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JB Reflections and Perspectives

The pioneering spirit of Takashi Sugimura: his studies of the biochemistry of poly(ADP-ribosylation) and of cancer

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Takashi Sugimura has accomplished many scientific achievements in the field of biochemistry and in cancer research. Sugimura's group identified the novel polymer poly(ADP-ribose) in parallel to P. Mandel's and O. Hayaishi's groups and demonstrated the presence of the enzyme poly(ADP-ribose) polymerase (PARP). He also discovered the cognate catabolic enzyme, poly(ADP-ribose) glycohydrolase (PARG) and further elucidated the biology of poly(ADP-ribose). The astonishing discovery of pierisin, an apoptogenic peptide that ADP-ribosyaltes DNA, profoundly illuminates his scientific character and curiosity as well. Sugimura's work in cancer research shows an extraordinarily wide range, which includes the establishment of new methods in chemical carcinogenesis, the identification of various environmental mutagens/carcinogens and new tumour promoters. He also established the concept that cancer is a disease of DNA and contributed to the development of the concept of the multi-step model of carcinogenesis.

Keywords: cancer/carcinogenesis/mutagenesis/pierisin/poly(ADP-ribose)/tumour promoter.

Abbreviations: 4HNQO, 4-hydroxynitroquinoline 1-oxide; 4NQO, 4-nitroquinoline 1-oxide; AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; ADPR, ADP-ribose; AF2, trans-2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; AMP, adenosine monophosphate; DiMeIQx, 2-amino-3,4,8-trimethyl-imidazo[4,5-*f*]quinoxaline; Gb3, glycolipid globotriaosylceramide; Gb4, globotetraosylceramide; Glu-P-1, 2-amino-6-methyldipyrido [1,2-*a*:3′,2′-*d*] imidazole; Glu-P-2, 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole; HCAs, heterocyclic amines; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; IQx, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole; MeIQ, 2-amino-3,4-dimethylimidazo-[4,5-*f*] quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline; NAD, nicotinamide adenine dinucleotide; NCCRI, National Cancer Center Research Institute; Nm, nicotinamide; NMN, nicotinamide mononucleotide; PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerase; PDE, phosphodiesterase; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine; Phe-P-1, 2-amino-5-phenylpyridine; PR-AMP, 2′-(5″-phosphoribosyl)-5′-AMP; Trp-P-1, 3-amino-1, 4-dimethyl-5*H*-pyrido[4,3-*b*]indole.

Takashi Sugimura graduated from the Faculty of Medicine, the University of Tokyo in 1949, where he then spent several years in the Department of Radiology. The professor of the Department at that time was M. Nakaidzumi, who was a scientist of originality and creativity. In the Cancer Clinic of Nakaidzumi at the University of Tokyo, Sugimura was one of the first doctors to use the cancer chemotherapeutic drug, 'nitromin', nitrogen mustard *N*-oxide, a masked form of nitrogen mustard. Although some tumour suppressive effects were observed, the chemotherapy did not cure the patients and the side effects of this drug were severe. These clinical experiences led him to pursue basic research of cancer and inspired in him a tough and resolute conviction that cancer control through prevention and treatment at early stages are important. Accordingly, he joined the Cancer Institute (Tokyo) where Waro Nakahara was Director.

Sugimura spent then 2 years at the National Cancer Institutes, National Institutes Health (NIH) in Bethesda, USA in 1957–1959, working under J. P. Greenstein on cancer and nutrition, using a quantitative, water-soluble, chemically defined diet developed by Greenstein. While at the NIH he purified L- and D-amino acids from racemic amino acids and studied *in vivo,* the conversion rates of essential D-amino acids to their L-forms. Since Greenstein prematurely died in 1959, he moved to the Biochemistry Division, at Case Western Reserve University headed by H. G. Wood. There he worked under H. Rudney on coenzyme Q biosynthesis in yeast cells during their subsequent aerobic adaptation after they were first cultured anaerobically. He was impressed by the democratic customs in the operation of that Biochemistry Laboratory. Soon after his return to Japan, the Director of the National Cancer Research Institute (NCCRI), W. Nakahara, who was a graduate of Cornell University and also had a quite democratic style of scientific management, appointed him Chief, Biochemistry Division. Sugimura's science was deeply influenced by four of his mentors; Nakaizdumi, Greenstein, Wood and Nakahara.

When I joined his group in the NCCRI in 1992, he was already a prominent figure in science and in the

administration of both cancer research and also of the life science programs in Japan. Sugimura was elected as the Member of the Japan Academy in 1982 and is currently the Vice President of the Japan Academy. He has been a Foreign Associate of the National Academy of Sciences, USA since 1982 and a Foreign Member of the Royal Swedish Academy of Sciences and of the Royal Netherlands Academy of Arts and Science since 1987. He has received numerous prizes for his scientific work, including the Order of Cultural Merit in 1978, the Charles S. Mott Prize of the General Motors Cancer Research Foundation, USA in 1981, the Ernst W. Bertner Memorial Award, USA in 1981, and has Honorary Doctorate Degrees from the Thomas Jefferson University, USA; Leiden University, The Netherlands; and the Karolinska Institute, Stockholm, Sweden.

Dr Sugimura's scientific achievements could be found in his brief, scientific autobiography in *Comprehensive Biochemistry* (*1*), and here I propose to describe how he and his works in the field of poly(ADP-ribose) and cancer research have affected science in these research fields.

Discovery of poly(ADP-ribose)

In the early 1960s, Sugimura was looking for control mechanisms of macromolecular synthesis, by a low-molecular weight metabolite as a possible explanation of the uncontrolled growth of cancer cells. In 1964, P. Mandel and P. Chambon in Strasbourg, France published a paper entitled 'Nicotinamide mononucleotide activation of a new NAD-dependent polyadenylic acid synthesizing nuclear enzyme' (*2*). Sugimura was stimulated by this article and he and S. Fujimura confirmed the large increase of the incorporation of (C-14)-labelled ATP into an acid-insoluble fraction by adding nicotinamide mononucleotide (NMN) (*3*, *4*). However, Sugimura carefully observed that the product was resistant to alkali and therefore thought that the product could not be poly(A) (*3*, *4*). The acid-insoluble product was successfully digested by snake venom phosphodiesterase and they showed that the product was in fact 2′-(5″-phosphoribosyl)-5′-AMP (PR-AMP) (*5*). Reconstruction from this new data allowed the correct structure of the novel polymer, poly(ADP-ribose) (Fig. 1) to be deduced (*6*). At that time, this new polymer was totally unknown. Meanwhile, in Strasbourg, J. Doly, who was a graduate student of Chambon, and M. F. Petek also demonstrated, that the ribose-ribose bond was in 2″-ribosyl-1′-ribose, using conventional sugar methylation analysis and hence they too correctly identified this new polymer (*7*). Meanwhile, in Kyoto University, Y. Nishizuka under O. Hayaishi also discovered the structure of poly(ADP-ribose). They had the advantage that they were studying the metabolic pathway from L-tryptophan to NMN and then to NAD (*8*). It was known at that time that NAD served as a cofactor in oxidation–reduction reactions, but these three reports were the first indications that NAD also participated in other biochemical reactions. Sugimura first met Mandel, who was an eminent neurochemist, on the occasion of the International Meeting of Biochemistry held in Tokyo in 1966. Later Sugimura visited Strasbourg (see the photograph of Fig. 2A). M. Futai and Sugimura (*9*) found that snake venom phosphodiesterase and phosphodiesterase from spleen can degrade poly(ADP-ribose). M. Miwa and Sugimura (*10*) further showed poly(ADP-ribose) glycohydrolase (PARG) is a specific degradation enzyme of poly(ADP-ribose). Sugimura's and Hayaishi's works encouraged Japanese scientists to study the field of poly(ADP-ribose) science.

However, at that time scientists were still skeptical and thought that poly(ADP-ribose) was an *in vitro* artifact. Sugimura and his colleague Y. Kanai showed that it was not an artefact, when they

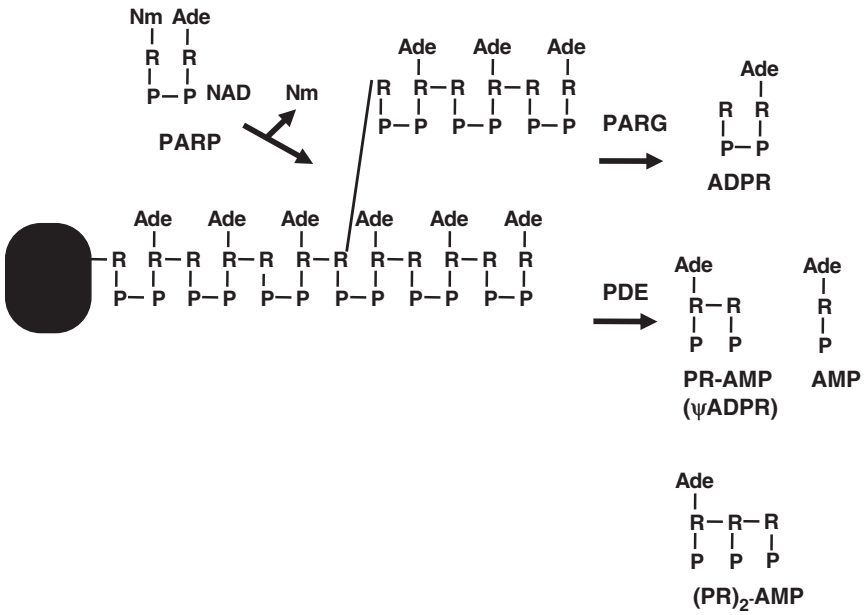

Fig. 1 Poly(ADP-ribosylation) reaction. Ade, adenine; P, phosphate; R. ribose; Nm, nicotinamide.

Fig. 2 Photos of poly(ADP-ribose) scientists. (A) A photo of Mandel (left) and Chambon (right), and K. Sugimura (the wife of Sugimura, behind Mandel) taken by Sugimura in the old town of Strasbourg, France. (B) A photo of the participants of the 'International Seminar on Poly ADP-ribose and ADP-ribosylation of Protein' held in Tomakomai in 1974. Adapted and reprinted by permission from the Fujihara Foundation of Science: 'Fujihara Kagaku Zaidan Kiroku' (the Proceedings of Fujihara Foundation of Science), 1980, p157. Mandel, Hayaishi, Sugimura, M.K. Jacobson, Shall, H. Hilz, (in the front raw), Miwa, K. Ueda, K. Yoshihara and M. Smulson (in the second raw) were seen.

demonstrated the presence of an antibody against poly(ADP-ribose) in the sera of patients with an autoimmune disease (*11*). This elegant work by Sugimura and colleagues convincingly showed the natural occurrence of poly(ADP-ribose) *in vivo*, even in human beings (*11*). Sugimura, Y. Kawamitsu and Miwa first generated a specific monoclonal antibody to poly(ADP-ribose), called 10H. Sugimura and Miwa distributed the hybridoma cells to scientists upon request. This antibody has been widely used over 20 years for detection of poly(ADP-ribose).

Sugimura has a great talent for friendly relations with all the scientists with whom he interacts. Hayaishi and he organized a meeting on poly(ADP-ribose) in Bethesda, Washington in 1973. And again, Sugimura and Hayaishi organized a meeting on poly(ADP-ribose) in 1974 in Tomakomai, Hokkaido sponsored by Fujihara Foundation (see photo, Fig. 2B). And then, when Sugimura received the prestigious Fogarty Scholar in Residence, NIH, he organized a symposium again in 1979 with M. Smulson (Georgetown University) in Bethesda. He also organized the international meetings of poly(ADP-ribose) in 1982, 1992 and 2004 in Japan.

Sugimura wrote the first comprehensive review on poly(ADP-ribose) in 1973 (*12*). Meanwhile, S. Shall (University of Sussex) had shown that 3-aminobenzamide, an inhibitor of poly(ADP-ribose) polymerase (PARP) enhanced the cell killing effect of dimethyl sulphate (*13*). This was a breakthrough that demonstrated a relationship between DNA repair and PARP. In the 2004 meeting in Tomakomai, clinical trials of potent PARP inhibitors used as adjuvants with chemotherapy was one of the hot subjects (*14*). In 2005, it was reported that PARP inhibitors showed 'synergistic lethality' against BRCA-mutated tumors by the groups of T. Helleday (*15*) and A. Ashworth (*16*). These two papers led to an explosion of interest in PARP inhibitors as anticancer agents. There are currently many clinical trials registered as novel modalities in cancer therapy (*17*). I recall that Miwa and Sugimura published a paper indicating the enhancement of the anti-tumour effect of bleomycin by benzamide on Ehrlich cancer cells transplanted in mice (*18*). When I joined H. Esumi in the NCCRI to study the role of PARP and PARG in carcinogenesis and cancer treatment, Sugimura encouraged us to develop knockout mouse models, although there

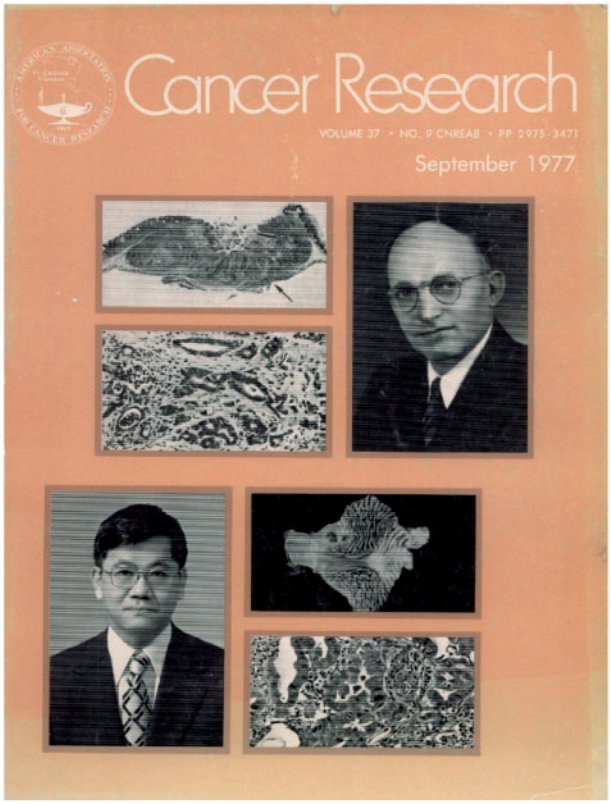

Fig. 3 The front cover of vol. 37, no. 9 of Cancer Research published in 1997. Sugimura and H. P. Morris developed chemical induction methods of gastric cancers in experimental animals. Adapted and reprinted by permission from the American Association for Cancer Research: Cancer Research, 1997, vol. 37, no. 9, the front cover.

were already some reports. His advice was, if you perform experiments with your own hands and methods, you will find unreported observations and reach new results. That was true and we observed that involvement of Parp-1 in DNA damage response and carcinogenesis (*19*, *20*), differentiation (*21*) and isolated rat Parg gene and found a potential nuclear export signal (*22*).

Chemical carcinogenesis and concept of multi-step carcinogenesis

Sugimura developed the hypothesis that carcinogenesis was related to mutagenesis. *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was a typical mutagen. Consequently, Sugimura injected MNNG subcutaneously into rats. He observed that the rats developed fibrosarcomas, in a dose-dependent manner (*23*). Then he simply gave the rats MNNG in drinking water. After a year, they observed the development of gastric cancers (adenocarcinomas) in the glandular stomach of the rats (*23*). Naturally, the importance of this major advance was readily and widely appreciated (Fig. 3). Thus, Sugimura was able to establish the concept for evolution of stomach carcinogenesis. The sequence is first, erosions, then a hyperplastic stage, progressing to an adenoma and eventually to adenocarcinoma with local invasion and even with metastasis. This concept of multi-step carcinogenesis (*24*) and the rodent cancer models have supported and greatly advanced cancer research from various aspects.

Sugimura also observed phenotypic instability in cancer, such as disdifferentiation and dedifferentiation of the cells. He demonstrated the occurrence of intestinalization, an abnormal differentiation, in human stomach and MNNG-treated rat stomach (*25*). Besides methylation of DNA bases, Sugimura found methylated proteins after exposure to methylating agents. Sugimura was especially excited to find that the ε-amino residue of lysines were nitroamidinated by MNNG, converting lysine to nitrohomoarginine (*26*). He showed that this modification of basic proteins such as cytochrome c and histones, made them more acidic. Sugimura was aware that modification of nuclear proteins might play a role in phenotypic instability and hence in carcinogenesis. More than 30 years later, researchers became interested in this point and histone modifications on lysine residues, especially methylation, and they have been shown to be important in regulating transcription and are involved in phenotypic changes in cancer cells.

Later M. Tatematsu demonstrated that MNNG in the presence of an infection by *Helicobacter pylori* produced gastric cancer in the glandular stomach of Mongolian gerbils (*27*). Human gastric cancer is likely to be produced by genetic and epigenetic changes in the presence of an infection by *H. pylori* in combination with certain chemical stimuli (*28*).

Identification of environmental chemical carcinogens

R. Doll and R. Peto (*29*) have famously concluded from their epidemiologic studies of cancer incidence, that food is the major culprit in causing human cancer. Sugimura and M. Nagao (NCCRI) showed that the smoke from cooking is mutagenic. They then characterized several heterocyclic amines (HCAs) that were previously unknown and demonstrated that these new compounds are mutagenic. IQ, MeIQ and MeIQx were identified with H. Kasai and his colleagues and Trp-P-1 and Glu-P-1 with T. Kosuge and his colleagues (*30*). The HCA compounds were synthesized and indeed, they were proved to be carcinogenic (Fig. 4). The tumours induced by HCA compounds include breast, pancreas, prostate, colon and liver cancers (*31*). HCA were shown to be carcinogenic also in primates (*32*). Sugimura has related that actually his wife deserves all the credit for these discoveries because the hypothesis that drove this work was inspired when he sat watching the smoke when his wife was broiling fish at their home. He then found that the production of these mutagenic compounds only occurred when cooking proteinaceous foods, such as meat and fish. M. Jägerstad (Lund University) found that the precursors of the HCA compounds were sugars, amino acids and muscle creati(ni)ne (*33*). Later, J. Felton (Lawrence Livermore National Laboratory) added PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine) to the list of HCA compounds (*34*). PhIP did not produce hepatomas at all, but was carcinogenic in the colon, breast and prostate, which are common

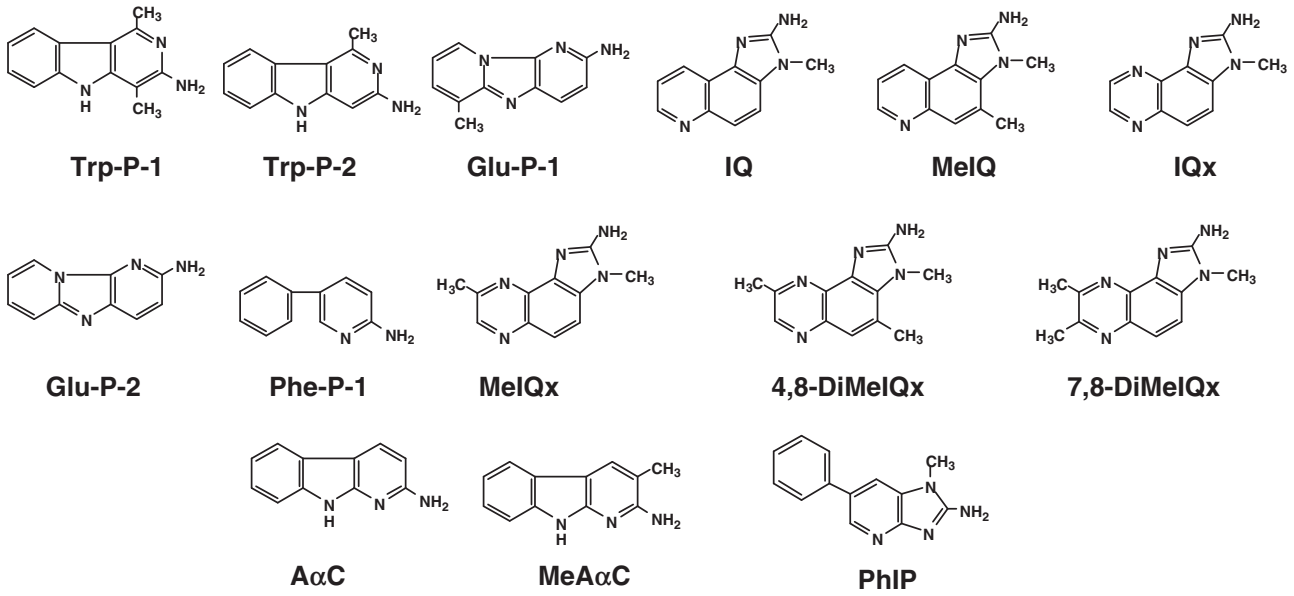

Fig. 4 Structures of heterocyclic amines.

sites of cancer in people in Western countries. The intake of HCA compounds from cooked foods is generally low, but the simultaneous presence of many mutagens/carcinogens should be seriously taken into consideration. In this way, Sugimura opened a new field and a new possible dimension in our collective search for the cause of human cancers (*35*, *36*). Many research groups started to use HCAs as carcinogens in animal models. Affecting factors and genes for HCA-induced carcinogenesis were later isolated with K. Wakabayashi and H. Nakagama. The findings of chemical carcinogens by Sugimura's group also activated the search of environmental carcinogens and DNA lesions that lead to mutations.

Discovery of pierisin from cabbage butterflies

An important and astonishing example of the scientific perspicacity of Sugimura was his discovery of 'pierisin' (*37*). It happened that Sugimura was awarded the prestigious Japan Prize in 1997, sharing it with B. Ames. His friend, K. Koyama (NCCRI), wanted to give Sugimura a memorable present, a framed picture of the National Flag made from the wings of butterflies. Sugimura was famous about as butterfly collector from his childhood, just as Nakahara. In the event, the central, red part of the flag was made from purchased wings of the butterfly, *Appias nero*. The white part was made from the wings of the cabbage butterfly, *Pieris rapae*, grown in the laboratory. Sugimura then asked Koyama just to check the remaining larvae and pupae for the possible presence of a specific substance. He suggested this because he had the inspired hypothesis that perhaps the metamorphosis of the larvae to adults would require the presence of a responsible substance, which would enforce apoptosis on the unwanted cells. Koyama immediately responded and demonstrated the presence of a substance in the larvae and pupae that killed cultured gastric cancer cells (*38*). The active substance was

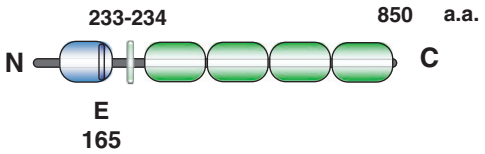

Fig. 5 The domain structure of pierisin-1. The N-terminal region contains a mono(ADP-ribosyl)transferase activity domain (*37*). The E165 is a crucial amino acid residue for the enzyme activity conserved in ADP-ribosylating toxin. The C-terminal region harbours four repeats that show similarity to the lectin domain of the ricin superfamily and is suggested to bind Gb3 and Gb4 receptors.

shown to be a peptide, which was then purified and the cDNA was then cloned by T. Watanabe and K. Wakabayashi. Further analysis allowed the deduction that the protein was composed of 850 amino acid residues (*37*).

Sugimura and Wakabayashi further discovered that the N-terminal half of this protein had a sequence which suggested an ADP-ribosylating property (Fig. 5). Sure enough, they showed that these proteins are enzymes that can ADP-ribosylate targets (*37*). This new protein did not ADP-ribosylate amino acid residues of protein, but it modified guanine bases in DNA (*39*). Y. Totsuka and Wakabayashi (*40*) showed that pierisin caused mutations, including GC to CG transversion. They also demonstrated that the C-terminal half of this protein has the property to bind to the cell surface receptors called Gb3 and Gb4 (*41*). Phylogenetic studies showed an interesting distribution of pierisin homologous genes among the butterfly family Pieridae (*42*). Until pierisin was discovered, most ADP-ribosylation reactions were against amino acid residues of proteins. Discovery of pierisin accelerated scientists to focus on the biological significance of ADP-ribosylation reactions on acceptor molecules other than proteins.

Table I. Sugimura's achievements.

Poly(ADP-ribose) science	
Identification of poly(ADP-ribose) and PARP	1966, 1967
Identification of PARG	1971
Anti-poly(ADP-ribose) antibody in autoimmune disease	1977
PARP inhibitor as a chemotherapeutic agent	1983
Development of animal models of carcinogenesis	
Gastric cancer model in rats using MNNG	1966, 1967
Modification of ε-amino residue of lysine by MNNG	1968
Environmental chemical carcinogens	
Discovery of HCAs	1977, 1986
Contributed to ban use of AF2 by proving its carcinogenicity	1978
Carcinogenicity of HCAs	1991
Co-mutagenic activity of aminophenylnorharman and aniline	1998
Multi-step carcinogenesis model	
Intestinal metaplasia of stomach epithelial cells	1971
Concept of multi-step carcinogenesis	1972, 1986
Expression pattern switching of aldolase during carcinogenesis	1972
Action mechanisms of chemical carcinogens	
Carcinogenicity of 4-nitroquinoline 1-oxide (4NQO)	1957
In vivo binding of 4NQO to DNA	1967
In vitro DNA scission by 4HAQO	1968
Hydrogen peroxide formation by 4HAQO	1969
Tumour promoters	
Identification of tumor promoter, teleocidin B	1979
Identification of tumor promoters, aplysiatoxin and palytoxin	1982, 1983
Phenotypic instability in cancer	
Concept of disdifferentiation and dedifferentiation of cancer	1972
Splicing aberration induced by carcinogen in albuminemic rat	1985
Pierisin	
Identification of pierisin as an apoptosis inducer	1999
DNA ADP-ribosylation by pierisin	2001
Mutation caused by pierisin	2003

Epilogue

One of the special features of Sugimura's style of work was his great care that experimental data should always be thoroughly reproducible. He has also been cautious in making wide generalizations. He always paid meticulous attention to exceptional cases in his observations. I mention one particular, striking case. He was investigating the mutagenicity of natural foodstuffs and he could demonstrate mutagenicity ubiquitously in fruits and vegetables. This observation was at the time unexpected. Only later was the mutagenic compound identified as quercetin, a flavonoid that is widely found in plant life (*43–45*). However, after intensive studies Sugimura clearly demonstrated that although quercetin was mutagenic, it is not carcinogenic (*46*). Moreover, he discovered rather surprisingly that this flavonoid is in fact anti-carcinogenic, against many types of carcinogens. Even *in vitro*, quercetin can often protect against the effects of DNA damaging agents.

Sugimura has always enjoyed talking with both young and senior scientists. He encourages younger scientists 'to dig out from the ground their own jewels'. It is still surprising for me that Sugimura carried out highly distinguished work in so many different fields (Table 1) (*47*). These achievements have been possible because of his intense and challenging mission to control cancer through science and a continuous curiosity in the subjects of cancer and biochemistry. Sugimura has emphasized that prevention of cancer by improving life-styles (the first line of preventive action) and early diagnosis of cancer (the second line of preventive action) will be much more effective in saving lives and will be much less costly than treating patients at advanced stages. I venture to think that perhaps his greatest satisfaction comes from his success and his advocacy of cancer prevention as our best means of conquering this terrible disease.

Conflict of interest
None declared.

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