were base substitutions; 20 G:C to A:T transitions (74.1%), three A:T to G:C transitions (11.1%), two G:C to T:A transversions and two G:C to C:G transversions (7.4%). These observations are consistent with the results obtained from mutation analyses of the *CTNNB1* gene in cancers from HNPCC patients; 12 reported base substitutions were as follows: TCT to TTT, CCT, or TGT at codon S45; ACC to GCC at codon T41; TCT to TGT at codon S37; GGA to GAA at codon G34; GAC to GGC or TAC at codon D32 (40). The G:C to A:T transition at codon G34 and G:C to C:G transversion at codon S37 were commonly observed in both human and mouse tumors. The difference observed between human and mouse mutation spectra may due to the different nucleotide sequence context in this locus of these two species. The similarity of the mutation types and spectra suggests that the $KBrO_3$-treatment of mice may mimic the oxidative stress in human to induce DNA damage in the intestine. We detected only two G:C to T:A transversions, thus suggesting that the DNA repair enzymes, including Ogg1 and Mutyh, may suppress 8-oxoG-related mutagenesis to some extent in $KBrO_3$-treated *Msh2*-deficient mice. Besides the one G:C to T:A transversion at the codon for S33, three types of mutations other than the G:C to A:T transition were observed at only the codon for S37; three A:T to G:C transitions, two G:C to C:G transversions, and one G:C to T:A transversion. Because these types of mutations were suppressed by the overexpression of MTH1, which hydrolyzes oxidized purine nucleotide triphosphates, in *MSH2*-deficient human cells (28, 29), the nucleotide sequence context around the codon for S37 might be competent to enhance the incorporation of oxidatively-damaged purine nucleotide triphosphates by DNA polymerase.

MMR factors are well known to be involved in the induction of apoptosis caused by O^6-methylguanine (O^6-mG), a type of DNA damage produced by alkylating agents (19-21). O^6-mG can pair with thymine during DNA replication, forming O^6-mG:T mispairs. MutSα recognizes this mispair and forms a complex with MutLα and PCNA to induce apoptosis (20). The *Msh2*-deficiency also caused a failure to induce apoptosis effectively in response to oxidative DNA damage (26). Thus, in the present study, we analyzed the cell death of the crypts of small intestines from wild type and *Msh2*-deficient mice treated with $KBrO_3$ using a TUNEL assay. A larger number of TUNEL-positive cells were observed in the crypts from wild type mice compared with *Msh2*-deficient mice, suggesting that the crypt cells with MMR-deficiencies showed an increased chance of surviving protracted exposure to $KBrO_3$. The better survival of MMR-deficient cells with pre-mutagenic lesions in the genome induced by oxidative stresses may contribute to the increased cancer risk characteristic of the hereditary non-polyposis colorectal cancer syndrome.

In conclusion, we herein demonstrated that oxidative stress enhanced the tumor formation in the small intestines of *Msh2*-deficient mice, thereby providing experimental evidence of the association between oxidative stress and hereditary non-polyposis colorectal cancer caused by MMR-deficiency in humans. We propose that MMR suppresses spontaneous tumorigenesis in the intestines of mammals by simultaneously preventing the occurrence of mutations and the removal of precancerous cells containing pre-mutagenic oxidative lesions in the genome.

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Competing Interests

The authors have declared that no competing interest exists.

References

1. Ames BN, Shigenaga MK Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA. 1993; 90: 7915-22.
2. Gajewski E, Rao G, Nackerdien Z, Dizdaroglu M. Modification of DNA bases in mammalian chromatin by radiation-generated free radicals. Biochemistry. 1990; 29: 7876-82.
3. Kasai H, Nishimura S. Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. Nucleic Acids Res. 1984; 12: 2137-45.
4. Fraga CG, Shigenaga MK, Park JW, Degan P, Ames BN. Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proc Natl Acad Sci USA. 1990; 87: 4533-7.
5. Shibutani S, Takeshita M, Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature. 1991; 349: 431-4.
6. Smith KC. Spontaneous mutagenesis: experimental, genetic and other factors. Mutat Res. 1992; 277: 139-162.
7. Radicella JP, Dherin C, Desmaze C, Fox MS, Boiteux S. Cloning and characterization of hOGG1, a human homolog of the OGG1 gene of Saccharomyces cerevisiae. Proc Natl Acad Sci USA. 1997; 94: 8010-5.
8. Rosenquist TA, Zharkov DO, Grollman AP. Cloning and characterization of a mammalian 8-oxoguanine DNA glycosylase. Proc Natl Acad Sci USA. 1997; 94(14): 7429-34.
9. Ohtsubo T, Nishioka K, Imaiso Y, et al. Identification of human MutY homolog (hMYH) as a repair enzyme for 2-hydroxyadenine in DNA and detection of multiple forms of hMYH located in nuclei and mitochondria. Nucleic Acids Res. 2000; 28:1355-64.
10. Shinmura K, Yamaguchi S, Saitoh T, et al. Adenine excisional repair function of MYH protein on the adenine:8-hydroxyguanine base pair in double-stranded DNA. Nucleic Acids Res. 2000; 28: 4912-8.
11. Slupska MM, Luther WM, Chiang JH, Yang H, Miller JH. Functional expression of hMYH, a human homolog of the Escherichia coli MutY protein. J Bacteriol. 1999; 181: 6210-3.
12. Takao M, Zhang QM, Yonei S, Yasui A. Differential subcellular localization of human MutY homolog (hMYH) and the functional activity of adenine:8-oxoguanine DNA glycosylase. Nucleic Acids Res. 1999; 27: 3638-44.

13. Mo JY, Maki H Sekiguchi M. Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool. Proc Natl Acad Sci USA. 1992; 89: 11021-5.
14. Sakumi K, Furuichi M, Tsuzuki T, et al. Cloning and expression of cDNA for a human enzyme that hydrolyzes 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. J Biol Chem. 1993; 268: 23524-30.
15. Fujikawa K, Kamiya H, Yakushiji H, et al. The oxidized forms of dATP are substrates for the human MutT homologue, the hMTH1 protein. J Biol Chem. 1999; 274: 18201-5.
16. Fujikawa K, Kamiya H, Yakushiji H, Nakabeppu Y, Kasai H. Human MTH1 protein hydrolyzes the oxidized ribonucleotide, 2-hydroxy-ATP. Nucleic Acids Res. 2001; 29: 449-54.
17. Bjelland S, Seeberg E. Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. Mutat Res. 2003; 531: 37-80.
18. Iyer RR, Pluciennik A, Burdett V, Modrich PL. DNA mismatch repair: functions and mechanisms. Chem. Rev. 2006; 106: 302-23.
19. Stojic L, Brun R, Jiricny J. Mismatch repair and DNA damage signalling. DNA Repair (Amst). 2004; 3: 1091–1101.
20. Hidaka M, Takagi Y, Takano TY, Sekiguchi M. PCNA-MutSalpha-mediated binding of MutLalpha to replicative DNA with mismatched bases to induce apoptosis in human cells. Nucleic Acids Res. 2005; 33: 5703-12.
21. Jiricny, J. The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol. 2006; 7: 335-46.
22. Fishel R, Lescoe MK, Rao MR, et al. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell. 1993; 75: 1027–38.
23. Leach FS, Nicolaides NC, Papadopoulos N, et al. Mutations of a MutS homolog in hereditary nonpolyposis colorectal cancer. Cell. 1993; 75: 1215–25.
24. Bronner CE, Baker SM, Morrison PT, et al. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. Nature. 1994; 368: 258–61.
25. Papadopoulos N, Nicolaides NC, Wei YF, et al. Mutation of a MutL homolog in hereditary colon cancer. Science. 1994; 263: 1625–9.
26. DeWeese TL, Shipman JM, Larrier NA, et al. Mouse embryonic stem cells carrying one or two defective Msh2 alleles respond abnormally to oxidative stress inflicted by low-level radiation. Proc. Natl. Acad. Sci. USA. 1998; 95: 11915-20.
27. Egashira A, Yamauchi K, Yoshiyama K, et al. Mutational specificity of mice defective in the *MTH1* and/or the *MSH2* genes. DNA Repair (Amst). 2002; 1: 881-93.
28. Colussi C, Parlanti E, Degan P, et al. The mammalian mismatch repair pathway removes DNA 8-oxodGMP incorporated from the oxidized dNTP pool. Current Biol. 2002; 12: 912-8.
29. Russo MT, Blasi MF, Chiera F, et al. The oxidized deoxynucleoside triphosphate pool is a significant contributor to genetic instability in mismatch repair-deficient cells. Mol Cell Biol. 2004; 24: 465-74.
30. Kasai H, Nishimura S, Kurokawa Y, Hayashi Y. Oral administration of the renal carcinogen, potassium bromate, specifically produces 8-hydroxydeoxyguanosine in rat target organ DNA. Carcinogenesis. 1987; 8: 1959-1.
31. DeAngelo AB, George MH, Kilburn SR, Moore TM, Wolf DC. Carcinogenicity of potassium bromate administered in the drinking water to male B6C3F1 mice and F344/N rats. Toxicol Pathol. 1998; 26: 587-94.
32. Kurokawa Y, Maekawa A, Takahashi M, Hayashi Y. Toxicity and carcinogenicity of potassium bromate--a new renal carcinogen. Environ Health Perspect. 1990; 87: 309-35.
33. Kawanishi S, Murata M. Mechanism of DNA damage induced by bromate differs from general types of oxidative stress. Toxicology. 2006; 221: 172-8.
34. Sakamoto K, Tominaga Y, Yamauchi K, et al. MUTYH-null mice are susceptible to spontaneous and oxidative stress-induced intestinal tumorigenesis. Cancer Res. 2007; 67: 6599-604.
35. Schlemper RJ, Riddell RH, Kato Y, et al. The Vienna classification of gastrointestinal epithelial neoplasia. Gut. 2000; 47: 251-5.
36. Polakis P. Wnt signaling and cancer. Genes Dev. 2000; 14: 1837-51.
37. Barker N, Ridgway RA, van Es JH, et al. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature. 2009; 457: 608-11.
38. Dolle ME, Snyder WK, Gossen JA, Lohman PH, Vijg J. Distinct spectra of somatic mutations accumulated with age in mouse heart and small intestine. Proc Natl Acad Sci USA. 2000; 97: 8403-8.
39. Al-Tassan N, Chmiel NH, Maynard J, et al. Inherited variants of MYH associated with somatic G:C-->T:A mutations in colorectal tumors. Nat Genet. 2002; 30: 227-32.
40. Miyaki M, Iijima T, Kimura J, et al. Frequent mutation of beta-catenin and APC genes in primary colorectal tumors from patients with hereditary nonpolyposis colorectal cancer. Cancer Res. 1999; 59: 4506–9.

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Short communication

A Pilot Study for the Mutation Assay Using a High-throughput DNA Sequencer

Tomonari Matsuda[1,3], Makiko Takamune[2], Yoko Matsuda[1] and Masami Yamada[2]

[1]Research Center for Environmental Quality Management, Kyoto University, Shiga, Japan
[2]National Institute of Health Sciences, Tokyo, Japan

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We present here a mutation assay with little bias which incorporates high-throughput DNA sequencing technology. Our strategy is simple: 1) expose cells to a test compound, 2) isolate colonies, and 3) carry out whole-genome sequencing of the clones. In this pilot study, we used *Salmonella typhimurium* TA100 as a tester strain and successfully detected mutations induced by the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2). We believe that this new mutation assay will be a very useful tool in hazard assessment of chemicals.

Key words: next-generation DNA sequencer, mutation assay, Ames test

Introduction

In conducting a hazard assessment for a newly developed chemical compound, mutation assays are regarded as among the most important toxicity tests. This is because mutation assays are often used as surrogate tests for carcinogenicity, and manufacturers tend to decide whether to quit or continue development based on the results of mutation assays. Thus, it is very important to develop a reliable mutation assay that is free from false positive and false negative results.

In most of the currently used mutation assays, mutations in a specific gene (target gene) are detected by monitoring a phenotypic change in the cells. As an example, the Ames test, which was developed in 1970's, is the most widely used mutation assay. The tester strain *Salmonella typhimurium* TA100 has a point mutation in its *hisG* gene at codon 69 and cannot grow and form colonies on a minimal agar plate lacking histidine. However, if a reverse mutation occurs in the same codon of the gene, the revertant is now able to form colonies on minimal medium. Thus, mutagenicity is evaluated by counting the revertant colonies induced by a test compound. Although this is a very simple and inexpensive test system it has many biases, mostly related to the base sequence and the types of mutations that can arise at the target site. For example, codon 69 of the *hisG* gene of TA100 is CCC, so that this tester strain preferentially detects mutations at G:C base pairs, not A:T base pairs, and because of this sequence bias, it is recommended to use several other strains in addition to TA100 which can detect different mutation spectrum such as TA98, TA102, and TA1537.

This inconvenience motivated us to develop a new forward mutation assay that does not rely on any phenotypic selection. Our strategy is simple: 1) expose cells to the test compound, 2) isolate colonies, and 3) carry out whole-genome sequencing of the clones. This strategy became possible only after recent technical advancements in performance of next-generation DNA sequencers and reductions in running costs. Although any cell type, including human cell lines, may be used in this system, we began with *Salmonella typhimurium* TA100 so that we could compare our new strategy and the conventional Ames test. Another reason for choosing a bacterial strain is its small genome size and monoploidy that facilitates this strategy.

Materials and Methods

The Ames test was carried out with TA100 as the tester strain and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2; 0.01 μg/plate) as the test chemical. AF-2 was purchased by Wako (Osaka, Japan). S9 mix was not applied in this test. Experimental details are provided at "supporting information" (http://www.j-ems.org/journal/supporting.html).

Ten revertant colonies were randomly isolated, five from the solvent control group and five from the AF-2-exposed group, and subjected to DNA purification. Whole-genome sequencing of each of the 10 clones was carried out in a single run by using a high-throughput DNA sequencer, MiSeq (Illumina, San Diego, CA). The sequencing data compiled to fastaq files were analyzed using CLC Genomics Workbench ver.5 software (CLC

[3]Correspondence to: Tomonari Matsuda, Research Center for Environmental Quality Management, Kyoto University, 1-2 Yumihama, Otsu, Shiga 520-0811, Japan. Tel: +81-77-527-6224, Fax: +81-77-524-9869, E-mail: matsuda.tomonari.8z@kyoto-u.ac.jp
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bio A/S, Aarhus, Denmark). First, the multiplex raw data were divided by sample name, and low-quality sequencing data were trimmed, depending on quality scores. The cleaned-up sequencing data were then mapped to the following reference sequences: NC_003197 (*Salmonella typhimurium* str. LT2 chromosome, complete genome, 4,857,432 bp), AY046276 (IncN plasmid R46, complete sequence, 50,969bp) and CP003387 (*Salmonella typhimurium* str. 798 plasmid p798_93, complete sequence, 93,877 bp). Using these mapped data, base substitution mutations were detected by the "SNP detection" command, small insertions and deletions were detected by the "DIP detection" command, and large insertions and deletions were detected by the "Structural Variation Detection—Coverage Analysis" command of CLC Genomics Workbench ver.5.

Results and Discussions

AF-2 showed mutagenicity in TA100 strain as expected. The mean number of revertant colonies obtained for the solvent control (DMSO) was 141 ± 14/plate ($n=3$), and for the AF-2-exposed group it was 530 ± 40/plate ($n=3$).

Whole-genome sequencing of randomly selected 10 clones was carried out. For all 10 clones, the sequence data were mapped over the whole genome, and the average coverage of each clone ranged from 18.4× to 42.7×, and the average of the ten clones was 31.5× as shown in Table S1 (Supporting information, http://www.j-ems.org/journal/supporting.html). The differences in genomic sequences between the reference strain *Salmonella typhimurium* LT2 and TA100 are shown in Tables S2 and S3 (Supporting information). In addition to these differences, a large deletion (49,906 bp) including DNA excision repair gene, *uvrB* was observed in TA100 which is consistent with the previous report (1). The genes encoded in this deleted region are shown in Fig. S1 (Supporting information).

The mutations detected in the AF-2 and solvent (DMSO) groups are listed in Tables 1 and 2, and the mapping data is shown in Fig. S2 (Supporting information). Table 1 lists the mutations in codon 69 of the *his*G gene, in which the TA100 sequence is CCC (Pro). Mutations were detected in all the clones tested, meaning that phenotypic reversion was confirmed by alterations of the genetic sequence. However, there was only one true revertant clone with the wild-type sequence CTC (Leu); the other 9 clones had codon-69 sequences that specify other amino acids. The observed base substitutions were C→A, C→T, C→G at base position 205 and C→A, C→T at base position 206. These base substitutions have been reported in *hisG* gene of some revertant colonies (2).

Table 2 lists the mutations observed at other genomic sites. Only one clone in the DMSO-treated group had an additional mutation, whereas five unique mutations were observed in three clones of the AF-2-treated group.

Table 1. Mutations observed in codon 69 of the *hisG* gene

Treatment	Clone ID	Frequency (%)	Coverage	Gene	Site	Mutation	Amino Acid Change
DMSO	1	100	14×	hisG	206	C>A	Pro69His
	2	100	17×	hisG	205	C>A	Pro69Ile
	3	100	14×	hisG	206	C>A	Pro69His
	4	100	14×	hisG	205	C>A	Pro69Ile
	5	100	17×	hisG	205	C>T	Pro69Phe
AF-2	6	100	29×	hisG	206	C>A	Pro69His
	7	100	15×	hisG	206	C>A	Pro69His
	8	100	37×	hisG	206	C>T	Pro69Leu
	9	100	10×	hisG	205	C>T	Pro69Phe
	10	100	26×	hisG	205	C>G	Pro69Val

Table 2. Mutations observed at other sites

Treatment	Clone ID	Frequency (%)	Coverage	Gene	Site	Mutation	Surronding seq.	Amino acid change
DMSO	2	100	31×	hypE	831	T>A	GATTGCC	
AF-2	6	100	29×	yraP	25	G>A	GCAGTCC	Val9Ile
	8	96.7	30×	cueO	964	C>T	CCGCTGC	
	8	100	29×	ybiR	832	C>A	GCACTGT	Leu278Met
	8	100	38×	xseA	996	C>G	GGCCAGG	
	10	100	27×	hisG	231	G>C	GCTGGAA	

Of these six mutations (AF-2 + control), four were silent mutations and so did not produce any amino acid changes. We also looked for small insertions/deletions and large insertions/deletions, but could not find any such changes induced by AF-2.

The mutational frequencies/genome were calculated as 0.2 in the DMSO-treated group, and 1.0 in the AF-2-treated group. These can be further expressed as 4.2 mutations/10^8 bp in the DMSO group and 21 mutations/10^8 bp in the AF-2 group. In this calculation, mutations in *hisG* gene codon 69 were not included.

As for the mutation spectrum, the data in Table 1 should also be omitted because the mutational target is limited to *hisG* codon 69, and this will cause strong bias. Looking at Table 2, there is only one mutation in the DMSO-treated group, at an A:T base pair. On the other hand, in the AF-2-treated group, all five mutations occurred at G:C base pairs and there were two G:C→A:T (40%), one G:C→T:A (20%) and two G:C→C:G (40%) mutations, which is somewhat consistent with a previous report about the mutation spectrum induced by AF-2 in the *lacI* gene of *Escherichia coli* (3) [G:C→A:T (33.8%), G:C→T:A (52.4%), G:C→C:G (6.9%), A:T→T:A (6.2%), A:T→G:C (0.7%)]. Also listed in Table 2 are the sequences surrounding the mutation sites. AF-2-induced mutations with some sequence preference (Fig. S3); however, more data will be required to determine the preference with precision.

As this new assay uses high-throughput DNA sequencing to detect mutations without phenotypic selection, it is thought to be a bias-free method. What further issues need to be considered so as to enhance the practicality of this new mutation assay? In this pilot study, we selected revertant colonies to confirm detectability of mutations by monitoring *hisG* codon-69 mutations, and we successfully detected those mutations in all 10 clones tested. Thus, having proved the principle of the method, we suggest that from now on it is better to produce colonies on a rich medium such as LB and randomly pick colonies for completely non-biased mutation detection.

Another issue is how to establish the criteria to define positive and negative results. To develop the criteria it is necessary to have an accurate estimate of the spontaneous mutation frequency. The only way to obtain this is to increase the denominator, i.e., combine all future data on spontaneous mutations. Assuming we are able to determine the 'real' spontaneous mutation frequency, we could then calculate the probability that all of the observed mutations are spontaneous mutations. Spontaneous mutation is rare event and occurs randomly, so that it follows the Poisson distribution [1] shown below:

$$P(X=k)=\frac{\lambda^k}{k!}e^{-\lambda} \quad [1]$$

Here, λ is the number of expected spontaneous mutations, and k is the number of observed mutations. The probability that all of the observed mutations are spontaneous mutations can be calculated by using equation [2]:

$$P(X\geq k)=1-P(X\leq k-1)=1-e^{-\lambda}\sum_{i=0}^{k-1}\frac{\lambda^i}{i!} \quad [2]$$

Assuming that the spontaneous mutation frequency observed in this pilot study is close to the true rate, it is one mutation per five clones (i.e., $\lambda=1$). On the other hand, five mutations were found in the AF-2-treated group per five clones (i.e., $k=5$; the *hisG* mutations were not included in this calculation). In this situation, the probability that all five mutations were of spontaneous origin is calculated as $P(X\geq 5)=0.0037$, meaning it is very likely that the mutations were actually induced by AF-2. An Excel sheet for calculating the probability is shown in Table S4 (Supporting information). If you input the λ value, $P(X\geq k)$ values are automatically calculated. By using the $P(X\geq k)$ value, we can decide positive or negative results, according to the proper threshold such as $P\leq 0.05$ or 0.01.

In summary, high-throughput next-generation DNA sequencing technology can be the basis of a practical mutation assay which delivers bias-free mutation spectra and frequencies. The same strategy as outlined here can be applied to other organisms such as yeasts or human cell lines. We predict that this strategy will become a gold standard for genotoxicity tests in the not-too-distant future.

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References

1 Porwollik S, Wong RM, Sims SH, Schaaper RM, DeMarini DM, McClelland M. The *ΔuvrB* mutations in the Ames strains of Salmonella span 15 to 119 genes. Mutat Res. 2001; 483: 1-11.

2 Knasmüller S, Zöhrer E, Kronberg L, Kundi M, Franzén R, Schulte-Hermann R. Mutational spectra of Salmonella

typhimurium revertants induced by chlorohydroxyfuranones, byproducts of chlorine disinfection of drinking water. Chem Res Toxicol. 1996; 9: 374–81.

3 Bertenyi KKA, Lambert IB. The mutational specificity of furazolidone in the lacI gene of Escherichia coli. Mutat Res. 1996; 357: 199–208.

Genes and Environment, Vol. 35, No. 3 pp. 63–68 (2013)

Commentary

Unconscious Exposure to Radiation

Takayoshi Suzuki[1]

[1]Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, Tokyo, Japan

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When we consider the risk of radiation caused by the Fukushima Daiichi Nuclear Plant accident, we may feel the situation to be much like the formation of rain spots on a car. The dirty spots are difficult to tolerate by the owner of a brand-new car but can be accepted by a used car owner who does not clean his or her car frequently. In the course of collecting information to prepare a webpage concerning radiation risk on the Japanese Environmental Mutagen Society (JEMS) homepage following the Fukushima accident, I have learned that we have already unconsciously been exposed to an unexpected level of radiation. Therefore, our body is not like that brand-new car affected by rain spots or, in this case, radioactive contamination. We are internally exposed to ^{40}K radiation through the foods we eat on a daily basis, and we have already been exposed to the 1,000–10,000 times higher background of the nuclear fallout that occurred during the 1960s because of world-wide nuclear bomb experiments. It is important to know these facts to consider the excess risk derived from the Fukushima accident and thereby learn to be more cautious. Obtaining a proper answer scientifically about the health effects of low-level radiation exposure is very difficult when using available data on radiation biology. Increasing risk awareness and communication is also important together with proving the real risk of low-level radiation. Radiation risk should be considered in a relative manner by comparing it with other confounding factors, which together can be treated as a total risk. The increased risk posed by radiation exposure can be traded-off by reducing other risk factors affecting our lifestyle. The most important task for us is to transfer available scientific knowledge to the public such that the information is more understandable to help people make their own decisions on how to face radiation risk.

Key words: radiation risk, risk communication, risk assessment, Fukushima nuclear accident, low dose radiation

Introduction

Following the accident at the Fukushima Daiichi Nuclear Plant on March 11, 2011, public concern was focused on radiation risk. Because of the tragedies of Hiroshima/Nagasaki and Chernobyl, people fear the invisible risk of radiation. The pacifying comments (hiding of truth) provided by radiation specialists regarding the safety of nuclear plants after the accident compromised their reliability. In addition, there is a conflict even among radiation biologists regarding the estimation of the radiation risk caused by the accident. Therefore, an important task for scientists is to explain the level of radiation risk that actually exists in a more understandable manner.

When the accident occurred, I was a member of the public relation office of the Japanese Environmental Mutagen Society (JEMS). I then decided to create a webpage on the risk of radiation to provide useful information to the public. Throughout the course of preparing the page, I have learned many important facts about radiation exposure that I did not know before because I am not a radiation biologist. The most striking piece of information for me was the high background of the nuclear fallout that occurred during the 1960s.

Unconscious External Exposure

During the late 1950s to early 1960s, many nuclear bomb experiments were carried out worldwide by the US, the Soviet Union, and the UK, followed by France and China. The experiments were initially performed in the atmosphere and released an enormous amount of radioactive nuclides all over the world. In Tokyo, the nuclear fallout level reached its maximum in 1963, which was 1,000–10,000 times higher than the normal background before the Fukushima accident. The high fallout level has gradually decreased but has persisted over the decades.

Data on the environmental radiation level in the past and present are available at the Japan Chemical Analysis Center, which is directed by the Nuclear Regulation Authority (1). Although the species of nuclear fallouts from the nuclear bomb or the nuclear plant accident are different, Cs-137 (^{137}Cs) is a common concern for a long-lived radioactivity. Figure 1 shows the annual changes

[1]Correspondence to: Takayoshi Suzuki, Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel/Fax: +81-3-3700-1926, E-mail: suzuki@nihs.go.jp
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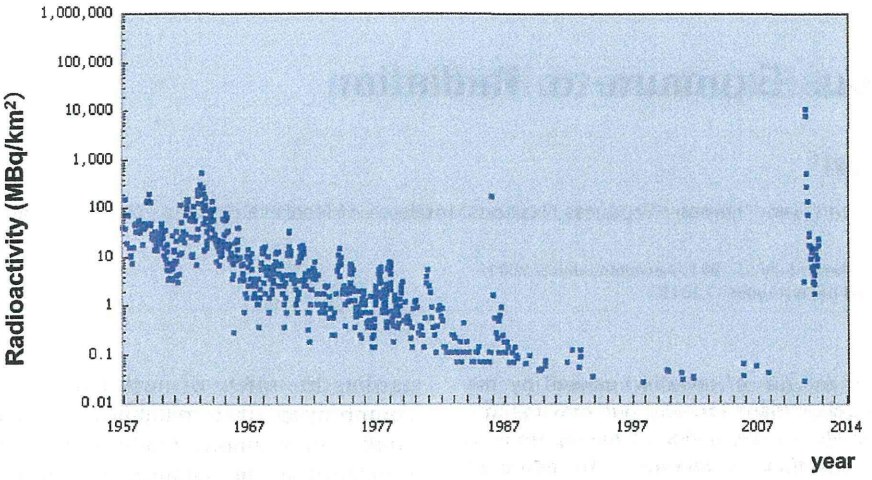

Fig. 1. (Color online) Annual changes in monthly nuclear fallout (^{137}Cs) level in Tokyo. The graph was created at the database site of the Nuclear Regulation Authority at http://search.kankyo-hoshano.go.jp/servlet/search.top (on June 5, 2013). Data are available for the period 1957–2012. Peaks in radioactivity are shown to have occurred in 1963 (atmospheric nuclear bomb experiments), 1986 (Chernobyl accident), and 2011 (Fukushima accident).

in the monthly nuclear fallout (^{137}Cs) from 1957 to 2011 in Tokyo. The data show that the peak after the Chernobyl accident (1986) is very similar to that in 1963, when the fallout from nuclear bomb experiments reached its maximum. However, after the Fukushima accident in 2011, the peak reached a level more than 10 times higher. Therefore, we have been exposed to the highest level of fallout in history in the wake of the Fukushima accident. This fact should have been announced at the time of the accident to reduce outdoor exposure as much as possible, especially during rain. Although the same data for Fukushima in 2011 were not available on the website of the Japan Chemical Analysis Center, the fallout level is considered to be at least higher than that in Tokyo. Fallout in Fukushima has nearly stopped now but the high level of fallout has already settled down in the area surrounding the nuclear plant, making it difficult for residents living within 20 km of the plant to return to their homes. Removing these sedimented radioactive nuclides remains an important task for the Japanese government.

I was born in 1962, when the nuclear fallout reached its maximum level. Therefore, I grew up under the high background radio contamination during the so-called sensitive younger generation. At that time, unfortunately, environmental pollution from industries was much worse than it is now in Japan. Thus, our generation has grown up in a dangerous environment but without any apparent health defects, such as increased incidence of cancer. There is a chance that a careful epidemiological survey according to annual birth groups to compare health defects in relation to radiation exposure may provide informative data on the effects of nuclear fallout in future.

In addition to artificial nuclear fallouts, we are also exposed externally to natural radiation sources such as radon, radium, and potassium from the ground and cosmic radiation from space. People believe that hot springs containing radon or radium are good for their health, but these springs exhibit a certain level of radioactivity (0.1–10 μSv/h). Annually, we are exposed to 0.48 mSv of radiation from the ground (natural rock). The radiation background is high at the Japanese parliament building due to the potassium-rich feldspar containing granite used for its construction. A transcontinental flight by aircraft causes approximately 0.1 mSv of exposure. In total, ordinary people are exposed externally to 2.1 mSv of radiation annually (world average), which exceeds the recommended maximum annual additional exposure level of 1 mSv set by the International Commission on Radiological Protection (ICRP) (2). Residents living in the high-radiation-background area in Kerala, India, are exposed to up to 5 mSv of radiation annually, but there is no evidence for increased incidence of cancer among the residents.

In addition, we are exposed to radiation when undergoing X-ray and computed tomography (CT) scans. A single chest CT scan causes 6.9 mSv of radiation and an average medical exposure in Japan is about 4 mSv, which is far beyond the recommended maximum annual exposure limit. The risk of medical radiation exposure should be a greater public concern because the dose level is relatively high.

Unconscious Internal Exposure

In addition to external exposure, we are exposed internally to natural sources of radiation. Potassium is a one of the essential elements for humans and one of

three of the most important nutrients for plants. Therefore, virtually every food contains potassium. Importantly, potassium has a radioactive isotope, potassium-40 (^{40}K), that is found naturally with an incidence of 0.0117%. Generally, we take more than 1 g of potassium from everyday foods, and a standard adult weighing 60 kg has approximately 4,000 Bq of radioactivity due to ^{40}K in his or her body, where the unit Bq denotes nuclear decay events per second; therefore, we are exposed to 4,000 ^{40}K decays every second. Approximately 89% of ^{40}K forms ^{40}Ca by beta decay, and the remaining 11% decays to ^{40}Ar by electron capture and the emission of gamma rays (Fig. 2). Therefore, our body is always internally exposed to a certain level of radiation.

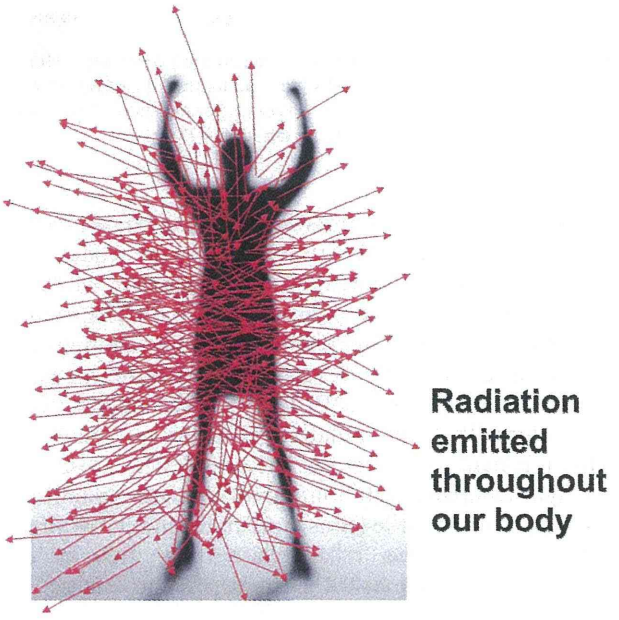

Fig. 2. (Color online) An illustration of gamma rays released throughout the human body. Because of internal exposure of ^{40}K, 4000 Bq radiation in human body (60 kg) is releasing. Among them, approximately 10% (400 Bq) is the γray which passes and released from the body as illustrated although they are not visible.

In addition to natural sources of radiation, we have been exposed to artificial radiation through foods contaminated by nuclear fallout. Data regarding the radioactivity of foods are available at the same Nuclear Regulation Authority Database site (http://search.kankyo-hoshano.go.jp/food). When the annual changes in the radioactivity (^{137}Cs) of tea leaves in Shizuoka is plotted (Fig. 3), for example, it is clearly seen that the level of radioactivity was high when the level of nuclear fallout was high. It exceeded the current food regulation level of 100 Bq/kg for ^{137}Cs in 1963, which is similar to the levels observed in the aftermath of the Chernobyl and Fukushima accidents. In contrast, the radioactivity of ^{40}K has been constant at approximately 100 Bq/kg. It is more difficult to suppress the radioactivity level in dried food by per kg unit than in normal foods. Considerations should be made based on the amount of food consumed.

In conclusion, we are exposed internally to ^{40}K radiation through everyday foods.

Relative Risk

In my presentation at the JEMS open symposium, 2013, I introduced an antique wine glass composed of what is called ''uranium glass'' as an example of familiar radiation sources. The glass contains uranium, which fluoresces under UV light (Fig. 4). Uranium glass

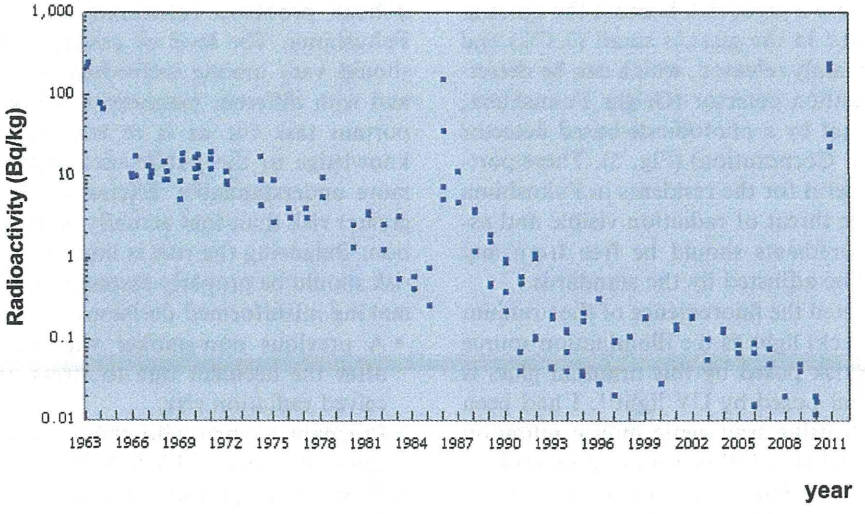

Fig. 3. (Color online) Annual changes in ^{137}Cs in tea leaves in Shizuoka. The graph was created at the database site of the Nuclear Regulation Authority at http://search.kankyo-hoshano.go.jp/servlet/search.top (on June 5, 2013). Data are available for the period 1963–2011.

Fig. 4. (Color online) Uranium glass which illuminating fluorescence under UV light exposure.

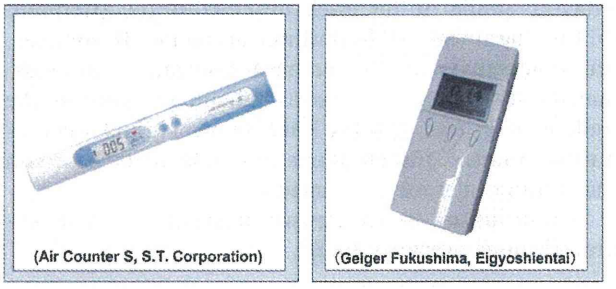

Fig. 5. (Color online) Portable radiation counters: (left) photodiode type (Air Counter S, S.T. Corporation) and (right) Geiger type (Geiger Fukushima, Eigyoshientai).

is popular among antique collectors and has been used frequently in the past. Uranium itself presents a real hazard by releasing beta and gamma rays. Using uranium glass does not pose a great risk because the amount of uranium contained in the glass is small (0.1%) and only beta rays are mainly released, which can be detected by a Geiger radiation detector (Geiger Fukushima, Eigyoshientai) but not by a photodiode-based detector (Air Counter S, S.T. Corporation) (Fig. 5). These portable detectors are useful for the residents in Fukushima to make the invisible threat of radiation visible and assessable. The measurements should be free from any miss-operation and be adjusted by the standards.

When I demonstrated the fluorescence of the uranium glass, I used UV (black) light as the illumination source and said that "the risk posed by this uranium glass is much lower than that posed by UV light". I had been involved in research using transgenic mouse mutation assays and understand that UV is a more powerful inducer of gene mutations than X-rays. Ono *et al.* reported on the mutagenicity of X-ray and UVB radiation in the transgenic MutaMouse (3). An increased mutation

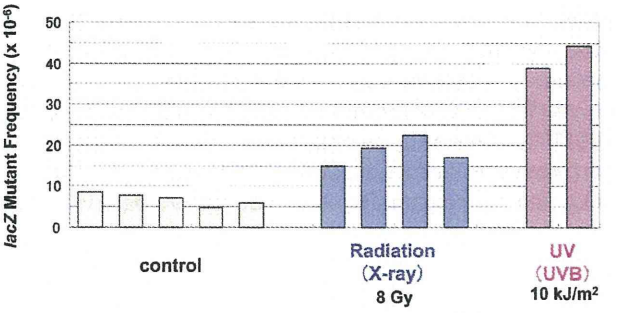

Fig. 6. (Color online) *LacZ* mutant frequency in skin of MutaMouse after X-ray (8 Gy) or UVB (10 kJ/m^2) radiation. The mutant frequency of *lacZ* gene was analyzed 1 week after exposure. The graph was adopted from data obtained by Ono *et al.* (3).

frequency (MF) (a few times) was observed in skin after applying a lethal dose (8 Gy) of X-ray radiation; however, an approximately 6-fold increase in MF was observed in skin after applying 10 kJ/m^2 of UVB radiation (Fig. 6), a dose level easily achieved by sunlight exposure for a few hours in summer. Therefore, the gene mutations induced by UVB light is much higher than that induced by X-ray radiation. However, not much attention is paid to the risk for the genotoxicity posed by UV light compared with that posed by X-ray radiation. The risk of low dose levels is more understandable when compared with other risks (relative risk) than as an absolute value, such as 0.1% excess risk for cancer.

Way of Considering Risk

After understanding the limitation of the current level of science in making a conclusion about the real risk of low-level radiation, I feel that specialists in risk assessment and communication or regulatory science are more important than radiation biologists in solving the difficult problems concerning radio contamination in Fukushima. The level of concern about the same risk should vary among individuals in different situations and with different manners of thinking. The most important task for us is to transfer available scientific knowledge to the public such that the information is more understandable. Excessive fear of radiation is a greater risk than that actually posed by low-level radiation. Balancing the risk is important (4), and radiation risk should be properly assessed to prevent people from making misinformed decisions, for example,

- A previous non-smoker who has started smoking after the accident due to stress caused by the perceived radiation risk;
- Pregnant women who choose to have abortions because of a fear of birth defects;
- Hospitalized patients in a serious condition who are forced to move away from the contaminated area.

Because the population in Fukushima is approximately

2 million, 2000 deaths are expected if an excess risk of mortality is 0.1% (although it is impossible to prove scientifically). If the risk is considered in this manner, the excess risk may not be considered acceptable. It is recommended that the situation be thought in a different way: 0.1% excess risk of mortality, for example, shortens the lifespan of a 40-year-old person by 2 weeks on average (although it may still not acceptable for a part of persons). The risk should be considered together with other risk factors. Therefore, the way in which risk is considered and communicated is important. In addition, the importance of risk education should be emphasized. We do not learn about the basics of radiation risk and biology at school. Thus, we are not good at considering and managing risk. The real risk of low-level radiation should be taught in school to be able to manage radiation risk in the future.

After the accident, I was embarrassed to see an article in a weekly magazine titled "20 years later in Japan, cancers, malformations, strange diseases, and mental retardations". Mass media in Japan has frequently overemphasized radiation risk and causing anxiety among citizens. Sohei Kondo said in his book (5) about the problem in mass media of aggravating the perceived risk of radiation, "However, I do not agree to punish the mass media for their exaggerated reports that caused a radiation phobia. The primary cause of the radiation phobia is a concept 'lowest level of radiation still has toxicity,' which got to be a common sense. This is a fundamental thinking for a specialist for radiation protection, for which mass media broadcasts extravagantly. Radiation specialists are lacking their efforts on telling exposed residents that there is no scientific evidence on the miss-concept and real information on the safety of the low level radiation by a plain and precise manner".

Unresolved Questions on Radiation Risk

What level of radiation risk was posed to those who grew up in the 1960s?: Evidence shows that there was no apparent adverse effect among children due to the global nuclear fallout, but an excess incidence of cancer was reported for residents living near the Nevada test site (6). Although the incidence of leukemia, which can be detected early, did not clearly show an increase due to the global nuclear fallout, solid cancers, which are late-onset, can only be analyzed among individuals who were exposed to the higher background during their childhood in the 1960s because they will enter cancer-prone ages in the coming years. The results of such analysis may provide a scientific basis for conducting a risk assessment of low-dose radiation in relation to the Fukushima accident.

It has also been recently reported that there was a 12 % excess relative risk of childhood leukemia per millisievert of cumulative red bone marrow dose from gamma radiation (including radon) in a control case study of natural background radiation in Great Britain (7).

What is the scientific evidence that supports the ICRP recommendation of 1 mSv as the acceptable annual effective dose for radiation exposure?: A Japanese regulatory decision against radiation protection was made based on the recommendation of the ICRP. The recommendation was made based on available scientific data. However, it should be noted that there is scientific uncertainty involved, such as that associated with the linear non-threshold model (LNT). People tend to believe the value to be an absolute and authoritative one, but the scientific uncertainty involved should be explained and resolved. This is an important task for regulatory scientists.

When a resident in Fukushima develops cancer, can we say that it has no relation to the excess radiation cause by the accident?: Because the incidence of cancer in normal Japanese is approximately 50%, one out of two persons in Fukushima develops cancer. Even if the involvement of excess radiation dosage is very low, it is impossible to be considered zero. It seems natural that residents in Fukushima feel that the radiation caused their cancer. We need to have a scientific tool to determine the involvement of radiation in the development of their tumors, such as a molecular signature of radiation damage such as a specific gain of chromosome band 7q11 (8), or sequence specific deletions.

Concluding Remarks

- A paradigm shift in risk assessment from "zero" to "acceptable" risk is necessary.
- Radiation risk should not be considered alone but as a total risk involving several factors.
- Relief and safety are independent. The concept of acceptable risk varies among individuals. Therefore, relief is more desired than safety.
- We should consider the unconscious exposure to radiation mentioned in this article.
- The most important task for us is to transfer available scientific knowledge to the public such that the information provided is more understandable and provides people the opportunity to make their own decisions.
- Within the 10-km area surrounding the nuclear plant, the ambient radiation dose is 10- to 100-fold higher than it was before the accident, with the area showing hot spots 1,000 times higher in intensity (June 2014). Continuous efforts should be undertaken to reduce ground contamination, possibly by inverting soil or covering it with concrete.

References

1 Nuclear Regulation Authority "Database on environmental radioactivity and radiation in Japan" http://search.kankyo-hoshano.go.jp/servlet/search.top,(ref 13-06-06).
2 ICRP, 2007. 2007 Recommendations of the International

Commission on Radiological Protection (Users Edition). ICRP Publication 103 (Users Edition). Ann. ICRP 37 (2–4).
3 Ono T, Ikehata H, Hosoi Y, Shung BS, Kurishita A, Wang X, et al. X-ray- and ultraviolet-radiation-induced mutations in Muta mouse. Radiat Res. 1997; 148: 123–8.
4 Kasamatsu T, Kohda K. Balancing risks. Regul Toxicol Pharmacol. 2006; 46: 100–4.
5 Kondo S. "Hito ha Housyasen ni Naze Yowaika (why human is weak against radiation) 3rd ed." (in Japanese), Blue Backs, Tokyo: Kodansha; 1998.
6 Simon SL, Bouville A, Land CE. Fallout from nuclear weapons tests and cancer. American Scientist. 2006; 94: 48–57.
7 Kendall GM, Little MP, Wakeford R, Bunch KJ, Miles JC, Vincent TJ, et al. A record-based case-control study of natural background radiation and the incidence of childhood leukaemia and other cancers in Great Britain during 1980–2006. Leukemia. 2013; 27: 3–9.
8 Hess J, Thomas G, Braselmann H, Bauer V, Bogdanova T, Wienberg J, et al. Gain of chromosome band 7q11 in papillary thyroid carcinomas of young patients is associated with exposure to low-dose irradiation. Proc Natl Acad Sci USA. 2011; 108: 9595–600.

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タンパク質・内在性代謝物バイオマーカーを利用した医薬品開発の活性化にむけて

斎藤嘉朗#，前川京子，齊藤公亮，佐藤陽治，鈴木孝昌

Toward acceleration of drug development with proteomic and metabolomic biomarkers

Yoshiro Saito#, Keiko Maekawa, Kosuke Saito, Yoji Sato, Takayoshi Suzuki

Biomarkers, reflecting disease states or predicting/assessing drug efficacy or adverse reactions, are expected to play pivotal roles in effective drug development and promoting proper usage of drugs. To accelerate biomarker identification and usage, administrative guidance can direct to design appropriate exploration, validation and utilization studies and show examination procedures. However, very limited number of guidance or its draft were released from Japanese, US and European regulatory authorities so far. From 2012, we have been conducting proteomic and metabolomic studies using blood and urine samples from human and rat, in order to establish draft guidance for sampling/storage of these biofluid and for extrapolation of biomarker candidates from animals in the non-clinical to humans in the clinical studies. The results are still partial and the rest of the analysis is ongoing. However, we developed sensitive proteomic system for urine and found large inter-sex differences in the proteomic profiles of rat. In addition, matrix-, sex- and generation-differences were also observed in the metabolite levels in human blood, some of which showed over 2-fold differences. We continue this regulatory science studies for contribution to accelerated novel biomarker findings and its usage by generation of the draft guidance.

Keywords: Biomarker, Biofluid, Drug development, Metabolomics, Proteomics

はじめに

バイオマーカーは，「客観的に測定され，評価される特性値であり，正常な生物学的プロセス，病理学的プロセス，または治療的処置に対する薬理学的反応の指標」と米国のバイオマーカー定義ワーキンググループにて定義されている[1]．臨床的な最終評価指標を，早期に，簡便に，かつ頑健に反映するサロゲート（代替え）マーカーとしての利用が医薬品開発において始まっている．疾患の状態や医薬品の有効性確保および安全性向上のための指標となり，医薬品開発が効率化されるため，バイオマーカーを利用した世界の臨床試験年間登録数は急増している[2]．さらに医薬品の市販後においても，各患者における有効性を最大限に確保し，副作用を最小限に抑えるために，その利用が期待されている．重篤な副作用は死亡や重い後遺症につながることもあり，バイオマーカーの診断費用を含めても医療経済学的に有用との研究結果もある[3]．

バイオマーカー自体は，新しい概念ではなく，例えば肝障害におけるアラニンアミノトランフフェラーゼ（ALT）や腎障害におけるクレアチニンなど，古くから用いられているものも多い．しかし臓器障害がある程度重篤になってからしか上昇が見られない，または臓器特異性が低いなど，問題が多いマーカーも使われており，臨床現場では問題となっている．そのため，より早期に軽症の段階で検出しうる新規バイオマーカーの探索が活発に行われている．

バイオマーカーとしては，遺伝子多型やmRNAレベル等のゲノムバイオマーカーに関する検討が多くなされており，近年ではマイクロRNAも注目されている．しかし，遺伝子多型に関しては，ヒトに固有であり，一部の代謝酵素等を除いて非臨床試験段階で検討することは難しく，臨床試験段階で初めて十分なデータが得られる

To whom correspondence should be addressed:
Yoshiro Saito; Division of Medicinal Safety Science, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; Tel: +81-3-3700-9528; Fax: +81-3-3700-9788; E-mail: yoshiro@nihs.go.jp

ため，医薬品開発の初期から利用することは難しい．一方で，血液や尿などの体液に含まれるタンパク質[4]や内在性代謝物[5]は，種差が報告されている分子が一部存在するものの，非臨床で用いられる動物から，臨床におけるヒトへの外挿は，多くの場合，理論的に可能と考えられる．

1．バイオマーカーとしての要件

医薬品開発において有用なバイオマーカーは，

1）種差なく共通して変化し，非臨床から臨床への外挿が可能，
2）疾病や医薬品の有効性および安全性と，その早期段階から感度・特異度高く相関，
3）食事や運動等の環境要因を含め，目的とする要因以外による影響を受けにくい，

ものと考えられる．さらにバイオマーカーを適切に評価するためには，正確度および精度高く測定することが必要であり，これには

4）生体試料を測定する機器や方法（バイオアナリシス）の要件
5）測定する試料の品質に関する要件

も重要となる．しかしバイオマーカーの利用はまだ緒に就いたばかりであり，各製薬会社がその利用方法を模索している状態である．

2．行政的な動向

各国の規制当局である日本の厚生労働省/医薬品医療機器総合機構，米国・食品医薬品庁（FDA），欧州・医薬品庁（EMA）もバイオマーカーについては注目しており，その積極的な利用を促進するため，いくつかのガイダンス等が公開されている．しかし，そのほとんどはゲノムバイオマーカーを対象にしており，タンパク質や内在性代謝物は対象外となっているものが多い．また，手続きに関する記載が多く，評価要件など技術的なものは少ない．

ゲノムバイオマーカーに関しては，その定義を「正常な生物学的過程，発病過程，及び／または治療的介入等への反応を示す指標となる，DNAもしくはRNAの測定可能な特性」としたゲノム薬理学における用語集について(ICH E15)[6]を始め，ゲノムデータの申請方法(FDA)[7]，バイオマーカーと診断法の同時開発（EMA）[8]，臨床試験の研究デザインやデータ解析方法（EMA，FDA）[9,10]，薬物動態解析におけるゲノムバイオマーカー利用のスキーム（EMA）[11]，医薬品の製造販売後監視におけるゲノムバイオマーカー利用の課題（EMA）[12]，試料採取や保管・測定法・データ処理（EMA，FDA）[13,14]，に関するガイダンス（または案，コンセプトペーパーやリフレクションペーパーを含む）が公開されている．

一方，タンパク質や内在性代謝物に関してのガイダンスは，ほとんど無い．適格性確認のための資料における用法の記載要領，構成及び様式を定めた「医薬品またはバイオテクノロジー応用医薬品の開発におけるバイオマーカー（ICH E16）」[15]はゲノム以外のバイオマーカーを明示的には適用範囲としていないが，この文書に記載される原則は，タンパク質等の他のバイオマーカーについても適用可能としている．これ以外には，組織学的知見を陽性対照としたFDAの概要的ガイダンス案[16]しかなく，実データを反映した明確な評価要件がないため，その探索・検証・利用は個別に模索している状態である．

このようにバイオマーカーの有用性を担保するための評価要件が確立していない状況では，不適切な試料の利用や不的確なバイオマーカーの利用により，かえって医薬品開発の遅延や混乱を招く可能性がある．今後は，ゲノム同様，タンパク質や内在性代謝物に関しても，測定試料の品質要件を始めとする多くのガイダンスを策定する必要がある．しかし，その策定の基礎となるデータは非常に乏しいのが現状である．

3．体液中のタンパク質および内在性代謝物をバイオマーカーとする場合の，ガイダンス案策定に向けて

3－1．研究班の目的，構成と期待される成果

平成24年度より厚生労働科学研究費補助金（医薬品・医療機器等レギュラトリーサイエンス総合研究事業)「血液・尿中バイオマーカーの非臨床・臨床適用に関する評価要件の確立研究（研究代表者：斎藤嘉朗，研究分担者：熊谷雄治（北里大学），鈴木孝昌，前川京子)」が開始された．本研究は，血液・尿中のタンパク質および内在性代謝物バイオマーカーの医薬品開発における利用を促進するための評価要件案作成の一環として，特に問題となる測定用試料の採取条件，および非臨床動物で見出されたバイオマーカーのヒトへの外挿性に関する評価要件案の作成を最終目標として，1）バイオマーカーの開発動向調査，2）測定用血液・尿の採取等に関する要件の明確化，3）動物モデルおよびヒト試料を用いた外挿性に関する実践的検討と，注意すべき評価要件の明確化，等を行うものである（図）．本研究の遂行により，血液・尿中のタンパク質および内在性代謝物バイオマーカーの測定用試料採取，および非臨床試験から臨床試験への外挿に関する評価要件案が作成され，ガイダンス等として発出されると，本邦におけるバイオマーカーの開発および利用推進につながり，医薬品開発を効率化して新薬創出の増加に結びつくと期待される．またこれら試料採取に関する要件案および非臨床から臨床への外挿に関する評価要件案は，世界的にも例がなく，国際的にも

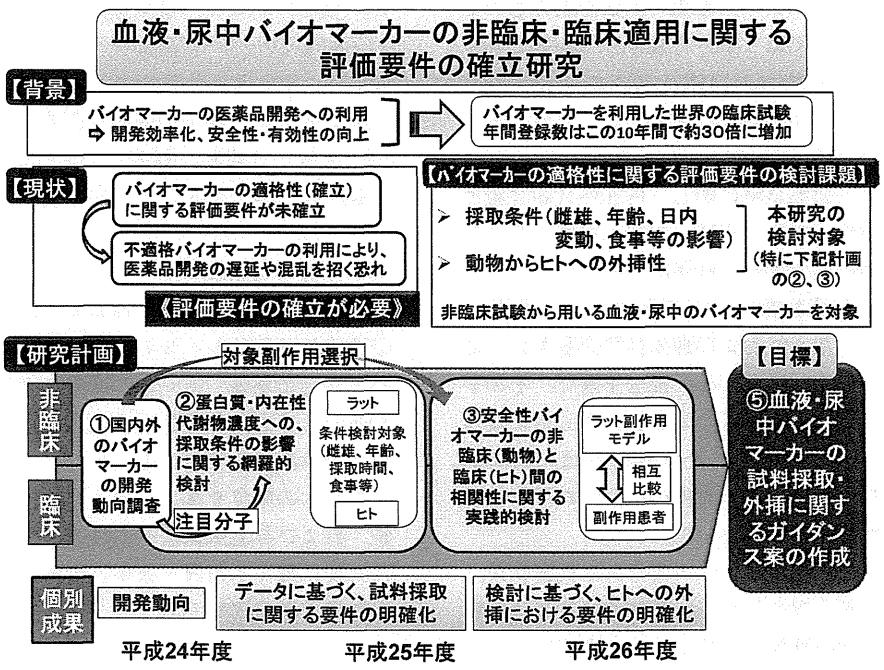

図　研究の背景，目的と計画

貢献できる研究である．

3－2．研究の進捗状況

現在，三年計画の一年目が終了した段階であるが，下記の成果を挙げている．

3－2－1．バイオマーカーの開発動向調査

開発動向としては，腎障害マーカーとして尿中Kim-1等の新規バイオマーカーが有用であることが報告されている[17]．さらにタンパク質マーカーとしては，抗がん剤ゲムシタビンによる重篤な血液毒性（好中球減少および血小板減少）の発症と血中のハプトグロビンのレベルが有意に相関すること[18]，血清中のアポリポ蛋白タンパク質Eのレベルが89％の正確性で薬物性肝障害患者と健常人を区別しうること[19]等が報告されている．代謝物マーカーとしては，薬物性肝障害に関して，血中ALT+γ-グルタミルシトルリンのレベルがマーカーとなりうること[20]，急性腎障害患者の血清を対象とした研究でアシルカルニチンや数種のアミノ酸レベルの増加，リゾホスファチジルコリンの減少[21]が報告されている．

3－2－2．プロテオミクス解析による尿中タンパク質に関する検討

ショットガンプロテオミクス法では，トリプシン消化後にタンパク質およびペプチド混合試料を，液体クロマトグラフ-質量分析計（LC-MS）で分析するが，尿中には高発現量のタンパク質があり，そのため微量のタンパク質は検出できない[22]．そこで，雄ラット尿を用いて，有機溶媒による簡便な高発現タンパク質の除去に関する前処理条件の検討を行った．収率は低くなるものの高発現のタンパク質を効率的に除去し，低濃度のタンパク質を中心に一回の分析で700種以上を同定しうる系を構築した．これを用いて，ラット尿中のプロテオーム解析を行った．雌雄間の比較では，予想されたように差が認められ，特に雌雄のいずれかのみで発現が認められるタンパク質が，同定タンパク質数の約1/3程度を占めた．食事影響については，比較的小さいものであった．従って，非臨床試験に用いるラットの尿を対象としたタンパク質バイオマーカーの探索と候補の選択に当たっては，性差を十分考慮に入れる必要があると示唆された．一方，食事影響については，重要でないと考えられた．

さらに平成25年度の解析としては，高齢群の一部の個体において，ばらつきの原因となる高発現量のタンパク質の存在が明らかとなり，注意が必要であることが示唆されている．

3－3．メタボロミクス解析による血中内在性代謝物に関する検討

内在性代謝物は親水性の高いもの（糖リン酸や核酸等）から低いもの（リン脂質やトリアシルグリセロール等）まで，その分子論的な性質は多様である．このため，現在の技術では一つの方法で全ての種類の内在性代謝物を

測定することは不可能であり，複数の方法を組み合わせる必要がある．本研究では，大きく親水性代謝物と疎水性代謝物に分類し，前者はLC/MSとガスクロマトグラフー質量分析計にて，後者はLC/MSにて，カラム等の条件を変えて測定を行った．

まず絶食後のヒト血液試料に関し，内在性代謝物濃度への採取・背景条件（性別，年齢，血漿・血清）および保管条件(凍結融解)の影響を，網羅的に明らかにした．測定可能であった代謝物数は，約550種である．血漿と血清間の比較では，親水性および疎水性代謝物共に，血液凝固に関係する代謝物群などの一部分子種で，そのレベルが血漿・血清間で大きく異なることが明らかになった．２倍以上のレベル差を示した代謝物数は，若年男性，老年男性，若年女性，老年女性のいずれの群でも30種程度で，親水性代謝物が多かった．

男女差に関しては，有意なレベル差のある代謝物が，老年よりも若年で多く認められた．若年ではアミノ酸類が男性において，脂肪酸類が女性において有意に高いレベルを示した．また年齢にかかわらず，女性でスフィンゴミエリンレベルが有意に高い傾向を示した．男女間で２倍以上の差があった代謝物は，若年血漿，老年血漿，若年血清，老年血清で，それぞれ数種であった．

年齢（30歳程度と60歳程度）に関しては，レベルに有意な差のある代謝物は，男性よりも女性で多く認められた．女性では，黄体ホルモン代謝物等が若年において，胆汁酸代謝物やトリアシルグリセロール等が老年において有意に高いレベルを示した．若年・老年間で２倍以上の差があった代謝物は，男性血漿，男性血清，女性血漿，女性血清で，それぞれ10種程度であった．

さらに若年男性の血漿と血清に関し，凍結融解回数（２回と10回）による相違について検討を行った．凍結融解を繰り返した場合，親水性代謝物では，血清と比べ血漿で大きなレベルの変化が認められる分子種が多かった．一方，疎水性代謝物では，血漿・血清ともに，ほぼすべてのリン脂質分子種等で，20-30％程度のレベル減少が認められた．

以上の結果，健常人というバックグラウンドレベルで，各条件につき２倍以上の差が認められた代謝物は，バイオマーカーの探索・診断の際に十分注意すべきであると考えられた．また，臨床におけるバイオマーカー診断に際しては，検体を選択することは不可能であり，検体間において普遍的なバイオマーカーが求められる．したがって，内在性代謝物をバイオマーカーとして測定する際には，１）血液試料として，血漿・血清のいずれでも利用可能だが，親水性代謝物の場合は凍結融解の影響を考慮すると血清が望ましく，疎水性代謝物の場合は，血液凝固過程の影響を受けにくい血漿が望ましいこと，２）検出率が高く，試料背景間および各試料背景内における差異の小さい代謝物をバイオマーカーとして選択すること，３）以上を満たせない場合には，これら背景的差異に対して，病気や薬剤反応性などによる差異が相対的に大きい代謝物（程度については，今後，検討予定）を選択すべきであること，が示唆された．

４．研究の将来展望

今後は，健常ラットと健常人におけるタンパク質および内在性代謝物レベルの相違（外挿性）について検討を行う予定である．さらに，特定の副作用に関し，ラットのモデル系と副作用患者の試料を比較して，副作用マーカーの外挿性を検討する．

これらの知見を基に，バイオマーカーの測定用試料採取，および非臨床試験から臨床試験へのバイオマーカーの外挿に関する血液・尿中バイオマーカーの評価要件案を作成する予定である．

５．おわりに

総合科学技術会議は平成25年４月17日の会議で，「個別化医療の世界的研究開発競争における日本の出遅れ，および創薬力の低下」を指摘している．本邦におけるバイオマーカー探索・同定とその医薬品開発への応用の早期実現は，まさに待ったなしの状況である．本研究の遂行によるガイダンス案の作成は，バイオマーカー評価の一部ではあるが，国としての基準を示すものとなり，本邦におけるバイオマーカー開発とその利用を通じた医薬品開発の活性化につながると期待される．今後とも医薬品の品質，有効性および安全性を確保するための研究機関として，医薬品の開発と適正使用の推進に向けた適切な規制のための研究という社会的な役割を十分に果たしていきたい．

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引用文献

１）Biomarkers Definitions Working Group.: *Clin. Pharmacol. Ther.* 69, 89-95 (2001).
２）Phillips, K.A., Van Bebber, S. and Issa, A.M.: *Nat. Rev. Drug Discov.* 5, 463-9 (2006).
３）Dong, D., Sung, C., Finkelstein, E.A.: *Neurology.* 79, 1259-67 (2012).
４）Feng, Z., Prentice, R. and Srivastava, S.: *Pharma-*

cogenomics. 5, 709-19 (2004).
5) Kell, D.B.: *Expert Rev. Mol. Diagn.* 7, 329-33 (2007).
6) ゲノム薬理学における用語集. 薬食審査発第0109013号, 薬食安発第0109002号, 平成20年1月9日
7) Pharmacogenomic Data Submission. FDA, March 2005.
8) Reflection paper on co-development of pharmacogenomic biomarkers and Assays in the context of drug development (Draft). EMA/CHMP/641298/2008, 24 June 2010.
9) Reflection paper on methodological issues associated with pharmacogenomic biomarkers in relation to clinical development and patient selection (Draft). EMA/446337/2011, 9 June 2011.
10) Clinical Pharmacogenomics: Premarket Evaluation in Early-Phase Clinical Studies and Recommendations for Labeling. FDA, January 2013.
11) Guideline on the use of pharmacogenetic methodologies in the pharmacokinetic evaluation of medicinal products. EMA/CHMP/37646/2009, 12 December 2011.
12) Concept paper on key aspects for the use of pharmacogenomic methodologies in the pharmacovigilance evaluation of medicinal products. EMA/CHMP/917570/2011, 15 December 2011.
13) Reflection paper on pharmacogenomic samples, testing and data handling. EMEA/CHMP/PGx-WP/201914/2006, 15 November 2007.
14) Pharmacogenomic Data Submission-Companion Guidance (Draft). FDA, August 2007.
15) 医薬品またはバイオテクノロジー応用医薬品の開発における バイオマーカー：適格性確認のための資料における用法の記載 要領, 資料の構成及び様式. 薬食審査発0120第1号, 薬食安発0120第1号, 平成23年1月20日.
16) Use of histology in biomarker qualification studies (Draft). FDA, December 2011.
17) Fuchs, T.C. and Hewitt, P.: *AAPS J.* 13, 615-31 (2011).
18) Matsubara, J., Ono, M., Negishi, A., Ueno, H., Okusaka, T., Furuse, J., Furuta, K., Sugiyama, E., Saito, Y., Kaniwa, N., Sawada, J., Honda, K., Sakuma, T., Chiba, T., Saijo, N., Hirohashi, S. and Yamada, T.: *J. Clin. Oncol.* 27, 2261-8 (2009).
19) Bell, L.N., Vuppalanchi, R., Watkins, P.B., Bonkovsky, H.L., Serrano, J., Fontana, R.J., Wang, M., Rochon, J., Chalasani, N.; US Drug-Induced Liver Injury Network (DILIN) Research Group.: *Aliment Pharmacol. Ther.* 35, 600-12 (2012).
20) Soga, T., Sugimoto, M., Honma, M., Mori, M., Igarashi, K., Kashikura, K., Ikeda, S., Hirayama, A., Yamamoto, T., Yoshida, H., Otsuka, M., Tsuji, S., Yatomi, Y., Sakuragawa, T., Watanabe, H., Nihei, K., Saito, T., Kawata, S., Suzuki, H., Tomita, M. and Suematsu, M.: *J Hepatol.* 55, 896-905 (2011).
21) Sun, J., Shannon, M., Ando, Y., Schnackenberg, L.K., Khan, N.A., Portilla, D., Beger, R.D.: *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 893-4, 107-113 (2012).
22) Decramer, S., Gonzalez de Peredo, A., Breuil, B., Mischak, H., Monsarrat, B., Bascands, J.L. and Schanstra, J.P.: *Mol. Cell. Proteomics.* 7, 1850-62 (2008).

Bull. Natl. Inst. Health Sci., **131**, 50-57 (2013) **Note**

ショットガンプロテオミクスによる加水分解小麦とその原料であるグルテンに含まれるタンパク質の網羅的解析

中村里香，酒井信夫，葩島由二，福井千恵，鈴木孝昌，中村亮介，蜂須賀暁子，安達玲子，手島玲子[#]

Comprehensive analyses of hydrolyzed wheat protein using shotgun proteomics

Rika Nakamura, Shinobu Sakai, Yuji Haishima, Chie Fukui, Takayoshi Suzuki, Ryosuke Nakamura, Akiko Hachisuka, Reiko Adachi, Reiko Teshima[#]

Hydrolyzed wheat protein (HWP; hydrolyzed gluten) is used in various types of products worldwide. Several cases of wheat-dependent, exercise-induced anaphylaxis following exposure to HWP (Glupearl 19S) in cosmetics have been reported. Glupearl 19S was produced from the gluten after partial hydrolysis with hydrogen chloride, and its allergenicity is larger than that of gluten (Adachi R., *Allergy* 2012;67:1392-9.). It is considered that provocation of allergic manifestations is caused by deamidated gluten in food and/or non-food products. Moreover, an increasing number of studies have shown that HWP can induce IgE-mediated hypersensitivity by skin contact and/or food ingestion. However, the essential molecular properties and profiles of HWP are still unknown. In this study, bioinformatic and multivariate analyses using shotgun proteomics have revealed that 27 proteins significantly decreased in Glupearl 19S compared with intact gluten as shown by the ratio of ion signal intensity of tryptic peptides. In contrast, a single protein significantly increased in HWP compared with intact gluten as shown by the ratio of ion signal intensity of tryptic peptides. Furthermore, we have identified six Glupearl 19S-specific peptides using shotgun proteomics, database searches on Mascot Sequence Query, and *de novo* sequencing. The six peptides were identified as the specific markers of Glupearl 19S.

Keywords: hydrolyzed wheat protein, shotgun proteomics, mass spectrometry, deamidation

緒言

近年，加水分解小麦（hydrolyzed wheat protein, HWP）を含有する洗顔用石鹸の長期使用において，小麦依存性運動誘発アナフィラキシー（Wheat-dependent, exercise-induced anaphylaxis；WDEIA）を発症した事例が数多く報告され，本邦にて大きな社会問題となっている[1-2]．このアレルギー病態の特徴は，当該石鹸の使用前には小麦アレルギーの既往症の無かった人が，石鹸中に含まれるHWP（グルパール19S®，Glupearl 19S）によって経皮・経粘膜的に感作され，その後の小麦の摂食によりWDEIAを発症することにある．

小麦タンパク質の主成分であるグルテンは，可溶性小麦タンパク質であるグロブリンを水で洗い流した後の残渣に含まれるエタノール可溶性画分に分画される．これを加水分解すると，タンパク質が部分的に切断されて可溶性が増大するため，起泡性や手触りを良くする目的で，シャンプー等の化粧品・医薬部外品にHWPが使用されている．グルパール19Sは，小麦グルテンを部分的に酸加水分解して製造された化粧品原料で，比較的分子量の大きなペプチド断片が残存している[1]．一般的にタンパク質を加水分解，低分子化することによりその抗原性は減弱すると考えられているが，HWPにおいては，酸加水分解によってグルテンよりも抗原性が増強することが明らかになっている[1-3]．酸加水分解によりグルテンの抗原性が増強する理由としては，可溶性の増大により生体内に侵入しやすくなっていること，更に酸によりタンパク質の一次構造に物理化学的変化が生じ，新たな

[#] To whom correspondence should be addressed:
Reiko Teshima; Division of Novel Foods and Immunochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; Tel: +81-3-3700-1349; Fax: +81-3-3700-7438; E-mail: rteshima@nihs.go.jp

エピトープが生じている可能性等が考えられている．グルテンタンパク質の物理化学的変化としては，セリアック病でグルタミン残基の脱アミド化が抗原性に寄与していることが報告されている[4]．我々は，グルタミン酸残基及びアスパラギン酸残基を特異的に切断する酵素であるV8プロテアーゼを用いて，グルパール19Sのタンパク質に含まれるグルタミン残基及びアスパラギン残基が脱アミド化修飾されていることを明らかにしているが[5]，ペプチドレベルでの研究は未だ行われていない．また我々は，グルテンを原料として酸加水分解を行ったHWPは，30分から1時間程度の部分的な分解によって抗原性が顕著に増強し，更に長時間分解を進めることにより，分子量の減少と共に抗原性が減弱することを明らかにしている[6]．これらの知見から，ある特定の加水分解条件によって生成されたHWPのみが抗原性を有することが示唆されているが，分子量以外に抗原性の指標となるファクターは明らかにされていない．

そこで本研究は，強い抗原性を有するグルパール19Sに特徴的なペプチドの探索を目的とし，液体クロマトグラフ質量分析計（LC-MS）を用いた網羅的解析を行った．同時に，グルテンとHWPのペプチドプロファイリングを比較するショットガンプロテオミクスにより，酸加水分解によるHWPの一次構造の物理化学的変化を推察した．

実験方法

1．試料

グルパール19S®は，株式会社片山化学工業研究所より入手した．グルテン（Sigma社）及びグルパール19S粉末に乾燥重量で100 mg/mLとなるよう1M Tris［tris (hydroxymethyl) aminomethane, pH 11.4］を加え，終夜室温で静置しストック懸濁液を調製した．また，抗原性が減弱したHWPとして，酸加水分解を24時間行った低分子化HWP（HWP24h）を調製した．酸加水分解は，0.1N塩酸中にグルテンストック懸濁液を終濃度1 mg/mLとなるよう加え，100℃のヒートブロック上で所定の時間（0-24h）加熱して行った．その後，経時的に0.1M水酸化ナトリウム水溶液で中和して酸加水分解反応を停止した．グルテン，グルパール19S，及び経時的な酸加水分解により調製した8種HWPsのSDS電気泳動パターンをFig. 1に示す．

LC-MSサンプルの調製は，以下に示す方法で行った．グルテン及びグルパール19Sのストック懸濁液に細胞溶解液［7M urea, 2M thiourea, 30 mM Tris, 4 %（w/w）CHAPS: pH 8.5］を加えてタンパク質を溶解させた後，2-D Quant Kit（GE Healthcare社）を用いてタンパク質量を定量し，その20 μgをトリプシン消化に供した．Dithiothreitolで還元，IodoacetamideでSH基のカルボキシメチル化を行った後，Trypsin Gold, Protease MAX（Promega社）を加えて37℃で終夜インキュベートした．10% Trifluoroacetic acid（TFA）を終濃度0.5%となるよう加えて酵素反応を停止し，得られたペプチドをバリアン社製OMIX Tip（C18, 100 μL）にて脱塩し，0.1% TFA含有2%アセトニトリルに再溶解した．

2．装置

・質量分析計

Thermo Scientific社製リニアイオントラップ／フーリエ変換ハイブリッド型質量分析計LTQ Orbitrap XL

測定前にTyrosine-1,3,6-Standard（CS Bio Co.）を用いてチューニング及び質量校正を行った．

・Nano-LC

HTC-PALオートサンプラー（CTC Analytics）を装備したADVANCE nano UPLC（AMR）

・トラップカートリッジ

CERI社製L-Trap（0.3×5 mm, L-C18, 5 μm, 12 nm）

・分析用逆相カラム

CERI社製L-column Micro（L-C18, 0.1×150 mm, 3 μm, 12 nm）

LC-MS/MS条件

イオン源にはCaptive Ion Sprayシステムを使用し，試料のイオン化はESI positive ion mode（スプレー電圧1.6 kV）により行った．MSスペクトルはFT analyser（分解能30,000, 測定質量範囲m/z 300-1,400, Lock mass＝フタル酸ジエチルヘキシル及びシロキサン, Profile mode）により取得し，XCalibur data dependent modeにより，スキャンにおけるイオン強度の高い3種のピークを順次選択してイオントラップによりMS/MSスペクトルを測定した（CID, Normalized collision energy 35 kV, Activation time 300 ms, Dynamic exclusion duration 60 s, Centroid mode）．測定時間は150分間とし，価数判別機能を利用して1価イオンのMS/MSスペクトルは測定しないように設定した．

Nano-LCの移動相には，A溶媒（0.1%ギ酸含有2%アセトニトリル）とB溶媒（100%アセトニトリル）を使用した．流速は300 nL/minとし，サンプル注入（1.0 μg）はオートサンプラーを使用して行った．1分析あたりの溶出時間は150分とし，サンプル注入後，0-40% B/125 min → 40-55% B/130 min → 55-100% B/135 min →100% B/145 min→0% B/160 minのグラジエントプログラムで溶出した．

3．試験操作

3．1．ショットガンプロテオミクスによる網羅的な構成タンパク質変動比較

グルテン及びグルパール19SのMSデータは各２回ずつ取得し，全データをi-RUBYソフトウェア（メディカルプロテオスコープ）にアップロードし，Mascot/Uni-Prot/NCBInr（Taxonomy; Green plants）データベースによるタンパク質同定，MS/MSスペクトル相同性に基づくピークマッチングと保持時間補正を行うことにより，タンパク質の比較定量解析を行った．

3．2．グルパール19Sのペプチド結合開裂及び脱アミド化の検討

ペプチド結合の開裂解析には，Proteome Discoverer ソフトウェア上でのMascot検索条件の消化酵素設定を“None”とし，同定されたペプチド配列のうち末端がトリプシン切断部位でない酸加水分解によるペプチド結合の切断の割合を算出した．

脱アミド化の分析には，Mascot検索条件のvariable modification設定に“deamidation（NQ）”を追加し，脱アミド化修飾されたペプチドを同定した．

3．3．多変量解析によるグルパール19Sに特徴的なペプチドの探索

グルテン，グルパール19S及びHWP24hのMSデータをプロテオーム定量解析ソフトウェアProgenesis LC-MS（Nonlinear Dynamics社）にアップロードし，Swiss-Prot（Taxonomy; Green plants）データベースによるタンパク質同定，イメージ解析によるピークマッチングと保持時間補正を行い，３サンプル間各ペプチドピークのシグナル強度を比較した．タンパク質の同定はProteome Discovererソフトウェア（Thermo Scientific社）を使用したMascot検索（NCBI nrデータベース，Taxonomy; Green plants）を並行して行った．

3．4．*de novo* sequencingによるペプチド配列の推定

de novo sequencingにはPEAKS Studio v6.0（インフォコム社）を使用した．消化酵素設定を“None”としてアミノ酸配列の推定を行った．TLC（Total Local Confidence）が60%以上のスコア値を示したアミノ酸配列を信頼度の高いデータと判断し，その配列をNCBI protein-protein BLAST（blastp）検索（Taxonomy; Green plants）に供した．

結果

1．SDS電気泳動による分子量の比較

グルテン，グルパール19S，及び経時的な酸加水分解により調製した８種HWPsのSDS電気泳動パターンをFig. 1に示す．グルテンでは構成タンパク質が明確なバンド状に現れるのに対し（lane G），グルパール19Sでは明確なバンドは認められず100 kDa以下にラダー状のパターンが認められた（lane 19S）．経時的な酸加水分解により調製したHWPsでは，0.5h加水分解物がグルパール19Sと同様に高分子領域にラダー状のバンドを呈していた．加水分解の時間経過と共にラダーが低分子領域にシフトし，24h経過後には15 kDa以上の明瞭なバンドが完全に消失した．

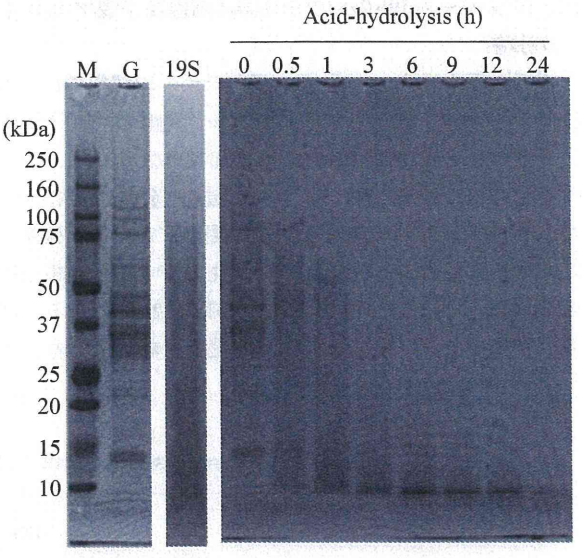

Fig. 1　SDS-PAGE pattern of gluten, Glupearl 19S and HWPs

Gluten, Glupearl 19S, and HWPs (2.5 μg protein/lane) were separated in a 10-20% acrylamide gel (D.R.C. Co., Ltd.) and the gel was stained with coomassie brilliant blue. Lane M, molecular weight marker; lane 19S, Glupearl 19S; lane G, native gluten

2．ショットガンプロテオミクスによるグルパール19Sとグルテンの網羅的な構成タンパク質変動比較

グルパール19Sとグルテンとの比較において，酸加水分解の過程でどのような構成タンパク質に変化が生じているかを明らかにするため，ショットガンプロテオーム解析を行った．グルテン及びグルパール19Sのトリプシン消化物より取得したMS/MSデータを，i-RUBYソフトウェアにてGreen plantsのタンパク質データベース内で検索したところ，発現比較の対象となるタンパク質の総数は5,074であった．そのうち変動比較の候補タンパク質をイネ科コムギ属（*Triticum*）としたところ，954のタンパク質を絞り込むに至った．これら候補タンパク質のうち，グルテンとグルパール19Sとの間の発現比が５倍以上または1/5以下であるタンパク質は268（28.1%）