor protein results in DNMT1 mRNA overexpression [38] and DNMT1 protein is stabilized in cultured cancer cells [39]. Aberrant DNA methylation is closely associated with cancers preceded by chronic inflammation and/or persistent viral infection, and cytokine interleukin-6 treatment [40] and induction of latent membrane protein 1 of Epstein–Barr virus [41], which is associated with nasopharyngeal and stomach cancers, induce DNMT1 overexpression in cultured cancer cells. The molecular mechanisms responsible for DNMT1 overexpression during multistage cervical carcinogeneis should be clarified.

DNMT1 targets replication foci, where DNA methylation patterns are copied from the mother strand, by binding to PCNA [9]. However, targeting of the substrate DNA by DNMT1 may be disrupted by mechanisms such as dysfunction of p21WAF1, which competes with DNMT1 for binding to PCNA, in cancer cells [42]. Moreover, it has recently been suggested that DNMT1 is capable of *de novo* methylation activity as well as having a maintenance function [10,11]. Therefore, it is feasible that, in cancers, DNMT1 overexpression participates in the *de novo* methylation of CpG islands. In fact, we have already reported that DNMT1 mRNA or protein overexpression is significantly correlated with CIMP of stomach [14], colorectal [16], and urinary bladder [17] cancers, and with accumulation of DNA hypermethylation of multiple tumor-related genes in pancreatic cancer [18]. DNMT1 protein overexpression may thus also become a background factor associated with silencing of tumor-related genes during multistage cervical carcinogenesis. In fact, reduction of DNMT1 expression results in re-expression of the E-cadherin gene in cultured cervical cancer cells [43]. We tried to evaluate the DNA methylation status of the promoter regions of multiple tumor-related genes in specimens microscopically dissected from formalin-fixed and paraffin-embedded tissue samples in which we had performed immunohistochemical examination for DNMT1. However, we were able to examine only a small number of genes and specimens because the microdissected specimens were tiny and the DNA was degraded. Further technical improvement will be needed in order to identify tumor-related genes that may be targeted by overexpressed DNMT1 in our cohort.

The level of DNMT1 protein expression initially increased until higher-grade CIN and microinvasive carcinoma, and then decreased in invasive carcinoma. In our previous study of the urinary bladder, although DNMT1 protein expression was lower in invasive carcinoma than in carcinoma *in situ*, CIMP was continuously maintained during progression from carcinoma *in situ* to invasive carcinoma [17]. DNMT1 overexpressed at an early stage may be related not only to DNA hypermethylation of tumor-related genes, but also to silencing of genes by recruiting the transcriptional repressor protein complex including DMAP1, DNMT1-associated protein, and histone deacetylases [44,45]. DNA hypermethylation on multiple CpG islands may be maintained even if DNMT1 expression is diminished to some extent during malignant progression.

Even after the peak of DNMT1 protein overexpression, heterogeneous expression was observed in some invasive carcinomas: cancer cells often showed particularly strong DNMT1 nuclear immunoreactivity at the invading front (Fig. 2). There appears to be a mechanism regulating DNMT1 protein expression, possibly one that depends on cancer–stromal interactions and/or the

microenvironment of cancer cells. Such findings are consistent with the particularly strong DNMT1 expression at the invading front of urinary bladder cancers with unfavorable clinical outcome [12]. DNMT1 reactivated at the invading front may locally silence an additional set of genes with anti-invasive function.

Acknowledgements

This work was supported by a Grant-in-Aid for the Third Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan, and a program for promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio). M. Sawada is a recipient of a research resident fellowship from the Foundation for Promotion of Cancer Research in Japan.

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Genome-wide array-based comparative genomic hybridization analysis of pancreatic adenocarcinoma: Identification of genetic indicators that predict patient outcome

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(Received August 1, 2006/Revised November 17, 2006/Accepted November 19, 2006/Online publication January 8, 2007)

We analyzed the subchromosomal numerical aberrations of 44 surgically resected pancreatic adenocarcinomas by array-based comparative genomic hybridization. The aberration profile ranged widely between cases, suggesting the presence of multiple or complementary mechanisms of evolution in pancreatic cancer, and was associated with lymph node metastasis and venous or serosal invasion. A large number of small loci, previously uncharacterized in pancreatic cancer, showed non-random loss or gain. Frequent losses at 1p36, 4p16, 7q36, 9q34, 11p15, 11q13, 14q32-33, 16p13, 17p11-13, 17q11-25, 18q21-tel, 19p13, 21q22 and 22q11-12, and gains at 1q25, 2p16, 2q21-37, 3q25, 5p14, 5q11-13, 7q21, 7p22, 8p22, 8q21-23, 10q21, 12p13, 13q22, 15q13-22 and 18q11 were identified. Sixteen loci were amplified recurrently. We identified novel chromosomal alterations that were significantly associated with a range of malignant phenotypes. Gain of LUNX, HCK, E2F1 and DNMT3b at 20q11, loss of p73 at 1p36 and gain of PPM1D at 17q23 independently predicted patient outcome. Expression profiling of amplified genes identified Smurf1 and TRRAP at 7q22.1, BCAS1 at 20q13.2-3, and VCL at 10q22.1 as potential novel oncogenes. Our results contribute to a complete description of genomic structural aberrations and the identification of potential therapeutic targets and genetic indicators that predict patient outcome in pancreatic adenocarcinoma. (*Cancer Sci* 2007; 98: 392–400)

Pancreatic adenocarcinoma is a leading cause of cancer-related death worldwide; the 5-year survival rate for patients that underwent surgery remains below 5%.[1] Pancreatic adenocarcinoma appears to successively acquire genetic aberrations in genes involved in the regulation of cell proliferation, the central ones being early activating mutations of the K-ras oncogene, followed by inactivation of the p53, p16 and DPC4 TSG.[2] The application of chromosome CGH,[3] karyotype and allelotype studies in pancreatic cancer has also revealed a large number of complex structural and numerical aberrations at the subchromosomal level.[4–11] Recurrent aberrations reported concern copy number gain on 3q, 5p, 7p, 8q, 11q, 12p, 17q, 19q and 20q and loss on 1p, 3p, 4q, 6q, 8p, 9p, 10q, 12q, 13q, 15q, 17p, 18q, 19p, 21q and 22q.[8,12] aCGH methods have recently been developed and used in studies of various malignancies, including pancreatic cancer. The latter used cell lines,[13–18] and a small number of primary cases[14,15] or xenografts,[19] to confirm previously described regional alterations and identify novel ones. Although some of these loci are known to contain oncogenes or TSG,[2] the role that copy number alterations of most of the above loci play in pancreatic cancer genesis or progression, if any, is far from being fully evaluated. From these and previous studies, it is also evident that there exists substantial variation in the reported aberrations between studies as well as between individual cases.

The aim of the present study was to examine the SNAP of pancreatic cancer to identify novel loci that contain genes for which copy number status is likely to be relevant to pancreatic carcinogenesis or associated with clinically relevant parameters. For this, we used aCGH to examine a comparatively large number of well-characterized primary cases and LCM to allow more accurate analysis. In addition, mRNA expression analysis of loci exhibiting amplifications was carried out to identify genes that are amplified recurrently and overexpressed in pancreatic cancer.

Materials and Methods

Tumor samples. Forty-four methanol-fixed pancreatic ductal adenocarcinomas from 43 patients were examined (Suppl. Table 1). These included 33 specimens from patients who had undergone surgery at the National Cancer Center Hospital between 1994 and 2003, and 11 xenografts that were produced following the orthotopic implantation of tumors in severe combined immunodeficient mice, as described previously.[20] Forty-two samples were of primary tumors, one of a liver metastasis and one of a pancreatic xenograft of a liver metastasis, the corresponding primary of which was also examined. Tumor classification was carried out according to the Japan Pancreas Society guidelines.[21] The study was approved by the institutional review board of the National Cancer Center.

LCM and whole-genome amplification. LCM was carried out with a PixCell II (Arcturus Engineering, Mountain View, CA, USA). At least 5000 tumor cells per sample were recovered. Genomic (test) DNA was extracted by standard procedures. Sex-matched high molecular weight human genomic DNA (Promega, Madison, WI, USA) was sheared randomly (HydroShear; Gene Machines, San Carlos, CA, USA) and used as reference DNA. Both test and reference DNA were amplified

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Abbreviations: aCGH, array-based comparative genomic hybridization; BAC, bacterial artificial chromosome; CGH, comparative genomic hybridization; HD, homozygous deletion; LCM, laser-capture microdissection; PCR, polymerase chain reaction; SNAP, subchromosomal numerical aberration profile; TSG, tumor suppressor gene.

doi: 10.1111/j.1349-7006.2007.00395.x

Fig. 1. Chromosomal copy number changes revealed by array-based comparative genomic hybridization. Representative array-based comparative genomic hybridization profile of a pancreatic adenocarcinoma. Copy number losses (ratio < 0.75) and gains (ratio > 1.25) were detected in both large fractions of the chromosome arms and small chromosomal regions. Amplifications (ratio > 2.00, arrowheads) and homozygous deletions (ratio < 0.25, arrow) were also identified in this tumor. The average signal ratios (test:reference) of two normalized signals from duplicated spots are given from chromosome 1p telomere (left) to Xq telomere (right). The vertical dotted and continuous lines indicate the position of the centromere and telomere of each chromosome, respectively.

using an adaptor ligation-mediated whole-genome PCR, as described previously.[22]

Array-based CGH. A custom-made CGH array ('MCG Cancer Array-800 ver. 2') was used, consisting of 800 duplicated target BAC clones that correspond to chromosomal loci of potential importance in various cancers (listed at http://www.cghtmd.jp/CGHDatabase/microarray/mcg800_array_e.htm). Labeling of the DNA probes, hybridization, data acquisition and data normalization were carried out as described previously.[23–25] Based on control experiments,[26] we considered a signal ratio <0.75 or >1.25 to indicate loss or gain, respectively, and a ratio of <0.25 or >2.00 to indicate HD or amplification, respectively.

The validity of our aCGH data was confirmed by fluorescence *in situ* hybridization, PCR (Suppl. Fig. 1), loss of heterozygosity analysis and immunohistochemistry for selected genes.[26]

Expression profiling of primary xenografts. We used xenografts for gene expression analysis due to their abundance in tumors cells compared with primary tumors. We focused on the relationship between amplification and overexpression; additional gene expression profiling results will be submitted in a subsequent publication.

Total RNA was extracted from frozen xenograft samples, biotin-labeled cRNA synthesized and hybridized to a probe array (HG-U95Av2, Affymetrix) and data acquired as described.[27] A probe set signal log ratio (SLR) of the gene expression level in the tumor relative to the control (normal pancreas) >1.5 was defined as indicating overexpression.

Statistical analysis. The χ^2 test was used to assess the statistical significance, set at 0.05, of intergroup differences in the frequency of aberrations of individual loci. The relationship between clinicopathological parameters and the number of aberrations per case was evaluated using Student's unpaired *t*-test. Survival curves were calculated using the Kaplan–Meier method, and differences in survival periods were analyzed with the log-rank test.

Results

Range of numerical aberrations. We constructed and analyzed the genomic profile of 44 pancreatic adenocarcinomas using aCGH. Subchromosomal numerical aberrations were revealed in all but two (42/44) of the tumors examined (Fig. 1). The number of aberrations differed widely between cases (Suppl. Table 2; Suppl. Fig. 2). Apart from the two cases in which no copy number changes were observed, a third case showed changes in only 11 loci (all gains), whereas nine cases (20%) had alterations in more than 50% of loci. In most cases (34/44), the number of gains was higher than the number of losses ($P < 10^{-7}$). Overall, however, the loss rate was similar to the gain rate (19% of loci altered on average per case for both). Similarly, amplifications were observed more frequently, in terms of number of cases and number of aberrations per case, than HD. Most loci showed aberrations in at least one case, the majority showing loss or gain in 2–25% and 0–20% of cases, respectively.

Loss. The most frequently lost loci were 17p13.3 (ABR, in 75% of cases), 18qtel (CTDP1, SHGC-145820, 68%) and 18q21 (SMAD7, 66%). The loci containing the p16 (9p21), p53 (17p13.1), SMAD4 and DCC (both at 18q21) genes were lost in 41, 55, 61 and 30% of cases, respectively. In total, 33 loci with frequent (>50%) losses were identified at 1p36, 4p16.3, 7q36, 9q34.3, 11p15, 11q13, 14q32-33, 16p13.3, 17p11.2, 17p13.1-3, 17q11-qter, 17q21.2, 17q25, 18q21, 18qtel, 19p13.2-3, 21q22.3, 22q11.23 and 22q12.1-2 (Fig. 2). The chromosome arms with the highest number of loci lost, taking into account only loci that were lost in >25% of cases, included, in descending order of frequency, 1p, 11q, 17p, 10q, 8p, 18q, 22q, 6q, 9p, 14q and 17q (Suppl. Table 3).

Homozygous deletions. Twenty-six loci with HD were detected, nine of which were in more than one case (Table 1). HD were detected in 11 cases (25%), seven of which in only one locus. The 1p35-36.33 region contained the highest number of loci deleted (six). The most frequently deleted locus was 9p21 spanning the p16 gene, whereas the locus containing SMAD4 (18q21) was deleted in one case.

Gains. Loci with frequent (>50% cases) gains were identified at 1q25.2-q25.3, 2p16, 2q21.2, 2q23-q37, 2q31, 2q33, 2q34, 3q25.1, 5p14.2, 5q11.2-q13.2, 7q21.1, 7p22; 8p22, 8q21, 8q22-q23, 10q21.1, 12p13.33, 13q22, 15q13-q22 and 18q11.2 (Fig. 2; Suppl. Table 4). The most frequently gained locus was 7q21.1 (71%) containing the HGF gene. The loci spanning the KRAS2 (12p12.1) and KRAG (12p11.2) genes were gained in 45 and 20% of cases, respectively. The NRAS (1p13), MYC (8q24), MDM2 (12q14.3) and AKT1 (14q32.2)[28] loci were gained in 45, 43, 36 and 18% of cases, respectively.

Amplifications. Amplifications were observed in 37 tumors. The seven cases in which no amplification was observed included six with few aberrations, and, interestingly, one case with 419 aberrations. Nineteen cases had amplifications in more than 1%, and three cases in more than 5% of loci examined.

Sixteen loci were amplified in five cases or more (>10%) (Table 2). The most frequently amplified locus was 18q11.2 containing RBBP8. 7q34 (BRAF) was amplified in four cases, whereas 12p12.1 (KRAS2), 1p13 (NRAS) and 8q24 (MYC) were amplified in two cases each.

Association of SNAP with clinicopathological parameters. A number of clinicopathological parameters were associated with the degree and type of aberration (Suppl. Table 5). Overall, cases with a phenotype indicating increased malignant potential had a higher degree of aberrations. Smaller tumors and tumors with higher venous or perineural invasion histological scores had a higher total number of aberrations than tumors that were larger or with lower invasion scores. No other clinicopathological parameters examined, such as the sex, primary tumor location, macroscopic type (infiltrative or nodular), degree of differentiation (Suppl. Table 6), infiltration or otherwise of certain neighboring tissues, pattern of such infiltration (INF α, β, or γ), or spread within the main pancreatic duct had significant correlation with SNAP (data not shown).

Association with venous invasion. Venous invasion-negative tumors had markedly different SNAP than venous invasion-positive tumors, although it should be noted that only a small number of negative tumors was examined. The loci lost or gained more frequently in the venous invasion-positive tumors are shown in

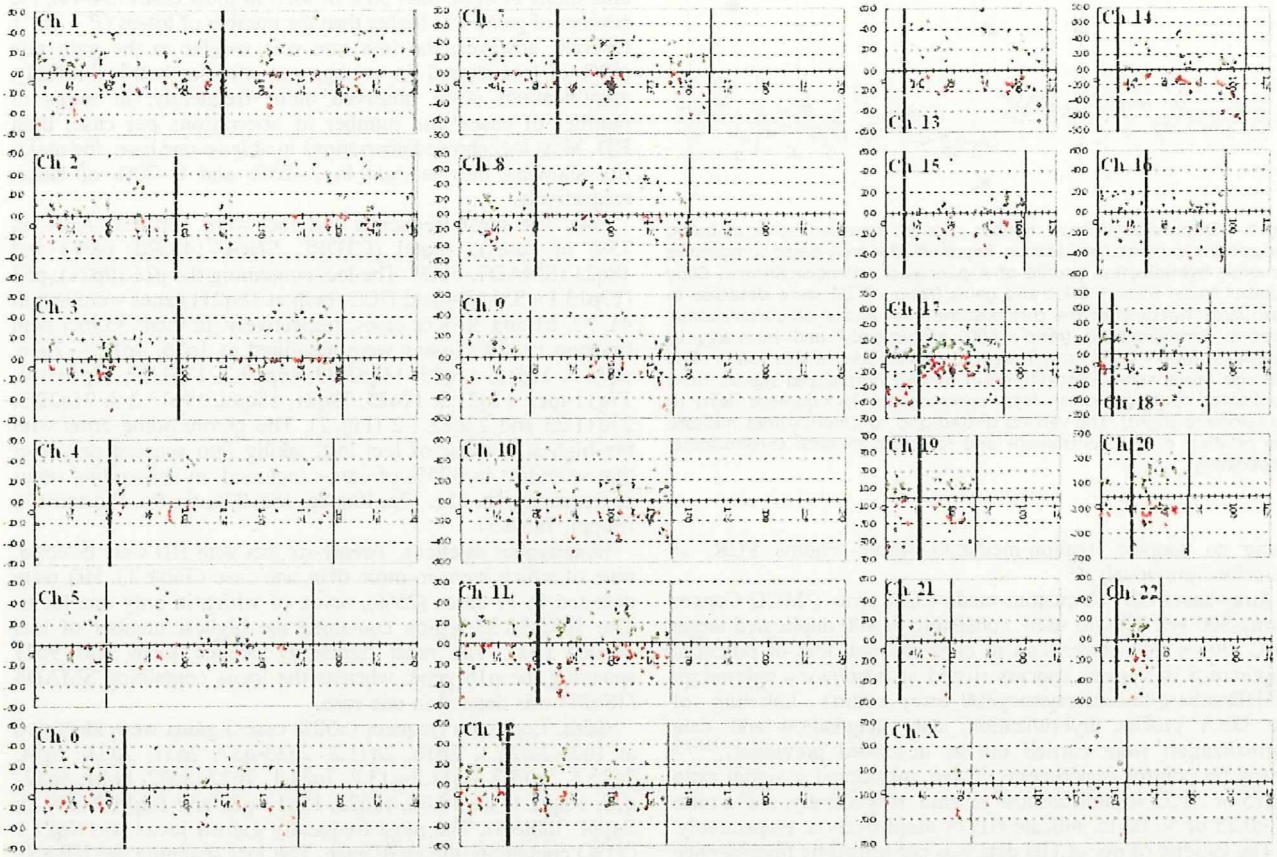

Fig. 2. Distribution of chromosomal copy number aberrations in pancreatic cancer. The horizontal axis indicates the physical distance (Mb) of the chromosomal loci from the telomere of the short arm. The vertical axis indicates the frequency (%) of tumors with chromosomal alterations (green, gain; red, loss). The vertical dotted and continuous lines indicate the positions of the centromere and telomere of each chromosome, respectively.

Table 1. Loci deleted homozygously in more than one case

Locus†	No. cases	Percentage of cases
9p21 (p16)	5	11
16p13.3 (ABCA3)	3	7
1p36.1 (p73)	2	5
5p15 (TERT)	2	5
11p15 (HRAS)	2	5
17q25 (MAFG)	2	5
18q21 (SMAD7)	2	5
18qtel (CTDP1,SHGC-145820)	2	5
19p13.3 (ABCA7)	2	5

†Known cancer-related genes contained in the respective clones are shown in parentheses.

Table 2. Loci amplified in more than 10% of cases

Locus†	No. cases	Percentage of cases
18q11.2 (RBBP8)	10	23
7q21.1 (HGF)	9	20
2q31 (PMS1)	7	16
11q13.3 (BCL1,FGF4)	7	16
2q34 (ERBB4)	6	14
11q13 (CCND1)	6	14
7q22.1 (Smurf1)	6	14
8q21 (NBS1)	5	11
2p16 (GTBP)	5	11
7p22 (ETV1)	5	11
2q21.2 (LRP1B)	5	11
2q35 (HUP2)	5	11
6q22 (ROS1)	5	11
8p11.2-p11.1 (FGFR1)	5	11
7q22.1 (CYP3A4)	5	11
7q22.1 (TRRAP)	5	11

†Known cancer-related genes contained in the respective clones are shown in parentheses.

Table 3. HD were not observed in the venous invasion-negative tumors (0/5 vs 19/37, $P = 0.03$). In the venous invasion-negative tumors, 178 and 84 loci, respectively, were lost or gained more frequently than in the positive tumors; these included frequently amplified loci (3/5) such as the ones containing FGF7 (15q13-q22), BRAF (7q34) ($P \leq 4.1 \times 10^{-5}$), ROS1 (6q22), GTBP (2p16) ($P = 0.0004$) and HGF (7q21.1) ($P = 0.02$).

Association with lymph node metastasis. Unlike venous invasion, few differences were observed when the genomic profiles of lymph node metastasis-positive and lymph node metastasis-negative tumors were compared, although only six negative tumors were examined. Only three loci showed significant differences in their signal ratios, 9p13 (SCYA21), 11q22 (ATM) and 17q12 (RAD51L3). Xq28 (MAGEA2) was lost more frequently in the lymph node metastasis-negative group (3/6 vs

doi: 10.1111/j.1349-7006.2007.00395.x

Table 3. Loci altered frequently in the venous invasion-positive pancreatic adenocarcinomas

Chromosomal locus	Contained cancer-related gene	Sub-chromosomal loss detected				*P* value†
		Venous invasion-positive cases		Venous invasion-negative cases		
		n	%	*n*	%	
19p13.3	ABCA7	26	70	0	0	0.002
9q34.3	ABCA2	25	68	1	20	0.040
1p36.33	TP73	22	59	0	0	0.012
11q13	FGF3	22	59	0	0	0.012
4p16	GAK	20	54	0	0	0.023
11q12	LTBP3	20	54	0	0	0.023
20q13	Livin	20	54	0	0	0.023
18q22	BCL2	19	51	0	0	0.030
5p14.2	CDH10	25	68	0	0	0.004
8q24	OPG	21	57	0	0	0.017
3q27-q29	TP63	20	54	0	0	0.023
8q24.1	NOV	20	54	0	0	0.023
14q22.3	RBBP1	19	51	0	0	0.030

†χ^2 test.

Table 4. Loci altered frequently in pancreatic adenocarcinoma cases with short-survival (<1 year) compared with long-survival periods

Chromosomal locus	Contained cancer-related gene	Sub-chromosomal loss detected				*P* value†
		Venous invasion-positive cases		Venous invasion-negative cases		
		n	%	*n*	%	
1p36.33	TP73	9	69	4	21	0.006
8q24.3	GLI4	5	38	1	5	0.018
Xq12	AR	5	38	1	5	0.018
11q13	STIP1, FOLR1	8	62	5	26	0.046
20q11.2	LUNX, TOP1	5	38	0	0	0.003
18p11.3	TGIF	6	46	1	5	0.006
4q13-q21	AREG	4	31	0	0	0.010
6q21	CCNC	4	31	0	0	0.010
10q21.1	PCDH15	10	77	6	32	0.012
1p32	RLF	5	38	1	5	0.018
2q36	Cul3	8	62	4	21	0.020
17q23	PPM1D	8	62	4	21	0.020
4q21	GRO1	6	46	2	11	0.022
1p36.2	KIAA0591(KIF1B)	7	54	3	16	0.023
4q21	GRO2	7	54	3	16	0.023
13q32	GPC5	7	54	3	16	0.023
8q22-q23	EIF3S6	9	69	6	32	0.036

†χ^2 test.

5/36, $P = 0.037$). Four loci, all on 7q21-22 (containing the HGF, DMTF1, MLL5 and CDK6 genes), were gained more frequently in the lymph node metastasis-positive group (all $P < 0.05$).

Association with survival. Thirty-two cases had survival data amenable to analysis. The genomic profiles of cases with a survival period shorter ($n = 13$) or longer than ($n = 19$) 1 year were compared. Four and 13 loci, respectively, were lost or gained more frequently in the short-compared with the long-survival group (Table 4). In contrast, only two loci were lost (6q25/ESR1 and 22q11.23/ADRBK2) and none gained more frequently in the long-compared with the short-survival group. Loss of 1p36 (p73) and 11q121-3 was associated with both short-term survival and evidence of venous invasion, whereas gain of 7q21-22 was associated with both short-term (<3 years) survival and the presence of lymph node metastases.

Kaplan–Meier analysis showed that loss of 1p36 (p73) ($P = 0.02$; Fig. 3a), gain of 17q23 (PPM1D) ($P < 0.05$; Fig. 3b) and particularly gain of the LUNX locus at 20q111-12 ($P < 0.0001$; Fig. 3c) were significantly associated with prognosis, whereas loss of the STIP1 or FOLR1 locus (11q13), gain of the TOP1 (20q11-12) and gain of MUC3 or Smurf1 loci (7q21-22) were not. Loci adjacent to LUNX on 20q11 were further analyzed; gain of the HCK ($P < 0.001$; Fig. 3d), E2F1 ($P < 0.005$; data not shown) and DNMT3b loci ($P < 0.05$; data not shown), but not TGIF2, were also associated with prognosis, albeit not as closely as LUNX.

Potential oncogenes revealed by expression profiling analysis. Eighty-one loci were amplified in at least one case in the group examined; these loci contained 15 genes that were overexpressed in at least one case (Table 5). Of the individual amplifications observed, 14.7% (20/136) resulted in overexpression. Only four genes were amplified and overexpressed in more than one case: Smurf1 (7q22.1), BCAS1 (20q13.2-3), which was the most frequently overexpressed, VCL (10q22.1) and TRRAP

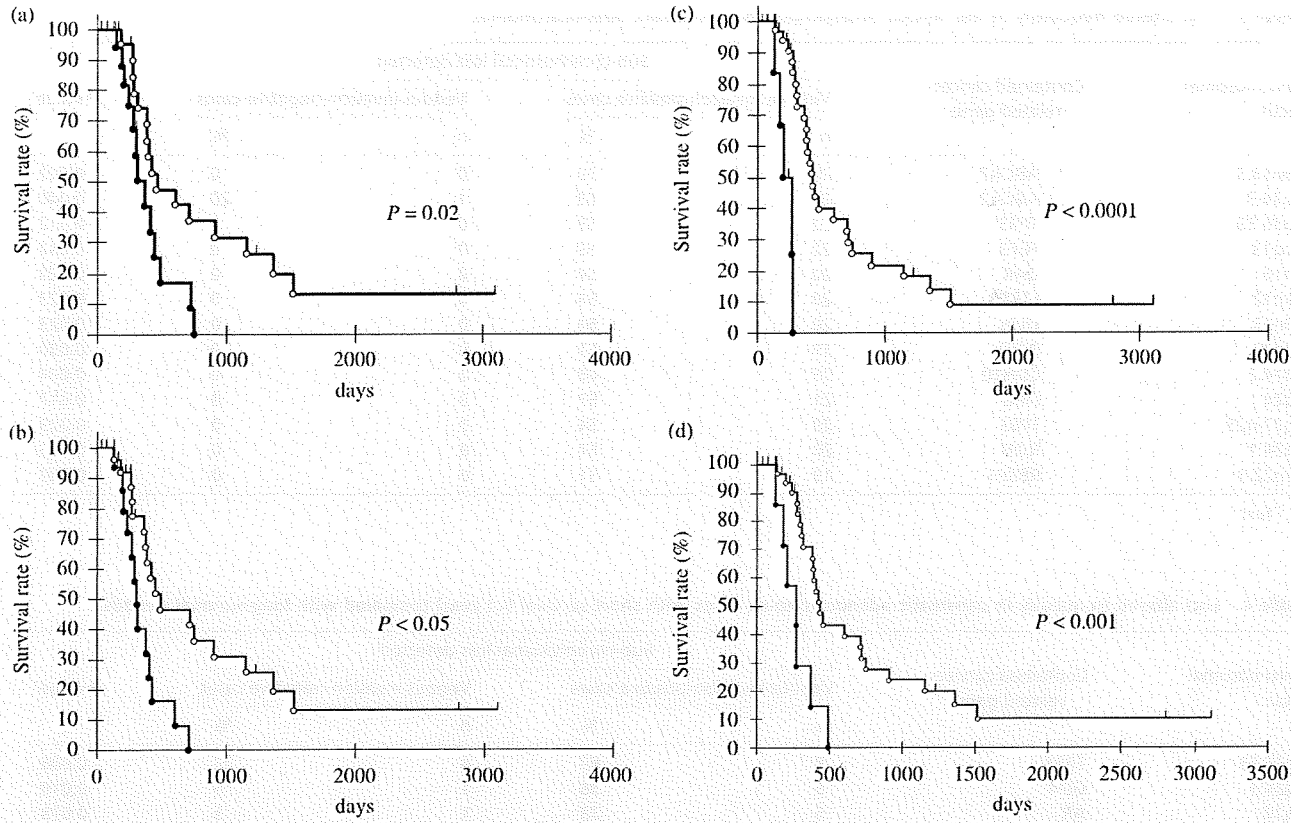

Fig. 3. Overall survival rate of pancreatic cancer patients according to the absence or presence of chromosomal abnormalities. (a) Overall survival rates of cases with chromosomal loss of the p73 locus on 1p36 (indicated as black dots) and cases without such loss (indicated as white dots). (b) Overall survival rates of cases with chromosomal gain of the PPM1D locus on 17q23 (indicated as black dots) and cases without such gain (indicated as white dots). Overall survival rates of cases with chromosomal gain of the (c) LUNX and (d) HCK loci on 20q11 (indicated as black dots) and cases without such gain (indicated as white dots). Survival curves were calculated by the Kaplan–Meier method.

(7q22.1) (Table 5). Genes that were contained in loci frequently amplified but not overexpressed included RBBP8 (18q11.2), LRP1B (2q21.2) and HGF (7q21.1). It should be noted that Smurf1 protein overexpression was also detected in pancreatic cancer clinical samples, as part of a separate study (F. Suzuki, T. Shibata, S. Hirohashi, J. Inazawa, I. Imoto, unpublished data).

The expression levels of genes on 20q11 were examined in more detail, because of the close association of four loci on 20q11 with survival. Eleven genes on 20q11 (BLCAP, RALY, GSS, ID1, NCOA6, TPX2, COX4I2, EPB41L1, BCL2L1, DNCL2A, CTNNBL1) were overexpressed. BCL2L1 (or BCL-x1) expression was also associated with lymph node metastasis of the xenografted tumors in mice (data not shown). It should be noted that two adjacent loci, TNFRSF6B and ZNF217 (20q13), were amplified in three cases each.

Discussion

This study represents the first genome-wide analysis of the subchromosomal numerical aberration profile (here designated SNAP) of a substantial number of pancreatic cancer cases by aCGH and is the first to establish its relationship with particular clinicopathological parameters of known prognostic value. It examined a number of primary tumors large enough to exclude randomly observed alterations from being considered as likely candidates, as would be the case in smaller-scale studies. In all previous studies except one,[29] case selection was based on the exclusion of samples that did not possess a high degree of neoplastic cellularity, which translates to a high copy number ratio error probability. In the present study, tumors were subjected to LCM so as to exclude non-tumor DNA from the analysis and thus increase both the number of available cases and the accuracy of the derived copy number ratio.

One of the striking findings of the study was the wide range in the number and pattern of aberrations observed between cases. Whereas many cases showed few aberrations and two had none whatsoever, 20% of cases showed alterations in more than 50% of loci examined. Importantly, the loss rate and range reported here (17%, 0–46%) is in very close agreement with the one reported in a comprehensive genome-wide allelic loss study of pancreatic cancer (15%, 1.5–32%).[30] It should be noted that it was not possible to know whether alterations of adjacent loci represented single amplicons or losses or whether they were independent events. Our results indicate that in the majority of pancreatic adenocarcinomas genomic instability occurs at the subchromosomal level, affecting a varying but large number of genes, and suggests the presence of multiple or complementary patterns of tumor evolution. Based on the association of SNAP with clinicopathological parameters revealed here, it is fair to assume that some of these aberrations contribute to tumor progression whereas others are the result of it. For the remaining cases showing a low SNAP or absence of aberrations, alternative mechanisms leading to tumor progression may be in place, such as DNA methylation or mismatch repair system aberrations,

doi: 10.1111/j.1349-7006.2007.00395.x

Table 5. Correlation of amplification with overexpression in pancreatic cancer genes both amplified and overexpressed in at least one xenograft

Gene	Locus	Amplified cases (%)	Overexpressed cases (%)	Amplified and overexpressed in the same case (%)	Amplified or gained and overexpressed in the same case (%)
Smurf1	7q22.1	36	25	25	25
BCAS1	20q13.2-q13.3	18	83	17	33
VCL	10q22.1	18	33	17	17
TRRAP	7q22.1	36	17	17	17
SRI	7q21.1	9	83	8	33
Cul3	2q36	27	42	8	33
TPD52	8q21	9	42	8	33
EFNB2	13q33	9	83	8	17
PDAP1	7q22	27	17	8	17
ZNF217	20q13	9	25	8	17
PLAU	10q24	18	17	8	8
WHSC1	4p16.3	9	17	8	8
CDK4	12q14	9	17	8	8
CYP3A4	7q22.1	36	8	8	8
CCNE1	19q11	18	8	8	8
OPG	8q24	9	33	0	33
BARD1	2q34	9	25	0	25
ELE1,MSMB	10q11.2	9	42	0	17
RAP1B	12q14	9	25	0	17
KRAS2	12p12.1	18	17	0	17
DHFR,MSH3	5q11.2-q13.2	9	17	0	17
TPR	1q25	9	17	0	8
MLL5	7q22.3	27	8	0	8
SSXT	18q11.2	27	8	0	8
PEG10	7q21.3	9	8	0	8
NBS1	8q21	9	8	0	8

mutations or small deletions, or chromosomal translocations and rearrangements not accompanied by numerical aberrations. These mechanisms may act in a way complementary to that of numerical aberrations in pancreatic carcinogenesis, so that in cases with high or low SNAP the above-mentioned mechanisms would be expected to play a minor role whereas other alternative mechanisms would be expected to play a minor or major role respectively.

A number of clinicopathological parameters was associated with SNAP, including, importantly, survival probability. Overall, cases with a phenotype indicating increased malignant potential had a higher SNAP. Specific loci, the loss or gain of which is associated with particular clinicopathological characteristics, were identified and are delineated in detail in the results section. Although only a small number of negative tumors was examined, it is noteworthy that loci associated with venous invasion were different from those associated with lymph node metastasis. Our results therefore appear to indicate that invasiveness and metastatic ability result from diverse and distinct molecular mechanisms in pancreatic cancer.

Despite the aforementioned genomic complexity, we identified genes the copy number status of which is associated with survival and may therefore be of prognostic value. Gains of the LUNX (20q11.2), AREG (4q13-q21) and CCNC (6q21) loci were detected exclusively in the short-survival group. Loss of 1p36 (p73) and 11q12-13 was associated with both short-term survival and evidence of venous invasion, whereas gain of 7q21-22 was associated with both short-term survival and the presence of lymph node metastases. Combining the above observations, we identified candidates most likely to yield clinically relevant results. A strong association was revealed between the copy number status of a number of loci at 20q11 and prognosis, mainly concerning the LUNX (PLUNC) locus ($P < 0.0001$) but also including adjacent loci containing HCK, E2F1 and DNMT3b. LUNX is upregulated and has been proposed as a marker for detection of micrometastases in non-small-cell lung cancer.[31] E2F1 activates the transcription of genes that encode proteins necessary for DNA replication, and is deregulated in most tumors.[32] DNMT3b may contribute to tumorigenesis by improper *de novo* methylation and silencing of the promoters of growth-regulatory genes, and its expression may be of clinical significance in breast cancer.[33] Although our data refer to loci rather than individual genes, the significance of the copy aberrations of the above loci has not been described previously in pancreatic or other cancers. Two loci on 20q13 were amplified in three cases each, whereas a further 12 genes on 20q11, including BCL2L1, were overexpressed. BCL2L1 is a BCL2-independent apoptosis regulator located in close proximity to LUNX. Its overexpression has already been linked to short survival times in pancreatic cancer[34,35] and other malignancies, and was also found to be associated with lymph node metastasis in the present study. Amplification and overexpression of BCL10 and BCL6 were also recently described in pancreatic carcinoma.[15] The 11q13.3 locus, containing another BCL family member, BCL1, was found to be amplified frequently in our study, which together with our findings on BCL2L1 described above may indicate a role for the BCL family in pancreatic carcinogenesis. The BCL2L1 overexpression and association with the metastatic phenotype may partially explain the effect the 20q11 region copy number status has on survival. However, we tend to think, in agreement with a similar proposal,[36] that our findings are more indicative of the fact that many (but not all) genes collectively confer selective advantage, in varying degrees of involvement, within the 20q11 region.

Loss of 1p36 (p73) and gain of 17q23 (PPM1D) were also significantly associated with prognosis. As mentioned earlier, 1p36/p73 loss was also associated with evidence of venous invasion in our study. p73, like its homolog p53, is able to induce apoptosis and has been reported to predict clinical outcome

in bladder cancer.[37] PPM1D amplification abrogates p53 tumor-suppressor activity. PPM1D is located within one of the most commonly amplified regions in breast cancer.[38] Gain of 17q21-q24 has also been associated with poor prognosis in ovarian clear cell adenocarcinomas, in which both PPM1D and APPBP2 were identified as likely amplification targets,[39] but, like p73, the PPM1D locus has not been previously reported to be of prognostic significance in pancreatic cancer.

Examination of the association between SNAP and expression provided a satisfactory filter for candidate genes. Only 15 of the 81 loci amplified and 14.7% (20/136) of individual amplifications observed contained genes that were overexpressed concurrently. This concordance level lies between those observed in breast cancer[40,41] and colon cancer,[42] in which 44–62% and 4%, respectively, of genes showing amplifications were overexpressed. It is, however, significantly lower than the one recently reported for pancreatic cancer cell lines, in which 60% of the genes within highly amplified genomic regions displayed associated overexpression,[14] a discrepancy that may partially be explained by the different source used (primary tumors vs cell lines) and the fact that we examined loci rather than genes. More than one target gene was overexpressed in some amplicons in our study, a finding not in disagreement with the above study.[14] We identified four genes contained in loci that were amplified and that were overexpressed recurrently: Smurf1 and TRRAP, both at 7q22.1, BCAS1 (20q13.2-3), and VCL (10q22.1). Smurf1 acts as a negative regulator of transfroming growth factor β signaling.[43] It was amplified in six cases overall and overexpressed concurrently in four. Although, as mentioned, gain of the Smurf1 locus was not associated with poor prognosis, 7q21-22 gain was associated with the presence of lymph node metastasis and was detected significantly more frequently in the short-term (<3 years) survival group. TRRAP is an essential cofactor for both the c-Myc and E1A/E2F oncogenic transcription factor pathways and interacts specifically with the E2F-1 transactivation domain. Its inclusion among the four genes both amplified and overexpressed lends further support to the association between E2F1 gain and poor survival revealed here. The fact that Smurf1 and TRRAP are amplified in pancreatic cancer was reported recently, albeit only in cell lines.[16] We show that amplifications of these genes also occurs in primary tumors and that they are recurrently accompanied by overexpression, therefore presenting as very likely novel oncogenes in pancreatic cancer. BCAS1 (20q13), reported to be amplified and overexpressed in breast cancer,[44] was the most frequently overexpressed gene among the ones contained in loci recurrently amplified, and may therefore have a similar role in pancreatic cancer; 20q13 was also one of the most frequently amplified loci in a recent aCGH study on pancreatic cancer.[14,15] Finally, 10q22-24 contained another novel candidate, vinculin, an intracellular protein with a crucial role in the maintenance and regulation of cell adhesion and migration.[45] KRAS2 and 20 other genes have recently been identified as potential target genes on 12p.[36] This finding is in partial agreement with our study, in which five loci on 12p were amplified and KRAS2 was amplified in two cases.

Numerous recurrent, non-random, patterns of subchromosomal aberrations have emerged through our analysis. Thirty-three loci with frequent losses (>50% cases) were identified. The chromosome arms found to contain the highest number of loci lost are in agreement with allelic loss[30] and chromosome CGH studies, with the additional detection of losses at 5p, 8q, 9q, 11p, 16p and 20q. The most frequently lost loci were: 17p13.3 (in 75% of cases), containing ABR, a multifunctional cellular signaling regulator and a putative TSG in medulloblastoma, 18qtel (CTDP1, SHGC-145820, 68%) and 18q21 (66%), containing SMAD7, a member of the SMAD family, although all three are close to either the p53 or the DPC4 locus. HD were detected in 25% of cases, affecting 26 loci. The 1p353-6.33 region contained the highest number of loci deleted (six) or lost (16). The most frequently deleted locus was 9p21 spanning the p16 gene, the inactivation of which is known to play an established role in pancreatic carcinogenesis. The above regions (17p, 18q and 1p353-6) have been reported previously to show frequent loss.[19] The most frequently gained locus was 7q21.1 (71%) containing the HGF gene, which encodes a cytokine involved in initiating cell migration. Some regions in which gains were observed frequently, at 6p21 (2 loci), 11q22 (7 loci) 12p12 (three loci) and 17q12 (Suppl. Table 4), have been previously proposed as novel amplicons.[16] Sixteen loci were amplified frequently (>10%), although, again, the possibility of another gene being amplified within these loci cannot be excluded. Two of the most frequently amplified loci were on 11q13, in agreement with a report by Holzmann *et al.* on 13 pancreatic cancer cell lines and six primary tumors.[15]

Novel loci likely to play important roles in pancreatic carcinogenesis and in the acquisition of certain malignant phenotypes were identified. Genes associated with prognosis or established histopathological indicators of malignancy, or showing both numerical aberrations and overexpression, may represent novel oncogenes. The copy number alterations of the p73 and PPM1D loci, the 20q11 region, including LUNX, and the loci amplified that contained genes concurrently overexpressed, particularly Smurf1, shown here may be of great importance for predicting clinical outcomes and setting new therapeutic targets in pancreatic cancer but will require prospective studies in order to be firmly established.

Acknowledgments

We thank T. Sakiyama for helping with the aCGH data analysis, T. Kondo for advice on statistical analysis, and Y. Arai, S. Uryu and Y. Kuwabara for advice on the hybridization technique. This study was supported in part by a Grant-in-Aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan; the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio), Japan; and by a Grant-in-Aid from CREST of JST. P. L. was a recipient of a Research Fellowship from the Program for Invitation of Foreign Researchers from the Foundation for Promotion of Cancer Research in Japan. H. K. was a recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

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Supplementary material

This material is available as part of the online article from:

The following supplementary material is available for this article:

Fig. S1. Homozygous deletions detected by array-based comparative genomic hybridization were validated by polymerase chain reaction and gel electrophoresis for selected cases and genes. Genes contained in the two most frequently deleted loci, p16 (left panel gel, cases 12, 18 and 41) at 9p21 and ABCA3 (right panel gel, cases 44 and 47) at 16p13.3 were examined (exons 2 and 28, respectively). Homozygous deletions were confirmed in all five cases examined, whereas wild-type products were detected in all control tissues used. The control tissue for p16 consisted of the corresponding normal tissue in one of three cases examined; corresponding normal tissue was not available for the other two cases (cases 12 and 18) as they derived from xenografts. The control tissues for the cases examined for homozygous deletions of the ABCA3 gene consisted of: (a) the corresponding normal tissues of both cases and (b) a third case (case no. 40), in which the array-based comparative genomic hybridization signal ratio indicated loss of heterozygosity of the ABCA3 gene, but not homozygous deletion, and its corresponding normal tissue.

Fig. S2. Range of numerical aberrations observed between cases. The total number of (a) numerical aberrations, (b) losses and (c) gains observed ranged widely between cases. In two cases no copy number changes were observed (a–c), whereas nine cases (20%) had alterations in more than 50% of loci (>400 loci) (a). Although the loss range was wider than the gain range (b,c), in most cases the number of gains was higher than the number of losses. Overall, however, the loss rate was similar to the gain rate (19% of loci altered on average per case for both).

Table S1 Clinicopathological parameters of 43 pancreatic cancer cases analyzed by array-based comparative genomic hybridization.

Table S2 Numerical aberrations observed in 44 pancreatic adenocarcinoma cases examined by array-based comparative genomic hybridization.

Table S3 Loci lost frequently (>25% cases) in 44 pancreatic adenocarcinoma cases examined by array-based comparative genomic hybridization, arranged by region.

Table S4 Loci gained frequently (>25% cases) in 44 pancreatic adenocarcinoma cases examined by array-based comparative genomic hybridization, arranged by region.

Table S5 Association of sub-chromosomal numerical aberrations with selected clinicopathological parameters in pancreatic cancer.

Table S6 (A) Loci altered more frequently in moderately compared with well differentiated pancreatic adenocarcinomas. (B) Loci altered more frequently in poorly compared with moderately differentiated pancreatic adenocarcinomas.

http://www.blackwell-synergy.com/doi/abs/10.1111/j.1349-7006.2006.00395.x

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doi: 10.1111/j.1349-7006.2007.00395.x