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REVIEW

FISH TUMORS AND THEIR IMPORTANCE IN CANCER RESEARCH

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Prologue

Although comparative oncologists two decades ago may have tended to be on the defensive when arguing that fish, which are water-dwelling, poikilothermic vertebrates of greatly diverse form, might be of interest to cancer investigators,[1] justification of their use no longer seems necessary. As testified by the increasing number of publications on fish tumors appearing in international journals, various fish species have now become widely accepted as useful tools for cancer research with, in some cases, distinct advantages over models using rodents or avian species.

In particular, the study of neoplasms in lower aquatic animals has recently gained considerable importance as a means of detecting injurious agents in the environment. Investigation of fish tumors is also integral part of the basic biological approach to understanding possible common mechanisms involved in cancer development at various phylogenetic levels. The finding that tumors can be readily induced at various sites in fish species by different chemical carcinogens or viruses was of central significance in this context.

Comparative Oncology of Fish

Since the turn of the century there has been a massive accumulation of information affirming that neoplasms occur in non-mammalian species and that they often bear a striking resemblance to those arising in man.[2] In searching for tumors in fish, efforts have been naturally directed to fish markets, public aquariums, private ponds and hatcheries, and early contributions have been comprehensively reviewed and discussed by Schlumberger and Lucké,[3] Wellings[4] and Mawdesley-Thomas.[5] However, a more systematic world-wide contribution is provided by the Registry of Tumors in Lower Animals (RTLA) at the Smithsonian Institution, started by Clyde J. Dawe and John C. Harshbarger in 1965.[6,7] The registry specializes in neoplasms of subhomeothermic animals from all parts of the world and is responsible for documenting discovery, generating opinions on the nature and significance of lesions and preserving material for future use. The RTLA makes about 200 accessions per year and distributes activity reports to interested investigators.[6] Over the past 20 years, tumors appearing in bony fish have been by far the most common (51%), with tumors in cartilaginous fish being in comparison rather rare (7%). Although the etiology is unknown for the most part, tumors were often found in clusters in the same species or family of bony fish and as will be mentioned below, some cases may be ascribed to genetic factors, viruses and chemical contaminants in the water or food.

Historical Note on Fish Tumor Studies in Japan

As is the case in most littoral countries, the Japanese people have long been familiar with fish both as a foodstuff and as pets. In Japan, fishing and fish culture are regarded as among the most important food industries and a large number of modern aquariums, fully-equipped fishery stations and hatcheries have been constructed. This has facilitated the collection of information concerning spontaneous fish tumors. Another favorable aspect for research in this country is that the goldfish (*Carassius auratus*) and multicolored carp (nishikigoi) (*Cyprinus carpio*) are raised commercially in abundance. These fish are particular variants established through repeated artificial mating for more than 200 years. Japanese comparative oncologists are thus in a unique position to study neoplasms in such artificially selected fish species. One of the early important contributions from Japan was the discovery and identification by the late Dr. Keizo Takahashi,[8] in 1929, of more than 100 neoplasms among 100,000 fish caught along the Japanese coast. Here, we would like to point out that at least 6 major papers on fish tumors appeared in this journal (*Jpn. J. Cancer Res.*) between 1920 and 1955.[9, 10] Thereafter, however, there was a decline in activity in this area of cancer research in Japan, until a resurgence of interest took place during the past decade. Recently, several groups have been organized to make contributions to the investigation of neoplasms in feral and domesticated fish.

Characteristics of Fish Tumors

1. Pathomorphology

Regarding the comparability of neoplasms in fish with those in man and other mammals, similarities and differences in general biological and morphological features require discussion. The structures of some normal fish tissues are considerably different from those of mammals. For instance, the typical lobular structure of mammalian liver is not a feature of fish liver tissue,[11] which consists of sheet-like arrangements of parenchymal cells with interlacing sinusoids and a few bile ducts. Histologically, however, tumors in fish do not generally differ markedly from equivalent mammalian tumors, and this enables the comparative oncologist to classify fish tumors on much the same bases as tumors in mammals. For example, various types of liver neoplasms develop which can be classified as trabecular hepatocellular carcinomas, poorly differentiated hepatocellular carcinomas, cholangiocellular carcinomas, etc.[11] Although fish ovarian tumors are diverse and exhibit great variation, they are mainly composed of similar cells to those observed in human dysgerminomas, granulosa-theca cell tumors or embryonal carcinomas, suggesting a range of histogenetic origins.[12] Furthermore seminomas in fish are composed of typical germ cells similar to those in human seminomas or embryonal carcinomas.[13] However, it must be borne in mind, as already pointed out by Dawe and Harshbarger[1] that diagnostic names are no substitute for a detailed knowledge of the individual biological features of a particular neoplasm in a particular species. For example, consider the kidney tumors. In man, these tumors (Wilms' tumor) develop only in children, frequently metastasize and are lethal, whereas in fish adults may also be affected.[7, 13] Thus, in fish nephroblastomas are not embryonal tumors as in man and whereas embryologically, the mesonephros is the functional kidney in fishes and amphibians, in reptiles, birds and mammals it regresses and is replaced by the metanephros in adult forms. Histologically, only a few fish neoplasms can be classified as renal adenocarcinomas equivalent to those developing in adult humans (Grawitz tumor). Other examples where fish neoplasms differ from those observed in man include the erythrophoromas or iridophoromas, peculiar pigment cell neoplasms which do not exist in mammals.[14] Several descriptions of either spontaneous[15, 16] or induced[17] tumors of the swim bladder, which is the fish counterpart of mammalian lung, have appeared. However, the lesions were papilloma-like growths or sarcomas, histologically quite different from mammalian lung tumors. Epithelial neoplasms can also be induced in the gills, the functional equivalent of the mammalian lung.[18] The examples above suggest that the types of neoplasms which develop in either fish or mammals purely reflect the range of normal cells possessed. Virtually all of the major organs and cell types have been observed to give rise to neoplasms in a wide range of fishes with the notable exception of brain tumors.[14] There are as yet no reports of fish intracranial tumors which are histopathologically equivalent to brain tumors such as gliomas in man.[14] The fact that fish lack the well-developed neopallial cortex which constitutes the largest portion of the brain in higher mammals may be an important factor in this context.

The question of whether there is a relationship between development of immunity, the lymphoid system and tumorigenesis remains to be answered, since conflicting data concerning the concept of immunological surveillance of tumorigenesis have been reported. However, it appears of interest that the prevalence of neoplasms increases markedly approximately in parallel to the appearance of thymic systems despite the fact that tumors have been described in *Drosophila* or *Mollusca* that lack thymic systems.[6,7] Phylogenetically, a thymus first appeared in jawless fish (lamprey and hagfish) and fish possess a more highly evolved antibody-forming capacity.[19]

2. Tumorigenesis

Temperature seems to be an important factor in tumorigenesis in poikilothermic animals. For example renal tumors (Lucké tumors)[20] in the leopard frog (*Rana pipiens*) and papillomas[21] in the Japanese newt (*Cynops pyrrhogaster*) are known to be sensitive to environmental temperature, with seasonal changes being ascribed to temperature-dependent viral activation. Although no such examples are known for fish neoplasia, chemical carcinogenesis studies have demonstrated that tumors appear later and their growth is retarded when the temperature is low.[22,23]

A number of reports have indicated that different species of fish differ greatly with regard to spontaneous tumor incidence or susceptibility to chemical carcinogens. For example, different varieties of salmonid fish show wide variation in their response to aflatoxins.[24] Two aspects may be of particular relevance to such inter-species variation. Firstly, the high susceptibilities of some species might be related to a high capacity for specific carcinogen metabolism and a correspondingly high rate of DNA modification. Comparative biochemical studies on the metabolism of carcinogens in various species give us clues for the solution of this problem in general.[25] Secondly, species differences could be due to some genetic resistance or proneness to cancer unrelated to carcinogen activation. Several factors might be involved, including the DNA repair systems, which are thought to play an important role in the initial steps of neoplastic development. This problem will be discussed in a separate section.

3. Tumors in "living fossil" fish

Extant lungfish and coelacanths are considered to be derived from Devonian ancestors with retention of basic morphologic structures for hundreds of millions of years. Given the generally assumed close relationship between mutagenesis and carcinogenesis, the question has been raised whether neoplasms develop in species such as so-called "living fossil" fish that show only a very slow rate of evolutionary change with regard to morphology.[1] In close cooperation with 5 aquariums in Japan, we had the opportunity to perform a complete autopsy survey and were able to find tumors, including 3 liver tumors, in 14 lungfishes.[26,27] The fossil record for fish species is perhaps more abundantly preserved than that of any other class of vertebrates. Yet no example of a tumor-bearing fossil fish has come to our attention.[1] Dose this imply that neoplasms are a modern disease in fish reflecting man's pollution of the environment? Since neoplasms develop at relatively high incidences in "living fossil" fish, closer attention to this question is warranted. The study of fossil fish for neoplastic development is a field lying completely fallow.

Chemical Contaminants and Fish Tumors

The study of neoplasms in lower aquatic animals is useful for detecting injurious factors in the environment. The accumulating evidence demonstrates unequivocally that wild fish taken from contaminated waters present with a variety of preneoplastic and neoplastic lesions. Prior to 1965, liver tumors were reported to be rare in feral fish.[6] More recently, however, hepatomas (adenomas, hepatocarcinomas, cholangiocarcinomas) have been found at high incidence in English sole (*Pleuronectes vetulus*) from Puget Sound[28] and in winter flounder (*Pleuronectes americanus*) from Boston Harbor[29] associated with increased levels of sediment contamination (including carcinogens). A similar range of lesions has been described in brown bullhead (*Ictalurus nebulosus*) living in rivers entering Lake Erie[30] and white perch

(*Morone americana*) from Chesapeake Bay.[31]

These hepatic neoplasms or preneoplastic lesions have been observed in bottom-dwelling fish inhabiting areas where sediments are known to contain toxic and potentially carcinogenic chemicals. Thus, Malins *et al.*[32] found a strong association between hepatic lesions in English sole and polycyclic aromatic hydrocarbons (PAHs). Hendricks *et al.*[33] further demonstrated that the PAH carcinogen benzo[*a*]pyrene is capable of producing hepatic neoplasms in rainbow trout (*Salmo gairdneri*). Liver tumors were also induced by benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene in medaka by Hawkins *et al.*[34]

In general, the fact that bottom dwelling/feeding fish species have the highest rates of neoplasia has therefore been interpreted as evidence that exposure to sediment-bound chemical carcinogens may play an essential role in tumor induction in these fishes. In Japan only few systemic field studies concerning the prevalence of particular neoplasms in polluted rivers, lakes or coastal waters have been made. However, the development of melanomas in the nibe croaker (*Nibea mitsukurii*) a common fish inhabiting shallow seawater in Japan was extensively investigated by Kimura and his associates.[35] Comparison of the geographic distribution of melanomas based on large samples at 25 different stations along the Pacific coast of Japan revealed marked variation in tumor incidences ranging from zero to 47% from one place to another. Kimura *et al.*[35] therefore concluded that environmental chemicals are involved in active induction of the melanomas. Thus, a relationship between chemical contaminants and tumors in fish has been inferred from fish tumor surveys, chemical analysis of sediments, studies on carcinogen metabolism in fish and laboratory carcinogenesis studies with chemical carcinogens. Recently, DNA adduct detection including a ^{32}P-postlabeling technique has also been attempted using tissues of fish collected from polluted waterways.

Chemical Induction of Tumors in Fish

Experimental induction of tumors in fish has been achieved with various carcinogens using different types of fish, leading to their increasing use as model systems.[36] For example, studies of fish liver tumors have been carried out extensively with cultured rainbow trout,[33, 37] which are particularly sensitive to dietary exposure to aflatoxin B_1. Stanton[38] first succeeded in inducing liver tumors in small aquarium fish (*Brachydanio rerio*) and subsequently studies of experimental hepatocarcinogenesis in guppy (*Poecilia reticulata*) have also been reported.[39, 40] In our laboratory it was established that a small aquarium fish, the medaka (*Oryzias latipes*), which has a wide range of habitats in Japan, is very sensitive to diethylnitrosamine (DENA).[11, 41] Since it is highly susceptible to a variety of carcinogens, and the time required for tumor induction is very short, this fish has been investigated by several researchers in both Japan[23, 42, 43] and the United States.[18, 34, 44] Neoplastic lesions including tumors of the liver,[11, 34, 42] gill[18] and eye (retina)[44] and melanoma[43] were readily induced. More recently, the use of rainbow trout[17, 45] or medaka[46] eggs to provide an accelerated and probably more sensitive system has been devised by several groups. This entails a single exposure of fertilized eggs (embryos) to an aqueous solution containing various carcinogens for approximately 1 hr or so. Various tumors including hepatocarcinomas and nephroblastomas were found to develop subsequently. This method seems in certain respects to simulate transplacental carcinogenesis in rodents. However, it has advantages over mammalian transplacental techniques in that chemicals are applied to embryos under direct observation and thus might prove amenable to application for both basic research on the mechanisms of carcinogenesis and for screening of potential environmental carcinogens.

DNA Repair Mechanisms in Fish

Capacity for DNA repair in higher animals is regarded as a cellular process of essential relevance to mutagenesis, carcinogenesis and also aging. However, relatively little is known about DNA repair in fish. DNA damage induced by ultraviolet (UV) radiation or carcinogens can be repaired by several different mechanisms including excision repair, photoreactivation, and O^6-methylguanine DNA methyltransferase (O^6-MT)-mediated repair. In fish, much attention has been directed to the photoreactivation repair pathway specific for pyrimidine dimers[47-50] because they contain a large amount of this enzyme[51] but considering the filtering properties of their environment UV would seem to have only marginal responsibility for neoplastic development in fish.

Most environmental carcinogens are present as procarcinogens requiring activation and fish have been found to metabolize procarcinogens to ultimate forms in the same way as higher vertebrates.[25] Adduct formation by carcinogen binding to DNA appears to be an important initial event for the process of initiation. DNA excision repair in response is the host defense mechanism for removal of adducts, occurring relatively rapidly and as long as the excision repair system works adequately cells are able to recover from carcinogenic damage. The following is a summary of data obtained so far concerning excision repair in fish cells. 1. Medaka ganglion cells showed positive unscheduled DNA synthesis (UDS) in response to various chemical carcinogens, but liver and intestinal cells did not.[41] UDS could be demonstrated in ganglion cells of several fish species in response to carcinogens.[41] 2. Fish thyroid cells[48] and fibroblasts[50] were found to be deficient in UDS in response to UV radiation. 3. Trout liver cells showed positive UDS in response to several carcinogens.[52] 4. Lungfish do not appear to have evolved beyond the fish stage with regard to UDS.[26] From the available information we can therefore conclude that most species of fish have the genetic information for excision repair, but this information may not be fully expressed in some cells. In general the extent of excision repair is significantly lower than in mammalian cells, including those of man.[26,41]

Finally, another important DNA repair pathway is the O^6-methylguanine DNA methyltransferase (O^6-MT) system. Among the alkylating carcinogen-induced DNA lesions, methylation at the O^6-position of guanine, termed O^6-methylguanine, is regarded as one of the most significant because it is mutagenic and potentially carcinogenic.[53] The lesion is known to be repaired by O^6-MT, which transfers the methyl group of DNA to one of its own cysteine residues[54,55] and the properties of this enzyme protein have been extensively studied in various species including bacteria,[54] rodents[55] and man.[56,57] As mentioned earlier, fish species are extremely susceptible to alkylating carcinogens, and so this repair pathway might be also of considerable importance for the fish case. We therefore used an *in vitro* assay system to compare the capacity of liver extracts from eight species of fresh water fish to repair O^6-methylguanine in DNA.[58] Among the different species of fishes tested, medaka liver extracts showed the highest level of activity, with values approximately equal to those established for mouse liver. The other fish species demonstrated rather lower activities with considerable interspecies variation. Thus, although the levels of O^6-methylguanine repair were comparatively low, unequivocal activity was present in all fish species examined. Experiments with *E. coli*[59] and rats[60] have shown that O^6-MT activity can be increased by pretreatment with low doses of alkylating carcinogens. However, when rainbow trout were treated continuously with a relatively low dose of dimethylnitrosamine and O^6-MT activity measured at various time-points, no adaptive response was observed.[58]

Viral Etiology of Fish Tumors

A role for viruses as etiological agents for fish neoplasms has been established in a few cases. For example the lymphosarcomas found in northern pike (*Esox lucius*), which are enzootic in North America,[1] Ireland,[61] and Sweden,[62] are transplantable with evidence of cell-free transmission, suggesting the involvement of an infectious agent.[61,63] Furthermore, Papas *et al.*[64,65] reported the presence of reverse transcriptase and C-type virus-like particles associated with these lymphosarcomas. In addition two groups in Japan independently reported that skin papillomas (epithelioma) in salmonids are induced by oncogenic viruses. Kimura *et al.*[66] isolated a new herpesvirus (*Onchorhynchus masou* virus, OMV) from the ovarian fluid of nontumorous cultured yamame (*Onchorhynchus masou*). OMV is primarily pathogenic to yamame fry causing severe necrosis of the liver and kidney. However, interestingly, skin tumors, mostly in oral areas, developed much later among the survivors of an artificial infection.

Tumors were also inducible in coho salmon (*Onchorhynchus kisutch*) and the virus was successfully recovered from a tumor sample. Sano *et al.*[67] also isolated a herpesvirus (yamame tumor virus, YTV) from a spontaneous tumor of the mandible of cultured yamame. Young fry inoculated with the virus developed similar tumors in the mandibular region and it was reported that chum salmon (*Onchorhynchus keta*) were also susceptible. However, at present it has not yet been confirmed whether OMV and YTV are identical or whether the tumors produced by these viruses are histopathologically the same.

Genetic Influences on Fish Tumors

Genetically determined fish melanomas (sometimes referred to as Gordon's melanoma) have been known for about 60 years and have contributed much to the understanding of genetic influences on tumor development. Following the original observation by Häussler[68] and Kosswig[69] of melanomas occurring in hybrid crosses of green swordtail with platyfish in 1928, Gordon and coworkers[70, 71] and a series of other investigators[71–75] systematically analyzed the genetic factors involved by classic breeding methods. The melanoma is thought to develop as follows. The platyfish has several external color patterns, each of which appears to be determined by at least one color gene located on the X chromosome. The activity of the color gene is controlled by a group of modifying genes and the swordtail-platyfish hybrid has fewer modifying genes than does the platyfish. Repeated backcrossing of the swordtail-platyfish hybrid with the swordtail successively reduces the number of modifying genes, which ultimately permits unlimited proliferation of melanocytes. More recently the color gene has been considered to be an oncogene by definition and was designated as "tumor gene" (Tu) by Anders and his associates.[76] Tu is normally under negative control by certain linked and nonlinked regulatory genes (R genes), and a number of physiological and chemical factors, such as ionic strength, mutagenic compounds and tumor promoters, can also influence melanoma development. Since there is evidence that expression of c-*src* (the cellular counterpart of the Rous sarcoma virus oncogene) might correlate with that of Tu[76] more studies of the latter appear warranted.

Genetic factors may also be involved in the development of some other fish neoplasms. For example, it is of interest that the goldfish or carp, in which many erythrophoromas[77] or ovarian neoplasms[12] have been recorded, are particular variants established through repeated artificial mating with the aim of highlighting colors. Etoh *et al.*[78] in 1983 reported that the incidence of erythrophoromas increased age-dependently within their investigated population of goldfish and a level of about 60% was finally attained in the oldest stock. This fact suggests that aging is as an important factor for the development of the erythrophoromas in goldfish, as for neoplasia in general in man.

Oncogenes and Possible Involvement in Fish Tumorigenesis

As stated above, Anders and his associates[76] have reported on the basis of a number of genetic analyses that the "tumor gene" (Tu) might correlate with the c-*src* gene. However, Tu was not elucidated at the molecular level. We have adapted a different approach to fish oncogenes.

The *ras* gene of goldfish was first cloned from normal liver DNA and the nucleotide sequences analyzed by Nemoto *et al.* of our group.[79] Comparison of nucleotide and predicted amino acid sequences between the *ras* gene from normal goldfish liver DNA and H-*ras*, K-*ras* and N-*ras* gene from human tissue revealed surprising homology. In particular, the goldfish *ras* gene and human K-*ras* gene showed 96% homology regarding the predicted amino acid sequence, whereas the degrees of homology between the former and human H-*ras and* N-*ras* genes were 87% and 88%, respectively. In mammalian *ras* genes from cancerous tissue, point mutations at the 12th or 61st codon have often been observed and therefore we examined goldfish erythrophoroma permanent cell lines established in our laboratory.[80, 81] No point mutation was apparent at the 12th codon of the erythrophoroma *ras* gene.[81] However, our examination was directed at only the first exon and thus it is not clear at present whether an activated oncogene may be involved in the development of this tumor type.

Almost at the same time another group isolated and sequenced the rainbow trout c-*myc* gene.[82] They similarly showed that the presumptive codon region of the trout c-*myc* gene shows extensive homology to the c-*myc* genes of chicken, mouse and man. At the predicted amino acid level, fish c-*myc* product showed considerable homology to vertebrate c-*myc* gene products. They further demonstrated that trout c-*myc* is expressed in normal rainbow trout cells as a single 2.3-kb mRNA, similar in size to other vertebrate transcripts.

Production of Inbred Strains of Fish

Because of the lack of immune compatibility in normal fish, transplantation studies have been restricted. This problem has, however, recently been overcome in various ways. For example inbred strains of medaka[44] and *Poecilia lucida*[83] were produced after repeated classical sister-brother matings and melanomas[44] or liver tumors[83] induced in these fish could be successfully transplanted to syngenetic hosts. Similarly, some researchers noted that the hermaphroditic fish *Rivulus marmoratus*[84, 85] or the gynogenetically reproducing fish *Poecilia formosa*[48, 86] were highly useful for such experiments. As a result of these unusual modes of reproduction, genetically identical offspring are obtainable as clones. More recently, large-scale production of homozygous diploid fish has been achieved in a variety of fish species.[87, 88] In these cases, eggs were activated with sperm made genetically impotent by exposure to UV light and the maternal haploid complement of chromosomes was permitted to duplicate. Since partitioning of the duplicated chromosomes into 2 cells during mitosis is prevented by hydrostatic pressure or heat shock treatment this treatment results in diploid cells with 2 identical sets of chromosomes, both derived from the mother. Subsequent cycles of chromosome replication and cell division proceed normally and give rise to diploid fish with 2 identical sets of chromosomes in each cell. Since both male and female homozygous fish were present in each generation, production of selected hybrid populations by mating was facilitated.

Production of Transgenic Fish

Development of transgenic mice by transfer of foreign genes into fertilized eggs has now become generally accepted as a useful tool in cancer research. Ozato *et al.*[89] first succeeded in producing transgenic fish by microinjecting recombinant plasmids containing the δ-crystallin gene into the oocytes of medaka. The gene and its products were detected in 7-day-old embryos at a relatively higher incidence than in transgenic mouse systems. The following characteristics of the medaka embryos favor the use of this species for foreign gene transfer. Eggs can be obtained throughout the year in the laboratory. The enormous volume of the oocytes nucleus, about 1000 times the mouse nucleus, is advantageous for the microinjection of DNA and the chorion of medaka eggs is transparent, which makes both the microinjection and subsequent observation easier. Of course, in addition, in contrast to transgenic mouse experiments, no pseudopregnant or foster mothers are needed.

EPILOGUE

Fish occupy a distinct segment of the phylogenetic tree and constitute the largest and most diverse class of vertebrates. With over 20,000 species and enormous spectra in life style, size and longevity, fish serve as an ideal material source for the comparative study of cancer. This range allows extensive comparisons and should perhaps allow a more penetrating insight into basic principles than investigations limited to mammalian models.

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