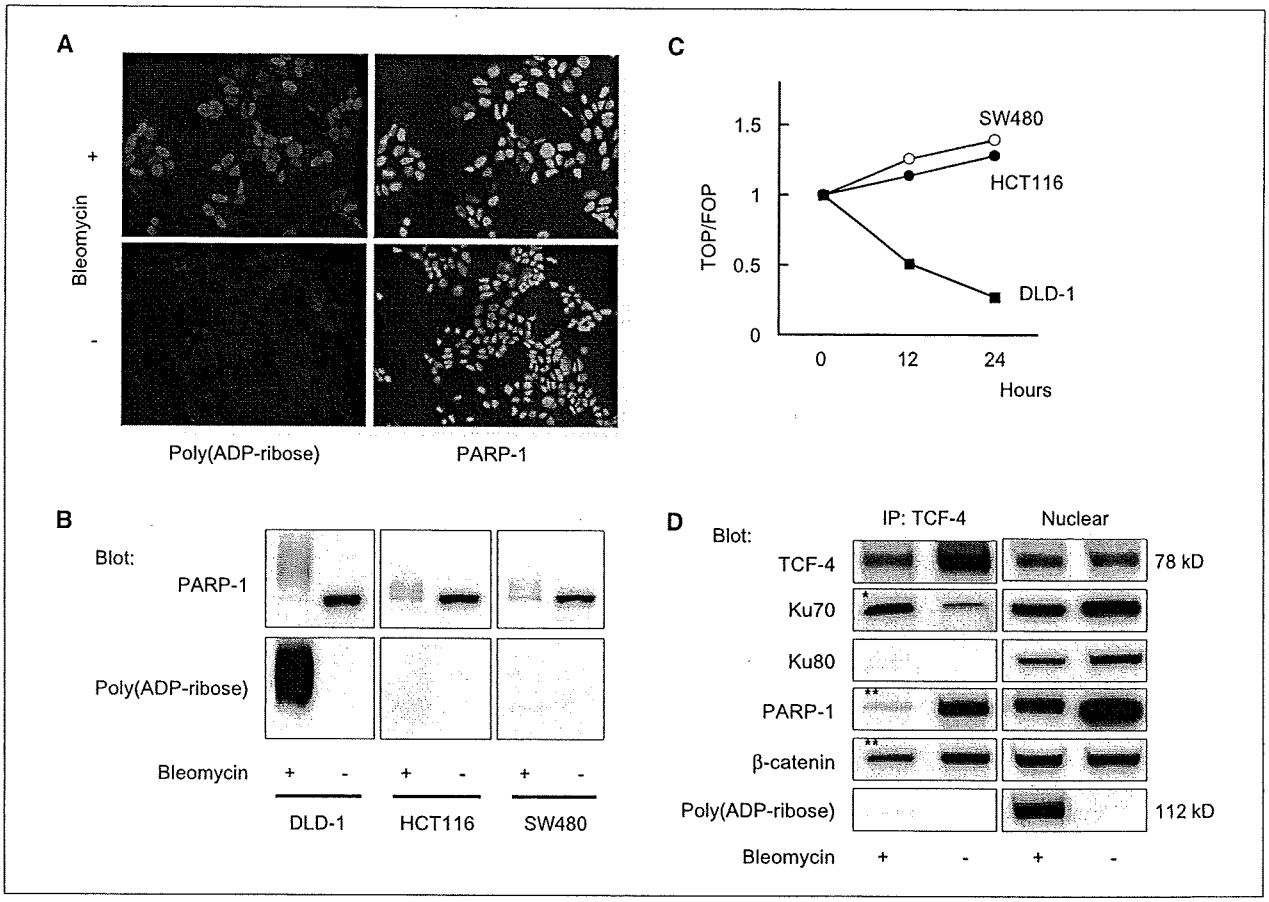

Figure 5. Possible linkage of DNA damage recognition and Wnt signaling. *A*, detection of polyADP-ribosylation in DLD-1 cells incubated with medium containing, or lacking, bleomycin for 6 h. Immunofluorescence staining was done using anti–poly(ADP-ribose) rabbit polyclonal antibody (*red*) and anti–PARP-1 mouse monoclonal antibody (*green*). *B*, detection of PARP-1 (*top*) and its polyADP-ribosylation (*bottom*) in DLD-1, HCT116, and SW480 cells untreated or treated with bleomycin for 6 h. Note that polyADP-ribosylated PARP-1 protein migrated more slowly and was less reactive with anti–PARP-1 antibody (*top*). *C*, TCF/LEF transcriptional activity of DLD-1, HCT116, and SW480 cells untreated (*0 h*) or treated only with bleomycin for 12 or 24 h. The ratio of TOP-FLASH to FOP-FLASH (*TOP/FOP*) was adjusted to that of the control (untreated) and expressed as a fold increase. *D*, DLD-1 cells were untreated (–) or treated with bleomycin for 3 h (+). Nuclear extracts (*Nuclear*) and immunoprecipitates with anti–TCF-4 antibody (*IP: TCF-4*) were blotted with anti–TCF-4, anti-Ku70, anti-Ku80, anti–PARP-1, anti–β-catenin, and anti–poly(ADP-ribose) antibodies.

interact with the Ku heterodimer (24), we used PARP-1–null MEF to investigate whether the interaction between TCF-4 and Ku proteins is mediated by PARP-1. Ku70 was coimmunoprecipitated with FLAG–TCF-4 even in the absence of PARP-1 (Fig. 4*A*), revealing that PARP-1 is not necessary for the interaction between TCF-4 and Ku. Restoration of PARP-1 did not affect the total amount of Ku70 in the nucleus (Fig. 4*B, Total*), but the amount of Ku70 coimmunoprecipitated with FLAG–TCF-4 was reduced (Fig. 4*B, IP: FLAG*), suggesting that PARP-1 competes with Ku70 for binding to TCF-4. Ku80 was barely coimmunoprecipitated with FLAG–TCF-4 in PARP-1–null MEF (data not shown).

We hypothesized that the transcriptional activity of TCF-4 is mutually regulated by the relative amount of Ku70, PARP-1, and β-catenin proteins binding to TCF-4. The enhancement of TOP-FLASH activity by transfection of Ku70 shRNA was further augmented by PARP-1 overexpression (Fig. 4*C*). Ku70 seems to suppress the transcriptional activity of TCF-4 by inhibiting the participation of β-catenin in the transcriptional complex containing TCF-4. Knockdown of Ku70 expression did not affect the total amount of β-catenin in the nucleus (Fig. 4*D, Total*), but the amount of β-catenin protein coimmunoprecipitated with FLAG-TCF4 was increased (Fig. 4*D, IP: FLAG*).

Possible linkage of DNA damage recognition and Wnt signaling. When DNA is damaged, PARP-1 polyADP-ribosylates several acceptor proteins. Treatment of colorectal cancer DLD-1 cells with bleomycin, a DNA-damaging alkylating agent, induced the accumulation of polyADP-ribosylated molecules in the nucleus (Fig. 5*A*). PARP-1 polyADP-ribosylates its own automodification domain in response to DNA damage. Bleomycin induced polyADP-ribosylation of PARP-1 protein most significantly in DLD-1 cells [Fig. 5*B*, poly(ADP-ribose)]. In parallel with the degree of PARP-1 polyADP-ribosylation, bleomycin inhibited the TCF/LEF activity of DLD-1 cells but not that of SW480 and HCT116 cells (Fig. 5*C*).

Because PARP-1 competes with Ku70 for binding to TCF-4 (Fig. 4*B*) and polyADP-ribosylation inhibits the interaction of PARP-1 with TCF-4 (11), we investigated how the polyADP-ribosylation of PARP-1 affects the composition of the TCF-4–containing transcriptional complex. Nuclear extracts from DLD-1 cells untreated or treated with bleomycin were immunoprecipitated

with anti–TCF-4 antibody. Although the total amount of Ku70 in the nucleus was not affected by bleomycin treatment (Fig. 5*D*, *Nuclear*), the amount of Ku70 coimmunoprecipitated with the anti–TCF-4 antibody was significantly increased (Fig. 5*D*, *). On the other hand, the amounts of PARP-1 and β-catenin coimmunoprecipitated with anti–TCF-4 antibody were decreased (Fig. 5*D*, **).

Immunohistochemical analysis revealed the frequent presence of nuclear poly(ADP-ribose) formation in the nuclei of colorectal adenoma cells (*T*) from FAP patients, whereas this was rarely observed in normal intestinal epithelial cells (Fig. 6, *N*).

Discussion

In this study, we showed that Ku70 and Ku80 are native components of the TCF-4 and β-catenin transcriptional complex (Fig. 1*D*). Ku70 physically interacts with a domain of TCF-4 containing the HMG box (Fig. 2*A*). Ku70 was an inhibitor of the TCF/LEF transcriptional activity (Fig. 3*B*; Supplementary Fig. S2). Down-regulation of Ku70 by RNA interference increased the expression of several known target genes of TCF/LEF (Fig. 3*C*), and the expression of Ku70 mRNA was frequently down-regulated in colorectal cancer tissues (Fig. 3*D*). Consistent with our findings, down-regulation of Ku70 protein expression has been reported previously in colorectal adenoma and carcinoma (25).

Ku has already been shown to work as a transcription factor that binds to promoter elements in a sequence-specific manner (26). Ku is capable of associating with the RNA polymerase II complex (27), but the entire Ku70/Ku80/DNA-PKcs complex is thought to be required for transcriptional regulation. DNA-PK phosphorylates RNA polymerase I (28) and II (29). Furthermore, DNA-PK interacts and/or phosphorylates other oncogenic transcription factors, including c-myc (30) and c-jun (31). However, these DNA-PK–induced phosphorylation mechanisms seem inadequate to explain the regulation of TCF-4 and β-catenin–mediated gene transactivation by Ku70, because the native TCF-4 and β-catenin complex contained mainly Ku70 (Fig. 1*D*). Ku80 is necessary for the recruitment and activation of DNA-PKcs (32), but knockdown of Ku80 by RNA interference did not affect the transcriptional activity of TCF-4 (data not shown). Ku70 is expressed in the nucleus, whereas Ku80 and DNA-PKcs are expressed either exclusively or predominantly in the cytoplasm of colorectal adenoma and carcinoma cells (25). A previous study has shown that the Ku heterodimer interacts with YY1 and suppresses α-myosin heavy-chain gene expression independently of DNA-PKcs (33).

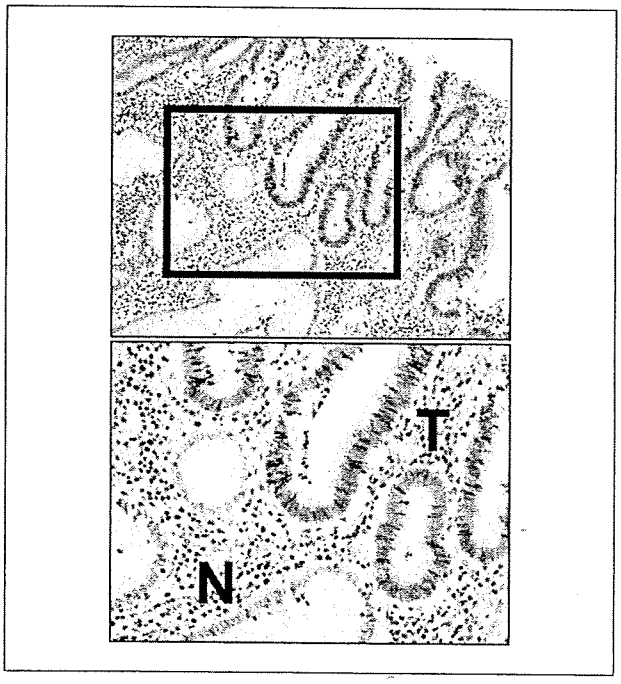

Figure 6. Nuclear poly(ADP-ribose) formation in colorectal adenoma. Immunohistochemistry of colorectal adenoma and normal glands of a FAP patient. Formation of poly(ADP-ribose) was detected with anti–poly(ADP-ribose) polyclonal antibody.

We previously reported that PARP-1 is a native component of the TCF-4 and β-catenin complex and that PARP-1 physically interacts with the region of TCF-4 distal to the HMG box (11). PARP-1 has already been reported to form a complex with the Ku heterodimer (34). However, the interaction of Ku70 with TCF-4 is not mediated by PARP-1, and, in fact, PARP-1 competes with Ku70 for binding to TCF-4. We observed that Ku70 was coimmunoprecipitated with TCF-4 even in PARP-1–null cells (Fig. 4*A*). Transfection of PARP-1 decreased the amount of Ku70 present in the immunoprecipitate with anti–TCF-4 antibody (Fig. 4*B*). The domain of TCF-4 binding to Ku70 (Fig. 2*A*) was physically close to the domain binding to PARP-1 (11). In contrast to Ku70, PARP-1 was overexpressed in colorectal cancer (11) and enhanced the transcriptional activity of TCF/LEF. Although transfection of Ku70 shRNA or cDNA alone had a small effect (~2- to 4-fold) on the TCF/LEF transcriptional activity (Fig. 3*B*; Supplementary Fig. S2), the combination of PARP-1 overexpression and Ku70 down-regulation markedly increased its activity (by >5-fold; Fig. 4*C*). The transcriptional activity of TCF-4 seems to be competitively regulated by the relative amount of Ku70 and PARP-1 proteins binding to TCF-4 (Fig. 5*D*).

PARP-1 is activated by DNA strand breakage and facilitates DNA repair by polyADP-ribosylating various acceptor molecules as well as its own automodification domain. Without DNA damage, the amount of polyADP-ribosylated proteins is kept at a low level (Fig. 5*A*). Poly(ADP-ribose) formation was barely observed in normal colon epithelial cells, whereas colorectal adenoma cells frequently accumulated nuclear poly(ADP-ribose) (Fig. 6). DNA damage is caused by endogenous free radicals produced as byproducts of oxidative metabolism. We previously reported that a key redox-status regulatory protein, manganese superoxide dismutase, was overexpressed even in small adenomas of FAP patients in parallel with the accumulation of β-catenin (22), indicating the occurrence of a certain type of DNA damage during the course of early colorectal carcinogenesis.

The protein composition of the TCF-4–containing nuclear complex is not fixed but regulated dynamically in response to DNA damage. Based on the present observations and previous studies, we propose a working hypothesis that the transcriptional activity of TCF-4 is regulated by polyADP-ribosylation of PARP-1 and subsequent recruitment of Ku70 to TCF-4 (Supplementary Fig. S3). In response to DNA damage, PARP-1 polyADP-ribosylates its own automodification domain (Fig. 5*B*). This modification inhibits the interaction between PARP-1 and TCF-4 (11), and the dissociation of PARP-1 from TCF-4 allows Ku70 to interact with TCF-4. The amount of β-catenin coimmunoprecipitated with TCF-4 was regulated by Ku70 (Fig. 4*D*). The recruitment of Ku70 into TCF-4 likely inhibits the interaction between TCF-4 and

β-catenin, the transcriptional activity of TCF-4, and the expression of target genes of TCF-4.

In summary, we have revealed that Ku70 and PARP-1 regulate TCF-4 and β-catenin–mediated gene transactivation in a competitive manner. Although our model may be oversimplified, identification of cross-talk between the Wnt signaling pathway and DNA damage recognition will provide a novel insight into the mechanism of colorectal carcinogenesis and suggest possible avenues of therapeutic intervention.

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Review Article

PolyADP-ribosylation and cancer

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The polyADP-ribosylation reaction results in a unique post-translational modification involved in various cellular processes and conditions, including DNA repair, transcriptional control, genomic stability, cell death and transformation. The existence of 17 members of the poly(ADP-ribose) polymerase (PARP) family has so far been documented, with overlapping functional consequences. PARP-1 is known to be involved in DNA base excision repair and this explains the susceptibility spectrum of PARP-1 knockout animals to genotoxic carcinogens. The fact that centrosome amplification is induced by a non-genotoxic inhibitor of PARP and in PARP-1 knockout mouse cells, is in line with aneuploidy, which is frequent in cancers. Genetically engineered animal models have revealed that PARP-1 and VPARP impact carcinogenesis. Furthermore, accumulating experimental evidence supports the utility of PARP and PARG inhibitors in cancer therapy and several clinical trials are now ongoing. Increasing NAD^+ levels by pharmacological supplementation with niacin has also been found to exert preventive effects against cancer. In the present review, recent research progress on polyADP-ribosylation related to neoplasia is summarized and discussed. (*Cancer Sci* 2007; 98: 1528–1535)

Cancer is a disease that is characterized by various genetic changes, with mutations occurring in many proto-oncogenes and tumor suppressor genes during a multistep process. These, together with epigenetic alterations, transcriptional deregulation, and aberrations in post-translational modification, are the forces driving carcinogenesis. Forty years have passed since poly(ADP-ribose), a biopolymer involved in a unique post-translational modification, the polyADP-ribosylation reaction, was first discovered.[1–3] PolyADP-ribosylation is an NAD^+-dependent enzymatic reaction resulting in covalent modification of acceptor proteins with repeating units of ADP-ribose residues (Fig. 1). The structure of the biopolymer was characterized some 30 years ago.[4] Originally poly(ADP-ribose) polymerase (PARP)-1 was found in the nuclei and shown to be activated by DNA strand breaks. The presence of a salvage pathway of NAD^+ synthesis in the nuclei points to the importance of the polyADP-ribosylation reaction. Subsequent to descriptions of monoADP-ribosylation of arginine residues of mammalian proteins, cyclic ADP-ribose formation from NAD^+ by ADP-ribosyl cyclase and NAD^+-dependent histone deacetylases, named sirtuins, is the other enzyme group that uses NAD^+ in an important regulatory system for gene transcription. Ironically, NAD^+ is also used as the substrate by various microbial toxins for monoADP-ribosylation reactions.[5] In clear contrast to many other post-translational modifications, poly(ADP-ribose) molecules covalently attached to acceptor proteins vary greatly in size, up to several hundred ADP-ribose residues with branching and large negative charges.[4,6] These underlie the unique structural and functional characteristics of polyADP-ribosylation.

The present review summarizes recent progress suggesting that polyADP-ribosylation is dynamic and important for the regulation of critical cell functions, including mechanisms suppressing carcinogenesis. Possible applications in cancer therapy and prevention are also discussed. A recent review of the molecular aspects of polyADP-ribosylation is helpful.[7]

PolyADP-ribosylation and related reactions

There are now 17 PARP members deduced from genome sequences[7] (Fig. 2). Their differences in the subcellular localizations of PARP and specific expression timing, in part associated with the mitotic apparatus including centrosomes and spindle body, suggest various functions[3,8] (Fig. 3, Table 1). Poly(ADP-ribose) (PAR) and poly(ADP-ribose) glycohydrolase (PARG) also localize in the spindle body and centrosomes during mitosis[9] (Fig. 3).

MonoADP-ribosylation reactions. Post-translational modification by single ADP-ribose residues is termed monoADP-ribosylation and is catalyzed by various viral and bacterial toxins,[5] and eukaryotic enzymes using NAD^+ as the substrate. Mammalian monoADP-ribosyl transferases (ART) 1–7 have already been reported and ART2 is located on cell surfaces and is able to catalyze autopolyADP-ribosylation.[10] Of note, pierisin, isolated from *Pierisis rapae*, is demonstrated to monoADP-ribosylate guanine residues of DNA and induce apoptosis of various cancer cells.[11]

DNA Repair

Single-strand breaks and base excision repair. PARP-1 is activated by single- and double-strand breaks (SSB and DSB, respectively) and binds to such DNA strand breaks with zinc finger motifs (Fig. 2). After hydrogen peroxide treatment or SSB induction, foci of poly(ADP-ribose) appear in nuclei within several minutes, followed by foci of X-ray repair cross-complementing I (XRCCI). In *PARP-1*$^{-/-}$ cells, these are not detected. XRCCI has a high affinity for poly(ADP-ribose), and polyADP-ribosylated PARP-1 is suggested to bind and recruit XRCCI to SSB.[12]

PARP-1 knockout cells show increased sensitivity to alkylating agents, topoisomerase (topo) I inhibitors and γ-irradiation.[13] Increased levels of SSB and DSB and a delay in DNA repair are observed in *PARP-1*$^{-/-}$ mouse embryonic fibroblast (MEF) after alkylating damage. When mutation frequency was measured in the *red/gam* gene using the *gpt*-Δ transgenic mouse system, the frequency of deletions, particularly those accompanying rearrangements, is increased in the livers of *PARP-1*$^{-/-}$ mice after treatment with *N*-bis(2-hydroxypropyl)nitrosamine (BHP).[14] After alkylation of bases in DNA, glycosylases active in base excision repair (BER) first remove alkylated DNA bases. During the BER process, strand breaks and gaps are produced, and PARP-1 is activated and

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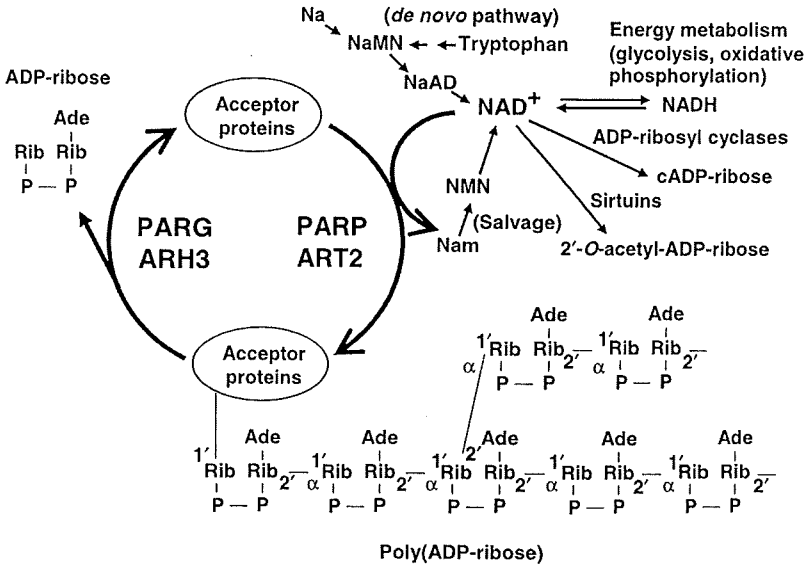

Fig. 1. PolyADP-ribosylation and related reactions. ARH3, ADP-ribose-(arginine) protein hydrolase 3; ART2, monoADP-ribosyl transferase 2; Na, nicotinic acid; NaAD, nicotinic acid adenine dinucleotide; NAD^+ (βNAD^+), nicotinamide adenine dinucleotide; Nam, nicotinamide; NaMN, nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide; PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerases.

Enzyme	Peptide structure	Gene locus	Function/property
PARP-1	DNA binding domain Zn fingers; Auto-modification domain; PARP domain; Z Z NLS BRCT; 1014; F54L M129T E251K V762A K940R; E	1q41-q42	DNArepair, genomic stability, cell death regulation, transcription, centrosome regulation
PARP-2	SAP; 570; E	14q11.2	DNA repair, genomic stability, telomere regulation, transcription
PARP-3	540; E	3p21	Centrosome regulation
VPARP (PARP-4)	BRCT; IHRP; IHRP; NLS; MVP-BD; 1724	13q11	Vault complex regulation, multidrug resistance
Tankyrase-1	HPS; E; 24 ankyrin repeats; SAM; 1327; E	8p23.1	Telomere regulation
Tankyrase-2	HPS; 16 ankyrin repeats; SAM; 1166; E	10q23.3	Golgi transport
PARP-9 (Bal-1)	macro-H2A macro-H2A; 854; T	3q13-q21	Overexpression in B cell agressive lymphoma
PARP-10	RRM; NES UIM; 1025; I	8q24.3	Suppression of transformation by c-MYC
PARG	NLS NES; NLS-like; catalytic domain; 976	10q11.23	DNA damage response, cell death regulation, transcription
ARH3	363; DD	1p34.3	PARG activity

Fig. 2. Poly(ADP-ribose) polymerases (PARP) family proteins relating to carcinogenesis, poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribose-(arginine) protein hydrolase 3 (ARH3). Peptide structures, domains, motifs, gene loci and functions/properties are shown. For PARP-1, positions of amino acids where single nucleotide polymorphisms (SNP) and mutations (italic) are shown. The caspase cleavage site is shown by the triangle. ARH, ADP-ribosyl protein hydrolase; BRCT, BRCA1 C-terminus; HPS, homopolymeric runs of His, Pro, and Ser; IHRP, inter-α-trypsin inhibitor family heavy chain-related protein motif; MVP-BD, major vault protein binding motif; NES, nuclear export signal; NLS, nuclear localization signal; RRM, RNA recognition motif; SAP, SAF-A/B, acinus, and PIAS motif; SAM, a sterile α motif; UIM, ubiquitin-interacting motif. The critical amino acid residue in the PARP domain of PARP-1 and the residues at the corresponding position for each PARP family protein are shown. The DD residues indicated in ARH3 are critical residues for PARG activity.

recruits XRCC1 and Ligase III-α complex in certain conditions, as illustrated in Fig. 4. PARP-1 may possibly protect the introduced DNA gaps (Fig. 4). In the absence of PARP-1, stalled BER may cause unligated SSB and may further induce DSB, and DNA fill-in reactions in short-patch or long-patch repair processes may be disturbed. The condensin I complex also supports BER through interactions with PARP-1 and XRCC1.[15] Furthermore, PARP-1 or PARP-2 can induce reactivation of

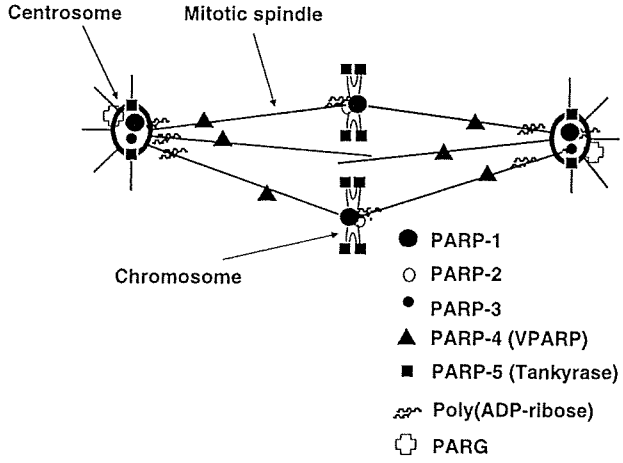

Fig. 3. Localization of poly(ADP-ribose) polymerase (PARP) family members, poly(ADP-ribose) glycohydrolase (PARG) and poly(ADP-ribose) at the mitotic apparatus.

stalled DNA topo I in covalent complexes and stimulate DNA strand break sealing.[16]

DSB and homologous recombination repair. DSB repair is mainly carried out by error-prone non-homologous end-joining (NHEJ) and error-free homologous recombination (HR) pathways (Fig. 4). Interactions of the DNA-dependent protein kinase (DNA-PK) complex, PARP-1 and Werner syndrome protein (WRN) seem to be involved in balancing these pathways.[17] Ku70/80 in the DNA-PK complex has a high affinity for DSB, and this affinity is reduced by polyADP-ribosylation.[17] In *PARP-1*$^{-/-}$ chicken DT 40 cells, the HR pathway is substantially inhibited by the Ku protein, indicating that PARP-1 functions in suppressing Ku protein blockage of HR repair.[18] WRN is recruited by interaction with Ku70/80 to DSB and is necessary for full activation of PARP-1.[17] The presence of an alternative DSB pathway involving PARP-1, PNK and DNA ligase III has also been suggested.

In HR repair, after bridging DSB by Rad50, Mre11 and the NBS-1 complex, the DSB terminus with a 3′-overhang structure is protected by Rad51 and BRCA2 (Fig. 4). In the absence of BRCA2, PARP-1 may possibly function to protect DSB ends from nuclease attack because *BRCA2*-deficient cancer cells are highly sensitive to PARP inhibitors and exhibit an increased frequency of DSB.[19,20] Some DSB-repair deficient cells, including examples that are *ATM*-deficient, also show hypersensitivity to PARP inhibitors.[21]

PARP-2 and DNA repair. PARP-2 is mainly present in centromeres during interphase and recognizes DNA loop structures. It is also activated by DNA damage.[7] After γ-irradiation, an increased level of DNA strand breaks was observed in the centromere regions of *PARP-2* knockout cells, these also demonstrating increased sensitivity to alkylating agents.[22] PARP-2 also supports BER and interacts with PARP-1, XRCC1, DNA polymerase-β and DNA ligase III. The functions of PARP-1 and PARP-2 in BER may be complementary only in a part and it remains to be determined whether their roles differ depending on the local chromatin structures.

PARG and DNA repair responses. PARP-1 and PARG are suggested to form a complex that breaks down poly(ADP-ribose) to ADP-ribose, resulting in regeneration of ATP molecules, which are required for DNA repair.[23] PARG has been found to re-localize at sites of DNA breaks induced by UV-A laser microirradiation in HeLa cells,[24] but further evidence of involvement in DNA repair needs to be obtained.

Transcriptional control

PARP-1 acts as a co-activator and co-repressor of transcription. In *PARP-1*$^{-/-}$ mice, NF-κB-dependent *inducible nitric oxide synthase* (*iNOS*) gene expression is substantially reduced.[25] Acetylation of lysine residues near the BRCT motif of PARP-1 is required for activation of transcription.[25] PARP-1 also acts as a co-activator of retinoic acid-inducible retinoic acid receptor (RAR)-dependent transcription of the *RARβ* gene, by binding to an inactive mediator that is then activated by RAR.[26] Subsequently the co-repressor complex is released, and recruited histone acetyltransferase complex activates transcription. PARP-1 also functions as a co-activator in β-catenin/TCF4-dependent transcription.[27] In estrogen receptor (ER)-dependent transcription of the *ER* gene, auto-polyADP-ribosylation stimulates formation of transcriptional complexes.[28] During transcriptional activation of the *ER* gene, PARP-1 interacts with topo II-β and transient DSB are induced, which is necessary for transcription.[29] The

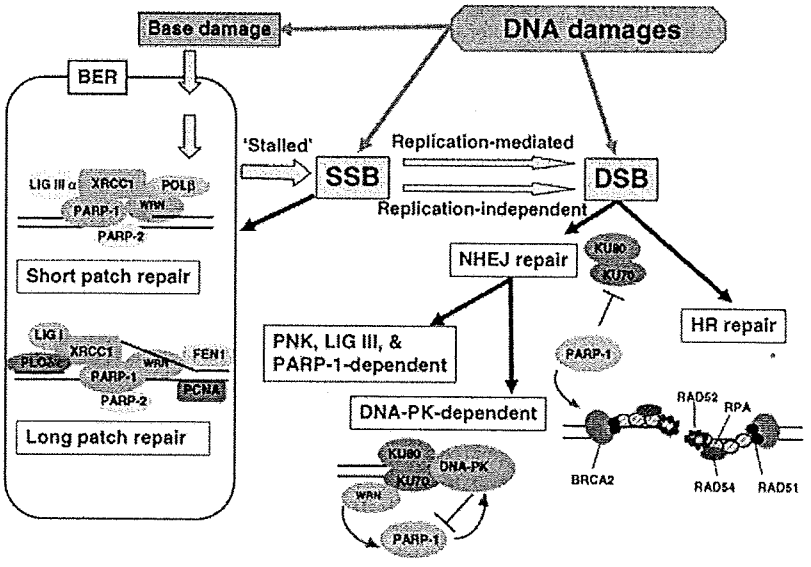

Fig. 4. Model for involvement of poly(ADP-ribose) polymerase (PARP) in DNA repair. DNA damage directly produces base damage, single-strand breaks (SSB) and double-strand breaks (DSB). SSB may be left unligated accidentally in the process of base excision repair (BER). SSB are possibly converted to DSB in a replication-mediated manner or replication-independently. Involvement of PARP in short-patch and long-patch repairs of BER, non-homologous end-joining (NHEJ) repair, and homologous recombination (HR) repair is shown. FEN1, flap endonuclease I; LIG, DNA ligase; PCNA, proliferating cell nuclear antigen; PNK, polynucleotide kinase; POL, DNA polymerase.

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Table 1. Phenotypic outcome of dysfunction in polyADP-ribosylation

Enzyme	Subject	Outcome	Method
PARP-1	Carcinogenesis	Susceptibility (induced by alkylating agents)↑	KO mice
		Susceptibility (in aged mice)↑	KO mice
	Genomic instability	SCE↑	KO mice
		Gene amplification↑	KO mice
		Micronuclei↑, chromosomal aberration↑	Antisense
		Centrosome amplification↑, Ploidy↑	KO mice
		Deletion mutation (after BHP treatment)↑	KO mice
		Transcriptional dysregulation↑	KO mice and *Drosophila*
	DNA damage response	DNA repair↓	KO mice, antisense
		Lethality of alkylating agents and γ-irradiation↑	KO mice
		Cell death induced by oxidative stress↓	KO mice
	Differentiation	Differentiation to trophoblast lineage↑	KO mice
PARP-2	Genomic instability	Chromosomal aberration↑	KO mice
		Ploidy↑	KO mice
		Aberration of spermatogenesis↑	KO mice
	DNA damage response	DNA repair↓	KO mice
		Lethality of alkylating agents and γ-irradiation↑	KO mice
	Differentiation	Adipocyte differentiation↓	KO mice
Tankyrase 1	Mitosis control	Aberration in chromosomal segregation↑	siRNA
PARG	DNA damage response	Lethality of alkylating agents and γ-irradiation↑	KO mice
	Neuronal dysregulation	Neuronal degeneration↑	KO *Drosophila*
VPARP	Carcinogenesis	Carcinogenesis induced by urethane↑	KO mice

KO, knock-out; PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerase; SCE, sister chromatid exchanges; VPARP, vault-associated PARP.

polyADP-ribosylation reaction is required for transcriptional activation of wide regions of chromatin through 'puff formation'.[30]

DNA methylation, imprinting and PARP. Hypomethylation of the global genome and local DNA hypermethylation frequently occur from the early stages of carcinogenesis. In this context the finding that PARP inhibitors enhance DNA methylation of the *HTF9* gene promoter is of interest.[31] DNA methyltransferase (DNMT) 1 possesses two poly(ADP-ribose) binding motifs and DNMT activity is repressed after its binding to poly(ADP-ribose).[32]

In cancer cells, loss of imprinting is also observed. CTCF (CCCTC binding factor), which binds to the non-methylated maternal allele of the insulator domain in the *H19* imprinting control region (ICR), is preferentially polyADP-ribosylated.[33] More than 140 CTCF target sites have been found to be poly-ADP-ribosylated and chromatin insulator functions are sensitive to PARP inhibition. It remains to be clarified which members of the PARP family are involved in the regulation of imprinting.

Macrodomain and PARP-1. The release of histone from chromatin by polyADP-ribosylation, in the so-called 'histone shuttle model', may enable dynamic conversion of local chromatin structures. Besides histones, various proteins bind poly(ADP-ribose) in cellular extracts. PARP-9 (Fig. 2), PARP-14 and PARP-15 all contain macrodomains that consist of hydrophobic amino acids and a helix structure. Recently, the macrodomains of macroH2A and PARP-9 were demonstrated to bind monoADP-ribose and poly(ADP-ribose).[34] There is thus a possibility that the local or cellular ADP-ribose metabolic state is translated into transcriptional regulation through macrodomains.

Differentiation control

In early studies, PARP inhibitors were shown to modulate differentiation processes. In human promyelocytic leukemia, HL-60 cells, stimulation of differentiation into granulocytes was accompanied by loss of *c-myc* gene amplification.[35] In *H-ras*-transformed NIH3T3 cells, PARP inhibitors also caused loss of amplified *H-ras* and *c-myc* oncogenes and reversal of the transformed phenotype.[35]

During teratocarcinoma-like tumor formation from mouse embryonic stem (ES) cells, induction of the trophoblast lineage, including trophoblast giant cells (TGC), was observed in tumors derived from *PARP-1*$^{-/-}$ ES cells.[36] The properties of TGC are similar to those of the syncytiotrophoblastic giant cells (STGC) observed in human germ cell tumors. *PARP-1* deficiency may be related to induction of STGC during human germ cell tumor development.

Cell-cycle controls

PARP-1 is also involved in cell-cycle check-point control after DNA damage. After γ-irradiation, p53-dependent induction of the *p21* and *mdm2* genes is attenuated by PARP inhibitors and suppression of G1 arrest and enhancement of G2 arrest are observed.[2,37] After treatment with neocarzinostatin, an increased level of γ-H2AX, a marker of DSB, was observed, accompanied by augmented p53 phosphorylation at the ser 18 residue in *PARP-1*$^{-/-}$ MEF. This accompanied enhancement of kinase activity of the ATM protein.[38] In addition, S-phase entry from G0 phase was found to be delayed in several cell types by *PARP-1* deficiency.[2]

Role of PARP-1 in cell death regulation

During the course of carcinogenesis, various types of cell death stress, including oxidative stress induced by inflammation and energy depletion, may be operating. Survival may be associated with mutations or epigenetic alteration of genes responsible for cell death pathways. PARP-1 dependent cell death occurs after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or massive oxidative stress. After MNNG treatment, NAD^+ depletion and translocation of apoptosis-inducing factor-1 (AIF-1) from mitochondria to nuclei is normally observed, but this type of cell death is lacking in *PARP-1* knockout cells resistant to oxidative stress,[39] and streptozotocin-induced pancreatic β-cell death.[40] PARP inhibitors enhanced development of streptozotocin-induced pancreatic insulinomas in rats,

suggesting that PARP-1 dependent cell death is involved in the prevention of carcinogenesis.[41] Because more than half of cancers feature mutation in the p53-dependent apoptosis pathway, PARP-1 dependent cell death could be a good target for cancer therapy.

PARG$^{-/-}$ mice lacking the 110 kDa isoform are hypersensitive to alkylating agents and γ-irradiation, with reduced automodification activity of PARP-1.[42] *PARG*$^{-/-}$ ES cells also exhibit increased sensitivity to MMS treatment and γ-irradiation, apoptosis occurring within a much shorter period than in *PARG*$^{+/+}$ ES cells.[43] This was linked to a marked increase of polyADP-ribosylated proteins in nuclei and a reduction in NAD^+ levels. The cytotoxicity of MNNG and menadione is increased in *PARG*$^{-/-}$ cells.[44] These results suggest that PARG activity is involved in survival after DNA damage. The poly(ADP-ribose) polymer itself induces cell-death through induction of AIF release from mitochondria.[45] This is consistent with a large accumulation of polyADP-ribosylated compounds and neuronal cell death in *PARG* knockout *Drosophila*.[46] Thus, there is a possibility that Parg inhibitors might enhance chemo- or radiation therapy of cancers.

Chromosomal stability

It is well known that cancer cells are generally characterized by extensive genomic instability. Centrosome amplification is frequently observed and could be a cause of chromosomal missegregation between daughter cells.

A century ago, a hypothesis was proposed that malignant tumors arise through defects of centrosome functions that lead to improper cell divisions resulting in aneuploidy.[3] Many reports have appeared documenting that certain post-translational modifications, including phosphorylation and ubiquitylation, occur in centrosomes and regulate their function.[3] Because PARP-1, PARP-3, PARP-4 and PARP-5, as well as polyADP-ribosylated proteins and PARG, are found in the mitotic apparatus (Fig. 3), polyADP-ribosylation might be involved in the regulation of fidelity of correct separation of chromosomes during mitosis.[3,47] It is interesting to note that 3-aminobenzamide (3-AB), an inhibitor of PARP, seems to be not mutagenic (non-genotoxic) to *Salmonella*, but does cause centrosome amplification in mammalian cells.[47] They also may induce sister chromatid exchanges.[5] These data suggest that non-genotoxic compounds like PARP inhibitors might induce chromosomal instability, which could promote carcinogenesis.

Cellular transformation

PARP inhibitors like benzamide decrease *in vitro* transformation of human fibroblasts induced by various types of carcinogens, such as benzo[a]pyrene and MNNG.[48] In contrast, transformation of mouse C3H10T1/2 cells by ethylnitrosourea (ENU) or ethylmethanesulfonate was elevated by PARP inhibitors.[5] These effects are possibly related to transcriptional control by PARP family members. PARP-10 was recently identified as a c-myc interacting protein, suppressing cellular transformation induced by c-MYC and E1A protein.[49] PARP-10 polyADP-ribosylates itself, as well as core histones, and may be indirectly involved in the regulation of c-myc.

Animal models of tumorigenesis

PARP-1$^{-/-}$ mice show increased susceptibility to carcinogenesis induced by alkylating agents, including BHP, and azoxymethane.[2,50] In contrast, there is no such difference regarding carcinogenesis induced by a heterocyclic amine, IQ (2-amino-3-methylimidazo [4,5-*f*]quinoline) and 4-nitroqinoline 1-oxide (4NQO), both of which give rise to bulky DNA adducts.[51,52] Therefore, there are carcinogen specific effects concerning the involvement of PARP-1 in carcinogenesis. Alkylation damage to DNA bases may be repaired mainly by BER, while bulky DNA adducts induced by IQ and 4NQO may be targeted by nucleotide excision repair (NER). Susceptibility to carcinogenesis might thus be explained by the involvement of PARP-1 in the repair pathway for BER, but not for NER.

In *PARP-1*$^{-/-}$*p53*$^{-/-}$ mice, an increased frequency of spontaneous development of carcinomas and lymphomas has been observed. Medulloblastomas also develop at the age of 16 weeks, accompanied by activation of the hedgehog pathway through overexpression of the *GLI* (*Greig cephalopolysyndactyly syndrome*) gene.[53] It is notable that medulloblastomas are also observed in *Ligase IV*$^{-/-}$*p53*$^{-/-}$ mice, which are defective in NHEJ repair.[2] The combination of DNA-PKc mutations (SCID mutations) with *PARP-1* deficiency has resulted in increased incidence of T-cell lymphoma and partial recovery of V[D]J recombination in T cells.[54] *Ku80* heterozygous mutations with *PARP-1* homozygous mutations have caused enhanced frequency of hepatocellular carcinoma development in aged mice.[55] In addition, *WRN*$^{\Delta hel1/\Delta hel}$ *PARP-1* null mice show an increased incidence of spontaneous tumors.[56] Furthermore, in aged *PARP-1*$^{-/-}$ mice, the incidence of spontaneous development of hepatocellular tumors is increased.[55] Recently, it was shown that *vault-associated PARP* (*VPARP*) knockout mice exhibit elevated susceptibility to carcinogenesis induced by urethane in the lungs and by dimethylhydrazine in the colon.[57]

Human cancer

In human cancers, increased expression of the *PARP-1* gene has been reported in Ewing's sarcomas,[2] and in malignant lymphomas.[2] In contrast, decreased expression has been observed in several gastric and colon cancer cell lines,[2] as well as in some breast cancers.[58]

Relations of genetic alterations in *PARP-1* gene with carcinogenesis have been reported by several authors. The heterologous *Met129Thr* mutation in the *PARP-1* gene has been reported in the germ cell tumor.[59] The *Val762Ala* single nucleotide polymorphism (SNP) was found to impact prostate cancer in Caucasians, the *Ala/Ala* allele being associated with a two-fold increase in susceptibility.[60] The 762Ala variant showed decreased PARP activity and reduced interaction with XRCC1 compared with the 762Val variant. With esophageal and lung cancers, a two-fold increase in risk with the *Ala/Ala* allele was observed in Chinese smokers.[61,62] It is also noteworthy that a combination effect of the *762Ala* allele of the *PARP-1* gene and the *399Gln* allele of the *XRCC-1* gene has been reported. It should be noted that the *762Ala* allele frequency is much higher in Asian compared to Caucasian populations. The relation of *Val762Ala* as well as *Lys940Arg* to the risk of lung cancer was investigated in Japan, but no associations were detected.[63] Genetic differences in the population or variation in the profile of environmental exposure to carcinogens may have exerted an influence.

PARP-9 contains two macroH2A domains that could repress transcription when localized sufficiently close to a promoter. This PARP was originally found as BAL1 (B-Aggressive Lymphoma 1) and is expressed at significantly higher levels in fatal high-risk diffuse large B-cell lymphomas (DLBCL) than in curable low-risk tumors.[64] Increased PARP-9 expression in DLBCL is associated with an activated peripheral B-cell phenotype and high rates of tumor cell migration.

Inhibition of PARP and PARG for potentiation of anticancer drugs

Earlier work in the 1980s was focused on the effects of PARP inhibitors, benzamide and 3-AB. 3-AB was demonstrated to enhance the cytotoxic effect of dimethylsulfate,[65] when used in

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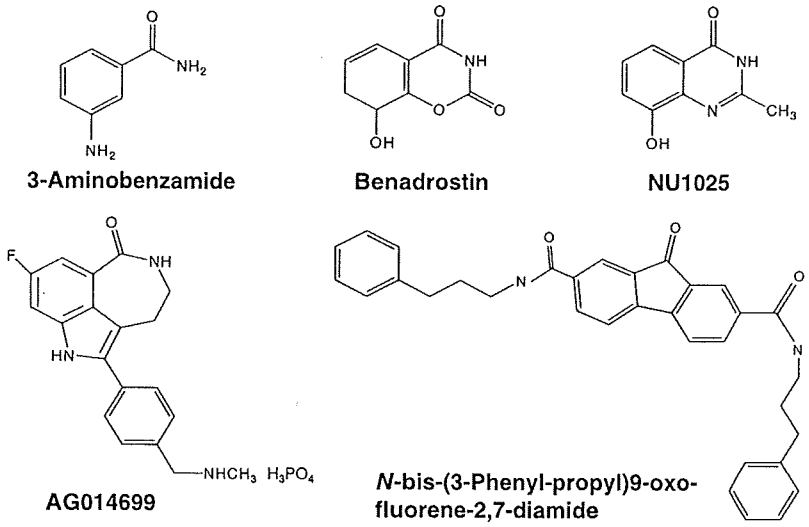

Fig. 5. Developed poly(ADP-ribose) polymerase (PARP) and poly(ADP-ribose) glycohydrolase (PARG) inhibitors. Structures of PARP inhibitors, 3-aminobenzamide,[65] benadrostin,[2] NU1025, AG014699,[67] and PARG inhibitor *N*-bis(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide[70] are shown.

combination with bleomycin as an anticancer drug in Ehrlich ascites mammary cancer cells.[66] Recently a more potent inhibitor, AG14361 and the AG014669 derivative (Fig. 5), were developed in England and are now undergoing Phase II clinical trials with the DNA methylating anticancer drug, temozolomide, for malignant melanomas.[67] The *BRCA2* gene required for HR repair is mutated in some cancer cells. Inhibitors of PARP that stall DNA replication forks and cause DSB might thus be expected to kill such mutated cells.[19,20] However, because the *BRCA2* deficient human cell line, CAPAN-1, was not found to be sensitive to PARP inhibition,[68] further studies are still necessary to understand what factors affect the consequences of PARP inhibition. A specific inhibitor for PARP5 (tankyrase 1) affecting telomerase function is also suggested to be a potential telomere-directed anticancer target.[69]

So far, PARG inhibitors have not been intensively studied as anticancer agents. Nobotanin B, adenosine diphosphate (hydroxymethyl)-pyrrolidinediol (ADP-HPD), as well as its cell-permeable derivative, 8-octylamino-ADP-HPD, have been reported. Pargamicin was also recently reported.[2] An approach to using a PARG inhibitor, *N*-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide (Fig. 5), in combination with temozolomide to treat temozolomide-resistant cancers, has been published.[70] Therefore, PARG could be a new molecular target for cancer chemotherapy.

Chemoprevention

The substrate of PARP, NAD^+, is synthesized using nicotinic acid mononucleotide by the kynurenic pathway starting with L-tryptophan or using niacin (nicotinic acid, vitamin B3) supplied in the diet (Fig. 1). It has been reported that in rats maintained under a niacin-deficient diet, the level of NAD^+ in bone marrow is decreased, with even more extensive reduction in poly(ADP-ribose).[71] The animals were found to show greatly elevated susceptibility to ENU, particularly regarding induction of leukemias. In contrast, rats supplemented with niacin or nicotinamide in the diet had increased NAD^+ and basal and ENU-treated poly(ADP-ribose) levels in bone marrow. ENU-induced carcinogenesis was furthermore slowed.[71] Niacin deficiency was also shown to enhance skin cancer development with preventive effects of supplementation. Tashtoush *et al.* showed that myristyl nicotinate, a derivative of nicotinic acid, can be used as a topical agent increasing NAD^+ content by 40%.[72] Because NAD^+ is used in various physiological processes, the preventive effects of niacin could be due to a combinatory influence on ADP-ribosylation enzymes and other NAD-requiring reactions.

Concluding remarks

Carcinogenesis is multistage and may involve not only gene mutations but also abnormal dynamics of chromosomal organization, possibly also caused by non-genotoxic factors. For a better understanding of the entirety of neoplasia, more needs to be learned from basic biological as well as clinical features. Research on polyADP-ribosylation has progressed rapidly, as evidenced by the multitude of details available on the website, PARP link (http://parplink.u-strasbg.fr/index.html). Considering the fact that many post-translational modifications are actively involved in regulation of key reactions, interplay among processes like polyADP-ribosylation, monoADP-ribosylation, phosphorylation, acetylation, methylation, and ubiquitination of key proteins deserves greater attention. This research field might best be termed 'Proteomodificomics (PMM)'. Progress of PMM in today's post-genome era, with collaboration from various scientists and clinicians, should help establish new concepts for understanding the characteristics of clinical cancer that should lead to better diagnosis, treatment and prevention.

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Association of a missense single nucleotide polymorphism, Cys1367Arg of the WRN gene, with the risk of bone and soft tissue sarcomas in Japan

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Bone and soft tissue sarcomas (BSTSs) are rare malignant tumors of mesenchymal origin. Although BSTSs frequently occur in some hereditary cancer syndromes with germline mutations of DNA repair genes, genetic factors responsible for sporadic cases have not been determined. In the present study we undertook a case-control study and analyzed possible associations between the susceptibility to BSTS and the single nucleotide polymorphisms (SNPs) in DNA repair genes. Genomic DNAs extracted from case and control peripheral blood leukocytes were genotyped by pyrosequencing. For candidate polymorphisms, we chose 50 non-synonymous missense SNPs, which we have previously been identified by resequencing 36 DNA repair genes among the Japanese population. In the first screening, we analyzed 240 cases and 685 controls and selected six SNPs at the significance level of $P < 0.1$ (Fisher's exact test). The six SNPs were further analyzed in the second genotyping on an additional set of 304 cases and 834 controls. In the joint analysis (the first and second genotyping combined) of 544 cases and 1378 controls, Cys1367Arg of the WRN gene was found to be a protective factor of BSTS (odds ratio = 0.66, 95% confidence interval = 0.49–0.88, $P = 0.005$). An exploratory subgroup analysis without multiple comparison adjustment suggested that the WRN-Cys1367Arg SNP is associated with soft tissue sarcomas, sarcomas with reciprocal chromosomal translocations and malignant fibrous histiocytoma. (*Cancer Sci* 2008; 99: 333–339)

Bone and soft tissue sarcomas (BSTSs) are nonepithelial, non-hematological malignant tumors of mesenchymal origin. Three characteristics of BSTS are that: (i) they are rare malignancies; (ii) they develop at a variety of sites; any mesenchymal tissues throughout the body; and (iii) show highly heterogeneous histological types. In the United States, the incidence of malignant bone tumors is estimated to be around 0.8 per 100 000 population,[1] and that of soft tissue sarcomas (STS) approximately 5.0 per 100 000.[2] It is estimated that 2370 bone and joint malignancies and 9220 malignant soft tissue tumors would newly develop in 2007.[3]

The rarity and the histological heterogeneity of BSTSs have hampered the identification of the risk factors and etiology. BSTSs show a slight male predominance, and the incidence of these rare tumors both in Japan and in other countries seems to be stable in the last several decades except for an increase in Kaposi sarcoma as reported in the United States.[4,5] This appears to be in contrast to several other types of cancers, such as gastrointestinal or gynecologic cancers, which have shown a significant change in incidence in Japan probably due to the change in environmental and life style factors. No significant racial variation has been noted in the overall incidence of sarcomas with some exceptions, such as Ewing sarcoma, which reportedly occurs more frequently in Caucasians.[6]

To date, some environmental and genetic factors for BSTS risk have been suggested. The environmental factors include external radiation therapy,[7] Thorotrast, arsenical pesticides and medications, phenoxyherbicides, dioxin, vinyl chloride, immunosuppressive drugs, alkylating agents, androgen-anabolic steroids, human immunodeficiency virus, and human herpes virus type 8.[5] Information on the genetic factors has so far been limited to certain monogenic hereditary cancer syndromes known to be associated with the incidence of BSTS, such as Li–Fraumeni syndrome, hereditary retinoblastoma, and Werner syndrome with a germline mutation of *TP53*, *RB1*, and *WRN*, respectively. These genes are involved in DNA repair and related systems. Other monogenic hereditary syndromes are also associated with the specific type of multiple benign tumors and their malignant transformation, such as multiple neurofibromas and malignant peripheral nerve sheath tumors in neurofibromatosis 1, and multiple osteochondromas and chondrosarcomas in hereditary multiple exostosis, that have a germline mutation of *NF1* and *EXT1/2*, respectively.

Recently, BSTSs have been considered to be divided into two distinct entities based on their somatic genetic aberrations. One group is characterized by reciprocal chromosome translocations resulting in tumor-specific fusion genes, which may be a critical step for pathogenesis. Another group of sarcomas tend to show complex abnormalities in the karyotypes, suggesting an overall increase in genetic and chromosomal instability. However, the precise mechanisms of tumorigenesis in both groups remain unclear.

The characteristic increase in the risk of BSTS in inherited diseases caused by germline mutations in the DNA repair and related systems has prompted us to investigate the association of the DNA repair gene polymorphisms with the risk of BSTSs. A case-control study was carried out on 544 cases with BSTS and 1378 controls at 50 non-synonymous coding single nucleotide polymorphisms (cSNPs), which we have identified by resequencing of 36 candidate genes on a Japanese population[8]. Associated studies of common polymorphisms of the DNA repair genes have already been reported in many types of cancer,[9] but to our knowledge, this study is the first report on BSTS.

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Table 1. **Fifty missense single nucleotide polymorphisms (SNPs) in DNA repair genes grouped by their representative pathways**

DNA repair gene	Gene name	SNP	Amino acid change
Base excision repair			
PARP-1/ADPRT1	Poly (ADP-ribose) polymerase family, member 1	T2444C	Val762Ala
		A2978G	Lys940Arg
APE1/APEX	APEX nuclease (multifunctional DNA repair enzyme) 1	A395G	Ile64Val
		T649G	Asp148Glu
MBD4	Methyl-CpG binding domain protein 4	G1212A	Glu346Lys
NUDT1	Nudix (nucleoside diphosphate linked moiety X)-type motif 1	G273A	Val83Met
OGG1	8-oxoguanine DNA glycosylase	C2243G	Ser326Cys
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1	C685T	Arg194Trp
		G944A	Arg280His
		G1301A	Arg399Gln
Nucleotide excision repair			
ERCC5/XPG	Excision repair cross-complementing rodent repair deficiency, complementation group 5	C3507G	His1104Asp
ERCC6/CSB	Excision repair cross-complementing rodent repair deficiency, complementation group 6	G1275A	Gly399Asp
XPC	Xeroderma pigmentosum, complementation group C	A2655C	Lys822Gln
XPD/ERCC2	Excision repair cross-complementing rodent repair deficiency, complementation group 2	G1615A	Asp312Asn
		A2932C	Lys751Gln
Mismatch repair			
MLH1	mutL homolog 1, colon cancer, non-polyposis type 2	A676G	Ile219Val
MLH3	mutL homolog 3 (*Escherichia coli*)	C2645T	Pro844Leu
		C2939T	Thr942Ile
MSH2	mutS homolog 2 (E. coli)	C91T	Thr8Met
MSH3	mutS homolog 3 (E. coli)	A3122G	Thr1036Ala
MSH6	mutS homolog 6 (E. coli)	G203A	Gly39Glu
DNA damage response genes			
TP53	Tumor protein p53 (Li–Fraumeni syndrome)	G466C	Arg72Pro
DNA double strand break repair			
BLM	Bloom syndrome	C967T	Thr298Met
		G4035A	Val1321Ile
BRCA2	Breast cancer 2, early onset	A1342C	Asn372His
KIAA0086	DNA cross-link repair 1A	C1867G	His317Asp
LIG4	DNA ligase IV	A2245G	Ile591Val
NBS1	Nijmegen breakage syndrome 1	G605C	Glu185Gln
RAD51L3	RAD51-like 3 (S. cerevisiae)	G501A	Arg126Gln
RAD54L	RAD54-like (S. cerevisiae)	A551G	Lys151Glu
RINT1	RAD50 interactor 1	G33C	Glu4Gln
WRN	Werner syndrome	C2573T	Thr781Ile
		T4330C	Cys1367Arg
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3	C1075T	Thr241Met
DNA polymerase			
POLD1	Polymerase (DNA directed), delta 1	G409A	Arg119His
POLH/XPV/RAD30	Polymerase (DNA directed), eta	A1840G	Lys535Glu
POLI/RAD30B	Polymerase (DNA directed) iota	A2180G	Thr706Ala
POLL	Polymerase (DNA directed), lambda	C1683T	Arg438Trp
POLZ/REV3	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	C4259T	Thr1146Ile
REV1	REV1 homolog (S. cerevisiae)	T982C	Phe257Ser
		A1330G	Asn373Ser
Other pathways			
FANCA	Fanconi anemia, complementation group A	G827A	Ala266Thr
		G1080A	Arg350Gln
		A1532G	Ser501Gly
		A2457G	Asp809Gly
		C3294T	Ser1088Phe
FANCE	Fanconi anemia, complementation group E	G451T	Arg89Leu
		G1213A	Arg343Gln
FANCF	Fanconi anemia, complementation group F	A983G	Lys324Glu
FANCG/XRCC9	Fanconi anemia, complementation group G	C1382T	Thr297Ile

ADP, adenosine diphosphate;

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Table 2. Case and control subjects

		1st screening		2nd genotyping		Joint analysis	
		Case	Control	Case	Control	Case	Control
Gender	Male	143	483	174	492	317	875
	Female	97	202	130	342	227	503
Age	<40	109	113	127	214	236	318
	≥40	131	572	177	620	308	1060
Institutions[†]	NCCH	240	242	127		367	242
	NCCE			19		19	
	KEIO		302	158	507	158	809
	IWT				327		327
	NNH		141				
Total		240	685[†]	304	834	544	1378[‡]

Distributions of gender, age and five institutions where case and/or control subjects were recruited are listed. [†]The 685 controls in the first screening are from our published data (8). [‡]The 141 NNH control data were not available for the joint analysis. KEIO, Keio University Hospital, Tokyo; IWT, Iwata Hospital, Shizuoka; NCCE, National Cancer Center East, Chiba; NCCH, National Cancer Center Hospital, Tokyo; NNH, National Nishigunma Hospital, Gunma.

Table 3. Histological distribution of the cases and subgroup classification

Bone sarcoma	
Osteosarcoma	105
Chondrosarcoma	41
EWS/PNET	18
MFH	11
Chordoma	8
Adamantinoma	3
Leiomyosarcoma	2
MPNST	1
Fibrosarcoma	1
	190

Soft tissue sarcoma	
Liposarcoma	111
MFH	92
Synovial sarcoma	38
Leiomyosarcoma	19
MPNST	19
Dermatofibrosarcoma protuberance	17
EWS/PNET	15
EMC	7
Osteosarcoma	6
Fibrosarcoma	5
Epithelioid sarcoma	5
Rhabdomyosarcoma	6
Angiosarcoma	6
Alveolar soft part sarcoma	4
Clear cell sarcoma	2
Mesenchymal chondrosarcoma	1
Hemangiopericytoma	1
	354

Subgroup	Total (%)
ALL	544 (100.0)
BS/STS	
BS	190 (34.9)
STS	354 (65.1)
Translocation	
(+)	154 (28.3)
(–)	390 (71.7)
Histopathology	
Osteosarcoma	111 (20.4)
MFH	103 (18.9)
Liposarcoma	111 (20.4)
Others	219 (40.3)

ALL, all samples; BS, bone sarcomas; EMC, extraskeletal myxoid chondrosarcoma; EWS/PNET, Ewing sarcoma and peripheral neuroectodermal tumor; MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumor; STS, soft tissue sarcomas; Translocation (+), sarcomas with reciprocal chromosomal translocations; Translocation (–), sarcomas without reciprocal chromosomal translocations.

Materials and Methods

Study design, case and control subjects. Genotype data of the 50 SNPs,[8] in Table 1 from 240 cases and 685 controls were analyzed as the first screening to select those SNPs to be subjected to the second genotyping in the additional 304 cases and 834 controls. In this study, we used a joint analysis,[10] in which the results of the second genotyping were combined with those of the first genotyping (Table 2).

The cases were recruited in three hospitals (National Cancer Center Hospital [NCCH], Tokyo, National Cancer Center East Hospital, Chiba [NCCE], and Keio University Hospital, Tokyo [KEIO]) from January 2004 to March 2005 (Table 2). The patients, all Japanese, were either newly diagnosed as BSTS or had been followed up in an outpatient clinic for the history of BSTS. The cases consisted of various histological subtypes, which were confirmed by histological examination in each hospital (Table 3).

We used 685 control subjects in the first screening, which were the same control population as those used in our previous study on the same set of SNPs but on a different type of malignancy, lung cancer (Table 2).[8] Those subjects consisted of 383 non-cancer patients in two hospitals (NCCH and National Nishigunma Hospital, Gunma [NNH] in Table 2) and 302 healthy volunteers in KEIO (Table 2). We did not, however, include the 141 control subjects from NNH (Table 2) in the subsequent joint analysis, because their individual genotyping data have not been published and are unavailable for analyses other than the lung cancer study. In the second genotyping, we analyzed additional samples from 834 people who participated in the health examination programs (KEIO and Iwata Hospital, Shizuoka [IWT] in Table 2). We consider that these subjects were suitable enough as controls for our cases; most of the control subjects in this study were also analyzed in our separate gastric cancer project involving a SNP-based genome scan, and its data suggested little if any population stratification (unpublished data, 2007). Therefore, the control subjects for the joint analysis totaled 1378 people with a criterion of no history of cancer during the study period (Table 2).

Subgroup analysis was carried out on the subgroups defined by Table 3. For the 'sarcomas with reciprocal chromosomal

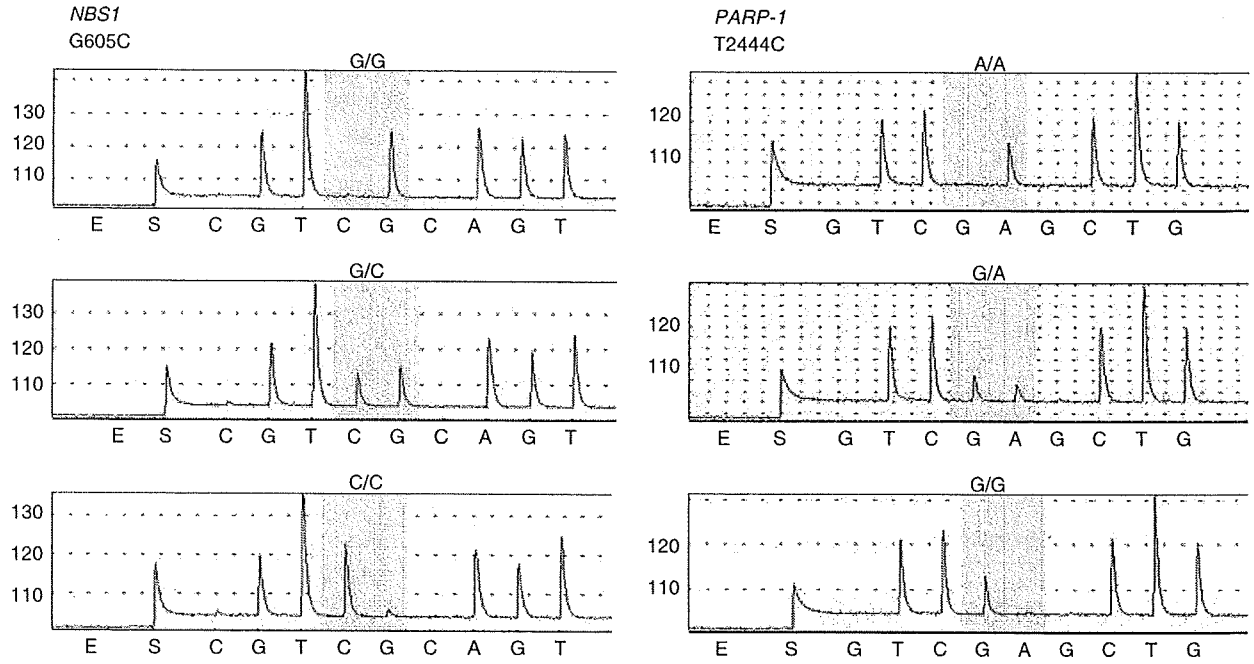

Fig. 1. Genotyping by pyrosequencing. (a) NBS1-Glu185Gln (G605C) and (b) PARP-1-Val762Ala (T2444C) (sequencing in reverse direction). Top, major homozygote; middle, heterozygote; and bottom, minor homozygote.

translocations', we included synovial sarcoma, Ewing sarcoma/peripheral neuroectodermal tumor, myxoid/round cell liposarcoma, clear cell sarcoma, dermatofibrosarcoma protuberance, extraskeletal myxoid chondrosarcoma, and alveolar soft part sarcoma. The specific fusion genes have been reported for those sarcomas, and cytogenetic examinations are routine for their histological diagnosis.[11]

This study was approved by Institutional Review Board of each institution, and all of the subjects signed an informed consent form to participate in the study.

DNA extraction and genotyping. From each individual we obtained a 10–20-mL sample of whole blood. Genomic DNAs were isolated directly from the samples using Blood Maxi Kit (Qiagen, Tokyo, Japan) or FlexiGene DNA Kit (Qiagen) according to the manufacturer's instructions. Ten nanograms of genomic DNA were subjected to genotyping for 50 SNPs by pyrosequencing using the PSQ96 system (Pyrosequencing, Uppsala, Sweden) as described previously.[12] Briefly, a genomic fragment containing an SNP site was amplified by polymerase chain reaction (PCR) with a set of PCR primers, one of which was biotinylated. The PCR products were purified using streptavidin-modified paramagnetic beads (Dynabeads M-280; Dynal, Skoyen, Norway), denatured and subjected to nucleotide sequencing by pyrosequencing chemistry. Quality of the SNP typing was confirmed by inspection of the sequence data and by Hardy–Weinberg equilibrium (HWE) tests.

Statistical analysis. Fisher's exact test, odds ratios (OR) and 95% confidence intervals (CI) were used to analyze association of the SNPs and BSTS risk in allele, dominant (i.e. aa + aA vs AA, where 'A' is major allele and 'a' is minor allele) and recessive (i.e. aa vs aA + AA) models.[13] Crude OR was used for the allele model, while the dominant and recessive models were analyzed using OR adjusted for age (≥40 vs <40, see Suppl. Fig. S1 online) and gender with 95% CI calculated using a logistic regression analysis. When statistical calculation is not applicable due to 0 subjects being in a cell of a contingency table, we indicate the result as 'NA'.

In order to identify disease-associated SNPs, the following criteria were used: candidate SNPs were selected by P-value < 0.10 on the allele model in the first screening, and then disease associated-SNPs were statistically identified by the allele model in the joint analysis with a multiple comparison adjustment using the Holm's method,[14] for six SNPs. Please note that the family wise error rate may be inflated by a few percent, depending on the number of the true SNPs present in the initial 50 SNPs, by this method of multiple comparison adjustment, because the first and second screenings are not independent in the joint analysis (see Suppl. Table S1 online).

A subgroup analysis was carried out as an exploratory, adjunct analysis without multiple comparison adjustment to address the histological heterogeneity of BSTS. Because the subgroups showed different age preferences, dominant and recessive models were used in order to adjust age and gender by multiple logistic regression.

Results

Results of the joint analysis. The typical pyrosequencing data are shown in Fig. 1, and the results of the two-stage genotyping are summarized in Table 4. Minor allele frequencies and P-values of the HWE tests are also listed in Table 4. The six SNPs were selected by the first screening, and the final statistical gene selection was made using all the samples genotyped in the first screening and the second genotyping combined, except 141 controls from Nishigunma Hospital (cases = 544 and controls = 1378 in total). We identified a SNP, WRN-Cys1367Arg, whose allele frequency was significantly different between all BSTS cases and the control subjects (OR = 0.66, 95% CI = 0.49–0.88, $P = 0.005$ and $P = 0.03$, before and after six-SNP multiple comparison adjustment by Holm's method, respectively), showing a protective effect. The minor allele frequency of the SNP is 8.2%.

Subgroup analysis. Since BSTS is characterized by the substantial heterogeneity in its histology, the effects of the DNA repair gene polymorphisms might differ among the subgroups. The results of the subgroup analysis of the WRN-Cys1367Arg SNP are shown in Table 5. Although under-powered and exploratory in nature, the subgroup analysis suggested that the difference in genotype frequency of WRN-Cys1367Arg between the cases and controls

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Table 4. Statistics of allele model analysis for the single nucleotide polymorphisms (SNPs) selected in the first screening by $P < 0.1$

Gene	SNP		Minor allele frequency		HWE (*P*-value)		OR	Allele model	
			Cases (*n*)	Controls (*n*)	Cases	Controls		95% CI	*P*-value
MBD4	G1212A	1st screening	0.304 (240)	0.349 (685)	0.545	0.207	0.82	0.65–1.02	0.083
	Glu346Lys	Joint analysis	0.335 (544)	0.353 (1378)	0.620	0.199	0.92	0.79–1.07	0.286
MSH6	G203A	1st screening	0.275 (240)	0.323 (685)	0.074	0.793	0.80	0.63–1.00	0.057
	Gly39Glu	Joint analysis	0.304 (544)	0.310 (1378)	0.434	0.621	0.97	0.84–1.13	0.750
PARP-1	A2978G	1st screening	0.077 (240)	0.050 (685)	1.000	1.000	1.58	1.04–2.38	0.044
	Lys940Arg	Joint analysis	0.066 (544)	0.052 (1378)	1.000	0.509	1.30	0.97–1.74	0.098
REV1	A1330G	1st screening	0.023 (240)	0.043 (685)	1.000	1.000	0.52	0.27–1.00	0.055
	Asn373Ser	Joint analysis	0.029 (544)	0.044 (1378)	0.042	0.680	0.67	0.45–0.99	0.049
WRN	T4330C	1st screening	0.056 (240)	0.090 (685)	1.000	0.480	0.61	0.40–0.93	0.024
	Cys1367Arg	Joint analysis	0.052 (544)	0.082 (1378)	0.213	0.619	0.66	0.49–0.88	0.005
XRCC1	C685T	1st screening	0.279 (240)	0.327 (685)	0.016	1.000	0.80	0.63–1.00	0.058
	Arg194Trp	Joint analysis	0.292 (544)	0.323 (1378)	0.587	0.811	0.87	0.74–1.01	0.073

CI, confidence interval; HWE, Hardy–Weinberg equilibrium; OR, odds ratio.

Table 5. Statistics of subgroup analysis using recessive and dominant models

SNP	Subgroup	(*n*)	Recessive model†			Dominant model‡		
			OR	95% CI	*P*-value	OR	95% CI	*P*-value
WRN	ALL	544	0.43	0.09–2.01	0.442	0.66	0.48–0.91	0.011
T4330C	BS	190	NA	NA–NA	0.489	0.85	0.53–1.37	0.589
Cys1367Arg	STS	354	0.72	0.16–3.32	0.998	0.58	0.40–0.85	0.005
	Translocation (+)	154	NA	NA–NA	0.632	0.52	0.29–0.95	0.034
	Translocation (–)	390	0.61	0.13–2.86	0.817	0.72	0.51–1.02	0.068
	Osteosarcoma	111	NA	NA–NA	0.826	1.00	0.56–1.80	1.000
	MFH	103	NA	NA–NA	1.000	0.45	0.22–0.94	0.032
	Liposarcoma	111	NA	NA–NA	0.911	0.72	0.40–1.31	0.346

Odds ratios (OR) adjusted for age (≥ 40, < 40) and gender with 95% confidence intervals (CI) were calculated using a logistic regression analysis for all subgroups. Cases = 544 and Controls = 1378 in total. †Recessive model is aa versus aA + AA. ‡Dominant model is aa + aA *versus* AA, where a is a minor allele. ALL, all samples; BS, bone sarcomas; MFH, malignant fibrous histiocytoma; NA, statistical calculation not applicable, because the number of the subjects is less than five in any cell in a contingency table; SNP, single nucleotide polymorphism; STS, soft tissue sarcomas; Translocation (+), sarcomas with reciprocal chromosomal translocations; Translocation (–), sarcomas without reciprocal chromosomal translocations.

appears to be significant in STS, sarcomas with reciprocal chromosomal translocations and malignant fibrous histiocytoma. It is noted, however, that the effect direction of the minor allele (Arg1367) of this SNP was protective in all of the subgroups, except osteosarcoma (OR = 1.00), irrespective of the statistical significance. The subgroup analysis data of the other five SNPs analyzed in the second stage is shown in Suppl. Table S2 online.

Discussion

This study attempted the first systematic survey on the possible role of DNA repair gene polymorphisms in the susceptibility to sporadic BSTS. From the joint analysis of the two-stage case-control study on total 544 cases with BSTS of various histology and 1378 controls, a missense SNP of the WRN gene, Cys1367Arg, was identified. WRN is a member of the RecQ family of DNA helicases, and mutations of the gene can give rise to a rare autosomal recessive genetic instability disorders, Werner syndrome (WS).[15] WS is a premature aging disease characterized by predisposition to cancer and the early onset of symptoms related to normal aging.[16] The types of cancer with elevated risk appear selective, including soft tissue sarcomas, thyroid carcinoma, malignant melanoma, meningioma, hematological malignancies, and osteosarcoma. A diversity of malignancies was found in WS in the literature from 1939 to 1995, but it is noteworthy that the ratio of epithelial to nonepithelial cancers was about 1:1, instead of the usual 10:1.[17] BSTS make up more than 20% of cancer arising in WS patients.[17] Soft tissue sarcomas that have been identified in WS patients include malignant fibrous histiocytoma, malignant peripheral nerve sheath tumor, fibrosarcoma, rhabdomyosarcoma, liposarcoma, and synovial sarcoma. The reason for the high representation of mesenchymal tumors in WS patients has not been clarified.

WRN encodes a protein with 1432 amino acids that possesses both 3′→5′ DNA helicase and 3′→5′ DNA exonuclease activities. DNA helicases are enzymes that unwind the energetically stable double-stranded structure of DNA to provide a single-stranded template for important cellular processes such as replication, base excision repair, homologous recombination, and telomere maintenance.[15,18,19]

Several epidemiological studies have already been carried out on WRN-Cys1367Arg. Most of those studies focused on the diseases relating to the WS phenotype, such as myocardial infarction, diabetes mellitus and lymphomas. The more frequent Cys1367 allele has been reported to be associated with a lower frequency of osteoporosis in postmenopausal Japanese women,[20] whereas the minor allele Arg1367 may be associated with a lower risk of myocardial infarction and type 2 diabetes mellitus in the Japanese population.[21,22] With regard to cancer risk, Arg1367 was reported to be associated with a decreased risk of non-Hodgkin lymphoma among women in Connecticut.[23] Of note, in three out of the four reports listed above, WRN-Cys1367Arg showed protective effects against the various diseases associated with WS phenotype, and the same held true for our observations on BSTS.

Based on the observations that all of the pathogenic *WRN* mutations identified so far result in truncation of the C-terminal

of the WRN protein, it has been proposed that a lack of the C-terminal nuclear localization signal is important in the pathogenesis of WS.[24,25] Although WRN-Cys1367Arg is located adjacent to the nuclear localization signal, a previous report has failed to detect any significant difference between WRN (Arg1367) and WRN (Cys1367) with respect to their nuclear localization,[26] or helicase and helicase-coupled exonuclease activity.[27] Other possible explanations for the observed association include the allelic difference of WRN-Cys1367Arg in the interactions with other proteins or the presence of unknown functionally responsible polymorphisms that are in linkage disequilibrium with WRN-Cys1367Arg.

Some of the SNPs on the genes responsible for a hereditary form of cancer have shown association with a sporadic form of the same type of cancer.[28–31] Our findings on the *WRN* SNP on BSTS may add another example of the possible sharing, at least in part, of the oncogenesis pathway between the monogenic and polygenic forms of the same type of cancer and a continuity of the genotype-phenotype spectrum.

It may be controversial to analyze the possible genetic backgrounds with all types of BSTS combined because of its highly heterogeneous nature in histology. However, little if any information is currently available on the genetic predisposition to the sporadic forms of BSTS, and genetic factors that are common to most BSTSs may exist, as well as those specific to certain subgroups. As one of the first exploratory analyses on the genetic susceptibility of sporadic BSTS, the primary role of this study is to generate hypotheses, which deserve further validation. To validate the hypothesis, evidence should be sought both in statistical replication and meta-analysis using future case-control panels and also in biological functional analyses.

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Supplementary Material

The following supplementary material is available for this article:

Fig. S1. Age distributions of cases and controls in this study. The case appears to have two peaks, before and after around 40 years of age.

A Monte Carlo simulation experiment to compare family wise error rate for the multiple testing correction by six single nucleotide polymorphisms (SNPs) (six hypotheses examined for the joint analysis) and that by 50 SNPs (initial candidate SNPs screened in the first screening). As the framework of simulation, we set the following conditions:

Condition 1.
The total number of SNPs to be examined is set as $m = 50$ and the number of 'true' disease-associated SNPs (positive SNPs) among the 50 SNPs as $N_p = 3$ or 5.

Condition 2.
The population allelic odds ratio for the disease-associated N_p SNPs is $\psi = 1.3$, 1.5 or 1.7, while the population odds ratio for the remaining m-N_p SNPs unrelated to the disease is 1.0.

Condition 3.
In the first stage, sample size is set as 240 cases and 685 control subjects. The second stage sample size is 304 cases and 834 control subjects. In a joint analysis, sample size is set as 544 cases and 989 control subjects.

Condition 4.
The proportion of allele X in the control population is a random variable uniformly distributed in unit interval (0.05, 0.95).

Condition 5.
The criteria to evaluate the performance of each method are two indicators, sensitivity and specificity in the joint analysis.

Condition 6.
The Monte-Carlo simulation to calculate sensitivity and specificity is repeated 10 000 times, and the mean values of indicators are calculated.

Table S1 shows a summary result of the simulation experiment, which suggests that our study was designed to contain the overall false-positive (type 1 error) rate (1-specificity) to 5% or 8% at the power of 90%, if there are five or three true SNPs, respectively, included in our starting 50 candidate SNPs. On the other hand, the false-positive rates for the multiple testing correction by the 50 SNPs with OR = 1.5 or 1.7 are less than 1%.

Table S1. Simulation results for each multiple testing correction method

Table S2. Statistics of subgroup analysis by recessive and dominant models on all the six single nucleotide polymorphisms (SNPs) analyzed in the second stage of genotyping

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Poly(ADP-ribose) Glycohydrolase Deficiency Sensitizes Mouse ES Cells to DNA Damaging Agents

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Abstract: Poly(ADP-ribose) glycohydrolase (Parg) is the main enzyme for degradation of poly(ADP-ribose) by splitting ribose-ribose bonds. *Parg*-deficient ($Parg^{+/-}$ and $Parg^{-/-}$) mouse ES cell lines have been established by disrupting both alleles of *Parg* exon 1 through gene-targeting. A transcript encoding a full length isoform of Parg was eliminated and only low amounts of Parg isoforms were detected in $Parg^{-/-}$ embryonic stem (ES) cells. Poly(ADP-ribose) degradation activity was decreased to one-tenth of that in $Parg^{+/+}$ ES cells. $Parg^{-/-}$ ES cells exhibited the same growth rate as $Parg^{+/+}$ ES cells in culture. Sensitivity of $Parg^{-/-}$ ES cells to various DNA damaging agents, including an alkylating agent dimethyl sulfate, cisplatin, gemcitabine, 5-fluorouracil, camptothecin, and γ-irradiation was examined by clonogenic survival assay. $Parg^{-/-}$ ES cells showed enhanced lethality after treatment with dimethyl sulfate, cisplatin and γ-irradiation compared with wild-type ($Parg^{+/+}$) ES cells ($p<0.05$, respectively). In contrast, a sensitization effect by *Parg*-deficiency was not observed with gemcitabine and camptothecin. These results suggest the possibility that functional inhibition of Parg leads to sensitization of tumor cells to some chemo- and radiation therapies.

Keywords: Poly(ADP-ribose) glycohydrolase, Knockout, ES cell, DNA damaging agent, Alkylating agent, γ-irradiation, cisplatin, 5-fluorouracil.

INTRODUCTION

Poly(ADP-ribose) glycohydrolase (Parg) [1], phosphodiesterase and ADP-ribosyl protein lyase [2, 3] are the three main groups of enzymes reported to be involved in poly(ADP-ribose) degradation. Parg specifically degrades poly(ADP-ribose) synthesized by poly(ADP-ribose) polymerase (Parp) family proteins into ADP-ribose through cleavage of the α(1''-2')glycosidic linkage [1]. Recently, an ADP-ribose-(arginine) protein hydrolase (ARH) 1 homolog, ARH3, has been identified to possess Parg activity [4]. Accumulating evidence indicates that Parg is the major enzyme for poly(ADP-ribose) degradation in cells. Poly(ADP-ribose) is suggested to promote repair DNA synthesis [5, 6].

Extensive studies suggest that inhibition of Parp-1 activity causes a sensitization effect to various types of DNA damaging agents, including alkylating agents, topoisomerase I inhibitors and γ-irradiation. Augmented sensitivity to DNA damaging agents could be explained by blockade of DNA repair pathways, including base excision repair and DNA strand break repair pathways [7, 8]. Potent Parp inhibitors are now in clinical trials to test their sensitizing effects for chemotherapeutic agents, including an alkylating agent, temozolomide, or γ-irradiation on cancers [9, 10].

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The proper equilibrium of poly(ADP-ribose) synthesis and its degradation may be required in DNA damage response. Inhibition of Parg activity is expected to result in accumulation of polyADP-ribosylated proteins and inactivation or functional alteration of these proteins, which may critically affect DNA damage response. In fact, some studies show that Parg inhibition sensitizes cells to DNA damaging agents. For example, *Parg* knockout mice disrupted at exons 2-3, lacking a full length 110 kDa isoform and expressing a mitochondrial 60 kDa isoform, showed an increased sensitivity to an alkylating agent and γ-irradiation [11]. $Parg^{-/-}$ mice lacking all isoforms exhibited early embryonic lethality and $Parg^{-/-}$ cells showed increased sensitivity to an alkylating agent and menadione [12]. Although *PARG* knockdown in human cancer cell lines and mouse embryonic fibroblasts showed protection of the cells from acute toxicity by hydrogen peroxide [13], another study reported that *PARG* knockdown in human cancer cells using siRNA resulted in decreased clonogenic survival after treatment with hydrogen peroxide [6].

Parg inhibitor *N*-bis-(3-phenyl-propyl)9-oxo-fluorene-2, 7-diamide augmented the anti-tumor effect of an alkylating agent, temozolomide, in a malignant melamoma model in mice [14]. This study is encouraging for the application of Parg pharmacological inhibition in sensitization to cancer chemotherapy. A previous study showed that Parg inhibitory compounds, gallotannin and nobotannin B, suppressed oxidative and excitotoxic neuronal cell death by preventing NAD depletion and suggested that Parg is positively in-

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volved in the Parp-1-mediated cell death pathway [15]. Mono-galloyl glucose derivatives is reported to inhibit Parg and potentially reduces *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine-induced cell death [16]. The effect of Parg inhibition on the action of various types of DNA damaging agents needs to be studied in different cell types to examine the benefit of Parg inhibition in chemo- or radiotherapies of cancer.

In the present study, we established hypomorphic *Parg*-deficient ($Parg^{-/-}$) mouse embryonic stem (ES) cell lines by gene-targeting. Poly(ADP-ribose) degradation activity is reduced to approximately one-tenth in $Parg^{-/-}$ ES cells compared to $Parg^{+/+}$ ES cells. Mouse ES cells are tumorigenic and retain properties similar to teratocarcinoma cells [17]. The sensitivity spectrum in ES cells should be therefore useful to evaluate the impact of Parg functional inhibition in the sensitivity of cancer cells to DNA damaging agents and chemotherapeutic agents. Here in this study, $Parg^{-/-}$ ES cells showed increased sensitivity to different types of DNA damaging agents including a monofunctional alkylating agent, dimethyl sulfate (DMS), cisplatin and γ-irradiation. Early and augmented formation of an oligonucleosomal DNA ladder after treatment with DMS in $Parg^{-/-}$ ES cells suggests that the apoptotic cell death process is accelerated under *Parg* deficiency. The findings suggest that Parg inhibition is useful for sensitization of cancer cells to particular types of chemotherapeutic agents as well as radiotherapy.

MATERIALS & METHODS

Generation of $Parg^{+/-}$ and $Parg^{-/-}$ ES Cell Lines

Mouse *Parg* genomic fragments were isolated by screening a 129Sv mouse BAC library using a mouse *Parg* cDNA fragment as the probe. The targeting vector was constructed as follows; a 7 kb *Eco*RV-*Xho*I fragment spanning *Parg* exon 1 was subcloned into pBlueScript with a negative selection marker, a *diphtheria toxin A* gene (*DT-A*) fragment. An N-terminal 86 bp fragment of the *β-galactosidase* gene (*lacZ*) was prepared by PCR reaction using a sense primer containing *Hin*dIII and *Nar*I sites, 5'-ACTCAGAAGCTT GGCGCCGTCGTTTTAC AACGTCGT G-3', corresponding to nucleotides 708-728 of pSV-β-gal (GenBank accession number: X65335), and an anti-sense primer, 5'-ATGGGATAGGTTACGTTG GTGTA G-3', corresponding to nucleotides 982-1005. The PCR fragment was digested with *Hin*dIII and *Eco*81I and inserted into *Hin*dIII-*Eco*81I digested pSV-β-gal (Promega) and thereby the *Nar*I site was introduced into the *lacZ* gene. A neo^r cassette (a neomycin-resistance gene driven by the MC1 promoter with SV40-derived polyadenylation signal at the 3'-terminus) from pMC1neopolyA was inserted into the *Bam*HI site downstream of the above modified *lacZ* gene and a plasmid containing the neo^r cassette flanked by the mutated *lacZ* and a *loxP* sequence was generated. The 4.5 kb fragment harboring $lacZ/loxP/neo^r$ was obtained from this plasmid by *Nar*I digestion. The 4.5 kb fragment harboring $lacZ/loxP/neo^r$ was inserted into the *Nar*I site 47 bases downstream of the first ATG in the *Parg* gene in the same orientation as transcription. This $lacZ/loxP/neo^r$ targeting vector allowed the in-frame fusion of the *lacZ* gene to *Parg* exon 1, 47 bases downstream of the translation initiation site. In addition, another targeting vector was constructed for the disruption of the remaining allele of the *Parg* gene using a puromycin resistance gene ($puro^r$) cassette [18], kindly donated by Dr. P. Laird. This 13 kb plasmid harbors the 7 kb *Eco*RV-*Xho*I fragment and the *DT-A* gene cassette as above, with replacement of the 0.4 kb *Nar*I fragment containing the first ATG with a $loxP/puro^r$ fragment. The neo^r targeting vector was linearized at the *Kpn*I site, electroporated into J1 ES cells, and then ES cells were cultured on a STO cell feeder layer in a medium [19] supplemented with 175 μg/ml G418 (GIBCO/BRL). The STO cells were kindly donated by Dr. P. Laird and STO feeder layer was prepared by treatment with 10 μg/ml mitomycin C (Sigma) for 3 hrs and washing the cells with phosphate-buffered saline (PBS) a for three times. G418-resistant clones were screened by Southern blot analysis to identify $Parg^{+/-}$ ES clones. To generate $Parg^{-/-}$ ES cell clones, $Parg^{+/-}$ ES cell clones were electroporated with the linearized $puro^r$ targeting vector. Selection was performed by culture in the presence of 0.5-1.0 μg/ml of puromycin (Sigma) and 175 μg/ml of G418.

To examine homologous recombination, Southern blot analysis was carried out using the "3'-probe" and "5'-probe", generated as follows. For the "3'-probe", a 264 bp fragment corresponding to exon 3 was prepared by PCR using a sense primer 5'-GACTCCATGATGAGTTCTGTGC-3' and an anti-sense primer 5'-ATCAGTGTGGGGTGACTGACC-3'. This fragment was hybridized to *Bam*HI-digested genomic DNA. For the "5'-probe", a 0.7 kb *Hin*dIII-*Pst*I fragment (see Fig. **1A**), was hybridized to the genomic DNA digested with *Pst*I.

To sequence the targeting junction present in intron 1 of *Timm23 (translocase of inner mitochondrial membrane 23 homolog)* gene, two primers (5'-CAGACTTCCAATTGTT ACACAAGCATC-3' and 5'-GGTCAGTTTGTC TTTAGA GTTGCAAG-3') were used to amplify the DNA fragment, and the fragment was directly sequenced using these primers. To sequence the boundary of the *Timm23* and *Parg* genes, the primers in intron 1 of the *Timm23* gene (5' -CTGTCCAGGAAGGAGTCAG-3') and in exon 1a/exon1 of *Parg* gene (5' -CCTCATTCACTAACCCGGACA-3') were used for the neo^r-allele. The above primer for intron 1 of the *Timm23* gene (5'-CAGACTTCCAATTGT TACACA AGCATC-3') and a primer for the promoter of $puro^r$ (5'-GCTGCTA AAGCGCATGCTCC-3') were used for the $puro^r$ allele.

Northern Blot and RT-PCR Analysis

Northern blot analysis of the *Parg* gene was performed using a 0.2 kbp C-terminal fragment of *Parg* cDNA using total RNA.

For RT-PCR, we used the first-strand cDNA synthesized for 5'RACE RT-PCR, and amplified using LA *Taq* with GC II buffer (TAKARA). Primer sets used to amplify specific sequences were 5'-TGCGGGTCCCAGCATGAGTGCG-3' (S1) and 5'- GAACACGCCTCTGCCTGCC-3' (A3); 5'-CGCTCCCGTCC AGTTCAGG-3' (S2) and 5'-TCTTG GGTCCTTTAGTATCCATCC-3'(A1). The primer set for amplifying *Timm23* was 5'-CAAGGAGCACTTTGGGC