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Research Article

From Chronic Feed-Induced Intestinal Inflammation to Adenocarcinoma with Metastases in Salmonid Fish

Ole B. Dale,[1] Brit Tørud,[5] Agnar Kvellestad,[1,2] Hanna S. Koppang,[3,4] and Erling O. Koppang[2]

[1]Section of Fish Diseases, National Veterinary Institute; [2]Section of Anatomy and Pathology, Institute of Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science; [3]Department of Pathology and Forensic Odontology, Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo, Blindern; [4]Laboratorium for Patologi AS, Oslo, Norway and [5]Fiskehelsa BA, Valsøyfjord, Norway

Abstract

Neoplasms in fish normally show poor abilities for metastasis, and there are no reports on intestinal cancer with metastasis to other organs. In aquaculture production, carnivorous salmonids in Northern Europe receive commercial feeds with plant ingredients. Such contents have been shown to cause chronic intestinal inflammation. Inflammation provokes carcinogenesis in the human gut, and here, we report a similar pathologic progression in salmonids. Nine commercially farmed groups of Atlantic salmon and rainbow trout (n = 39,160) and one experimental positive group (n = 789) fed the same commercial feed and two negative control groups (n = 3009) were investigated for the occurrence of intestinal tumors and metastases. Exposure period, gender, and sexual maturation were registered. Autopsy revealed an overall intestinal tumor occurrence of 10.62%, of which liver metastasis varied from 0% to 11.35% between the groups. Intestinal cancer prevalence increased from 0.50% to 14.81% during 4 months of feeding in the experimental group. A significant gender effect was registered in the commercially farmed groups but not in the experimental group. Histologic examination showed adenocarcinomas evolving through progressive epithelial dysplasia associated with severe chronic inflammation. One intestinal tumor was registered in one individual in the negative control groups. This is the first report on feed-induced intestinal carcinogenesis and metastasizing adenocarcinomas in fish fed an approved commercial diet. The pathogenesis was associated with a certain commercial diet provoking the inflammation-dysplasia-carcinoma sequence. The histologic progression was analogous to that of human colorectal cancer associated with inflammatory bowel disease. [Cancer Res 2009;69(10):4355–62]

Introduction

Inflammation and cancer are linked conditions, and this association has especially been recognized in colorectal cancer occurring in patients with inflammatory bowel disease (IBD; refs. 1–3). Typically, IBD-associated colorectal cancer develops through an inflammation-dysplasia-carcinoma sequence (3, 4). In addition, the dietary effect on intestinal cancer development seems highly associated with inflammation (5–7). Primary intestinal cancer is relatively rare in most domestic animal species in comparison with humans (8). The featured disease model organisms include mutated or carcinogen-exposed rodents (9). Nevertheless, also bony fish species, or teleosts, represent promising model organisms with respect to intestinal cancer research (10). Mutated zebrafish (*Danio rerio*) may develop sporadic intestinal adenomas, but transformation to malignancy has yet not been shown (11). However, chemical carcinogens added to the feed, and chronic inflammation caused by parasites, have led to the development of intestinal adenocarcinomas in this species (12–14), whereas the rainbow trout (*Oncorhynchus mykiss*) has been remarkably resistant to developing intestinal neoplasia in carcinogenesis experiments (15). Neoplasms in fish are reported to be less metastatic compared with those of mammals (10, 16), but some studies show that this phenomenon indeed occurs (17–20).

Experimental teleost models for intestinal cancer are appealing. The intestinal anatomy is similar to that of mammals, but some differences exist. In zebrafish, Atlantic salmon (*Salmo salar*) and rainbow trout, a solid collagen-rich layer, the stratum compactum, is embedded within the submucosa and surrounded by eosinophilic granular cells, a teleost mast cell analogue (21, 22). The mucosa is arranged as short folds with interlocated epithelial stem cells rather than as villi, and crypts of Lieberkühn and Peyer's patches have not been reported. The intestine is divided into an anterior, mid, and posterior portion, the latter diverges from the colon of mammals with raised intestinal folds and no glands invaginating into the mucosa (22).

Farmed salmonids are fed pellets. Their eggs are stripped during late fall or early winter and hatched in springtime. The fry receive a conservatively formulated fish meal and oil-dominated feed. After smoltification and transfer to seawater, Northern European feed regimes frequently include an on-growth diet in which the content of proteins and oils from terrestrial plants such as soy and maize has increased significantly year by year (23–25). To mimic natural migratory behavior, brood fishes are not fed from the summer before spawning when they are transferred to fresh water to mature for reproduction.

Salmonids are genuine carnivores, and plant ingredients in their feed induce chronic intestinal inflammatory conditions characterized by increased mucosal leukocyte accumulations and epithelial cell proliferation in a dose-dependent manner (26–32). Further, vegetable oils rich in n-6 polyunsaturated fatty acids (PUFA) are known to facilitate intestinal inflammation in mammals (5), and their effect in salmon includes lower transcription levels of certain stress and antioxidant-related genes (33).

We here report epithelial changes preceding cancer in the inflamed intestinal tract of farmed salmonids receiving a particular commercial feed. We classify the tumors as adenocarcinomas and describe metastases to other organs in affected fish.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Ole B. Dale, Section of Fish Diseases, National Veterinary Institute, Box 750 Dep, 0033 Oslo, Norway. Phone: 47-23216138; Fax: 47-23216101; E-mail: ole.b.dale@vetinst.no.

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Table 1. Fish groups investigated for the occurrence of tumors

Group	Species	*n*	Investigated	Location
A	A.s	4,367	Fall 2005	I
B	A.s.	5,288	Fall 2005	II
C	R.t.	2,632	Winter 2006	I
D	R.t.	3,461	Winter 2006	II
E	A.s.	3,000	Fall 2005	III
F	A.s.	172 + 6,458	June 2006 + fall 2006	I
G	A.s.	259 + 5,715	June 2006 + fall 2006	II
H	A.s.	100 + 3,778	June 2006 + fall 2006	III
I	R.t.	3,095	Winter 2007	I
J	R.t.	3,835	Winter 2007	II
K	A.s.			IV
	F.m.	0 + 63	June 2007 + October 2007	
	F.i.	298 + 43	June 2007 + October 2007	
	M.m.	0 + 40	June 2007 + October 2007	
	M.i.	302 + 43	June 2007 + October 2007	
L	A.s.	9	October 2007	V

NOTE: Locations I to III, different fish farms, Western Norway. Location IV, experimental fish farm, Northern Norway. Location V, Hellefossen, river Drammenselva, Eastern Norway. Fall indicates from mid-September to and including December, as sampling is prolonged over a period. Winter indicates January to and including March, as production cycle for rainbow trout is delayed to that of salmon. All groups received a brand feed diet from the same producer, except for groups E and L. Only group K was on feeding in the period between the samplings, and groups A to J were starved to mimic natural behavior.
Abbreviations: A.s., Atlantic salmon; R.t., rainbow trout; F.m., female mature; F.i., female immature; M.m., male mature; M.i., male immature.

Materials and Methods

Study groups. Occurrence of intestinal and liver tumors of broodstock salmonid groups, ages 4 y after hatching and weighing between 9 and 15 kg, were observed at health surveillance controls in four fish groups (A–D) located at three different commercial Norwegian fish farms in 2005 and winter 2006. Following smoltification, the fish had been routinely i.p. injected with an oil-based vaccine and transferred to sea cages. In the seawater period, all fish received an identical feed brand for 24 to 29 mo. After spawning, all individuals were autopsied and pathologic changes were recorded (n = 12,748). A fifth group (E, negative control group 1) being kept at the same locality as group H, but fed another commercial diet, was included as a control (n = 3,000; Table 1).

The findings in 2005 and winter 2006 highlighted tumor problems in broodstock salmon. Therefore, in June 2006, an initial investigation of three groups (F–H) of broodstock salmonids, ages 4 y after hatching, weighing between 9 and 15 kg and being fed the same feed brand as groups A to D for 33 to 40 mo, was performed in June, 4 mo before spawning (n = 531). In fall the same year, and in winter 2007, after spawning, the remaining individuals of these three groups and an additional two groups (I and J; n = 22,881), also having been fed the identical feed brand, were investigated (Table 1). Gender was registered (groups A and F–J).

Intestinal inflammatory changes in salmon are rapidly reduced when soy-containing feeding regimes halt (26). To mimic natural behavior, feeding was stopped in the summer for the above-mentioned groups (A–J). Therefore, one group (K, experimental positive control) was allowed to mature sexually while on active feeding with the particular diet until registration of pathologic effects. Gender and sexual maturation status were registered. Two samplings from this group were performed, one in June 2007 (n = 600) after 39 mo of feed exposure and the remaining fish in October (n = 189) following 42 mo of continuous feed exposure (Table 1).

For comparison with salmon living from a natural diet, three male and six female wild sexually mature Atlantic salmon (group L, negative control group 2; 3.8–8.7 kg) were examined by autopsy and histologic samplings. These fishes were caught in November 2007 for river cultivation purposes (Hellefossen, river Drammenselva, Buskerud, Norway) and autopsied after stripping for sperm and ova (Table 1).

All fishes were euthanized according to regulations for fish in aquaculture issued by the Norwegian Directorate of Fisheries (Forskrift om drift av akvakulturanlegg. § 28). Macroscopically identifiable intestinal changes from all groups were sampled for histologic examination in addition to samples from control fish, but only tumors with a distinct, solid appearance were registered, excluding velvety and diffusely elevated changes.

Histology, histochemistry, and immunohistochemistry. Histologic examination was performed on 89 intestinal and liver samples from groups A to J, 228 samples from group K, and 18 samples from group L according to standard procedures with tissue fixation in 10% phosphate-buffered formalin, embedding in paraffin, and staining of sections from all blocks with H&E. Hearts, kidneys, and spleen were in addition sampled in severely affected individuals. Several serial sections from selected material were stained by procedure periodic acid-Schiff (PAS) and Alcian blue for detection of mucins and stained with Martius scarlet blue and elastin/van Gieson for thrombi, collagen, and elastin.

Immunohistochemistry was performed to clarify whether neoplastic cells carried the epithelial cell marker cytokeratin. Further, occurrence of proliferating cell nuclear antigen (PCNA) was investigated in addition to reaction for MHC class II molecules. For detection of cytokeratin, the following primary antibodies were used: anti–pan-keratin (AE1/AE3/PCK26 primary antibody, diluted 1:100), Confirm anti-keratin (AE3 primary antibody, diluted 1:100), Confirm anti-cytokeratin (AE1 primary antibody, diluted 1:100; all Ventana Medical Systems), and mouse anti-cytokeratin

Table 2. Tumor occurrence macroscopically observed in the groups investigated

Group		% intestinal tumor	% additional liver metastasis
A		5.24	11.35
B		1.50	ND
C		0.49	ND
D		0.43	ND
E		0.03	ND
F		12.8 + 25.33	ND + 3.85
G		8.50 + 28.89	ND + 2.06
H		11.00 + 12.68	ND + 1.67
I		7.30	8.41
J		14.19	0.92
K			
	F.m.	ND + 12.70	ND
	F.i.	ND + 13.95	ND
	M.m.	ND + 17.50	ND
	M.i.	1.00 + 16.28	ND
L		ND	ND

NOTE: The frequency of intestinal tumors is estimated from the total group population, and the frequency of liver tumors is estimated from the fraction of the groups also positive for intestinal tumors. Liver tumors were invariably detected associated with occurrence of intestinal tumors. The splitting of figures in groups F to H and K refers to samplings at different times as accounted for in Table 1.
Abbreviations: ND, not detected; 0, data not available.

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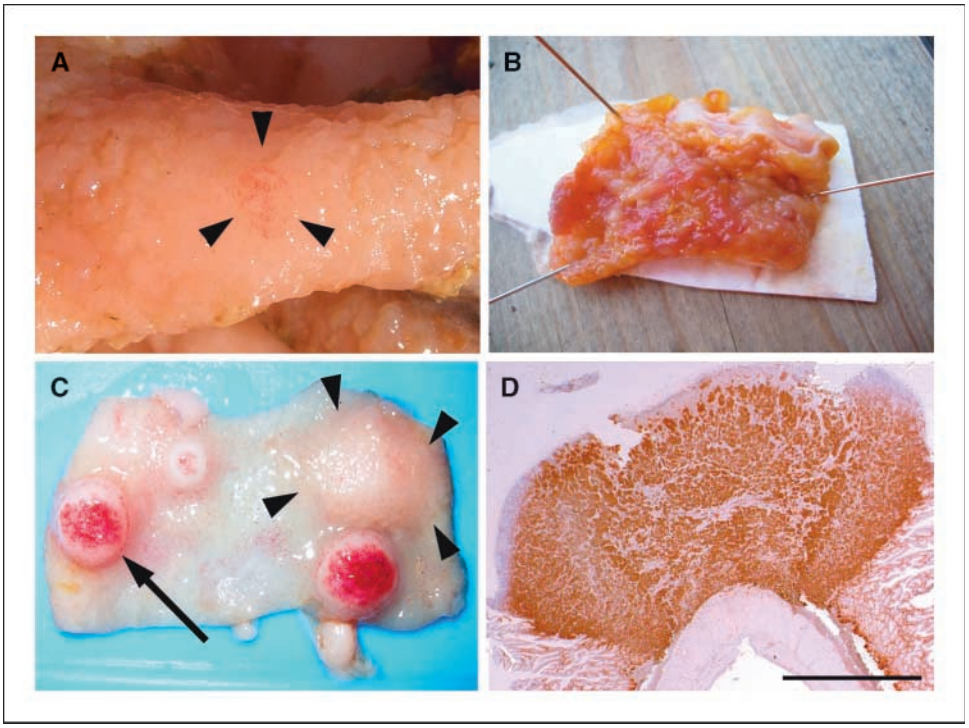

Figure 1. Representative gross appearance of salmonid intestinal tumor as observed in the study groups. *A,* modest, early intestinal changes appear as flat, slightly reddish patches (*arrowheads*). *B,* more advanced changes with velvet-like, thickened, and erythematous gut surface. *C,* well-advanced tumor manifestations with large, discrete neoplastic nodules, sometimes with an ulcerated surface (*arrow*), but also more diffuse, thickened mucosal elevations (*arrowheads*). *D,* a PCNA-stained whole-mount section of a tumor as indicated by arrow in *C* shows strong immunoreactivity throughout the tissue mass. *Bar,* 0.5 cm.

(pan, clone AE1/AE3, diluted 1:100; Zymed Laboratories). PCNA was detected using anti-PCNA clone PC10, diluted 1:400 (Dako). The immunohistochemistry procedure for keratin and PCNA detection was as described previously (34), with the exception that antigen retrieval was performed with sodium citrate (pH 6) for 10 min in microwave treatment. Initial dilution experiments identified optimal concentrations for the antibodies used for the subsequent analysis. Immunohistochemistry for cytokeratin and PCNA included positive control sections of human origin (material from Laboratorium for Patologi AS, Oslo, Norway) on the slides together with the fish sections. Negative controls were also performed without the primary antibody. Reaction for MHC class II was performed as previously described (34).

Results

Macroscopic examination. There were no signs of clinical disease in any of the investigated fish. Tumors were registered in all groups of farmed fish at all samplings and were located both in the anterior and posterior intestines and were identified both in sexually mature and immature fish. In affected fish, more than two tumors were frequently observed, and in individuals with well-advanced tumors, multiple manifestations of different premalignant and malignant developmental stages were frequently encountered throughout the entire intestine.

In the 2005/2006 samplings (groups A–D), the intestinal tumor occurrence varied from 0.43% to 5.24%, and liver tumors in intestinal tumor–positive individuals from "not detected" to 11.35%. In group E fed a different commercial diet (negative control group 1), one intestinal tumor was registered in one individual (Table 2).

In the 2006/2007 samplings, intestinal tumors were detected in fish from three groups sampled twice, both in June and in fall and winter (groups F–H), and in an additional two groups investigated in fall and winter only (groups I and J). The overall tumor prevalence for groups F to H increased from 10.36% in June to 23.60% in the later samplings. Additionally, liver metastases were detected in all groups (F–J) in fall and winter (Table 2). Very few other neoplasms were identified, including two individuals with nephroblastoma, one individual with spleen hemangioma, one individual with renal ganglioneuroma, and finally one individual with gill chondroma (data not shown). In groups A and F to J (Supplementary Table S1), in a pooled assessment, male fish had a higher incidence of tumors (Pearson $\chi^2 = 19.46$; $P < 0.0001$).

In the continuously fed fish group (K), the overall intestinal tumor prevalence increased from 0.50% in June to 14.81% in October. Only one single liver metastasis was detected in this group. There was no effect of sexual maturation on tumor occurrence, but male fish had a slightly higher incidence of tumors, although statistically not significant (Pearson $\chi^2 = 0.47$; $P = 0.50$; Table 2). The gender effect on the overall tumor incidence (groups A and F–K) was not statistically significant (Pearson $\chi^2 = 1.09$; $P = 0.30$).

In the wild salmon group (L, negative control group 2), no macroscopically visible changes or tumors were detected in any organ.

In the June samplings (groups F–H and K), changes were predominantly of a mild character, observed as multiple flattened changes or focal lesions prominating into the lumen with plaques and a velvety surface (Fig. 1*A* and *B*). Tumor size was age dependent and varied between individuals from barely visible smaller tumors in the June samplings to prominent tumors with bleedings in the fall and winter samplings (groups A–D and F–K; Fig. 1*C* and *D*), but flattened changes could also be observed in such individuals. In addition, macroscopically visible liver tumors (Supplementary Fig. S1*A* and *B*) were detected but restricted only to fish displaying well-developed intestinal tumors (Table 2), consistent with primary intestinal tumors with liver metastases.

Histology, histochemistry, and immunohistochemistry. A set of histologic and histochemical approaches was applied to classify the tumors. Mucin presence was confirmed by both PAS

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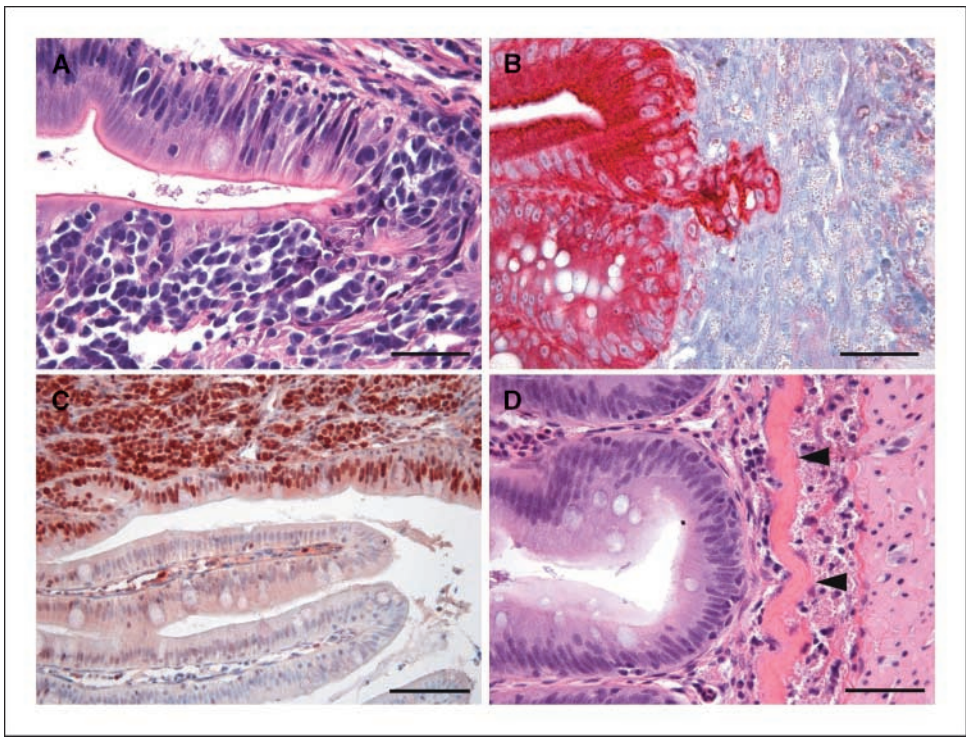

Figure 2. Micrographs of salmonid gastrointestinal epithelial dysplastic changes and adenocarcinoma. *A,* epithelial changes ranging between severe dysplasia and *in situ* carcinoma. H&E staining. *Bar,* 50 μm. *B,* bud of cytokeratin-positive cells in the basal part of a fold. Pan-keratin immunostain. *Bar,* 50 μm. *C,* the epithelium covering the tumors almost invariably displayed PCNA-positive nuclei (*brown red*), as did the nuclei of the tumor cells. The epithelium of the lower two folds is negative. PCNA immunostain. *Bar,* 100 μm. *D,* normal salmon intestinal wall from control wild-caught fish illustrates differences from the mammalian counterpart, as the muscularis mucosae is missing, and a thick, collagen-rich structure, the stratum compactum (*arrowheads*), is embedded in the submucosa, which is covered by a simple columnar epithelium with basally located nuclei. H&E staining. *Bar,* 50 μm.

and Alcian blue staining of the epithelial cells with a morphology consistent with mucous cells. All antibodies for cytokeratin and PCNA detection labeled epithelial cells in accordance with the control sections from human tissue; however, anti–pan-keratin (AE1/AE3/PCK26) gave a weak reaction also in endothelial cells, whereas the other keratin antibodies did not label other than epithelial cells.

Intestines from fish in all groups with barely detectable, moderate, and well-advanced changes were selected for histologic examination, and the following description of results will be in accordance with this sequence. In mildly affected fish, folds appeared thickened and cell rich with confluent epithelium. Irregular stratification and loss of polarity of the epithelial cells were present in both fold base regions and distal parts. In fish with moderate and severe macroscopic changes, nuclei were located both in the basal and apical half of the enterocytes, displaying hyperchromasia and nuclear pleomorphism including elongation of nuclei and mitotic figures, consistent with the diagnosis "high-grade precancerous dysplasia" (4). In portions of the epithelium, the changes occasionally included a complete dearrangement and nuclear depolarization with full-thickness nuclear stratification, justifying an *in situ* carcinoma diagnosis (Fig. 2*A*; Supplementary Fig. S2*A–F*).

In sections with moderate macroscopic changes (Fig. 1*A* and *B*) and in epithelium adjacent to and covering more advanced tumors (Fig. 1*C* and *D*), individual or small groups of cells were seen to have penetrated the basal membrane between the folds (Fig. 2*B*; Supplementary Fig. S2*G*). These findings are in accordance with carcinogenesis from epithelia in mouse models and humans (4, 9). The invading cells showed moderate to severe variation in form and size, displayed a basophilic cytoplasm frequently containing mucin, and had pleomorphic nuclei. They were invariably located in the proximity of MHC class II$^+$ cells and cells with eosinophilic granula [suggested to be the teleost fish mast cell analogue (21); Supplementary Fig. S2*H* and *I*]. Transformed epithelial cells were often strongly PCNA$^+$ (Fig. 2*C*), showing high mitotic activity. The interfold base of the wild-caught Atlantic salmon showed no signs of dysplastic changes (Fig. 2*D*).

In group K, which was under constant feeding, inflammation in both spring and fall samplings was much more prominent compared with the starved groups. When examining the submucosa in flattened and velvety macroscopic changes (Fig. 1*A* and *B*), severe infiltrations of inflammatory cells were revealed. The folds appeared thickened and were dominated by lymphocytes and eosinophilic granulated cells (Fig. 3*A*; Supplementary Fig. S3*A*). In serial sections of severely inflamed parts investigated with different techniques, it became apparent that early carcinogenesis occurred at such sites (Fig. 3*A–D*). Tumor cells in such manifestations could be seen apparently halting against the stratum compactum (Fig. 3*D*). Tumor cells were also present in the lumen of submucosal vessels (Supplementary Fig. S3*B*). In sum, these results showed an intimate association between epithelial morphology, inflammation, and tumor development.

Well-advanced tumors consisted of basophilic cells with polymorphous nuclei and showed an intense mitotic activity as shown by PCNA staining (Figs. 1*D* and 2*C*; Supplementary Fig. S1*B*), suggesting malignancy and potential for invasiveness (35). A large proportion of the tumor cells was positive for cytokeratin immunostaining, and toward tumor centers, a reduction of the stratum compactum thickness was evident (Supplementary Fig. S3*C*). Frequently, this structure was penetrated by tumor cells seemingly lysing their way through the barrier in a trabecular pattern (Supplementary Fig. S3*D* and *E*). Abundant granulated eosinophilic cells were intermingled within the tumors (Supplementary Fig. S3*D*). Cytokeratin- and mucin-positive tumor cells either had a homogenous cytoplasm with a centrally located nucleus or appeared as vacuolated signet-ring cells, frequently encountered in clusters (Supplementary Fig. S3*F*). In a subset of 100 randomly selected tumors, 9 contained >50% mucous cells out

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of the tumor cell population, which is in keeping with a mucoid adenocarcinoma diagnosis (Supplementary Fig. S3*G*). Vacuoles within the tumors contained mucin (Supplementary Fig. S3*H*). Sometimes, abnormal glandular structures were detected deeply within well-advanced tumors (Supplementary Fig. S3*I*). Seen together, these results gave evidence for aggressive tumor growth with mucin-producing cells of epithelial origin, conformal with an adenocarcinoma diagnosis.

Histologic examination revealed the presence of variably sized liver tumors, sometimes expanding through most of the liver tissue and being highly $PCNA^+$ (Supplementary Fig. S1*B*). Cytokeratin staining revealed strong reaction in tumor cells (Fig. 4*A*). Tumor cells were also found scattered as solitary dissociated or minute groups of cells throughout the liver parenchyma (Supplementary Fig. S4*A*). The tumors sometimes contained vacuoles positive for mucin and with cells displaying a signet-ring appearance (Fig. 4*B*; Supplementary Fig. S4*B* and *C*). In liver vessels, tumor cells could be seen arranged in solid structures (Fig. 4*C*) or scattered in the peripheral bloodstream (Supplementary Fig. S4*D* and *E*). Tumor cells were also seen associated with the muscle fiber meshwork within the teleost cardiac lumen and in the kidney interstitium and spleen parenchyma in affected fish. PAS staining revealed positive reactions in occasional cells in these metastases, which all were strongly cytokeratin positive (Fig. 4*D*; Supplementary Fig. S4*F*). Due to their content of mucin and positive cytokeratin reaction, these tumors were diagnosed as adenocarcinoma metastases originating from primary tumors in the intestinal tract.

Discussion

This is the first report of intestinal adenocarcinoma with metastases in fish. The condition was associated with a special feed brand and with inflammation. The tumors were found in farmed brood fishes that have a much longer exposure to commercial feed than fish for food production. In humans, intestinal cancer prevalence is positively correlated both to the duration and the severity of inflammation (3, 36). Previous reports show that inflammation and epithelial dysplasia in salmonids initiated by plant feed ingredients appear after just weeks of exposure (26, 29, 32).

Following the findings of intestinal tumors, the Norwegian Food Safety Authority analyzed the particular feed brand and visceral organs of affected fish for presence of known carcinogens. Their results showed that the level of heavy metals and other environmental contaminants was significantly below limit values (37, 38). Further, 300 randomly sampled individuals, each from six additional broodstocks of salmon fed other feed brands, were likewise investigated without any finding of tumors (39).

Modest macroscopic changes were characterized by an elevated mucosa, of which some had a velvety appearance. Polyps were never encountered. Thus, the macroscopic sequence of development differed from those of mutated zebrafish, which spontaneously develop intestinal polyps (11). The onset of carcinogenesis from multiple locations of flattened, dysplastic epithelium is common in IBD-associated adenocarcinoma in humans and not in the hereditary or sporadic forms, which usually develop from adenomatous polyps (3, 4). The gross distribution and macroscopic characteristics of the salmonid intestinal tumors thus resembled those of IBD-associated adenocarcinoma in humans.

Histologic examination of macroscopically modest changes revealed profoundly altered intestinal architecture, dominated by blunted and fused folds and with the submucosa infiltrated by mononuclear cells as in chronic inflammation. The haphazardly arranged enterocyte nuclei were conformal with a high-grade dysplasia diagnosis (3, 4). Carcinogenesis occurred from the bottom of the folds (crypts in mammals) similar to that described in humans and mice models (9). In humans, dysplasia is present in >90% of cases of IBD with carcinoma, frequently adjacent to cancer

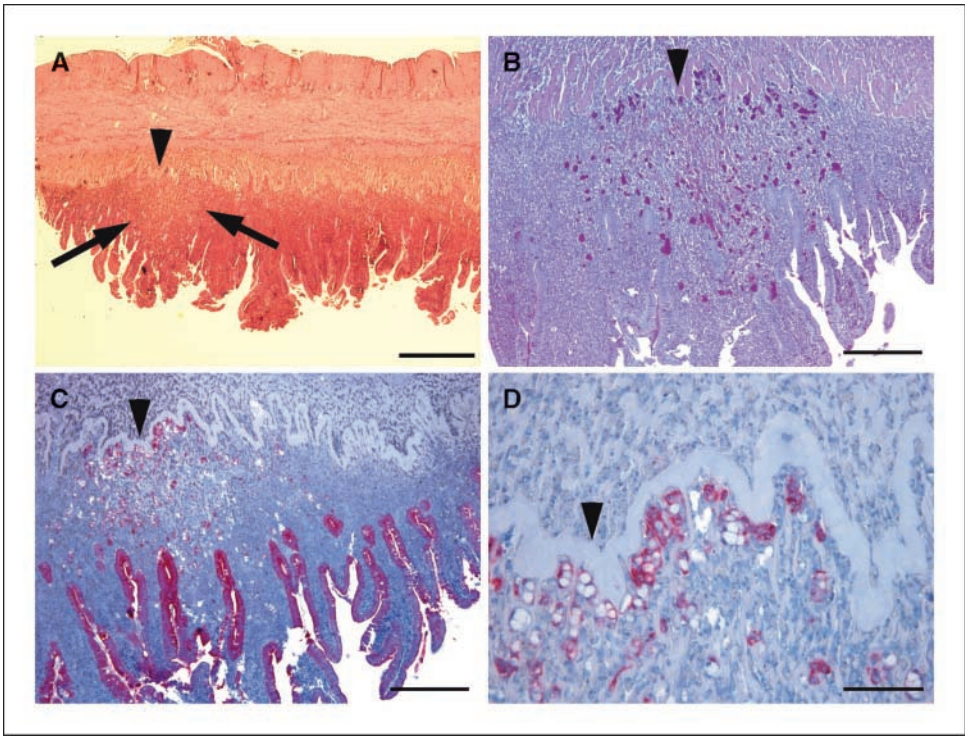

Figure 3. Inflammation and carcinogenesis in the salmonid gut. Early tumor in inflamed intestine (*arrows*; *arrowheads* indicate identical position), serial sections. *A,* site of carcinogenesis. H&E staining. *Bar,* 1,000 μm. *B,* small, dispersed groups of PAS-positive cells and vacuoles. PAS staining. *Bar,* 400 μm. *C,* loosely dispersed tumor cells within inflamed mucosa. Pan-keratin immunostain. *Bar,* 200 μm. *D,* higher magnification with tumor cells apparently halted against the stratum compactum. Pan-keratin immunostain. *Bar,* 100 μm.

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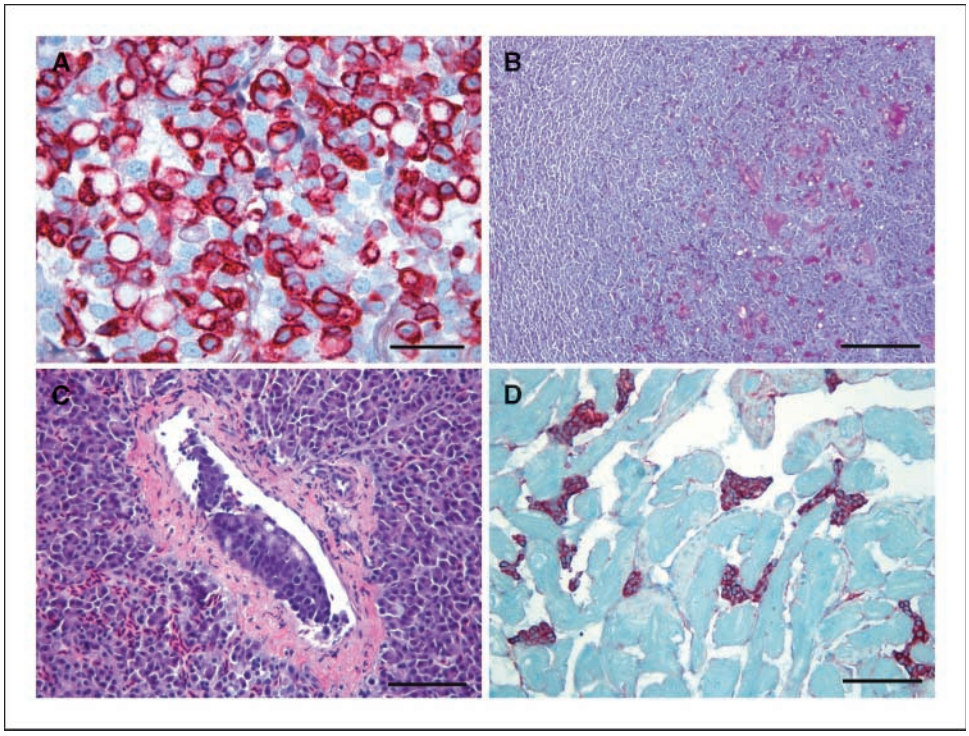

Figure 4. Micrographs of metastases of adenocarcinoma in salmonids. *A,* cytokeratin-positive cells in a liver tumor, several with a signet-ring appearance. Pan-keratin immunostain. *Bar,* 30 μm. *B,* PAS-positive cells and vacuoles in liver tumor. PAS staining. *Bar,* 200 μm. *C,* a solid aggregate of tumor cells within a liver vessel. H&E staining. *Bar,* 100 μm. *D,* cytokeratin immunostain with strong reaction in cardiac metastases. Cytokeratin AE1 immunostain. *Bar,* 100 μm.

(4). This concurs with our findings of dysplastic epithelium adjacent to and covering the tumors.

Carcinogenesis is strongly influenced by cell signaling and an oxidative microenvironment caused by inflammation (1–4). Intestinal inflammation in salmonids caused by plant-containing feed (26–33) probably leads to cytokine release and an oxidative environment caused by activated epithelium-located leukocytes as seen in mammals. Although not specifically accounted for by the feed producers (23–25), the type and content of plant meal and oils in different feed brands vary, and consequently, the fish will respond differently. Other components, not accounted for by the feed producers, may also be added. Increased enterocyte proliferation combined with decreased apoptosis is a carcinogenetic event that dietary long-chained *n*-3 PUFAs seem to counter in humans (5). Soy meal in salmonid diet may induce intrafold epithelial proliferation but also distal fold apoptosis (31). Further, the *n*-6 PUFAs found in several vegetable oils used in fish feed (23–25, 33) seem to enhance the development of colon carcinogenesis in mammals (5). Salmonids are themselves poor synthesizers of long-chained *n*-3 PUFAs and rely on feed supplement to obtain high tissue levels (40). Dietary supplement of long-chained *n*-3 PUFAs has preventive effects against tumor cell metastasis from colon carcinoma in mice (41). The partial replacement of marine-obtained oils with vegetable oils combined with plant meal in the current fish feed brand may thus have contributed to cancer development. Despite feeding regimes adapted to industrialized fish production, farmed salmonids still remain a most valuable source for long-chained *n*-3 PUFAs in the human diet (42).

Adenocarcinomas are the most common form of intestinal carcinomas developing in association with IBD in humans, and signet-ring cell adenocarcinomas are 10 times more common in IBD patients than in the general population (4). In the fish, mucinous adenocarcinomas were not uncommon, but the portion of signet-ring cells in most advanced tumors was insufficient to justify the diagnosis of signet-ring adenocarcinoma. The liver tumor cells were positive for cytokeratin and mucin, which strongly suggested an intestinal cell origin. Liver metastases were occasionally dominated by variably sized mucin-containing vacuoles. In liver vessels, tumor cells were found both as dispersed cells and solid cell clusters, indicating prominent capacity for metastasis. Tumor cells in the heart, spleen, and kidney metastases were strongly positive for cytokeratin, but rarely containing mucin, possibly reflecting a loss of differentiation following metastasis to more peripheral organs.

Previous experiments have shown cross-reactivity of antibodies raised against mammalian cytokeratin or PCNA with corresponding antigens in fish (11, 31). PCNA staining has been used to assess the invasiveness of tumors of the digestive tract (35) and was applied to characterize the fish tumors. The epithelial *in situ* tumor cells and those in the intestinal and liver neoplasms were highly positive for PCNA, suggesting high proliferating activity of these tumors. The aggressive invasiveness of the tumors was further shown by their capacity to destroy and penetrate the thick, collagenous stratum compactum, their invasion of small vessels in the submucosa, and their presence in the peripheral blood.

In farmed salmonids, we report a highly metastatic cancer developing in the intestinal tract. The genetic background, geographic location, and sexual maturation did not seem to explain the sudden emergence of high cancer frequency, whereas a particular feed brand was the common factor. Withdrawal of this particular feed from the generation of brood fishes that mature now in the fall of 2008 seems to have lowered the cancer frequency to the negligible level seen in our farmed negative control group. Gender effects on the carcinogenesis incidence have been observed in mammals and have been attributed to sex hormonal influences (2). In the commercially farmed brood fish in this study, a gender effect was observed as a higher tumor frequency in the males. A higher frequency of tumors in males was also observed in the

experimental positive group but not statistically significant. In the commercially farmed fish groups, the gender effect may in part be due to different handling. The males were kept alive longer and stripped for sperm several times before being euthanized and autopsied, whereas the females were stripped, euthanized, and autopsied in one operation. As the tumors seemed to grow quite rapidly in this period, and the males due to repeated handlings also were subjected to more stress than the females, such differences could lead to a higher number of detectable tumors in the male groups. In contrast, there was no different treatment of the genders in the experimental positive group.

Our findings strongly suggest that chronic inflammation and dysplasia were initiated and maintained by plant feed ingredients, and due to the prolonged time of exposure in the broodstock salmon, this condition finally resulted in cancer in an analogous sequence as that of human IBD-associated colorectal cancer. However, we cannot exclude that factors other than the feed brand may have contributed to the pathogenesis reported here. All fishes except the wild individuals were vaccinated with i.p. injections of oil-adjuvanted vaccines, which are known to induce chronic and generalized inflammatory responses, aberrant cytokine expression, and systemic autoimmunity (43, 44). In mice, mineral oil or its component pristane is known to induce plasmacytoma in an interleukin-6–dependent manner in susceptible strains of mice (45, 46). Cytokines induced by vaccination may have significant effects on development and progression of intestinal inflammation and tumors. Further, carcinogenic contaminants have been identified in fish feed and farmed salmon from many countries (47), and an influence from possible water-borne carcinogens cannot be excluded.

We are currently performing experiments to identify risk factors for carcinogenesis in salmon feed as this clearly needs attention from an animal welfare point of view. In addition, experimental induction of the inflammation-dysplasia-carcinoma sequence in salmonids by feeding with plant-enriched meal may represent an interesting new animal model for colonic cancer in man.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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