AN ABSTRACT OF THE THESIS OF

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Michael L. Kent

Zebrafish (*Danio rerio)* have become an increasingly important model organism for cancer research. There are several cancer models in which neoplasms are induced chemically or genetically. However, spontaneous (unknown etiology) neoplasms are rather common in zebrafish, particularly in older fish. For over a decade, spontaneous intestinal neoplasia and preneoplastic intestinal changes including hyperplasia, dysplasia and enteritis have been observed in zebrafish from several laboratories submitted to the ZIRC (Zebrafish International Resource Center) diagnostic service. A retrospective analysis showed that 2% of the total fish submitted to the service demonstrate these lesions. These affected fish were submitted from 18 facilities from laboratories in the United States and other countries. One facility (referred to as the primary facility) was particularly affected, representing approximately 74% of the fish with lesions. Tumor prevalence appeared similarly distributed between sexes and generally occurred in zebrafish greater than one year of age, although neoplastic changes were observed in fish as young as 6 months. Eleven lines displayed these preneoplastic and neoplastic changes, including wild-types and mutants. Zebrafish submitted as normal sentinel fish affected with these intestinal lesions demonstrated that these lesions are most often subclinical and emphasize that as an extra-experimental variable these lesions could have underappreciated effect in research.

Given the distribution of the affected fish defined from the retrospective study, possible etiologic agents include a variety of environmental elements or infectious agents. To further investigate potential etiologic agents versus a carcinogen in the diet, a diet challenge was conducted previously by feeding the same diet fed at the primary facility to fish held at a different location. No appreciable preneoplastic or neoplastic lesions developed in the experimental fish from this study, indicating that diet is not the cause. Furthermore, lesion prevalence between different populations of age and strain matched fish at the primary facility were dramatically different. This indicates that a water-borne carcinogen in the juvenile and adult rearing laboratories not the cause, as all of the affected fish were held in the same water supplies in recirculating systems at each facility.

An infectious agent, therefore, could be the cause, as both bacteria and viruses are recognized as causes of cancers in fishes. I conducted a transmission study to investigate this etiology. The experiment included subjecting naïve fish to cohabitation with affected fish, feeding of a tissue homogenate from a pool of affected fish, and intraperitoneal (IP) injection of filtered and non-filtered affected tissue homogenate. Two fish from the cohabitation study sampled at 8 mo. and two fish from the injected filtrate study sampled at 7 mo. exhibited early hyperplastic changes. However, comprehensive histological evaluation at the termination (10 mo. post-exposure [11 mo. post-exposure for cohabitation fish]) of the study did not recognize any preneoplastic or neoplastic intestinal lesions. However, the study was terminated earlier than the original protocol due to high mortality attributed to *Piscinoodinium pillulare* infections amongst the fish in the study.

I conducted a comprehensive histological review of these neoplasms through a retrospective examination of 9,508 zebrafish provided by the ZIRC diagnostic service. The neoplasms were classified either as adenocarcinoma or

small cell carcinoma, with a few exceptions (carcinoma not otherwise specified, tubular adenoma, and tubulovillous adenoma) based upon histomorphologic presentation. In mammals, these neoplasms usually arise from the gastrointestinal epithelium or neural-endocrine cells (e.g., carcinoids). Hence we proposed that cells of origin for these neoplasms in zebrafish were either intestinal epithelial or gut-derived neural endocrine origin. We subjected tissue sections of several of these neoplasms to a panel of mammalian antibodies directed toward epithelial (Cytokeratin Wide Spectrum Screening [WSS], AE1/AE3) or neural (S100, and chromogranin A) tissues. We also investigated the specificity of these antibodies using Western blot analysis, comparing human and zebrafish profiles. WSS and AE1/AE3 were relatively reactive with approximately half of the neoplasms analyzed (staining positive with these markers). S100 and chromogranin A (neural markers) did not specifically stain the cells within the neoplasms. The positive cytokeratin association (WSS and/or AE1/AE3) for most of the neoplasms, while negative for neural and neuroendocrine markers (S100 and chromogranin A respectively), indicates that these intestinal neoplasms are of common epithelial origin. Perhaps those that were negative for cytokeratin may have further progressed to a state of dedifferentiation as most of the neoplasms that were negative were classified as small cell carcinomas and the cell type of these neoplasms are characterized as cells with a small amount of cytoplasm and lacking features of typical epithelial cells.

In conclusion, based on data to date, the neoplasms are epithelial in origin, and three plausible causes for the lesions should be considered; 1) a microorganism in adult facilities, 2) a microorganism in larval nurseries, and 3) a chemical carcinogen in individual nursery tanks.

Intestinal Hyperplasia and Neoplasms in Zebrafish (*Danio rerio*)

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Colleen Elizabeth Paquette

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Colleen Elizabeth Paquette, Author

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CONTRIBUTION OF AUTHORS

Cari Buchner provided daily support for the preliminary diet study reported under the retrospective study in Chapter 2. Robert L. Tanguay provided materials for that same diet study. Karen Guillemin contributed to experimental design and data analysis. Timothy J. Mason provided technical support for the transmission studies and training for histological specimen preservation. Tracy S. Peterson assisted with the histological interpretation of hundreds of zebrafish specimens Christiane V. Löhr contributed to the experimental design and data interpretation of the immunohistochemical study. Kay Fischer provided technical support for the immunostaining. Rong Wang instructed and assisted on the Western blots. Roderick H. Dashwood provided lab facilities for the Western blots.

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Chapter 1: Introduction

Cancer in the Mammalian Gastrointestinal Tract

Cancer is the second leading cause of death worldwide after heart disease[1]. The gastrointestinal tract serves as the primary site for a large proportion of these cancers, including the third and fourth most common ones worldwide, colorectal and gastric respectively[2]. Adenocarcinoma is the most common form of these cancers and risk factors include smoking, diet, genetic factors, inflammatory bowel disease, *Helicobacter pylori* infections[1], and more recently bile acid exposure[3]. Colorectal and gastric cancers are the second and third most common cause of cancer-related death, respectively[4], and socio-economic influence aside; there is still necessity for improvement regarding screening and therapeutic options[1].

Spontaneous neoplasia of the gastrointestinal tract is not very common among non-human mammals, and very rare in laboratory species. Gastric cancer has been reported most commonly in the dog. Other species noted for gastric cancer, although rare, include non-human primates, ferrets, and rodents (Syrian hamster, mouse, and rat). These species have demonstrated varying levels of success as in vivo models when induced using chemicals and/or *Helicobacter* species[5]. Spontaneous adenocarcinoma of the intestine is also most common among dogs. Other similarly affected species include cats and sheep. However, rodents have demonstrated chemical induced intestinal adenocarcinomas similar to those observed in the spontaneous models[6]. Several zebrafish lines have been developed to model human gastrointestinal cancers, including *tp53*^{M214K} (wild-type mutant), which presents with malignant peripheral nerve sheath tumors[7], and the *apc*/+ (AB mutant), which develops liver and intestinal tumors[8]. My thesis

investigates the etiology, distribution, and cell of origin of a common intestinal neoplasm in zebrafish.

Etiologic Agents of Intestinal Cancer in Fishes

Parasites

The nematode parasite, *Pseudocapillaria tomentosa*[9] , which commonly infects cyprinid fishes, is a suspected cofactor in promoting intestinal lesions as result of experiments conducted using zebrafish. It is reasonable to believe, that this parasite may play a similar role in its various species of cyprinid hosts.

Bacteria

Recent evidence suggests *Mycobacterium marinum* promotes hepatocellular proliferation in Japanese medaka (*Oryzias latipes*) via chronic inflammation[10]. And whereas *Helicobacter* species have been associated with gastrointestinal cancer in mammalian systems, there is currently no evidence of a similar role of this genus of bacteria in fishes.

Viruses

Viruses have been connected to certain fish cancers, eleven with documented retroviral involvement. Most of these retroviral diseases are associated with various cofactors, most commonly seasonal variability of water temperature. None of the tumors associated with these viruses have been reported to have primary sites in the intestinal tract. Angelfish lip fibroma is the only reported aquarium fish affected by a viral induced tumor[11].

Water-borne Carcinogens

Fishes have long served as tools of water quality bioassays. Various epithelial tissues have been commonly afflicted by water-borne carcinogens, the intestinal tract included. Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are two classic examples of these environmental contaminants and have been associated in a number of both wild and captive fish[12].

Diet

Diet has been implicated in the progression from chronic inflammation to neoplasia in the gastrointestinal tract of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) fed a commercial diet rich in plant products[13].

Zebrafish as a Cancer Model

Zebrafish entered the field of cancer research in the 1960's when Stanton used them as an in vivo vertebrate model to test carcinogens, but it has only been since the mass genetic screens of the 1990's that zebrafish have really begun to compete with mice as a viable cancer model organism. Zebrafish found increasing popularity in this field as they offer increased brood size, decreased generation rates, and can be housed in a more concentrated manner per square foot than mice[14]. These traits, combined with the ease of forward and reverse genetic screens allowed innumerable cancer models to be generated. Cancer models are no longer restricted to chemically induced phenotypes. In recent years, transgenic and xenotransplantation models have also become optional methods of inducing cancer in zebrafish, again increasing the potential of zebrafish as a model

organism exponentially[15]. Most cancers in zebrafish are induced with chemicals or genetically, although spontaneous neoplasms are not uncommon in zebrafish older than two years of age[16, 17, 18]. Increased incidence of intestinal tumors have been observed in zebrafish exposed to DMBA (7, 12 dimethylbenz[a]anthracene)[19.] As previously mentioned, the nematode parasite, *P. tomentosa*[7] has also been shown to serve as a cofactor in promoting intestinal lesions. Endogenous retroviruses have been identified in zebrafish[20], but no oncogenic viruses have been currently implicated as the cause of intestinal neoplasia in zebrafish[21, 22].

Aims of Research

For over a decade, spontaneous intestinal neoplasia and preneoplastic intestinal changes including hyperplasia, dysplasia and enteritis have been observed in zebrafish submitted to the ZIRC (Zebrafish International Resource Center) diagnostic service. The aim of my thesis was to profile these intestinal lesions in zebrafish. In Chapter 2, I examine over a decade of diagnostic records to discern the historical prevalence of these lesions among the zebrafish submitted to the ZIRC diagnostic service and they are classified based upon histomorphologic presentation. Risk factors including sex, genetic predisposition as an effect of genetic background or specific strain/line of the fish (hereafter referred to simply as genetics), and age are also evaluated. A preliminary study regarding diet as a possible etiologic agent is also included here. Chapter 3 progresses from the information gleaned from the retrospective study, to consider the possibility of an infectious agent etiology for these intestinal lesions. I conducted a transmission study which challenged naïve fish with fish affected with the preneoplastic and neoplastic lesions through methods of cohabitation,

feeding of affected tissue, and intraperitoneal injection of affected tissue homogenate (either unfiltered or filtered). In Chapter 4, I revisit the histomorphological classifications of the tumors, as established in Chapter 2, and using immunohistochemistry to determine if they are in fact of intestinal epithelial or gut-derived neural endocrine origin.

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Chapter 2

A Retrospective Study of the Prevalence and Classification of Intestinal Neoplasia in Zebrafish (*Danio rerio*)

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Abstract

For over a decade, spontaneous intestinal neoplasia has been observed in zebrafish (*Danio rerio)* submitted to the ZIRC (Zebrafish International Resource Center) diagnostic service. In addition, zebrafish displayed preneoplastic intestinal changes including hyperplasia, dysplasia and enteritis. A total of 195 zebrafish, representing 2% of the total fish submitted to the service, were diagnosed with these lesions. Neoplastic changes were classified either as adenocarcinoma or small cell carcinoma, with a few exceptions (carcinoma not otherwise specified, tubular adenoma, and tubulovillous adenoma). Tumor prevalence appeared similarly distributed between sexes and generally occurred in zebrafish greater than one year of age, although neoplastic changes were observed in fish 6 months of age. Eleven lines displayed these preneoplastic and neoplastic changes, including wild-types and mutants. Affected zebrafish originated from 18 facilities, but the majority of fish were from a single zebrafish research facility (hereafter referred to as the primary facility) that has submitted numerous samples to the ZIRC diagnostic service. Zebrafish from the primary facility submitted as normal sentinel fish demonstrate that these lesions are most often subclinical. Fish fed the diet from the primary facility and held at another location did not develop intestinal lesions, indicating that diet is not the etiologic agent.

Introduction

Zebrafish have become an increasingly important model organism in the field of cancer research[1,2,3,4]. Most cancers in zebrafish models are induced with chemicals or genetically, although spontaneous neoplasms are not uncommon in zebrafish two years of age or older[5,6,7]. Specific mutants created as cancer models include *tp53*^{M214K} (wild-type mutant), which presents with malignant peripheral nerve sheath tumors[8], and the *apc/+* (AB mutant), which develops liver and

intestinal tumors[9]. Increased incidence of intestinal tumors have been observed in zebrafish exposed to DMBA (7, 12-dimethylbenz[a]anthracene)[10] and incipient intestinal preneoplastic lesions may undergo enhanced promotion when there are co-morbid conditions present, such as the nematode parasite, *Pseudocapillaria tomentosa*[11]. The normal zebrafish intestine has been well-characterized previously[12,13,14] as an agastric simple tubular structure, the mucosa of which is formed by longitudinal folds, that is in many ways similar to the mammalian counterpart, with the exceptions of a submucosa, Peyer's patches and villi. The anatomic organization of the intestine demonstrates a rostral-to-caudal decreasing of the luminal diameter, lined by columnar epithelium interspersed with mucus (goblet) cells that increase in number caudally. Myenteric neurons and enteroendocrine cells also form components of the zebrafish intestine.

We have observed spontaneous intestinal neoplasia in zebrafish submitted to the ZIRC diagnostic service from several facilities since 2000, shortly after the diagnostic center at ZIRC was established. This retrospective study was aimed to provide analysis of the prevalence of these spontaneous intestinal neoplasms identified within the ZIRC diagnostic database over the last twelve years and a descriptive histologic classification of the preneoplastic and neoplastic lesions.

Materials & Methods

Review of Historical Prevalence and Characterization of Lesions

The records of 9,539 zebrafish that were submitted to the ZIRC diagnostic database between January 4, 2000 and July 3, 2012 were reviewed using the following parameters provided within the ZIRC diagnostic service submitting form: date of submission, submitting facility, age, sex, genetic background, clinical or subclinical submission, and preneoplastic or neoplastic diagnosis within the intestine. Records omitted from the review include those involving

species other than zebrafish and those which were found to be incomplete and therefore unable to be properly evaluated. Fish identified through the database to have preneoplastic or neoplastic changes of the intestine were selected for further review by histopathology at low magnification (200x total magnification) and high magnification (400x total magnification) in order to confirm the original diagnoses and to further characterize the preneoplastic intestinal lesions as hyperplasia and/or dysplasia, as well as to determine whether inflammatory changes were present and to classify the tumor type. The entire digestive tract, focusing on the intestine, of each fish was examined from oropharynx to excretory vent for three or four serial H&E stained sections.

Diet Study

To investigate the possible role of diet, in 2009 we obtained some of the formulated diet mixture and individual components from the facility where we observed a very high prevalence of intestinal lesions. We conducted the following experiment at the Sinnhuber Aquatic Research Center (SARL) at Oregon State University, a laboratory with no history of the lesions described here[15]. A total of 200, 44 day old, 5D strain zebrafish were divided into 5 groups each with 2 replicate tanks containing 20 fish/tank. Fish were held at 28 C in the SARL recirculating systems. Diet groups were as follows, representing the diet mixture and individual components of the standard diet formulation used at the primary facility: 1) Silver Cup® tropical fish food (Sterling Silver Cup Fish Feeds, Murray, UT, 2) Ziegler® adult zebrafish food Ziegler Bros, Gardners, PA), 3) TetraMin® tropical fish flakes, 4) Equal mixture of diets 1-3, and 5) the SARL zebrafish diet [comprised of 72% Aquatox Flake (Ziegler Bros, Gardners, PA), 13% Cyclop-eeze® (Argent Chemical Laboratories, Redmond, WA) and 15%

Golden Pearl 300-500 micron diet (Artemia International, Fairview, TX)]. Fish were fed 2-3 times/day to satiation. The study was terminated 6 mo. later; all fish were processed for histology and examined for the presence of intestinal lesions. Prevalence of intestinal lesions were compared between fish fed the various diets at SARL to sentinel fish at the primary facility that were fed Diet 4 over the same time periods (2009 and 2010) using an analysis of variance (ANOVA).

Results

The ZIRC diagnostic service, which performs routine post mortem diagnosis of apparently healthy and diseased fish from an average of 26 zebrafish research facilities per year[16], has observed an increasing prevalence of intestinal neoplasia and associated pathology. To better understand this trend, we undertook a systematic survey of all records to characterize these intestinal neoplasms by histopathology and prevalence as described below.

Histopathology

In our re-evaluation of archival samples we observed the following intestinal pathologic changes that were summarily categorized. Intestinal preneoplastic changes were generally observed in the absence of and concurrent with frank neoplastic disease. Within the course of this study, the most common preneoplastic changes involving the intestinal mucosal epithelium included hyperplasia and dysplasia that often lead to extensive folding and formation of pseudocrypts (Fig. 2.1). Hyperplastic and dysplastic changes in the mucosal epithelium were characterized as follows: hyperplasia was denoted by an increase in the number of epithelial cells within mucosal folds, which often formed pseudocrypts, while retaining normal microanatomic structure as compared to

control fish intestine. Dysplasia was defined by progressive loss of the normal microanatomic structure which may involve disorganization or absence of pre-existing histoanatomic architecture, loss of nuclear polarity, nuclear atypia, cellular pleomorphism, and aberrant mitotic figures (Fig. 2.1). Hyperplasia and dysplasia occurred independently and occasionally together in fish displaying preneoplastic lesions. Enteritis was typified by intraproprial and intraepithelial (mucosal) infiltrates of intermixed lymphocytes, eosinophilic granule cells and histiocytes within the affected portion of intestine.

Intestinal tumor types included adenocarcinoma, small cell carcinoma/carcinoid-like tumor, carcinoma not otherwise specified, tubular adenoma, and tubulovillous adenoma. Adenocarcinoma was characterized by randomly oriented and invasive pseudocrypts derived from mucosal epithelium, often resembling pseudoacinar structures replete with intraluminal cellular detritus, as well as, nests of polygonal cells within the lamina propria that displayed moderate to extreme cellular and nuclear atypia including aberrant mitotic figures (Fig. 2.2). Small cell carcinoma/carcinoid-like tumor was comprised of small sheets and nests of round, fusiform or pleomorphic tumor cells that demonstrated a high degree of nuclear and cytological atypia, as well as an absence of mitotic figures, that occasionally formed insular or organoid patterns suggestive of a neuroendocrine origin (Fig. 2.2). Both adenocarcinoma and small cell carcinoma/carcinoid-like tumor frequently elicited intense peri- and intratumoral fibroplasia (scirrhous response) and chronic inflammation. Carcinomatosis, defined as extraintestinal spread of tumor cells throughout the coelomic cavity, was observed occasionally with both adenocarcinoma and small cell carcinoma/carcinoid-like tumor (Fig. 2.3). Carcinoma not otherwise specified was classified as such because this neoplastic entity was much less differentiated

and organized than either adenocarcinoma or small cell carcinoma/carcinoid-like tumor, and indeed in some cases shared characteristics similar to both (Fig. 2.2). Tubular adenoma and tubulovillous adenoma were rare. Tubular adenomas (Fig. 2.2) were identified as a focal polypoid mass comprised of tubuloglandular-like structures within the lamina propria formed by hyperplastic epithelium with normal intestinal mucosa immediately adjacent to the mass, while tubulovillous adenomas had a combined pattern**.** Table 2.1 summarizes the various histological presentations, emphasizing characteristics that differ.

Review of Historical Prevalence of Lesions

The prevalence of preneoplastic changes and neoplastic changes within the intestine among zebrafish submitted to the ZIRC diagnostic database between January 4, 2000 and July 3, 2012 is summarized in Table 2.2 and involved approximately 2% of the total fish submitted within this period. Of the 2% total fish affected by these intestinal lesions, 1.7% of the fish were submitted as subclinical and 0.3% as clinical. Fish submitted as clinical were those that exhibited clinical signs of any disease when they were collected. Fish submitted as subclinical were healthy-appearing fish; they demonstrated no clinical signs of disease and may have been either from sentinel tanks or collected randomly from main facility tanks as a general health check. Fish classified as neoplastic often displayed preneoplastic changes, but were not counted among the fish with preneoplastic changes for this study, as we considered the tumor formation to be a notable progression following the preneoplastic lesions.

It was not uncommon for more than one fish to be affected by preneoplastic or neoplastic changes within a single case submission. By a case basis (several individual fish from one population), 32.2% of the cases included

both preneoplastic changes and neoplastic changes amongst the submitted specimens. The mean age of zebrafish with preneoplastic changes was 402 dpf (days post fertilization), with a range of 188-731 dpf. The mean age of zebrafish with neoplastic changes was 477 dpf, with a range of 188-1071 dpf. The affected fish included 107 females and 88 males. Within the affected female population 58.9% were classified as neoplastic and 41.1% were preneoplastic. The affected male population was classified as 55.7% neoplastic and 44.3% preneoplastic. Eleven genetically distinct lines of zebrafish were connected to the affected populations. The affected fish came from a total of 18 labs, both domestic and international.

A single zebrafish facility in the USA submits a large volume of diagnostic and normal sentinel zebrafish cases to ZIRC on a regular basis and so it was described as the primary facility for the purposes of this study. Approximately 74% of the fish affected by these intestinal changes, or 144 fish, came from the primary facility. The majority of these fish were part of the facility's sentinel program. The prevalence of intestinal changes amongst this subpopulation of affected fish occurred continuously from 2002 to 2012 (Fig. 2.4) at an average of approximately 32% of the sentinels affected each year, with a range of 9.1%-62.6%. Preneoplastic and neoplastic intestinal changes occurred at comparable proportion each year.

Comparisons of percent subclinical fish with the lesions amongst 15 facilities with a history of the lesions are reported in Figure 2.5. Close to 80% of the subclinical fish from these facilities that had the intestinal lesions were from the primary facility, while the facility with the next highest prevalence was responsible for less than 10% of the affected fish. The other affected facilities submitted 0.5-11.7% of the total subclinical fish amongst the affected facilities,

with a mean of 3.1% submissions per facility. Another facility submitted over 55% of the samples, but showed about 2% prevalence of the lesions.

Table 2.3 summarizes the prevalence of various intestinal presentations amongst all the fish examined. A total of 82 fish from the entire data set had preneoplastic changes; the majority of which showed only hyperplasia, and some exhibited a combination of hyperplasia and dysplasia. The majority of the 113 tumors were classified as adenocarcinomas or small cell carcinomas/carcinoid-like tumors, whereas the remaining lesions were classified as carcinoma not otherwise specified, tubular adenoma or tubulovillous adenoma (Table 2.3). The progression of the neoplastic process to carcinomatosis was observed in 1.5% of fish with neoplastic changes. The majority of the tumors and preneoplastic changes were observed between the anterior and mid-intestine, with rare occurrence in the distal third of the intestine. A total of 14 of 82 (17.1%) fish with preneoplastic lesions exhibited enteritis. Enteritis was observed in 5 fish with neoplasia, and hence over all prevalence of the former lesion was 9.7% in fish with either preneoplastic or neoplastic lesions. Enteritis was not observed in fish without lesions.

Diet Study

Most of the fish in all groups survived and appeared healthy after 6 months feeding the various diets. A total of 32 fish (16 fish/tank) were examined from each group, except one tank fed Diet 5 (the SARL zebrafish diet) contained only 13 fish. None of the fish exhibited histological changes consistent with the preneoplastic or neoplastic lesions reported here. The complete lack of lesions in fish fed Diet 4 at SARL was significantly different ($P< 0.001$) compared to the fish fed the same diet prepared at the primary facility, as these fish showed

approximately 33% and 63% prevalence of intestinal lesions in 2009 and 2010, respectively (Figure 2.3).

Discussion

Our systematic retrospective survey of the ZIRC diagnostic survey database and histological analysis of archived samples revealed a high incidence of intestinal neoplasia among laboratory reared zebrafish. Intestinal neoplasia identified in the ZIRC diagnostic database was primarily adenocarcinoma and small cell carcinoma/carcinoid-like tumor. Histomorphologic characteristics of these tumors were used in classification and identification at the tissue and cellular level. These intestinal tumors shared many of the common microscopic characteristics observed in their human counterparts, including small cell carcinoma and adenocarcinoma[17,18,19].

Although the cell of origin for zebrafish small cell carcinoma/carcinoid-like tumor is currently unknown, it is reasonable to postulate that the enteroendocrine cell of the zebrafish intestine may be a likely source, because this intestinal cell type is indicated as a progenitor cell of small cell carcinoma in mice[20]. This is further supported by the observation that similar to mice; mitotic figures are not present in these tumors of zebrafish as well, because terminally differentiated enteroendocrine cells do not undergo cell division[21]. Although zebrafish and humans share many conserved cancer gene sequences, the molecular studies already conducted in zebrafish tumor models do not conclusively prove that identical molecular mechanisms are responsible for tumor development or more importantly, that zebrafish tumors have the same histogenesis as the human counterpart[22].

Further descriptive work at the tissue and cellular levels are prerequisites to molecular based studies. Immunohistochemical identification and confirmation of the cell of origin for these intestinal tumors is imperative and would provide a useful adjunct to histomorphologic classification. For most zebrafish intestinal tumors, there is remarkable conservation of protein antigens that closely parallel human tumors, for which there are current zebrafish models. As an example, both human and zebrafish adenocarcinoma and small cell carcinoma/carcinoid-like tumors retain identical specific protein antigen and cell proliferation markers that are important in identifying and characterizing them, including cytokeratins, chromogranin A[23, 24], S100, synaptophysin, insulin, glucagon, somatostatin, PCNA and cdx2[25,26]. Therefore it is essential to more fully characterize zebrafish tumors not only at the histomorphologic and cellular levels, but also at the tumor protein (i.e. antigen) level before more fully investigating molecular aspects, such as gene expression, in zebrafish tumors.

Whether spontaneous or induced, zebrafish tumors must be initially approached in a phylogenetic context if they are to be generalized to similar human tumors[22]. Generation and development of monoclonal antibodies has advanced since the early experimental procedures[25], which involved using whole tumor cells or protein fractions as immunogens, to molecular approaches using known amino acid sequences that allow creation of immunogens from specific tumor cell peptides. Exploitation of this peptide generated from the amino acid sequence of the antigen of interest would be critical for establishing a zebrafish-specific tumor antigen immunostain panel and potential antigen-directed research modalities applicable to human medicine, such as experimental anti-neoplastic therapies. The intestinal tumors described in this study are currently under immunohistochemical evaluation within our lab. Zebrafish develop common

spontaneous neoplasia associated with aging, including spermatocytic seminoma and ultimobranchial gland adenoma that occur in zebrafish at 1.5 to 2 years of age[7,10] and embryonal neuroectodermal tumors of the central nervous system in both juvenile and adult fish[5,27]. Although many zebrafish tumors recapitulate their human counterparts in terms of basic histologic appearance, certain molecular characteristics (increased cell proliferation, nuclear atypia, and cellular differentiation) and mechanisms of regulation (cell cycle and apoptosis)[3,22,28], there is relatively little understanding of how conserved tumor antigens are between the two species. Some researchers developing zebrafish tumor models consider histologic evaluation as an unnecessary step[29], which would lead to potentially erroneous conclusions because without it as a starting point, obvious tissue and cellular similarities cannot be determined.

Although these intestinal tumors were observed in several facilities, the definitive causative agent is unknown. Possible etiological factors include genetics, water-borne carcinogens, infectious agents or some combination of these. Knockout mutants in zebrafish are well established as cancer models[1], and these mutants demonstrate an increased propensity towards developing cancerous lesions. For example, *apc/+* (AB mutant) zebrafish have been reported to develop intestinal tumors[9]. Genetics as a cause of tumorigenesis in this study is unlikely given that 11 different genetic lines displayed preneoplastic and/or neoplastic changes, including wild-type lines. Additionally, intestinal proliferative lesions and subsequent neoplasia do not appear to be sex-linked, as both males and females are similarly affected.

Whereas the primary facility had the greatest number of affected fish with preneoplastic or neoplastic intestinal lesions, this does not appear to be due to an increased frequency of subclinical submissions. For example, adjusting for this

factor, we found that the prevalence was indeed much higher in the primary facility than the others. Moreover, facility 15 submitted the most subclinical fish (approximately 60%) amongst the affected facilities, but showed a low prevalence of the lesions (Fig. 2.5). We cannot suggest a potential cause of the high prevalence in the primary facility as it appears to be managed and operated no differently than traditional zebrafish facilities with large recirculating systems.

Diet has been implicated in the progression from chronic inflammation to tumorigenesis in the gastrointestinal tract of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) fed a commercial diet rich in plant products[30]. Diet as a cause of the intestinal lesions described here was unlikely as a potential source of carcinogenesis based upon the experiment carried out at SARL. Data from the retrospective study suggests that intestinal lesions may be observed as early as 6 months of age and the sampling at 6 mo. yielded no sign of preneoplastic or neoplastic intestinal changes. Although not all fish develop the lesions this young, it would be expected at minima some progression towards these intestinal lesions in some of the fish would have occurred if diet was the cause. The Diet 4 fed at SARL was the exact same diet in regards to formulation and source material that was used at the primary facility, which on average has a prevalence of intestinal changes of 32% per annum. Moreover, the experiment at SARL was conducted with diet prepared at the primary facility in 2009, and the sentinel fish at this location showed approximately 33% prevalence of lesions in 2009 and 63% prevalence in 2010. Nevertheless, our results excluding diet as the cause of these lesions should be considered preliminary at this time because the experiment was terminated after only 6 mo.

A water-borne carcinogen must also be considered as chemical carcinogens such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG),

methylazoxy-methanol acetate (MAMA), and DMBA have all been previously demonstrated to cause neoplasia in zebrafish[31]. Proliferative lesions similar in pattern and location to some of the tumors were observed in fry and juvenile zebrafish exposed to DMBA by bath and diet exposure, respectively[10]. Although we tended to see the lesions in older fish compared to the findings from the DMBA study, this may be explained by a lower exposure dosage or a less tumorigenic water-borne carcinogen. The typical zebrafish facility also has a very rigorous water filtration system, involving any combination of sand or bead filtration systems, activated carbon filters, reverse osmosis, and UV filtration resulting in very pure water. Any carcinogen must get past these complexes of filters or be introduced downstream of the filtration process, either as a component of the material used to transport the water or to house the fish.

Several parasites have been implicated as promoters of neoplasia, most notably the nematode *Spirocerca lupi*, infection with which has been associated with osteosarcoma and esophageal fibrosarcoma in dogs[32]. Another agent associated with intestinal neoplasia in zebrafish, while not established as a causative agent, is the nematode *Pseudocapillaria tomentosa*. Zebrafish that were exposed to both DMBA and *P. tomentosa* demonstrated a higher prevalence of intestinal tumors than uninfected fish exposed to DMBA[11]. Whereas this nematode was implicated in the original diagnosis of several affected fish, it was not prevalent amongst the affected fish in our study.

Other infectious agents are also suspected, whether bacterial or viral. *Helicobacter pylori* has been previously associated with human gastroesophageal neoplasia and similar gastric carcinogenesis has been modeled in the Mongolian gerbil[33]. Although experimental evidence has not linked bacteria to carcinogenesis in zebrafish to date, the chronic inflammation elicited by certain pathogenic

strains of bacteria, and even the natural microbiota of zebrafish could potentially serve as promoters of intestinal carcinogenesis. Viruses have been connected to certain fish cancers[34], such as SLV (salmon leukemia virus) in Chinook salmon[35] and WDSV (walleye dermal sarcoma virus) in walleye[36]. Although endogenous retroviruses have been identified in zebrafish[37], no oncogenic viruses have been currently implicated as the cause of intestinal neoplasia in zebrafish. To date, no naturally occurring pathogenic virus has been isolated from zebrafish[38,39]. Transmission studies are currently underway within our lab to evaluate the possibility of an infectious etiology for these intestinal lesions.

Our survey demonstrates that intestinal neoplasia and preneoplastic pathology are common among zebrafish research facilities. The fish surveyed in this study are not a random selection and there is a bias by the volume of cases submitted by the primary facility, but these fish do represent many different research facilities and fish genotypes commonly used in zebrafish research. Based on the continuity of cases through the years and the fact that many of these lesions occur in subclinical fish, we suggest that these lesions could introduce an underlying, unappreciated variable into zebrafish research.

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TABLE 2.1. DEFINING HISTOLOGICAL SIGNS OF INTESTINE PRESENTATIONS AS OBSERVED WITHIN ZEBRAFISH SUBMITTED TO THE ZEBRAFISH INTERNATIONAL RESOURCE CENTER DIAGNOSTIC SERVICE 2000-2012

Intestinal Presentation	Defining Signs
Normal Intestine	One cell thick layer of columnar epithelial cells lining mucosal folds with basally-oriented oval nuclei; mucosal folds become progressively shorter caudally, causing "villi" (the normal undulating structure of the intestinal wall appears villous, but lacks the true anatomic characteristics of villi) to appear shorter as the intestine approaches the excretory vent (anus); lamina propria, but no submucosa; inner circular and outer longitudinal smooth muscle layers invest the intestine throughout its length. Mucosal mucus (goblet) cells can be observed and increase in number distally.
Hyperplastic Intestine	Multilayered and increased numbers of epithelial cells, especially within basilar mucosal folds; "piling-up" of mucosal epithelial cells; nuclear pseudostratification; enhanced nuclear basophilia; pseudocrypt formation resulting from increased mucosal folding; anisokaryosis frequently observed and increased mitotic figures.
Dysplastic Intestine	Features of hyperplastic intestine in addition to increased nuclear and cellular pleomorphism, and occasionally aberrant mitotic figures, the loss of nuclear polarity and disorganization or absence of pre-existing histoanatomic architecture.
Intestinal adenocarcinoma	Features of dysplastic intestine plus formation of disorganized pseudocrypts with invasion deep into the lamina propria and frequently through the basement membrane into the underlying muscularis layers; bizarre mitotic figures; neoplastic epithelial cells are pleomorphic and may be columnar, cuboidal or attenuated; hyperchromatic nuclei; annular strictures and fibroplasia frequently accompany tumorigenesis; pseudocrypts formed by the folding of neoplastic mucosal epithelium often resembled pseudoacinar structures that contained intraluminal sloughed rafts of necrotic neoplastic cells.
Intestinal small cell carcinoma/carcinoid-like tumor	Sheets and nests of round, polygonal or fusiform cells with minimal cytoplasm; hyperchromatic nuclei with granular chromatin and inconspicuous nucleoli; extensive fibroplasia; tumor cells occasionally formed an insular or organoid pattern characteristic of neuroendocrine tumors.
Intestinal tubular/tubulovillous adenoma	Focal adenomatous polypoid structures with clusters of proprial pseudocrypts resembling mammalian glandular colonic crypts. The pseudocrypts often are lined by hyperplastic mucosal epithelium where the cells are crowded and have hyperchromatic nuclei. Increased mitotic figures are observed. Tubulovillous adenoma is essentially similar to tubular adenoma with a combination of both villous and pseudocrypt structures.

TABLE 2.2. PREVALENCE OF PRENEOPLASTIC & NEOPLASTIC LESIONS IN ZEBRAFISH SUBMITTED TO THE ZEBRAFISH INTERNATIONAL RESOURCE CENTER DIAGNOSTIC SERVICE 2000-2012

Year	Number of Positive Cases/Total Submitted Cases	Number of Positive Clinical Fish/Total Clinical Fish	Number of Positive Subclinical Fish/Total Subclinical Fish	Number of Affected Labs/Total Submitting Labs	Lines of Fish Affected
2000	2/41	0(0)[1]/119	1(1)/60	2/13	Albino, Brass (AB)
2001	3/38	3(0)/90	0(0)/57	2/14	Albino, 5D
2002	3/57	1(1)/184	4(5)/176	2/19	AB
2003	2/47	0(0)/157	2(0)/432	1/16	AB
2004	4/61	0(1)/201	3(5)/208	2/29	Golden Tupfel Long Fin, AB
2005	3/53	0(0)/154	3(8)/218	2/24	AB
2006	4/63	0(4)/282	4(3)/456	2/25	AB, Ekkwill
2007	6/68	4(2)/237	1(0)/511	5/31	AB, SJA, Wageningen ZF WT Zodiac F5 Line, Coagulation Factor II Mutagenized Transgenic
2008	6/71	1(0)/143	13(6)/1024	5/28	AB, Tupfel Long Fin
2009	8/78	2(3)/153	10(9)/1064	6/30	AB, WIK
2010	4/54	3(1)/ 57	11(5)/1179	2/25	AB
2011	12/95	0(0)/191	39(21)/1277	8/44	AB, Tuebingen, WIK
2012	3/53	0(0)/49	7(8)/860	2/25	AB, Tuebingen
Study Totals	60/779	14(12)/2017	98(71)/7522		

[1]Positive clinical and subclinical fish totals are distinguished by fish with neoplastic changes (without parenthesis) and preneoplastic changes (within parenthesis).

TABLE 2.3. PREVALENCE OF INTESTINE PRESENTATIONS AS OBSERVED WITHIN ZEBRAFISH SUBMITTED TO THE ZEBRAFISH INTERNATIONAL RESOURCE CENTER DIAGNOSTIC SERVICE 2000-2012

	Type	Prevalence[1] (%)
Preneoplastic Changes	Hyperplasia Only	67.1
	Hyperplasia & Dysplasia	32.9
Neoplastic Changes	Adenocarcinoma	50.4
	Small Cell Carcinoma/Carcinoid-like	37.2
	Carcinoma Not Otherwise Specified	9.7
	Tubular Adenoma	1.8
	Tubulovillous Adenoma	0.9

[1]Fish classified as neoplastic often displayed preneoplastic changes, but are not counted among the fish with preneoplastic changes for this study, as we considered the tumor formation a notable progression following the preneoplastic lesions. For this reason, prevalence is calculated relative to either the preneoplastic or neoplastic population affected.

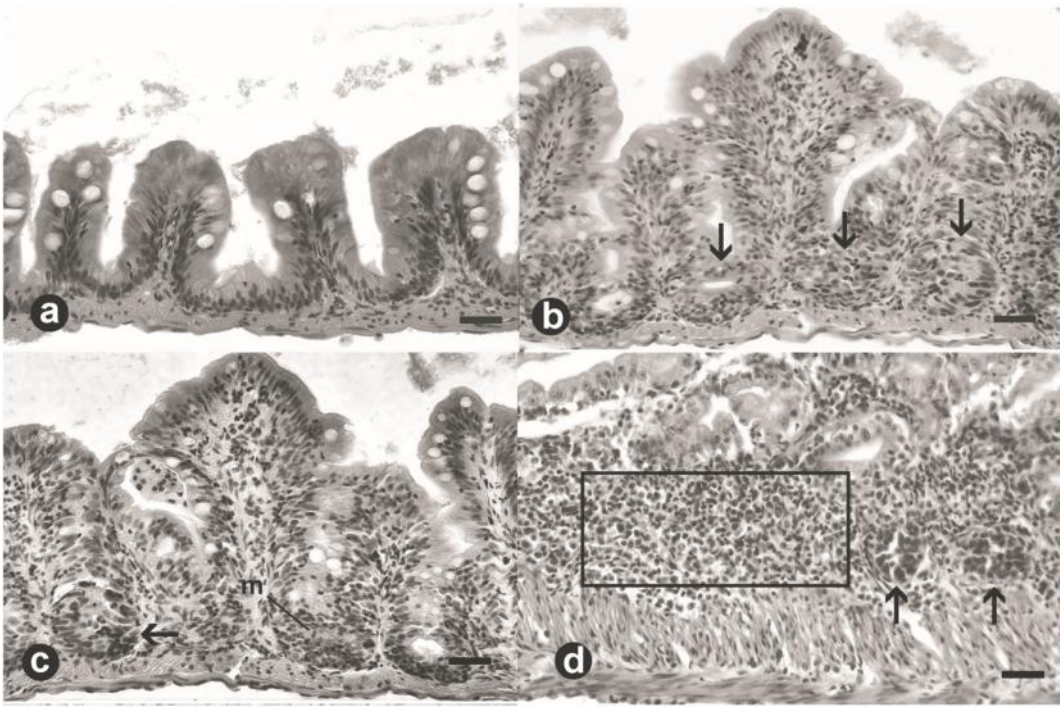

Figure 2.1. Normal and preneoplastic lesions in zebrafish intestines. H&E. Bar = 25µm. **(A)** Normal zebrafish intestine, lined by a single layer of columnar epithelium. **(B)** Hyperplasia, with multilayered columnar epithelium and formation of mucosal inter-fold pseudocrypts involving the basal epithelium (arrows). Note pseudostratification of nuclei, but nuclei retain polarity. **(C)** Dysplasia, with nuclear atypia and cellular pleomorphism. Also, there is loss of normal histological architecture, loss of nuclear polarity, and aberrant mitotic figures (arrow). **(D)** Enteritis, chronic inflammatory cell infiltrate within the lamina propria (indicated by box). Note two presumptive aberrant pseudocrypt foci (arrows).

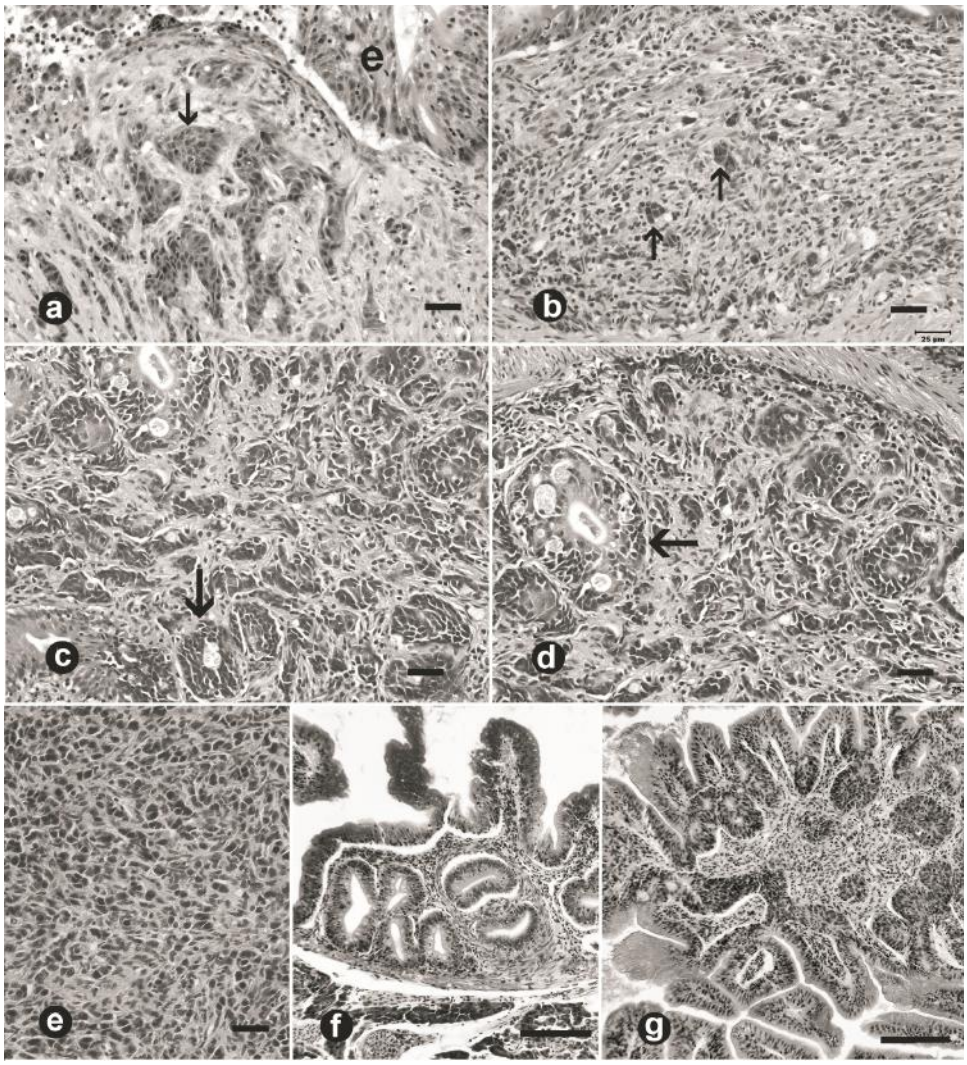

Figure 2.2. Intestinal neoplasia in zebrafish. H&E. Bar = 25µm, unless otherwise indicated. **(A, B)** Small cell carcinoma, with small nests of fusiform to pleomorphic tumor cells (arrows) in the lamina propria that occasionally form organoid patterns. E = epithelium. (**C, D)** Adenocarcinoma, with tumor cells forming pseudoacinar structures (arrows), complete with a lumen in the most advanced tumors. **(E)** Carcinoma not otherwise specified in the lamina propria. Less differentiated and organized than the adenocarcinoma and small cell carcinoma. **(F)** Tubular adenoma, with glandular-like pattern. Bar = 100 µm. **(G)** Tubulovillous adenoma, with the villotubular pattern. Bar = 100 µm.

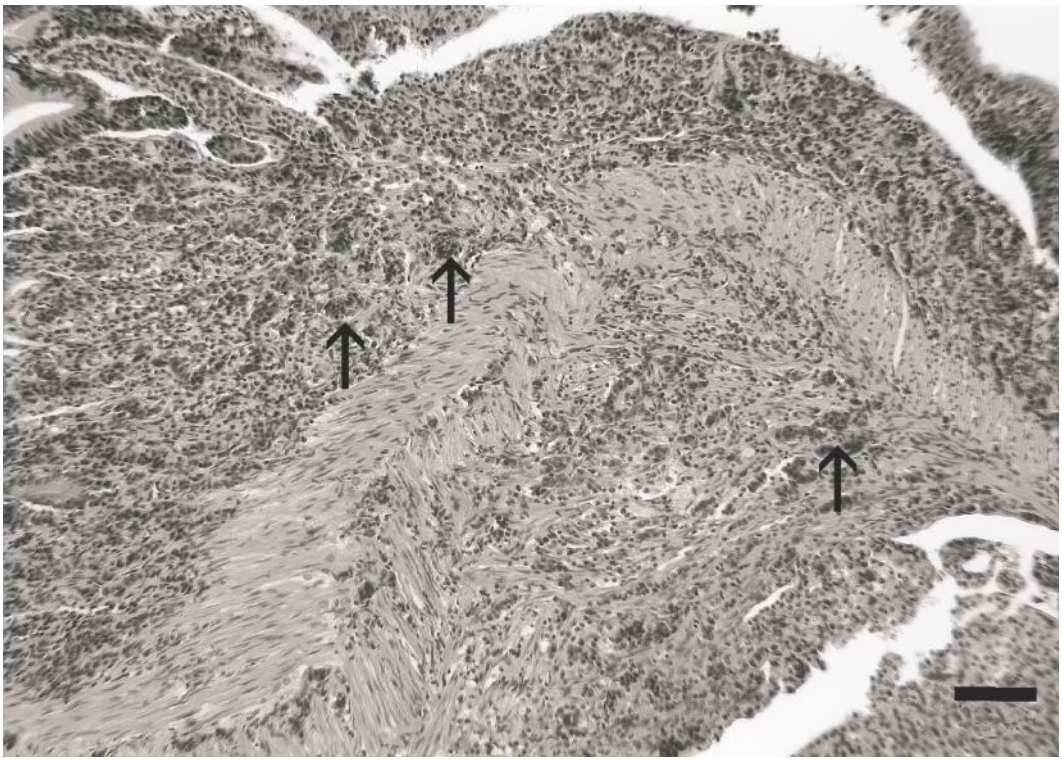

Figure 2.3. Carcinomatosis (adenocarcinoma), characterized by disorganized nests of tumor cells (arrows) infiltrating through the layers of the anterior intestine, with extension into the coelomic cavity. H&E. Bar = 50µm.

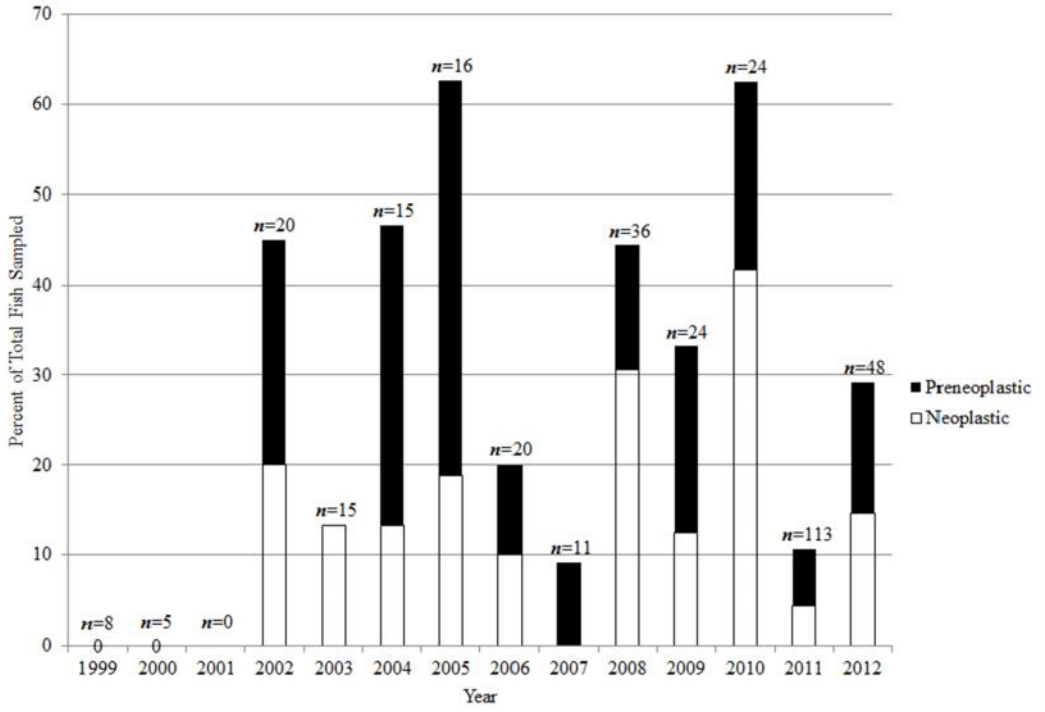

Figure 2.4. Prevalence of preneoplastic and neoplastic changes amongst the sentinel fish from a single, large zebrafish research facility in the USA (the primary facility as cited in the text). There were no positive sentinel fish in 1999 or 2000. No sentinel fish were sampled in 2001.

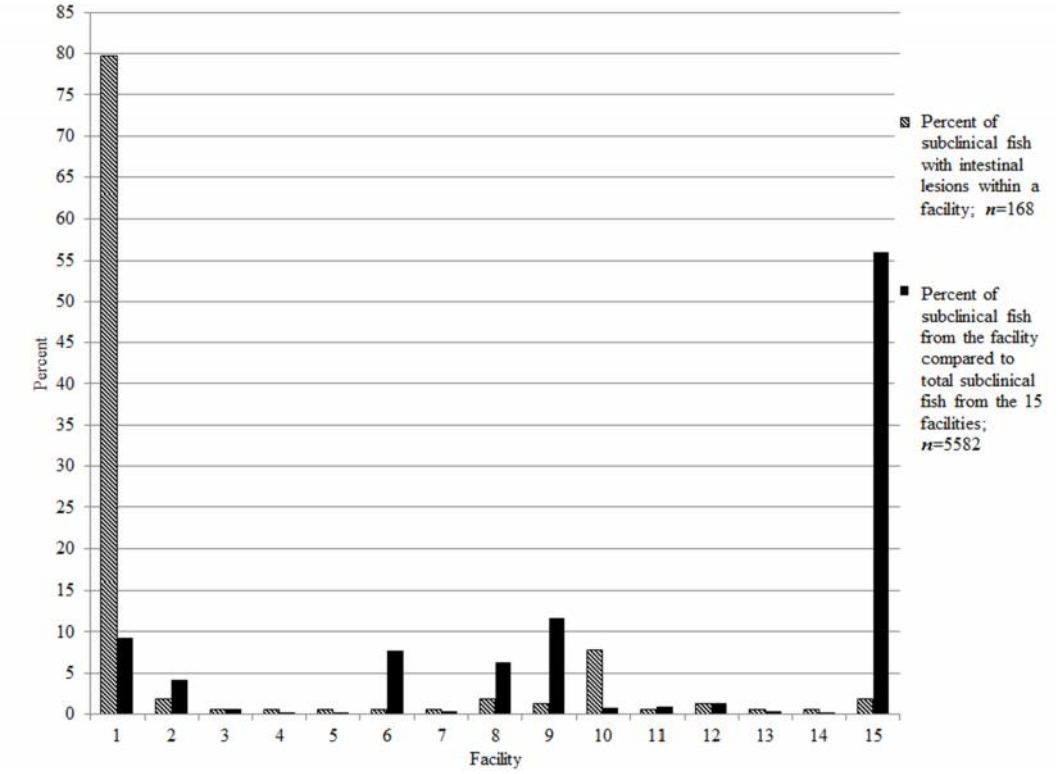

Figure 2.5. Prevalence of intestinal preneoplastic and neoplastic changes in subclinical zebrafish relative to total subclinical fish submitted from 15 facilities from 2000-2012. The single, large zebrafish research facility (the primary facility as cited in the text) is included here as facility 1.

Chapter 3

A Study on the Transmissibility of Intestinal Neoplasia in Zebrafish (*Danio rerio*)

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Abstract

Spontaneous intestinal neoplasms and preneoplastic lesions have been observed in many zebrafish (*Danio rerio*) research facilities, and with a high prevalence in some laboratories. The etiology of these lesions is unknown, but research to date suggests that diet or genetic background is not related. Hence, an infectious agent should be considered. To elucidate a possible infectious etiology, we conducted transmission trials via cohabitation with affected fish, feeding of affected tissue homogenate, and intraperitoneal (IP) injection of filtered and non-filtered affected tissue homogenate cohabitation with affected fish, feeding of affected tissue homogenate, and intraperitoneal injection of filtered and non-filtered affected tissue homogenate. Fish were held for 10 or 11 mo. post-exposure, and samples were taken throughout the experiment. Mild to moderate hyperplastic changes were identified in two fish from the cohabitation study sampled at 8 mo. and two fish from the injected filtrate study sampled at 7 mo. However, comprehensive histological evaluation at the termination of the study did not recognize any preneoplastic or neoplastic intestinal lesions. Fewer fish were available at the end of the study than anticipated due to high mortality due to *Piscinoodinium pillulare* infections amongst the fish in the study. Results from the study were equivocal, and hence this study reasons to be repeated.

Introduction

Spontaneous neoplasms are not uncommon in zebrafish two years of age or older[1, 2, 3]. Amongst these spontaneous neoplasms, intestinal neoplasms were previously reported with a historical prevalence of approximately 2% in 18 of 130 zebrafish research facilities[4]. Possible etiological factors correlated with piscine neoplasms lesions include genetics (as it relates to a genetic predisposition as an

effect of genetic background or specific strain/line of the fish), diet, water-borne carcinogens, infectious agents or some combination of these[5,6,7, 8]. Genetics and diet were previously investigated, but failed to establish any correlation with the incidence of these proliferative intestinal lesions[4]. Age and strain matched fish on the same water system at a large zebrafish facility with an extremely high incidence rate exhibited dramatic differences in prevalence of lesions. This indicates that an infectious agent, rather than a waterborne carcinogen, is the cause of these lesions.

Several parasites have been demonstrated to be initiators or promoters of neoplasia in mammals, including gastrointestinal cancers in humans[9]. With fish, the capillarid nematode *Pseudocapillaria tomentosa* is a cofactor (probably a promoter) for intestinal neoplasia in zebrafish exposed to 7,12-dimethylbenz[*a*]anthracene (DMBA)[8]. Several oncogenic viruses cause cancer in fishes[10]. Regarding bacteria, *Helicobacter pylori* is a well-recognized cause of human gastrointestinal neoplasia, and similar gastric carcinogenesis has been modeled in the Mongolian gerbil[11]. Experimental evidence has not yet linked bacteria or viruses to carcinogenesis in zebrafish, but the chronic inflammation elicited by certain pathogenic strains of bacteria or the natural microbiota of zebrafish could potentially serve as promoters of intestinal carcinogenesis.

In this study we investigated a possible infectious etiology of the intestinal lesions commonly seen in zebrafish through multiple modes of transmission previously attributed to infectious agents: cohabitation with affected fish, feeding of affected tissue homogenate, and IP injection of filtered and non-filtered affected tissue homogenate.

Materials and Methods

Donor fish were identified by screening samples of fish from various tanks at a facility that we previously identified with a high prevalence of these lesions[4]. Unaffected recipient fish (AB line) were sourced from Sinnhuber Aquatic Research Laboratory (SARL), Oregon State University. This laboratory has no history of the intestinal lesions of interest[12].

Fish were screened by histology. The water supply for all treatment groups was the same source; dechlorinated city water followed by reverse osmosis. Water temperature was maintained at 28 C. Assessment for occurrence of intestinal lesions was conducted by preserving fish in Dietrich's, cutting the fish in half, and preparing sagittal sections stained with hematoxylin and eosin. The entire digestive tract, focusing on the intestine, of each fish prepared for histological evaluation was examined from oropharynx to excretory vent for three or four serial hematoxylin and eosin (H&E) stained sections. All fish sampled were examined by blind read by at least two of the authors (C.P., M.K., or T.P.), with no knowledge of the treatment regime.

Cohabitation Study

Two 8.0L breeding tanks (Tecniplast USA, Inc.) were fitted with perforated dividers. In each breeding tank, fish from a positive tank (100% affected control) were initially placed on one side of the divider, two additional fish from a positive tank were added within a month of the experiment start date based upon availability. Fourteen unaffected fish (AB line) were placed on the other side of the divider of each breeding tank. Fish were observed at least twice a day. Moribund fish were removed, euthanized, and preserved for histological evaluations.

Feeding & Injection Studies

Thirty donor fish from the positive tanks (8 from the 100% affected and 22 from the 40% affected) were euthanized using ice water[13]. Intestines of these fish were removed aseptically and homogenized with 1x PBS. The homogenate was divided into three equal volumes. One third was fed to 36 fish: Feed Group-Gross Hot Feed. Ten negative fish from SARL were similarly prepared and the entire volume fed to 36 fish: Feed Group-Gross Clean Feed which will serve as the feed control.

The remaining 2/3 of homogenate was passed through a 40 µm Nitex metal screen. Additional PBS was added to assist with the filtration rate. The homogenate was then split into two equal volumes. Half was used for Injection Group 1-Tissue Homogenate/No Filter. The other half of the homogenate was then pre-filtered with a 5 µm syringe filter. Again PBS was added to assist with the filtration rate. The homogenate was then passed through a 0.45 µm tangential flow syringe filter (Costar µStar). This served as the inoculum for Group 2-Filtered Homogenate. Sterile PBS was injected to Group 3-Injected and Cohabitation Control.

Fish were anesthetized with MS-222 bath at 100 ppm in water. A total of 36 fish/group were injected with 25 µl/fish of the appropriate inoculum using a 26 gauge needle. For the duration of the experiment, each group of 36 fish was divided into 2 tanks of 18 fish each, for a total of 10 tanks. Fish were monitored at least twice a day. Moribund fish were removed, euthanized, and preserved for histological evaluations. Fish were sampled at 7 mo. post-exposure and 12 mo. post-exposure.

Results

High mortality occurred in the fish receiving non-filtered IP inoculate, with 58% mortality in the first month. These 21 fish were not examined by histology. Mild changes were identified in two fish from the cohabitation study (Fig. 3.3) sampled at 8 mo. and two fish from the injected filtrate study (Fig. 3.4) sampled at 7 mo. (Table 3.1). As these lesions were mild, or perhaps equivocal, we provide examples of normal intestine (Fig. 3.1) and a fish with hyperplasia from our previous study[4] (Fig. 3.2). High mortality occurred in various tanks beginning in the third month post-exposure due to gill infections by *Piscinoodinium pillulare* (Fig. 3.5) (Table 3.1). The experiments were terminated at 10 mo. for the feed and injection studies and 11 mo. for the cohabitation study. All available fish sampled throughout the course of the studies were again evaluated, and all fish collected at 10 or 11 mo. were scored as negative for the preneoplastic and neoplastic intestinal lesions.

Discussion

Our cohabitation, feed, and injection studies yielded equivocal results. Four fish showing mild hyperplastic lesions were identified at the 7 or 8 month sampling time point from the cohabitation and injection with filtered homogenate experimental groups. This finding suggests a possible infectious agent, even a virus. However, no affected fish were seen at the later time points. The level of exposure material in relation to the mode of transmission could account in part for the transmission inconsistencies. Also the experiments were terminated at earlier than originally planned (1.5 years), due to the significant loss of fish due to a *P. pillulare* infection. The experiment fish were held in a room also housing pet-

shop fish. This dinoflagellate parasite is very common in pet fish and is not uncommon among research fishes, including zebrafish[14]. Whereas it is possible that contaminated equipment may have allowed transmission of these microscopic parasites between tanks, *Aeromonas salmonicida* and *Ichthyophthirius multifiliis* pathogens have been shown to be capable of traveling upwards of several feet by aerosol between tanks within a laboratory[15, 16].

A few parasites have been implicated as promoters of neoplasia in fish. The microsporidian parasite, *Nucleospora salmonis*, has been associated with Chinook salmon plasmacytoid leukemia[17], and the capillarid nematode *P. tomentosa* has also been associated as a cofactor in intestinal neoplasia in zebrafish. Whereas the nematode is occasionally seen in zebrafish, including those in research facilities[8], this parasite was not observed in many of the fish and facilities with the intestinal lesions[4].

Bacterial agents should be considered as potential agents or co-factors with these lesions as *Helicobacter pylori* has been previously associated with human gastroesophageal neoplasia[18]. *Helicobacter* species have not been reported from fish, but Broussard et al. showed that another common bacterium of fish, *Mycobacterium marinum*, acts a cancer promoter in Japanese medaka (*Oryzias latipes*)[19]. Although experimental evidence has not linked bacteria to carcinogenesis in zebrafish to date, the chronic inflammation elicited by certain pathogenic strains of bacteria, and even the natural microbiota of zebrafish could potentially serve as promoters of intestinal carcinogenesis[19, 20].

Viruses have been connected to certain fish cancers, particularly herpes viruses[21] and retroviruses. These cancers include lymphoid or dermal neoplasms. Most of the retroviral-induced tumors in fishes are associated with various cofactors, most commonly seasonal variability of water temperature[10], a variable

not commonly introduced to laboratory zebrafish. Endogenous retroviruses have been identified in zebrafish[22], but no oncogenic viruses have been currently implicated as the cause of intestinal neoplasia in zebrafish. In addition, naturally occurring pathogenic viruses have not been isolated from zebrafish[20, 23].

A water-borne carcinogen must also be considered either as a primary or cofactor etiologic agent. Chemical carcinogens are a standard method for inducing chemical tumorigenesis. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), methylazoxy-methanol acetate (MAMA), and DMBA have all been previously demonstrated to cause neoplasia in zebrafish[24]. Proliferative lesions similar in pattern and location to some of the tumors were observed in fry and juvenile zebrafish exposed to DMBA by bath and diet exposure, respectively[25]. The pattern of prevalence in the primary facility points away from a water borne carcinogen as we have seen dramatic differences in the prevalence of the lesions amongst age and strain matched tanks on the exact same water system.

The typical zebrafish facility has a recirculating water system with a very rigorous water filtration system, involving any combination of sand or bead filtration systems, activated carbon filters, reverse osmosis, and UV filtration resulting in very pure water. Any carcinogen must get past these complexes of filters or be introduced downstream of the filtration process, either as a component of the material used to transport the water or to house the fish. While juvenile and adult fish are commonly maintained in the laboratory facilities on these recirculating systems, all sharing the same water, the larval fish are most often reared in static tanks in a separate nursery facility. Zebrafish exposed to carcinogens at embryonic and larval stages may not develop consequential proliferative lesions until much later in life[23] and typically undergo several tank

transfers between these time points, potentially accounting for an incidence dispersal pattern similar with an infectious agent etiology.

Another factor to consider is diet. Our previous studies have indicated that the actual components of the diet are not the primary etiologic agent[4]. Contamination after formulation should be considered, particularly with waste food in the tanks. Fungi grow on feeding receptacles of tanks and on uneaten food within the water. Fungi have been shown to form carcinogenic compounds (e.g., aflatoxin) known to induce cancer of the liver and/or esophagus in humans[26] . Aflatoxin occurs in rancid feeds and is a potent liver carcinogen in rainbow trout [27], and to a lesser degree zebrafish[28]. Whereas the incident rate of a series of events similar to this amongst the facilities affected by these proliferative lesions is unknown, it stands to reason this could be a potential cofactor to consider in future transmission trials.

Stress and density may also be cofactors in the etiology of these intestinal lesions, given the laboratory environment common amongst the reported affected fish[10]. These lesions could introduce an underlying, unappreciated variable into zebrafish research. Resolution of the etiology of these intestinal lesions is essential to minimize extra-experimental variability, and as a means to possibly introduce a new zebrafish cancer model.

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TABLE 3.1. ADJUSTED SAMPLING SCHEDULE FOR TRANSMISSION STUDIES OF INTESTINAL LESIONS IN ZEBRAFISH IN RESPONSE TO HIGH MORTALITY DUE TO *PISCINOODINIUM PILLULARE* INFECTION STARTING 3 MO. POST-EXPOSURE[1]

	Tank 1 7 mo.[2]	Tank 2 7 mo.[2]	Tank 1 10 mo.[3]	Tank 2 10 mo.[3]	Moribunds[4]
Treatment					
Cohabitation	NS[5]	NS	NS	3	5
Fed Affected Tissue	5	6	6	NS	1
Fed Unaffected Fish (Control)	5	5	NS	NS	2
IP Unfiltered Homogenate	NS	NS	NS	NS	1
IP Filtered Homogenate	4	15	NS	NS	NS
IP PBS (Control)	NS	NS	NS	NS	13

[1]18 fish/tank at time of exposure, cohabitation tanks 14 fish/tank
[2]Cohabitation treatment sample was at 8 mo.
[3]Cohabitation treatment sample was at 11 mo.
[4]Moribunds are fish that were sampled as-necessary; time points range from approximately 1 mo. to 10 mo. post-exposure. Not all moribund fish could be preserved due to cannibalistic nature of zebrafish, and the rapidity of autolysis.
[5]NS=Not sampled due to limited or lack of fish

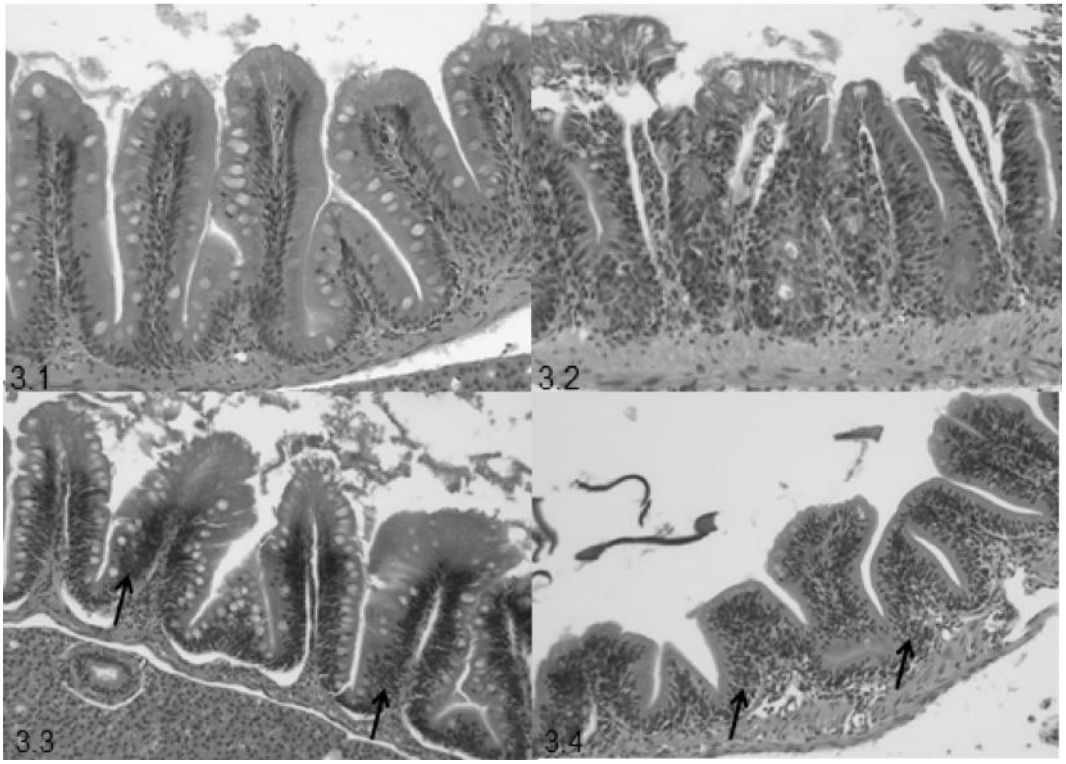

Figure 3.1. Intestine; adult zebrafish. Normal intestinal structure. H&E. **Figure 3.2.** Intestine; adult zebrafish. Well progressed hyperplastic changes, notable by pseudostratification of the nuceli indicated by the arrows within the intestine of a fish in the cohabitation experimental group. H&E. **Figure 3.3.** Intestine; adult zebrafish. Hyperplastic changes, notable by pseudostratification of the nuceli indicated by the arrows within the intestine of a fish in the cohabitation experimental group. H&E. **Figure 3.4.** Intestine; adult zebrafish. Hyperplastic changes, notable by pseudostratification of the nuceli indicated by the arrows within the intestine of a fish in the injected with filtered homogenate experimental group. H&E.

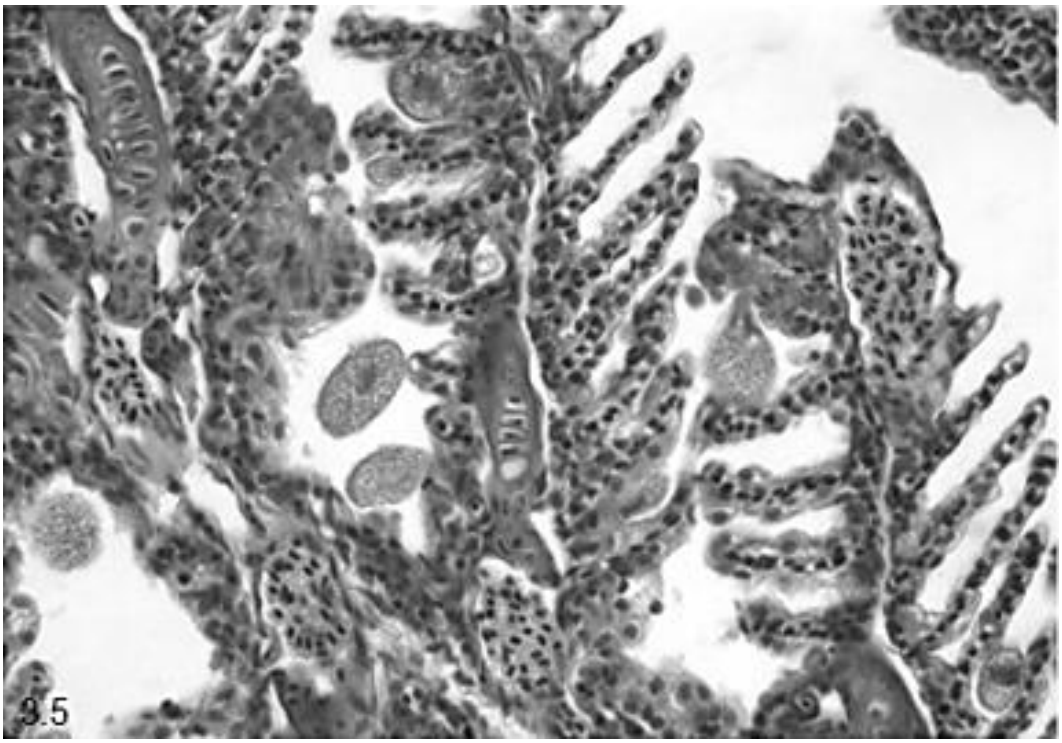

Figure 3.5. Gill; adult zebrafish. *Piscinoodinium pillulare* amidst the gill lamellae. H&E.

Chapter 4

Immunohistochemical Characterization of Intestinal Neoplasia in Zebrafish (*Danio rerio*) Indicates Epithelial Origin of Tumor Cells

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Abstract

Spontaneous neoplasia of the intestinal tract in sentinel and moribund zebrafish are common in some zebrafish (*Danio rerio*) facilities. We previously classified these tumors as adenocarcinoma, small-cell carcinoma, or carcinoma not otherwise specified based on histomorphologic characteristics. Mammalian gastrointestinal neoplasms most frequently arise from epithelium or neuroendocrine cells (e.g., carcinoids). Therefore, we propose that the cells of origin for these neoplasms in zebrafish neoplasms were derived from either intestinal epithelial or gut-derived neuroendocrine tissue. Select tissue sections were exposed to a panel of mammalian antibodies directed toward epithelial (Cytokeratin Wide Spectrum Screening [WSS], AE1/AE3) or neural (S100, chromogranin A) tissues. We also investigated antibody specificity using Western blot analysis, comparing human and zebrafish profiles. WSS and AE1/AE3 were relatively reactive with approximately half of the tumors immunopositive for epithelial markers. S100 and chromogranin A demonstrated non-specific immunostaining of the cells in all of the tumors examined. The positive cytokeratin association (WSS and/or AE1/AE3) for most of the tumors, while negative for neural and neuroendocrine markers (S100 and chromogranin A, respectively) suggests that intestinal neoplasia in the zebrafish arises from epithelial cells, and perhaps those tumors negative for cytokeratin may have dedifferentiated.

Introduction

For over a decade, spontaneous intestinal neoplasia has been observed in zebrafish (*Danio rerio*) submitted to the ZIRC (Zebrafish International Resource Center) diagnostic service[1, 2]. Many of the fish from these populations also

displayed preneoplastic changes in the intestine, including hyperplasia and dysplasia. Based on routine histology, the tumors have been classified either as adenocarcinoma or small cell carcinoma, with few exceptions (carcinoma not otherwise specified, tubular adenoma, and tubulovillous adenoma).

The cell of origin/cell type of these neoplasms has not been fully elucidated. Considering the location and morphology, an epithelial origin would be likely. However, in mammals many gastrointestinal neoplasms arise from gut derived neuroendocrine cells[3]. These are often classified as carcinoids either small cell carcinoma or carcinoid tumors, but neuroendocrine cells may be a component of other intestinal tumors, as well[4]. Therefore, in an attempt to resolve this question with zebrafish intestinal tumors, a subset of paraffin-embedded sentinel and moribund zebrafish that were submitted to the ZIRC (Zebrafish International Resource Center) diagnostic service were evaluated by immunohistochemistry. The select subset of zebrafish had intestinal tumors previously classified as adenocarcinoma, small-cell carcinoma, or carcinoma not otherwise specified based on histomorphologic characteristics.

There is close conservation between human and zebrafish tumorigenic mechanisms at the molecular, cellular and tissue levels, including expression of tumor antigen target epitopes[5, 6]. Based upon interspecies target epitope conservation and the limited availability of zebrafish-specific antibodies, we chose to evaluate tumor antigen expression using mammalian antibodies. WSS and AE1/AE3 are both cytokeratin markers expressed in human intestinal adenocarcinomas[7]. S100 is a marker for neural crest-derived-cells and highlights tumor antigens expressed in human neurogenic tumors (i.e. schwannoma at multiple anatomic sites) and gastrointestinal stromal tumors (GISTs)[8]. Chromogranin A is expressed in cells of endocrine and neuroendocrine, origin. It

is commonly expressed in tumors of neuroendocrine origin, such as small-cell carcinomas[9]. In addition, WSS and AE1/AE3 cytokeratins, S100 and chromogranin A antibody protein profiles were evaluated using Western blots, in order to compare and contrast differences, if any, between the human and zebrafish antibodies.

Materials and Methods

Western Blot

Five whole frozen zebrafish approximately 30 dpf were processed for Western blot analysis. The animals were pooled and homogenized with IP lysis buffer (Roche) and incubated overnight at 4 °C. Protein concentration was determined using the Thermo Scientific Pierce Micro BCA protein assay using bovine serum albumin as the standard. Zebrafish protein samples were then prepared and analyzed by electrophoresis on a SDS-PAGE gel using MagicMark™ XP Western Protein Standard and human HTP-1 cells for a positive control. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and blocked by immersion in phosphate buffered saline (PBS) containing 0.1% Tween-20. The membranes were then incubated at 4 °C overnight with the primary antibodies against WSS (CK(WSS)-Dako Z0622) 1:2000, AE1/AE3 (AE1/AE3 -Dako M3515) 1:400, S100 (S100-Dako Z0311) 1:500 or chromogranin A (Chromogranin A-Dako A0430) 1:2000. After incubation, the membranes were double washed with PBS containing 0.1% Tween-20 and incubated for 1 h with a secondary antibody of rabbit (WSS, S100, and chromogranin A) or mouse (AE1/AE3). Membranes were subsequently washed with PBS containing 0.1% Tween-20. Substrate development for photo

documentation was performed using the ECL chemiluminescent substrate (Pierce).

Immunohistochemistry

Representative unstained tissue sections containing tumor, 4-5 µm thick, were placed on charged slides (Tanner Scientific) and heated at 60^{o}C for 1hour. Slides were rehydrated through 2 changes of xylene, 2 changes of 100% ethanol, 1 change of 80% ethanol and water. Antibodies requiring high temperature antigen retrieval (Chromogranin A-Dako A0430) 1:1000; (S100-Dako Z0311) 1:400 were treated as follows: High temperature antigen retrieval was performed in a microwave pressure cooker (Viking Tender cooker) using Dako Target Retrieval solution (s1699) for 10 minutes after pressure was reached. The pressure cooker was slowly vented and the slides were allowed to sit for 20 minutes at room temperature. Slides were placed on the Dako Autostainer and washed in tris-buffered saline containing Tween 20 (TBST, Biocare Medical, TWB945M) followed by 3% hydrogen peroxide (H_2O_2,Sigma) in TBST for 10 minutes. The Cytokeratin (CK(WSS)-Dako Z0622) 1:500 and Cytokeratin AE1/AE3, Dako M3515) 1:100 were enzymatically digested with Proteinase K (Dako S3020) for 5 minutes, substituting for the high temperature antigen retrieval. Dako serum-free protein block (x0909) was applied for 10 minutes and air-dried. Primary antibodies were diluted in Dako antibody diluent (s3022) and applied for 30 minutes at room temperature. MaxPoly-One polymer HRP rabbit or mouse (MaxVision Biosciences) was applied for 10 minutes at room temperature and again washed in TBST. The chromogen Nova Red (Vector Laboratories, SK-4800) was applied for 5 minutes and washed in distilled water (dH20) followed by Dako hematoxylin (s3302) diluted 1:3 in dh20 for 5 minutes, rinsed in dH20,

rinsed in TBST to blue, run down to xylene and cover slipped. Considering the subjectivity in evaluation of immunohistochemistry results, the slides were read independently by three of us (C.P., M.K., and C.L.). Results were recorded as follows for each antibody; + = positive, +/- weak staining or few neoplastic cells showing positivity, and - = negative (Table 4.1).

Results

Western Blot

Specific protein bands for WSS, AE1/AE3, S100, and chromogranin A were detected in the prepared homogenates of adult zebrafish and human HTP-1 cells via Western blot. While the S100 protein band appeared as expected, the other three antibodies consistently reacted to produce protein bands 11-16 kDa below their expected molecular weights against the zebrafish tissue[9, 10, 11, 12]. WSS recognized two distinct bands at approximately 40 kDa for zebrafish, while a series of bands 48-59 kDa expected and observed with the human cells[7, 13] (Fig. 4.1). AE1/AE3 recognized several bands between approximately 38 and 41 kDa, while series of bands approximately 40 and 48-67 kDa expected and observed with the human cells[7, 14] (Fig.4. 2). S100 recognized a less distinct band that appears to be approximately 10 kDa, compared to the approximate 21 kDa for human[15] (Fig. 4.3). Chromogranin A recognized a band at approximately 44 kDa in the zebrafish tissue and at the expected 74 kDa in the human cells[9] (Fig. 4.4).

Immunohistochemistry

Based upon Western blot validation of the WSS, AE1/AE3, S100, and chromogranin A antibodies in zebrafish tissue, we analyzed their expression in normal tissue and tissue from intestinal tumors from 14 individual fish. Overall,

there was agreement between the three independent evaluations (Table 4.1). The intestinal tumors within these fish were classified according to the protocol that we previously outlined[1] as adenocarcinoma (Fig. 4.5), small-cell carcinoma (Fig. 4.6), or carcinoma not otherwise specified based upon histomorphologic characteristics in H&E sections .

The WSS antibody, a mammalian cytokeratin marker, showed strong staining specificity with normal epithelial cells of the intestine (4.7), skin, nares, and gills (Fig. 4.8) of adult zebrafish. Skeletal muscle stained weakly, as well. Neural tissue and exocrine pancreas were negative. AE1/AE3, also a mammalian cytokeratin marker, demonstrated similar staining specificity in normal tissues (Fig. 4.9, Fig. 4.10). However, WSS produced a stronger staining reaction in the nares, gills, and skin while AE1/AE3 stained more intensely in the intestine (including both the luminal epithelial cells and the mesothelial cells in the serosa). Seven of the fourteen (50%) of the intestinal neoplasms scored positive for WSS (Fig. 4.11), while nine of them were positive for AE1/AE3 (Fig. 4.12) (Table 4.1).

S100 antibody is used as a mammalian neural marker. In normal zebrafish tissue it showed strong immunoreactivity in brain tissue (Fig. 4.13) and the vertebrae of the spinal column and the myenteric neurons of the intestine, but was weakly reactive in the pituitary. Normal intestinal tissue was negative for S100. The intestinal tumors were scored negative for S100, except for two of the carcinomas designated "+/-" by two of the evaluators (Fig. 4.14).

Chromogranin A, a mammalian neuroendocrine marker, reacted with normal brain, spinal cord, pituitary and nerve ganglia (Fig. 4.15). Specificity and staining intensity was stronger with the latter two. Normal intestinal epithelium was negative. All of the intestinal tumors were regarded as negative for chromogranin A (Fig. 4.16).

Discussion

We recently reported, in a retrospective survey of the ZIRC diagnostic database, a high incidence of intestinal tumors among laboratory zebrafish[1]. The majority of the intestinal tumors within that study were classified as adenocarcinomas, small-cell carcinomas, or carcinomas not otherwise specified based upon histomorphologic characteristics and patterns. Immunohistochemical analysis reported here indicates that most, if not all, of the neoplasms are of epithelial origin. About 50% of the tumors were positive with the WSS and AE1/AE3, while none stain strongly positive with neural tissue markers. The small size of zebrafish allows for preparing one histologic slides containing all representative tissues from entire organ systems. This provides an excellent format for positive and negative controls for immunohistochemistry, as appropriate normal tissues are present in the exact specimen as the tissue of interest. In our study, a wide variety of epithelial cells were strongly positive with both cytokeratin stains.

The difference in histomorphologic patterns and cytokeratin expression amongst the intestinal tumors does not indicate entirely different tumor cell origins, but rather possible dedifferentiation and progression towards anaplasia. . It is typical in mammalian tumors to observe progression towards dedifferentiation as the tumor grows and matures[16] and to observe stratification of expression even amongst neoplastic cells within the same tumor[7]. Also, zebrafish, like all teleostean and chondrostean fishes, normally retain pluripotent blast cell populations as adults. These cell populations may form akin to an anaplastic reserve resembling tumor cells. The dedifferentiated tumor cells may, therefore, actually resemble these pluripotent blast cells[17]. In our study, neoplastic cells in the small cell carcinomas were more often negative for the two epithelial

antibodies. These cells are morphologically less differentiated, with a small nucleus and minimal cytoplasm. In contrast, the neoplastic cells in the adenocarcinomas appear more consistent with intestinal epithelial cells, with stippled chromatin nuclei and a visible nucleolus.

Both neural markers, S100 and chromogranin A, were negative in tumor sections examined, indicating that the tumors are most likely not of neuroendocrine origin. Indeed, gut derived neural endocrine neoplasms are often positive with these antibodies, particularly with the latter[3, 18, 19,20]. Mammalian S100 antibody has been shown to cross-react with zebrafish schwannomas[21], and also cross-react with neural tissues in other fishes[22, 23, 24, 25, 26, 27]. However, it did not cross with a dysembryoplastic neuroepithelial tumor of zebrafish that we recently described[28]. Our Western blot analysis demonstrates immunoreactivity with a ~10 kDa protein, and the human cell line was weakly reactive at the expected ~21 kDa[15].

Nevertheless, neural tissues (e.g., brain and spinal cord) were positive with S100, but weaker compared to the other immunomarkers tested. The dilution factor of both of these could be improved upon in future work, such that it is more easily distinguishable using conventional light microscopy to ensure an unequivocal negative within zebrafish tissue, normal or neoplastic. Molecular characterization of zebrafish tumor antigen orthologs has been previously documented[9, 10].

Chromogranin A rabbit antibodies have not previously been investigated with the zebrafish model. Here, we observed specific staining of neural tissues, particularly nerve ganglia and the pituitary gland. In contrast, none of the reviewers scored any of the neoplasms as positive for this antibody. With Western blot analysis, chromogranin A expressed a distinct band ~44 kDa, which was

lower than the predicted zebrafish chromogranin A molecular weight, based on sequence data[9], although similar to what was observed in brown bullhead[25]. However, the human cell line ran under the same conditions reacted strongly, although non-specifically (Figure 4.4).

Our experience with Western blot analysis, sans S100, repeatedly resulted in protein bands ~11-16 kDa below their expected molecular weights. AE1 and AE3 have been previously characterized by complimentary keratin blot-binding analysis[11] and S100 by Western blot[10]. Most zebrafish proteins have molecular weight estimations based solely upon the amino acid sequence and do not reflect cross-reactivity ability. Any level of reduction in the cross-reactivity between the mammalian antibodies and the targeted fish proteins may explain the bands correlating to lower than expected molecular weights. Mammalian antibodies used for zebrafish tumor diagnosis are not optimized for use in zebrafish and even antibodies generated against other teleost fish have shown markedly reduced affinity for zebrafish antigens[12]. Moreover, the few zebrafish-specific antibodies that have been developed to date have not been optimized or validated and they are either polyclonal or not directed at specific tumor antigens[29].

While there is significant conservation between vertebrate tissue and cellular antigens, thus demonstrating potential utilization in zebrafish, particularly cytokeratins[11, 30], establishing a series of zebrafish-specific antibodies is still needed. The creation of a zebrafish-specific tumor antigen panel would enable more definitive identification of zebrafish tumors and by relation other anatomic structures. This would allow broad screening of these tumors to evaluate and characterize antigen expression and provide further antibody validation, as well as potentially using antibody-directed anti-neoplastic therapy in zebrafish models of human neoplasia. Nevertheless, we can conclude from this study that most, if not

all, of the commonly observed intestinal tumors seen in zebrafish are most likely derived from epithelial cell origin.

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TABLE 4.1. SUMMARY OF WSS, AE1/AE3, S100, AND CHROMOGRANIN A EXPRESSION IN INTESTINAL TUMORS OF ZEBRAFISH SUBMITTED TO THE ZEBRAFISH INTERNATIONAL RESOURCE CENTER DIAGNOSTIC SERVICE 2001-2011[1]

	WSS	AE1/AE3	S100	CHROMOGRANIN A
Adenocarcinoma				
F1	+ : + : +	+ : + : +	- : - : -	- : - : -
F2	+ : + : +	+ : +: +	- : - : -	- : - : -
F3	- : - : -	- : - : -	- : +/- : -	- : - : -
F4	- : - : -	- : - : -	- : - : -	- : - : -
Small-Cell Carcinoma				
F5	- : - :-	+/- : +/- : +/-	- : - : -	- : - : -
F6	- : - : -	+/- : +/- : +/-	- : - : -	- : - : -
F7	- : - : -	-/-:-:-	- : - : -	- : - : -
F8	+ : + : +	+ : + : +	- : - : -	- : - : -
F9	- : - : +/-	+ : + : +	- : - : -	- : - : -
Carcinoma NOC[2]				
F10	+ : + : +	+ : + : +	- : - : -	- : - : -
F11	- : - : -	+ : +/- : +	- : - : -	- : - : -
F12	+ : +/- :+	+ : + : +	- : - : -	- : - : -
F13	+ : + : +	+ : + : +	+/- : - : +/-	- : - : -
F14	+ : +/- : +	+ : +/- : +	+/- : +/- : -	- : - : -

[1]The three symbols separated by colons per antigen in a given tumor represent three separate readings ; + denotes a positive interaction, - denotes a negative interaction, +/- denotes an equivocal interaction

[2]Carcinoma Not Otherwise Classified

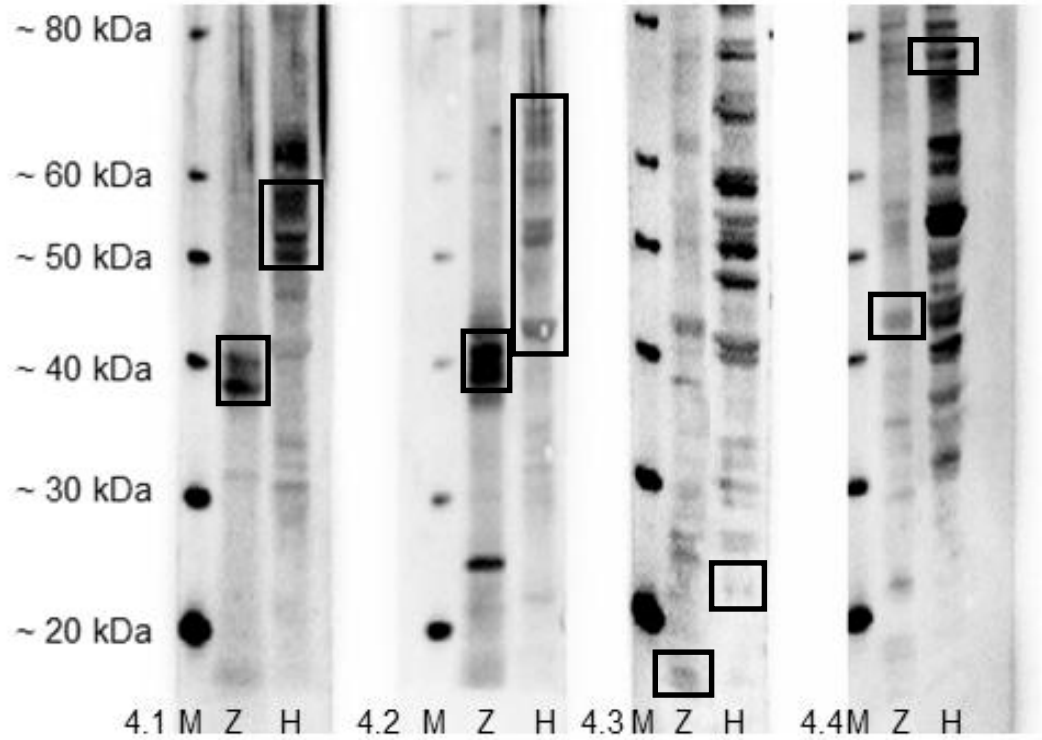


Figure 4.1. Western blot against WSS compared to expected targeted protein sizes (indicated by box) where M=marker protein, Z=normal whole adult zebrafish tissue homogenate, H=HTP-1 cells (human). **Figure 4.2.** Western blot against AE1/AE3 compared to expected targeted protein sizes (indicated by box) where M=marker protein, Z=normal whole adult zebrafish tissue homogenate, H=HTP-1 cells (human). **Figure 4.3.** Western blot against S100 compared to expected targeted protein size (indicated by box) where M=marker protein, Z=normal whole adult zebrafish tissue homogenate, H=HTP-1 cells (human). **Figure 4.4.** Western blot against chromogranin A compared to expected targeted protein size (indicated by box) where M=marker protein, Z=normal whole adult zebrafish tissue homogenate, H=HTP-1 cells (human).

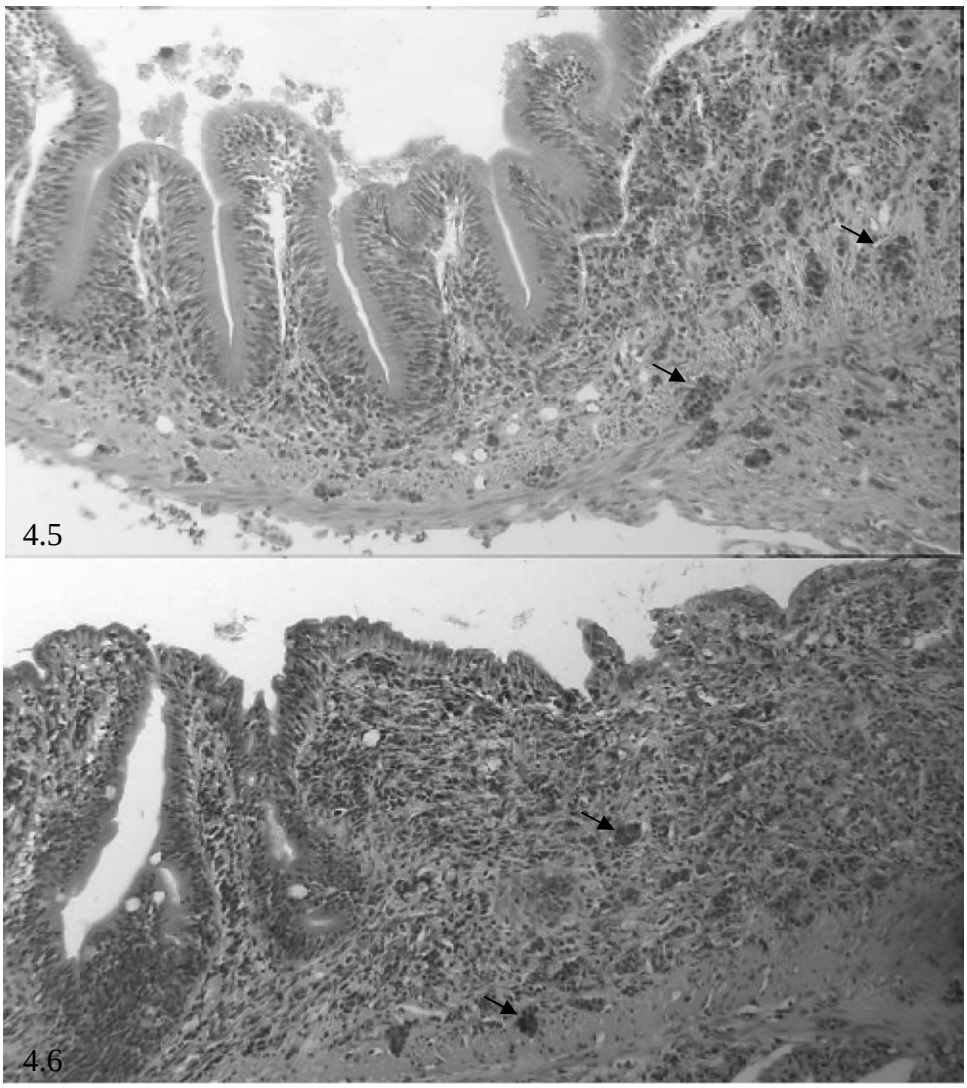
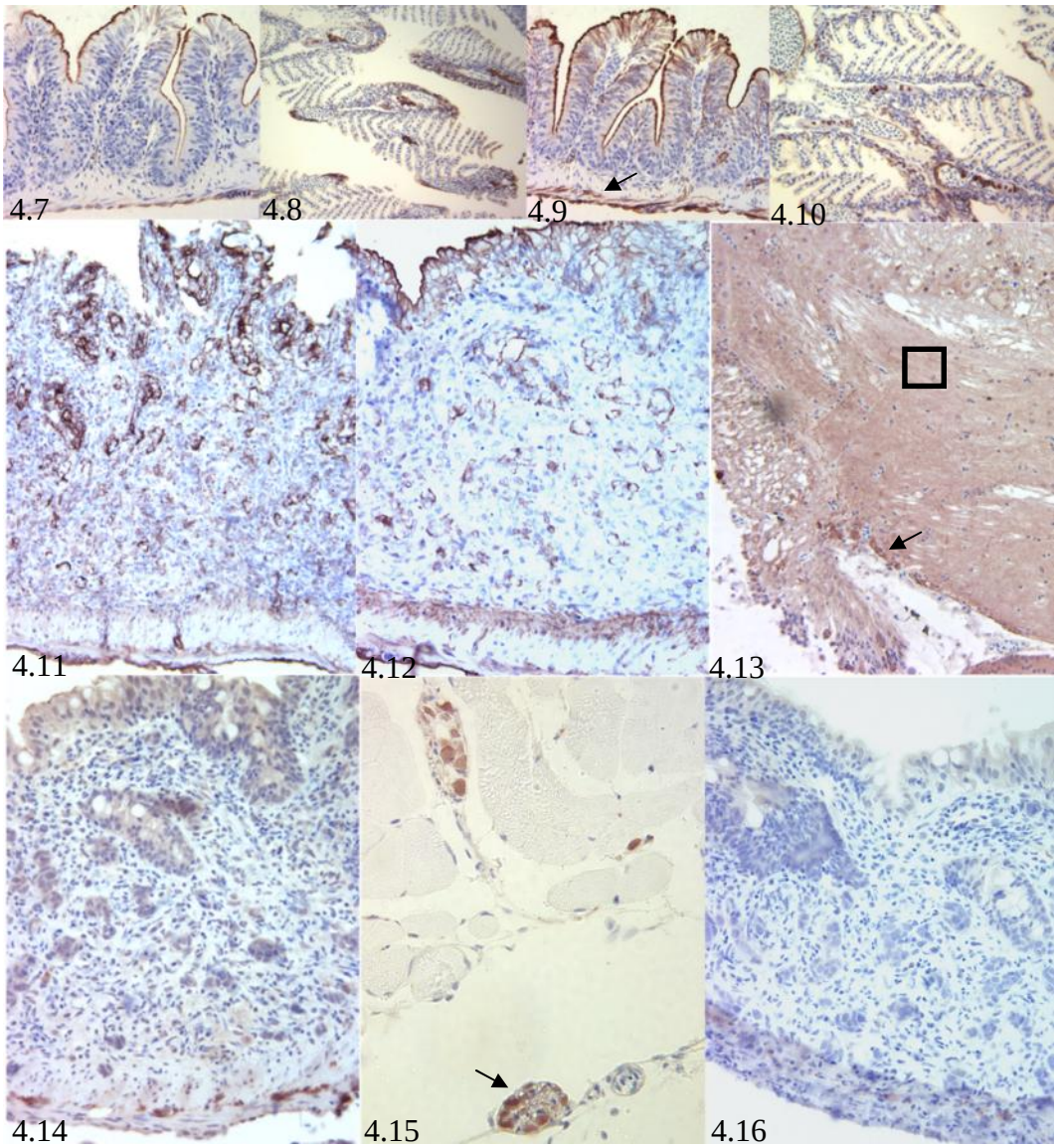


Figure 4.5. Intestine; adult zebrafish, Fish 1. Adenocarcinoma with neoplastic cells (arrows) forming pseudoacinar structures within the lamina propria and muscularis layer, extending through the serosal layer into the coelomic cavity. Hematoxylin and eosin. **Figure 4.6.** Intestine; adult zebrafish, Fish 6. Small cell carcinoma with fusiform cells forming small intraproprial aggregated nests of tumor cells (arrows), with invasion into the muscularis layer.

Figure 4.7. Intestine; adult zebrafish, Fish 6. Cytokeratin expression in the normal cells of the intestinal epithelium. WSS. **Figure 4.8.** Gill; adult zebrafish, Fish 6. Cytokeratin expression in the gill epithelium. WSS. **Figure 4.9.** Intestine; adult zebrafish, Fish 6. Cytokeratin expression in the normal cells of the intestinal epithelium including mesothelial cells (arrow). AE1/AE3. **Figure 4.10.** Gill; adult zebrafish, Fish 6. Cytokeratin expression in the gill epithelium. AE1/AE3. **Figure 4.11.** Intestine; adult zebrafish, Fish 2. Cytokeratin expression in intestinal tumor (arrows) previously classified as an adenocarcinoma based upon histomorphologic characteristics. WSS. **Figure 4.12.** Intestine; adult zebrafish, Fish 2. Cytokeratin expression in intestinal tumor (arrows) previously classified as adenocarcinoma based upon histomorphologic characteristics. AE1/AE3. **Figure 4.13.** Brain; adult zebrafish, Fish 6. Astrocytes (box) and ependymal cells (arrow) staining in normal brain tissue. S100. **Figure 4.14.** Intestine; adult zebrafish, Fish 2. Intestinal tumor previously classified as adenocarcinoma based upon histomorphologic characteristics demonstrating negative staining. S100. **Figure 4.15.** Autonomic ganglia; adult zebrafish, Fish 6. Normal ganglion cells expressing positive staining (arrows). Chromogranin A. **Figure 4.16.** Intestine; adult zebrafish, Fish 2. . Intestinal tumor previously classified as adenocarcinoma based upon histomorphologic characteristics demonstrating negative staining. Chromogranin A.



Figures 4.7-4.16

Chapter 5: Conclusion

The aim of this thesis was to profile spontaneous preneoplastic and neoplastic lesions occurring in the intestine of the zebrafish (*Danio* rerio) on a historical, etiological, and cellular level. Historically, there was a high prevalence of these intestinal lesions among laboratory reared zebrafish. Preneoplastic lesions included hyperplasia, dysplasia, and enteritis. Neoplastic changes were classified either as adenocarcinoma or small cell carcinoma, with a few exceptions (carcinoma not otherwise specified, tubular adenoma, and tubulovillous adenoma) based upon histomorphogenic presentation. The intestinal lesions appeared similarly distributed among the sexes, and genetic backgrounds. There was an increase in incidence with fish greater than one year of age, although fish as young as six months were affected. Based on the continuity of cases through the years and the fact that many of these lesions occur in subclinical fish, these lesions could introduce an underlying, unappreciated variable into zebrafish research. This would be a particular concern in studies using zebrafish as a model of gastrointestinal diseases and carcinogenesis.

Regarding potential etiologic agents, diet was eliminated based upon a preliminary study. An infectious agent etiology was suspected although cohabitation, feed, and injection studies yielded equivocal results. Marginal hyperplastic changes were identified fish from the cohabitation study and in fish from injected filtrate study sampled rather early in the experiment. However, comprehensive histological evaluation at the termination of the study at 10 mo. post-exposure for fed and injected fish and 11 mo. post-exposure for cohabitation fish did not recognize any preneoplastic or neoplastic intestinal lesions. The study was terminated earlier than anticipated due to high mortality in various groups

attributed to *Piscinoodinium pillulare* infections. Whereas evidence for an infectious agent etiology is equivocal, this study reasons to be repeated. The potential of a water-borne carcinogen should also be investigated in the future as a potential etiologic agent or promoter. The typical zebrafish facility maintains juvenile and adult fish on a recirculating water system with a very rigorous water filtration system and any carcinogen must get past these complexes of filters or be introduced downstream of the filtration process, either as a component of the material used to transport the water or to house the fish. Another downstream addition is food. And while preliminary studies eliminated diet as the primary etiologic agent, depending on the feeding protocol and tank maintenance schedule of a facility it has been observed that fungus may develop around the feeding receptacle. Fungi have been shown to produce carcinogenic compounds known to induce cancer intestinal neoplasia in zebrafish. Larval fish are usually reared in static tanks in a separate nursery setting prior to being transferred to the main recirculating water system used for adult fish at a given laboratory facility. Given the varying lesion prevalence between different populations of age and strain matched fish at the primary facility, and that larval zebrafish exposed to carcinogens often do not develop until later in life, microorganisms and chemical carcinogen exposure within these nursery facilities offer yet another source to consider for the potential etiologic agents of these lesions. Stress and density may also be cofactors in the etiology of these intestinal lesions, given the laboratory environment common amongst the reported affected fish.

Immunohistochemical analysis indicated that most, if not all, of the neoplasms are of epithelial origin. About 50% of the tumors subjected to our series of mammalian antibodies were positive with the WSS and AE1/AE3, while none stained strongly positive with the neural antibodies (S100 and chromogranin

A). In our study, a wide variety of epithelial cells were strongly positive with both cytokeratin stains. The difference in histomorphologic pattern and cytokeratin expression amongst the intestinal neoplasms likely does not indicate entirely different tumor cell origins, but rather different manifestations on a path towards increasing dedifferention. Hence, I concluded that most, if not all, of the common intestinal neoplasms seen in zebrafish are derived from epithelial cell origin.

In conclusion, based on data to date, the neoplasms are epithelial in origin, and three plausible causes for the lesions should be considered; 1) a microorganism in adult facilities, 2) a microorganism in larval nurseries, and 3) a chemical carcinogen in individual nursery tanks.

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APPENDIX

TABLE A.1. PREVALENCE OF PRENEOPLASTIC AND NEOPLASTIC INTESTINAL LESIONS IN ZEBRAFISH ≥6 MO. SAMPLED FROM THE PRIMARY FACILITY[1]

	Gender	Total Fish	Preneoplastic	Neoplastic	Negative
Summer 2011	Male	52	21	4	27
	Female	47	23	2	22
Winter 2012	Male	110	25	3	82
	Female	24	5	5	14
Survey Totals		233	74	16	143

[1]A single, large zebrafish research facility in the USA (the primary facility as cited in the text).

[2]Genetic backgrounds of the fish surveyed include AB and/or Tuebingen.