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Primary Intestinal and Vertebral Chordomas in Laboratory Zebrafish (Danio rerio)

Primary Intestinal and Vertebral Chordomas in Laboratory Zebrafish (*Danio rerio)*

Timothy K Cooper, Katrina N. Murray, Sean Spagnoli, and Jan M Spitsbergen

Penn State Hershey Medical Center, Departments of Comparative Medicine and Pathology (TKC)

Zebrafish International Resource Center, Pathology and Health Services (KNM, SS)

Oregon State University, Department of Neuroscience (SS)

Oregon State University, Department of Microbiology (JMS)

Abstract:

Chordomas are uncommon neoplasms arising from notochord remnants, most commonly occurring in the axial skeleton. Extraskeletal soft tissue chordomas are rare primary tumors, and primary intestinal chordomas have not been reported. Herein we report 24 cases of spontaneous primary intestinal chordomas in zebrafish, as well as 9 spontaneous vertebral chordomas. Both intestinal and vertebral tumors showed invasive behavior, although more commonly in the latter. In all cases of primary intestinal chordomas, there was no axial or peripheral skeletal or other non-visceral involvement. Although uncommon, intestinal chordomas represent a unique background lesion in aged zebrafish.

Introduction:

Chordomas are uncommon primary malignant neoplasms of notochord origin that most frequently affect the axial skeleton in humans. The overall incidence is reported to be 8.4 per ten million, with a higher incidence in males and individuals older than 40 years.[11,16] Tumors are reported to arise in the cranium (frequently clival, 32%), spine (33%) and sacrum (29%).[11,16] Chordomas are typically lobulated with fibrous trabeculae, and the presence of numerous vacuolated (physaliphorous) cells is characteristic.[12] Although they infrequently metastasize, tumors are typically locally invasive with frequent recurrence, often owing to the difficulty of complete resection.[16] Extra-axial skeletal chordomas are rare in people; extra-skeletal soft tissue chordomas are exceptionally rare, with less than twenty reported cases.[9]

Chordomas are uncommon skeletal tumors in most animals. Chordomas are relatively more frequent in ferrets, occurring as axial tumors of the caudal vertebrae and skull base.[3] A number of tumors have also been reported in F344 rats, with a notably high rate of pulmonary metastases (75%).[19] No spontaneous extraskeletal chordomas have been reported in animals. Parachordoma, a variant of myoepithelioma with myxoid differentiation that resembles chordoma, has only been reported once in

the veterinary literature, in the gluteal muscle of a dog. However, the distinction between parachordoma and chordoma in that case was largely informed by the extra-axial location rather than morphologic characteristics, and the authors acknowledged uncertainty in the diagnosis.[6] No spontaneous extraskeletal soft-tissue chordomas originating in the gastrointestinal tract have been reported to date in any species. Primary vertebral chordomas have been previously reported as a rare tumor in zebrafish (2% incidence), but no spontaneous chordomas in any location have been reported in any other species of fish.[17] Recently a transgenic zebrafish model of vertebral chordoma was reported, via expression of the *HRAS* oncogene in the notochord.[1] Herein we describe spontaneous primary intestinal and vertebral chordomas in 33 laboratory zebrafish, the former representing a novel tumor type not previously described in any species.

Materials and Methods:

Thirty-two of the cases came from the archives of the Zebrafish International Resource Center (ZIRC) or from the case files of Dr. Jan Spitsbergen from her studies of spontaneous and carcinogen-induced neoplasia. One of the cases was identified prospectively in a mutant screen (Mullins and Fisher (UPenn) and Cheng (PSUHMC)).

Zebrafish were processed for histology by several different methods, depending on the source or study. For some carcinogenesis studies, fish were euthanatized in tricaine methanesulfonate (MS 222; Argent Laboratories) pH 7.4 in phosphate buffer, the tail was removed, and an incision was made through the ventral abdominal wall from the heart to the anus to promote internal fixation. Fish were fixed in Bouin's fixative for 24 hr. Fish were dehydrated in a graded series of ethanol solutions, then embedded in paraffin. Sagittal step sections were cut from the fish's left side. Three 4-6 micron sections were saved and placed onto a single glass slide, one section through the lens of the left eye, one just medial to the left eye, and one from midline. Sections were stained with hematoxylin and eosin (H & E). For broodstock and more recent carcinogenesis bioassays, fish were fixed in buffered zinc formalin for 24 hr, decalcified for 48 hr in Cal X II (formic acid/formalin; Fisher Scientific), and both halves of the fish were sectioned for histology. Nine step sections were cut between the middle of the lens of the left eye and the middle of the lens of the right eye. Three sections were placed onto each of 3 slides (nine sections total) and stained routinely with H & E. For diagnostic cases submitted to the Zebrafish International Resource Center (ZIRC) at the University of Oregon, fish were routinely fixed in Dietrich's fixative and decalcified overnight in 5% trichloroacetic acid. Fish were bisected for embedding by cutting sagittally, just to the left of midline, using a razor blade. The two halves were placed into a single cassette. Detailed histology protocols are available on the ZIRC website (http://zebrafish.org/zirc/health/diseaseManual.php). Sections from seven cases were stained with Masson's trichrome or Alcian blue (pH 2.5)-periodic acid Schiff (PAS) with and without hyaluronidase digestion (H3884, Sigma-Aldrich, St. Louis, MO). All images were obtained with an Olympus BX51 microscope and DP71 digital camera using cellSens Standard 1.6 imaging software (Olympus America, Center Valley, PA).

Results:

Most fish had no reported history of experimental manipulation, and many tumors were incidental findings in fish submitted as colony sentinels. Demographic characteristics of fish with intestinal tumors are reported in Supplemental Table 1. Average age was 454 days and there were ten females and twelve males (sex was not reported for two fish). One fish (I22) was only 74 days old. Only a single fish (I11) was exposed to a known carcinogen, diethylnitrosamine. Three fish had genetic manipulations of possible relevance. One fish had morpholino knockdown of the tumor suppressor *brca2* (fish I9)*,* however a tumor was also identified in a scrambled morpholino control (fish I10). Morpholinos are modified antisense oligonucleotides targeted to specific mRNA transcripts commonly used to silence gene expression in zebrafish (http://www.ncbi.nlm.nih.gov/genome/probe/doc/TechMorpholino.shtml). One fish (fish I17) had an inactivating M214K missense mutation in the DNA-binding domain of the tumor suppressor *tp53* (ZFIN ID: ZDB-ALT-050428-2), and lecithin retinol acyltransferase b (*lratb,* fish I13) is a gene involved in retinol metabolism that is specifically expressed in the notochord (ZDB-GENE-060810-31).

Tumors were well-differentiated and resembled normal notochord present in the spine of adult zebrafish (Fig. 1). Intestinal tumors formed sessile polypoid exophytic and expansile masses projecting into the intestinal lumen (Fig. 2). Subgrossly, tumors were distinctly multilobular, separated by fibrous bands, and expansile, consisting of multiple well-circumscribed and encapsulated nodules. Tumors were more commonly located in the cranial half of the intestine. In four fish (cases I7, I9, I19 and I24), multiple individual nodules were scattered widely throughout the intestine (Fig. 3). Within the lobules, there were one to several thin layers of plump spindloid cells arranged parallel to the capsule. The centers of the lobules contained numerous individual or clustered large round cells with compressed peripheral ovoid nuclei and a large single clear cytoplasmic vacuole with sharp margins (resembling physaliphorous cells, Fig. 4). Less commonly these cells had abundant pale eosinophilic cytoplasm and central nuclei. Clusters of small round (glomoid) cells were absent. There were moderate to abundant amounts of glassy eosinophilic acellular matrix between the cells. Mitotic figures were absent. The overlying intestinal epithelium was often attenuated, but only rarely ulcerated. Both normal vertebral and neoplastic intestinal matrix was weakly positive with Alcian blue and resistant to hyaluronidase digestion (Fig. 5). In case I10, multiple nodules were present in the exocrine pancreas (Fig. 6). In case I11, multiple nodules were present in the dorsal wall of the cranial intestine as well as within the adjacent liver.

Demographic characteristics of fish with vertebral tumors are reported in Supplemental Table 2. Average age was 482 days, similar to intestinal tumors. There were three females and two males (sex was not reported for four fish). Two fish (V4 and V5) were exposed to known carcinogens, diethylnitrosamine and dibenzo[a,l]pyrene. A skeletal deformity was clinically identified in only one fish.

Spinal tumors were morphologically similar to intestinal tumors. Four of the nine vertebral tumors were invasive into CNS and one additional tumor was invasive into pancreas. Invasion tended to be protrusive, expansile and nodular rather than infiltrative or finger-like. Fish with vertebral tumors did not show any evidence of intestinal involvement, nor did those with intestinal tumors show any evidence of vertebral involvement.

In zebrafish that are 1.5 years of age or older, vertebrae often show mineralization multifocally in notochord material, especially in and around the dorsal and ventral protrusions of the "hourglass-shaped" structures containing notochord material (Supplemental Figure 1). In these areas of mineralization, hyperplasia of notochord cells often occurs, resulting in clusters of small round cells containing scant cytoplasm. Amorphous masses of mineralized material containing scattered notochord cells occasionally ruptures through the notochord sheath in the areas of dorsal and ventral protrusions of notochord in the spine. These degenerative changes are easily differentiated from chordoma because chordoma does not contain the mineralization seen in aging vertebrae.

Discussion:

Based upon histologic and histochemical characteristics, the tumors of the zebrafish intestine and vertebrae described are appropriately classified as chordomas. The major differential diagnosis for extra-skeletal soft tissue chordoma is parachordoma. Parachordomas are mixed tumors classified as variants of myoepithelioma, but have vacuolated cells similar to chordoma.[8] Small round glomoid cells are present in parachordoma, but absent in chordoma.[4,5] Parachordomas have a myxoid matrix which is Alcian blue-positive but hyaluronidase-sensitive.[5] Chordomas, in contrast, have Alcian blue-positive, hyaluronidase-resistant matrix. Although some have used the axial versus extra-axial location to distinguish between chordoma and parachordoma, respectively, this is not an appropriate diagnostic criterion.[9] Extraskeletal soft-tissue chordoma does exist, is extremely rare, and is histologically, immunohistochemically and behaviorally distinct from parachordoma, the former having metastatic potential that is absent in the latter.[9,15] The distinction between chordoma and parachordoma also takes on clinical significance in humans, as the former is invasive and likely to recur following surgical excision. It should be noted, however, that post-resection local recurrence of parachordomas has been reported.[15] Gastrointestinal involvement by parachordoma is uncommon in humans, with only a single report of a primary tumor in the gastric serosa.[18]

Expression of brachyury, a nuclear T-box transcription factor (*T, brachyury homolog,* HGNC:11515), is necessary and sufficient to distinguish chordoma from parachordoma in humans, as the tumors share a number of other markers including cytokeratin (CK) 8/18, CK 7, S100, and epithelial membrane antigen.[7,9,15,21] Chordomas are also variably positive for CK20, CK 1/5/10/14, and carcinoembryonic antigen.[15] Brachyury is a regulator of embryonic notochord formation, and chordomas are positive for brachyury expression, whereas parachordomas are negative.[13] Attempts to detect brachyury in formalin-fixed paraffin embedded zebrafish were unsuccessful. Further complicating the matter, zebrafish, like many teleost fish, have two functional brachyury orthologues, *bra* and *ntla*.[10] Although ultrastructural studies could assist in the differentiation of chordoma and parachordoma, the nature and availability of specimens in this series excludes this possibility.

Another significant differential for vertebral chordoma is hyperplasia secondary to degeneration of the notochord, a common age-related finding in laboratory zebrafish. Although both processes include proliferation of notochord cells, notochord degeneration is characterized by prominent mineralization of the notochord material. Alternatively, while some would perhaps classify the lesions

described in these fish as non-neoplastic ectopic notochord rests (choristomas), the size, multiplicity and invasive behavior demonstrated by these nodules is consistent with a neoplasm.

Although generally considered uncommon tumors, chordomas do occur as spontaneous background lesions in zebrafish. Primary intestinal chordoma, a condition not previously reported in any species, appears to be more common in zebrafish than chordoma of the axial skeleton. Overall, the tumor frequency is low. Chordomas were identified in only 25 of the 11,902 zebrafish examined between 2003 and the end of 2013. Over 20,000 fish were evaluated for carcinogenesis studies, but less than 75 fish have been evaluated to date in the UPenn study. Lesions disproportionately affected older fish (average age >400 days), consistent with observations in humans with chordoma. Both sexes were approximately equally represented in this dataset. There were no significant commonalities in the experimental or clinical histories, with most fish presenting as unmanipulated wild types. Fish with mutations in connexin 41.8 and longfin ($cx41.8^{t1}$; lof^{t2}) appear to be over-represented in our dataset, with two spinal chordomas and one intestinal chordoma. The reasons for this are not clear, but may be due to sampling bias related to the lines of fish submitted for evaluation. Interestingly, one case (fish I3) had a null allele for the *ntla* orthologue, although chordoma has not been described as a phenotype in those mutants.[14] However, genetic mutations affecting the brachyury *T* gene in human chordomas are characterized by amplification events, rather than silencing or loss of function.[22] Silencing of brachyury expression by shRNA has inhibited chordoma growth *in vitro,* consistent with the proposed central role of this transcription factor in driving tumorigenesis.[13]

Because intra-abdominal metastases of chordomas have been reported in humans[9,20], we felt it important to determine whether there were primary vertebral chordomas in the cases with intestinal tumors. In the fish examined, no involvement of vertebrae, other bones, or soft tissue was observed, with the exception of local invasion into adjacent viscera. The reason for the occurrence of primary intestinal chordomas is potentially explained by Burger *et al.* in their transgenic zebrafish model.[1] In that model, the *sonic hedgehog b* promoter *(shhb (*ZDB-GENE-980526-41), previously *tiggy winkle hedgehog (twhh))* was used to drive *HRAS* expression in the intestine. Because *shhb* expression also occurs in the notochord, abnormalities in that organ were also present. It is interesting to speculate that spontaneous *shhb* mutations may drive tumorigenesis in both sites, as alteration of the sonic hedgehog signaling pathway in human chordoma formation has been previously suggested.[2]

Herein we describe the demographic and morphologic characteristics of primary intestinal and vertebral chordomas in laboratory zebrafish. Uniquely, intestinal chordomas exist as a distinct clinical entity in this species.

Figure Legends:

Fig 1. Spinal column, sagittal section; zebrafish. Normal notochord. HE.

Fig 2. Intestinal chordoma; zebrafish. Expansile and polypoid multilobular encapsulated tumor within the lamina propria. HE.

Fig 3. Intestinal chordomas; zebrafish. Multiple tumor nodules are present throughout the intestinal wall. HE.

Fig 4. Intestinal chordoma; zebrafish. Tumor contains numerous highly vacuolated (physaliphorous) cells in a pale eosinophilic amorphous hyaline matrix. HE.

Fig. 5. Intestinal chordoma; zebrafish. The tumor matrix is Alcian blue-positive and hyaluronidase-resistant. Alcian blue (pH 2.5) and periodic acid Schiff with hyaluronidase digestion.

Fig 6. Pancreatic chordoma; zebrafish. Multiple tumor nodules are present in the exocrine pancreas. HE.

Supplemental Figure 1. Notochord degeneration; zebrafish, sagittal section of vertebrae. Hyperplasia and mineralization of degenerate notochord material at the dorsal and ventral portions of the vertebra, with protrusion of degenerate notochord material from the dorsum of the vertebra. Inset shows higher magnification mineralized hyperplastic notochord material. HE.

References:

1 Burger A, Vasilyev A, Tomar R, Selig MK, Nielsen GP, Peterson RT, et al.: A zebrafish model of chordoma initiated by notochord-driven expression of HRASV12. *Dis Model Mech* 2013:In press.
2 Cates JM, Itani DM, Coffin CM, Harfe BD: The sonic hedgehog pathway in chordoid tumours. *Histopathology* 2010:56(7)**:**978-979.
3 Dunn DG, Harris RK, Meis JM, Sweet DE: A histomorphologic and immunohistochemical study of chordoma in twenty ferrets (Mustela putorius furo). *Vet Pathol* 1991:28(6)**:**467-473.
4 Fisher C: Parachordoma exists--but what is it? *Adv Anat Pathol* 2000:7(3)**:**141-148.
5 Folpe AL, Agoff SN, Willis J, Weiss SW: Parachordoma is immunohistochemically and cytogenetically distinct from axial chordoma and extraskeletal myxoid chondrosarcoma. *Am J Surg Pathol* 1999:23(9)**:**1059-1067.
6 Forsyth SF, Thompson KG, French AF, Halsey TR: Possible parachordoma in a dog. *N Z Vet J* 2009:57(5)**:**299-302.

7   Jambhekar NA, Rekhi B, Thorat K, Dikshit R, Agrawal M, Puri A: Revisiting chordoma with brachyury, a "new age" marker: analysis of a validation study on 51 cases. *Arch Pathol Lab Med* 2010:134(8)**:**1181-1187.

8   Kilpatrick SE, Limon J: Mixed tumor/Myoepithelioma/Parachordoma. In: Fletcher CDM, Unni KK, Mertens F, eds. *WHO Classification of Tumours: Pathology and Genetics of Tumors of Soft Tissue and Bone*. Lyon, France: IARC Press; 2002: 198-199.

9   Lauer SR, Edgar MA, Gardner JM, Sebastian A, Weiss SW: Soft tissue chordomas: a clinicopathologic analysis of 11 cases. *Am J Surg Pathol* 2013:37(5)**:**719-726.

10 Martin BL, Kimelman D: Regulation of canonical Wnt signaling by Brachyury is essential for posterior mesoderm formation. *Dev Cell* 2008:15(1)**:**121-133.

11 McMaster ML, Goldstein AM, Bromley CM, Ishibe N, Parry DM: Chordoma: incidence and survival patterns in the United States, 1973-1995. *Cancer Causes Control* 2001:12(1)**:**1-11.

12 Mirra JM, Della Rocca C, Nelson SD, Mertens F: Chordoma. In: Fletcher CDM, Unni KK, Mertens F, eds. *WHO Classification of Tumours: Pathology and Genetics of Tumors of Soft Tissue and Bone*. Lyon, France: IARC Press; 2002: 316-317.

13 Nibu Y, Jose-Edwards DS, Di Gregorio A: From notochord formation to hereditary chordoma: the many roles of Brachyury. *Biomed Res Int* 2013:2013**:**826435.

14 Schulte-Merker S, van Eeden FJ, Halpern ME, Kimmel CB, Nusslein-Volhard C: no tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. *Development* 1994:120(4)**:**1009-1015.

15 Scolyer RA, Bonar SF, Palmer AA, Barr EM, Wills EJ, Stalley P, et al.: Parachordoma is not distinguishable from axial chordoma using immunohistochemistry. *Pathol Int* 2004:54(5)**:**364-370.

16 Smoll NR, Gautschi OP, Radovanovic I, Schaller K, Weber DC: Incidence and relative survival of chordomas: the standardized mortality ratio and the impact of chordomas on a population. *Cancer* 2013:119(11)**:**2029-2037.

17 Spitsbergen JM, Buhler DR, Peterson TS: Neoplasia and neoplasm-associated lesions in laboratory colonies of zebrafish emphasizing key influences of diet and aquaculture system design. *ILAR J* 2012:53(2)**:**114-125.

18 Spivach A, Zanconati F, Tirabosco R, Falconieri G: Parachordoma of the gastric serosa: report of a myxoid mimicry in an unusual location. *Int J Surg Pathol* 2007:15(3)**:**307-310.

19 Stefanski SA, Elwell MR, Mitsumori K, Yoshitomi K, Dittrich K, Giles HD: Chordomas in Fischer 344 rats. *Vet Pathol* 1988:25(1)**:**42-47.

20 Takeyama J, Sasano H, Fukumoto M: Giant chordoma occupying the whole abdominal cavity. *Pathology* 2008:40(3)**:**313-314.

21 Tirabosco R, Mangham DC, Rosenberg AE, Vujovic S, Bousdras K, Pizzolitto S, et al.: Brachyury expression in extra-axial skeletal and soft tissue chordomas: a marker that distinguishes chordoma from mixed tumor/myoepithelioma/parachordoma in soft tissue. *Am J Surg Pathol* 2008:32(4)**:**572-580.

22 Yang XR, Ng D, Alcorta DA, Liebsch NJ, Sheridan E, Li S, et al.: T (brachyury) gene duplication confers major susceptibility to familial chordoma. *Nat Genet* 2009:41(11)**:**1176-1178.