of previously unreported missense mutations, and examined their ability to transactivate marker genes through distinct p53 binding sites when the mutants were expressed in yeast. We determined the functional effect of each mutant p53 and found that the p53 function correlated well with the structure and mutations (18).

Temperature-sensitive (ts)[1] p53 mutations have been reported and used as tools for conditional p53 expression in mammalian cells. We identified previously four distinct ts p53 mutations in eight of the 91 human tumor cell lines using a yeast-based transcription assay and predicted that 5–10% of the tumor-derived missense mutations should be ts mutations (19). To date, 61 p53 ts mutations have been isolated by using several different methods, including a yeast-based functional assay (Table I). Among these, the V272M ts mutant was reactivated by a small molecule, aminothiol WR1065 (20), at a non-permissive temperature, suggesting that ts mutants may be functionally rescued by small molecules.

The purpose of this study was the screening and isolation of a large number of ts mutations from a comprehensive missense mutation library, mapping them to the p53 structure, and considering the function-structure relationship through the ts mutants. To isolate a number of ts p53 mutations, we screened the p53 library containing 2,314 p53 missense mutations using a yeast-based p53 functional assay and found 142 ts p53 mutants, including previously unreported 131 mutants. We confirmed that most were also ts in p53-less mammalian cells. The ts mutants were preferentially mapped on one of the β-sheets, and there were hot spot sites for ts mutations. Because a fairly significant fraction of the p53 mutants in the *TP53* mutation databases were ts mutants, these ts p53 mutant proteins may be novel molecular targets through the ts mechanism and structure-dependent restoration of p53 function.

EXPERIMENTAL PROCEDURES

p53 Missense Mutation Library—2,314 p53 missense mutations were constructed recently through a 96-well, formatted, site-directed mutagenesis and stably expressed in a haploid yeast strain harboring a p53-responsive $p21^{WAF1}$ reporter plasmid (pAS03G) (21) or in diploid yeast strains harboring p53-responsive reporter plasmids with a *MDM2* promoter or p53 binding sequences derived from *BAX* (pKS07R), *14-3-3σ* (pKS09R), *p53AIP1* (pKS11R), *GADD45* (pKS13R), *Noxa* (pKS15R), and *p53R2* (pKS17R) as described previously (18).

Screening ts p53 Mutants Using a Yeast Assay—The 2,314 yeast clones expressing the mutant p53 were grown on 25 96-well formatted plates containing synthetic complete (SC) media lacking leucine and tryptophane (SC −Leu −Trp) in the case of the haploid strains, or SC media lacking leucine, tryptophane and histidine (SC −Leu −Trp −His) in the case of the diploid strains.

Fluorescent Intensity—To evaluate the transactivity of each mutant p53 quantitatively, the yeast clones (haploid cells) were replicated on SC −Leu −Trp solid media using a 96-pin replicator and grown at 37 or 32 °C for 2 days. The plates were then directly processed in a 96-well formatted fluorometer (Fluoroskan Ascent FL, Labsystems) to measure the fluorescent intensity (excitation, 485 nm; emission, 538 nm) of p53-dependent enhanced green fluorescent protein expression through a human $p21^{WAF1}$-derived p53 binding sequence. The diploid cells, selected by mating reaction, were incubated on SC −Leu −Trp −His plates at 37 or 30 °C for 2 days, and the fluorescent intensity of Ds-Red was measured using the same fluorometer (excitation, 544 nm; emission, 590 nm) to evaluate the p53-dependent Ds-Red expression through other p53-binding sequences. At least two independent experiments were performed for each reporter, and the fluorescence intensities were averaged. The averaged values were standardized in each p53 binding sequence, clustered, and visualized using the CLUSTER and TREEVIEW programs. The standardized data were also spotted on a two-dimensional graph for 30 and 37 °C. We defined the following criteria to select ts mutants from the p53 mutant library, namely $M_{30}/W_{30} \geq 0.7$, $M_{37}/W_{37} \leq 0.5$, and $M_{30}/M_{37} \geq 2$, where M_{30} and M_{37} indicate the fluorescent intensities of the p53 mutants at 30 and 37 °C, respectively, and W_{30} and W_{37} indicate the fluorescent intensities of the wild-type p53 at 30 and 37 °C, respectively.

Cell Culture and Transfection—A *TP53*-deficient human osteosarcoma cell line, Saos-2, was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated (56 °C for 30 min) fetal calf serum (JRH Bioscience) in the presence of 5% CO_2. For luciferase assays, the cells were grown to 60–90% confluence in 96-well tissue culture plates at 37 °C and then cultured at 32 or 37 °C for another 24 h. For immunoprecipitation, the cells were grown in 90 × 20-mm tissue culture plates at 37 °C in the presence of 5% CO_2 and further incubated at 32 or 37 °C for another 18 h. Transient transfections were performed using the Effectene (Qiagen) transfection reagent. For luciferase assays, the cells were co-transfected with 12.5–50 ng of the expression vector (pCR259-p53WT, pCR259-p53MT, or a p53-less control pCR259 vector) (18) and 50–87.5 ng of the p53-responsive luciferase plasmid (p21Ps-luc, pMDMPs-luc, pBAXPs-luc, pSIGMAPs-luc p53R2Ps-luc, or p53GADD45Ps-luc) (18, 21) and incubated for a further 24 h. For immunoprecipitation, the cells were transfected with 2 μg of the expression vector (pCR259-p53WT, pCR259-p53MT, or a control pCR259 vector) and further incubated for 36 h.

Luciferase Assay—After 24 h of transfection, luciferin (Steady-Glo luciferase assay system, Promega), a substrate of luciferase, was added to the culture media and further incubated for 60–120 min according to the manufacturer's instructions. The fluorescent intensity was measured using the Fluoroskan Ascent FL (see above). The relative fluorescent intensity to the wild-type control was calculated from three sets of independent experimental data at 32 and 37 °C. The value differences at the two temperatures were statistically evaluated by *t* test. The ts mutants were defined when the *p* value was <0.001.

Immunoprecipitation and Immunoblotting of p53—Saos-2 cell lysates were prepared in 100 μl of NET buffer (150 mM NaCl, 50 mM Tris-HCl (pH8.0), 5 mM EDTA, and 1% Nonidet P-40) containing 0.1 μg/μl phenylmethylsulfonyl fluoride. Fifty microliters of the cell lysates were immunoprecipitated with 10 μl of the PAb1620 (Ab-5; Oncogene) or the PAb240 (Ab-3; Oncogene) monoclonal antibody against human p53. The lysates, with 8 μl of the crude lysate, were fractionated by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to Optitran BA-S83 membranes (Schleicher & Schuell), and the expressed p53 mutants were detected using a HRP-conjugate anti-p53 antibody (p53(FL393)HRP, Santa Cruz Biotechnology). The proteins were visualized and quantitatively analyzed using an ECL Western blotting detection system (Amersham Biosciences), a lumino-image analyzer (LAS1000, Fuji Film) and ID image analysis software (Kodak Digital Science).

Drawing p53 Peptide Structures—To map the ts p53 mutants on the p53 core domain, the NCBI structure file, 1TUP, was customized for our purpose and visualized using Cn3D 4.0 software (22).

RESULTS

Clustering of 2,314 Mutations on Transactivities at Two Distinct Temperatures—An unsupervised, hierarchical one-dimensional cluster analysis allowed us to cluster the 2,314 p53 mutants on the basis of similar measured transactivities for eight distinct p53 binding sequences (p53 binding sites) at 30 and 37 °C (Fig. 1*A*). The mutants are divided into two major clusters. In one of these clusters the mutants retain transactivities; in the other they lose activity, and these clusters are mostly temperature-independent. Notably, there is one temperature-dependent sub-cluster within the latter cluster (Fig. 1*B*). The cluster consists of 64 p53 mutants, and the transactivities of the mutants are inactive on almost all p53 binding sites at 37 °C but active on some p53 binding sites at 30 °C, indicating that a large number of mutants are ts for transactivation in yeast cells.

Isolation of ts p53 Mutants in Yeast—Although the cluster analysis found the typical ts mutants that represent temperature sensitivity for most p53 binding sites, there are mutants that show temperature sensitivity on limited types of p53 binding sites and, therefore, are not clustered. To also isolate such clones, the transactivities of the 2,314 mutant clones at 30 and 37 °C were standardized and overviewed by a scatter plot for each p53 binding site (Fig. 2). Among the 18,512 data points (8 × 2,314 clones), the majority had similar transcriptional

[1] The abbreviations used are: ts, temperature-sensitive; SC, synthetic complete; HRP, horseradish peroxidase.

TABLE 1
Summary of the 61 reported p53 mutations

p53 mutant[a]	Experimental system[b]	p53BS or promoter[c]	Reference
S99F***	1	BAX, CDKN1A, PIG3	27
A119V	1	BAX, CDKN1A, PIG3	27
Y126S	1	BAX, CDKN1A, PIG3	27
Y126D	1	BAX, CDKN1A, PIG3	27
K132N	3	CON	28
K132R	3	CON	28
M133T	1	RGC	29
V135* (mouse p53)	2	CDKN1A	30, 31
A138V***	2	BAX, BCL2, CDKN1A, MDM2	32
T140Y	3	CON	28
V143A	1, 2	BAX, GADD45A, CCNG1, CDKN1A, CON, IGFBP3, MDM2, RGC	33–35
P152L***	1	BAX, CDKN1A, PIG3	27
P152T**	1	BAX, CDKN1A, PIG3	27
G154V**	2	GAL4	36
T155I**	1	BAX, CDKN1A, PIG3	27
M160I/A161T*	1	BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC	35
I162F	1	BAX, CDKN1A, PIG3	27
T170R	1	BAX, CDKN1A, PIG3	27
V172F***	1	RGC	29
R175K*	2	BAX, FOS, IGFBP3	37
R175I*	2	BAX, FOS, IGFBP3	37
R175P***	2	BAX, FOS, IGFBP3	37
R175Q*	2	BAX, FOS, IGFBP3	37
R175S***	2	BAX, FOS, IGFBP3	37
R175M*	2	BAX, FOS, IGFBP3	37
H179Q	2	GAL4	36
E180K	1	BAX, CDKN1A, PIG3, RGC	27, 38
R181G	1	BAX, CDKN1A, PIG3	27
R181H	1	BAX, CDKN1A, PIG3	27
H193R***	1	BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC	35
V197L***	1, 2	BAX, CDKN1A, FOS, TGFA, RGC	39, 40
Y205N***	1	BAX, CDKN1A, PIG3	27
H214R**	1	RGC	19
P219L***	1	BAX, CDKN1A, PIG3, RGC	27, 38
Y220C	1	BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC	35
Y220H	1	BAX, CDKN1A, PIG3	27
E224K	1	BAX, CDKN1A, PIG3	27
D228V	1	BAX, CDKN1A, PIG3, RGC	27, 38
Y234C**	1	RGC	19
Y234H***	1	BAX, CDKN1A, PIG3	27
M237R**	2, 3	CON, FOS, RGC	28
N239S	3	CON	28
M246V**	3	CON	28
N247I***	2, 3	CON, GAL4	28, 36
R248W	3	CON	28
P250L**	1	BAX, CDKN1A, PIG3, RGC	27, 38
L252F***	1	BAX, CDKN1A, PIG3	27
I254F**	1	BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC	35
T256A**	3	CON	28
D259N	1	BAX, CDKN1A, PIG3, RGC	27, 38
G266E	1	BAX, CDKN1A, PIG3	27
V272M***	1–3	BAX, CDKN1A, CON, GADD45A, MDM2, PIG3, RGC	19, 28, 38, 41
R273H	3	CON	28
R273L	1, 3	BAX, CDKN1A, CON, PIG3	27, 28
A276G	1	BAX, CDKN1A, PIG3	27
D281Y	3	CON	28
R283I***	1	BAX, CDKN1A, PIG3, RGC	38
E285K**	1	RGC	19
E286G***	1	BAX, CDKN1A, PIG3	27
E286K***	1	BAX, CDKN1A, PIG3	27
286K/287D*	1	BAX, CDKN1A, PIG3	27

[a] The meaning of the asterisk symbols used in this column is as follows: *, ts mutants not constructed in this study; **, ts mutants also isolated in this study; ***, distinct substitution(s) at the same residue were ts mutants in this study.

[b] The meaning of the numbers used in this column is as follows: 1, yeast system; 2, mammalian cell system; 3, cell-free system.

[c] All but three of the gene names used in this column refer to those used in the Online Mendelian Inheritance in Man (OMIM) site (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM). The three exceptions are: CON, p53-binding consensus sequence; GAL4, yeast GAL4-binding sequence; and RGC, human ribosomal gene cluster sequence. The study on the GAL4 was performed by GAL4-binding domain and p53 fusion protein.

activity (either active or inactive) at both 30 and 37 °C, indicating that they were not ts. Obviously, there were significant numbers of p53 mutant clones that represented higher transactivity at 30 °C than at 37 °C, showing ts mutants for the transactivation function (*circled spots* in Fig. 2). On the other hand, only a limited number of clones represented higher transactivity at 37 °C than at 30 °C, showing cold-sensitive mutants. As there is no clear boundary between ts and non-ts mutants, we defined the borders for convenience as described under "Experimental Procedures." According to the definition, 142 p53 mutants were selected as ts for yeast transactivation assay (Fig. 3*A*), indicating that 6.1% (142 of 2,314) of the p53 mutants were ts for at least one of the p53 binding sites. The 142 mutants, including 131 previously unreported ts mutants,

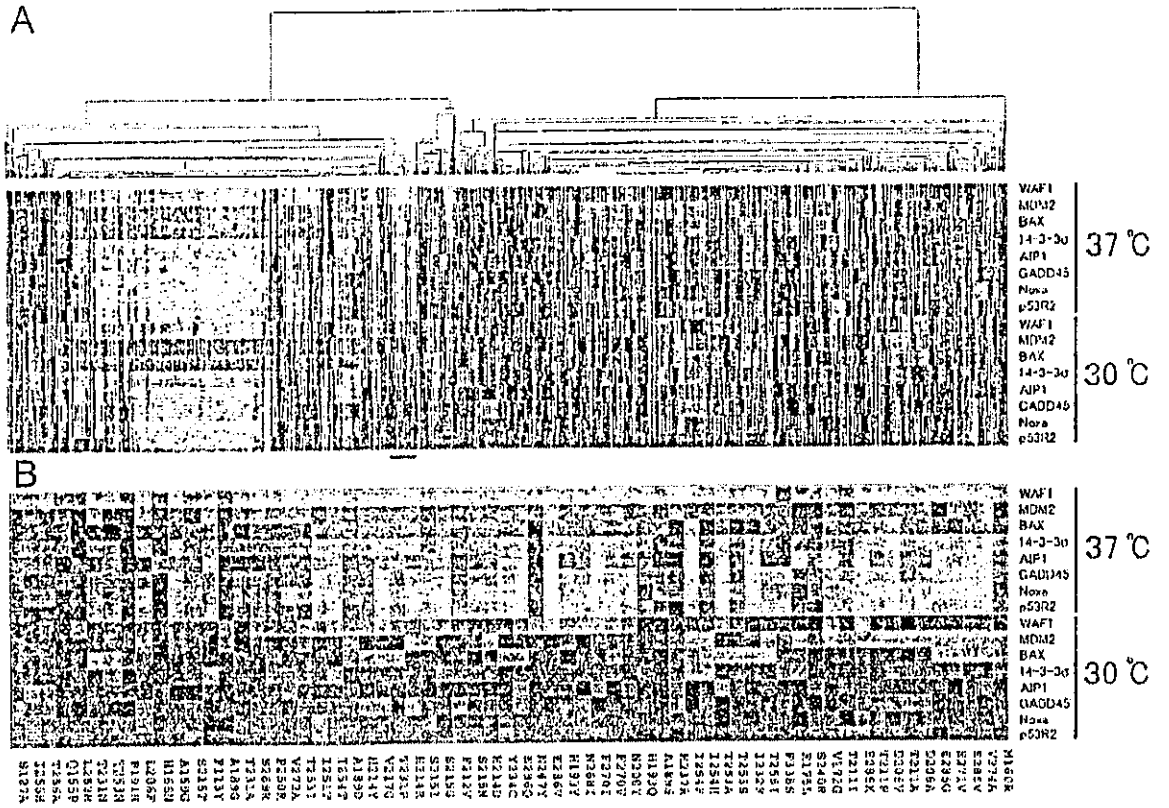

FIG. 1. **Cluster analysis of mutant p53 transactivities at 30 and 37 °C.** *A*, one-dimensional hierarchical cluster analysis of 2,314 p53 mutants. Standardized data for the indicated p53 binding sites and temperatures were shown as the color gradation of *red* (high), *green* (low), and *black* (intermediate). A ts sub-cluster was *underlined*. *B*, the ts sub-cluster containing 64 p53 mutants was magnified.

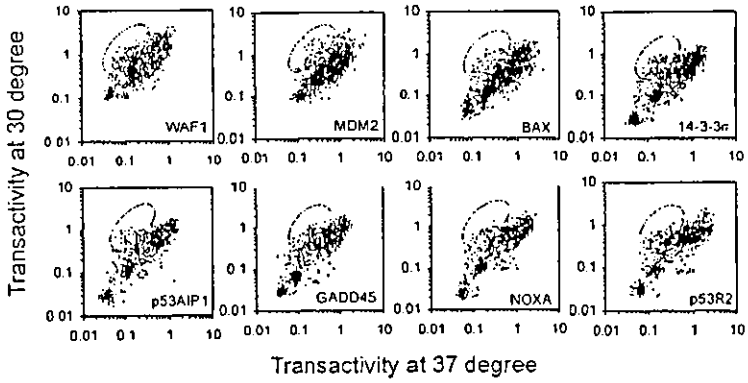

FIG. 2. **Scatter plot of the transactivities of the 2,314 p53 mutants for eight distinct p53 binding sequences at 30 and 37 °C.** Standardized data for the indicated p53 binding sites and temperatures were used. *Circled spots* contained ts clones.

were mapped on 82 residues of p53 (82 of 393; 20.9%) and 131 (92%) were within the core DNA-binding domain (residues 97–286).

Mapping of ts p53 Mutants on p53 Core DNA-binding Domain—To characterize the isolated ts mutants in the structural context, we mapped the 131 mutants on the core DNA-binding domain (Fig. 4, *A* and *B*). Among these, 70 mutants (53.4%) were mapped on the β-strands. The frequency of ts mutants in the constructed mutants differed among the 10 β-strands; the frequency was relatively higher in S4 (14 of 49, 28.6%), S7 (11 of 34, 32.3%), S9 (16 of 49, 32.7%), and S10 (15 of 61, 24.6%), whereas it was lower in S1 (1 of 17, 5.9%), S2 (1 of 24, 4.2%), S3 (1 of 33, 3.0%), S5 (2 of 22, 9.1%), S6 (2 of 24, 8.3%), and S8 (7 of 43, 16.3%). In particular, residues 158 to 161 (S4), 211 to 217 (S7), 251 to 256 (S9), and 268 to 272 (S10) were hot areas for ts mutants because there were 10 or more mutants in every four contiguous residues. Three or more ts mutants were observed in residues Thr^{155}, Arg^{158}, Met^{160}, Ala^{161}, Val172, His^{214}, Ser^{215}, Pro^{223}, Thr^{231}, Thr^{253}, Ile^{254}, Thr^{256}, Ser^{269}, Glu^{271}, and Glu^{286}, and those residues should be designated ts hot spots.

Amino Acid Substitutions of the ts Mutants—Amino acid residues before and after substitution of the ts mutant are summarized in Table II. Isoleucine (21.4%), threonine (21.1%), and tryptophane (20%) were the most frequent residues among the original p53 residues. Glycine (20%), isoleucine (18%), alanine (16.6%), leucine (16.2%), and proline (13.2%) were the most frequent residues among the residues after substitution,

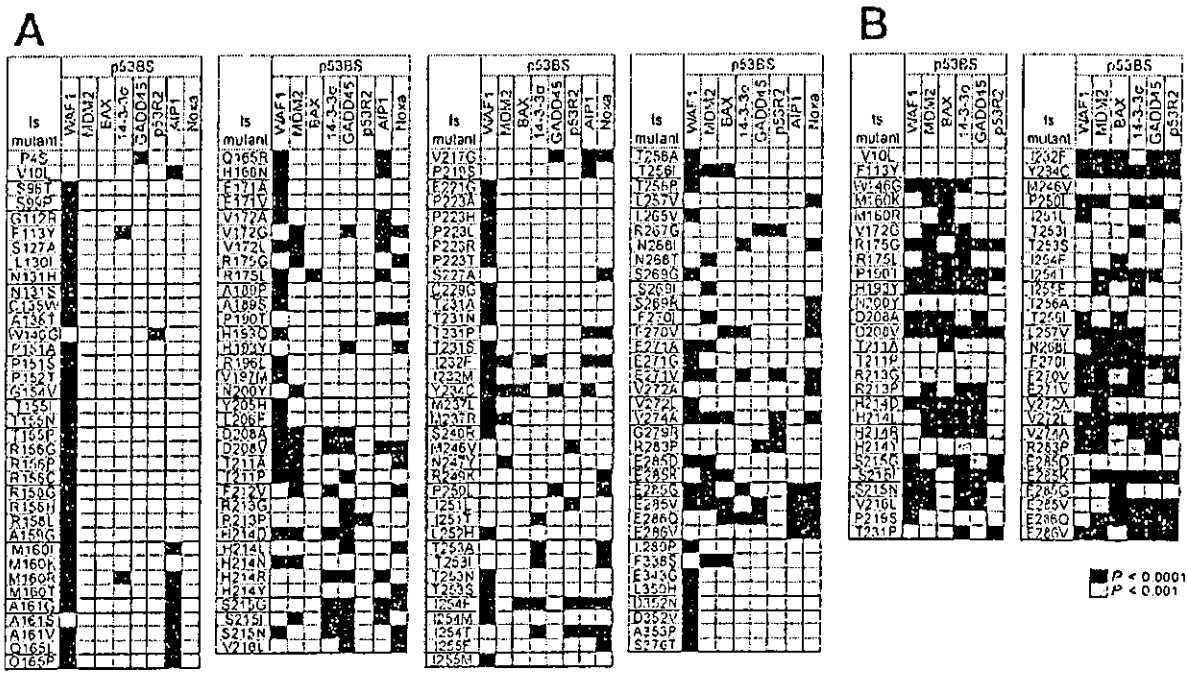

FIG. 3. **Panels of ts p53 mutants.** *A*, the ts p53 mutants isolated by yeast-based functional assay. 142 mutants were listed from the NH_2 terminus to the COOH terminus of p53. *Filled boxes* represent temperature sensitivity that satisfied the defined criteria (see "Experimental Procedures"). *B*, 54 of 142 ts mutants were examined for ts transactivities in Saos-2 cells. When, according to *t* test, the luciferase activities at 32 and 37 °C were statistically different with a *p* value <0.001 or <0.0001, the corresponding *boxes* in the panel were colored *gray* or *black*, respectively.

whereas aspartic acid (3.5%) and glutamic acid (0%) were found less frequently.

Evaluation of the ts p53 Mutants in Mammalian Cells—To evaluate whether the isolated p53 mutants in yeast were also ts for sequence-specific transactivation in mammalian cells, we randomly chose 54 p53 mutants from the 142 ts mutant p53 cDNA clones (Fig. 3*B*), and constructed expression vectors for mammalian cell experiments. Each mutant p53 was expressed in a p53-deficient human osteosarcoma cell line, Saos-2, and examined for the sequence-specific transactivation at both 32 and 37 °C by luciferase assay. When the values of the three independent experiments relative to the wild-type p53 at 32 °C were significantly ($p < 0.001$; *t* test) different from those at 37 °C in at least one of the six promoters ($p21^{WAF1}$, *MDM2*, *BAX*, *14-3-3σ*, *p53R2*, and *GADD45*), the mutant clone was defined as a ts mutant in mammalian cells. Among the 54 mutants, 48 (89%) were ts mutants in at least one of the six promoters. The results indicated that most ts mutants isolated in the yeast assay are also ts mutants in mammalian cells, suggesting that many of the remaining 88 clones may also be ts mutants in mammalian cells. Among the 48 clones, 16 were ts in all 6 promoters, whereas 32 clones were ts in a limited number of promoters, although many retained weak ts phenotypes for other promoters (data not shown).

Epitope Analyses of the p53 Protein Expressed in Saos-2 Cells Using Conformation-sensitive Antibodies—To examine whether the ts mutants display ts changes in their epitopes against conformation-sensitive antibodies, PAb1620 for wild-type-like conformation and PAb240 for denatured mutant conformation, six randomly selected ts mutants, M160R, H193Y, T211A, P219S, T253I, and V274A, were expressed in Saos-2 cells at both 32 and 37 °C. The cell lysates were immunoprecipitated using the two antibodies, detected by Western blot analysis using an HRP-conjugated anti-p53 antibody, and quantitatively analyzed using a lumino-image analyzer. In the case of wild-type p53, the PAb1620 epitope was exclusive, and only a trace of the PAb240 epitope was detected (Fig. 5*A*). Similar to wild-type p53, the PAb1620 epitope was dominant in R273H, although the PAb240 epitope was also detected. On the other hand, the PAb240 epitope was dominant, and the PAb1620 epitope was less abundant in R175H. The ratios of the epitope expressions of PAb1620 to PAb240 are shown in Fig. 5*B*. R175H and R273H were not ts because there were no significant differences in the ratios between 32 and 37 °C. Among the ts mutants, P219S and T253I showed an obvious ts increase in ratio. The remaining ts mutants showed no change or only a slight change in ratio.

DISCUSSION

Comparing 142 ts p53 Mutants with the Previously Reported p53 Mutant—Among the 142 ts mutants, 131 were previously unreported mutants. In our survey of previous papers, including our own, 61 human ts p53 mutants have been reported (Table I). These obviously include ts mutants not isolated in our system. We speculate that there are two reasons for the discrepancy. First, they were isolated using experimental systems different from those in our study, including a reporter assay for sequence-specific transactivation in mammalian cells, similar yeast assays with different p53 binding sites, an electrophoretic mobility shift assay (EMSA) in a cell-free system, and monitoring changes in structure-sensitive antibody reactivity. Therefore, it is possible that there are many potential ts mutants not isolated by the method adopted in this study. For example, a known ts mutant, V143A, did not appear as ts in the yeast cells because the ts phenotype may be mediated by ts interaction with human ASPP2 (p53BP2), a positive modulator of p53 transactivation (23, 24) that does not exist in yeast cells. Obviously, there may be mechanisms not directly affecting p53 binding to DNA. We are now planning to screen such novel ts mutants by using protein-protein interactions that may modify

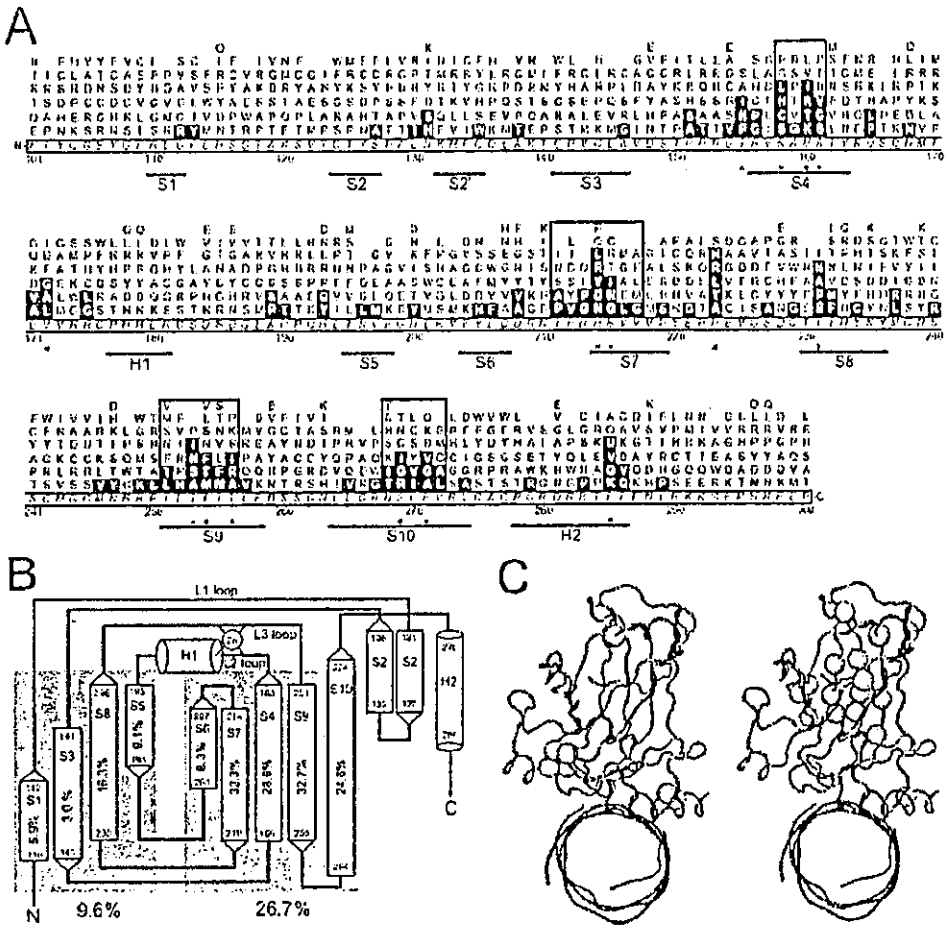

FIG. 4. **Location of the ts p53 mutants in the core DNA-binding domain.** *A*, precise map of the constructed p53 mutants and ts mutants (*white characters* in *black boxes*) in codons from 101 to 300. *Italicized characters*, original residues observed in wild-type p53; *open boxes*, four hot spots for ts mutants; *asterisk*, 15 hot spot residues for ts mutants; *bold lines*, secondary structures (11 β-strands and 2 α-helices). *B*, schematic representation of the core DNA-binding domain (14) and the fraction of the ts mutants in the two β-sheet structures. *Percentages*, fraction of the ts mutants within the constructed mutants in the indicated secondary structures; *small numbers*, codon numbers showing the NH_2-terminal and COOH-terminal ends of the indicated secondary structures. *C*, three-dimensional structure of p53 core DNA-binding domain with double-strand DNA oligonucleotides. Positions of the 10 tumor-derived hot spot residues (*left panel*) were compared with 15 representative ts hot spot residues (*right panel*). p53 and the interacting DNA structure were derived from Protein Data Bank file 1TUP (14), and views from the longitudinal axis of DNA are shown using CD3n 4.0 software (22).

p53 structure by their post-translational mechanisms. Second, as shown in Fig. 2, there is distinct strength in ts transactivation, and some reported ts mutants have been eliminated from our criteria because of a weak ts phenotype. In fact, several mutants clustered in Fig. 1*B* were not selected in our defined criteria. We also note that many previously identified ts mutants had a weak ts phenotype in our yeast screening (data not shown).

Promoter Specificity of the ts p53 Mutants—We have shown that several p53 mutants differ in transactivity spectrum in different p53 binding sites (18). Similarly, ts mutants differed in the ts transactivity spectra in different p53 binding sites (Fig. 3). We speculate that there are subtle differences in structural alterations caused by specific mutations and temperatures and that such alterations are responsible for the partial inactivation or reactivation of p53-binding to the distinct DNA sequences. In fact, there are similarities in the transactivity spectra among mutants in the same or contiguous residues (Fig. 3*A*), suggesting similar structural alterations. In particular, some showed ts in only one or two promoters, suggesting the possible application of such mutants in the conditional transactivation of specific promoters to study p53 downstream gene functions. Various ts transactivity spectra on different p53-responsive promoters were also observed in mammalian cells (Fig. 3*B*). The promoter selectivity of wild-type p53 by Ser^{46} phosphorylation has been shown as the mechanism of *p53AIP1* transactivation (25). Overall, from the results of this study and our previous observations (18), we propose that there may be other unknown potential mechanisms determining the promoter selectivity of wild-type p53 on p53 downstream promoters other than the *p53AIP1* gene. The ts transactivity against different promoters was similar in part but significantly different between human and yeast cells (data not shown). We speculate that there are several reasons for this discrepancy. First, the p53 binding elements, other than $p21^{WAF1}$ and *MDM2* used in the yeast study, were three copies of the specific p53-binding elements and differed from the genomic sequences used in the mammalian cell study. Second, the temperature for the identification of ts mutants in yeast was 30 °C, whereas it was 32 °C in mammalian cells. Third, post-translational modification and the interaction of other proteins may differ in yeast cells and mammalian cells. Finally,

TABLE II
Frequency of residue of ts mutant p53 in the DNA-binding domain before and after substitution

Residue	No. of constructed mutations	No. of ts mutants	Frequency of ts mutant
Before substitution (original residue at mutation site)			
Ile	42	9	21.43
Thr	76	16	21.05
Trp	5	1	20.00
Met	36	7	19.44
Glu	66	12	18.18
Ala	42	7	16.67
His	49	8	16.33
Arg	99	14	14.14
Pro	78	11	14.10
Phe	30	4	13.33
Val	76	9	11.84
Ser	86	9	10.47
Asn	63	6	9.52
Lev	75	6	8.00
Gln	36	2	5.56
Tyr	48	2	4.17
Gly	78	3	3.85
Asf	56	2	3.57
Cys	60	2	3.33
Lys	36	0	0
After substitution (substituted residue at mutation site)			
Gly	75	15	20
Ile	50	9	18
Ala	84	14	16.67
Leu	74	12	16.22
Pro	68	9	13.24
Asn	49	6	12.24
Met	33	4	12.12
Val	99	12	12.12
Thr	67	8	11.94
Tyr	47	5	10.64
Arg	86	9	10.47
Phe	43	4	9.30
His	55	5	9.09
Ser	98	7	7.14
Lys	42	3	7.14
Gln	38	2	5.26
Trp	21	1	4.76
Cys	43	2	4.65
Asp	56	2	3.57
Glu	35	0	0

the criteria to define ts mutants were strict and differed between yeast and mammalian cell systems. Therefore, there may be ts mutants defined by the yeast system but not by the mammalian cell system and *vice versa*, in addition to those showing ts phenotypes in both systems.

Ts Mutants and the Structure of the p53 Protein—We isolated 142 (6.1%) ts mutants from 2,314 p53 missense mutations. Most were mapped in the core DNA-binding domain (131, 91.5%), and a few were in the NH_2-terminal (4, 3.5%) or COOH-terminal (7, 4.9%) domains. The results indicated that the ts mutants isolated in this study may be mutants that directly affect sequence-specific DNA binding rather than mutants affecting the p53 function through post-translational modifications and protein-protein interactions. Within the DNA-biding domain, 50% (71 of 142) of the ts mutants were mapped on β-strands (S1–S10). As only 16% of the residues in the DNA-binding domain form β-strands, it is clear that the β-strands have more ts substructure than α-helices and loops in the DNA binding domain. Among the 11 β-strands, four areas in S4, S7, S9, and S10 are hot areas for ts mutants (see above), and, therefore, the ts mutants tended to be concentrated in one of the two β-sheets (Fig. 4*B*). In particular, residues Thr^{155}, Arg^{158}, Met^{160}, Ala^{161}, Val^{172}, His^{214}, Ser^{215}, Pro^{223}, Thr^{231},

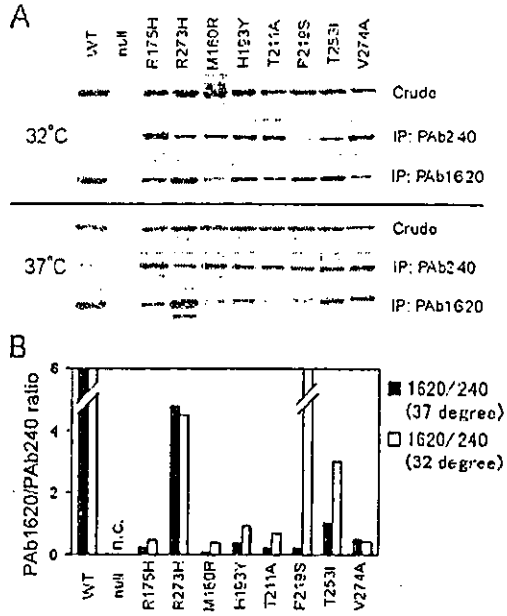

FIG. 5. **Expression of conformation-sensitive epitopes.** *A*, the indicated p53 proteins were expressed from the corresponding p53 expression vectors in the human osteosarcoma cell line, Saos-2, at 32 °C and 37 °C. The crude cell lysate was separated by SDS-polyacrylamide gel electrophoresis immediately or after immunoprecipitation (*IP*) using PAb240 or PAb1620. The p53 proteins were detected using an HRP-conjugated anti-p53 polyclonal antibody. *WT*, wild-type. *B*, the ratio of PAb1620 expression to PAb240 expression at 32and 37 °C. The expression of either PAb1620- or PAb240-reactive p53 proteins (shown in *panel A*) was quantitatively analyzed, and the ratio of the values are shown as graphs. *n.c.*, not calculated.

Thr^{253}, Ile^{254}, Thr^{256}, Ser^{269}, Glu^{271}, and Glu^{285} are ts hot spots (Fig. 4*A*). The ts hot spot mutants are spatially located relatively far from the p53-DNA interface, whereas the tumor-derived 10 hot spot mutations (except Thr^{220}) are formed or located close to the interface (Fig. 4*C*). Although the structural reason why the second β-sheet was more susceptible to ts is still unclear, this observation indicated that the β-sheet was a key structural element controlling the p53 function, suggesting the existence of a potential intramolecular mechanism in normal p53 regulation of the promoter selectivity after post-translational modification such as damage-sensitive phosphorylation.

The consideration of ts-specific amino acid residues before and after substitution is of great interest, because such information may provide a better understanding of the structure of ts mutants. In p53 protein, the majority of the sensitive residues before substitution comprised isoleucine, threonine, and tryptophane, all of which are hydrophobic residues preferentially used in the β-strands of many other proteins (26). On the other hand, the most frequent residues after substitution were glycine, isoleucine, alanine, leucine, and proline. Because these residues are smaller hydrophobic residues, it is possible that the structure of the β-strands is largely undisrupted. Less frequent residues were negatively charged, *i.e.* aspartic acid and glutamic acid. This result was reasonable, because these are known to be β-strand-disrupting residues (26). An unexpected result was frequent proline substitution, because proline is known to be a structurally stable residue and, therefore, should not be vulnerable to subtle structural change due to temperature shift.

The results of immunoprecipitation using conformation-sensitive antibodies were unexpected, because only a limited num-

ber of the mutations examined showed ts changes in the expression of epitopes. We speculate that most ts mutants partially recovered their structural alteration, but their structure and transactivation function were not completely restored. It will be interesting to examine whether such partial restoration of p53 function is sufficient to suppress tumor formation and/or progression when expressed under physiological conditions.

Frequency of ts Mutants in TP53 Mutation Databases—According to the latest International Agency for Research on Cancer (IARC) data base for tumor-derived somatic mutations (17), 1,135 distinct missense mutations, including 1,066 missense mutations with a single nucleotide substitution, are registered. These mutations have been reported 12,032 times in total. Among them, 10.3% (110 of 1,066) of mutants were thought to be ts mutants, and such ts mutations comprised 10.4% (1,254 of 12,032) of the total number of mutations. Therefore, we conclude that ts p53 mutation is not as rare as it was previously thought to be (19), and it may be a molecular target for the pharmacological rescue of p53 protein.

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Breast Cancer
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Oral Session

Collaboration of Breast Cancer Clinic and Genetic Counseling Division for BRCA1 and BRCA2 Mutation Family in Japan

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Background: BRCA1 and BRCA2 mutations cause high breast cancer incidence rates as high as 80%. Although prophylactic therapy is still controversial, several prophylactic therapies have been proposed and tried for BRCA1 and BRCA2 mutation carriers. Prophylactic surgery, chemo-prevention and precise screening have been proposed as prophylactic therapy. All BRCA1 and BRCA2 mutation carriers need knowledge about their disease and the countermeasures that are used to protect against onset of disease. Counseling plays an important role in this regard for people with genetic diseases. Therefore, collaboration between breast cancer clinics and genetic counseling services is the most important issue in clinical practice. Our group consists of three national universities and a general hospital. In this article we describe our trial to construct a clinical system against hereditary breast cancer as an interim report for the Japanese Ministry of Health, Labour and Welfare.

Patients and Methods: Twenty familial breast cancer patients were registered in this study. The whole sequence of BRCA1 and BRCA2 were analyzed. If pathological mutations were detected, their first degree families were introduced to the counseling division at each institute when candidates visited counseling divisions.

Results and Discussion: Four cases of a deleterious mutation in BRCA1 or BRCA2 were detected among 20 cases. Their first degree relatives are now under consideration for visiting counseling divisions. The clinical system described in this study should play a role to protect BRCA1 or BRCA2 mutation carriers in Japan.

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Key words: Hereditary breast cancer, Familial breast cancer, BRCA1, BRCA2

Breast cancer is the most common malignancy observed in Japan and western countries. Treatment of groups at high-risk for breast cancer is also a challenging problem. Familial breast cancer is an important factor impacting the early and bilateral onset of breast cancer. Five to 10% of breast cancer patients meet the definition of familial breast cancer[1,2]. BRCA1 and BRCA2 mutations have been reported as major causes of familial breast cancer. Twenty to thirty percent of Japanese familial breast cancer patients show deleterious BRCA1 or BRCA2 mutation[3]. Further, it has been reported that 80-90 percent of BRCA1 and BRCA2 mutation carriers suffer from breast cancer in their lives[4]. This high risk of breast cancer incident suggests the importance of screening for BRCA1 and BRCA2 mutations and preventive measures for familial breast cancer. Counseling and prophylactic therapies are of importance in prevention of such genetic diseases. Familial breast cancer patients and their first degree relatives are anxious about their genetic status. There is no standard clinical system and method for prophylactic therapy for BRCA mutation carriers in Japan. Hospitals or medical systems that provide proper knowledge of familial breast cancer and preventive therapies are needed. A larger number of patients are required to establish interventions for Japanese BRCA1 or BRCA2 mutation carriers. In this study we aimed to construct a multi-center

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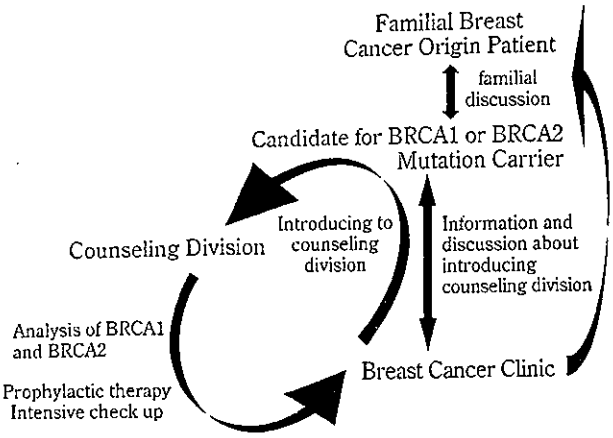

Fig 1. Counseling system for BRCA1 or BRCA2 mutation careers.

and inter-division cooperative system to smoothly introduce breast cancer clinic patients to the counseling division. This is an interim report for the grant of Japanese Health, Labour and Welfare.

Patients and Methods

Twenty familial breast cancer patients were registered between March 2002 and March 2003. Blood sampling and BRCA1/BRCA2 mutation analysis were done with informed consent according to the guidelines of the Japanese Ministry of Health, Labour and Welfare. Whole sequences of BRCA1 and BRCA2 were analyzed by the direct sequence method at Myriad Genetic Laboratories, Inc.

Clinical System

If obvious pathological mutations were detected, the patients were informed about the genetic counseling division at breast cancer clinics. Their first degree relatives were also informed about their genetic background and invited to visit genetic counseling services. When the first degree relatives visited genetic counseling divisions, they were informed about familial breast cancer and given the option to undergo genetic examination at breast cancer clinics.

Results

Deleterious Mutations of BRCA1 and BRCA2

Four cases of apparent deleterious BRCA1 and BRCA2 mutations were detected from among the total 20. These four carriers of deleterious mutations are now undergoing counseling to discuss along with their first degree relatives options for BRCA1 and BRCA2 examination and prophylactic treatments at the counseling divisions (Fig 1). An uncertain mutation thought to be a normal variant was also found in four cases.

Discussion

Treatment for BRCA1 and BRCA2 mutation carriers is a controversial issue. Prophylactic interventions for pathological BRCA1 and BRCA2 mutation carriers include prophylactic surgery, medication, irradiation and intensive follow-up. Prophylactic surgery consists of mastectomy and oophorectomy. Prophylactic mastectomy shows a prevention rate of 90%[5], while prophylactic oophorectomy shows a 50% prevention rate[6,7]. Although prophylactic mastectomy shows a high rate of prevention as high as 90%, prophylactic surgical treatments are invasive interventions not only physically but also mentally for healthy people. Contant *et al.* proposed prophylactic mastectomy accompanied by immediate breast reconstruction (IBR). IBR can be one of solution for those hesitant to undergo prophylactic mastectomy[8]. Medication and intensive check-up as conservative treatments show relatively low prevention rate. Treatment with tamoxifen has an approximately 50% prevention rate[9]. Intensive check-ups may also allow BRCA mutation carriers to undergo breast-conserving surgery and have a better prognosis as they can be diagnosed at an earlier clinical stage.

A nonsense mutation of BRCA1 that was reported to be a Japanese founder mutation was detected as reported by Sekine *et al.*[10]. In addition, a variation in BRCA2 detected in four cases may be related to breast cancer onset.

There have been very few reports of prophylactic therapy in Japan. Proper prophylactic therapy has to be established for Japanese BRCA mutation carriers and prognosis and outcome of prophylactic therapy needs to be clarified. Our study group consists of two national university hospitals and a general hospital that cover each area. We have also been studying BRCA1 and BRCA2 mutations before this study. Together with the data from the former study, we are now beginning to analyze potential for BRCA1 and BRCA2 mutation carriers. Each institute in our study group has

a breast cancer clinic and a counseling division. If BRCA1 and BRCA2 mutation carriers decide to visit our breast cancer clinics and receive counseling about their genetic status, they can accept our introduction to clinicians in the counseling division (Fig 1). When the candidates decide to undergo genetic examination, we analyze their BRCA1 and BRCA2 sequences. The results of the examinations are reported to us. The results are given to the breast cancer clinics in each institute and the patient is informed. If they have a deleterious mutation, they visit the genetic counseling division again and receive information about familial breast cancer and prophylactic treatments.

Prophylactic mastectomy, oophorectomy, irradiation, medication and intensive check-ups have been proposed for the treatment of deleterious BRCA1 and BRCA2 mutation carriers. Prophylactic mastectomy may seem to be a radical therapy even if they show the highest prevention rate. Since the general prognosis of breast cancer occurring in BRCA mutation carriers is not different from that of sporadic breast cancer patients. Diagnosis before the onset of breast cancer should positively influence outcomes. In addition, Hoogerbrugge *et al.* reported that the breast tissue of BRCA mutation carriers has atypical hyperplastic change. Careful follow-up for BRCA1 and BRCA2 carriers is required unless they undergo prophylactic surgery[10].

Long term observation should be continued to clarify the actual onset rate and clinical outcome of breast cancer among Japanese BRCA1 and BRCA2 mutation carriers. Eventually this counseling system may be available for all Japanese families with deleterious BRCA1 and BRCA2 mutation carriers.

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SHORT COMMUNICATION

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Identification and evaluation of 55 genetic variations in the *BRCA1* and the *BRCA2* genes of patients from 50 Japanese breast cancer families

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Abstract We sequenced approximately 23 kb genomic regions containing all the coding exons and their franking introns of two breast cancer susceptibility genes, *BRCA1* and *BRCA2*, of 55 individuals from 50 unrelated Japanese breast cancer families. We identified 55 single-nucleotide polymorphisms (SNPs) (21 in *BRCA1* and 34 in *BRCA2*) containing nine pathogenic protein-truncating mutations (four in *BRCA1*and five in *BRCA2* from ten patients). Among the remaining 46 SNPs, allele frequencies of 40 were examined in both the breast cancer patients and 28 healthy volunteers with no breast cancer family history by PCR-RFLP or by direct DNA sequencing. Twenty-eight SNPs were common and were also found in the healthy volunteers and/or a SNP database. The remaining 18 were rare (allele frequency <0.05) and were not found in the healthy volunteers and/or the database. The pathogenic significance of these coding SNPs (cSNPs) remains to be clarified. The SNP information from this study will be useful in the future genetic testing of both *BRCA1* and *BRCA2* genes in the Japanese population.

Keywords Single-nucleotide polymorphism (SNP) · Breast cancer susceptibility gene · *BRCA1* · *BRCA2* · Japanese population · Direct DNA sequencing

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Introduction

Mutations in the *BRCA1* and the *BRCA2* genes have been linked with the susceptibility to breast and ovarian cancer (Miki et al. 1994; Wooster et al. 1995; Tavtigian et al. 1996). Mutation carriers of these genes are at high risk of breast and ovarian cancer (Narod et al. 1995; Ford et al. 1998; Thorlacius et al. 1998; Neuhausen 1999; Rebbeck 1999; Struewing et al. 1997; Anglian Breast Cancer Study Group 2000). The two genes have large coding sequences consisting of 48 exons in total, and a large number of mutations and SNPs are reported in the Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/) and the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html). The majority of mutations described are protein-truncating mutations containing frame-shift mutations and nonsense mutations. In the BIC database, 55.9% of genetic variants are reported as pathogenic mutations containing mainly protein-truncating mutations and a small fraction of pathogenic missense mutations, and 39.3% of variants are categorized as "unclassified variants." These variants contain coding SNPs (cSNPs) that result in amino-acid substitutions or SNPs located at exon–intron boundaries. We have also reported five protein-truncating mutations and 12 "unclassified variants" that have been found only once in 24 breast cancer families (Sakayori et al. 2003). To elucidate whether detected SNPs are pathogenic mutations or not, it is important to accumulate SNP information (both the type and allele frequency) in patients and the general population.

Table 1 Sequence variations detected in the *BRCA1* and the *BRCA2* genes

SNP ID[a]	Location	Variation[b]	Flanking sequence (5′ to 3′)	dbSNP ID[c]	BIC[d]	Volunteers[e]
*BRCA1*gene						
BRCA1-1	Intron 1	IVS1-115C > T	tggtttgtat C/T attctaaaac	rs3765640		+
BRCA1-2	Exon 3	K38 (silent) (114G > A)	tctccacaaa G/A tgtgaccaca	rs8176099	+	+
BRCA1-3	Intron 8	IVS8-58delT	tacatttttt T/– aaccctttta		+	+
BRCA1-4#	Exon 11	G275D (824G > A)	gagccatgtg G/A cacaaatact			
BRCA1-5	Exon 11	S694 (silent) (2082C > T)	gacatgacag C/T gatactttcc	rs1799949	+	+
BRCA1-6	Exon 11	L771 (silent) (2311T > C)	cagtatttca T/C tggtacctgg	rs16940	+	+
BRCA1-7#	Exon 11	2389–2390delGA*	ggcaaaaaca GA/– accaaataaa			
BRCA1-8	Exon 11	P871L (2612C > T)	tcatttgctc C/T gttttcaaat	rs799917	+	+
BRCA1-9	Exon 11	E1038G (3113A > G)	gtttttaaag A/G agccagctca	rs16941	+	+
BRCA1-10	Exon 11	K1183R (3548A > G)	agcgtccaga A/G aggagagctt	rs16942	+	+
BRCA1-11#	Exon 12	C1372X (4116T > A)*	catctgggtg T/A gagagtgaaa			
BRCA1-12	Exon 13	S1436 (silent) (4308T > C)	taagtgactc T/C tctgcccttg	rs1060915	+	+
BRCA1-13#	Intron 14	IVS14 + 14A > G	agaaacatca A/G tgtaaagatg		+	
BRCA1-14	Exon 16	S1613G (4837A > G)	atctgcccag A/G gtccagctgc	rs1799966	+	+
BRCA1-15#	Exon 16	M1628T (4883T > C)	tataatgcaa T/C ggaagaaagt	rs4986854	+	
BRCA1-16	Intron 18	IVS18 + 66G > A	tacacctaac G/A tttaacacct	rs3092994	+	+
BRCA1-17#	Intron 22	IVS22 + 33A > T	gagagggagg A/T cacaatattc			
BRCA1-18#	Intron 23	IVS23 + 8G > T	atggtaaggt G/T cctgcatgta		+	
BRCA1-19	Exon 5	L63X (188T > A)*	cagtgtcctt T/A atgtaagaat		+	
BRCA1-20	Exon 3	H41R (122A > G)	aagtgtgacc A/G catattttgcaaa			
BRCA1-21	Exon 8	470–471delCT*	tccaactct CT/– aaccttggaa		+	
*BRCA2*gene						
BRCA2-1	Exon 2	5′UTR-26G > A	tatttaccaa G/A cattggagga	rs1799943	+	+
BRCA2-2#	Intron 2	IVS2-16T > A	taaggtggga T/A ttttttttta			
BRCA2-3#	Intron 2	IVS2-9T > G	ggatttttttt T/G ttaaatagat			
BRCA2-4	Intron 4	IVS4 + 67A > C	tgttctataa A/C gatgaatctg			+
BRCA2-5	Intron 4	IVS4-89T > C	acaatttata T/C gaatgagaat		+	+
BRCA2-6	Intron 7	IVS7 + 183T > A	caaatacatt T/A agtggtagtc			+
BRCA2-7	Intron 8	IVS8 + 56C > T	tttggaatgc C/T ttgttaaatt		+	+
BRCA2-8#	Exon 10	F266 (silent) (798T > C)	gtcatggatt T/C ggaaaaacat			
BRCA2-9	Exon 10	N289H (865A > C)	gtcaatgcca A/C atgtcctaga	rs766173	+	+
BRCA2-10	Exon 10	H372 N (1114C > A)	aaatgtagca C/A atcagaagcc	rs144848	+	+
BRCA2-11	Exon 10	S455 (silent) (1365A > G)	taccaaaatc A/G gagaagccat	rs1801439	+	+
BRCA2-12#	Exon 10	T582P (1744A > C)	tttaatatcc A/C ctttgaaaaa		+	
BRCA2-13	Exon 11	H743 (silent) (2229T > C)	cagtacaaca T/C tcaaaagtgg		+	+
BRCA2-14	Exon 11	M784V (2350A > G)	aaacctagtc A/G tgatttctag		+	+
BRCA2-15	Exon 11	N991D (2971A > G)	tgattacatg A/G acaaatgggc	rs1799944	+	+
BRCA2-16	Exon 11	K1132 (silent) (3396A > G)	agtttagaaa A/G ccaagctaca	rs1801406	+	+
BRCA2-17#	Exon 11	S1140 (silent) (3420T > C)	tgcagaagag T/C acatttgaag			
BRCA2-18#	Exon 11	3830delA*	aagatagaaa A/– tcataatgat			
BRCA2-19#	Exon 11	L1522F (4566G > T)	ctactctgtt G/T ggttttcata			
BRCA2-20#	Exon 11	G2044V (6131G > T)	tcccaaaaag G/T cttttcatat		+	+
BRCA2-21	Exon 14	S2414 (silent) (7242A > G)	aaactaaatc A/G cattttcaca	Rs1799955	+	+
BRCA2-22#	Exon 11	6491–6495delAGTTG*	gacaaacaac AGTTG/– gtattaggaa			
BRCA2-23	Intron 16	IVS16 + 47A > G	gtattcccte A/G tccctctttc			
BRCA2-24	Intron 16	IVS16-14T > C	aatattctac T/C tttatttgtt		+	+
BRCA2-25	Exon 11	V2109I (6325G > A)	acttcctcgt G/A ttgataagag		+	
BRCA2-26#	Exon 20	S2835X (8504C > A)*	gagaagacat C/A atctggatta			
BRCA2-27	Intron22	IVS22-147A > G	cagataaagt A/G taaagttagt			
BRCA2-28	Exon 25	R3128X (9382C > T)*	AacctccagtggC/Tgaccagaatcc		+	
BRCA2-29	Exon 10	1278delA*	ttcagaaaa A/– gacctattag			
BRCA2-30	Exon 10	K322Q (964A > C)	aaatctacaa A/C aagtaagaactagc		+	
BRCA2-31	Exon 10	E425 (silent) (1275A > G)	aaatatttcaga A/G aaagacct			
BRCA2-32	Exon 11	V1269 (silent) (3807T > C)	catgattctgt T/C gtttcaatgt		+	
BRCA2-33	Exon 11	E1455 (silent) (4365A > G)	cagaaaccaga A/G gaattgcata			
BRCA2-34	Exon 18	K2729N (8187G > T)	tatgctgttaa G/T gcccagttagatcct		+	

[a]The SNP ID that have been found in this study. #Published previously (Sakayori et al. 2003)

[b]The nucleotide number in the coding region indicates the position downstream of the first nucleotide of ATG (initiation codon) in the *BRCA1* gene or the *BRCA2* gene. *: Nonsense or frame-shift mutation

[c]National Center for Biotechnology Information (NCBI) dbSNP database

[d]Breast Cancer Information Core (BIC) database. +: listed in BIC

[e]28 Japanese healthy volunteers. +: found at least one individual

Table 2 Allele frequencies of SNPs in the *BRCA1* and the *BRCA2* genes

SNP ID[a]	Allele frequency		
	Breast cancer patients[b]	Volunteers[c]	dbSNP[d]
*BRCA1*gene			
BRCA1-1	*C*=0.43, *T*=0.57	*C*=0.52, *T*=0.48	*C*=0.343, *T*=0.657
BRCA1-2	*G*=0.93, *A*=0.07	*G*=0.98, *A*=0.02	*G*=0.994, *A*=0.006
BRCA1-3	*T7*=0.65, *T6*=0.35*	*T7*=0.50, *T6*=0.50	
BRCA1-4	*G*=0.98, *A*=0.02	*G*=1.00, *A*=0.00	
BRCA1-5	*C*=0.62, *T*=0.38	*C*=0.56, *T*=0.44	*C*=0.657, *T*=0.343
BRCA1-6	*T*=0.62, *C*=0.38	*T*=0.54, *C*=0.46	*T*=0.678, *C*=0.322
BRCA1-8	*C*=0.62, *T*=0.38	*C*=0.54, *T*=0.46	*C*=0.619, *T*=0.381
BRCA1-9	*A*=0.62, *G*=0.38	*A*=0.54, *G*=0.46	*A*=0.725, *G*=0.275
BRCA1-10	*A*=0.62, *G*=0.38	*A*=0.54, *G*=0.46	*A*=0.703, *G*=0.297
BRCA1-12	*T*=0.62, *C*=0.38	*T*=0.54, *C*=0.46	*T*=0.747, *C*=0.253
BRCA1-13	*A*=0.98, *G*=0.02*	*A*=1.00, *G*=0.00	
BRCA1-14	*A*=0.62, *G*=0.38	*A*=0.54, *G*=0.46	*A*=0.696, *G*=0.304
BRCA1-15	*T*=0.98, *C*=0.02	*T*=1.00, *C*=0.00	*T*=0.995, *C*=0.005
BRCA1-16	*G*=0.63, *A*=0.37	*G*=0.54, *A*=0.46	*G*=0.693, *A*=0.307
BRCA1-17	*A*=0.98, *G*=0.02*	*A*=1.00, *G*=0.00	
BRCA1-18	*G*=0.99, *T*=0.01	*G*=1.00, *T*=0.00	
BRCA1-20	*A*=0.99, *G*=0.01	ND	
*BRCA2*gene			
BRCA2-1	*G*=0.55, *A*=0.45	*G*=0.49, *A*=0.51	*G*=0.762, *A*=0.238
BRCA2-2	*T*=0.97, *C*=0.03*	*T*=1.00, *C*=0.00	
BRCA2-3	*T*=0.98, *G*=0.02*	*T*=1.00, *C*=0.00	
BRCA2-4	*A*=0.84, *C*=0.16*	*A*=0.84, *C*=0.16	
BRCA2-5	*T*=0.84, *C*=0.16*	*T*=0.84, *C*=0.16	
BRCA2-6	*T*=0.32, *A*=0.68*	*T*=0.58, *A*=0.42	
BRCA2-7	*C*=0.96, *T*=0.04*	*C*=0.96, *T*=0.04	
BRCA2-8	*T*=0.98, *C*=0.02	*T*=1.00, *C*=0.00	
BRCA2-9	*A*=0.86, *C*=0.14	*A*=0.84, *C*=0.16	*A*=0.838, *C*=0.024, *G*=0.009, *T*=0.129
BRCA2-10	*A*=0.80, *C*=0.20	*A*=0.82, *C*=0.18	*A*=0.607, *C*=0.281, *G*=0.026, *T*=0.085
BRCA2-11	*A*=0.86, *G*=0.14	*A*=0.84, *G*=0.16	*A*=0.875, *G*=0.125
BRCA2-12	*A*=0.99, *C*=0.01	*A*=1.00, *C*=0.00	
BRCA2-13	*T*=0.86, *C*=0.14	*T*=0.84, *C*=0.16	
BRCA2-14	*A*=0.93, *G*=0.07	*A*=0.95, *G*=0.05	
BRCA2-15	*A*=0.85, *G*=0.15	*A*=0.84, *G*=0.16	*A*=0.970, *G*=0.030
BRCA2-16	*A*=0.47, *G*=0.53	*A*=0.50, *G*=0.50	*A*=0.705, *G*=0.295
BRCA2-17	*T*=0.99, *C*=0.01	*T*=1.00, *C*=0.00	
BRCA2-19	*G*=0.98, *T*=0.02	*G*=1.00, *T*=0.00	
BRCA2-20	*G*=0.97, *T*=0.03	*G*=0.98, *T*=0.02	
BRCA2-21	*A*=0.58, *G*=0.42	*A*=0.50, *G*=0.50	*A*=0.758, *G*=0.242
BRCA2-23	*A*=0.96, *G*=0.04*	*A*=1.00, *G*=0.00	
BRCA2-24	*T*=0.36, *C*=0.64	*T*=0.27, *C*=0.73	
BRCA2-25#	*G*=0.99, *A*=0.01	*G*=1.00, *A*=0.00	
BRCA2-27#	*A*=0.98, *G*=0.02*	*A*=1.00, *G*=0.00	
BRCA2-30	*A*=0.97, *C*=0.03	ND	
BRCA2-31	*A*=0.97, *C*=0.03	ND	
BRCA2-32	*T*=0.86, *C*=0.14	ND	
BRCA2-33	*A*=0.98, *G*=0.02*	ND	
BRCA2-34	*G*=0.98, *T*=0.02	ND	

[a]Identical to the SNP ID listed in Table 1, #: found in a relative but not in the proband
[b]110 alleles from 55 patients, *: data derived from 68 alleles (34 patients)
[c]56 alleles from 28 healthy volunteers, ND: not determined
[d]National Center for Biotechnology Information (NCBI) dbSNP database

In this study, we extended our study on the *BRCA1* and *BRCA2* sequencing project to 55 breast cancer patients from 50 Japanese breast cancer families. We evaluated the detected SNPs by comparing the allele frequency of the SNPs in healthy volunteers.

Subjects and methods

Fifty-five enrolled patients (including 50 probands and five relatives) with a history of breast cancer from 50 unrelated high-risk breast cancer families were selected according to the criteria defined by the Tohoku Familial Cancer Society (Sakayori et al. 2003). An additional 28 Japanese volunteers with no breast cancer family history were also enrolled to analyze the specific *BRCA1* and *BRCA2* variations detected in the breast cancer patients. We obtained informed consent from all patients and volunteers, and the independent studies were approved by the Familial Cancer Society and the Ethical Committee of Tohoku University Graduate School of Medicine. To identify the sequence variations in the *BRCA1* and *BRCA2* genes of the familial breast cancer patients, we sequenced approximately 23 kb genomic regions of the *BRCA1* (8.4 kb) and the *BRCA2* (14.6 kb) containing all coding exons and their franking introns using a method described previously (Sakayori et al. 2003). The identified SNPs found in the familial breast cancer

patients were also examined in the genomic DNA from the healthy volunteers by PCR-RFLP analysis using PCR primers, cycle conditions and restriction enzymes for PCR-RFLP analysis (http://www.idac.tohoku.ac.jp/dep/co/data/saka/brca02.htm) or by DNA sequence analysis. We used Genbank (U14680 and U61268 for *BRCA1*, U43746 and X95152-77 for *BRCA2*) and the BIC database as the reference sequences of *BRCA1* and *BRCA2* genes.

Results and discussion

By DNA sequencing analysis of the *BRCA1* and *BRCA2*genes for 55 patients from 50 unrelated breast cancer families; we detected 55 SNPs (21 SNPs in *BRCA1* and 34 SNPs in *BRCA2*). Among these SNPs, we found nine protein-truncating mutations (four in *BRCA1*and five in *BRCA2*) in ten patients containing six novel mutations that were not found in the BIC database (http://research.nhgri.nih.gov/bic/) (Table 1). The existence of mutations were also confirmed by the stop codon assay in yeast (Ishioka et al. 1997; Sakayori et al. 2003). The percentage of protein-truncating mutations in the examined families was 20% (ten of 50), comparable with several reports describing the frequency of protein-truncating mutations of the two genes in Japanese breast cancer families (Inoue et al. 1995; Takano et al. 1997; Inoue et al. 1997; Ikeda et al. 2001). Although the frequency was also similar to results from Western populations, the number of patients studied in our and other Japanese populations was smaller than that in Western countries. Therefore, it is necessary to study a larger number of patients to clarify the mutation frequency in Japanese familial breast cancer.

Among the remaining 46 SNPs (17 SNPs in *BRCA1* and 29 SNPs in *BRCA2*), 18 and 31 have been reported in the dbSNP database and in the BIC database, respectively, (Table 1). To evaluate whether the SNPs are also found in Japanese healthy volunteers, all but six SNPs were further examined by PCR-RFLP analysis or by DNA sequence analysis. Twenty-six SNPs were found in the volunteers at least once with different allele frequencies (Table 2). In these 40 SNPs, we predicted that 27 SNPs were common polymorphisms and probably played no direct role in the tumorigenesis of breast cancer. The remaining 13 SNPs were quite low in allele frequency (<0.05) and were not found in the healthy volunteers or the dbSNP database. In addition, there were six SNPs that failed to examine in the volunteer group. In these six SNPs, one was predicted to be common and five to be rare from the allele frequencies in the patients. Overall, we predicted the 18 rare SNPs are candidates of pathogenic mutations and that the remaining 28 were common polymorphisms and probably have no direct roll in tumorigenesis of breast cancer. In the 18 rare SNPs, five (BRCA1-13, BRCA1-17, BRCA1-18, BRCA2-2, BRCA2-3) were located at the exon–intron boundaries and two (BRCA2-23, BRCA2-27) were located in introns far from exon–intron boundaries. Although SNPs at the exon–intron boundaries may affect normal RNA splicing, our RT-PCR analysis showed negative data for splicing abnormalities. In the remaining 11 rare cSNPs, seven (BRCA1-4, BRCA1-20, BRCA2-12, BRCA2-19, BRCA2-25, BRCA2-30 and BRCA2-34) were nonsynonymous substitution resulting in the amino-acid substitutions, and four (BRCA2-8, BRCA2-17, BRCA2-31 and BRCA2-33) were synonymous (silent) changes. These SNPs may directly affect the functions of BRCA1 and BRCA2 proteins or affect normal splicing by acting as possible cryptic splicing sites. Unfortunately, we have failed to clarify this issue mainly because there are no reliable functional assays of either BRCA1 or BRCA2 protein for many nonsynonymous changes.

To confirm the pathogenic effect of rare SNPs, both the development of functional assays for these gene products and more intensive SNP analysis including an investigation into whether these SNPs cosegregate with breast cancer onset in families are necessary.

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Preparation of silica encapsulated CdSe quantum dots in aqueous solution with the improved optical properties

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Abstract

Silica encapsulated CdSe quantum dots (QDs) have been prepared by the use of 3-mercaptopropyl trimethoxysilane (MPS) in a weak alkaline solution in ambient atmosphere at room temperature. The average size of the multicore-shell structured CdSe/SiO_2 is 28.0 nm and that of the CdSe QDs is 3.4 nm from the observation of the transmission electron micrographs (TEMs). The enhanced photoluminescence (PL) intensity of the QDs has been observed by passivation of silica shell. The increased photochemical and -physical stability of the encapsulated CdSe/SiO_2 QDs has also been demonstrated.

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Keywords: Encapsulation; CdSe; Quantum dots; SiO_2; Passivation; Photoluminescence (PL); Stability

1. Introduction

In recent years, interests have been greatly increased in the scientific and technological aspects of semiconductor quantum dots (QDs). Due to small size, such zero-dimensional structures demonstrate unique chemical and physical properties which are different from those of bulk solids [1,2]. That the number of atoms on the surface becomes comparable or even higher than inside leads to the changes in the electronic structure, state density and optical properties as consequence. However, semiconductor becomes chemically active especially while the size decreases to the quantum size range (several nanometers). Therefore, the specific physical and chemical properties are eliminated and the application is greatly limited. A way to remove the influence is a process named as passivation, which consists of

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surface atoms bonding to a different material with inert chemical properties. W.L. Wilson and P. Mulvaney et al. have concluded that silica is a quite suitable material to be a coating or encapsulating inert substance [3,4]. It has been successfully applied in passivation of Au [5], Ag [6], and CdS particles [7].

Owing to the potential and practical possibility of cadmium selenide (CdSe) with a suitable band gap (1.76 eV), for applications in industrial and medical fields, currently, the preparation, absorption, and exciton luminescence processes in CdSe nanoparticles have been extensively studied [8–11]. In particular, CdSe semiconductor quantum dots have greatly interested biologists and pharmaceutical scientists [12,13]. By now, quantum dot quantum wall (QDQW) nanocrystals heterostructurally coated with materials with higher band gap of CdS [14,15], ZnS [16], and even multilayers of HgSe/CdSe have been successfully fabricated and their physical and chemical properties have been apparently improved [17]. Moreover, CdSe nanoparticles have been encapsulated with silica as a film and their electronic properties can be improved obviously [18–21]. However, there is no any report on the preparation of core-shell structured CdSe/SiO_2 quantum dots (QDs) except the work reported by Rogach et al. [22] in which the PL intensity of CdSe Qds dramatically decreased after the passivation of SiO_2 in relatively complicated conditions.

In this work a citrate-stabilized aqueous suspension of quantum sized CdSe nanoparticles was prepared at room temperature based on a simple chemical precipitation in quite mild conditions. Also, these QDs were successfully encapsulated with silica by a slow deposition of silica solute from a silicate solution while being transferred into ethanol. Moreover, the valid passivation was confirmed with the absorbance and the photoluminescence of the CdSe particles was promoted after being coated with SiO_2. Meantime, the possible mechanisms for the passivation and the promotion were simply discussed.

2. Experimental

2.1. Materials

Special grade reagents (Wako Pure Chemical Ind.) of sodium silicate solution ($Na_2O(SiO_2)_{2.0-2.31}$, 52.0–57.0%), 3-mercaptopropyl trimethoxysilane (MPS), cadmium sulfate ($CdSO_4 \cdot 8/3H_2O$, >99%), selenium powder (Se, >99%), sodium nitrilotriacetate (Na_3-NTA:$N(CH_2COONa)_3$), sodium sulfite, ethanol, and sodium citrate were used as received. Water was double-distilled and deionized to have an electrical resistance higher than 18 MΩ cm^{-1}.

2.2. Preparation of the stable suspension of CdSe quantum dots

Firstly, 1.0 ml of 0.15 M $CdSO_4$ was mixed with Na_3NTA at a mole ratio of $[Cd^{2+}]:[NTA^{3-}] = 5:4$ to form a Cd–NTA complex. Then, 1 ml of 1.6 wt.% Na-citrate was added to the solution of the Cd–NTA complex. Finally, 0.75 ml of aqueous solution of 0.2 M sodium selenosulfate (Na_2SSeO_3) with excess Na_2SO_3 that was prepared by stirring 0.79 g of Se powder in 50 ml of 0.5 M Na_2SO_3 at 70 °C for 24 h were added to the above solution to form a solution of 0.006 M cadmium ion and then aged it at room temperature for 5 days. The reaction equation is shown as the following:

$$Cd^2 + 2OH^- + SeSO_3^{2-} \rightarrow CdSe + SO_4^{2-} + H_2O$$

2.3. Preparation of silica encapsulated CdSe nanoparticles

The established standard conditions for preparation of silica encapsulated CdSe QDs were shown as following.

Two milliliter of the formed CdSe stable suspension was diluted with 17 ml water and treated ultrasonically for 30 min. Then, 0.2 ml of freshly prepared 0.02 wt.% MPS and 0.8 ml of 0.54% sodium silicate solution at pH 10.5 were added to the above suspension and then aged at room temperature for 5 days with vigorous magnetic stirring. Hence, the silica slowly polymerizes onto the MPS-modified CdSe QDs. Finally, the resulting dispersion was transferred to ethanol at a volume ratio of 1:5, and then the excessive dissolved silicate precipitates out mainly on the existing cores, which leads to an increase in the thickness of the silica shell.

2.4. Electron microscopy

The products were observed with a JEM-2000EX II transmission electron microscope (TEM) with an

acceleration voltage of 200 kV and an EDMAX scanning electron microscope (SEM). Also, atomical composition of the products was analyzed with energy dispersive X-ray (EDX) measurements equipped with the SEM.

2.5. Optical measurements

UV spectrometry of CdSe nanoparticles in suspension before and after coating silica was conducted with an UV–vis spectrophotometer, Hitachi U-2000, where the light path length was 1 cm. In addition, the photoluminescence of these particles was investigated with a JASCO FP-750 Spectrofluorometer.

3. Results and discussion

3.1. Formation of CdSe quantum dots without and with silica encapsulating

After mixing Cd–NTA complex with Na_2SeSO_3 solution and aging it at room temperature for 5 days, a very stable deep red suspension was formed. This suspension was stable kinetically at least for 1 year. Since citrate ions act as protect agents for a variety of inorganic colloids including CdS particles [7], probably the citrate ions also seemed to be of the same function for the current system. This was confirmed simply with the fact that the suspension was fairly unstable in the absence of Na-citrate.

Fig. 1a shows transmission electron micrograph of the solid product from the stable suspension. Obviously uniform aggregates of CdSe particles with an average size of 22.5 nm were observed together with several tens of primary particles. The wet chemical analysis measurement of the solid sample showed 1:1 stoichiometry of Cd:Se. From powder XRD measurements on dried nanoparticles, the nanoparticles were in CdSe cubic structure which is typical for low temperature prepared CdSe particles.

Fig. 1b shows transmission electron micrograph of the silica encapsulated CdSe QDs. It obviously revealed that plural QDs were encapsulated completely with silica matrix. The average sizes of the microcapsules and the QDs were 28.0 and 3.4 nm, respectively. It should be noted that no aggregates

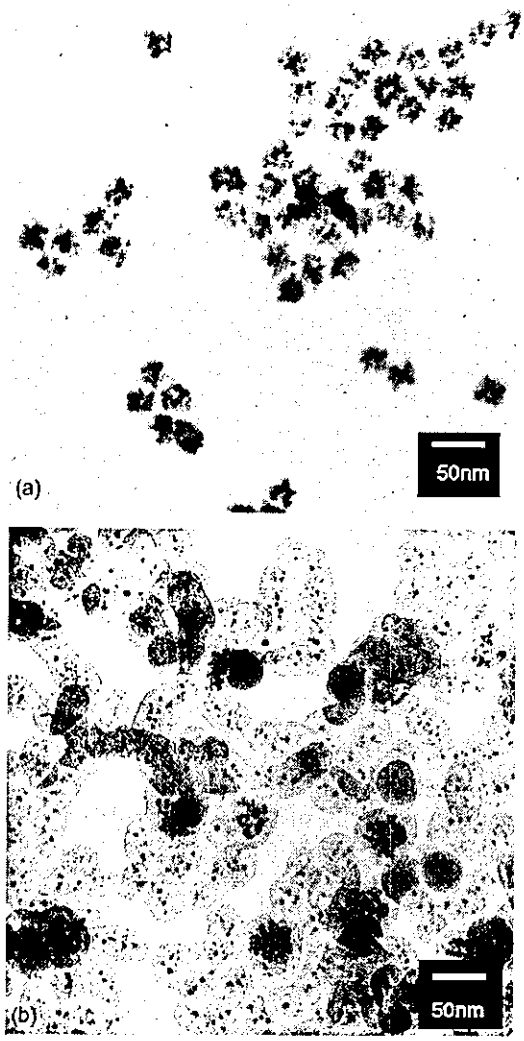

Fig. 1. Transmission electron micrographs of CdSe nanoparticles before (a) and after silica encapsulating (b).

were observed in the encapsulated particles. Thus, improvement of the particle dispersity was achieved by encapsulation.

It is hardly possible for silica to deposit directly on the surface of CdSe nanoparticles because of low affinity between them. MPS is a bifunctional coupling agent to bind with Cd atom on the surface in its mercapto group as well as a silane coupling agent. It has been successfully applied to the formation of silica coated CdS particles [7]. The adhesion of silicate

moieties to the particle surface can only be made effective when a silane coupling agent is present in the solution. Such a coupling agent acts as a surface primer for making the colloid surface vitreophilic and facilitating silicate deposition [11]. In the case of CdSe, MPS was chosen since it contains a mercapto group, which can directly bind to surface Cd^{2+} sites, leaving the silane groups pointing toward solution phase, from where the silicate ions approach the particle surface. These silicate ions build up a first silica shell which permits the transfer into ethanol without particle coagulation and Ostwald Ripening [4].

3.2. Stabilization against photodegradation

Since CdSe QDs are chemically active in comparison to the bulk solid, the Se atoms on the surface are easily oxidized to SeO_2 for direct exposure to air [16]. ($Se^{2-} + 2O_2 + 2H_2O \rightarrow SeO_2 + 4OH^-$). Such instability was also obtained for CdS QDs [7]. However, when these QDs are coated with some inert substance, they are expected to be photochemically stable. It was observed that a very thin silica shell did not prevent photodegradation, because O_2 molecules diffused through the thin shell easily. While such nanoparticles with a thin silica coating were transferred to methanol phase, almost all the silicate moieties were precipitated out due to the sudden decrease in solubility owing to the change in the polarity of the solvent. Hence, the QDs with thick silica encapsulating were formed.

Fig. 2 shows the time evolution of the absorbance of the citrate-stabilized and silica encapsulated CdSe QDs suspensions. After coating with thick silica, the absorbance kept constant, while the absorbance of the QDs without coating decreased markedly with time compared to the silica coated CdSe. These facts indicate the silica shell was rigid enough to prevent O_2 from reaching the surfaces of the QDs. Such a protection will be of great significance in the preparation of stable nanostructured materials with practical applications.

Additionally, the particle size of 3.4 nm estimated from the peak position as followed by the literature [8] was in a good agreement with TEM observation. Furthermore, the sharp absorption peak shown in Fig. 2b displays another advantage of the silica coating.

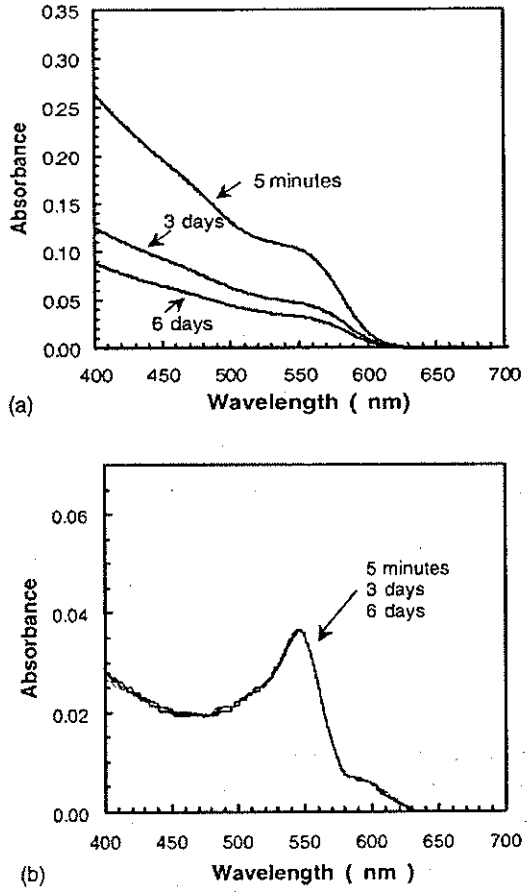

Fig. 2. UV–vis spectra at different times after preparation of citrate stabilized particles (a) and silica-coated particles (b).

3.3. Promotion of photoluminescence of CdSe QDs by silica encapsulating

It is not unusual that the PL intensity could be enhanced if the semiconductor nanoparticles are properly modified or coated with materials of a higher band gap. This has come true for Cd_3As_2 modified with $N(Et)_3$ [23], CdTe with thioglycolic acid [24] or dodecylamine [25], CdSe with CdS [14,15], with ZnS [16] or with ZnSe [26], etc.

In order to investigate the change in the PL intensity of the CdSe QDs after silica encapsulating, the PL intensity was measured for the initial CdSe QDs before coating, the medium CdSe QDs modified by MPS with a thin shell of silica (before being transferred to ethanol), and the final ones with silica

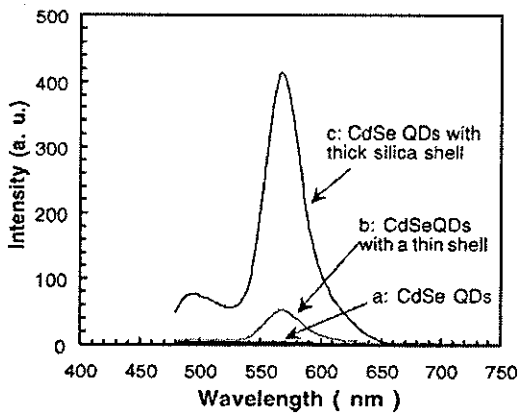

Fig. 3. Photoluminescence intensity (a.u.) of (a) CdSe QDs suspension; (b) CdSe QDs with a thin silica shell; (c) QDs with a thick silica shell. (λ_{exc} = 400 nm, $[Cd^{2+}]$ = 0.0006 M).

encapsulating (after being transferred to ethanol). Fig. 3 shows the improvement of the PL properties of the QDs in the coating process. Obviously, the PL intensity of the final QDs with a thick rigid shell of silica was much stronger than that of the initial QDs. The reason for the increased PL intensity is the formation of a thick silica shell, which may have a higher potential for the ground-state electron. This makes the shell of silica analogous to the CdS [14] and ZnS [16] around the CdSe core, the CdSe [17] shell around HgSe core, since CdSe semiconductor has a lower band gap than that of CdS, ZnS, and SiO_2, a higher band gap than that of HgSe. This conclusion is possibly further supported from the opposite side by the fact that formation of HgSe shell around CdSe core decreased the PL intensity of the CdSe nanoparticles [17], since the band gap of HgSe is lower than that of CdSe semiconductor.

Here, it has been found that the PL intensity increased after the addition of ethanol in the cases with different concentrations of MPS and/or different pH. That is to say, the PL intensity of the QDs with a thick shell of silica is always higher than that with a thin silica shell (before addition of ethanol). However, in comparison to the initial QDs, the PL intensity of MPS capped and then silica thinly coated CdSe QDs (before addition of ethanol) became weak at high pH or in the presence of high concentrated MPS. Virtually, the PL became undetectable at pH > 10 or with 10 times concentrated MPS of the current one. Rogach et al. [22] has obtained the similar results. Actually, it has been found that the existence of MPS negatively affected the PL intensity of the QDs. Then, the enhancement of silica shell on the PL intensity was so strong that the QDs exhibited a higher PL intensity than the initial QDs even after the formation of a thin shell of silica under the standard conditions, as revealed from Fig. 3. The details about the effect of MPS on PL will be shown elsewhere.

3.4. Stabilization against intense illumination

Fig. 4 shows the changes in PL intensity at different illumination times after exposure to Ar laser (532 nm, 700 mW). It was firstly found that the PL intensity of the bare CdSe QDs increased rapidly in the initial stage of the illumination. This seemed to result from the effect of photo annealing, and thereby, the defect sites on the surface of the QDs decreased. Then, under a long strong illuminating, the PL intensity greatly decreased. It can be considered that the QDs absorbed so many photons with a great energy and then decomposition into the elements Cd and Se took place under the extreme conditions. This has previously been pointed out by Spanhel et al. in the case of CdS [27].

However, in case of silica encapsulated CdSe QDs, the PL intensity was kept almost constant except the slightly increase in the quite early stage of the illumination. Probably, the silica shell was rigid

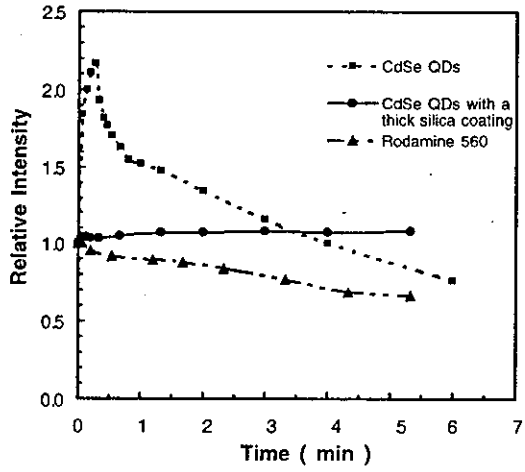

Fig. 4. Changes in photoluminescence intensity at different illumination (700 mW, Ar Laser) times. (a) CdSe QDs; (b) silica-encapsulated CdSe QDs; (c) Rhodamine 560.