Multiple Genetic Alterations in Human Carcinogenesis

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Cancer development in man is to be a multistage process as suggested by epidemiological studies on commonly occurring gastric, colon, and breast cancers and also on human retrovirus-related leukemia, and by the finding by physicians and surgeons of precancerous lesions for many types of neoplasias. In the last 10 years it has become evident that human cancers have multiple genetic alterations caused by point mutations, recombinations, amplifications, and/or deletions. The genes affected include both oncogenes and tumor-suppressor genes and genes that accelerate cell proliferation and metastasis. Cancers with more malignant properties and poorer prognosis are generally associated with larger numbers of genetic alterations. These multiple genetic alterations are considered to be a direct reflection of the multiple steps involved in carcinogenesis. The multiple genetic alterations are caused by multiple environmental carcinogenic substances or factors, each of which usually exists only at minute concentrations and does not exert any major impact alone except under particular occupational, iatrogenic, and locally geographic conditions. The fact that carcinogenesis is a multistep process involving multiple genetic alterations clearly needs to be taken into consideration in assessing the risks of environmental carcinogenic substances or factors. The increasing incidence of multiple primary cancers is also most easily understood from the viewpoint of multiple steps in carcinogenesis. Possible multiple approaches to cancer prevention should therefore be considered in relation to multistep carcinogenesis and multiple carcinogenic factors.

Introduction

The existence of multiple steps in carcinogenesis was predicted by Rous and his associates from careful observations in animal experiments almost a half century ago (1). Armitage and Doll also reached the same conclusion from analysis of the age distributions of common cancers in humans in 1954 (2). More recently, it was similarly suggested on the basis of age distribution of patients that several events are required between initial HTLV-1 infection and onset of adult T-cell leukemia (ATL) (3). Moreover, two-step carcinogenesis in the dorsal skin of mice by a single application of a limited amount of carcinogenic polycyclic aromatic hydrocarbon followed by repeated application of croton oil was demonstrated by Berenblum and Shubik in 1947 (4).

The importance of genetic alterations in somatic cells in cancer development was described by Bauer in 1928 (5). However, in 1914 Boveri predicted the function of oncogenes, using the term “chromosomes which promote division,” and the inactivation of tumor-suppressor genes by saying that “inhibition chromosomes were eliminated” in his book (6). In the last 20 years, molecular biological studies have clearly revealed the existence of many oncogenes and tumor-suppressor genes and their respective activation and inactivation among many types of cancers.

Multiple Genetic Alterations

The occurrence of more than one alteration of cancer-related genes, namely, N-ras activation and c-myc amplification in cultured HL-60 cells, was first observed by Weinberg and associates (7). Subsequently, Taya et al. demonstrated K-ras mutation and c-myc amplification in a human lung giant-cell carcinoma, Lu-65 (8). Yamada et al. first demonstrated the coexistence of a mutation in K-ras, amplification of mutated K-ras, and amplification of c-myc in human pancreatic cancer without passing through cell culture (9). Loss of heterozygosity (LOH) of chromosomes 3p, 13q, and 17p was first observed by Yokota et al. in human small-cell lung cancers (10). The genes responsible for the alterations of chromosomes 13q and 17p were the Rb and p53 genes, respectively, while that on chromosome 3p was suggested to be a gene for a receptor-type protein tyrosine phosphatase γ (11), although the possible contribution of other genes was also envisaged. Vogelstein et al. demonstrated the involvement of multiple genes in human colon carcinogenesis, detecting point mutations of K-ras,
N-ras, and allelic deletion of chromosomes 5q, 17p, and 18q (12). The development of convenient laboratory methods such as RFLP [restriction fragment length polymorphism (13)], PCR [polymerase chain reaction (14)], and SSCP [single-strand conformation polymorphism (15)] analyses has greatly facilitated accumulation of information on multiple genetic alterations in various types of human cancers. Indeed, application of these techniques to small amounts of clinical materials has opened a new field of clinical molecular pathology. As a result there are now more than 50 reports available on multiple genetic alterations in human cancers (16).

**Multistep Carcinogenesis**

Fearon and Vogelstein reported the accumulation of genetic alterations during human colorectal carcinogenesis documenting the presence of multiple steps at the molecular level (17). The involvement of a gene on chromosome 5q in familial polyposis of the colon was first reported by Bodmer’s group (18). During in vivo progress from normal epithelium to hyperproliferation epithelium, early adenoma, intermediate adenoma, late adenoma, and carcinoma sequence, the frequencies of LOH of chromosome 5q, K-ras activation, and LOHs of chromosomes of 17p and 18q increase (17). The gene on chromosome 18q was identified by Fearon et al. as DCC (deleted in colorectal cancer), which encodes for a cell adhesion protein (19). More recently, altered genes on chromosome 5 were identified as MCC (mutated in colorectal cancer) and APC (adenomatous polyposis coli) by Vogelstein’s (20,21), Nakamura’s (22), and White’s (23,24) laboratories.

Tumor growth is thought to be monoclonal, originating from a single cell. This was difficult to prove in human cancers in vivo, but Tsuda et al. confirmed it using surgically resected hepatocellular carcinomas (25). They were able to obtain many specimens of early hepatocellular carcinomas that were surgically removed from patients diagnosed by a recently developed ultrasonic system for tumor detection. The hepatitis B virus genome integrates into the genome of hepatic cells at random sites, but DNA isolated from hepatomas of different patients and from different foci in individual patients gave separate, distinct signals for integration site bands (25–27). In addition, Tsuda et al. succeeded in demonstrating a stepwise carcinogenic process in a nodule in which hepatocellular carcinoma cells were clonally growing. For this, they examined DNA of a small nodule composed of more malignant cells in a nodule composed of less malignant cells. This is known as a tumor within a tumor. DNA isolated from both the peripheral, less malignant part and the central, more malignant part showed the same single, distinctive band of integrated HBV genome, whereas DNA from the noncancerous portion surrounding the nodule gave only a smear with an HBV genome on Southern blot analysis (25). In a hepatocellular nodule containing two small nodules composed of more malignant cells, the DNA of these two malignant carcinomas both showed mutations of p53 genes, but in different exons. Furthermore, mutations of p53 genes were not observed in the peripheral, less malignant parts (Oda and Hirohashi, personal communication). In hepatocellular carcinomas, frequencies of LOHs on chromosomes 4, 16q, and 17p increase in general with the acquisition of more malignant phenotypes; i.e., from early, well-differentiated, through moderately differentiated to poorly differentiated lesions (28).

There is much circumstantial evidence that amplifications of various genes accumulate during the late phase of carcinogenesis. Chromosomal aberrations are also often found in clinically advanced cases of cancers. Amplification of multidrug-resistance genes is an example of further genetic alteration after establishment of malignancy (29). Genetic alterations are often found more frequently in metastatic foci than in primary tumors (10), and several genes have been proposed as being causally related to metastasis (30). Genomic instability would be expected to play a role in more rapid accumulation of further genetic alterations. A genetic alteration resulting in genomic instability, which might correspond to mutator mutations in microbial genetics (31), would be crucial in human carcinogenesis and could be called a “carcinogenizer mutation.”

**Specificity in Genetic Alterations with Regard to Organs, Histopathology, and Clinical Stages of Human Cancers**

Genetic alterations show some specificities depending on the organ of origin, histopathology, and clinical stage of cancers. For example, K-ras mutations are known to be common in pancreatic and colon cancers, but not in breast and liver carcinomas (32). The c-erbB-2 oncogene, encoding a tyrosine kinase receptor, is amplified in scirrhous-type adenocarcinomas, signet-ring-cell carcinomas, and mucinous adenocarcinomas, but not in the intestinal type of stomach cancer (34,35). This K-sam gene was originally isolated as an amplified gene in KATO III cells, derived from a signet-ring-cell carcinoma of the stomach (34,36). In 50% of the esophageal cancers and 15% of the breast cancers examined, the chromosome 11q13 locus, containing the HST1 and INT2 genes, was found to be amplified, but almost none were found in the stomach cancers (37,38). Study of molecular mechanisms underlying genetic alteration is important for establishing etiology of cancers among various organs.

Information on specificities of multiple genetic alterations should thus provide clues as to why cancer development occurs in particular organs. For instance, analysis of mutation site and base change for hepatomas in aflatoxin B1-contaminated and noncontaminated areas suggested different patterns in the mechanism of mutation in p53 genes (39,40). Interestingly, cholangiocarcinomas in Japan are frequently associated with K-ras activation, but those in Thailand, which resulted in part from liver fluke infection, have no K-ras mutation, whereas both show the same frequency of mutation of the p53 gene (Tsuda and Hirohashi, unpublished data).
Multiple Environmental Carcinogens, Carcinogenic Factors, and Carcinogenic Conditions

The multiple genetic alterations in carcinomas are presumably caused by multiple kinds of environmental xenobiotics and autobiotics. As xenobiotics, polycyclic aromatic hydrocarbons, N-nitrosamines, mycotoxins, and heterocyclic amines are typical genotoxic carcinogens. However, the extent of exposure to any one of such chemicals is generally thought to be very small in comparison with their effective doses for producing cancers in rodents in standard long-term carcinogenesis experiments. It should be true for exposures to ionizing radiation and ultraviolet light. Autobiotic genotoxic agents such as oxygen radicals can also modify DNA bases, as represented by the formation of 8-hydroxyguanine and thymine glycol (41,42). In addition, nitric oxide synthase produces NO from L-arginine, this NO being involved in endogenous formation of nitrosamine compounds (43–45) as well as inducing deamination of 5-methylcytosine residues of DNA to produce thymine residues, resulting in transition of C to T after replication of DNA (46). With all the compounds described above as examples, DNA damage is caused, which could lead to genetic alteration in somatic cells.

The fidelity of DNA replication and equal distribution of the genome between daughter cells both involve some possibility of genetic alterations, and cell proliferation itself might be expected to increase the chances of these genetic alterations occurring. Cell turnover in healthy resting-cell populations can be induced by viral or bacterial infection, mechanical tissue damage, and inflammatory reactions. The structure of the hepatitis C virus (HCV) was recently fully elucidated (47,48), and HCV infection causes chronic hepatitis associated with death of hepatic cells and their compensatory regeneration. However, genomic integration of the HCV genome has not so far been observed. Instead, hepatic cells that undergo many generations through repeated cell divisions are more likely to accumulate genetic alterations by chance. This condition could be termed “local aging” or “topographic senescence” limited to the liver, because in healthy adults, hepatic cells rarely divide, probably once a year (49). Hepatocytes that divide 30 times a year represent a hepatic cell population aging 30 times faster than the liver in healthy condition.

There is also a class of nongenotoxic carcinogens called tumor promoters. The best known tumor promoter is 12-O-tetradecanoylphorbol-13-acetate (TPA), isolated from croton oil. Other compounds with similar strong tumor-promoting activity in two-step carcinogenesis experiments in the dorsal skin of mice have also been reported. These are teleocidin from mycelia of Streptomyces medioacetidicus, lyngbyatoxin A from a blue-green alga, Lyngbya majuscula and aplysia toxin from a blue-green alga, Lyngbya majuscula (50,51). All these tumor promoters share the same surface receptors and activate protein kinase C (51).

Okadaic acid, a polyether compound of a C36 fatty acid obtained from a black sponge, was also found to be a tumor promoter in mouse skin experiments, having the same potency as TPA (52). However, unlike TPA and teleocidin, okadaic acid is a potent inhibitor of protein phosphatases, modulating phosphorylation of intracellular proteins, including some related to the cell cycle (53). All these tumor promoters stimulate prostaglandin E2 synthesis (54).

TPA, teleocidin, lyngbyatoxin A, aplysia toxin, and okadaic acid are effective at microgram levels in carcinogenesis experiments when applied to mouse skin and exert many biological activities in vitro even at nanogram levels. However, there is, ironically, no evidence that these specifically functioning tumor promoters which have been most intensively studied in the international cancer research community have, in fact, any relation to human carcinogenesis, except for one paper describing a relationship between drinking bush tea and esophageal cancer in Caribbean people (54).

More probably, common food components act as tumor promoters. For example, sodium chloride might enhance development of gastric cancer through damage of gastric mucosa and production of chronic gastritis (55). In rat experiments, sodium chloride is reported to show a promoting effect in gastric carcinogenesis and to increase levels of malondialdehyde in both gastric mucosa and urine (56,57). Higher fat intake should yield a tumor-promoting condition for colon cancer development through more formation of bile acid (58) and for breast cancer development through increased generation of estrogen by aromatase in adipose tissues (59).

Recently, Long-Evans rats with a cinnamonlike coat color (LEC) were established. These LEC rats develop hepatitis spontaneously at 4 months old, and in those that overcome the acute phase and survive with chronic hepatitis, hepatomas develop at 1–1.5 years of age without exception (60). It was found that copper accumulates in their livers and causes cell death and compensatory regeneration (61). It is probable that this excess copper increases oxygen radical production, thereby causing continued turnover of hepatic cells and creating a favorable environment for hepatocarcinogenesis. It is noteworthy that hepatomas in LEC rats lack activation of any of the ras oncogenes (62).

Multiple Genetic Backgrounds

Among many reports of cancer-prone families, one of the oldest records is probably that of gastric cancers in the Napoleon family (63). In xeroderma pigmentosum, characterized by a deficiency in the molecular mechanism of DNA repair of pyrimidine dimers produced by UV, multiple skin cancers develop on exposure to sunlight (64). Familial adenomatous polyposis coli, Wilms’ tumor, and retinoblastoma families are also well documented as cases of neoplasia with a genetic background, and the genes responsible for these diseases have recently been identified (21,23,65–67). Li Fraumeni syndrome is associated with a germ line mutation in the p53 gene. The mutated sites are in the region from amino acid codons 245 to 258 (68,69). Many
other genetic diseases, including the Lynch syndrome and SBLA syndrome, have also been reported (70,72), but the genes responsible for these diseases have not been identified as yet. More or less nonspecific genetic abnormalities may also be involved in cancer development in cancer-prone families, although the nature of the affected genes in cancer cells among families has not been completely elucidated.

In addition to hereditary alterations of genes in somatic cells, host factors are also important. These include genetic control of drug-metabolizing enzymes, such as cytochrome P450 (72,73), genetic polymorphism of acetylating enzymes (74,75), and the genetic background of histocompatibility (78).

Risk Estimation and Multiple Carcinogenic Steps Involving Multiple Genetic Alterations

Although evidence has been accumulating that multiple carcinogenic steps and multiple genetic alterations are involved in carcinogenesis, it has also become clear that there are many carcinogens in the ordinary human environment. Their number, especially of genotoxic carcinogens, is enormous, but each one is usually present at only very low levels. Risk estimation once seemed relatively simple and very promising, but with increasing awareness of the situation, assessment of actual risk has proved complicated and difficult. It is now generally accepted that there must be many cells with some genetic alterations in the bodies of adult and aged people. The number of genetic alterations may not be sufficient to yield fully malignant cancer cells, but the affected populations could probably be quite easily converted to malignant cells by additional genetic lesions caused by environmental mutagens/carcinogens. The extent of exposure to such compounds necessary to convert these intermediate cells to malignant cells would probably be much smaller than that necessary to drive the whole process from a normal cell base level. Various mathematical formulas have been proposed to calculate increase in risk. For instance, assuming the necessity of 10 genetic alterations for fully malignant conversion, cells with 8 and 9 genetic alterations have, respectively, 1 million and 1 billion times higher sensitivity than normal to conversion to fully malignant cells by further exposure to environmental mutagens/carcinogens (Sakai et al., unpublished data). Cells with some, but not sufficient, genetic alterations are called in clinical terms precancerous cells, and regions composed of these cells are called precancerous regions. Some of them, however, may not be necessarily distinguishable from normal tissue in their appearance.

It is often claimed that the actual intake (exposure size) of environmental mutagens/carcinogens is very small in comparison with the dose necessary to induce tumors in adult healthy rodents in long-term feeding experiments. However, recent molecular biological studies have revealed the monoclonal nature of carcinogenesis. During monoclonal growth of cells with some genetic alterations, additional genetic alterations occur. If tumor cells really originate from a single cell, the tumor risk depends on the target size, namely, the cell number from which the cancer could originate. Cancer cells arise from a certain size of stem cell population, and the number of these cells is proportional to the total number of cells in the body. In other words, the risk of cancer development probably depends on the size of the body, or the total number of cells. A human weighs $10^7$ orders (300 times) more than a rat and $10^8$ orders (3000 times) more than a mouse. Therefore, at the same concentrations of environmental carcinogens/mutagens, the human risk of developing cancer cells is $10^2$ orders (900 times) more than for the rat and $10^3$ orders (3000 times) more than for the mouse. Moreover, the normal lifespan of man is about 50 times that of rodents. Because the mean generation time of cells may be almost the same in humans and rodents, the risk of developing cancer cells may be 15,000 and 150,000 times higher in humans than in rats and mice, respectively. The above calculation is clearly based on oversimplifications, but, if based on this calculation, the actual intake of heterocyclic amines in cooked meat and fish by humans in normal, daily life corresponds to the doses that are carcinogenic in rodents (77). This is true not only for heterocyclic amines, but also for other environmental mutagens/carcinogens including polycyclic aromatic hydrocarbons, N-nitrosamines, and mycotoxins. Because oxygen radicals may play an important role (78), the specific metabolic rate of humans, at 30 calories/g/day, about one-tenth of the 200 calories/g/day in mice (79), needs to be carefully considered to explain their different contribution to cancer development between humans and rodents.

The presence of many types of anticarcinogenic substances in diet also requires inclusion in real risk estimation (80). These substances are antioxidants, retinoids, indoles, aromatic isothiocyanates, and plant-originated polyphenols. Consideration of these factors is important to avoid overestimation of the risk of carcinogens. On the other hand, factors effecting tumor promotion should also not be overlooked. In the two-step initiation/promotion experiments, the carcinogenic effect of the genotoxic substance 7,12-dimethylbenz[a]anthracene painted on mouse skin could easily be enhanced about 100 times by subsequent treatment with croton oil or its active principle, TPA (81). Similar promotion was observed for treatment of the dorsal skin of mice with a heterocyclic amine, 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), and then TPA (82).

Long-term experiments in animals on the effects of coadministrations of various kinds of genotoxic carcinogens are difficult to conduct. Ito et al. devised a new approach, “Ito’s method,” for medium-term tests using the rat liver (83). Diethylnitrosamine treatment, partial hepatectomy, and then administration of a test substance results in the formation of glutathione S-transferase placental form (GST-P)-positive foci in the liver, the number and area of which can be measured using a color video image processor. This method requires a shorter period and fewer animals compared to long-term carcinogenesis experiments and is useful for screening hepatocarcino-
gens and their inhibitory substances and for estimating risk of carcinogens. Ito et al. have used the method to examine the effects of combinations of five heterocyclic amines each at 1/25 and 1/5 of their carcinogenic doses, and found that in combination, these compounds exerted additive effects (84). Probably many other carcinogens present in minute amounts in our environment are acting on our body in a similar way.

Another factor that requires consideration is the age at which exposure takes place. Thus it should be mentioned that neonatal mice are much more sensitive to heterocyclic amines than are adult mice (85).

**Multiple Primary Cancers**

Recently, the cure rate of cancer has markedly increased, but second primary cancers often develop in patients whose first primary cancer has been completely cured. This second primary cancer is not a metastasis or a recurrence of the first primary cancer. Moreover, it is not necessarily due to chemotherapy or radiotherapy of the first tumor. Furthermore, a third primary cancer appears not rarely after cure of the second cancer and subsequently fourth and even fifth primary cancers have been observed (86). The age of onset of these primary cancers increases with increase in their number. About 8% of the patients being treated at the National Cancer Center Hospital from 1962 to 1989 were cases of such multiple primary cancer (87). Multiple primary cancer occurs metachronously as described above. However, synchronous multiple cancers are also often found in single organs (also called multifocal cancers), such as the lung, stomach, liver, prostate, or urinary bladder, as well as simultaneously in two or more organs. A typical cause of multiple primary cancers is exposure to cigarette smoke. For example, there are noticeable linkages of laryngeal cancer with lung cancer and of pharyngeal cancer with esophageal cancer (87). It seems likely that these cancers of the respiratory and upper digestive tracts are due to heavy exposure to cigarette tar, and many other cells in the tract should have some, but not sufficient, numbers of genetic alterations for their conversion to cancer cells. These cells could be buds or precursors of subsequent primary cancers. Multiple primary cancers are not a new phenomenon, but only recently has attention been called from scientists of cancer-specializing institutes and hospitals and officers in governmental agencies to this health issue. Serious consideration of suitable methods to prevent cancers including multiple primary cancers are urgently needed in light current knowledge of multiple genetic alterations in cancer cells and of multistep carcinogenesis.

**Multiple Approaches for Cancer Prevention**

As discussed above, there are multiple genetic alterations, multiple carcinogenic steps, multiple environmental factors, and multiple primary cancers. This means that multiple approaches are not only necessary but also possible for cancer prevention. As multiple carcinogenic factors are involved, there is no single royal road to cancer prevention. The risk factor for any single compound determined even from exhaustive animal experiments is hard to apply in reality to humans. Even the background of the human populations is highly heterogeneous, not only genetically but also in cellular composition with regard to somatic cells with previous genetic alterations. Moreover, humans are exposed to many different types of carcinogenic factors simultaneously and/or successively.

A realistic and practical approach is to try to reduce the extent of exposure of humans to carcinogenic factors as far as possible, unless this results in serious discomfort at the individual level or serious inconvenience at the community level, including economic burden to the nation. In the case of individuals, educated individual decision making should be encouraged with acceptance, at their own risk, of certain levels of exposure. In the case of the community, well-balanced committees of aware scientists, realistic economists, and critical sociopsychologists should decide pragmatically on suitable measures to prevent cancers without seriously affecting the development of societies, even though such decisions may be the targets of idealistic people. In other words, in some cases, it may be necessary to accept the presence of minute amounts of some carcinogens in our environment.

In fact, there are many examples of carcinogens in our ordinary, daily life that we must accept, irrespective of whether we are conscious of their existence (88,89). Well-known examples are aflatoxin B1 in peanuts and their products and heterocyclic amines ubiquitously present in cooked meat and fish. However, exposure to these compounds could be reduced by more strict regulation of aflatoxin B1 and by appropriate methods to lessen the formation of the latter compounds in cooked meat and fish such as by use of microwave ovens and by wrapping meat and fish in aluminum foil to prevent charring. The avoidance of meat and fish contact with a naked flame and the removal of charred areas are also effective for lessening the intake of heterocyclic amines.

In cases of cancers due to viral infection, block of the route of viral infection is extremely useful: for instance, stopping breast milk feeding for preventing HTLV-I infection, careful screening of donor blood for HBV and HCV, and hygienic sexual intercourse for preventing HPV infection. In addition, vaccination is definitely an effective approach.

Generally speaking, the holistic approaches to improvement of lifestyle are much more important than decreasing the extent of exposure to a single carcinogenic factor. For instance, the prevalence of gastric carcinomas, which are still a main form of cancer in many countries of the world, has been decreasing markedly in advanced Western countries, indicating that it can indeed be prevented. In countries where its prevalence is still high, avoidance of a high intake of sodium chloride, reductions in nitrite and nitrate intake, the use of a refrigerator rather than food preservatives, consumption of green-yellow vegetables and fruit rich in vitamin C and carotenoids, and maintenance of hygienic oral and tooth conditions are all recommendations requiring implementation. If *Helicobacter*
pyrolidin can be proved more clearly to be related to gastric carcinogenesis, as recently reported (90), effective measures should also be taken to eliminate this bacteria from gastric gland crypts, although Helicobacter is opportunistic and may reappear after antibiotic and other drug treatment. Thus, there are multiple pragmatic approaches to cancer prevention that could be adopted based on recent findings regarding the multiple genetic alterations, multiple steps, and multiple environmental factors involved in carcinogenesis.

**Epilogue**

The realization that carcinogenesis involves multiple genetic alterations of cells, multiple steps, and multiple environmental factors has greatly enhanced our understanding of cancer cells and the cancer problem and has also provided the rationale for realistic approaches to cancer prevention. An important point to be considered is the impact of information on multiple genetic alterations and multiple carcinogenic steps on cancer therapy. There should be many target molecules for cancer drugs (91). Remedy for one among many genetic alterations may suppress the malignant phenotype. In the next 10 years, the development of multiple methods for cancer therapy can be envisaged as a rational approach to control of neoplasia.

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