medium that lacked leucine and uracil (SC-Leu-Ura), or for 5FOA resistance, by growing them on synthetic medium containing 5FOA (1mg/ml). If 80% or more of transformants were $Ura^+/5FOA^S$, the sample was scored as negative for the protein-truncating mutation, indicating a homozygous wild type. If less than 80% of transformants were $Ura^+/5FOA^S$, we amplified the PCR fragment again and repeated the SC assay to confirm the final result. If the result showed less than 80% $Ura^+/5FOA^S$ again, the sample was positive for the presence of the heterozygous protein-truncating mutation.

DNA sequencing

We sequenced approximately 23.0kb of the *BRCA1* and *BRCA2* genes that contained all the coding exons and their flanking exon–intron boundaries. Except for exon 11 of both the *BRCA* genes, each coding exon and the flanking intron was amplified with a set of upstream and downstream primers and was sequenced by 1–3 sequence primer(s). Exon 11 of each of the *BRCA1* and *BRCA2* genes was sequenced using 16 and 18 primers, respectively. All of the primer sequences are listed at our website (http://www.idac.tohoku.ac.jp/dep/co/data/saka/brca01.htm) and all sequencing reactions were performed using a CEQ DTCS Kit or CEQ DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) and a GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, CT, USA), and then processed by an automated capillary sequencer CEQ2000 or CEQ2000EX (Beckman Coulter).

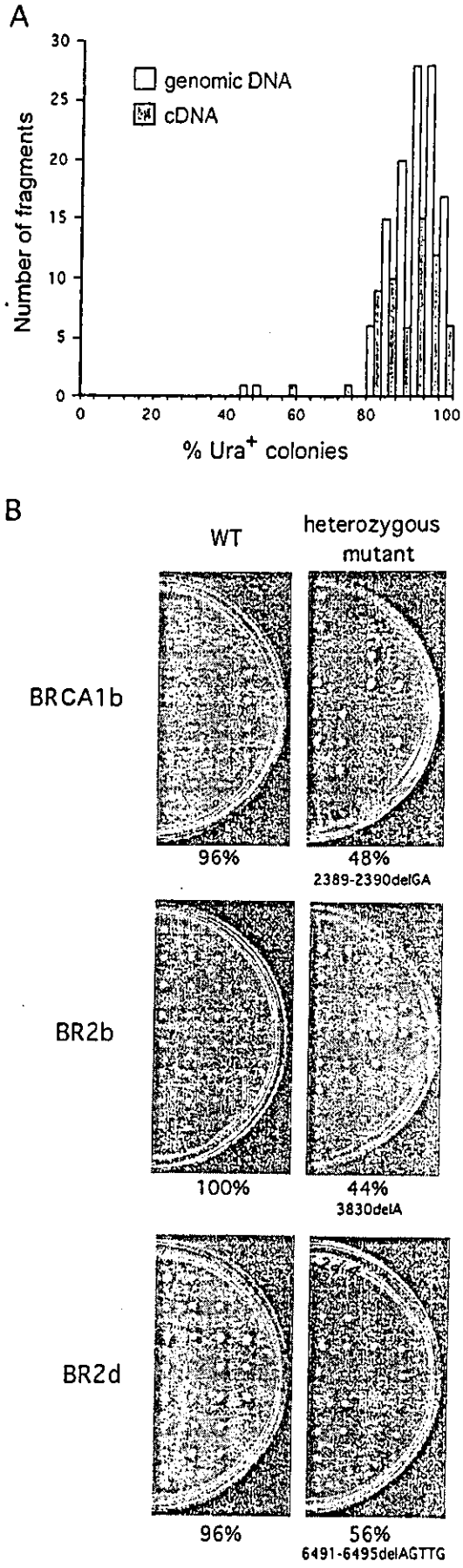
Results and discussion

We have described previously the SC assay for the *BRCA1* gene and the *APC* gene, a gene that is responsible for familial adenomatous polyposis (Ishioka et al. 1997). In the SC assay, a PCR-amplified DNA fragment derived from patient cDNA or genomic DNA was recombined into a specific gap vector harboring the yeast *URA3* gene, and the yeast transformant was selected as a colony using an appropriate selection medium (SC-Leu). The inability to express the URA3-fusion protein depends on the existence of a protein-truncating mutation within the inserted PCR fragment. Therefore, most of the colonies (≥80%) are able to grow on a medium lacking uracil (Ura^+) when the patient DNA fragment has neither nonsense nor frame-shift mutations. If the patient's DNA contains a heterozygous protein-truncating mutation, approximately half of the colonies cannot grow in the same medium (Ura^-). So far, the same or a similar yeast-based assay has been applied to other genes (Ishioka et al. 1997; Suzuki et al. 1998).

In this study, we constructed a plasmid system for an SC assay of the *BRCA2* gene (Fig. 1B), which divides the open reading frame of the *BRCA2* gene into six fragments (Fig. 1A) and inserts each of them into a *Bam*HI fragment of the pCI-HA(URA3)-2 (Ishioka et al. 1997; Suzuki et al. 1998) (Fig. 1B). When these plasmids are introduced into *ura3*$^-$ yeast cells, the yeast cells can be positively selected on the plate lacking uracil (Ura^+) or negatively selected on the plate containing 5FOA ($5FOA^S$) (data not shown). These vectors were digested by one or two restriction endonucleases (Fig. 1B) and used as gap vectors. We confirmed previously that cotransformation of each of the gap vectors with corresponding PCR fragments with or without a known protein-truncating mutation resulted in a $Ura^-/5FOA^R$ or $Ura^+/5FOA^S$ yeast phenotype, respectively.

Screening protein-truncating mutations by SC assay

Using the SC assay of *BRCA1* (Ishioka et al. 1997) and *BRCA2* (earlier), we screened a total of nine fragments covering the coding sequences of both *BRCA1* (three fragments) and *BRCA2* (six fragments) in 29 breast cancer patients (24 families) whose *BRCA* status had not been examined. A technician who was not informed about the patients' clinical background and the results of the DNA sequencing performed the SC assay. In 12 cases, both genomic DNA and RNA were available and, therefore, we examined all nine DNA fragments. In the remaining 17 cases, we examined only four (BR1b, BR2/ex10, BR2b, and BR2c) fragments that covered approximately 57% of the coding sequences (Fig. 1A) because RNA was not available and it was impossible to perform reverse transcriptase (RT)-PCR to amplify the remaining fragments. Among the 176 fragments we analyzed, the majority (172) were negative for the SC assay; 80% or more of the colonies were Ura^+ (mean ±SD = 90.0 ± 6.5%), which showed that there were no protein-truncating mutations in these fragments (Fig. 2A). The remaining four fragments that were derived from different patients were positive for the SC assay because a significantly lower number of Ura^+ colonies were found (Ura^+ fraction <80%) (Fig. 2A). To confirm the results, we reexamined the SC assay using independently amplified PCR fragments. Three of the four fragments (cases BR007, BR005, and BR001) again were positive, and, therefore, were thought to have retained a protein-truncating mutation within each fragment. Representative results are shown in Fig. 2B. The remaining fragment, which was derived from cDNA (BR2a fragment) and previously had a 72% Ura^+ fraction, had a negative score (88%) in the second experiment. Although we did not pursue the cause of low fidelity in this case, we had found previously that the RT reaction sometimes caused unexpectedly lower fidelity and that this issue was usually resolved by repeating the experiment. Therefore, we concluded finally that the BR2a fragment was negative for the SC assay. Because, in general, it is impossible to check and control the quality of RNA strictly, we stress that the repeat analysis and confirmation of the mutations by DNA sequence analysis are important, especially when the PCR fragment is derived from RT-PCR and the Ura^+ fraction is close to the cut-off value (80%). So far, 424 *BRCA1* or *BRCA2* fragments have been screened using the SC assay, and the Ura^+ fraction (mean ± SD) was 52.4 ± 8.9% (n = 17) or 89.3 ± 7.1% (n = 407) in fragments



with or without a heterozygous mutation. To evaluate the cut-off value (80%) of the SC assay, we drew the ROC (receiver operating characteristic) plots (Zweig and Campbell 1993) based on the individual data of this study. The plots indicated that any values from 60% to 80% of the rational cut-off value warranted good sensitivity (=1.00) and specificity (>0.99). Therefore, the value of 80% should be a reasonable cut-off value and may minimize possible false-negative cases in future studies.

Detection of sequence variations in the coding sequences by direct DNA sequencing

To confirm the sequence variations, we sequenced the full-length coding sequences and the flanking introns of the *BRCA1* and *BRCA2* genes using genomic DNA in all of the cases by automated direct sequencing, a standard method for mutation detection. Two technicians who were blind to the patients' clinical backgrounds and the results of the SC assay performed the DNA sequencing. We found five protein-truncating mutations, including three frameshift mutations (2389-2390delGA in exon 11 of *BRCA1*, 3830delA in exon 11 of *BRCA2*, and 6491-6495delAGTTG in exon 11 of *BRCA2*) and two nonsense mutations (C1372X in exon 12 of *BRCA1* and S2835X in exon 20 of *BRCA2*) (Table 1). During the sequencing analysis, we also found 40 other sequence variants (16 in *BRCA1* and 24 in *BRCA2*) within the coding regions and their flanking introns (data not shown). We concluded that most of these variants are common polymorphisms because they are found repeatedly in other patients and because some of them have been reported in an SNP database. However, they also contain 12 SNPs (5 in *BRCA1* and 7 in *BRCA2*) that were found only once in this study and not reported previously (Table 1). In the case of the five SNPs that substitute a single amino acid, there is a possibility that they contain pathogenic missense mutations, but there is no reliable functional assay at present.

Diagnostic accuracy of the SC assay for detecting protein-truncating mutations

To examine the diagnostic accuracy of the SC assay, we compared the results of the SC assay with the DNA sequencing data (Fig. 3). We found no protein-truncating

Fig. 2A,B. Distribution of Ura$^+$ fractions (Ura$^+$ colonies/total colonies assayed). **A** The result of the SC assay of both *BRCA1* and *BRCA2* genes is shown for a total of 176 fragments derived from specimens (116 genomic DNA and 60 cDNA) of this study. **B** Representative results of the SC assay showing heterozygous mutations. *Right panel*, The yeast transformants (25 clones) derived from the BR1b fragment of cases BR007 (*top*), BR2b fragment of BR005 (*middle*), and BR2d fragment of BR001 (*bottom*) were evaluated for their ability to grow in synthetic media lacking uracil. Approximately one half of the transformants (44%–56%) did not grow. A specific mutation shown *under each panel* was finally detected by DNA sequencing. *Left panel*, Negative controls for the corresponding DNA fragments derived from patients without any protein-truncating mutation. More than 80% of colonies grew. *WT*, Wild type

Table 1. Sequence variants (polymorphism and mutation) of *BRCA1* and *BRCA2* genes

Case number	Allele ID	Location	Nucleotide change	Sequence (5' to 3')	Interpretation
5 protein-truncating mutations					
BR007	BRCA1-7	Exon 11	2389-2390delGA	ggcaaaaaca GA/-accaaataaa	Frame-shift mutation
BR017	BRCA1-11	Exon 12	4116T>A	catctgggtg T/A gagagtgaaa	Nonsense mutation (C1372X)
BR005	BRCA2-18	Exon 11	3830delA	aagatagaaa A/- tcataatgat	Frame-shift mutation
BR001	BRCA2-22	Exon 11	6491-6495delAGTTG	gacaaacaac AGTTG/- gtattaggaa	Frame-shift mutation
BR018	BRCA2-26	Exon 20	8504C>A	gagaagacat C/A atctggatta	Nonsense mutation (S2835X)
12 sequence variations found once in this study					
BR028	BRCA1-4	Exon 11	814G>A	gagccatgtg G/A cacaaatact	G275D
BR027	BRCA1-13	Intron 14	IVS14+14A>G	agaaacatca A/G tgtaaagatg	Close to exon-intron boundary
BR013	BRCA1-15	Exon 16	4883T>C	tataatgcaa T/C ggaagaaagt	M1628T
BR027	BRCA1-17	Intron 22	IVS22+31A>T	gagagggagg A/T cacaatattc	Close to exon-intron boundary
BR006	BRCA1-18	Intron 23	IVS23+8G>T	atggtaaggt G/T cctgcatgta	Close to exon-intron boundary
BR017	BRCA2-2	Intron 2	IVS2-16T>A	taaggtggga T/A ttttttttta	Close to exon-intron boundary
BR026	BRCA2-3	Intron 2	IVS2-9T>G	ggatttttt T/G ttaaatagat	Close to exon-intron boundary
BR028	BRCA2-8	Exon 10	798T>C	gtcatggatt T/C ggaaaaacat	F266 silent
BR025	BRCA2-12	Exon 10	1744A>C	tttaatatcc A/C ctttgaaaaa	T582P
BR020	BRCA2-17	Exon 11	3420T>C	tgcagaagag T/C acatttgaag	S1140 silent
BR023	BRCA2-19	Exon 11	4566G>T	ctactctgtt G/T ggttttcata	L1522F
BR030	BRCA2-20	Exon 11	6131G>T	tcccaaaaag G/T cttttcatat	G2044V

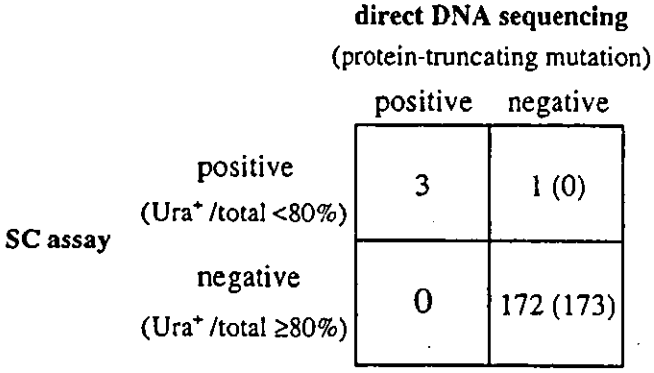


Fig. 3. Diagnostic accuracy of the SC assay for detection of protein-truncating mutations in both *BRCA1* and *BRCA2* genes. The results of the SC assay were compared with that of direct DNA sequencing. *Values in the box* indicate the number of fragments used in the SC assay. *Values in parentheses* indicate the results after reassay of fragments that were positive in the first screening

mutation in the DNA sequences of the 172 fragments that were negative for the SC assay, indicating that the negative predictive value was 100%. Of the remaining four fragments that were positive for the SC assay, three fragments contained protein-truncating mutations (Fig. 2B, Table 1). Only one fragment that was positive in the first screening had no protein-truncating mutation (false-positive case), but this was eventually scored as negative after repeating the assay (see earlier). Among the five protein-truncating mutations detected by DNA sequencing, two mutations could not be compared with the SC assay because no RNA was available. Therefore, the SC assay detected all protein-truncating mutations within the examined DNA fragments (sensitivity, 100%). Because there was one false-positive case in the first screening, the specificity of the assay was 99% (172 of 173) but the value finally became 100%. These results indicate that the SC assay is sufficiently sensitive and specific to screen protein-truncating mutations of the *BRCA1* and the *BRCA2* genes, even in a population that contains small fractions of *BRCA* mutations.

We have reported previously on the analytical validity of the SC assay for the *BRCA1* gene using blood samples with or without known *BRCA1* truncating mutations, and we have also discussed the technical advantages of the SC assay over the PTT (Ishioka et al. 1997). In this study, we expanded the application of the SC assay to the *BRCA2* gene, and confirmed the diagnostic accuracy of the SC assay for both *BRCA1* and *BRCA2* genes by analyzing specimens with unknown *BRCA* status in a blind manner. Once an examined specimen is positive in the SC assay, only 0.8–3.4kb DNA sequencing is needed to detect a protein-truncating mutation. We conclude that the SC assay is a useful and reliable screening method to detect pathogenic protein-truncating mutations in both *BRCA* genes. Apart from the technical advantages, we also note that the SC assay has an economical advantage. We have calculated the net cost (labor cost not included) for detecting protein-truncating mutations with or without the use of the SC assay. The cost of the SC assay for screening plus DNA sequencing only for SC assay-positive fragments was significantly lower than that for full-length DNA sequencing in all specimens; e.g., if the fraction of BRCA-truncating mutation is 20% in a population, the former costs only 11.4% of the latter. The advantage of the SC assay over the PTT should be validated by comparing the two assays in a large number of clinical samples.

We also showed that the frequency of the *BRCA1* and *BRCA2* gene mutations in our Japanese familial breast cancer patients was similar to that among similar patients in Western countries, which confirms previous reports (Ikeda et al. 2001; Inoue et al. 1995; Inoue et al. 1997; Takano et al. 1997). However, care should be taken in interpreting the data because there were some differences, for example, the number of breast cancer patients in the families of the two populations. Furthermore, there may be mutations that could not be detected by the current screening system and

DNA sequencing, such as a largely deleted mutant allele that has been observed in other ethnic populations (Petrij-Bosch et al. 1997; Puget et al. 1999a; Puget et al. 1999b; Puget et al. 1997; Swensen et al. 1997), and that could not be described as missense mutations because there is no functional assay available. In fact, it has been reported that at least 10%–15% of deleterious *BRCA1* mutations are missense mutations. These issues need to be resolved by developing other methods, including a reliable, rapid, and accurate functional assay.

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References

Anglian Breast Cancer Study Group (2000) Prevalence and penetrance of *BRCA1* and *BRCA2* mutations in a population-based series of breast cancer cases. Br J Cancer 83:1301–1308

Easton DF, Bishop DT, Ford D, Crockford GP (1993) Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. Am J Hum Genet 52:678–701

FitzGerald MG, Bean JM, Hegde SR, Unsal H, MacDonald DJ, Harkin DP, Finkelstein DM, Isselbacher KJ, Haber DA (1997) Heterozygous *ATM* mutations do not contribute to early onset of breast cancer. Nat Genet 15:307–310

Ford D, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P, Bishop DT, Weber B, Lenoir G, Chang-Claude J, Sobol H, Teare MD, Struewing J, Arason A, Scherneck S, Peto J, Rebbeck TR, Tonin P, Neuhausen S, Barkardottir R, Eyfjord J, Lynch H, Ponder BA, Gayther SA, Zelada-Hedman M, The Breast Cancer Linkage Consortium (1998) Genetic heterogeneity and penetrance analysis of the *BRCA1* and *BRCA2* genes in breast cancer families. Am J Hum Genet 62:676–689

Hogervorst FB, Cornelis RS, Bout M, van Vliet M, Oosterwijk JC, Olmer R, Bakker B, Klijn JG, Vasen HF, Meijers-Heijboer H, Menko FH, Cornelisse CJ, den Dunnen JT, Devilee P, van Ommen GJ (1995) Rapid detection of *BRCA1* mutations by the protein truncation test. Nat Genet 10:208–212

Ikeda N, Miyoshi Y, Yoneda K, Shiba E, Sekihara Y, Kinoshita M, Noguchi S (2001) Frequency of *BRCA1* and *BRCA2* germline mutations in Japanese breast cancer families. Int J Cancer 91:83–88

Inoue R, Fukutomi T, Ushijima T, Matsumoto Y, Sugimura T, Nagao M (1995) Germline mutation of *BRCA1* in Japanese breast cancer families. Cancer Res 55:3521–3524

Inoue R, Ushijima T, Fukutomi T, Fukami A, Sugimura H, Inoue S, Okonogi H, Sugimura T, Matsumoto Y, Nagao M (1997) *BRCA2* germline mutations in Japanese breast cancer families. Int J Cancer 74:199–204

Ishioka C, Suzuki T, FitzGerald M, Krainer M, Shimodaira H, Shimada A, Nomizu T, Isselbacher KJ, Haber D, Kanamaru R (1997) Detection of heterozygous truncating mutations in the *BRCA1* and *APC* genes by using a rapid screening assay in yeast. Proc Natl Acad Sci USA 94:2449–2453

Kuklin A, Munson K, Gjerde D, Haefele R, Taylor P (1997) Detection of single-nucleotide polymorphisms with the WAVE DNA fragment analysis system. Genet Test 1:201–206

Mazoyer S, Dunning AM, Serova O, Dearden J, Puget N, Healey CS, Gayther SA, Mangion J, Stratton MR, Lynch HT, Goldgar DE, Ponder BA, Lenoir GM (1996) A polymorphic stop codon in *BRCA2*. Nat Genet 14:253–254

Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, Bell R, Rosenthal J, Hussey C, Tran T, McClure M, Frye C, Hattier T, Phelps R, Haugen-Strano A, Katcher H, Yakumo K, Gholami Z, Shaffer D, Stone S, Bayer S, Wray C, Bogden R, Dayananth P, Ward J, Tonin P, Narod S, Bristow PK, Norris FH, Helvering L, Morrison P, Rosteck P, Lai M, Barrett JC, Lewis C, Neuhausen S, Cannon-Albright L, Goldgar D, Wiseman R, Kamb A, Skolnick MH (1994) A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. Science 266:66–71

Narod SA, Ford D, Devilee P, Barkardottir RB, Lynch HT, Smith SA, Ponder BA, Weber BL, Garber JE, Birch JM, Cornelis RS, Kelsell DP, Spurr NK, Smyth E, Haites N, Sobol H, Bignon Y, Chang-Claude J, Hamann U, Lindblom A, Borg A, Piver MS, Gallion HH, Struewing JP, Whittemore A, Tonin P, Goldgar DE, Easton DF, The Breast Cancer Linkage Consortium (1995) An evaluation of genetic heterogeneity in 145 breast-ovarian cancer families. Am J Hum Genet 56:254–264

Neuhausen SL (1999) Ethnic differences in cancer risk resulting from genetic variation. Cancer 86:2575–2582

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci USA 86:2766–2770

Petrij-Bosch A, Peelen T, van Vliet M, van Eijk R, Olmer R, Drusedau M, Hogervorst FB, Hageman S, Arts PJ, Ligtenberg MJ, Meijers-Heijboer H, Klijn JG, Vasen HF, Cornelisse CJ, van't Veer LJ, Bakker E, van Ommen GJ, Devilee P (1997) *BRCA1* genomic deletions are major founder mutations in Dutch breast cancer patients. Nat Genet 17:341–345

Powell SM, Petersen GM, Krush AJ, Booker S, Jen J, Giardiello FM, Hamilton SR, Vogelstein B, Kinzler KW (1993) Molecular diagnosis of familial adenomatous polyposis. N Engl J Med 329:1982–1987

Puget N, Torchard D, Serova-Sinilnikova OM, Lynch HT, Feunteun J, Lenoir GM, Mazoyer S (1997) A 1-kb Alu-mediated germ-line deletion removing *BRCA1* exon 17. Cancer Res 57:828–831

Puget N, Sinilnikova OM, Stoppa-Lyonnet D, Audoynaud C, Pages S, Lynch HT, Goldgar D, Lenoir GM, Mazoyer S (1999a) An Alu-mediated 6-kb duplication in the *BRCA1* gene: a new founder mutation? Am J Hum Genet 64:300–302

Puget N, Stoppa-Lyonnet D, Sinilnikova OM, Pages S, Lynch HT, Lenoir GM, Mazoyer S (1999b) Screening for germ-line rearrangements and regulatory mutations in *BRCA1* led to the identification of four new deletions. Cancer Res 59:455–461

Rebbeck TR (1999) Inherited genetic predisposition in breast cancer. A population-based perspective. Cancer 86:2493–2501

Shih HA, Couch FJ, Nathanson KL, Blackwood MA, Rebbeck TR, Armstrong KA, Calzone K, Stopfer J, Seal S, Stratton MR, Weber BL (2002) *BRCA1* and *BRCA2* mutation frequency in women evaluated in a breast cancer risk evaluation clinic. J Clin Oncol 20:994–999

Struewing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, Timmerman MM, Brody LC, Tucker MA (1997) The risk of cancer associated with specific mutations of *BRCA1* and *BRCA2* among Ashkenazi Jews. N Engl J Med 336:1401–1408

Suzuki T, Ishioka C, Kato S, Mitachi Y, Shimodaira H, Sakayori M, Shimada A, Asamura M, Kanamaru R (1998) Detection of *APC* mutations by a yeast-based protein truncation test (YPTT). Genes Chromosomes Cancer 21:290–297

Swensen J, Hoffman M, Skolnick MH, Neuhausen SL (1997) Identification of a 14 kb deletion involving the promoter region of *BRCA1* in a breast cancer family. Hum Mol Genet 6:1513–1517

Szabo CI, King MC (1997) Population genetics of *BRCA1* and *BRCA2*. Am J Hum Genet 60:1013–1020

Takano M, Aida H, Tsuneki I, Takakuwa K, Hasegawa I, Tanaka H, Saito M, Tsuji S, Sonoda T, Hatae M, Chen JT, Takahashi K, Hasegawa K, Toyoda N, Saito N, Yakushiji M, Araki T, Tanaka K (1997) Mutational analysis of *BRCA1* gene in ovarian and breast-ovarian cancer families in Japan. Jpn J Cancer Res 88:407–413

Tavtigian SV, Simard J, Rommens J, Couch F, Shattuck-Eidens D, Neuhausen S, Merajver S, Thorlacius S, Offit K, Stoppa-Lyonnet D, Belanger C, Bell R, Berry S, Bogden R, Chen Q, Davis T, Dumont M, Frye C, Hattier T, Jammulapati S, Janecki T, Jiang P, Kehrer R, Leblanc JF, Mitchell JT, McArthur-Morrison J, Nguyan K, Peng Y, Samson C, Schroeder M, Synder SC, Steele L, Stringfellow M, Stroup C, Swedlund B, Swensen J, Teng D, Thomas A, Tran T, Tran T, Tranchant M, Weaver-Feldhaus J, Wong AKC, Shizuya H, Eyfjord JE, Cannon-Albright L, Labrie F, Skolnick MH, Weber B, Kamb A, Goldgar DE (1996) The complete *BRCA2* gene and mutations in chromosome 13q-linked kindreds. Nat Genet 12:333–337

Thorlacius S, Struewing JP, Hartge P, Olafsdottir GH, Sigvaldason H, Tryggvadottir L, Wacholder S, Tulinius H, Eyfjord JE (1998)

Population-based study of risk of breast cancer in carriers of *BRCA2* mutation. Lancet 352:1337–1339

van der Luijt R, Khan PM, Vasen H, van Leeuwen C, Tops C, Roest P, den Dunnen J, Fodde R (1994) Rapid detection of translation-terminating mutations at the adenomatous polyposis coli (*APC*) gene by direct protein truncation test. Genomics 20:1–4

Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G (1995) Identification of the breast cancer susceptibility gene *BRCA2*. Nature 378:789–792

Xiao W, Oefner PJ (2001) Denaturing high-performance liquid chromatography: a review. Hum Mutat 17:439–474

Zweig MH, Campbell G (1993) Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 39:561–577

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Loss of Heterozygosity Analyses of Asynchronous Lesions of Ductal Carcinoma *in situ* and Invasive Ductal Carcinoma of the Human Breast

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Background: Ductal carcinoma *in situ* (DCIS) of the breast is known to possess characteristics of the pre-invasive stage of breast cancer and is the precursor to invasive ductal carcinoma (IDC). However, the natural history of the progression from DCIS to IDC remains unknown at the molecular level.

Methods: We investigated the loss of heterozygosities (LOHs) in tumors of seven patients with a history of breast biopsy. The seven specimens were diagnosed as DCIS on histopathological re-examination. These patients were diagnosed with ipsilateral breast cancer a few years after biopsy. We used thirteen selected microsatellite markers that were mapped to and/or very close to the tumor suppressor genes or regions with frequent LOHs in breast cancer. DNA isolated from microdissected formalin-fixed, paraffin-embedded tissues was subjected to a PCR-LOH analysis for these chromosome loci, and the pattern of LOHs was compared between the two asynchronous lesions for the seven cases.

Results: In all patients except one, the LOHs were concordant at 91% as the informative chromosome loci in cases 1 to 6 were 56, and the concordance in LOH pattern between DCIS and IDC was detected at 50 loci. The LOHs had accumulated in accordance with the tumor progression from DCIS to IDC. The recurrent lesion occurred at or near the site of the primary biopsy and had similar or identical histopathologic features.

Conclusions: These recurrences observed were probably residual disease rather than true recurrences. Our results suggest the following: (i) genetic alternations accumulate during cancer progression from DCIS to IDC, (ii) DCIS is a lesion that has a high risk of developing invasive transformation and (iii) after approximately 5 years without treatment, DCIS may develop into IDC.

Key words: breast cancer – LOH – DCIS – IDC – natural history

INTRODUCTION

Genetic alternations, including activation of oncogenes and inactivation of tumor suppressor genes, are involved in the development of human breast cancer (1,2). Several studies have reported the loss of heterozygosities (LOHs) in invasive ductal carcinoma (IDC) and ductal carcinoma *in situ* (DCIS) (3–6). LOHs is also frequently found on several chromosome arms in IDC and DCIS. To date, specific allelic losses have been reported in chromosome arms 6q, 8p, 11q, 13q, 16q, 17p and 17q.

Many IDCs have histopathologically spread as a result of intraductal components (DCIS) (7). IDC is believed to generally arise from DCIS. Therefore, it is important to understand the genetic alternations that lead to the transformation of DCIS into IDC. We had previously analyzed the LOHs in patients harboring synchronous atypical ductal hyperplasia (ADH), DCIS and IDC and found that higher frequencies of LOHs were observed in parallel with the tumor progression from ADH to DCIS and then to IDC (8). Fujii et al. analyzed LOH in patients harboring synchronous DCIS and IDC and found that LOH in DCIS was also observed in synchronous IDC (9). The data supported the idea of a stepwise progression from DCIS to IDC.

An understanding of the natural history of DCIS is thus the basis of prognostication and therapeutic recommendation. Despite the abundant molecular data on breast cancer in general, the natural history of the progression from DCIS to IDC remains unknown at the molecular level because it is difficult to follow the pathological progression from DCIS to IDC in the

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Table 1. Summary of the clinical characteristics and pathologic data

Case	Site	Lesion	Date of biopsy	DCIS	Surgical margin	Date of operation	Intraductal components	Interval time (months)
				Necrosis			Necrosis	
				Nuclear grade			Nuclear grade	
				Architectural pattern			Architectural pattern	
Case 1	Right	SL	1977/12/01	necrosis (–)	positive	1980/04/09	necrosis (–)	28
				NG 1			NG 1	
				sol, crib			sol, crib	
Case 2	Left	SL	1982/08/20	necrosis (–)	positive	1985/05/14	necrosis (–)	33
				NG 2			NG 2	
				pap, crib			pap, crib	
Case 3	Right	SL	1986/11/14	necrosis (–)	positive	1992/02/07	necrosis (–)	63
				NG 2			NG 2	
				low pap			low pap	
Case 4	Right	SL	1988/02/29	necrosis (–)	positive	1995/07/10	necrosis (–)	89
				NG 1			NG 1	
				crib, sol			crib, sol	
Case 5	Left	SL	1989/11/17	necrosis (–)	unknown	1994/11/09	necrosis (–)	60
				NG 2			NG 2	
				crib			crib	
Case 6	Left	SL	1992/11/18	necrosis (–)	unknown	1997/06/24	necrosis (–)	55
				NG 2			NG 2	
				sol, pap			sol, pap	
Case 7	Right	AL	1987/04/10	necrosis (–)	unknown	1992/11/18	necrosis (–)	67
				NG 2			NG 1	
				low pap			sol	

SL, same lesion; AL, another lesion; NG, Nuclear Grade; NG 1, low nuclear grade; NG 2, intermediate nuclear grade; NG 3, high nuclear grade; sol, solid; crib, cribriform; pap, papillary; low pap, low papillary.

same patient. The diagnosis of DCIS cannot be established without first removing a breast lesion for microscopic examination. A follow-up of such patients after the biopsy would be the best way to predict the clinical outcome of the disease. We, therefore, decided to evaluate and compare the molecular and clinicopathologic features of a selected number of patients with primary DCIS, who were treated only by biopsy and later had recurrent IDC lesions (asynchronous lesions) of the breast, using the LOH analysis to characterize the relationship between the two events at the molecular level.

PATIENTS AND METHODS

Samples Analyzed

We selected patients with a history of breast biopsy, which was histopathologically diagnosed as benign. These patients were diagnosed with ipsilateral breast cancer, IDC, approximately 2 years after the biopsy. In each case, paraffin-embedded, hematoxylin–eosin stained sections were re-examined by three authors (MA, TM, NO) in accordance with the Consensus conference on the classification of DCIS (10). In this study, we examined samples from seven patients diagnosed with DCIS (non-comedo type).

The seven cases comprised Japanese patients with sporadic breast cancer. Three of them were from Tohoku University Hospital in Sendai, two from other hospitals in Sendai and two were from Kurashiki. Informed consent regarding the usage of their samples for genetic experiments was obtained from the patients who were treated in Tohoku University Hospital. All the tumor specimens (primary DCIS and recurrent IDC) were fixed in formalin and embedded in paraffin. These paraffin-embedded tissues were sectioned at 3 μm, stained with hematoxylin and eosin and then re-examined histopathologically. In recurrent IDCs, intraductal components of IDC were also examined and evaluated and then compared with primary DCISs. The clinical characteristics and pathologic data of the seven cases are summarized in Table 1.

Microdissection and DNA Extraction

In each case, the formalin-fixed and paraffin-embedded tissues were sectioned at 10 μm. *In situ* cancer (primary DCIS) cells,

Table 2. Results of LOH analysis of primary DCIS and recurrent IDC

	Case 1		Case 2		Case 3		Case 4		Case 5		Case 6		Case 7	
	DCIS	IDC	DCIS	IDC	DCIS	IDC	DCIS	IDC	DCIS	IDC	DCIS	IDC	DCIS	IDC
D3S1300	+	+	+	+	+	+	–	–	+	–	n	n	–	+
D6S262	+	+	+	+	n	n	–	–	+	+	n	n	n	n
D8S137	n	n	n	n	–	–	–	–	n	n	+	–	–	+
D8S339	+	+	+	+	–	–	+	–	+	+	n	n	+	–
D9S126	n	n	+	+	+	+	–	–	–	–	+	+	+	+
D11S899	n	n	+	+	+	+	+	+	n	n	+	+	+	+
D11S528	n	n	+	+	+	+	–	–	–	–	n	n	–	–
D13S267	–	–	+	+	+	+	+	+	n	n	+	+	–	+
D16S514	n	n	+	–	n	n	+	+	–	–	n	n	–	–
D16S422	+	+	n	n	–	–	–	–	n	n	–	–	+	–
D17S928	+	+	+	+	–	–	+	+	+	+	n	n	n	n
TP53	+	–	+	+	n	n	+	+	+	+	+	+	+	+
D17S800	+	+	+	+	n	n	n	n	+	–	+	+	+	+

+, retained heterozygosity; –, loss of heterozygosity; n, not informative due to homozygosity.

invasive cancer cells (recurrent IDC) and normal cells were collected by microdissection using the Laser Captured Microdissection system, LM100 (Olympus, Japan) (11). DNA was extracted by a standard procedure, according to methods described previously (12).

LOH Analysis

Thirteen selected microsatellite markers, which were mapped to and/or very close to the tumor suppressor genes or regions with frequent LOHs in breast cancer, were used in this study. Primers for PCR amplification of these markers were designed based on the nucleotide sequences obtained from the GenBank database. Nucleotide sequences of the primers and annealing temperatures of PCR amplifications have been described previously (8). One out of each pair of primers was labeled with Cy 5 (Pharmacia Biotech, Uppsala, Sweden), and PCR amplifications and electrophoreses were performed according to methods described previously (13) using an ALFred DNA Sequencer with Fragment Manager software (Pharmacia Biotech, Piscataway, NJ). An allelic loss was defined as a more than a 50% reduction in the area of a peak calculated in the tumor as compared to that of a corresponding normal tissue. PCR reactions were performed twice per marker to confirm LOHs in both primary DCIS and recurrent IDC.

RESULTS

All the seven primary lesions were histopathologically diagnosed as a benign proliferative disease, but the re-examined diagnosis for all revealed DCIS (non-comedo type), van Nuys classification Group 1 (14). A total of seven cases with primary DCIS and recurrent IDC were analyzed using 13 selected microsatellite markers that are the predicted loci for localization of tumor suppressor genes (TSGs) for breast cancer. Microscopic examination of the biopsy specimens for primary DCIS revealed that the surgical margins of these specimens were clearly positive in cases 1 to 4. However, the surgical margins in cases 5 to 7 were undetermined because only one slice of the specimen was used and other aspects of the surgical edge were unclear. The time interval between the data of the first biopsy (primary DCIS) and that of the curative operation (recurrent IDC) was 28~89 months; the mean time was 55 months. None of the women, who had a positive family history of breast cancer, received any form of radiation therapy, hormonal therapy or chemotherapy after the biopsy of the primary lesions.

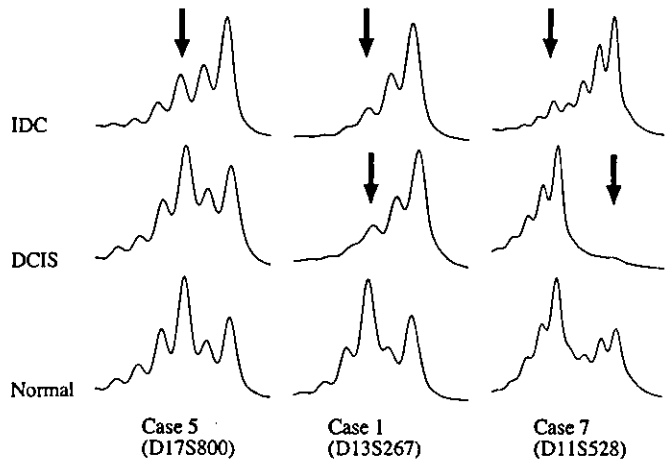

Figure 1. Examples of microsatellite analyses. Case 5 showed no allelic loss in ductal carcinoma *in situ* (DCIS) and showed a new allelic loss in invasive ductal carcinoma (IDC). Case 1 showed allelic loss in both DCIS and IDC. Case 7 showed the loss of a shorter allele in IDC, and a longer allele in DCIS. Arrows indicate lost alleles.

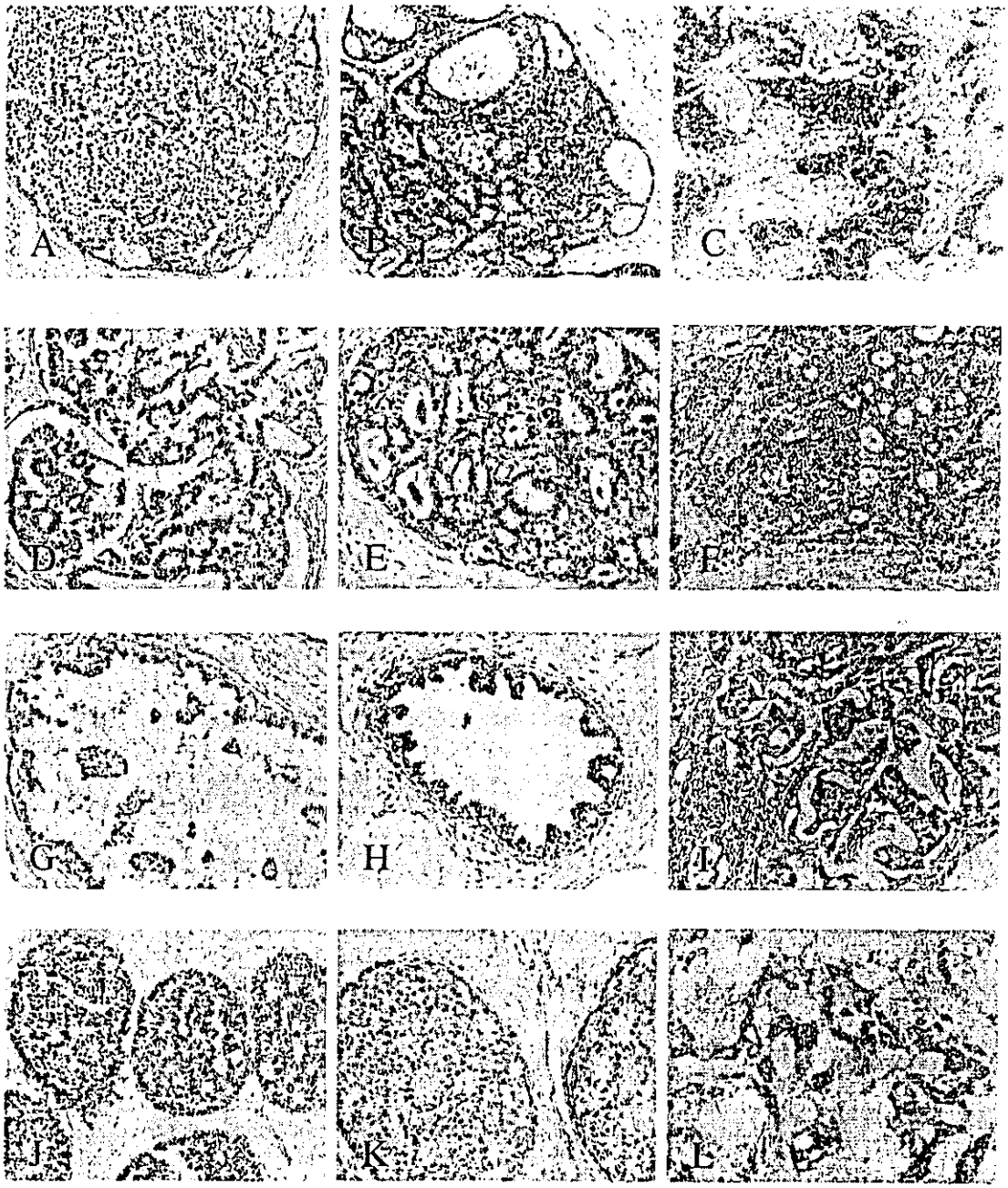

Figure 2. Histopathological appearances of primary DCIS and recurrent IDC. Case 1 (DCIS: A, intraductal component of IDC: B, IDC: C), Case 2 (DCIS: D, intraductal component of IDC: E, IDC: F), Case 3 (DCIS: G, intraductal component of IDC: H, IDC: I), Case 4 (DCIS: J, intraductal component of IDC: K, IDC: L). Cases 1 to 4 showed similar nuclear grades and architectural patterns for primary DCIS and recurrent IDC. LOHs in the primary DCIS were also observed in the recurrent IDC.

LOHs were observed at one or more of the tested loci in all seven cases. The incidence of LOH in each locus is shown in Table 2. Typical examples of LOH analyses are shown in Fig. 1. In all cases except one (case 7), the LOHs were concordant at 91% (50/56) as the informative chromosome loci in cases 1 to 6 were totally 56, and the concordance in LOH pattern between DCIS and IDC was detected at 50 loci. Even if the LOH pattern differed, the LOHs had accumulated in accordance with the tumor progression from DCIS to IDC. The recurrent lesion occurred at or near the site of the primary biopsy and had similar or identical histopathologic features.

Case 1 showed one LOH at 13q in primary DCIS and an additional LOH at p53 in recurrent IDC. Recurrent IDC appeared at the edge of the primary operation scar; the time interval from progression of primary DCIS to recurrent IDC was 28 months. Comedo-necrosis was not observed, and the nuclear grade was low (NG 1) in both primary DCIS and recurrent IDC. Similarly, the architectural pattern of DCIS was cribriform + solid in both primary DCIS (Fig. 2A) and recurrent IDC (Fig. 2B).

Case 2 showed no allelic loss in primary DCIS, however, a new allelic loss was observed at 16q in recurrent IDC. Recur-

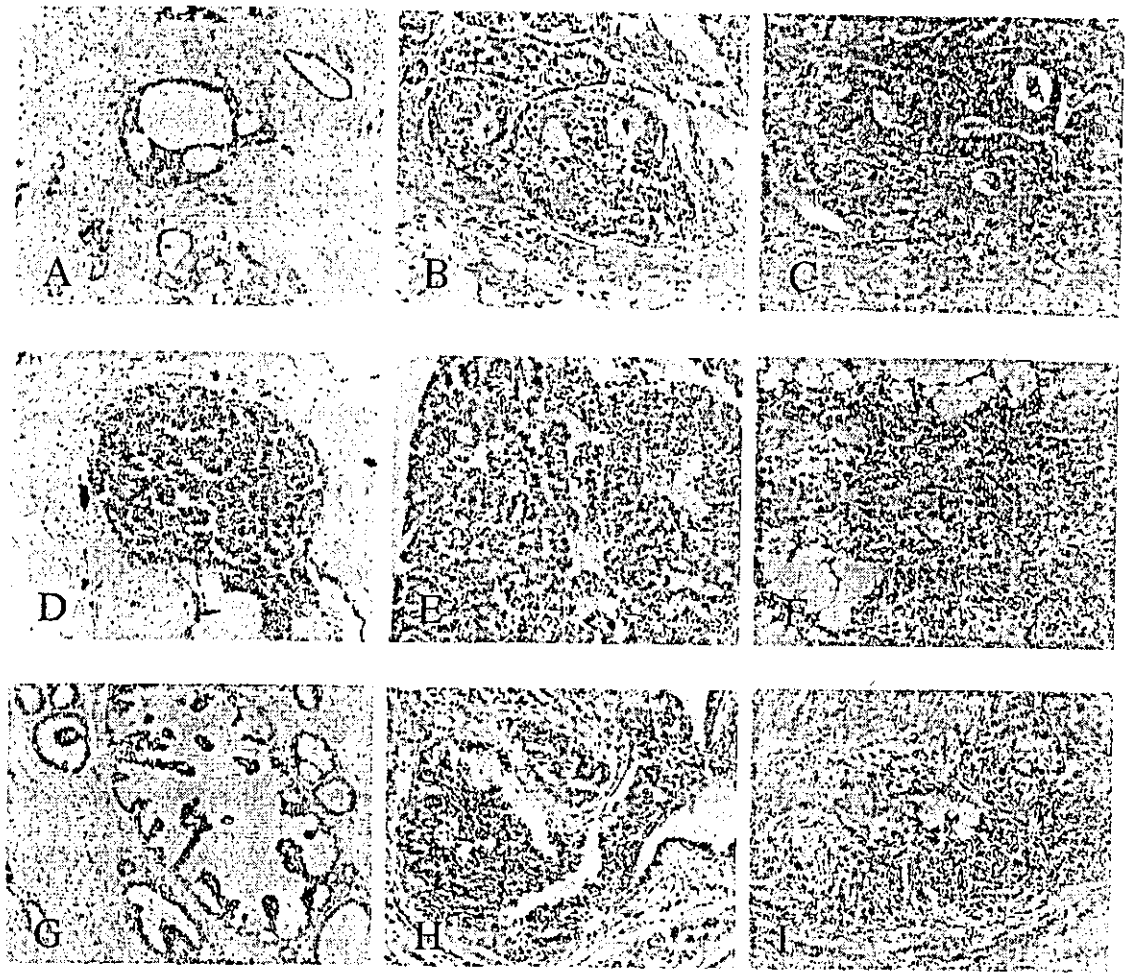

Figure 3. Histopathological appearances of primary DCIS and recurrent IDC. Case 5 (DCIS: A, intraductal component of IDC: B, IDC: C), Case 6 (DCIS: D, intraductal component of IDC: E, IDC: F), Case 7 (DCIS: G, intraductal component of IDC: H, IDC: I). Cases 5 and 6 showed similar nuclear grades and architectural patterns for primary DCIS and recurrent IDC. LOHs in the primary DCIS were also observed in the recurrent IDC. Case 7 showed different nuclear grades, architectural patterns, and allelic losses between DCIS and IDC.

rent lesions appeared at the edge of the biopsy scar, and the time interval was 33 months. Comedo-necrosis was not observed, the nuclear grades were intermediate (NG 2), and the architectural patterns were papillary + cribriform in both lesions (Fig. 2D, E).

Case 3 showed identical LOHs at 8p, 16q and 17p and no new LOHs in the recurrent lesions. Recurrent lesions appeared at the biopsy scar, and the time interval was 63 months. Comedo-necrosis was not observed, and the nuclear grades and architectural patterns were similar in both lesions (Fig. 2G, H). Case 4 showed identical allelic losses at 3p, 6q, 8p, 9p, 11q and 16q and a new loss at 8p in the recurrent lesion. The recurrent lesion appeared near the primary lesion, and the time interval was 89 months. Comedo-necrosis was not observed, and the nuclear grades and architectural patterns were similar in both lesions (Fig. 2J, K).

Case 5 showed identical allelic losses at 9p, 11q and 16q and new losses at 3p and 17q in the recurrent lesion. The recurrent lesion appeared at the primary scar, and the time interval was 60 months. Comedo-necrosis was not observed, and the nuclear grades and architectural patterns were similar in both lesions (Fig. 3A, B).

Case 6 showed LOH at 16q and a new LOH at 8p in recurrent IDC. The recurrent lesion appeared at the edge of the primary lesion, and the time interval was 55 months. Comedo-necrosis was not observed, and the nuclear grades and architectural patterns were similar in both lesions (Fig. 3D, E).

Case 7 showed LOHs at 3p, 8p, 11q, 13q and 16q in primary lesions, however, LOHs were observed at 8p, 11q and 16q in the recurrent lesion. The recurrent and primary lesions appeared in different quadrants. Comedo-necrosis was not observed in both lesions. The nuclear grade was NG II in primary DCIS but NG I in IDC. The architectural pattern was micropapillary in DCIS (Fig. 3G) and solid in IDC (Fig. 3H). This case is considered to have different clonality in IDC and DCIS because the appearance of lesions; the nuclear grades, architectural patterns and allelic losses for DCIS and IDC were all different.

In all the cases, the architectural pattern of DCIS associated with IDC was non-comedo, and the nuclear grade was low or intermediate. Cases 1 to 6 not only showed the same clonality,

but also exhibited similar nuclear grades, architectural patterns and appearance of lesions between the primary DCIS and recurrent IDC. Furthermore, all the allelic losses observed in the primary DCIS were also observed in the recurrent IDC. In all these cases, the time interval between diagnosis of primary DCIS and the recurrent IDC was 28~89 months, and the mean time interval was 55 months.

DISCUSSION

The natural history of breast carcinogenesis, progression from DCIS to IDC, has important prognostic and therapeutic implications. This is the first report on longitudinal molecular analysis from primary DCIS (non-comedo type) to recurrent IDC. We investigated LOHs using 13 microsatellite markers in seven cases of breast cancer. These chromosomal loci were selected on the basis of various previous studies (3–6). In all cases except one, LOHs were observed in parallel with the tumor progression from DCIS to IDC, suggesting a common genetic pathway for the development of both lesions and the continuous proliferation of the residual disease. These cases occurred at or near the site of the primary biopsy and had similar or identical histopathologic features. The observed recurrences were probably residual disease rather than true recurrences because surgical margins were clearly positive in four of the six cases.

According to the data obtained from this study, it is important to discuss the genetic criteria for differentiating between locally recurrent tumors and metachronous multicentric tumors. All the tumors except that of case 7 were considered to originate near the primary lesion, suggesting continuous genetic accumulation from the residual disease. They carried a maximum of two out of nine chromosomal loci (22%, case 5) with different LOH patterns at the asynchronous lesions. However, the lesion in case 7, which originated in another quadrant with different histological characteristics, showed a different LOH pattern at five (45%) of 11 chromosomal loci, suggesting that the threshold of discordance should lie between 22% and 45%.

In conclusion, hypothetically, recurrent IDC develops from residual primary DCIS that has accumulated one or more additional genetic alternations. Lininger et al. reported a comparison of LOH in primary DCIS and recurrent DCIS, in three cases with ipsilateral recurrent DCIS. LOH observed in primary DCIS was also observed in recurrent DCIS, and at least one additional LOH was observed in recurrent DCIS (15). Page et al. reported that the natural history of small, non-comedo DCIS lasts for at least two decades, with invasive carcinoma developing at the site of biopsy (16). However, our results showed that the natural history of small, non-comedo DCIS developed into IDC after approximately 5 years of treatment by biopsy only.

These results suggest that the following: (i) genetic alternations accumulate during cancer progression from DCIS to IDC, and (ii) DCIS is a lesion that has a high risk of developing invasive transformation, and (iii) non-comedo DCIS may develop into IDC after approximately 5 years without radiation therapy, hormonal therapy or chemotherapy.

BRCA1 and *BRCA2* are breast cancer susceptibility genes on chromosome bands 17q21 and 13q12-q13, respectively (18,19). LOH on the *BRCA1* locus is observed in IDC (case 5). On the other hand, LOH at the *BRCA2* locus is observed in DCIS and IDC (case 1). In our previous study (8), inactivation of *BRCA1* played an important role in the early stage of breast carcinogenesis, and the inactivation of *BRCA2* was a late event.

The loss of D16S422 at the locus for *CDH13* was high in DCIS and IDC (cases 3, 4, 6). This gene, located on chromosome bands 16q24, encodes the adhesion molecule H-cadherin (*CDH13*) and is recognized as a TSG responsible for breast cancer. The expression of *CDH13* is significantly reduced in breast cancer (20), and its frequent inactivation in lung cancer has also been reported (21). Further, the introduction of wild-type DNA to breast cancer cells inhibited cell growth (20). Our previous study (8) and results suggested that H-cadherin might play an important role even in the early stages of breast carcinogenesis.

The *CDH1* gene, located on chromosome 16q22, encodes the adhesion molecule E-cadherin, which suppresses invasion *in vitro*. A decreased expression in breast carcinomas correlates with the presence of invasion and a shorter disease-free survival (22). Our previous study (8) suggested that E-cadherin might be a rate event of breast carcinogenesis. In our results, LOH on the E-cadherin locus is observed in IDC (case 1) and in DCIS and IDC (case 5).

The loss of chromosome 8p is also frequently observed not only in IDC, but also in DCIS. Putative TSG in this region probably plays an important role in the early stages of breast carcinogenesis (cases 3, 4). The linkage analysis indicated that a positive LOD score at D8S137 was observed in some familial breast cancer pedigrees that were neither linked to 13q nor to 17q (23). There may be a third breast cancer susceptibility gene (*BRCA3*) on this chromosome arm, and our results may provide an important clue for identifying the putative *BRCA3* gene.

ATM, the gene responsible for ataxia telangiectasia, is located on chromosome 11q22-q23 and the LOH of this gene (cases 4, 5) might be an early event. The frequent LOH of *p53* has been reported in a variety of tumors (24). In this study, however, the LOH of *p53* was not very frequent (case 1). Many investigators have analyzed the mutations of *p53* in breast cancer tumors and have found that this gene was not frequently mutated in this disease (25). Our present study is in agreement with these results.

Though the data on genetic progression of breast cancer is increasing, the mechanisms of carcinogenesis (natural history) from DCIS to IDC at the molecular level are still unknown. The genome-wide search for LOH showed 56 regions with consistent LOH (17). In an LOH analysis of 75 different breast cancers at multiple chromosome loci, every cancer showed a different pattern of deletions. In our previous study on LOH analysis in 70 DCIS lesions at the same chromosome loci, frequent LOH was observed at 8p, 16q and 17q, but the inci-

dences of LOH at loci on 3p, 6q, 9p, 11p, 11q and 17p were marginal. It is not confirmed whether the mutations of genes on these loci play a major role in the genesis of breast cancer. The results of this study also show a different pattern of deletions in each case (Table 2). However, we could not completely exclude these regions as the loci for the responsible genes since the number of samples used in this study were limited.

To summarize, the progression from DCIS to IDC may involve a pathway consisting of stepwise genetic alterations. DCIS is likely to have a high risk of developing invasive transformation. Further studies are necessary to identify the genetic alterations of breast tumor progression from DCIS to IDC in order to develop appropriate clinical management of breast cancer patients.

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References

1. Bièche I, Lidereau R. Genetic alterations in breast cancer. *Genes Chromosom Cancer* 1995;14:227–51.
2. Black DM. The genetics of breast cancer. *Eur J Cancer* 1994;30:1957–61.
3. Sato T, Tanigami A, Yamakawa K, Akiyama F, Kasumi F, Sakamoto G, et al. Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res* 1990;50:7184–9.
4. Fujii H, Szumel R, Marsh C, Zhou W, Gabrielson E. Genetic progression, histological grade, and allelic loss in ductal carcinoma *in situ* of the breast. *Cancer Res* 1996;56:5260–5.
5. Radford DM, Fair KL, Phillips NJ, Ritter JH, Steinbrueck T, Holt MS, et al. Allelotyping of ductal carcinoma *in situ* of the breast: deletion of loci on 8p, 13q, 16q, 17p and 17q. *Cancer Res* 1995;55:3399–405.
6. Driouch K, Briffod M, Bièche I, Champème MH, Lidereau R. Location of several putative genes possibly involved in human breast cancer progression. *Cancer Res* 1998;58:2081–6.
7. Moriya T, Silverberg SG. Intraductal carcinoma (ductal carcinoma *in situ*) of the breast. A comparison of pure noninvasive tumors with those including different proportions of infiltrating carcinoma. *Cancer* 1994;74:2972–8.
8. Amari M, Suzuki A, Moriya T, Yoshinaga K, Amano G, Sasano H, et al. LOH analyses of premalignant and malignant lesions of human breast: Frequent LOH in 8p, 16q, and 17q in atypical ductal hyperplasia. *Oncol Rep* 1999;6:1277–80.
9. Fujii H, Marsh C, Cairns P, Sidransky D, Gabrielson E. Genetic divergence in the clonal evolution of breast cancer. *Cancer Res* 1996;56:1493–7.
10. The Consensus Conference Committee. Consensus conference on the classification of ductal carcinoma *in situ*. *Cancer* 1997;80:1798–802.
11. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, et al. Laser captured microdissection. *Science* 1996;274:998–1001.
12. Kimura M, Abe T, Sunamura M, Matsuno S, Horii A. Detailed deletion mapping on chromosome arm 12q in human pancreatic adenocarcinoma: identification of a 1-cM region of common allelic loss. *Genes Chromosom Cancer* 1996;17:88–93.
13. Sato M, Mori Y, Sakurada A, Fukushige S, Ishikawa Y, Tsuchiya E, et al. Identification of a 910-kb region of common allelic loss in chromosome bands 16q24.1-q24.2 in human lung cancer. *Genes Chromosom Cancer* 1998;22:1–8.
14. Silverstein MJ, Poller DN, Waisman JR. Prognostic classification of breast ductal carcinoma *in situ*. *Lancet* 1995;345:1154–7.
15. Lininger RA, Fujii H, Man Y-G, Gabrielson E, Tavassoli FA. Comparison of loss of heterozygosity in primary and recurrent ductal carcinoma *in situ* of the breast. *Mod Pathol* 1998;11:1151–9.
16. Page DL, Dupont WD, Rogers LW, Jensen RA, Schuyler PA. Continued local recurrence of carcinoma 15–25 years after a diagnosis of low grade ductal carcinoma *in situ* of the breast treated only by biopsy. *Cancer* 1995;76:1197–200.
17. Kerangueven F, Noguchi T, Coulier F, Allione F, Wargniez V, Simony-Lafontaine J, et al. Genome-wide search for loss of heterozygosity shows extensive genetic diversity in human breast carcinomas. *Cancer Res* 1997;57:5469–74.
18. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, et al. A strong candidate for breast and ovarian cancer susceptibility gene *BRCA1*. *Science* 1994;266:66–71.
19. Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, et al. Identification of the breast cancer susceptibility gene *BRCA2*. *Nature* 1995;378:789–92.
20. Lee SW. H-cadherin, a novel cadherin with growth inhibitory functions and diminished expression in human breast cancer. *Nat Med* 1996;2:776–82.
21. Sato M, Mori Y, Sakurada A, Fujimura S, Horii A. The H-cadherin (*CDH13*) gene is inactivated in human lung cancer. *Hum Genet* 1998;103:96–101.
22. Driouch K, Dorion-Bonnet F, Briffod M, Champeme MH, Longy M, Lidereau R. Loss of heterozygosity on chromosome arm 16q in breast cancer metastases. *Genes Chromosom Cancer* 1997;19:185–91.
23. Seitz S, Rohde K, Bender E, Nothnagel A, Kölble K, Schlag PM, et al. Strong indication for a breast cancer susceptibility gene on chromosome 8p12-p22: linkage analysis in German breast cancer families. *Oncogene* 1997;14:741–3.
24. Jones MH, Nakamura Y. Detection of loss of heterozygosity at the human TP53 locus using a dinucleotide repeat polymorphism. *Genes Chromosom Cancer* 1992;5:89–90.
25. Mazars R, Spinardi L, Bencheickh M, Simony-Lafontaine J, Jeanteur P, Theillet C. *p53* mutations occur in aggressive breast cancer. *Cancer Res* 1992;52:3918–23.

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Original Article

Invasive micropapillary carcinoma of the breast: Clinicopathological and immunohistochemical study

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Invasive micropapillary carcinoma (IMPCa) of the breast refers to a unique variant of invasive ductal carcinoma, but its biological behavior has not been elucidated well. We analyzed 16 IMPCa cases (10 pure type, six mixed type). The incidence of IMPCa was 1.0% of all primary breast carcinoma. High nuclear grade (75.0%), as well as poorly differentiated histological grade (81.3%), was frequently seen. Lymph node metastases were evident in 92.9% of the examined cases, and about half of them showed more than 10 positive nodes. Comparison between serially experienced invasive ductal carcinoma, not otherwise specified (IDC-NOS), revealed that both high nuclear grade and poor histological grade were significantly more frequent ($P < 0001$), there was a lower frequency of positive estrogen receptor/progesterone receptor ($P < 0.05$, $P < 0.01$), a higher frequency of HER-2 overexpression ($P < 0.025$), and more frequent lymph node metastases ($P < 0.05$) in IMPCa. The comparison between lymph node positive IDC-NOS did not show any statistically significant differences in frequency for positive p53, matrix metalloproteinase protein-2 (MMP-2), vascular endothelial growth factor (VEGF) or E-cadherin. However, IMPCa showed a significantly increased number of blood vessels counted by CD34 immunostains ($P < 0.05$). These results suggest that IMPCa is, at least, the same or more aggressive than lymph node positive cases of IDC-NOS. Hence, not only the high incidence of lymph node metastases but also distant, blood-borne metastases may be important.

Key words: breast carcinoma, ductal carcinoma, immunohistochemistry, invasive micropapillary carcinoma, pathology

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Invasive micropapillary carcinoma (IMPCa) of the breast is considered to be a recently recognized, unusual type of invasive ductal carcinoma with unique morphology. Characteristically, this variant of carcinoma shows tumor cells arranged in small clusters with a central lumen usually present, and an image of a micropapillae within clear spaces, which appear to be empty, but in some instances mucinous materials have been seen with special stains.[1] Siriaungkul and Tavassoli identified nine cases of IMPCa, and came to the tentative conclusion that the behavior was not significantly different from that of invasive ductal carcinoma, not otherwise specified (IDC-NOS).[2] However, further investigations revealed that this tumor has a highly malignant potential because IMPCa had a high incidence of lymph node metastases, and tended to recur earlier.[3–7] Additionally, Paterakos and colleagues mentioned that the survival rate of patients with IMPCa was similar to patients with carcinoma with equivalent numbers of lymph node metastases.[8]

Immunohistochemical studies had been performed in some reports.[3,4,8,9] However, the precise clinicopathological characteristics have not been elucidated well, especially in Japanese women. Thus, we examined IMPCa, clinicopathologically and immunohistochemically, and compared them with IDC-NOS.

MATERIALS AND METHODS

We reviewed the case files from September 1998 to December 2001 in Tohoku University Hospital and Tohoku Kousai Hospital, and from January 1997 to December 2001 in Chugoku Chuo Hospital. Re-examination of the glass slides was done by two of the authors (CDLC and TM). To identify IMPCa cases, we followed the criteria: 'epithelial tufts forming

micropapillae without a fibrovascular core located within clear spaces, which are usually empty, and epithelial cells exhibiting reverse polarity with serrated peripheral borders' (Fig. 1).[1,3,8] The cases with obvious mucin within the empty space, which is sometimes allowed by some authors,[3] were eliminated from the series in this study.

Eleven patients were considered as IMPCa: six cases were considered as pure type IMPCa, with more than 90% of the invasive micropapillary carcinoma composed of characteristic features (Fig. 2); and five cases were considered as mixed type, with 33% to 90% of invasive carcinoma composed of IMPCa. A characteristic pattern of less than 33% invasive components was included in invasive ductal carcinoma, not otherwise specified (IDC-NOS). During the same periods, we have experienced 1056 cases of primary breast carcinomas. Thus, the overall incidence of all IMPCa cases in our series was 1.0%, and the pure IMPCa was 0.6% of all primary breast carcinomas. An additional five cases (four pure and one mixed) were added from the previous files and, finally, a total of 16 IMPCa cases were analyzed. For comparison, 150 cases of serially experienced IDC-NOS from the files of the Pathology Department of Tohoku University Hospital in 2002 were used. For immunohistochemical analysis, another 23 cases with positive lymph node metastases at the initial operation, with available follow-up data (43–149 months, mean 108 months, with eight cases (34.8%) dead from disease), were selected as control cases.

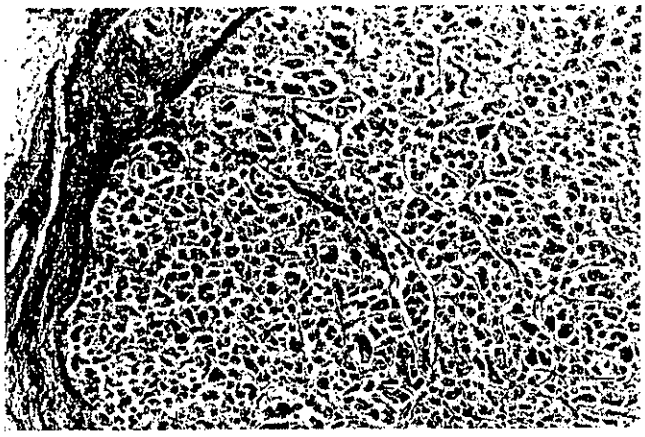

Figure 1 Invasive micropapillary carcinoma showing a micropapillary pattern of carcinoma cells floating within the empty space. HE, ×20.

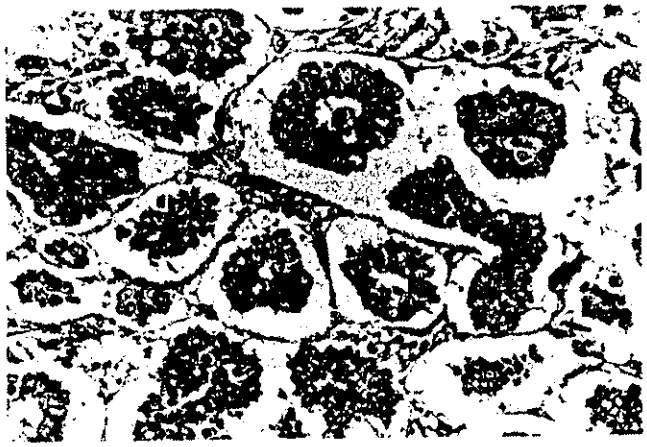

Figure 2 Pure-type invasive micropapillary carcinoma with an extensive characteristic histological pattern. HE, ×100.

All specimens were fixed in 10% formalin and embedded in paraffin, and 3 μm-thick sections were cut and mounted on glass slides. On the hematoxylin–eosin (HE) slides, the maximum diameter of the invasive carcinoma, presence or absence of intraductal components, presence or absence of comedonecrosis within the intraductal carcinoma, the nuclear/histological grading, and lymph node status, were evaluated. For nuclear grading, the criteria of the Japan National Surgical Adjuvant Study of Breast Cancer (NSAS-BC) Pathology Section[10] was used, and for histological grading, a modified Bloom & Richardson's method (Nottingham's classification)[11] was used.

Immunohistochemical staining for estrogen receptor, progesterone receptor, HER-2, and p53 was performed on the Ventana Bench Mark Automated Staining System. Manual immunostaining was used for matrix metalloproteinase protein-2 (MMP-2), vascular endothelial growth factor (VEGF), E-cadherin, Ki-67, CD34, Factor VIII related antigen, and type IV collagen. The source of the primary antibodies, dilution, and methods of pretreatment are listed in Table 1. The primary antibodies for manual staining were kept overnight at 4°C. After that, the Histofine SAB-PO kit (Nichirei, Tokyo, Japan) was applied. The positive staining was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB), and lightly counterstained with hematoxylin.

Estrogen and progesterone receptors were evaluated with a proportion score (PS), which represents the estimated proportion of positive tumor cells (range 0–5), and an intensity score (IS), which estimates the average staining intensity of positive tumor cells (range 0–3). The PS and IS were added to obtain a total score (TS) (range 0–8).[12] A TS greater than 4 was considered positive, and a TS less than 4 was considered negative. For VEGF, the cytoplasm of the carcinoma cells was compared with background staining to decide if they were positive or negative. To evaluate p53, more than 10% positive cells was considered as weakly positive (+), between 30% and 70% was considered moderately positive (++), and more than 70% was considered strongly positive (+++). For HER-2, the Hercep Test (DAKO) scoring criteria was used.[13] A weak to moderate complete membrane staining in more than 10% of tumor cells (score 2+) and a strong complete membrane staining in more than 10% of tumor cells (score 3+) were considered positive. The Ki-67 index was calculated as the number of Ki-67 positive cells per 100

Table 1 Immunohistochemical reagents and methods used

Antigen	Antibody	Source	Dilution	Antigen retrieval
ER	6F11	Novocastra, Newcastle upon Tyne, UK	1:50	Heating†
PR	MAB429	Chemicon, Temecula, CA, USA	1:30	Heating†
HER-2	(Polyclonal)	DAKO, Glostrup, Denmark	1:800	Heating†
p53	DO-7	Biomeda, Foster City, CA, USA	1:40	Heating†
Ki-67	MIB-1	Immunotech, Marseille, France	1:300	Autoclave
CD34	NV4A1	Nichirei, Tokyo, Japan	1:100	None
VEGF	(Polyclonal)	R&D Systems Inc., Minneapolis, MN, USA	1:100	Autoclave
E-cadherin	4A2C7	Zymed, San Francisco, CA, USA	1:400	Autoclave
MMP-2	42–5D11	Fuji, Tokyo, Japan	1:30	None
Factor VIII-R Ag	(Polyclonal)	DAKO, Glostrup, Denmark	1:200	Protease
Type IV collagen	CIV22	DAKO, Glostrup, Denmark	1:100	Pepsin

ER, estrogen receptor; PR, progesterone receptor; R Ag, related antigen; VEGF, vascular endothelial growth factor. †Performed by the Ventana Bench Mark Automated Staining System.

Table 2 Clinicopathological features of 16 invasive micropapillary carcinoma cases

Case	Age	Distribution	Size (mm)†	Nuclear grade	Histological grade‡	Comedo necrosis§	LN status	Follow up
1	39	Pure	24	High	III (Poor)	–	0/8	18 months, NED
2	43	Pure	30	Intermediate	III (Poor)	+	1/7	54 months, NED
3	35	Pure	26	High	III (Poor)	+	6/9	17 months, NED
4	37	Pure	8	High	III (Poor)	–	27/27	34 months, DOD
5	59	Pure	84	High	III (Poor)	+	29/29	216 months, AWD
6	50	Pure	35	High	III (Poor)	–	2/15	16 months, NED
7	44	Pure	14	Intermediate	II (Moderate)	–	27/29	3 months, NED
8	43	Pure	25	High	III (Poor)	–	25/34	83 months, DOD
9	38	Pure	32	High	III (Poor)	–	18/21	56 months, DOD
10	42	Pure	27	Intermediate	II (Moderate)	–	3/19	24 months, NED
11	65	Mixed	37	Intermediate	II (Moderate)	+	8/19	61 months, DOD
12	55	Mixed	25	High	III (Poor)	–	3/16	88 months, AWD
13	67	Mixed	17	High	III (Poor)	+	NA	46 months, AWD
14	44	Mixed	12	Intermediate	II (Moderate)	–	NA	30 months, NED
15	56	Mixed	45	High	III (Poor)	+	12/24	14 months, NED
16	38	Mixed	28	High	III (Poor)	–	5/22	30 months, NED

AWD, alive with disease; DOD, dead of disease; LN, lymph node; NA, data not available; NED, no evidence of disease. †Microscopic maximum diameter of invasive component. ‡Modified Bloom–Scharf–Richardson scoring system.[11] §Presence (+) or absence (–) within intraductal components.

tumor cells (expressed as a percentage). E-cadherin was considered as positive when staining was present in at least 10% of the tumor cells' membranes. For other markers, the presence of a single positive cell was considered a positive result. The number of blood vessels was counted by CD34 immunostains in a 1 mm^2 area, at least 4 times, and then a percentage promedium was made.

Statistical analysis to compare IMPCa and IDC-NOS were done by either the chi-squared test or standard *t*-test.

RESULTS

Clinical and pathological findings of 16 IMPCa are listed in Table 2. The age distribution at initial operation was between 38 and 67 years, with the average 50.9 years. Ten cases (62.5%) were pure type, and six were mixed type (37.5%). The tumor size, calculated by the maximum diameter of the invasive component on microscopy, was 7–84 mm (average 31.0 mm). The nuclear grade was high in 11 cases (68.8%) and intermediate in five cases (31.2%). Histological grade was III (poorly differentiated) in 12 cases (75.0%), and grade II (moderately differentiated) in four cases. Generally, both nuclear and histological grading was identical between IMPCa and the IDC-NOS area in mixed-type cases. Associated intraductal components was revealed in 10 cases, and, among them, comedonecrosis was seen in five cases. Extensive intraductal components were not evident. Lymphatic invasion was seen in 15 cases (93.4%), and was mostly extensive. A total mastectomy was performed for 10 cases, quadrantectomy for three cases, and lumpectomy for three cases. Lymph node dissection at the initial operation was performed in 14 cases. Lymph node metastases were seen in 13 cases (92.9%), and six of them (46.2%) showed more than 10 positive nodes. After the operation, chemotherapy was used for 13 cases, irradiation was used for five cases, and hormonal therapy was used for six cases as the adjuvant therapy. Follow up after the operation was evident for 2–204 months (mean 38 months); four cases (25.0%) were dead of disease (at 34, 56, 61, and 83 months), and three cases were alive with disease. During follow up, metastases was seen in the pleura (four cases), skin (three cases), bone

(two cases), chest wall (one case), axillary lymph node (one case), and pericardium (one case).

Table 3 shows the comparison with serially obtained IDC-NOS cases. The incidences of both high nuclear grade and poor histological grade were significantly higher in IMPCa (both $P < 0001$, respectively). The ratio of cases with positive hormone receptors was significantly low ($P < 0.05$, $P < 0.01$), but HER-2 positive cases were more frequent ($P < 0.025$). Lymph node metastases were more frequently seen, significantly, in IMPCa (13/14; 92.9%) than IDC-NOS (94/150; 65.3%) ($P < 0.05$). Additionally, half of the node positive IMPCa cases showed more than 10 positive lymph nodes, and the frequency was significantly higher than IDC-NOS ($P < 0.001$).

The results of immunohistochemistry can be seen in Figs 3,4,5,6, and the comparison between node positive control cases is summarized in Table 4. CD34 was positive in the endothelial cells of blood vessels (Fig. 3). However, neither micropapillary nests or the inner surface of empty space were positive for CD34, Factor VIII related antigen, nor type IV collagen. Invasive micropapillary carcinoma cases were more frequently positive for p53, but not statistically significant. CD34 showed a significantly increased number of blood vessels within the area of IMPCa ($P < 0.05$). Blood vessel counts by VEGF, E-cadherin MMP-2, and the Ki-67 index did not show any significant differences between the two groups. Immunohistochemical

Table 3 Comparison of histopathological features between IMPCa and IDC-NOS

	IMPCa (16 cases)	IDC-NOS (150 cases)
Age (average)	50.9 years	54.1 years
Nuclear grade		
1 (Low)	0	5 (3.3%)
2 (Intermediate)	4 (25.0%)	86 (57.3%)
3 (High)*	12 (75.0%)	59 (39.3%)
Histological grade		
I (Well differentiated)	0	27 (18.0%)
II (Moderately differentiated)	3 (18.8%)	71 (47.3%)
III (Poorly differentiated)**	13 (81.3%)	52 (34.7%)
ER***		
Positive	8 (50.0%)	112 (74.3%)
Negative	8 (50.0%)	38 (24.7%)
PR****		
Positive	5 (31.2%)	97 (64.7%)
Negative	11 (68.8%)	53 (35.3%)
HER-2*****		
Positive	8 (50.0%)	33 (20.3%)
Negative	8 (50.0%)	117 (79.7%)
LN status***		
Positive	13 (92.9%)	94 (65.3%)
Negative	1 (7.1%)	50 (34.7%)
10 or more +******	7/14 (50.0%)	10/114 (6.9%)

ER, estrogen receptor; IMPCa, invasive micropapillary carcinoma; IDC-NOS, invasive ductal carcinoma, not otherwise specified; LN, lymph node; PR, progesterone receptor. *Comparison between high and non-high grade, $P < 0.001$; **comparison between III (poor) and non-III grade, $P < 0.001$; ***$P < 0.05$; ****$P < 0.01$; *****$P < 0.025$; ******$P < 0.001$.

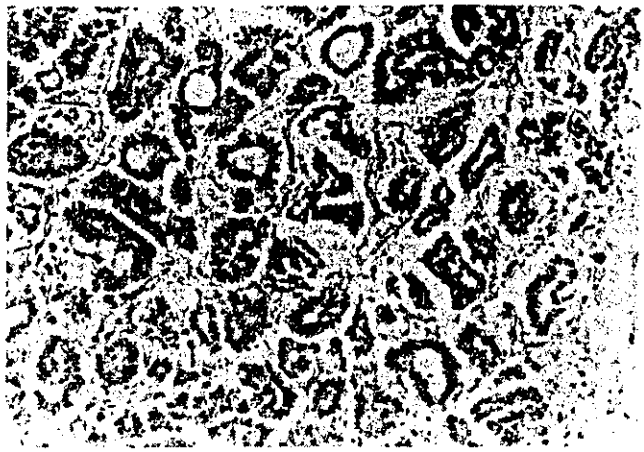

Figure 3 Invasive micropapillary carcinoma. Vascular endothelial growth factor was positive in the cytoplasm. LSAB, ×150.

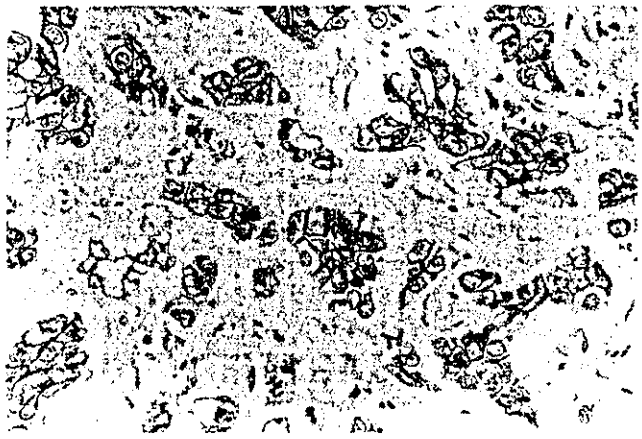

Figure 4 E-cadherin was positive in most of the cell membrane of carcinoma cells in the invasive micropapillary carcinoma cases. LSAB, ×150.

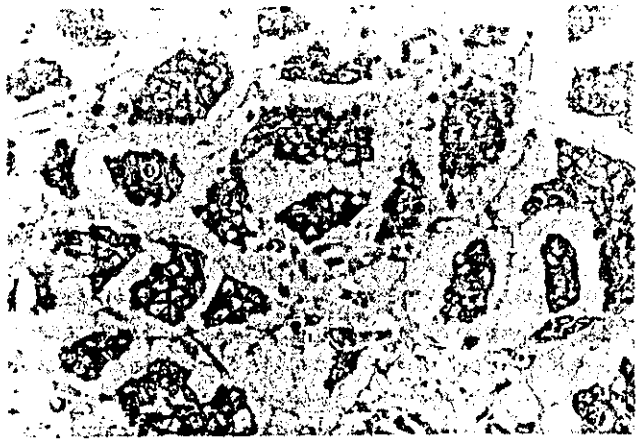

Figure 5 Matrix metalloproteinase-2 was positive in the cytoplasm of carcinoma cells in the invasive micropapillary carcinoma cases. LSAB, ×150.

Figure 6 CD34 revealed abundant capillary-sized vessels within the intervening stroma, but the empty spaces were not surrounded by endothelial cells. LSAB, ×80.

Table 4 Immunohistochemical findings of IMPCa and node positive control cases

	IMPCa (16 cases)	n+ IDC-NOS (23 cases)
p53		
Positive	9 (56.3%)	7 (30.4%)
Negative	7 (43.7%)	16 (69.6%)
Ki-67(%)	25.1 ± 14.1	23.5 ± 14.2
CD34 (MVC/mm^2)*	63.1 ± 36.0	33.6 ± 27.8
VEGF		
Positive	16 (100%)	22 (95.7%)
Negative	0	1 (4.3%)
E-cadherin		
Positive	16 (100%)	22 (95.7%)
Negative	0	1 (4.3%)
MMP-2		
Positive	15 (93.8%)	22 (95.7%)
Negative	1 (6.2%)	1 (4.3%)

IMPCa, invasive micropapillary carcinoma; IDC-NOS, invasive ductal carcinoma, not otherwise specified; MMP-2, matrix metalloproteinase protein-2; MVC, microvessel count; n+, node positive; VEGF, vascular endothelial growth factor. *$P < 0.05$.

features were generally identical in the IMPCa and IDC-NOS areas in mixed cases.

DISCUSSION

The incidence of IMPCa in our series was 1.0% of all primary breast carcinomas. It was much lower than any other previous studies, which showed 3.4%, 6%, and 7.6%, respectively.[6,7,14] One of the possible reasons for this low incidence is that the minimal requirement of the diagnostic criteria, namely the proportion of IMPCa in mixed-type cases, is different among the reported articles. Indeed, the incidence of pure type was reported as 1.7%,[6] and 0.8%,[14] which was not much different from the present study (0.6%). Additionally, several studies have stated that the presence of the IMPCa pattern within the invasive breast carcinoma, regardless of the proportion, shows the unfavorable nature of the tumor.[7,14,15]

Histologically, it is not difficult to notice this characteristic subtype of breast carcinoma. They are a variant of invasive ductal carcinoma, and a frequent association of intraductal carcinoma (10 of 16 in our series). E-cadherin, a marker of ductal carcinoma,[16] was consistently positive. Furthermore, they showed a high incidence of high nuclear/histological grade, both of which were more frequent, and statistically significant, than IDC-NOS, as in previous studies.[4,6,8,14] Immunohistochemical findings supported these features, with a tendency for a lower incidence of estrogen receptor (ER)/progesterone receptor (PR) positivity and a higher incidence of HER-2 positivity, compared to IDC-NOS. Previous studies reported a relatively high frequency of hormone receptor positivity in IMPCa (i.e. approximately 70% are ER positive by two authors[3,14]), but that might be associated with the staining procedure and/or counting methods. The proportions of the positive cases for c-erbB-2 (36.3%) and p53 (12.1%) were reported in one manuscript.[3]

One of the unique characteristics of this tumor type is a frequent association with lymph node metastases, especially with a large number of positive nodes.[7,15] Frequent (15/16 in the present series) and massive lymphatic vessel invasion is also revealed.[15] Lymph node metastases were not associated with the proportion of the IMPCa area within the tumor,[2,14,15] and frequently seen in cases of smaller tumors,[6] most likely because the events of lymphatic invasion occur earlier. Empty spaces, surrounding the micropapillae of carcinoma cell nests, were not surrounded by endothelial cells (CD34 and Factor VIII related antigen were totally negative in our series), a basement membrane (type IV collagen was totally negative), or epithelial cells. They were surrounded by fibrocollagenous stroma with spindle shaped stromal cells.[2,7] The empty spaces were not lymphatic vessels, and not seen in frozen sections,[5,7] and, thus, considered as an artifact at the time of fixation. The spaces may resemble peudoangiomatous stromal hyperplasia (PASH).[16] However, the association of steroid hormones (progesterone), frequently detected in the cases of PASH, were not seen considerably in IMPCa because the incidence of the presence of hormone receptors was relatively low. Additionally, IMPCa will occur in any ages (average 50.9 years old), and it is not like premenopausal deviation in PASH cases. Although the empty spaces themselves may exist in the same areas, the pathogenesis will be totally different between the two diseases.

These findings may suggest that IMPCa morphology is correlated with aggressive behavior of tumors, especially for metastatic potential. As IMPCa in general show a high frequency of lymph node metastasis, we have compared IMPCa with node-positive IDC-NOS without an IMPCa pattern. However, there were no significant differences of staining results

for p53, Ki-67, VEGF, E-cadherin and MMP-2 between the two groups. Hence, it is still unclear whether IMPCa histology is one of the significant unfavorable features among carcinomas with lymph node metastases. However, IMPCa showed significantly large numbers of small vasculatures within the stroma between the empty spaces, by microvessel densities using CD34 immunostains. Large numbers of small vasculatures will be associated with blood-borne distant metastases. In actual fact, IMPCa frequently express bone, lung and/or liver metastases.[7] Hence, the large numbers of small vasculatures may be one of the strong prognostic indicators of IMPCa, which has not been previously elucidated.

Finally, it is very interesting to investigate what kinds of findings are strongly associated with a poorer prognosis in these patients. As mentioned above, the proportion of the IMPCa area in a single mass may not affect the prognosis. Even the smaller tumors, less than 1 cm or even less than 0.5 cm, may show extensive lymph node metastases,[6] as in the present study, case 4 (8 mm in maximum diameter, lymph node status 27/27). Blood-borne metastases may also be important. Cases that died from IMPCa in the present series were of various ages (37, 38, 43 and 65 years) and sizes (8, 25, 32 and 37 mm), with high nuclear grade and poor differentiation. Positive lymph nodes for metastases were surprisingly high in number (8, 18, 25, and 27). Immunohistochemical profiles were variable, and specific features were not evident (data not shown). Some authors have estimated that negativity for estrogen receptors, more than four positive nodes, and high mitotic activity were of prognostic significances.[8,9] Although we did not analyse pure and mixed subtypes separately, there were three pure and one mixed IMPCa cases that were dead of disease. The significant differences between pure and mixed type, and whether the presence of a minor proportion of IMPCa is an unfavorable factor, still seems to be controversial. There are several possibilities for the explanation of the significant biological differences according to the proportion of IMPCa (within the tumor). One is that the aggressive behavior is associated with the total volume of IMPCa, regardless of the proportion. Another is that the non-IMPCa area of mixed cases may have the same aggressive manner with IMPCa areas, and the presence of IMPCa in any area is an unfavorable sign. However, the number of analyzed cases and the periods of follow up were limited, and uni- or multivariate analysis was not always significant, so further investigations are necessary for final conclusions. Tentatively, we consider that the IMPCa histology itself will be a strong indicator of the aggressive behavior of the carcinoma.

In conclusion, IMPCa itself, in any amount, should be considered as a poor prognostic sign of invasive breast carcinoma. The IMPCa may at least be more aggressive than IDC-NOS, and show significantly higher vasculature than node-positive IDC-NOS, according to the results of the present study. As these tumors show distant, blood-borne metastases, high vasculature in the intervening stroma is important, as well as their extensive lymphatic spread.

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REFERENCES

1 Elston CW, Ellis IO. Invasive micropapillary carcinoma. In: Elston CW, Ellis IO, eds. *Systemic Pathology, The Breast*, **Vol. 13**, 3rd edn. Edinburgh: Churchill Livingstone, 1998; 322–3.
2 Siriaungkul S, Tavassoli FA. Invasive micropapillary carcinoma of the breast. *Mod Pathol* 1993; **6**: 660–62.
3 Luna-More S, De los Santos F, Breton JJ, Canadas MA. Estrogen and progesterone receptors, C-ERBB-2, p53, and BCL-2 in thirty-three invasive micropapillary breast carcinomas. *Pathol Res Pract* 1996; **192**: 27–32.
4 Middleton LP, Tressera F, Sobel ME *et al.* Infiltrating micropapillary carcinoma of the breast. *Mod Pathol* 1999; **12**: 499–504.
5 Tresserra F, Grases PJ, Fabregas R, Fernandez CA, Dexeus S. Invasive micropapillary carcinoma. Distinct features of a poorly recognized variant of breast carcinoma. *Eur J Gynaecol Oncol* 1999; **20**: 205–8.
6 Walsh MM, Bleiweiss IJ. Invasive micropapillary carcinoma of the breast. Eighty cases of an underrecognized entity. *Hum Pathol* 2001; **32**: 583–9.
7 Tsumagari K, Sakamoto G, Akiyama F, Kasumi F. The clinicopathological study of invasive micropapillary carcinoma of the breast. *Jpn J Breast Cancer* 2001; **16**: 341–8 (in Japanese).
8 Paterakos M, Watkin WG, Edgerton SM, Moore IIDH, Thor AD. Invasive micropapillary carcinoma of the breast. A prognostic Study. *Hum Pathol* 1999; **30**: 1459–63.
9 Luna-More S, Casquero S, Perez-Mellado A, Rius F, Welill B, Gornemann I. Importance of estrogen receptors for the behavior of invasive micropapillary carcinoma of the breast. Review of 68 cases with follow-up of 54. *Pathol Res Pract* 2000; **196**: 35–9.
10 Tsuda H, Akiyama F, Kurosumi M, Sakamoto G, Watanabe T. Establishment of histological criteria for high-risk node-negative breast carcinoma for a multi-institutional randomized clinical trial of adjuvant therapy. Japan National Surgical Adjuvant Study of Breast Cancer (NSAS-BC) Pathology Section. *Jpn J Clin Oncol* 1998; **20**: 486–91.
11 Elston CW, Ellis IO. Pathologic prognostic factors in breast cancer. I. The value of histologic grade in breast cancer. Experience from a large study with long-term follow-up. *Histopathol* 1991; **19**: 403–11.
12 Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998; **11**: 155–68.
13 Tsuda H, Tani Y, Hasegawa T, Fukutomi T. Concordance in judgment among c-erbB-2 (HER-2/*neu*) overexpression detected by two immunohistochemical tests and gene amplification detected by Southern blot hybridization in breast carcinoma. *Pathol Int* 2001; **51**: 26–32.

14 Nassar H, Wallis T, Andea A, Dey J, Adsay V, Visscher D. Clinicopathologic analysis of invasive micropapillary differentiation in breast carcinoma. *Mod Pathol* 2001; **14**: 836–41.

15 Luna-More S, Gonzalez B, Acedo C, Rodorigo I, Luna C. Invasive micropapillary carcinoma of the breast. A new special type of invasive mammary carcinoma. *Pathol Res Pract* 1994; **190**: 668–74.

16 Acs G, Lawton TJ, Rebbeck TR, Livolsi VA, Zhang PJ. Differential expression of E-cadherin in lobular and ductal neoplasms of the breast and its biologic and diagnostic implications. *Am J Clin Pathol* 2001; **115**: 85–98.

17 Vuitch MF, Rosen PP, Erlandson RA. Pseudoangiomatous hyperplasia of mammary stroma. *Hum Pathol* 1986; **17**: 185–91.

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Isolation of Temperature-sensitive p53 Mutations from a Comprehensive Missense Mutation Library*

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Temperature-sensitive (ts) mutations have been used as a genetic and molecular tool to study the functions of many gene products. Each ts mutant protein may contain a temperature-dependent intramolecular mechanism such as ts conformational change. To identify key ts structural elements controlling the protein function, we screened ts p53 mutants from a comprehensive mutation library consisting of 2,314 p53 missense mutations for their sequence-specific transactivity through p53-binding sequences in *Saccharomyces cerevisiae*. We isolated 142 ts p53 mutants, including 131 unreported ts mutants. These mutants clustered in β-strands in the DNA-binding domain, particularly in one of the two β-sheets of the protein, and 15 residues (Thr^{155}, Arg^{158}, Met^{160}, Ala^{161}, Val^{172}, His^{214}, Ser^{215}, Pro^{223}, Thr^{231}, Thr^{253}, Ile^{254}, Thr^{256}, Ser^{269}, Glu^{271}, and Glu^{286}) were ts hot spots. Among the 142 mutants, 54 were examined further in human osteosarcoma Saos-2 cells, and it was confirmed that 89% of the mutants were also ts in mammalian cells. The ts mutants represented distinct ts transactivities for the p53 binding sequences and a distinct epitope expression pattern for conformation-specific anti-p53 antibodies. These results indicated that the intramolecular β-sheet in the core DNA-binding domain of p53 was a key structural element controlling the protein function and provided a clue for finding a molecular mechanism that enables the rescue of the mutant p53 function.

p53 tumor suppressor is a 393-amino acid transcription factor that activates the transcription of a number of downstream genes through p53 binding to two copies of the specific consensus DNA sequence 5′-RRRC(A/T)(T/A)GYYY-3′ (in which R is a purine nucleoside and Y is a pyrimidine nucleoside) in their regulatory regions (1). These molecular switches are activated by post-translational modifications, including phosphorylation, acetylation, and prolyl isomerization (2–5) of p53 in response to genotoxic or non-genotoxic stresses. The resulting biological effects are cell cycle arrest, apoptosis, DNA repair, and angiogenesis (6–10). A growing number of p53 downstream genes have been isolated, and p53 has been structurally and functionally divided into three portions, namely the NH_2-terminal portion containing the transactivation domain, the central core portion corresponding to the DNA-binding domain, and the COOH-terminal portion containing the oligomerization domain. The evolution of the DNA-binding domain is highly conserved in p53 orthologues (11) and also in the conserved human homologues p63 and p73 (12, 13).

The structure of the DNA-binding domain (residues 94–312) was resolved by x-ray crystallography (14). The domain consists of two α-helixes (H1 and H2) and 11 β-strands (S1, S2, S2′, and S3–S10) that were interconnected by loops (long L1–L3 loops and other short loops). Two anti-parallel β-sheets containing four (S1, S3, S5, and S8) and five (S4, S6, S7, S9, and S10) β-strands make up a large β-sandwich that serves as a scaffold for a loop-sheet-helix (LSH) motif (L1, S2, S2′, S10, and H2) and two large loops (L2 and L3). The loop-sheet-helix consists of two separate regions as follows: (i) the L1 loop (residues 113–123) and the S2-S2′ β-hairpin (residues 124–135) that correspond to evolutionary conserved region II (residues 117–142) (11); and (ii) the end of the S10 strand (residues 264–274) and the H2 helix (residues 278–286) that correspond to conserved region V (residues 270–286). In the loop-sheet-helix, the L1 loop and the H2 helix contact with a DNA major groove formed by the RRRC region of the consensus sequence. One of the large loops, the L2 (residues 164–194), is interrupted by a short helix (H1) and contains conserved region III (residues 171–181). Another large loop, L3 (residues 237–250), coincides with conserved region IV (residues 234–258) and makes contact with the DNA minor groove formed by the A/T rich region of the consensus sequence. The L2 loop stabilizes the L3 loop by packing through a side-chain interaction and a zinc atom tetrahedrally coordinated on residues Cys^{176}, His^{179} of the L2 loop and Cys^{238} and Cys^{242} of the L3 loop.

Mutations in the *TP53* gene are the most frequent genetic alterations in the various human tumors (15). According to the latest TP53 mutation databases (16, 17), more than 15,000 somatic mutations have been reported to date. The mutations are clustered in the DNA-binding domain, and the majority (~80%) are missense mutations. Among tumor-derived mutations, those at residues Arg^{175}, Gly^{245}, Arg^{248}, Arg^{249}, Arg^{273}, and Arg^{282} have frequently been reported, and all missense mutations were unable to bind the specific p53 binding sequences and the inactive transactivation for downstream genes. These are structurally important residues, because they directly involve DNA binding or stabilization of the L2 and L3 loops of the protein. However, the majority of remaining missense mutations have not yet been examined. Recently, we constructed 2,314 missense mutations that covered almost all of the tumor derived missense mutations, as well as a number

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