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Multistep Carcinogenesis: A 1992 Perspective

Takashi Sugimura

Cancer is the leading cause of death in Japan. Recent changes in cancer incidence patterns may reflect the trend toward a more Western diet and life-style. Among the dietary factors that contribute to carcinogenesis are the heterocyclic amines, a group of mutagenic compounds present in cooked meat and fish. Carcinogenesis is a multistep process in which cells accumulate multiple genetic alterations as they progress to a more malignant phenotype. Recognition of the growing number of interacting factors that contribute to carcinogenesis may force reconsideration of current methods of risk assessment.

Since 1981, cancer has been the major cause of death in Japan, followed by cardiovascular disease (*1*). The Japanese Ministry of Health and Welfare recorded 223,604 cancer-related deaths in 1991 (*2*). The incidence of gastric cancer has been declining, whereas cancers of the colon, pancreas, and breast have been increasing in incidence (*3*). These changes in organ site distribution may be due to recent changes in the nation's dietary habits, including reduced salt intake and increased fat intake (*4*). A similar link between diet and cancer incidence patterns has been demonstrated previously in studies of Japanese emigrants to Western countries (*5*). The incidence of lung cancer is rising steadily in Japan because the anti-smoking movement is still at an early stage (*3*).

As with the other modern sciences, the opening of the gates of Japan to foreign countries at the end of the Edo era (1603–1867) facilitated progress in cancer research and clinical practice; this progress followed the introduction of knowledge and state-of-the-art technology, first from Germany and later from the United States, especially after World War II. Since that time, international research efforts have led to vast improvements in cancer detection and treatment. These improvements are reflected in the improved survival rates of patients treated at the National Cancer Center in Tokyo. Thirty years ago, only 37% of the patients treated at the Center survived 5 years or more, whereas this figure is now estimated to be about 50% (*6*). Indeed, we have become accustomed to seeing cancer patients successfully return to their normal daily activities after treatment.

Despite this tremendous progress, many problems in cancer detection and treatment remain. Certain groups of cancers are still almost impossible to diagnose early enough to permit effective treatment. Among these are cancers of the pancreas and gallbladder, small-cell lung cancer, scirrhous-type gastric cancer, and ovarian cancer. Prostate cancers also pose a particular problem because, without sophisticated molecular analyses, it is difficult to distinguish between occult disease and disease that will progress and therefore require aggressive treatment. Another, more recently recognized problem is the occurrence of second primary cancers among patients who had previously been cured of their first cancers by medical intervention. Many of these second cancers are neither metastases nor recurrences of the earlier disease but rather are examples of "multiple primary neoplasia." The development of these second tumors is not necessarily treatment-related since they are observed in patients who have never received chemo- or radiotherapy. About 8% of the patients at the National Cancer Center in Tokyo are now being treated for such tumors, as compared with less than 2% in 1962 (*7*). As discussed below, these cancers may originate from "primed" cells that harbor some, but not all, of the genetic alterations required for cancer development. New genetic alterations—and potentially a new malignancy—may arise from exposure of these primed cells to environmental and endogenously produced carcinogenic factors.

In this article, I consider several aspects of current research on the causes and treatment of cancer in Japan. First, I describe studies on diet and cancer, particularly

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those concerned with the detection of carcinogens in cooked foods. Second, I briefly review recent studies on genetic alterations and genomic instability in cancer cells and discuss the possible consequences of this instability in tumor progression. Finally, I discuss new ideas on risk assessment of cancer, in light of the existence of multiple interacting carcinogenic factors.

Diet and Cancer

Diet is now recognized as a major factor in carcinogenesis (8). A positive correlation between fat intake and cancer development has been reported for cancers of the breast, uterus body, pancreas, and colon (8, 9). In addition, ingestion of excess salt appears to promote development of gastric cancers.

Certain foods are contaminated with genotoxic (DNA-damaging) carcinogens such as mycotoxins, plant alkaloids, nitrites, and nitrosamines. Contamination of food with the mycotoxin aflatoxin B_1 can be reduced, but complete elimination of this agent is not yet possible (10). Since the detection of carcinogenic plant alkaloids [for example, ptaquiloside in bracken fern (11) and petasitenine in *Petasites japonicus* Maxim (12)], efforts have been made to limit human exposure to these substances (13, 14). Dietary intake of nitrites and nitrosamines has been reduced by restricting the use of nitrite as a food additive. In contrast, because *N*-nitrosamines can be formed from endogenous nitric oxide in the body, it is not feasible to eliminate human exposure to these compounds.

Our own studies on the role of diet in carcinogenesis followed from our work in the 1950s on 4-nitroquinoline 1-oxide (4NQO) (15), a carcinogen that was mutagenic in the fungus *Aspergillus* (16). This compound served as an excellent model for demonstrating that molecules with mutagenic activity in microbes had carcinogenic activity in rodents. Many carcinogens react with DNA only after metabolic conversion to an activated form by mammalian enzymes such as the cytochrome P-450s. Metabolic activation of 4NQO does not require the cytochrome P-450 system; rather, 4NQO is converted to 4-hydroxylaminoquinoline 1-oxide (4HAQO) by $NAD(P)H_2$ oxidoreductases including DT diaphorase. The 4HAQO intermediate is further activated in the presence of seryl-tRNA synthetase, serine, and adenosine triphosphate to yield seryl-4HAQO, through which quinoline-DNA adducts are produced (17). Studies on the molecular mechanisms of DNA damage caused by 4NQO were greatly facilitated because its metabolic activation pathway is shared by prokaryotes and eukaryotes. Mutation induction in microbes and cultured mammalian cells, DNA adduct formation, DNA strand scission, and oxidative agent formation could be more efficiently studied with 4NQO and 4HAQO than with many other carcinogens (17).

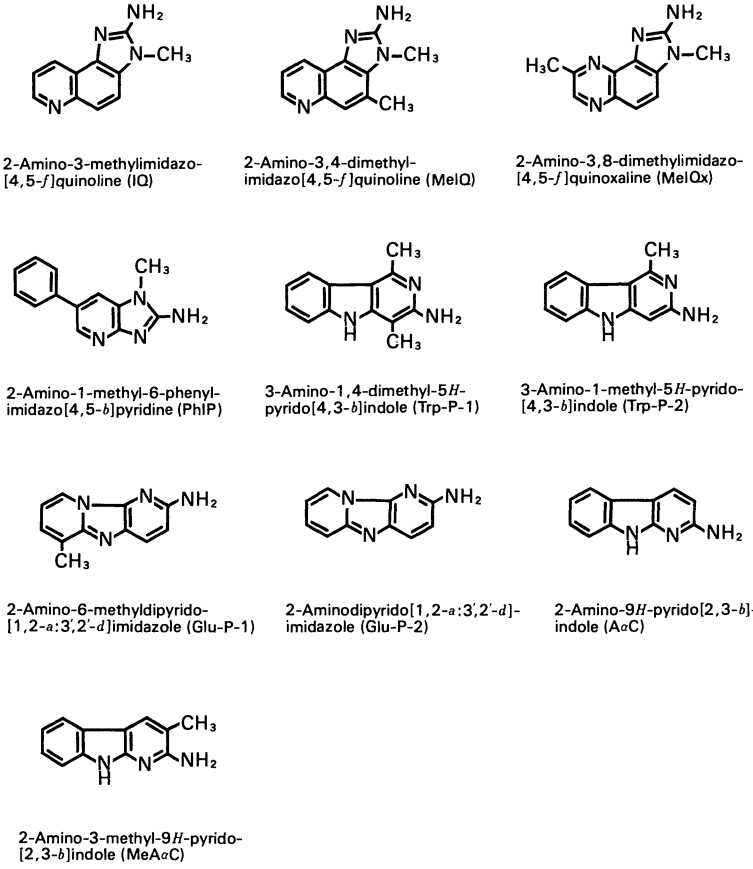

Fig. 1. Carcinogenic heterocyclic amines in cooked foods.

Another example of the link between carcinogenicity and mutagenicity was our demonstration that fibrosarcomas could be induced in rats by subcutaneous injection of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (18), a widely used mutagen in microbial genetic experiments. We later showed that this agent produced adenocarcinomas in the rat glandular stomach when administered as a solution in drinking water (19), thus proving that gastric cancer could be produced by a genotoxic substance. These results are consistent with recent evidence that human gastric cancers display multiple genetic alterations (20).

A compound that was once used as a food preservative in Japan, AF-2 [2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide], was found to induce chromosomal aberrations in cultured human lymphoblasts and to be mutagenic in *Escherichia coli*. Animal studies confirmed that AF-2 was carcinogenic, and its usage in food was subsequently banned (21).

Heterocyclic Amines as Carcinogenic Factors

Our studies on the relationship between mutagens and carcinogens, especially with regard to food additives, led to our discovery of a series of new mutagenic and carcinogenic compounds generated in cooked food such as grilled or broiled fish and meat (13, 21, 22). We used the Ames test [a mutagenicity assay in which *Salmonella typhimurium* bacteria are treated with test chemicals that have been metabolically activated in vitro with rat liver enzymes (23)] as a monitoring system for purifying the mutagenic compounds and identified them as new heterocyclic amines (HCAs), imidazoquinoline (IQ) and methylimidazoquinoxaline (MeIQx) derivatives. These compounds are produced by heating proteinaceous food of animal origin and are detectable under ordinary cooking conditions. The precursors of IQ and MeIQx compounds are creatin(in)e, which is abundant in fish and meat, and amino acids and sugars (24). Another HCA, phenylimidazopyridine (PhIP), was later detected in fried beef by Felton's group (25). Although the specific mutagenic potential of PhIP in the Ames test was lower than that of other HCAs, its carcinogenic potential was similar (26,

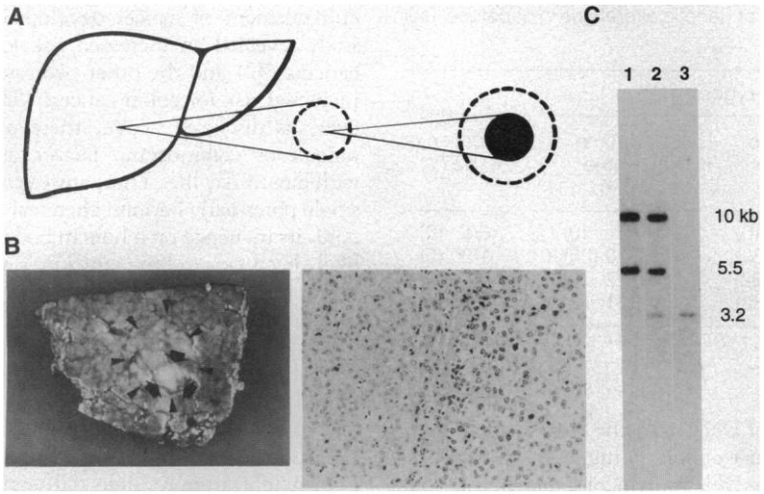

Fig. 2. Monoclonal growth expansion of hepatocellular carcinoma (HCC). (**A**) This schematic illustrates the development of an early HCC, which is weakly malignant but already monoclonal, in a liver that is damaged by chronic hepatitis or cirrhosis. A more malignant HCC often develops as a subclone within the early HCC. (**B**) Gross findings (left) and histological appearance (right) of a surgically resected HCC with features of "a tumor within a tumor." The outer tumor, indicated by arrowheads, is well differentiated, and the inner tumor, indicated by arrows, is poorly differentiated. The histological section shows the border between the two tumors. (**C**) Southern blot analysis of HBV DNA integration in cellular DNA extracted from the inner (lane 1) and the outer (lane 2) tumors in (B). DNA samples were digested with Eco RI and subjected to electrophoresis, and the separated fragments were transferred to a nitrocellulose filter that was then hybridized to a HBV-specific probe. In DNA from the outer tumor and cirrhotic tissue (lane 3), the bands at 3.2 kb correspond to free HBV DNA.

27). Heated foods contain much higher amounts of PhIP than other HCAs (*27*).

Additional experiments revealed that pyrolysis of proteins and amino acids can produce mutagenic HCAs. For example, pyrolysis of tryptophan and glutamic acid generates pyridoindole (Trp-P-1 and Trp-P-2) and dipyridoimidazole (Glu-P-1 and Glu-P-2) derivatives, respectively (*13, 21*). Two amino-α-carboline compounds (AαC and MeAαC), which are also HCAs, were identified as mutagens in pyrolysates of soybean globulin (*28*). Several of these HCAs are also found in broiled fish and meat.

We have shown that all ten HCAs tested thus far (Fig. 1) are carcinogenic in rodents (*21, 26, 27*). Although most of these compounds produced hepatocellular adenomas and carcinomas in rats and mice, PhIP did not induce hepatic lesions in either species. Rather, PhIP caused lymphomas in both male and female mice, colon cancers preferentially in male rats, and mammary cancers in female rats (*29*). Monkeys also developed hepatocellular carcinomas after administration of IQ by nasal-gastric intubation (*30*).

Other mutagenic HCAs recently identified in cooked meat are aminomethylimidazofuropyridine (MeIFP) (*31*) and 2-amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine (4′-OH-PhIP) (*32*).

The HCAs have been classified into non–IQ-types and IQ-types. The amino groups of the non–IQ-types, the pyridoindole and dipyridoimidazole derivatives, are deaminated by treatment with 2 mM sodium nitrite to form hydroxy groups, which results in the loss of their mutagenic activity. By contrast, the amino groups of IQ-type HCAs are not affected by this treatment. Thus, the differential response to 2 mM sodium nitrite can be used to estimate the relative contribution of IQ-type and non–IQ-type HCAs to the total mutagenicity in crude fractions of test material (*33*). It was in this way that the mutagenicity of tobacco smoke was found to derive primarily from non–IQ-type HCAs.

Amino groups of both IQ and non–IQ-type HCAs are metabolically activated to *N*-hydroxyamino groups by cytochrome P450IA2 (*34, 35*). The *N*-hydroxyamino derivatives are further modified by esterification with acetic acid, sulfuric acid, and proline, and it is these forms that produce DNA adducts (*34*). The ultimate reactive forms of Trp-P-2, Glu-P-1, IQ, and PhIP form adducts at the C-8 position of guanine (*36*). This base specificity is consistent with the finding that in HCA-induced tumors, mutations in the *ras* and *p53* genes occur most often at G:C base pairs (*37*).

The frequency of HCA-induced DNA adducts in various tissues may provide information useful for risk evaluation. In rats treated with HCAs, DNA adducts are present in relatively high amounts in the kidney, heart, and pancreas, but no tumors develop in these organs (*38*). DNA adduct formation may therefore be necessary but in itself is not sufficient for carcinogenesis. For rational evaluation of human cancer risk attributable to HCAs, to which we are exposed daily, we must learn more about the complex molecular events occurring during carcinogenesis. In so doing, we must consider possible interplays between HCAs and other environmental carcinogens.

Multiple Genetic Alterations and Multistep Carcinogenesis

Since the discovery by Weinberg and others of mutationally activated H-*ras* genes in human bladder cancer cells (*39*), the concept of somatic cell mutation has become very relevant to carcinogenesis. There is now much information available on the multiple genetic alterations that underlie various human cancers. These alterations lead to activation or overexpression, or both, of oncogene products and inactivation or loss of tumor suppressor gene products. In one case of human pancreatic cancer, for example, our laboratory detected simultaneous amplification of both the activated K-*ras* oncogene and the c-*myc* gene (*40*). Several groups showed that nearly all cases of small-cell lung cancer display loss of heterozygosity (LOH) on chromosome 3p, chromosome 13q (the site of the *Rb* gene), and chromosome 17p (the site of the *p53* gene) (*41, 42*). LOH involves two genetic alterations, usually a mutation in one allele and loss of the other allele. Thus, induction of small-cell lung cancers may require at least six genetic alterations. A few specimens of small-cell lung cancer also exhibit amplification of *myc* family oncogenes (*41, 43*).

The existence of multiple genetic alterations in human colon cancer has been elegantly demonstrated by Vogelstein, White, Nakamura, and their co-workers (*44*). The critical genes identified thus far are K-*ras*, *APC*, *DCC*, *MCC*, and *p53*. The progression to a more malignant phenotype is accompanied by an increase in the number of genetic alterations. Precancerous lesions such as adenomas already display some genetic changes, but these changes appear to be insufficient for expression of the fully malignant phenotype (*45*).

A direct correlation between malignancy and accumulation of genetic changes has also been shown for human hepatocellular carcinoma. Infection of liver cells with hepatitis B virus (HBV) is thought to be an early event in hepatocarcinogenesis. The viral DNA may mediate its effects at least in part through its ability to integrate at random sites in liver DNA of infected individuals (*46*). When HBV sequences are used to probe DNA from normal and cirrhotic regions of the liver in Southern blots, the

Table 1. Correlation between histological grade and number of genetic alterations in hepatocellular carcinoma.

Histological grade	Loss of heterozygosity*			Amplification of *HST1*†
	Chromosome 4	Chromosome 16q	Chromosome 17p	
Early	0/2 (0)	0/6 (0)	0/5 (0)	0/5 (0)
Well differentiated	2/13 (15)	4/20 (20)	7/20 (35)	1/20 (5)
Moderately differentiated	8/14 (57)	12/23 (52)	16/22 (73)	2/22 (9)
Poorly differentiated	15/24 (63)	28/32 (88)	24/31 (77)	6/31 (19)

*Number in parentheses is frequency expressed as percentage. †*HST1* is the DNA stretch on chromosome 11q encompassing *INT2-HST1-EXP1-EXP2/PRAD1*.

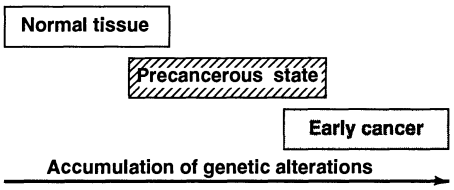

Fig. 3. Relationship between the appearance of genetic alterations and the development of cancer. In the future, information on the number and type of genetic alterations in various cancers is likely to have an impact on treatment decisions including, for example, the prevention of unnecessary surgery.

hybridizing DNA appears as a smear. Hybridizing DNA from cancerous regions of the liver, by contrast, appears as separate but distinct bands (Fig. 2), indicating that each tumor originates from a single cell (*47*). Furthermore, although there is often histopathological evidence of a "tumor within a tumor," where the central part of the tumor is composed of more malignant cells than the peripheral part, the DNA from the two parts nevertheless shows identical hybridization patterns. This result demonstrates that all the tumor cells originate from the same cell. Molecular analyses of one such tumor specimen that appeared to be "two tumors within a tumor" revealed that the peripheral (less malignant) lesion had a wild-type *p53* gene, whereas the two internal (more malignant) lesions had mutations in the *p53* gene, each at different codons (*48*). Thus, the hepatoma developed from a single cell and accumulated mutations during the course of monoclonal growth expansion. Like other cancers, hepatocellular carcinomas exhibit an increasing number of genetic alterations as they progress toward a more malignant phenotype (Table 1) (*49*).

It has been suggested that the high frequency of genetic alterations in malignant cells is associated with an increase in genomic instability. One possible manifestation of such instability is the chromosomal aberrations that are common in malignant cells. Genomic instability may be caused by mutations that affect the cellular machinery necessary for accurate replication and distribution of DNA into the two daughter cells (*50*). For example, a mutation in the gene encoding DNA polymerase may produce an enzyme with less fidelity, and mutations in the genes encoding proteins related to chromosome integrity, such as p53, could result in genomic instability. Enhanced cell growth itself increases the likelihood that genetic alterations will occur. In addition, the production of active oxygen species or nitric oxide in inflammatory cells during chronic inflammation or in precancerous lesions may provide favorable conditions for genetic alterations that predispose cells to malignancy. Loss of gap junctional communication may also lead to a sudden expression of altered phenotypes caused by the presence of genetic changes whose effects had previously been masked by normal intercellular communication.

The term "field cancerization" has been used to describe the development of cancers in a multicentric fashion in the same or adjacent organs (*51*). This phenomenon is often observed in tobacco-related cancers of the upper aerodigestive tract. For example, patients who present with pharyngeal cancer have an eight times higher risk of subsequently developing esophageal cancer compared to the normal population. Similarly, patients who present with laryngeal cancer have a seven times higher risk of developing lung cancer (*7*).

Evaluation of Carcinogenic Factors

The risk hazard of environmental carcinogens has been estimated mainly on the basis of data from animal studies and epidemiological analyses. The animal studies generally involve experiments with a single chemical tested at several different dosages. The epidemiological analyses are powerful primarily in cases in which there is heavy exposure of a defined population to a single chemical or to a mixture of a somewhat specialized group of chemicals, such as in tobacco smoke. With regard to the risk hazard associated with consumption of cooked meat and fish, three epidemiological studies have indicated a slight but significant enhancement of cancer development; one study revealed an increased risk for gastric cancers (*52*) and the other two revealed an increased risk for colon cancers (*53*). However, as discussed above, there are many ubiquitous carcinogenic factors associated with day-to-day life. Thus, any exposure to a single potentially harmful chemical superimposes its influence on a human body that has likely been exposed to many kinds of carcinogens in the past. Although each of these carcinogens may have only a minor impact on cancer risk, their cumulative impact could be substantial.

Other factors also need to be considered for biologically meaningful risk assessment. Because cancer is a disease that originates, in principle, from a single cell, carcinogenic toxicology is different from general toxicology. In the latter case, the concentration of the risk factor is expressed in terms of weight per unit-body-weight or per unit-body-surface. This approach is not appropriate for carcinogenic factors. A dose that would produce a single cancer cell in 1 rat among 250 is theoretically equivalent to the dose that would produce a single cancer cell in one human. Although this dose would prove fatal for only 1 of the 250 rats, it would be fatal for the one human; in other words, the risk in man is 250 times higher than that in the rat. Carcinogenic toxicology should therefore be viewed as "quantum toxicology." Given these considerations, we may be underestimating cancer risks with our current approaches. Of course, this is an oversimplification that does not take into account other factors such as natural defense mechanisms against cancer cell growth, which may be more pronounced in humans.

The existence of multiple carcinogenic factors, multiple genetic alterations in cancer cells, and multiple steps in carcinogenesis also underscores the uncertainties inherent in estimation of cancer risk. A pragmatic approach to eliminating carcinogenic factors that does not impose excessive burden on the individual or community seems the most rational approach at this stage of understanding.

Finally, prevention of carcinogenesis is of the utmost importance, especially for patients who have a high risk of developing a second neoplasm. Derivatives of β-carotene and ascorbic acid, antioxidants, polyphenolic compounds of plant origin, and polyunsaturated fatty acids of fish origin are among the most promising chemopreventive agents currently being investigated (*54*).

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Intracellular Signaling by Hydrolysis of Phospholipids and Activation of Protein Kinase C

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Hydrolysis of inositol phospholipids by phospholipase C is initiated by either receptor stimulation or opening of Ca^{2+} channels. This was once thought to be the sole mechanism to produce the diacylglycerol that links extracellular signals to intracellular events through activation of protein kinase C. It is becoming clear that agonist-induced hydrolysis of other membrane phospholipids, particularly choline phospholipids, by phospholipase D and phospholipase A_2 may also take part in cell signaling. The products of hydrolysis of these phospholipids may enhance and prolong the activation of protein kinase C. Such prolonged activation of protein kinase C is essential for long-term cellular responses such as cell proliferation and differentiation.

Protein kinase C (PKC) takes part in cellular responses to various agonists including hormones, neurotransmitters, and some growth factors. The enzyme is activated by increased amounts of diacylglycerol in membranes that result from agonist-induced hydrolysis of inositol phospholipids (*1*). However, hydrolysis of other phospholipids, particularly phosphatidylcholine, produces diacylglycerol at a relatively later phase in cellular responses, and a possible function of phospholipase D in cell signaling has been suggested (*2*). In fact, sustained activation of PKC is essential for subsequent responses such as cell proliferation and differentiation (*3*, *4*). It is also plausible that phospholipase A_2 is activated by most of the agonists that induce inositol phospho-

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