by the ethical committees of the Faculty of Medical Sciences, Kyushu University, and of all but two of the participating hospitals. The two hospitals had no ethical committees at the time of survey. Details of the methods have been described elsewhere (25).

Participants. Cases were a consecutive series of patients with histologically confirmed incident colorectal adenocarcinomas who were admitted to two university hospitals or six affiliated hospitals for surgical treatment during the period October 2000 to December 2003. Other eligibility criteria were: age of 20 to 74 years at the time of diagnosis, residence in the study area, no prior history of partial or total removal of the colorectum, familial adenomatous polyposis or inflammatory bowel disease, mental competence to give informed consent and to complete the interview. Of 1,053 eligible cases, a total of 840 cases (80%) participated in the interview, and 685 out of them gave an informed consent to genotyping.

Eligibility criteria for controls were the same as described for cases except for the two items, i.e., having no diagnosis of colorectal cancer and age of 20 to 74 years at the time of selection. A total of 1,500 persons were selected as control candidates by two-stage random sampling. The number of control candidates by sex and 10-year age class were determined in accordance to sex- and age-specific numbers of estimated incident cases of colorectal cancer. The first step was a random selection of 15 small areas out of 178 in total, and then ~100 persons were randomly selected in each small area using the municipal resident registry on the basis of proportions of population in the small areas by sex and 10-year age class. A letter of invitation was sent to each candidate, and at most three additional letters of invitation were mailed to nonrespondents. A total of 833 persons participated in the survey, and 778 gave an informed consent to genotyping. The net participation rate was calculated as 60% (833/1,382), after exclusion of 118 persons for the following reasons: death (n = 7), migration from the study area (n = 22), undelivered mail (n = 44), mental incompetence (n = 19), history of partial or total removal of the colorectum (n = 21), and diagnosis of colorectal cancer after the survey (n = 5).

In both cases and controls, older persons and women were less likely to give consent to genotyping, whereas there was no material difference in residence, smoking habit, and alcohol use between individuals giving consent and those who did not (Table 1).

Procedures. DNA was extracted from the buffy coat by using a commercial kit (Qiagen GmbH, Hilden, Germany). Genotyping was done by one of the authors (T. Hagiwara) using the PCR-RFLP method. The PCR was done in a reaction mixture of 10 μL containing 0.5 units of Taq and 1 μL of template DNA with a concentration of ~50 to 150 ng/μL. The *CYP7A1* genotype was determined, as described by Han et al. (24) using primers 5′-AATGT TTTTC CCAGT TCTCT TTC-3′ (sense) and 5′-AATTA GCCAT TTGTT CATTC TATTA G-3′ (antisense). After the initial denaturation at 94°C for 4 minutes, 30 cycles of PCR were done for 30 seconds at 94°C, for 30 seconds at 53°C, and for 30 seconds at 72°C, with a final extension at 72°C for 7 minutes. The PCR product of 393 bp fragment was digested with 10 units of *BsaI* in a reaction mixture of 20 μL for 3 hours at 50°C. The digestion results in fragments of 300 and 93 bp for the *A* allele, and those of 261, 93, and 39 bp for the *C* allele. The digested PCR products were applied to electrophoresis of 3% agarose gel (NuiSieve GTG, Rockland, ME), and visualized by ethidium bromide.

The polymorphism was referred to as *A-204C* by Couture et al. (23), but the actual site of the polymorphism is located 203 bp upstream of the transcription start site according to the latest report of the sequence http://www.ncbi.nlm.nih.gov/Genomes). This was also confirmed by our sequencing of the relevant fragment.

Statistical analysis. The association of *CYP7A1* genotypes with the risk of colorectal cancer was examined in terms of odds ratio (OR) and 95% confidence intervals (CI). ORs were obtained from multiple logistic regression analysis, including indicator variables for gender, 5-year age class, and resident area (Fukuoka City or suburban area) as covariates. Statistical significance was declared if 95% CI did not include unity. All statistical analyses were done using the SAS version 8.2 (SAS Institute, Inc., Cary, NC).

Results

Proportions of the *AA*, *AC*, and *CC* genotypes in cases of colorectal cancer were 24%, 56%, and 20%, respectively (Table 2). The corresponding proportions in the control group were 25%, 51%, and 24%, respectively. The distribution in the control group was in agreement with the Hardy-Weinberg equilibrium (P = 0.59). The *CC* genotype was slightly less frequent in the case group, and the adjusted OR for the *CC* versus *AA* genotype was slightly lower than unity, with the 95% CI including unity. When the *AA* and *AC* genotypes were combined as the referent, the adjusted OR for the *CC* genotype was 0.81 (95% CI, 0.63-1.04).

The association with *CYP7A1* polymorphism was further examined for cancers of the proximal colon, distal colon, and rectum separately (Table 3). A nearly significant decrease in the OR for the *CC* versus *AA* genotype was observed for proximal colon cancer, but not for the other sites of cancer. When the *AA* and *AC* genotypes were combined as referents, the adjusted ORs of proximal colon cancer for the *CC* genotype was significantly lower than unity.

Discussion

The present study was the first that examined the relation between a functional *CYP7A1* polymorphism (*A-203C*) and

Table 1. Characteristics of cases and controls by consent to genotyping

Variable	Cases			Controls		
	With consent	Without consent	P^*	With consent	Without consent	P^*
Number	685	155		778	55	
Mean age (y)	60.2	61.9	0.03	58.6	62.9	0.004
Male (%)	62.2	48.4	0.002	63.0	45.5	0.01
Fukuoka City (%)	61.3	57.4	0.37	64.4	67.3	0.67
Ever-smoking (%)†	56.3	53.1	0.22	59.7	41.8	0.18
Alcohol use (%)†,‡	58.9	55.4	0.22	59.3	45.0	0.33

*P values (two-sided) were based on t test or χ^2 test unless otherwise specified.
†Adjusted for sex and 5-year age class by the direct method with total number of cases or controls as standard population. P values were based on the Mantel-Haenszel method.
‡Drinking alcohol at least once per week ~5 years ago.

Table 2. Adjusted ORs and 95% CI of colorectal cancer according to *CYP7A1 A-203C* polymorphisms

CYP7A1 A-203C genotype	Number (%)		Adjusted ORs (95% CI)*
	Cases (n = 685)	Controls (n = 778)	
AA	163 (24)	193 (25)	1.00 (referent)
AC	385 (56)	399 (51)	1.13 (0.88-1.46)
CC	137 (20)	186 (24)	0.88 (0.65-1.20)

*Adjusted for gender, 5-year age class, and resident area.

colorectal cancer, and showed a decreased risk of cancer of the proximal colon, but not of the distal colon and rectum, among individuals having the *CC* genotype. This genotype is probably associated with lowered capability of synthesizing bile acids (22, 23), the findings provide further evidence to the role of bile acids in colorectal carcinogenesis.

An advantage in this large-scale case-control study is that controls were derived from free-living residents in the community. It is also notable that participation rates of eligible cases and controls were fairly high. Genotyping was done in 82% of the cases and 93% of the controls who participated in the survey. It is generally considered that selection and confounding are less likely to occur in studies of genetic polymorphisms (26, 27). It is, however, possible that use of hospital controls may cause selection bias even in the gene-disease association. For instance, individuals with high blood cholesterol levels may have been included or excluded differentially in the controls if selection had occurred in patients with cholesterol-related diseases. The study subjects were an ethnically homogenous population of Japanese, and the concern over population stratification would be negligible (28).

Since the first report by Rose et al. (29), many prospective studies have observed an inverse association between serum total or low-density lipoprotein cholesterol and colon cancer (30). Although this inverse association is generally ascribed to the effect of preclinical cancer existing at the baseline (30), an increased risk of proximal colon cancer associated with low levels of serum total cholesterol persisted 10 to 20 years later in a prospective study in Hawaii (31). Furthermore, a case-control study observed lower levels of total and low-density lipoprotein cholesterol in cases of proximal colon cancer, but not of distal colon cancer, than in controls (32). These findings are congruent with decreased risk of proximal colon cancer associated with the *CYP7A1 CC* genotype.

High-fat diets are shown to increase fecal excretion of secondary bile acids as well as of total bile acids in humans (33), and to enhance chemically induced colon carcinogenesis in animals (34). Although fat intake is strongly positively correlated with colon cancer rates among countries (35), and over time in Japan (36), studies of individuals have consistently failed to find a positive association between fat intake and colon or colorectal cancer (37). The lack of an association with fat in studies of individuals may be due to a limited variation of fat intake within populations. In this regard, the present findings emphasize the usefulness of studying functional genetic polymorphisms when study populations are homogeneous with respect to exposure to environmental factors such as nutrient and food intake.

In conclusion, a large case-control study in Japan showed a decreased risk of proximal colon cancer in individuals having the *CC* genotype of *CYP7A1 A-203C*, which probably renders less activity of the enzyme converting cholesterol to bile acids. The findings add to evidence for the role of bile acids in colorectal carcinogenesis.

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Table 3. Adjusted OR and 95% CI of colorectal cancer according to *CYP7A1 A-203C* genotypes by subsite

CYP7A1 A-203C genotype	Proximal colon (n = 150)		Distal colon (n = 232)		Rectum (n = 290)	
	No.	OR (95% CI)*	No.	OR (95% CI)*	No.	OR (95% CI)*
Model 1						
AA	39	1.00 (referent)	52	1.00 (referent)	69	1.00 (referent)
AC	88	1.09 (0.71-1.65)	129	1.18 (0.81-1.70)	159	1.09 (0.78-1.53)
CC	23	0.63 (0.36-1.10)	51	1.01 (0.65-1.57)	62	0.93 (0.62-1.39)
Model 2						
AA + AC	127	1.00 (referent)	181	1.00 (referent)	228	1.00 (referent)
CC	23	0.59 (0.37-0.96)	51	0.90 (0.63-1.29)	62	0.87 (0.63-1.22)

*Adjusted for gender, 5-year age class, and resident area.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: GLOBOCAN 2000. Int J Cancer 2001;94:153–6.
2. Kono S. Dietary factors for gastrointestinal cancers: a worldwide overview. Gann Monogr Cancer Res 1996;44: 29–39.
3. Parkin DM, Whelan SL, Ferlay J, Teppo L, Thomas DB, editors. Cancer incidence in five continents, Vol. VIII, IARC Scientific Publications, No. 155. IARC, Lyon; 2002.
4. Narisawa T, Magadia NE, Weisburger JH, Wynder EL. Promoting effect of bile acids on colon carcinogenesis after intrarectal instillation of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine in rats. J Natl Cancer Inst 1974;53: 1093–7.
5. Reddy BS, Narasawa T, Weisburger JH, Wynder EL. Promoting effect of sodium deoxycholate on colon adenocarcinomas in germ-free rats. J Natl Cancer Inst 1976;56:441–2.
6. Qiao D, Chen W, Stratagoules ED, Martinez JD. Bile acid-induced activation of activator protein-1 requires both extracellular signal-regulated kinase and protein kinase C signaling. J Biol Chem 2000;275:15090–8.
7. Milovic V, Teller IC, Faust D, Caspary WF, Stein J. Effects of deoxycholate on human colon cancer cells: apoptosis or proliferation. Eur J Clin Invest 2002;32:29–34.
8. Reddy BS, Wynder EL. Large-bowel carcinogenesis: fecal constituents of populations with diverse incidence rates of colon cancer. J Natl Cancer Inst 1973; 50:1437–42.
9. Wynder EL, Reddy BS. Metabolic epidemiology of colorectal cancer. Cancer 1974;34:801–6.
10. Hill MJ, Drasar BS, Williams RE, et al. Faecal bile-acids and clostridia in patients with cancer of the large bowel. Lancet 1975;1:535–9.
11. Reddy BS, Wynder EL. Metabolic epidemiology of colon cancer. Fecal bile acids and neutral sterols in colon cancer patients and patients with adenomatous polyps. Cancer 1977;39:2533–9.
12. Bayerdörffer E, Mannes GA, Richter WO, et al. Increased serum deoxycholic acid levels in men with colorectal adenomas. Gastroenterology 1993;104:145–51.
13. Bayerdörffer E, Mannes GA, Ochsenkühn T, Dirschedl P, Wiebecke B, Paumgartner G. Unconjugated secondary bile acids in the serum of patients with colorectal adenomas. Gut 1995;36:268–73.
14. Blackwood A, Murray WR, Mackay C, Calman KC. Fecal bile acids and clostridia in the aetiology of colorectal and breast cancer. Br J Cancer 1978;38:175.
15. Mudd DG, McKelvey ST, Norwood W, Elmore DT. Carcinoma of the large bowel and faecal bile acids. Br J Surg 1979;66:355.
16. Murray WR, Blackwood A, Calman KC, Mackay C. Faecal bile acids and clostridia in the aetiology of colorectal cancer. Br J Surg 1979;66:364.
17. Costarelli V, Key TJ, Appleby PN, Allen DS, Fentiman IS, Sanders TA. A prospective study of serum bile acid concentrations and colorectal cancer risk in postmenopausal women on the island of Guernsey. Br J Cancer 2002;86:174–4.
18. Giovannucci E, Colditz GA, Stampfer MJ. A meta-analysis of cholecystectomy and risk of colorectal cancer. Gastroenterology 1993;105:130–41.
19. Reid FD, Mercer PM, Harrison M, Bates T. Cholecystectomy as a risk factor for colorectal cancer: a meta-analysis. Scand J Gastroenterol 1996;31:160–9.
20. Pomare EW, Heaton KW. The effect of cholecystectomy on bile salt metabolism. Gut 1973;14:753–62.
21. Hepner GW, Hofmann AF, Malagelada JR, Szczepanik PA, Klein PD. Increased bacterial degradation of bile acids in cholecystectomized patients. Gastroenterology 1974;66:556–64.
22. Wang J, Freeman DJ, Grundy SM, Levine DM, Guerra R, Cohen JC. Linkage between cholesterol 7α-hydroxylase and high plasma low-density lipoprotein cholesterol concentrations. J Clin Invest 1998;101:1283–91.
23. Couture P, Otvos JD, Cupples LA, Wilson PW, Schaefer EJ, Ordovas JM. Association of the A-204C polymorphism in the cholesterol 7α-hydroxylase gene with variations in plasma low density lipoprotein cholesterol levels in the Framingham Offspring Study. J Lipid Res 1999;40:1883–9.
24. Han Z, Heath SC, Shmulewitz D, et al. Candidate genes involved in cardiovascular risk factors by a family-based association study on the island of Kosrae, Federated States of Micronesia. Am J Med Genet 2002;110:234–42.
25. Kono S, Toyomura K, Yin G, Nagano J, Mizoue T. A case-control study of colorectal cancer in relation to lifestyle factors and genetic polymorphisms: design and conduct of the Fukuoka colorectal cancer study. Asian Pac J Cancer Prev 2004;5:393–400.
26. Smith DG, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 2003;32:1–22.
27. Wheatley K, Gray R. Mendelian randomization—an update on its use to evaluate allogeneic stem cell transplantation in leukaemia. Int J Epidemiol 2004;33: 15–7.
28. Cardon LR, Palmer LJ. Population stratification and spurious allelic association. Lancet 2003;361:598–604.
29. Rose G, Blackburn H, Keys A, et al. Colon cancer and blood-cholesterol. Lancet 1974;1:181–3.
30. Law MR, Thompson SG. Low serum cholesterol and the risk of cancer: an analysis of the published prospective studies. Cancer Causes Control 1991;2:253–61.
31. Nomura AM, Stemmermann GN, Chyou PH. Prospective study of serum cholesterol levels and large-bowel cancer. J Natl Cancer Inst 1991;83:1403–7.
32. Kervinen K, Södervik H, Mäkelä J, et al. Is the development of adenoma and carcinoma in proximal colon related to apolipoprotein E phenotype? Gastroenterology 1996;110:1785–90.
33. Reddy BS. Diet and excretion of bile acids. Cancer Res 1981;41:3766–8.
34. Reddy BS. Dietary fat and colon cancer: animal model studies. Lipids 1992;27:807–13.
35. McKeown-Eyssen GE, Bright-See E. Dietary factors in colon cancer: international relationships. Nutr Cancer 1984;6:160–70.
36. Kono S. Secular trend of colon cancer incidence and mortality in relation to fat and meat intake in Japan. Eur J Cancer Prev 2004;13:127–32.
37. World Cancer Research Fund and American Institute for Cancer Research. Food, nutrition and the prevention of cancer: a global perspective. Washington, DC: American Institute for Cancer Research; 1997.

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Two modes of microsatellite instability in human cancer: differential connection of defective DNA mismatch repair to dinucleotide repeat instability

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ABSTRACT

Microsatellite instability (MSI) is associated with defective DNA mismatch repair in various human malignancies. Using a unique fluorescent technique, we have observed two distinct modes of dinucleotide microsatellite alterations in human colorectal cancer. Type A alterations are defined as length changes of ≤6 bp. Type B changes are more drastic and involve modifications of ≥8 bp. We show here that defective mismatch repair is necessary and sufficient for Type A changes. These changes were observed in cell lines and in tumours from mismatch repair gene-knockout mice. No Type B instability was seen in these cells or tumours. In a panel of human colorectal tumours, both Type A MSI and Type B instability were observed. Both types of MSI were associated with *hMSH2* or *hMLH1* mismatch repair gene alterations. Intriguingly, *p53* mutations, which are generally regarded as uncommon in human tumours of the MSI$^+$ phenotype, were frequently associated with Type A instability, whereas none was found in tumours with Type B instability, reflecting the prevailing viewpoint. Inspection of published data reveals that the microsatellite instability that has been observed in various malignancies, including those associated with Hereditary Non-Polyposis Colorectal Cancer (HNPCC), is predominantly Type B. Our findings indicate that Type B instability is not a simple reflection of a repair defect. We suggest that there are at least two qualitatively distinct modes of dinucleotide MSI in human colorectal cancer, and that different molecular mechanisms may underlie these modes of MSI. The relationship between MSI and defective mismatch repair may be more complex than hitherto suspected.

INTRODUCTION

Microsatellites are repetitive DNA sequences comprising short reiterated motifs dispersed throughout the eukaryotic genome (1). Microsatellite lengths are highly polymorphic in human populations, but appear stable during the life span of the individual. Somatic instability of microsatellite sequences has initially been reported in human colorectal cancer (2,3), and particularly in the familial cancer-prone syndrome, hereditary non-polyposis colorectal cancer (HNPCC) (4,5). In 1993, mutations in one of the genes encoding proteins essential for DNA mismatch repair (MMR) were found in

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

HNPCC individuals (6,7). MMR is an important editing system. It counteracts the base mismatches and strand misalignments that occur during DNA replication and recombination (8). Repetitive sequences such as those comprising microsatellites are particularly prone to polymerase slippage and, consequently, strand misalignment. If these errors remain uncorrected, the mutations are fixed during subsequent replication as addition or deletion of one (or more) repeat units. The phenomenon of unstable microsatellites, i.e. microsatellite instability (MSI), in which tumour cells accumulate this type of repeat length alterations in microsatellites, is considered to reflect MMR deficiency. The MSI^+ phenotype is frequently associated with various human malignancies (9). As defective MMR is regarded as a risk factor for familial predisposition or second malignancies, analyses of microsatellite instability have been prevalent, particularly in the field of oncology. However, the reported frequency for MSI^+ tumours in each malignancy differs widely in the literature (9).

Although analysis of MSI is now commonplace, a designation of MSI^+ may sometimes be a difficult decision. The 1997 National Cancer Institute (NCI) workshop, 'Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition', suggested that the variety of microsatellites used was a major cause of discrepancies among data from various laboratories, and recommended a panel of five microsatellites as 'working reference panel' (10). We believe that, in addition to selection of targets for analysis, methodological problems also account for some of the variability in results. Changes in microsatellite lengths are sometimes minor—as small as loss or gain of a single repeat unit. In addition, cells carrying changes in microsatellite sequences are not always major in a given sample. However, in an assay system using the conventional sequencing gel electrophoresis and autoradiography, it appears difficult to resolve microsatellite PCR products precisely and quantitatively. PCR itself has an intrinsic variability. The most widely used thermostable DNA polymerase (*Taq*) has a terminal deoxynucleotidyl transferase (TDT) activity, which adds one additional base to PCR products in a sequence-dependent manner. TDT activity of *Taq* polymerase is variably expressed, depending on the conditions used. This property, in addition to intrinsic strand misalignment during amplification of microsatellite repeats, increases the complexity of PCR products. In the conventional microsatellite analysis, intrinsic caution and the desire to avoid scoring false-positives may have led to an underestimate of the frequency of minor, more subtle microsatellite changes, such as alterations of limited numbers of repeat units. We have applied our fluorescent technique for microsatellite instability analysis (11) to address these problems. Here, we report that relatively subtle alterations in microsatellites are indeed generally associated with MMR deficiency. In contrast, most HNPCC tumours display much more extensive microsatellite changes. Our findings suggest that there are previously unrecognized aspects of microsatellite instability in human cancer.

MATERIALS AND METHODS

Cells and tissue specimens

Msh2$^{-/-}$ mouse embryonic fibroblast (MEF) cell line, RH95021 (12) and *Mlh1*$^{-/-}$ MEF cell line, MC2, were kindly provided by Dr Hein te Riele, Amsterdam Cancer Center and Dr Michael Liskay, University of Oregon, respectively. Cells were cultured in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Samples of cancer tissues and the corresponding normal mucosa were obtained from 79 patients with colorectal carcinoma who underwent surgery in the Department of Surgery and Science, Kyushu University Hospital from 1996 to 1999. Written informed consent for studies using the tissues was obtained from each patient. Ethical approval was obtained from the IRB of Kyushu University. Specimens, taken immediately after resection, were placed in liquid nitrogen. High molecular weight DNA was extracted and subjected to microsatellite analyses.

Microsatellite instability

Microsatellite analysis using fluorescence-labelled primers and an automated DNA sequencer has been described in detail (11). Briefly, five human dinucleotide microsatellites, D2S123, D5S107, D10S197, D11S904 and D13S175, in genomic DNA from tissue specimens were amplified by PCR. 5′ primers were labelled with the fluorescent compound, ROX (6-carboxy-x-rhodamine) or HEX (6-carboxy-2′,4′,7′,4,7,-hexachloro-fluorescein). PCR reactions were done using TaKaRa *Taq* (TaKaRa Co. Ltd., Tokyo, Japan). T4 DNA polymerase was added to the PCR products, followed by incubation at 37°C for 10 min. To compare electrophoretic profiles between two samples, 1.2 μl of ROX-labelled product and 0.3 μl of HEX-labelled product were mixed. Samples were denatured and loaded onto the ABI 373A sequencer (Applied Biosystems, Foster City, CA, USA). The data were processed using the GeneScan software (Applied Biosystems). For mice, three dinucleotide microsatellites, D1Mit62, D6Mit59 and D7Mit91, were analysed.

DNA sequencing

All the exons and exon–intron junctions of *hMSH2* and *hMLH1* were amplified by PCR using *Taq* polymerase with 3′ exonuclease activity, TaKaRa *Ex Taq* (TaKaRa Co. Ltd., Tokyo, Japan). Primer sequences are the same as reported by Kolodner *et al.* (13,14), except that the additional sequence complementary for M13 universal primer was deleted, and that one-step PCR was mainly employed. PCR products were used as a template for cycle sequencing reactions using BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Mutations found in one PCR product were verified by reverse sequencing and finally confirmed in two independently amplified PCR products. Sequencing analyses of *p53* gene (exon 5–9) were performed using *p53* primers (Nippon Gene, Tokyo, Japan).

Immunohistochemistry

Tissue specimens were fixed in buffered 10% paraformaldehyde and embedded in paraffin. Prior to the assay, the specimens were sectioned at 4 μm and deparaffinized using xylene. Immunohistochemistry was performed using the streptavidin–biotin–peroxidase complex method (Histofine SAB kit, Nichirei, Tokyo, Japan) using an automated stainer (VENTANA Discovery System, Ventana Medical Systems Inc., Tucson, AZ, USA). At least, two independent antibodies

were used to confirm the status of negative staining. Sections prepared from *Msh2*- and *Mlh1*-knockout mice were also used as negative controls. Antibodies used were as follows: anti-MSH2; NA27 and NA26 (Oncogene Research Products, Cambridge, MA, USA), anti-MLH1; PM-13291A (Phar Mingen, Hamburg, Germany), NA28 (Oncogene Research Products) and sc-581 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

RESULTS

Two modes of dinucleotide microsatellite instability in human cancer

We have established a sensitive fluorescent technique for microsatellite analysis (11). Application of this technique to human cancers revealed a number of previously unrecognized aspects of MSI. In particular, we observed two distinct patterns of alterations at dinucleotide microsatellites in human malignancies (15–17). Examples are shown in Figure 1. In some cases, length changes are relatively small and affect ≤6 bp (Type A, Figure 1A–D). In the other, more dramatic changes involving ≥8 bp are observed (Type B) (Figure 1E–H). Because Type B alterations involve large differences in microsatellite length, it can sometimes appear as if a 'third' allele is present in addition to the parental alleles (Figure 1E–H). Throughout the analyses using this technique, results were highly reproducible in several independent experiments. Neither additional peaks nor changes in the ratio between peaks were noted.

Microsatellite instability observed in mismatch repair gene-knockout mice

To analyse MSI in a defined genetic background, we used the *Msh2*$^{-/-}$ MEF cell line RH95021 (12). Alterations in the lengths of three dinucleotide microsatellites were analysed in RH95021 subclones. The majority of subclones (14/21) exhibited the same configuration at the D6Mit59 locus (exemplified by clone a, Figure 2A). In clones that deviated from this predominant pattern (Figure 2A, clones b–d), the microsatellite length was altered by ≤4 bp. In other words, the microsatellite changes were invariably Type A. A similar pattern of small-scale microsatellite changes was observed at

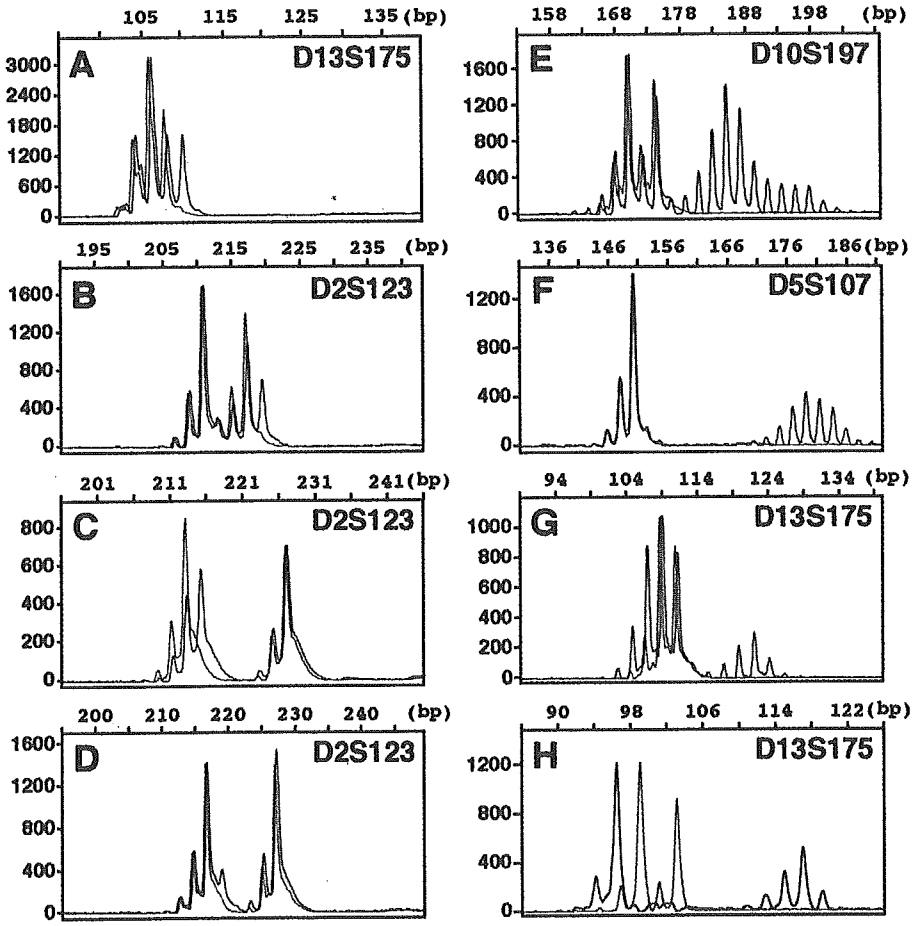

Figure 1. Type A and Type B microsatellite instability observed in human colorectal cancer. Using genomic DNA samples prepared from cancer and the corresponding normal mucosa, microsatellite sequences, indicated at the right top of each panel, were amplified by PCR with primers differentially labelled with fluorescence, then mixed and run on a same lane in an automated DNA sequencer. The amount of each DNA fragment was quantitatively detected and its size was estimated with accuracy of 1 bp, by standardization with size markers run in each lane. Results representative for each mode of microsatellite instability are shown: red lines, cancer; green lines, normal mucosa; Type A, (**A**) (IC678), (**B**) (IC810), (**C**) (IC721) and (**D**) (IC793); Type B, (**E**) (IC790), (**F**) (IC733), (**G**) (IC690) and (**H**) (NoTa). Patient codes in the parentheses correspond to those used in Table 1.

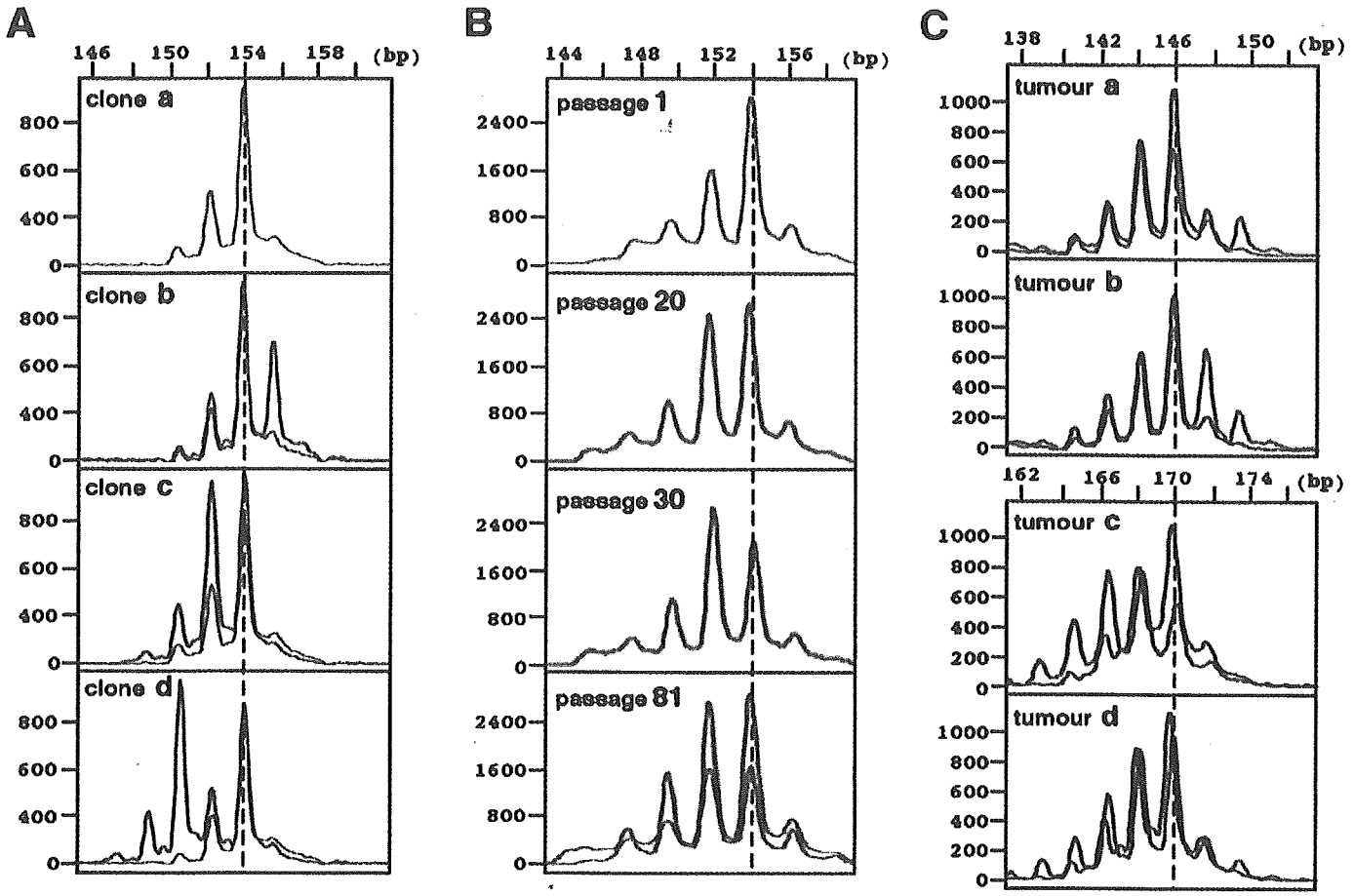

Figure 2. Microsatellite changes in *Msh2*$^{-/-}$ mouse embryonic fibroblast (MEF) cells and in tumours that arose in *Msh2*-knockout mice. (**A**) More than 20 subclones were isolated from RH95021 (*Msh2*$^{-/-}$) MEF cells and microsatellite changes were compared among these subclones. The majority exhibited the same configuration (clone a) and a few deviated from this predominant pattern (clone b–d). In each clone, the pattern of clone A has been superimposed with green lines, to facilitate comparison. Results obtained in D6Mit59 microsatellite are shown. (**B**) RH95021 cells were continuously cultured and sampled at different passages. Results obtained in D6Mit59 microsatellite at passage 1, 20, 30 and 81 are shown. In passage 81, the initial profile at passage 1 has been superimposed with a green line. (**C**) Tumours that arose *in vivo* in *Msh2*-knockout mice were analysed. Representative results obtained in D7Mit91 (tumours a and b) and D6Mit59 (tumours c and d) microsatellites are shown: red lines, tumour; green lines, the corresponding normal tissue.

Table 1. *hMSH2* and *hMLH1* alterations found in tumours exhibiting Type A and Type B MSI

PATIENT	MSI A/B	NUCLEAR EXPRESSION hMSH2	NUCLEAR EXPRESSION hMLH1	SEQUENCE exon *hMSH2* 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16	SEQUENCE exon *hMLH1* 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19	SUMMARY
IC669	A	P	N			cytoplasmic hMLH1
IC678	A	P	P		●	L521I (CTC to ATC)
IC692	A	P	P	□		G132G (GGC to GGA)
IC721	A	P	P	●		L390F (CTT to TTT)
IC724	A	P	P	●		G178R (GGA to AGA)
IC759	A	P	P			
IC793	A	P	N			*hMLH1* silencing
IC807	A	P	P		△	I219V (ATC to GTC)
IC810	A	P	P	●	△	*hMSH2* : L390F / *hMLH1* : I219V
IC815	A	P	P			
IC824	A	P	P			
IC860	A	P	P			
IC622	B	P	P			
IC653	B	P	P			
IC676	B	P	P			
IC690	B	P	N		●	R226Q (CGA to CAA) / cytoplasmic hMLH1, HNPCC
IC698	B	P	P		△	I219V (ATC to GTC)
IC733	B	P	P			
IC790	B	P	P			
IC853	B	P	P	□		G132G (GGC to GGA)
IC873	B	P	P			Q604Q (CAA to CAG)
NoTa	B	P*	N*			*hMLH1* silencing, HNPCC

MSI, microsatellite instability; P, positive nuclear staining in immunohistochemistry; N, negative; *, determined by immunoblotting; Closed circle, base substitution with amino acid change; open triangle, possible polymorphism; Open rectangle, base substitution without amino acid change.

two other dinucleotide microsatellites, D1Mit62 and D7Mit91 (data not shown). To investigate whether Type B variations might simply reflect the accumulation of numerous smaller alterations over many generations, RH95021 cells were continuously cultured, and sampled periodically at different passages. As shown in Figure 2B, even after undergoing 81 population doublings (2^{81} corresponds to 10^{24}), there was no detectable appearance of shorter or longer D6Mit59 alleles. Similar patterns were observed at the two other microsatellite loci (data not shown). Thus, there was no evidence of the accumulation of changes consistent with Type B instability at any of the three microsatellites in these MMR-defective MEFs. Similar data were obtained using a second MMR-deficient MEF cell line, MC2, which derived from an *Mlh1*$^{-/-}$ mouse (data not shown).

We also examined microsatellite instability in lymphomas and adenocarcinomas that arose in various organs of *Msh2*$^{-/-}$mice. Among 16 tumours that were analysed at the three microsatellite loci, each contained alterations at one or more locus. In all cases, changes were limited to ≤6 bp (examples are shown in Figure 2C) and no Type B alteration was observed. Intriguingly, an examination of published microsatellite changes in cells of MMR gene-knockout mice clearly indicates that most changes are of Type A (12,18,19).

Analysis of microsatellites in MEFs and tumours from MMR-defective animals therefore indicates that Type A MSI is a direct consequence of defective MMR. The absence of more extensive microsatellite length changes may indicate further that an *Msh2* or *Mlh1* defect is insufficient for the development of Type B instability.

Mismatch repair gene inactivation is associated with both Type A and Type B MSI in human colorectal cancer

HNPCC patients inherit mutations in MMR genes (6,7). More than 90% of HNPCC tumours are MSI$^+$ (20). The MSI$^+$ phenotype is also frequent among sporadic colorectal carcinomas (2–4,6,21). Inspection of published data derived from the conventional microsatellite analysis reveals that microsatellite changes thus far reported in various tumours, including those in HNPCC individuals, are largely Type B (2–4,20–22). We considered the possibility that the more subtle Type A MSI might have remained undetected in some cases. Using our fluorescent assay with a panel of five dinucleotide repeat microsatellites, we found that the frequencies of Type A and Type B MSI among 79 colorectal carcinomas were 30% and 17%, respectively. In agreement with previous observations that Type B instability is common in HNPCC colon tumours, the IC690 tumour and the colorectal carcinoma cell line, NoTa (Table 1), both of which were derived from patients who fulfilled the Amsterdam Criteria II for HNPCC (23), exhibited Type B instability (Figure 1G and H). Our finding that 17% of colorectal tumours display Type B MSI is consistent with the generally reported figure of around 20% for MSI among colorectal carcinomas (20,24,25). The observation that a further 30% of tumours displayed Type A MSI suggests that the frequency of MSI, at least in colorectal carcinomas, has previously been underestimated.

The relationship between MSI and defective MMR in our set of colorectal tumours was investigated further. *hMSH2* and

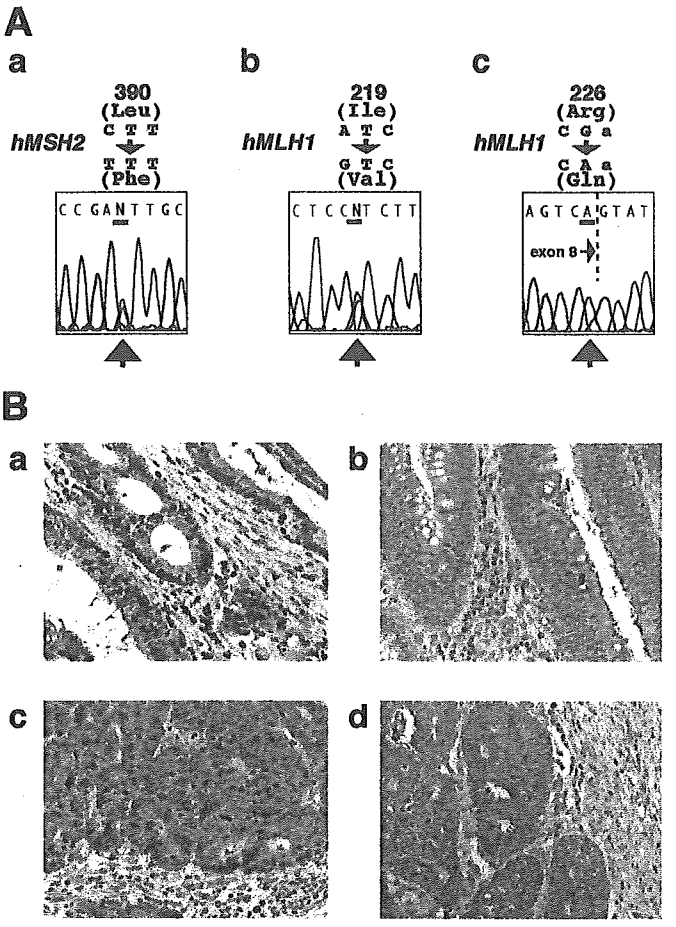

Figure 3. *hMSH2* and *hMLH1* alterations in tumours exhibiting Type A or Type B microsatellite instability. (**A**) Sequences for all the exons including exon–intron boundaries of *hMSH2* and *hMLH1* were determined using an automated sequencer. Sequence alterations found at (a) codon 390 of *hMSH2* (patient IC810), (b) codon 219 of *hMLH1* (patient IC810) and (c) codon 226 of *hMLH1* (patient IC690) were shown. (**B**) Abnormal expression of hMLH1 proteins observed in a panel of human colorectal carcinomas. (Panel a) A typical result with a strong nuclear staining implying normal hMLH1 expression (IC853). (Panel b) Complete loss of hMLH1 expression in tumour cells, which suggest a possible epigenetic silencing (IC793). (Panels c and d) Results without evident nuclear staining, but with an accumulation of hMLH1 antigens in the tumour cytoplasm, which may suggest an abnormal intracellular distribution of this protein (IC690 and IC669).

hMLH1 MMR genes of 12 tumours with Type A and 9 with Type B MSI were sequenced. The same genes in the NoTa cell line were also sequenced (Figure 3A and Table 1). Sequence alterations causing amino acid substitutions were identified in 5 of the 21 tumours. Four of these (80%) were associated with Type A MSI. In addition, one patient with Type A MSI (IC793) in whom no mutation was identified was negative for immunohistochemical staining of hMLH1 (Figure 3B, panel b). This is consistent with a possible epigenetic *hMLH1* gene silencing (26–28). In one other Type A case (IC669) and one Type B case (IC690), there was an abnormal intracellular distribution of hMLH1 which remained predominantly cytoplasmic (Figure 3B, panels c and d). Among the 10 tumours displaying Type B MSI, there was an example of base substitutions causing amino acid change in *hMLH1*. In this case, IC690, the failure of hMLH1 to localize to the nucleus was associated with the codon 226 mutation in exon 8 (Figure 3A,

Table 2. *p53* mutations found in 79 colorectal carcinomas

No.	Patient	EX05	EX06	EX07	EX08	EX09	Codon change	Base substitution	(type)	AA change	MSI
1	IC628				**273**		CGT → CAT	G:C → A:T	TS	Arg → His	N
2	IC630		196				CGA → TGA	G:C → A:T	TS	Arg → stop	A
3	IC634				306		CGA → TGA	G:C → A:T	TS	Arg → stop	N
4	IC668		193				CAT → CGT	A:T → G:C	TS	His → Arg	N
5	IC669	**175**					CGC → CAC	G:C → A:T	TS	Arg → His	A
6	IC673	176					TGC → AGC	A:T → T:A	TV	Cys → Ser	N
7	IC674				285		GAG → AAG	G:C → A:T	TS	Glu → Lys	N
8	IC680		ND	255			ATC → ACC	A:T → G:C	TS	Ile → Thr	A
9	IC693	179					CAT → CTT	A:T → T:A	TV	His → Leu	A
10	IC694				273		CGT → CAT	G:C → A:T	TS	Arg → His	A
11	IC711			239			AAC → GAC	A:T → G:C	TS	Asn → Asp	N
12	IC721	**175**					CGC → CAC	G:C → A:T	TS	Arg → His	A
13	IC748		190			ND	CCT → CTT	G:C → A:T	TS	Pro → Leu	A
14	IC754		196				CGA → CCA	G:C → C:G	TV	Arg → Pro	N
15	IC763	151					CCC → CAC	G:C → T:A	TV	Pro → His	N
16	IC772	**175**					CGC → CAC	G:C → A:T	TS	Arg → His	A
17	IC778	**175**	ND				CGC → CAC	G:C → A:T	TS	Arg → His	N
18	IC784		214		ND	ND	CAT → CGT	A:T → G:C	TS	His → Arg	A
19	IC808		205				TAT → GAT	A:T → C:G	TV	Tyr → Asp	A
20	IC812		190				CCT → CTT	G:C → A:T	TS	Pro → Leu	N
21	IC816				**273**		CGT → CAT	G:C → A:T	TS	Arg → His	A
22	IC819			**248**			CGG → CAG	G:C → A:T	TS	Arg →Gln	N
23	IC860				**273**		CGT → CAT	G:C → A:T	TS	Arg → His	A

MSI, microsatellite instability; N, negative; A, Type A MSI; TS, transition; TV, transversion; ND, not determined. Bold codon numbers indicate the acknowledged hot-spots for mutation.

panel c). These findings suggest that Type A instability, as well as Type B, is indeed associated with MMR defects.

p53 mutation is strongly associated with Type A MSI in human colorectal cancer

One view of the involvement of MMR defects in cancer development is that the 'microsatellite mutator phenotype (MMP)' (29,30) in mismatch repair-defective cells offers an alternative to chromosomal instability as a mechanism for genetic instability in cancer (31). On this model, MSI and chromosomal instability represent mutually exclusive pathways of tumour development. This reasoning is based partly in the observation that *p53* mutations, commonly associated with chromosomal instability, are infrequent among MSI^+ tumours (2,32–34). To examine the relationship between Type A/B instability and *p53* mutation, we sequenced the *p53* gene in our panel of 79 colorectal tumours. *p53* mutations resulting in an amino acid substitution were detected in 23 tumours (29.1%). The mutations were predominantly transitions in acknowledged hot spots; codons 175, 248 and 273 (Table 2). Of the *p53* mutations that were found in MSI^+ tumours, all were associated with Type A MSI (Tables 2 and 3). No *p53* mutations were detected among the 14 Type B tumours. Among Type A tumours, the frequency of *p53* mutation approached 50% (12/25). These findings confirm that *p53* mutations are rare in tumours with Type B MSI. More importantly, they suggest that, in contrast to prevailing opinion, defective MMR is significantly associated with *p53* mutation, at least in human colorectal cancer.

Table 3. *p53* mutation highly correlates with Type A MSI

		MSI Type A	Type B	Negative	Subtotal
p53	Wild type	13	14	29	56
	Mutant	12	0	11	23
	Subtotal	25	14	40	79

$p = 0.006$. MSI: Microsatellite instability.

DISCUSSION

The fluorescent technique we used here allows the unequivocal designation of Type A and Type B MSI, and has revealed a previously unrecognized complexity in the relationship between dinucleotide MSI and defective DNA MMR in human cancer. Type A MSI (changes $\leq$ 6 bp) is clearly linked with MMR inactivation in both mice and humans, which implies that Type A MSI is a direct consequence of defective MMR. Since we found no evidence of Type B instability (changes $\geq$ 8 bp) in MMR-defective animals, it is possible that changes in addition to repair deficiency contribute to, or are responsible for, Type B MSI. One important finding of this study is that Type A instability is frequent among human tumours. Type A MSI predominated in our large panel of colorectal carcinomas. We suggest that, because the changes associated with Type A instability are more subtle, the frequency of MSI among colorectal tumours may have been considerably underestimated. Our findings also reveal a hitherto unrecognized association between defective MMR and *p53* mutation. Significantly, Type A MSI was strongly associated with *p53* mutation in human colorectal tumours. Since Type A instability is unequivocally associated with MMR deficiency, this novel finding implies that, in contrast to prevailing opinion, *p53* mutations are common in MMR-defective tumours, at least in human colorectal cancer.

Established guidelines for classification of MSI utilize the frequency of changes in a defined set of microsatellite

markers, i.e. MSI-H and -L (10). However, qualitative differences in microsatellite changes are not widely discussed. In one of the earliest report of the MSI phenomenon, however, Thibodeau *et al.* divided microsatellite changes into two categories; Type I and II mutations (3). The former was defined a 'significant increase (expansion) or decrease (deletion) in the apparent fragment size' and the latter as a 'single 2 bp change'. This distinction has received little attention since then. Our data indicate that Type A MSI, which appears similar to their Type II mutation, is more frequent than hitherto suspected, and suggest that it represents the bona fide MMR-deficient phenotype. On the other hand, Type I mutations may correspond to our Type B instability. The problem is that mutations in MMR genes have been reported in tumours displaying this type of instability. More than 90% of HNPCC tumours are MSI^+ (20), and this type of MSI can be categorized as Type B/Type I (2–4,20–22). However, the frequencies of mutation in the two major MMR genes, *hMSH2* and *hMLH1*, in HNPCC kindred are not high in some reports (35–40). Among the panel of tumours displaying Type B MSI, we found a base substitution mutation in *hMLH1* and one case with a possible *hMLH1* silencing. This incidence of MMR gene inactivation in the Type B group is not unduly low, compared with the reported frequencies in the literature (25,36,41–43). The relationship between Type B MSI and defective MMR is probably more complex than hitherto suspected.

Type B MSI may involve molecular abnormalities in addition to defective MMR. We suggest that whereas Type A MSI probably reflects the uncorrected DNA polymerase slippage events that accumulate in MMR-defective cells, inappropriate processing of damage by recombinational DNA repair may contribute to Type B MSI. This hypothesis may be supported partly by our observation that there was no evidence of the emergence of Type B instability in MMR gene-knockout animals. It is known that microsatellite alterations occur via several independent mechanisms, including recombination (44–46), and MMR counteracts incorrect strand alignment during homologous recombination (47). The drastic microsatellite changes in Type B MSI may be more consistent with dynamic events, such as recombination, than with replication slippage. In this context, defective MMR might be a promoting, and consequently highly coincidental, but insufficient factor for Type B changes. Connection between MSI and the recombinational repair pathway in tumours, particularly in HNPCC, may warrant attention.

Type A MSI is also strongly associated with *p53* mutation in human colorectal tumours. This observation may be compatible with several recent reports (36,48,49) that have shown a connection between *p53* mutation and the MSI-L phenotype, since in colorectal cancer Type A MSI tends to be observed in a limited number of markers and, consequently, categorized as MSI-L. This finding may also provide an insight to the mechanism of genetic instability in tumours. Genetic instability in tumours has been regarded as deriving from two mutually exclusive pathways, chromosomal instability (CIN)—frequently associated with mutations in various oncogenes or tumour suppressor genes such as *p53*—and 'microsatellite mutator phenotype (MMP)' (29,30), in which *p53* mutations are rare and, instead, mutations are found in mononucleotide repeats within genes of a different variety. Several recent reports suggest that there might be an oversimplification in this distinction (50–53). From our observations, dinucleotide MSI in tumours can be divided into two modes, Type A and Type B, and Type A instability is the direct consequence of defective MMR. A close association of Type A MSI with *p53* mutation may suggest a hitherto unrecognized causal relationship between *p53* mutation and defective MMR. *p53* mutations may derive from a state with an elevated mutation rate, i.e. MMR-deficient phenotype, as initially suspected. Thus, our observations suggest added complexities to the relationship between MMR defects and MSI, and also shed light on previously unrecognized fundamental processes in the molecular mechanisms of genetic instability underlying tumour development.

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REFERENCES

1. Weissenbach,J., Gyapay,G., Dib,C., Vignal,A., Morissette,J., Millasseau,P., Vaysseix,G. and Lathrop,M. (1992) A second-generation linkage map of the human genome. *Nature*, **359**, 794–801.
2. Ionov,Y., Peinado,M.A., Malkhosyan,S., Shibata,D. and Perucho,M. (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature*, **363**, 558–561.
3. Thibodeau,S.N., Bren,G. and Schaid,D. (1993) Microsatellite instability in cancer of the proximal colon. *Science*, **260**, 816–819.
4. Aaltonen,L.A., Peltomaki,P., Leach,F.S., Sistonen,P., Pylkkanen,L., Mecklin,J.P., Jarvinen,H., Powell,S.M., Jen,J., Hamilton,S.R. *et al.* (1993) Clues to the pathogenesis of familial colorectal cancer. *Science*, **260**, 812–816.
5. Peltomaki,P., Lothe,R.A., Aaltonen,L.A., Pylkkanen,L., Nystrom-Lahti,M., Seruca,R., David,L., Holm,R., Ryberg,D., Haugen,A. *et al.* (1993) Microsatellite instability is associated with tumors that characterize the hereditary non-polyposis colorectal carcinoma syndrome. *Cancer Res.*, **53**, 5853–5855.
6. Fishel,R., Lescoe,M.K., Rao,M.R., Copeland,N.G., Jenkins,N.A., Garber,J., Kane,M. and Kolodner,R. (1993) The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell*, **75**, 1027–1038.
7. Leach,F.S., Nicolaides,N.C., Papadopoulos,N., Liu,B., Jen,J., Parsons,R., Peltomaki,P., Sistonen,P., Aaltonen,L.A., Nystrom-Lahti,M. *et al.* (1993) Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell*, **75**, 1215–1225.
8. Modrich,P. and Lahue,R. (1996) Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Ann. Rev. Biochem.*, **65**, 101–133.
9. Arzimanoglou,I.I., Gilbert,F. and Barber,H.R. (1998) Microsatellite instability in human solid tumors. *Cancer*, **82**, 1808–1820.
10. Boland,C.R., Thibodeau,S.N., Hamilton,S.R., Sidransky,D., Eshleman,J.R., Burt,R.W., Meltzer,S.J., Rodriguez-Bigas,M.A., Fodde,R., Ranzani,G.N. *et al.* (1998) A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res.*, **58**, 5248–5257.

11. Oda,S., Oki,E., Maehara,Y. and Sugimachi,K. (1997) Precise assessment of microsatellite instability using high resolution fluorescent microsatellite analysis. *Nucleic Acids Res.*, **25**, 3415–3420.
12. de Wind,N., Dekker,M., Berns,A., Radman,M. and te Riele,H. (1995) Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell*, **82**, 321–330.
13. Kolodner,R.D., Hall,N.R., Lipford,J., Kane,M.F., Rao,M.R., Morrison,P., Wirth,L., Finan,P.J., Burn,J. and Chapman,P. (1994) Structure of the human MSH2 locus and analysis of two Muir-Torre kindreds for msh2 mutations. *Genomics*, **24**, 516–526.
14. Kolodner,R.D., Hall,N.R., Lipford,J., Kane,M.F., Morrison,P.T., Finan,P.J., Burn,J., Chapman,P., Earabino,C. and Merchant,E. (1995) Structure of the human MLH1 locus and analysis of a large hereditary nonpolyposis colorectal carcinoma kindred for mlh1 mutations. *Cancer Res.*, **55**, 242–248.
15. Maehara,Y., Oda,S. and Sugimachi,K. (2001) The instability within: problems in current analyses of microsatellite instability. *Mutat. Res.*, **461**, 249–263.
16. Tokunaga,E., Oki,E., Oda,S., Kataoka,A., Kitamura,K., Ohno,S., Maehara,Y. and Sugimachi,K. (2000) Frequency of microsatellite instability in Breast cancer determined by high-resolution fluorescent microsatellite analysis. *Oncology*, **59**, 44–49.
17. Ikeda,Y., Oda,S., Abe,T., Ohno,S., Maehara,Y. and Sugimachi,K. (2001) Features of microsatellite instability in colorectal cancer: comparison between colon and rectum. *Oncology*, **61**, 168–174.
18. Edelmann,W., Cohen,P.E., Kane,M., Lau,K., Morrow,B., Bennett,S., Umar,A., Kunkel,T., Cattoretti,G., Chaganti,R. *et al.* (1996) Meiotic pachytene arrest in MLH1-deficient mice. *Cell*, **85**, 1125–1134.
19. Baker,S.M., Bronner,C.E., Zhang,L., Plug,A.W., Robatzek,M., Warren,G., Elliott,E.A., Yu,J., Ashley,T., Arnheim,N. *et al.* (1995) Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis. *Cell*, **82**, 309–319.
20. Liu,B., Parsons,R., Papadopoulos,N., Nicolaides,N.C., Lynch,H.T., Watson,P., Jass,J.R., Dunlop,M., Wyllie,A., Peltomaki,P. *et al.* (1996) Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nature Med.*, **2**, 169–174.
21. Gryfe,R., Kim,H., Hsieh,E.T., Aronson,M.D., Holowaty,E.J., Bull,S.B., Redston,M. and Gallinger,S. (2000) Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *New Engl. J. Med.*, **342**, 69–77.
22. Aaltonen,L.A., Peltomaki,P., Mecklin,J.P., Jarvinen,H., Jass,J.R., Green,J.S., Lynch,H.T., Watson,P., Tallqvist,G., Juhola,M. *et al.* (1994) Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. *Cancer Res.*, **54**, 1645–1648.
23. Vasen,H.F., Watson,P., Mecklin,J.P. and Lynch,H.T. (1999) New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology*, **116**, 1453–1456.
24. Lothe,R.A., Peltomaki,P., Meling,G.I., Aaltonen,L.A., Nystrom-Lahti,M., Pylkkanen,L., Heimdal,K., Andersen,T.I., Moller,P., Rognum,T.O. *et al.* (1993) Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history. *Cancer Res.*, **53**, 5849–5852.
25. Liu,B., Nicolaides,N.C., Markowitz,S., Willson,J.K., Parsons,R.E., Jen,J., Papadopolous,N., Peltomaki,P., de la Chapelle,A., Hamilton,S.R. *et al.* (1995) Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nature Genet.*, **9**, 48–55.
26. Thibodeau,S.N., French,A.J., Roche,P.C., Cunningham,J.M., Tester,D.J., Lindor,N.M., Moslein,G., Baker,S.M., Liskay,R.M., Burgart,L.J. *et al.* (1996) Altered expression of hMSH2 and hMLH1 in tumors with microsatellite instability and genetic alterations in mismatch repair genes. *Cancer Res.*, **56**, 4836–4840.
27. Herman,J.G., Umar,A., Polyak,K., Graff,J.R., Ahuja,N., Issa,J.P., Markowitz,S., Willson,J.K., Hamilton,S.R., Kinzler,K.W. *et al.* (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc. Natl Acad. Sci. USA*, **95**, 6870–6875.
28. Cunningham,J.M., Christensen,E.R., Tester,D.J., Kim,C.Y., Roche,P.C., Burgart,L.J. and Thibodeau,S.N. (1998) Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Res.*, **58**, 3455–3460.
29. Perucho,M. (1996) Cancer of the microsatellite mutator phenotype. *Biol. Chem.*, **377**, 675–684.
30. Perucho,M. (1996) Microsatellite instability: the mutator that mutates the other mutator. *Nature Med.*, **2**, 630–631.
31. Lengauer,C., Kinzler,K.W. and Vogelstein,B. (1998) Genetic instabilities in human cancers. *Nature*, **396**, 643–649.
32. Cottu,P.H., Muzeau,F., Estreicher,A., Flejou,J.F., Iggo,R., Thomas,G. and Hamelin,R. (1996) Inverse correlation between RER+ status and p53 mutation in colorectal cancer cell lines. *Oncogene*, **13**, 2727–2730.
33. Simms,L.A., Radford-Smith,G., Biden,K.G., Buttenshaw,R., Cummings,M., Jass,J.R., Young,J., Meltzer,S.J. and Leggett,B.A. (1998) Reciprocal relationship between the tumor suppressors p53 and BAX in primary colorectal cancers. *Oncogene*, **17**, 2003–2008.
34. Salahshor,S., Kressner,U., Pahlman,L., Glimelius,B., Lindmark,G. and Lindblom,A. (1999) Colorectal cancer with and without microsatellite instability involves different genes. *Genes Chromosome Cancer*, **26**, 247–252.
35. Froggatt,N.J., Brassett,C., Koch,D.J., Evans,D.G., Hodgson,S.V., Ponder,B.A. and Maher,E.R. (1996) Mutation screening of MSH2 and MLH1 mRNA in hereditary non-polyposis colon cancer syndrome. *J. Med. Genet.*, **33**, 726–730.
36. Konishi,M., Kikuchi-Yanoshita,R., Tanaka,K., Muraoka,M., Onda,A., Okumura,Y., Kishi,N., Iwama,T., Mori,T., Koike,M. *et al.* (1996) Molecular nature of colon tumors in hereditary nonpolyposis colon cancer, familial polyposis, and sporadic colon cancer. *Gastroenterology*, **111**, 307–317.
37. Weber,T., Conlon,W., Petrelli,N., Rodriguez-Bigas,M., Keitz,B., Pazik,J., Farrell,C., O'Malley,L., Oshalim,M., Abdo,M. *et al.* (1997) Genomic DNA-based hMSH2 and hMLH1 mutation screening in 32 Eastern United States hereditary nonpolyposis colorectal cancer pedigrees. *Cancer Res.*, **57**, 3798–3803.
38. Scartozzi,M., Bianchi,F., Rosati,S., Galizia,E., Antolini,A., Loretelli,C., Piga,A., Bearzi,I., Cellerino,R. and Porfiri,E. (2002) Mutations of hMLH1 and hMSH2 in patients with suspected hereditary nonpolyposis colorectal cancer: correlation with microsatellite instability and abnormalities of mismatch repair protein expression. *J. Clin. Oncol.*, **20**, 1203–1208.
39. Wahlberg,S.S., Schmeits,J., Thomas,G., Loda,M., Garber,J., Syngal,S., Kolodner,R.D. and Fox,E. (2002) Evaluation of microsatellite instability and immunohistochemistry for the prediction of germ-line MSH2 and MLH1 mutations in hereditary nonpolyposis colon cancer families. *Cancer Res.*, **62**, 3485–3492.
40. Pucciarelli,S., Agostini,M., Viel,A., Bertorelle,R., Russo,V., Toppan,P. and Lise,M. (2003) Early-age-at-onset colorectal cancer and microsatellite instability as markers of hereditary nonpolyposis colorectal cancer. *Dis. Colon Rectum*, **46**, 305–312.
41. Kuismanen,S.A., Holmberg,M.T., Salovaara,R., de la Chapelle,A. and Peltomaki,P. (2000) Genetic and epigenetic modification of MLH1 accounts for a major share of microsatellite-unstable colorectal cancers. *Am. J. Pathol.*, **156**, 1773–1779.
42. Percesepe,A., Borghi,F., Menigatti,M., Losi,L., Foroni,M., Di Gregorio,C., Rossi,G., Pedroni,M., Sala,E., Vaccina,F. *et al.* (2001) Molecular screening for hereditary nonpolyposis colorectal cancer: a prospective, population-based study. *J. Clin. Oncol.*, **19**, 3944–3950.
43. Potocnik,U., Glavac,D., Golouh,R. and Ravnik-Glavac,M. (2001) Causes of microsatellite instability in colorectal tumors: implications for hereditary non-polyposis colorectal cancer screening. *Cancer Genet. Cytogenet.*, **126**, 85–96.
44. Sia,E.A., Jinks-Robertson,S. and Petes,T.D. (1997) Genetic control of microsatellite stability. *Mutat. Res.*, **383**, 61–70.
45. Richard,G.F. and Paques,F. (2000) Mini- and microsatellite expansions: the recombination connection. *EMBO Rep.*, **1**, 122–126.
46. Lenzmeier,B.A. and Freudenreich,C.H. (2003) Trinucleotide repeat instability: a hairpin curve at the crossroads of replication, recombination, and repair. *Cytogenet. Genome Res.*, **100**, 7–24.
47. Evans,E. and Alani,E. (2000) Roles for mismatch repair factors in regulating genetic recombination. *Mol. Cell. Biol.*, **20**, 7839–7844.
48. Jass,J.R., Biden,K.G., Cummings,M.C., Simms,L.A., Walsh,M., Schoch,E., Meltzer,S.J., Wright,C., Searle,J., Young,J. *et al.* (1999) Characterisation of a subtype of colorectal cancer combining features of the suppressor and mild mutator pathways. *J. Clin. Pathol.*, **52**, 455–460.
49. Kambara,T., Matsubara,N., Nakagawa,H., Notohara,K., Nagasaka,T., Yoshino,T., Isozaki,H., Sharp,G.B., Shimizu,K., Jass,J. *et al.* (2001)

High frequency of low-level microsatellite instability in early colorectal cancer. *Cancer Res.*, **61**, 7743–7746.
50. Hawkins,N.J., Tomlinson,I., Meagher,A. and Ward,R.L. (2001) Microsatellite-stable diploid carcinoma: a biologically distinct and aggressive subset of sporadic colorectal cancer. *Br. J. Cancer*, **84**, 232–236.
51. Young,J., Simms,L.A., Biden,K.G., Wynter,C., Whitehall,V., Karamatic,R., George,J., Goldblatt,J., Walpole,I., Robin,S.A. *et al.* (2001) Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis. *Am. J. Pathol.*, **159**, 2107–2116.
52. Jass,J.R., Walsh,M.D., Barker,M., Simms,L.A., Young,J. and Leggett,B.A. (2002) Distinction between familial and sporadic forms of colorectal cancer showing DNA microsatellite instability. *Eur. J. Cancer*, **38**, 858–866.
53. Goel,A., Arnold,C.N., Niedzwiecki,D., Chang,D.K., Ricciardiello,L., Carethers,J.M., Dowell,J.M., Wasserman,L., Compton,C., Mayer,R.J. *et al.* (2003) Characterization of sporadic colon cancer by patterns of genomic instability. *Cancer Res.*, **63**, 1608–1614.

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Akt is frequently activated in HER2/neu-positive breast cancers and associated with poor prognosis among hormone-treated patients

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Akt/PKB is a serine/threonine kinase that plays an important role in survival when cells are exposed to different apoptotic stimuli. Aberrant activation of Akt/PKB in breast carcinoma is associated with poor prognosis and resistance to endocrine therapy and chemotherapy. The Akt signaling pathway currently attracts considerable attention as a new target for effective therapeutic strategies. We therefore investigated the relationship between activation of Akt and clinicopathologic variables including hormone receptor and HER2/neu status. Breast cancer tissues obtained from 252 patients were utilized for this study. We evaluated Akt activation by immunohistochemical assessment of the expression of phosphorylated Akt (pAkt) at Ser-473. Eighty-four cases (33.3%) were diagnosed as positive for pAkt expression. pAkt was significantly associated with HER2/neu overexpression ($p < 0.0001$). There was an inverse correlation between pAkt and PR expression ($p = 0.0321$); however, there was no association between pAkt and ER expression. Survival analysis showed that pAkt positivity was associated with poor disease-free survival in cases with postoperative hormone therapy; however, there was no association in cases without hormone therapy. Our results indicate that Akt activation induced poor prognosis in patients who received adjuvant hormone therapy. This finding suggests that inhibition of the Akt signaling pathway may increase the efficacy of hormone therapy and improve the prognosis of patients who receive adjuvant hormone therapy.

Key words: Akt; HER2/neu; breast cancer; endocrine therapy

Akt, also known as PKB, is a serine/threonine protein kinase and has emerged as a crucial regulator of widely divergent cellular processes, including apoptosis, proliferation, differentiation and metabolism. Disruption of normal Akt/PKB signaling occurs frequently in several human cancers, and the enzyme appears to play an important role in cancer progression and cell survival.[1] Akt is activated by a variety of stimuli, through growth factor receptors, in a PI-3 kinase–dependent manner. The mechanisms by which Akt promotes cell survival include phosphorylation of the proapoptotic proteins BAD, caspase-9, Forkhead transcription factors and IκB kinase α. These reduce the binding of BAD to Bcl-x_L, inhibit caspase-9 protease activity and Fas ligand gene transcription and activate the nuclear factor-κB cascades.[1]

ErbB2 (HER2/neu) is a member of the type I subclass of receptor tyrosine kinases, which has been associated with several types of human cancer. Numerous studies have demonstrated that *erbB2* is amplified and overexpressed in 20–30% of primary breast cancers and generally associated with poor prognosis.[2–6] In addition, HER2/neu overexpression is associated with resistance to chemotherapy and endocrine therapy.[7–9]

One of the major signaling pathways utilized by the erbB families is the PI-3K/Akt pathway, as well as the ras-/mitogen-activated protein kinase pathway. The ligand of erbB2 has not been identified; however, erbB2-containing heterodimers are potent activators of multiple signaling pathways involved in proliferation, invasion and survival.[10] Studies in breast cancer cells, primary breast tumors and transgenic mice all indicate that when erbB2 is overexpressed, it is constitutively associated with erbB3.[11] These ErbB2/erbB3 dimers strongly activate the PI3K-PKB/Akt pathway. This is supported by previous evidence that tumor cells overexpressing HER2/neu exhibit constitutive PKB/Akt activity.[12] Experimental studies have demonstrated that the malignant phenotypes of breast carcinomas with HER2/neu overexpression are partially due to PI-3K /Akt signaling.[8,9,13] In addition, constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab or tamoxifen in breast cancer cells.[14] These findings suggest that Akt activation could be used as a predictive marker for sensitivity to various therapies.

Activation of Akt/PKB has been positively associated with HER2/neu overexpression in breast carcinoma obtained from human material[15,16] and with a worse outcome among endocrine-treated breast cancer patients.[15,17] Among premenopausal patients treated with tamoxifen and/or goserelin, those with activated Akt were more prone to relapse with distant metastasis.[17] However, among postmenopausal patients, those negative for Akt showed significant benefit from tamoxifen.[15] These results support the findings from basic research that activated Akt promotes resistance to tamoxifen in breast cancer cells.[14]

In the present study, we investigated the incidence of Akt activation in breast carcinomas and correlated it with HER2/neu overexpression, other clinicopathologic variables and survival in 252 breast carcinomas in Japanese women. Akt/PKB activation was elevated significantly in cases with HER2 overexpression and associated with poorer prognosis in patients who received adjuvant hormone therapies.

Material and methods

Patient population and tumor specimens

A total of 252 primary human breast carcinoma specimens were obtained from patients who underwent surgery at the Department of Surgery and Science, Kyushu University Hospital, from 1991 to 2002. Informed consent was obtained from each patient prior to tissue acquisition. Clinical data were obtained from medical records. Resected tissues were routinely processed for histopathologic analyses by histopathologic specialists at our hospital. Histopathologic diagnosis was determined according to the criteria of the Japanese Breast Cancer Society.[18]

Antibodies

MAbs 6F11 and 1A6 (Ventana, Tucson, AR) were used for ER and PR staining. For HER2/neu evaluation, MAb CB11 (Ventana) was used. pAkt was detected using polyclonal antibodies against phosphorylated Ser-473 (Cell Signaling Technology, Beverly, MA).

Abbreviations: DAB, 3,3′-diaminobenzidine; DFS, disease-free survival; ER, estrogen receptor; LH-RH, luteinizing hormone–releasing hormone; MAb, monoclonal antibody; MPA, medroxyprogesterone acetate; pAkt, phosphorylated Akt; PI-3K, phosphatidylinositol-3 kinase; PI-3 kinase, phosphoinositide-3-OH kinase; PKB, protein kinase B; PKC, protein kinase C; PR, progesterone receptor.

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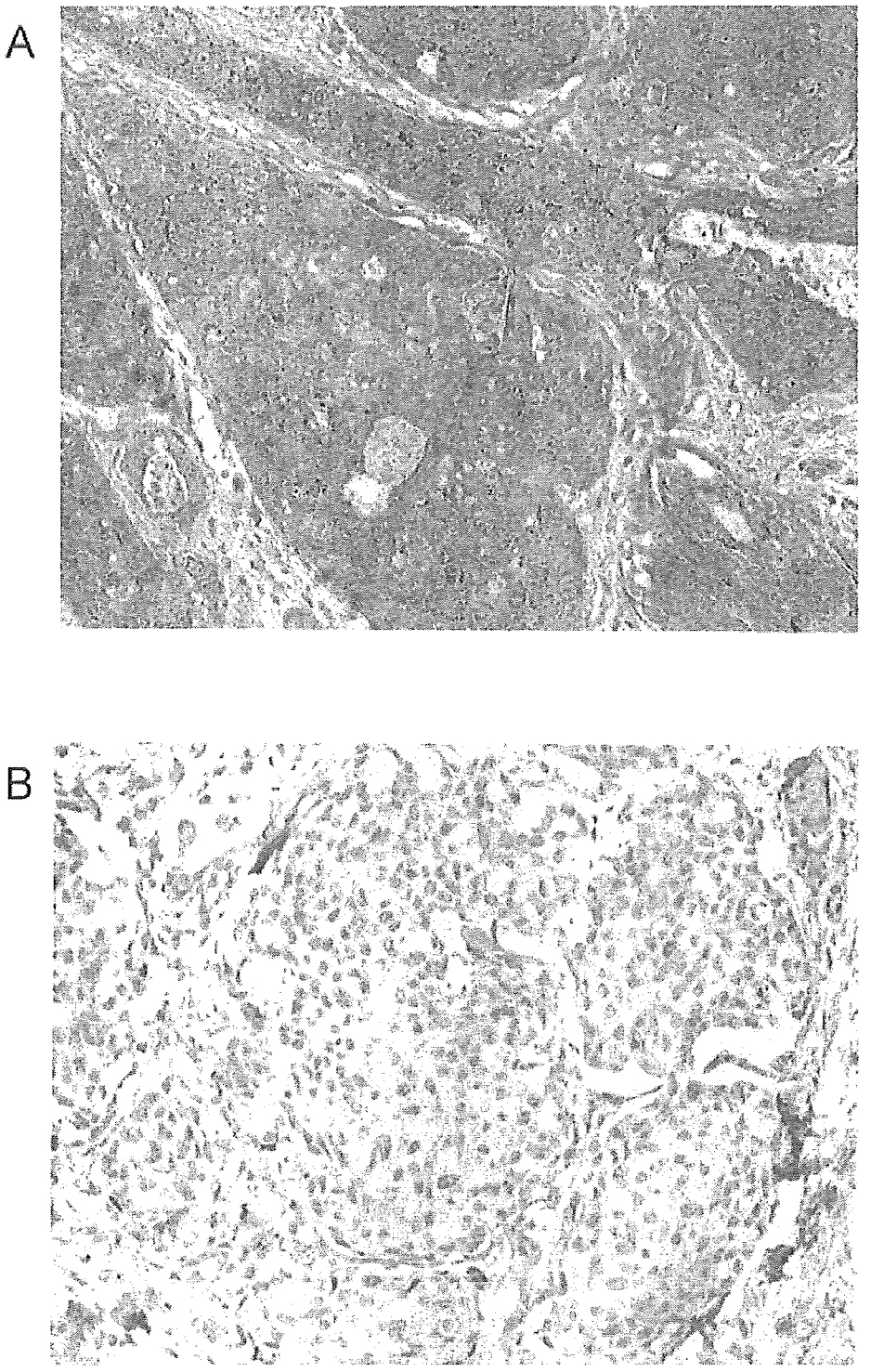

FIGURE 1 – Immunostaining of pAkt. Representative immunohistochemical staining of pAkt in breast carcinoma. pAkt was detected in the cytoplasm of tumor cells. (*a*) Positive immunostaining for pAkt. (*b*) Negative immunostaining for pAkt.

Immunohistochemistry and evaluation

Tissue samples were fixed by immersion in buffered formalin and embedded in paraffin. Sections (4 μ) were placed onto charged slides and dried at 60°C for 1 hr. Sections were deparaffinized and hydrated in water. Immunostaining of these paraffin sections was performed using the Ventana Discovery automated staining instru-

TABLE I – CORRELATIONS OF Akt ACTIVATION WITH HER2/neu EXPRESSION

pAkt	*n*	HER2/neu		*p* value
		Negative (%)	Positive (%)	
Negative	168	140 (83.3)	28 (16.7)	< 0.0001
Positive	84	48 (57.1)	36 (42.9)	

TABLE II – CORRELATIONS OF Akt ACTIVATION WITH HORMONE RECEPTOR EXPRESSION

pAkt	*n*	ER		*p* value	PR		*p* value
		Negative (%)	Positive (%)		Negative (%)	Positive (%)	
Negative	168	55 (32.7)	113 (67.3)	0.1632	82 (48.8)	86 (51.2)	0.0321
Positive	84	35 (41.7)	49 (58.3)		53 (63.1)	31 (36.9)	

TABLE III – CORRELATION BETWEEN pAkt EXPRESSION AND CLINICOPATHOLOGIC VARIABLES

Variables	pAkt		*p* value
	Negative (n = 168)	Positive (n = 84)	
Age (years)	54.7 ± 12.6	54.1 ± 11.0	N.S.
Tumor size (cm)	3.2 ± 2.0	3.2 ± 2.1	N.S.
Clinical stage			
0 (%)	1 (50)	1 (50)	N.S.
I (%)	38 (66.7)	23 (33.3)	
IIA (%)	60 (72.3)	33 (27.7)	
IIB (%)	45 (66.2)	23 (33.8)	
IIIA (%)	18 (56.3)	14 (43.7)	
IIIB (%)	6 (60.0)	4 (40.0)	
Axillary lymph node metastasis			
Negative (%)	112 (72.7)	42 (27.3)	0.0081
Positive (%)	53 (56.4)	41 (43.6)	
Pathologic classification			
Noninvasive ductal carcinoma (%)	4 (80.0)	1 (20.0)	N.S.
Papillotubular carcinoma (%)	43 (60.6)	28 (39.4)	
Solid tubular carcinoma (%)	35 (71.4)	14 (28.6)	
Scirrhous carcinoma (%)	65 (63.7)	37 (36.3)	
Mucinous carcinoma (%)	9 (90.0)	1 (10.0)	
Others (%)	9 (75.0)	3 (25.0)	

N.S., not significant.

ment (Ventana), and hematoxylin (Ventana) was employed as a nuclear counterstain. Immunostaining was visualized with a streptavidin peroxidase reaction using DAB as the chromogen (Ventana). A negative control reaction with no primary antibody was always performed alongside the reaction-containing sample.

Immunostaining was evaluated without knowledge of the clinical and pathologic parameters. ER and PR were recorded as positive if 10% or more of the nuclei in the invasive component of the tumor were stained.[19] HER2/neu was scored by widely accepted criteria that assessed the intensity and completeness of membrane staining.[20,21] The intensity of membrane staining was evaluated according to the following criteria: 0, none or up to 10% membrane staining; 1+, partial and/or faint membrane staining present in >10% of tumor cells; 2+, weak to moderate, complete membrane staining present in >10% of tumor cells; and 3+, strong, complete membrane staining present in >10% of tumor cells. Scores 0 and 1+ were considered normal (*i.e.*, negative for overexpression), and 2+ and 3+ were considered positive for HER2/neu overexpression. A specimen was considered positive for pAkt if 10% or more of the cytoplasm in the invasive component of the tumor stained positive for pAkt.

Statistical analysis

Associations between categorical variables were assessed by means of χ^2 tests. DFS was determined from the date of surgery to the date of relapse or last follow-up. DFS was estimated using the Kaplan-Meier method. The 2-sided log-rank test was used to test the association between variables and survival. The cut-off for significance was set at $p < 0.05$. Rates of recurrence in relation to expression of pAkt and other variables were estimated and tested using Cox's proportional hazards model.

Results

Expression of pAkt in primary breast cancer tissues

Phosphorylation of threonine-308 and serine-473 is required for activation of Akt1, and the phospho-Ser-473 Akt antibody recognizes only the phoshorylated/active form of Akt.[22,23] According to the manufacturer's information, the phospho-Akt (Ser-473) antibody used in our study detects Akt1 only when phosphorylated at serine-473 and Akt2 and Akt3 only when phosphorylated at equivalent sites. It does not detect Akt phosphorylated at other sites or related kinases such as PKC and p70 S6 kinase. pAkt was observed in the tumor membrane and cytoplasm, which is consistent with a previous report[23] (Fig. 1). A specimen was considered positive for pAkt if 10% or more of the cytoplasm in the invasive component of the tumor stained positive for pAkt. Representative positive and negative cases are shown in Figure 1. Eighty-four cases (33.3%) were diagnosed as positive for pAkt expression. We examined the correlation between pAkt expression and HER2/neu status. pAkt was expressed in significantly more of the HER2/neu-positive cases ($p < 0.0001$) (Table I). No significant correlation was observed between pAkt and ER expression; however, an inverse correlation was observed between pAkt and PR expression ($p = 0.032$) (Table II). Correlations between pAkt expression and other clinicopathologic variables are shown in Table III. Phosphorylation of Akt is associated with lymph node metastases ($p = 0.008$). No significant correlation was observed between pAkt expression and other clinicopathologic variables, such as age, tumor size, clinical stage and pathologic classification (Table III).

Prognostic value of phosphorylation of Akt in breast cancers

Because it has been suggested that high Akt activity in breast carcinoma is associated with resistance to hormone therapies and chemotherapy[14] and with poor prognosis,[15–17] we investigated whether pAkt might be associated with poor prognosis in our data set. We performed univariate survival analysis to show the association of DFS with pAkt in 240 patients whose clinical courses were available. There was no difference between pAkt-positive and -negative groups in terms of DFS for all cases (Fig. 2*a*). Of these 240 patients, 107 received postoperative hormone therapy, while the remaining 133 were not treated with hormone therapy. Interestingly, in the analysis of cases with postoperative hormone therapy, pAkt positivity was significantly associated with a higher risk of recurrence ($p = 0.0161$) (Fig. 2*b*). The hormone therapies received by these patients were as follows: selective estrogen receptor

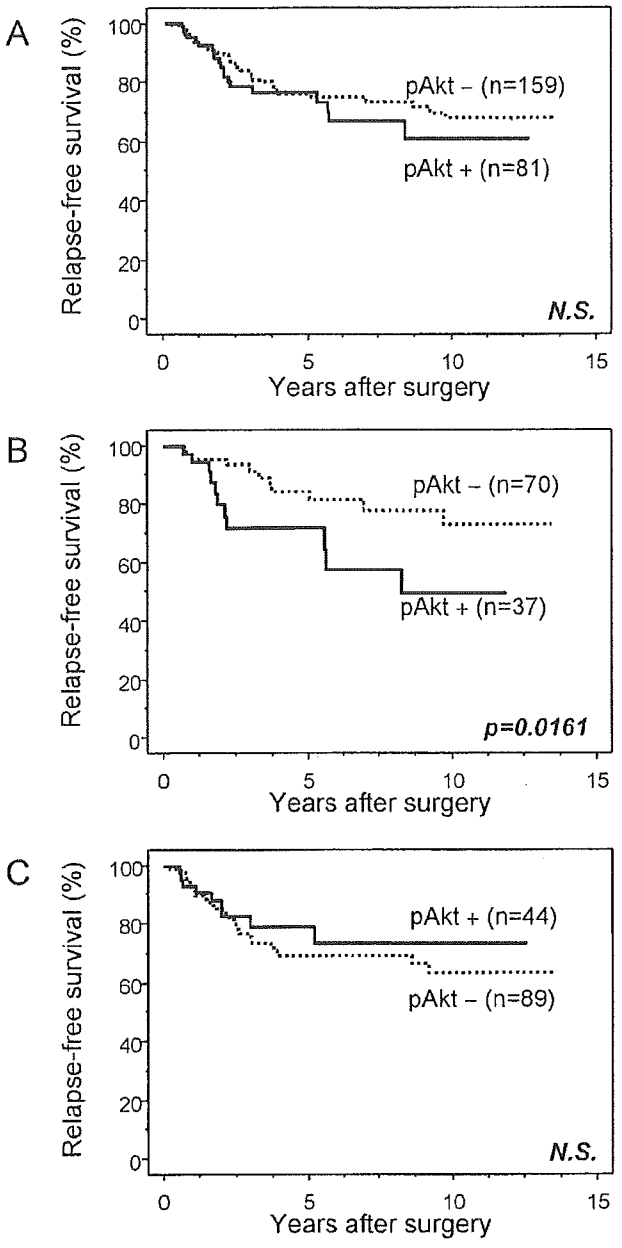

FIGURE 2 – Kaplan-Meier DFS curves of breast cancer patients. There was no difference between pAkt-positive and -negative groups in terms of DFS for all cases (*a*). In the analysis of cases with postoperative hormone therapy, pAkt positivity was significantly associated with a higher risk for recurrence (*b*). pAkt positivity had no prognostic value in cases without postoperative hormone therapy (*c*).

modulator (SERM) (tamoxifen or toremifene) in 81 patients, aromatase inhibitor (anastrozole or fadrozole) in 11 patients, LH-RH agonist (goserelin) in 8 patients, LH-RH agonist (goserelin) + tamoxifen in 5 patients and MPA in one patient. However, pAkt positivity had no prognostic value in cases without postoperative hormone therapy (Fig. 2*c*). Multivariate analysis of pAkt and traditional prognostic factors failed to indicate that pAkt was an independent prognostic factor (Table IV). However, in cases with postoperative hormone therapy, there was a tendency for higher risk in the pAkt-positive group compared to the pAkt-negative group ($p = 0.10$) (Table V).

TABLE IV – MULTIVARIATE ANALYSIS OF RECURRENCE IN ALL PATIENTS INCLUDING THE VARIABLES LYMPH NODE STATUS, HORMONE RECEPTOR STATUS AND pAkt

Variables	Rate ratio (95% CI)	Significance
Nodal status		
Negative	1	$p < 0.0001$
Positive	4.12 (2.2–7.6)	
ER		
Negative	1	$p = 0.95$
Positive	1.02 (0.5–2.0)	
PR		
Negative	1	$p = 0.95$
Positive	0.98 (0.5–1.9)	
pAkt		
Negative	1	$p = 0.40$
Positive	1.28 (0.7–2.3)	

CI, confidence interval.

TABLE V – MULTIVARIATE ANALYSIS OF RECURRENCE IN PATIENTS WHO RECEIVED POSTOPERATIVE HORMONE THERAPY INCLUDING THE VARIABLES LYMPH NODE STATUS, HORMONE RECEPTOR STATUS AND pAkt

Variables	Rate ratio (95% CI)	Significance
Nodal status		
Negative	1	$p = 0.0021$
Positive	4.98 (2.2–7.6)	
ER		
Negative	1	$p = 0.54$
Positive	0.7 (0.3–2.0)	
PR		
Negative	1	$p = 0.59$
Positive	0.77 (0.2–2.2)	
pAkt		
Negative	1	$p = 0.10$
Positive	2.08 (0.9–5.0)	

CI, confidence interval.

Discussion

Akt/PKB is a serine/threonine kinase and a downstream effector of PI-3K.[1] The major functions of the PI-3K/Akt signal pathway are to promote growth factor–mediated cell growth, proliferation, migration and survival.[1] This pathway has been intensively investigated in various malignancies. Because activation of the PI-3K/Akt pathway induces resistance to endocrine therapy and chemotherapy, inhibition of this pathway is now considered a promising strategy to improve the effect of therapies for various kinds of cancer (reviewed in Thompson and Thompson[24]).

erbB2/HER2/neu is a receptor tyrosine kinase, which has been most studied in breast cancer. Overexpression of erbB2/HER2/neu occurs in approximately 30% of human breast cancers and is generally associated with poor prognosis[3] and with resistance to systemic and local radiation therapies.[7]

Cell lines that overexpress HER2/neu exhibit high levels of Akt1.[25] In addition, a significant association has been demonstrated between the expression of HER2/neu and pAkt in 20 adenocarcinomas. Previous studies have shown that erbB2, when overexpressed, is constitutively associated with erbB3. Since erbB3 possesses 7 tyrosine residues that could be phosphorylated and act as binding sites for the SH2 domains of the p85 regulatory subunit of PI-3K, erbB2–erbB3 dimers strongly activate the PI-3K–PKB/Akt pathway. This provides a strong basis for studies that have demonstrated that tumor cells overexpressing erbB2 display constitutive PKB/Akt activity. These data implicate HER2/neu overexpression in activation of the Akt/PKB pathway and that the PKB/Akt pathway may play a major role in stimulating proliferation and survival in HER2/neu-overexpressing cells.

In the present study, we found that pAkt expression correlated significantly with HER2/neu overexpression. This finding was consistent with many *in vitro* studies using established cell lines

and breast cancer tissues.[15,16] We examined 252 breast cancer cases, which is considered sufficient power to draw a reliable conclusion.

Although we found no significant correlation between pAkt and ER expression, we found an inverse correlation between pAkt and PR expression ($p = 0.0321$). A recent study demonstrated that PR expression was reduced *via* the PI-3K/Akt pathway,[26] and this finding may support our results.

Because it has been shown that patients with high pAkt expression have a poor prognosis compared to other patients,[16] we first investigated the prognosis of patients analyzed in terms of pAkt expression. There was no difference in DFS among all patients. Then, we divided the patients into 2 groups, those who did and those who did not receive postoperative endocrine therapy. Interestingly, in the analysis of patients who received postoperative endocrine therapy, pAkt positivity was significantly associated with higher risk of recurrence ($p = 0.0161$) (Fig. 2*b*). Multivariate analysis, including pAkt and traditional prognostic factors, failed to indicate that pAkt was an independent prognostic factor in all cases (Table IV). However, in cases with postoperative hormone therapy, there was a tendency for higher risk in the pAkt-positive group compared to the pAkt-negative group ($p = 0.10$) (Table V). So far, there have been a few reports indicating the correlation between Akt activity and the effect of endocrine therapy using human material. Perez-Tenorio *et al.*[17] revealed that pAkt-positive patients were more prone to relapse with distant metastasis. These patients were premenopausal and treated with tamoxifen and/or goserelin. However, in the study of postmenopausal breast cancer patients, the benefit of tamoxifen was analyzed in ER-positive patients.[15] Patients with a negative Akt status showed significant benefit from tamoxifen, whereas there was no significant benefit from tamoxifen in patients with positive Akt status.[15] In the present study, we did not divide the patients according to menopausal status because of the paucity of patients. We found that DFS was worse only in patients who received postoperative endocrine therapy. They were administered a variety of agents; however, this result was consistent with the previous report. Activated Akt induces chemoresistance[13,14] in *in vitro* analyses; thus, it was expected that pAkt would be associated with poor prognosis in patients who received chemotherapy. However, we could not find any difference in patients who did or did not receive chemotherapy (data not shown). One possible reason for this observation is that many of these patients were treated with oral fluoropyrimidines. There is no evidence that oral fluoropyrimidines have sufficient efficacy as adjuvant chemotherapy. Thus, in the future, it would be interesting to study the association between pAkt and chemotherapy in chemotherapy regimens proven to have sufficient efficacy.

In the present study, pAkt was associated with positive nodal status, although there was no significant correlation between pAkt and tumor size, clinical stage and histopathologic classification. This suggests that pAkt may induce a more malignant phenotype *via* its role in antiapoptosis and proliferation.

In this study, we demonstrated that Akt/PKB activation was significantly elevated in cases of primary breast carcinoma with HER2/neu overexpression. Moreover, it is likely that evaluation of pAkt status, in addition to the status of hormone receptors and HER2/neu, will be useful in the prediction of the efficacy of postoperative endocrine therapy for breast cancer. However, to elucidate the significance of Akt/PKB activation in clinical outcome, we must utilize well-designed, prospective studies. The data obtained from such studies will likely provide very useful information about treatment for breast cancer patients.

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References

1. Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002;14:381–95.
2. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, McGuire WL. Studies of the *HER-2/neu* proto-oncogene in human breast and ovarian cancer. Science 1989;244:707–12.
3. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. Science 1987;235:177–82.
4. Paterson MC, Dietrich KD, Danyluk J, Paterson AH, Lees AW, Jamil N, Hanson J, Jenkins H, Krause BE, McBlain WA. Correlation between c-erbB-2 amplification and risk of recurrent disease in node-negative breast cancer. Cancer Res 1991;51:556–67.
5. Gullick WJ, Love SB, Wright C, Barnes DM, Gusterson B, Harris AL, Altman DG. c-erbB-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. Br J Cancer 1991;63:434–8.
6. McCann AH, Dervan PA, O'Regan M, Codd MB, Gullick WJ, Tobin BM, Carney DN. Prognostic significance of c-erbB-2 and estrogen receptor status in human breast cancer. Cancer Res 1991;51:3296–303.
7. Alaoui-Jamali MA, Paterson J, Al Moustafa AE, Yen L. The role of ErbB-2 tyrosine kinase receptor in cellular intrinsic chemoresistance: mechanisms and implications. Biochem Cell Biol 1997;75:315–25.
8. Kurokawa H, Arteaga CL. Inhibition of erbB receptor (HER) tyrosine kinases as a strategy to abrogate antiestrogen resistance in human breast cancer. Clin Cancer Res 2001;7:4436s–42s.
9. Kurokawa H, Arteaga CL. ErbB (HER) receptors can abrogate antiestrogen action in human breast cancer by multiple signaling mechanisms. Clin Cancer Res 2003;9:511S–5S.
10. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 2000;19:3159–67.
11. Siegel PM, Ryan ED, Cardiff RD, Muller WJ. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. EMBO J 1999;18:2149–64.
12. Zhou BP, Hu MC, Miller SA, Yu Z, Xia W, Lin SY, Hung MC. *HER-2/neu* blocks tumor necrosis factor–induced apoptosis via the Akt/NF-κB pathway. J Biol Chem 2000;275:8027–31.
13. Knuefermann C, Lu Y, Liu B, Jin W, Liang K, Wu L, Schmidt M, Mills GB, Mendelsohn J, Fan Z. HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. Oncogene 2003;22:3205–12.
14. Clark AS, West K, Streicher S, Dennis PA. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. Mol Cancer Ther 2002;1:707–17.
15. Stal O, Perez-Tenorio G, Akerberg L, Olsson B, Nordenskjold B, Skoog L, Rutqvist LE. Akt kinases in breast cancer and the results of adjuvant therapy. Breast Cancer Res 2003;5:R37–44.
16. Zhou X, Tan M, Stone Hawthorne V, Klos KS, Lan KH, Yang Y, Yang W, Smith TL, Shi D, Yu D. Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers. Clin Cancer Res 2004;10:6779–88.
17. Perez-Tenorio G, Stal O,Southeast Sweden Breast Cancer Group. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. Br J Cancer 2002;86:540–5.
18. Japanese Breast Cancer Society. General rules for clinical and pathological recording of breast cancer,14th ed. Tokyo: Kanehara, 2001.
19. Ellis MJ, Coop A, Singh B, Mauriac L, Llombert-Cussac A, Janicke F, Miller WR, Evans DB, Dugan M, Brady C, Quebe-Fehling E, Borgs M. Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial. J Clin Oncol 2001;19:3808–16.
20. Jacobs TW, Gown AM, Yaziji H, Barnes MJ, Schnitt SJ. Specificity of HercepTest in determining *HER-2/neu* status of breast cancers using the United States Food and Drug Administration–approved scoring system. J Clin Oncol 1999;17:1983–7.
21. Seidman AD, Fornier MN, Esteva FJ, Tan L, Kaptain S, Bach A, Panageas KS, Arroyo C, Valero V, Currie V, Gilewski T, Theodoulou M, et al. Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by *HER2* immunophenotype and gene amplification. J Clin Oncol 2001;19:2587–95.

22. Chan TO, Rittenhouse SE, Tsichlis PN. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. Annu Rev Biochem 1999;68:965–1014.
23. Sun M, Wang G, Paciga JE, Feldman RI, Yuan ZQ, Ma XL, Shelley SA, Jove R, Tsichlis PN, Nicosia SV, Cheng JQ. AKT1/PKBα kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. Am J Pathol 2001;159:431–7.
24. Thompson JE, Thompson CB. Putting the rap on Akt. J Clin Oncol 2004;22:4217–26.
25. Ahmad S, Singh N, Glazer RI. Role of AKT1 in 17β-estradiol- and insulin-like growth factor I (IGF-I)–dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells. Biochem Pharmacol 1999;58:425–30.
26. Cui X, Zhang P, Deng W, Oesterreich S, Lu Y, Mills GB, Lee AV. Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: progesterone receptor as a potential indicator of growth factor activity in breast cancer. Mol Endocrinol 2003;17:575–88.

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