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Meeting Report

Multistage Carcinogenesis: The Twenty-Second International Symposium of the Princess Takamatsu Cancer Research Fund

The Twenty-Second International Symposium of the Princess Takamatsu Cancer Research Fund on the topic "Multistage Carcinogenesis" was held in Tokyo, Japan, on November 19–21, 1991. The opening address was given by H. I. H. Princess Kikuko Takamatsu who welcomed and encouraged the invited participants to pursue a joint strategy of basic research and clinical research for cancer prevention and therapy. This sound strategy was reflected in the high quality content of the symposium including the vigorous discussion among all of the participants. In his introductory remarks, Dr. T. Sugimura (National Cancer Center Research Institute, Tokyo, Japan) discussed the impact of the concept of multistage carcinogenesis on cancer clinics and prevention and control programs. He emphasized both the value of cancer prevention in light of new second malignancies occurring in patients cured of their initial cancers and the multiple aspects of carcinogenesis including the importance of inherited predisposition of cancer.

The epidemiological, clinical, and laboratory evidence supporting the multistage nature of carcinogenesis was presented by Dr. C. Harris (National Cancer Institute, Bethesda, MD) in the keynote address. The recent identification of the multiple oncogenes and tumor suppressor genes involved in carcinogenesis is leading to a better understanding of the molecular mechanisms of carcinogenesis and providing molecular targets for the development of innovative therapy. The analysis of mutational spectra of these cancer-related genes, *e.g.*, *p53* and Ki-*ras*, also suggests molecular linkage between environmental carcinogens and carcinogenesis.

Clinical Observation of Multistage Carcinogenesis

Colorectal tumors provide an excellent system in which to search for and to study the genetic alterations involved in the development of a common human cancer. Dr. E. Fearon (Johns Hopkins University, Baltimore, MD) presented the results of the productive strategy of first identifying chromosomal regions harboring putative tumor suppressor genes by allelic loss analysis and then pinpointing the specific suppressor genes including *p53* on chromosome 17p, *DCC* on 18q, and *MCC* and *APC* on 5q. Missense mutation of the *p53* gene usually occurs before loss of the second normal allele of *p53*. A positive association was also found between the amount of allelic loss on multiple chromosomal sites and a poor prognosis. Breast cancer is another common tumor that progresses through multiple stages. Studies by Dr. M. Lippman (Georgetown University, Washington, DC) and coworkers suggested that hormone-dependent breast cancer cells release growth factors, including insulin-like growth factors 1 and 2, TGF-α,[1] platelet-derived growth factor, FGF, and *erb*-B2 ligands, that are under estrogenic control. Secreted FGF molecules participate in both autocrine and paracrine loops which appear pathogenetically significant in tumor progression. Conversion to the hormone-independent phenotype is often accompanied by both constitutive growth factor production, such as K-FGF, and the resultant enhanced angiogenesis.

Cancer sometimes clusters within specific families. Dr. S. Friend (MGH Cancer Center, Charleston, MA) and coworkers have discovered the genetic basis of this predisposition in retinoblastoma and the Li-Fraumeni syndrome. Germ line mutations in the *p53* tumor suppressor gene have been found in about two-thirds of the families clinically classified as belonging to the Li-Fraumeni syndrome. The remaining one-third of the families may be either misclassified or have defects in other genes involved in the *p53* pathway that negatively regulates cell proliferation. Tumors arising in the patients with a germ line *p53* mutation have lost the normal second *p53* allele which is consistent with the sequence of genetic alterations in colon carcinogenesis discussed by Dr. Fearon. The germ line *p53* mutations were found in exons 5–8 and the variation in their pathobiological effects is being experimentally tested at the biochemical and cellular levels. *De novo* germ line *p53* mutations were also described in other high risk groups. The remaining question of the genetic alterations in those patients exhibiting the Li-Fraumeni syndrome which are involved in neoplastic development remains as yet unanswered.

Allelic deletion analysis has also been a productive strategy in the study of lung cancer. Dr. J. Yokota (National Cancer Center Research Institute, Tokyo, Japan) presented results indicating that allelic deletion was significantly higher in brain metastasis (85% on chromosome 3p, 71% on 13q, and 86% on 17p) when compared to the primary non-small cell lung cancer (56% on 3p, 39% on 13q, and 38% on 17p). The acquisition of metastatic potential in colorectal carcinoma was accompanied by allelic deletions on chromosomes 13q, 14q, and 18q.

Cholangiocarcinoma is geographically clustered in areas where the liver flukes, *Clonorchis sinensis* and *Opisthorchis viverrini*, are endemic. Dr. C. Pairojkul (Khon Kaen University, Khon Kaen, Thailand) described the pathogenesis of liver fluke-associated cholangiocarcinoma including speculation that *N*-nitrosamines may be etiological factors. Mutations in Ki-*ras* were less frequent in tumors from Thailand (8%) than in those from Japan (58%). The frequency of *p53* mutations were similar: 35% in Thailand and 25% in Japan. Dr. S. Hirohashi (National Cancer Center Research Institute, Tokyo, Japan) discussed the pathology and molecular mechanisms of a more common liver cancer, HCC. The majority of HCCs are associated with chronic active viral hepatitis. Hepatitis C virus has been recently recognized for its etiological importance in Japanese HCC. The mutational spectrum of the *p53* tumor suppressor gene is different in HCC from Japan when compared to HCC in Qidong, China; *i.e.*, there are fewer codon 249 mutations in Japan. When compared to rodent HCC, mutations in the *ras* protooncogenes in human HCC are rare.

Experimental Observation of Multistage Carcinogenesis

Cancer researchers, such as Peyton Rous, who were investigating animal models in the early 20th century recognized the

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[1] The abbreviations used are: TGF, transforming growth factor; HCC; hepatocellular carcinoma; FGF, fibroblast growth factor.

multistage nature of carcinogenesis. The molecular alterations underlying this multistage process in the classical mouse skin carcinogenesis model were discussed by Dr. A. Balmain (Beatson Institute for Cancer Research, Glasgow, United Kingdom). The predominant sequence of genetic events is: (*a*) mutation in Ha-*ras* during tumor initiation; (*b*) duplication of the mouse chromosome 7 carrying the mutant Ha-*ras* as the papilloma forms; (*c* mutation in *p53* and loss of the second allele on mouse chromosome 11 during conversion of the papilloma to a squamous cell carcinoma; and (*d*) loss of a putative tumor suppressor locus on mouse chromosome 7, located at or near Ha-*ras*, which is involved in the transition from squamous to spindle cell carcinoma.

The role of hormones in multistage prostatic carcinogenesis was described by Dr. M. Bosland (New York Medical Center, New York, NY). A high frequency (70%) of Ki-*ras* mutations (G_{35} to A base substitutions) occurs in rat prostatic carcinomas induced by *N*-methyl-*N*-nitrosourea followed by chronic exposure to testosterone. A putative DNA adduct associated with chronic 17β-estradiol exposure in combination with low-dose testosterone was found by ^{32}P postlabeling analysis in the dorsolateral and anterior prostate which are the tissue regions giving rise to low-grade carcinomas in a second model of rat prostatic carcinogenesis. As with many such experiments, the structure of the DNA adduct is unknown and may or may not involve the steroid hormone directly.

An additional rat model of prostatic carcinogenesis has been investigated by Dr. N. Ito (Nagoya City University Medical School, Nagoya, Japan) and coworkers. Chronic administration of testosterone has been shown to enhance spontaneous and carcinogen-induced prostatic carcinoma. Although 3,2′-dimethyl-4-aminobiphenyl alone induced only noninvasive carcinomas in the ventral prostate of F344 rats, when testosterone propionate was given with and after 3,2′-dimethyl-4-aminobiphenyl administration, invasive adenocarcinomas with metastasis developed. The molecular bases of these pathological changes are being investigated; no changes in Ha-*ras* or *p53* genes have thus far been detected.

Regulation of Cell Growth and Differentiation in Multistage Carcinogenesis

Abnormalities in cell growth and differentiation pathways have long been recognized in carcinogenesis. Recent comparative studies of normal preneoplastic and neoplastic mammalian cells as well as simpler eukaryotic organisms have revealed critical genes involved in signal transduction, cell cycle control, and carcinogenesis. Dr. M. Yanagida (Kyoto University, Kyoto, Japan) and others have recently demonstrated the importance of protein phosphatases in cell division cycle and genomic stability in yeast. There are at least five genes (*dis2, scls21, ppa1, ppa2*, and *ppx1*) for serine(threonine) phosphatase, one (*pyp1*) for tyrosine phosphatase, and one for serine(threonine,tyrosine) phosphatase (*cdc25*) in fission yeast. Except for *cdc25* which dephosphorylates *cdc2* kinase(s), the target phosphoprotein substrates essential for cell division have not been identified for the other phosphatases. Mutant *dis* genes can cause aneuploidy including a 600-fold increase in the frequency of chromosomal loss. Analysis of human homologues of these and related phosphatase genes in human cancers may provide clues concerning the molecular basis of genomic instability during carcinogenesis and tumor progression.

Dr. D. Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) has also significantly contributed to the yeast paradigm of cell division control. *cdc2* kinase(s) is dephosphorylated and activated by *cdc25* tyrosine phosphatase(s) at the initiation of mitosis. *cdc25* is a multigene family in human cells. When antibodies to one of the human *cdc25* homologues was microinjected into mammalian cells, they arrested in mitosis. Biochemical studies revealed that complexes between *cdc25* and cyclin B_1 or B_2, but not cyclin A, were required for *in vitro* phosphatase activity.

The *trk* oncogene was first identified in a colon carcinoma biopsy by using gene transfer assays and is frequently activated by chromosomal rearrangement, deletion, or point mutation in thyroid papillary carcinoma. Dr. M. Barbacid (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ) presented data indicating that *trk* is a 3-member receptor family which binds 5 different neutrophic ligands with varying affinities; nerve growth factor is the primary ligand of *trk*, brain-derived neurotropic factor (BDNF) is the primary ligand for *trk*-B, and NT3 is the primary ligand for *trk*-C. Exposure of NIH 3T3 cells transfected with *trk*, *trk*-B, or *trk*-C to their primary ligands leads to the transformed phenotype. *trk*-B and *trk*-C have not as yet been implicated in human cancer.

Several protooncogenes have been shown to be amplified in human cancers. Dr. M. Terada (National Cancer Center Research Institute, Tokyo, Japan) described the isolation and biological characteristics of amplified K-*SAM* and N-*SAM* which are tyrosine kinase membrane receptors activated by FGF ligands. K-*SAM* is amplified in 20% of poorly differentiated stomach carcinoma but not in well differentiated carcinoma. In contrast, c-*erb*B2 is amplified in 40% of well differentiated stomach carcinoma but not in poorly differentiated tumors. Frequently in esophageal carcinoma, an amplicon containing *hst1*, *int2*, and *prad1* genes is found on chromosome 11q13. Whereas *hst1* and *int2* are not expressed in these carcinomas, *prad1* mRNA expression has been detected. The role of *prad1*, the product of which is a cyclin D, in esophageal carcinogenesis is being investigated.

Dr. P. Nettesheim (National Institute of Environmental Health Sciences, Research Triangle Park, NC) described abnormalities of growth factor systems in transformed rat tracheal epithelial cells. Evidence was presented that TGF-α functions as an autocrine growth factor in both normal and transformed cells but is down-regulated at confluence only in normal cells. Normal cells were found to be highly responsive to the growth-inhibitory effects of TGF-β and to secrete and activate TGF-β in late log and plateau phases of growth. In contrast, transformed cells are hyporesponsive to TGF-β and secrete no measurable amounts of active TGF-β. Dr. H. Moses (Vanderbilt School of Medicine, Nashville, TN) continued the discussion of TGF-β. Inhibition of skin keratinocyte proliferation by TGF-β appears to involve the retinoblastoma tumor suppressor gene protein that leads to suppression of c-*myc* mRNA expression via a non-retinoblastoma *p106* protein. The *p53* tumor suppressor gene also down-regulates transcription of c-*myc* in mink lung cells. In contrast, certain mutant *p53* products enhance c-*myc* transcription.

Dr. Y. Takai (Kobe University School of Medicine, Kobe, Japan) described a stimulatory type of GDP/GTP exchange protein, smgGDS, that activates Ki-*ras* p21, smgp21A, smg21B, rhop21, and rhoB p21 but not c-Ha-*ras* p21 and smg25A. Overexpression of both smgGDS and Ki-*ras* transforms NIH 3T3 cells. Whether smgGDS can act as an oncogene in human cancer is currently an open question.

Genetic Instability and Multistage Carcinogenesis

Aneuploidy and chromosomal aberrations are characteristic genetic abnormalities in human cancer. Deamination of 5-methylcytosine at CpG dinucleotide sites in DNA forms a T and this endogenous mutagenic mechanism is considered to be responsible for about 40% of all human germ line point mutations. Dr. P. Jones (University of Southern California Comprehensive Cancer Center, Los Angeles, CA) concluded that deamination of 5-methylcytosine is a major contributor of *p53* mutations in colorectal tumors but not lung cancers. When low-grade bladder carcinoma cells were transfected with certain mutant *p53* genes, the cells increased their invasive potential in an athymic nude mouse model.

Dr. L. Loeb (University of Washington, Seattle, WA) postulated that a mutator phenotype may be required for tumor progression. Potential sources of endogenous mutagenesis are errors in DNA replication and damage to DNA by oxygen-free radicals. When the spectrum of mutations caused by oxygen-free radicals in a *lacZ*-x fragment was examined, the most frequent base substitutions were C → T, G → C, and CC → TT transitions.

Chromosomal aberrations can be caused by deregulation of the cell division cycle by abnormalities in expression and/or function of checkpoint genes such as *cdc25*. Dr. H. Okayama (Osaka University, Osaka, Japan) has recently cloned two human homologues of *cdc25* from the fission yeast, *Schizosaccharomyces pombe*. One of these *cdc25* homologues is frequently overexpressed in neoplastic human cells and in cells infected by SV40 virus or transfected with *E6* gene of human papillomavirus 16. Overexpression of this *cdc25* homologue is also found in colorectal carcinomas from patients with adenomatous polyposis coli; carcinoma > adenoma > "normal"-appearing mucosa.

Insight into the molecular mechanisms by which DNA tumor viruses transform cells has come from the recognition that the virus-encoded oncoproteins interact specifically with important cell-regulatory proteins such as *p53* and pRb tumor suppressor gene products. Dr. P. Howley (National Cancer Institute, Bethesda, MD) compared the binding affinity of E7 transforming protein of human papillomavirus to the pRb and found "high cancer risk" human papillomavirus 16 or 18 E7 proteins for pRb. A single amino acid substitution in E7 is responsible for this difference between high and low cancer risk human papillomavirus. E6 transforming protein promotes the degradation of *p53* via an ubiquitin-dependent protease system and provides a novel mechanism of action for dominant-acting oncoproteins.

Transgenic mice have substantially increased our understanding of the *in vivo* pathobiological effects of oncogenes. Dr. M. Katsuki (Tokai University, Kanagawa, Japan) described 3 independent murine lines carrying the human c-Ha-*ras* transgene in which about 50% of the mice spontaneously developed tumors at restricted tissue sites. Point mutations in the transgene were frequently observed. The transgenic mice also were very susceptible to an alkylating carcinogen that caused the expected GGC → GAC point mutation at the 12th codon in the transgene.

Dr. S. Hinrichs (University of Nebraska, Omaha, NE) continued the discussion of transgenic models of human cancer. Transforming adenovirus *E1A* and *E1B* transgenes resulted in squamous cell carcinoma and adenocarcinoma in the stomach. The tumors arose at the squamous columnar junction via a multistage morphological sequence and may reflect the high rate of cell proliferation at this site. "Gene knockout" experiments will also contribute to our understanding of carcinogenesis.

Nakahara Memorial Lecture

Dr. H. Pitot (University of Wisconsin, Madison, WI), who reviewed the contributions of animal models to definition of the multistage nature of the development of neoplasia, described characteristics of the stages of initiation, promotion, and progression in such models. Evidence has been generated to indicate a genetic basis for the stage of initiation, as well as that of progression, with the former involving simple mutations in one or a few genes while the latter is correlated with demonstrable chromosomal abnormalities. A transgenic rat model programmed with the dominant *SV40* viral oncogene expressed specifically in hepatocytes mimics hereditary cancers resulting from homozygous recessive mutations of tumor suppressor genes but may lack a true intermediate reversible stage of promotion. While the carcinogenic risk of complete carcinogens, as well as many promoting agents, can be implied from such animal models, their use to identify putative progressor agents, active only during the development of the final stage of neoplasia, has not yet been fully utilized.

Promotion, Progression, Invasion, and Metastasis

The basic tenet of tumor promotion is selective clonal expansion of initiated cells. Protein kinase C, a family of lipid-regulated enzymes, has been implicated for several decades in tumor promotion by phorbol esters during mouse skin carcinogenesis. Dr. I. B. Weinstein (Columbia University, New York, NY) discussed the roles of specific isoforms of protein kinase C in signal transduction and growth control. Overexpression of transfected protein kinase C genes has different pathobiological effects depending on cell type; *e.g.*, transfected protein kinase $C_{\beta 1}$ inhibits growth of human colonic carcinoma cells, but stimulates growth of fibroblasts. Therefore, it will be interesting to determine if inactivating mutations in protein kinase C genes occur in human tumors.

Dysregulation of cell growth control and clonal expansion to form multiple polyps is characteristic of adenomatous polyposis coli. Dr. Y. Nakamura (Cancer Institute, Tokyo, Japan) was one of the international group of investigators who recently discovered the *APC* gene responsible for this autosomal dominant disease. The mutational spectrum of the *APC* gene is replete with small deletions and chain-terminating point mutations that result in a truncated or no gene product. Both germ line and sporadic mutations were of this type which suggests that the APC protein may be part of a multicomponent protein machine.

Protein tyrosine kinases are also signal transducers involved in control of cell proliferation. Dr. T. Yamamoto (University of Tokyo, Tokyo, Japan) discussed the *src*-like family of protooncogenes (c-*src*, c-*yes*, c-*fgr*, *fyn*, *lyn*, *lck*, *hck*, and *blk*). Two members of this family, *fyn* and *lyn*, are preferentially expressed in B- and T-lymphocytes, respectively, in peripheral lymphoid system and are associated with their antigen receptors. Evidence was presented that fyn and lyn proteins are important in activating resting lymphocytes to proliferate.

Nuclear transcription factors controlling gene expression are at the afferent end of the signal transduction pathway leading to cell proliferation. Dr. K. Alitalo (University of Helsinki, Helsinki, Finland) discussed the role of transcription factors, *myc* and *max*, in lung cancer. An alternatively spliced and truncated

form of *max*, *δmax*, enhances 4-fold the transformation of rat embryo fibroblasts by cotransfected *myc* and *ras*. In contrast, *max* decreases the transformation frequency in this *in vitro* system. Presumably these modulations by *max* and *δmax* are in part the result of heterodimer formation with *myc* and altering its functional activity.

Metastasis is the primary cause of cancer morbidity and mortality. Dr. V. Castronovo (National Cancer Institute, Bethesda, MD) discussed molecular approaches to inhibit cancer invasion and metastasis. *TIMP-2* may function as a tumor suppressor protein by inhibiting metalloproteinases such as type IV collagenase, required for invasion. Locomotion is a necessary component for tumor cell invasion. In animal models using a variety of human tumors, carboxylamidoimedazole (CAI) inhibited growth of primary tumors and their metastasis. Clinical phase I trials with CAI are in progress.

Tumor growth and metastasis are angiogenesis dependent. Dr. J. Folkman (Harvard Medical School, Boston, MA) reviewed the mechanisms of switching to the angiogenic phenotype during tumorigenesis. In a transgenic mouse model with a bovine papilloma virus transgene, angiogenic fibrosarcomas develop from nonangiogenic precursors called fibromatosis and are associated with release of fibroblast growth factor. Neovascular counts in human breast and prostate carcinomas can be predictive of metastasis and prognosis. An ongoing prospective study to examine the metastatic predictive value of measurement of basic fibroblast growth factor in sera and urine is in progress. The importance of this growth factor in the scirrhous reactions of some carcinomas such as breast and pancreas will be of considerable future interest.

Transgenic mice have also been useful to study the pathogenesis of carcinomas. Dr. T. Kitagawa (Cancer Institute, Tokyo, Japan) described multistep hepatocarcinogenesis in transgenic mice harboring an *SV40*-T-antigen transgene. Point mutations in c-Ha-*ras* occurred in about 40% of the tumors and were considered late events. In some tumors, the *SV40* T-transgene was absent indicating that continued presence of the transgene is not required for tumor growth. Aneuploidy and an increased frequency of sister chromatid exchanges were cited as evidence of genomic instability associated with *SV40* T-antigen gene.

Hepatocellular carcinomas develop in Long-Evans cinnamon rats with hereditary hepatitis. Dr. N. Takeichi (Hokkaido University, Sapporo, Japan) has found that copper is concentrated in the rat hepatocytes somewhat as it is to Wilson's disease in humans and is closely associated with the appearance of hepatitis. Administration of a copper chelation agent, D-penicillamine, prevented both the hepatitis and the hepatocellular carcinomas. Since copper accumulation could lead to oxygen radical formulation and DNA damage, point mutations in protooncogenes and tumor suppressor genes may also occur. The isolation and characterization of the genes responsible for Wilson's disease in humans and hereditary hepatitis in rats have not as yet been reported.

A population perspective on multistage carcinogenesis was presented by Dr. S. Moolgavkar (University of Washington). Cancer incidence data in populations may provide some insight into the number and nature of stages in malignant transformation. For example, a three-mutation model was more consistent with the genetic alterations observed in colon carcinogenesis than was a two-mutation model. Cell kinetic data, including rate of cell death, are needed to improve modeling of carcinogenesis.

The concluding remarks were given by Dr. I. B. Weinstein who emphasized a global approach to studies of carcinogenesis. Questions include: (*a*) what type of mutations accumulate during carcinogenesis; (*b*) what drives the formation of these mutations; (*c*) what are the functional consequences of these mutations; and (*d*) what is the extent that the tumor cell phenotype is epigenetic. He also cautioned to be open to models other than the traditional tumor initiation, promotion, and progression. He argued that the complex network of signal transduction pathways involves cross-talk, redundancy, and high fidelity so that mutations in multiple genes are required in multistage carcinogenesis.

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