

Zebrafish Xenotransplant Colon Cancer Model for Drug Screening

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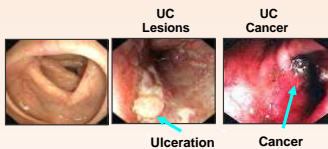
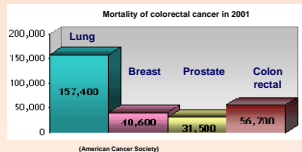
Abstract

Colorectal cancer is common worldwide and the second largest cancer killer in the United States. Although immunodeficient mice have played an important role as hosts for colorectal cancer xenografts, a simple and cost-effective xenotransplant animal cancer model is needed for more efficient drug screening. In this study, approximately 1500 human colon cancer Colo320 cells were microinjected into 3 day post fertilization (dpf) zebrafish yolk sac by microinjection and cancer cells proliferated, metastasized and formed tumor masses in zebrafish. We then successfully developed a quantitative microplate-based whole xenotransplant zebrafish chemiluminescence ELISA using an anti-human Survivin monoclonal antibody which did not cross react with zebrafish tissues. The chemiluminescence ELISA was highly sensitive and enabled detection of signal from as few as 10 human colon cancer Colo320 cells *in vitro*. After xenotransplant, zebrafish were treated with 5 FDA-approved anti-colorectal cancer drugs (5-Fluorouracil, Oxaliplatin, Camptothecin and Leucovorin) or a drug combination (Leucovorin + 5-Fluorouracil), respectively, by soaking. Drug effects on human colon cancer cell proliferation in zebrafish were assessed 3 days after drug treatment using the chemiluminescence ELISA. Drug concentration-dependent cancer cell inhibition was observed after treatment with: 5-Fluorouracil (3% - 43% inhibition), Oxaliplatin (3% - 60% inhibition), 5-Fluorouracil + Leucovorin (22% - 31% inhibition). Reduction in chemiluminescence signal (cancer inhibition) from treatment with 1000 µM 5-Fluorouracil, 1000 µM Oxaliplatin, and 250 - 1000 µM (each drug), 5-Fluorouracil + Leucovorin combination was statistically significant over vehicle controls ($p < 0.05$ and $p < 0.01$). As expected, no drug effect was observed for Leucovorin treatment alone; however, combination treatment with 5-Fluorouracil and Leucovorin significantly inhibited Colo320 cancer cells, even at 250 µM concentration. Camptothecin did not inhibit cancer cells at the test concentrations up to LC_{50} (0.24 µM). The overall prediction success rate in zebrafish xenotransplant colon cancer model was 80% (4/5) compared to results in mammalian models and humans. Our results demonstrate that the microplate-based whole xenotransplant zebrafish chemiluminescence ELISA is a highly specific and sensitive high throughput method for quantitating signal from cancer cells *in vivo*. Furthermore, xenotransplant zebrafish is a reproducible and predictive animal model for drug screening.

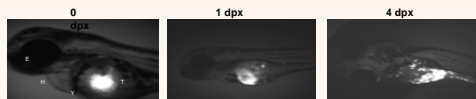
Introduction

Colorectal Cancer

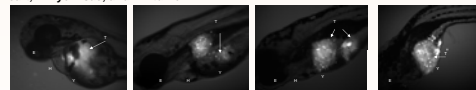
- # 2 cancer killer in the United States
- Median survival: 12-13 Months



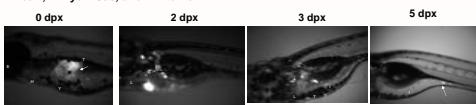
Zebrafish Colon Cancer Xenotransplant Model



Human Colo320 colon cancer cells xenotransplanted into 2 dpf zebrafish. Images were taken in the same zebrafish: 0, 1 and 4 days post xenotransplant (dpx). E = eye, H = heart, Y = yolk sac, and T = tumor.



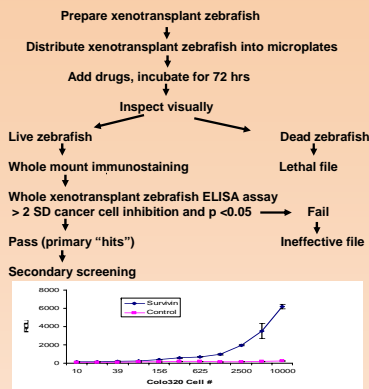
Human SW620 colon cancer cells xenotransplanted into 2 dpf zebrafish. Images were taken from the same zebrafish: 0, 1, 2 and 5 days post xenotransplant (dpx). E = eye, H = heart, Y = yolk sac, and T = tumor.



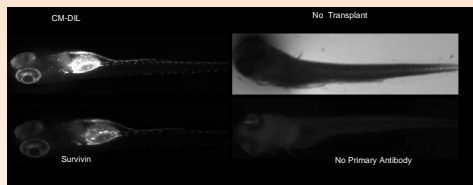
Human lymphoblastoid TK6 cells xenotransplanted into 2 dpf zebrafish. TK6 cells (non-cancer control cells) did not proliferate in zebrafish, and by 5 dpx, most xenotransplanted TK6 cells were not visible. E = eye, H = heart, Y = yolk sac, and T = tumor.

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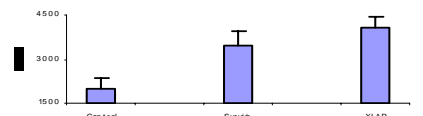
Whole Xenotransplant Zebrafish ELISA



Cell number-dependent increase in chemiluminescence signal in anti-human Survivin antibody-based *in vitro* Colo320 cell ELISA. The data are presented as Mean \pm SE from 5 measurements. Control: cells without Survivin primary antibody reaction. RCU: relative chemiluminescence units.

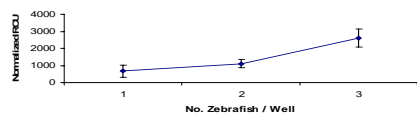


Whole mount immunostaining for zebrafish xenotransplanted with human Colo320 cancer cells. Anti-human Survivin monoclonal antibody specifically stained xenotransplant human colon cancer Colo320 cells, but showed essentially no cross reactivity with zebrafish. Zebrafish without xenotransplant (no transplant) and xenotransplant zebrafish without primary antibody staining were used as negative controls. CM-Dil-labeled xenotransplanted cells were visualized under rhodamine channel and antibody-stained zebrafish were visualized under FITC channel.



Human colon cancer Colo320 cell xenotransplant zebrafish ELISA for quantitating cancer cells. 2 dpf zebrafish were xenotransplanted with 1500 Colo320 cells and fixed 3 days post xenotransplant (dpx) for the ELISA. Data were expressed as Mean \pm SE from 5 measurements, 3 zebrafish per well. Control: wild-type zebrafish. RCU: relative chemiluminescence units. * $p < 0.05$, and ** $p < 0.01$

Survivin Quantitative Whole Zebrafish ELISA for Xenotransplanted Colo320 Cells

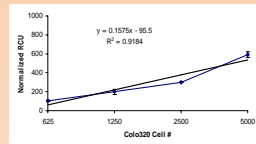


Linear Relationship between increase in chemiluminescence signal and number of xenotransplanted zebrafish per well. The data were presented as mean \pm SE from 5 measurements. Normalized RCU: normalized relative chemiluminescence units.

Whole Zebrafish ELISA for Xenotransplant Colo320 Cells: S/N Ratio*

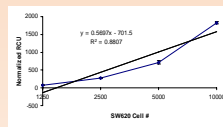
Primary Antibody	1 Zebrafish / Well	2 Zebrafish / Well	3 Zebrafish / Well
Control**	1	1	1
Survivin	1.5	1.6	2.3
XIAP	1.6	1.7	2

*S/N Ratio = signal in xenotransplant zebrafish stained with a primary antibody / signal in xenotransplant zebrafish control without primary antibody staining
** Control: xenotransplant zebrafish without primary antibody staining



Cell #/Well	Normal RCU (Mean \pm SD)*
625	101 \pm 6
1250	199 \pm 29
2500	301 \pm 3
5000	592 \pm 33
Xeno-Zebrafish**	378 \pm 27***

Estimate of number of Colo320 cancer cells in xenotransplant zebrafish based on *in vitro* cancer cell ELISA using anti-human Survivin monoclonal antibody. *In vitro* cell ELISA and *in vivo* whole xenotransplant zebrafish ELISA were performed simultaneously in the same microplate. Based on the dose-response curves generated from the *in vitro* cell ELISA, there were approximately 3006 Colo320 cells in a single zebrafish 3 days post xenotransplant. *Normalized RCU: normalized relative chemiluminescence units from 4 measurements. ** Human Colo320 xenotransplant zebrafish, 1 xenotransplant-zebrafish/well. *** Mean \pm SE from 12 measurements



Cell # / Well	Normal RCU (Mean \pm SD)*
1250	77 \pm 6
2500	271 \pm 16
5000	715 \pm 44
10000	1828 \pm 32
Xeno-Zebrafish**	634 \pm 38***

Estimate of number of SW620 cancer cells in xenotransplant zebrafish based on *in vitro* SW620 cancer cell ELISA using anti-human Survivin monoclonal antibody. Based on the dose-response curve produced from the *in vitro* cell ELISA, there were approximately 2344 SW620 cells in a single xenotransplant zebrafish 3 days post xenotransplant. *Normalized RCU: normalized relative chemiluminescence units from 4 measurements. ** Human SW620 xenotransplant zebrafish, 1 xenotransplant-zebrafish/well. *** Mean \pm SE from 12 measurements

Intra-Plate variability of xenotransplant zebrafish ELISA after vehicle-treatment

Colo320 cells	Treatment	Well #	RLU* Mean	RLU* SD	CV
Colo320 cells	0.4% DMSO	14	5157	746	14.5%
	1% DMSO	14	4560	1060	23.2%
	0.24% DMSO	14	4010	1179	29.4%
	Water	14	4755	549	11.5%
SW620 cells	0.4% DMSO	14	3975	703	17.7%
	Mean				19.3%
	0.4% DMSO	14	6999	1706	24.4%
	1% DMSO	14	4202	1280	30%
SW620 cells	0.24% DMSO	14	4883	773	15.8%
	Water	14	5145	1238	24.1%
	0.4% DMSO	14	4689	1142	24.4%
	Mean				23.7%

*Relative chemiluminescence units

Inter-Plate variability of xenotransplant zebrafish in different vehicle treatment experiments

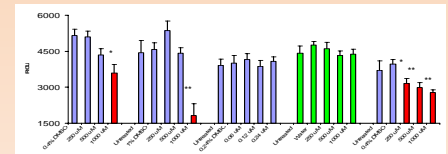
Experiment #	RCU* in Colo320 Cell Xenotransplant Zebrafish (Mean)	RCU* in SW620 Cell Xenotransplant Zebrafish (Mean)
1	5157	6999
2	4560	4202
3	4010	4883
4	4755	5145
5	3975	4689
Mean	4491	5184
SD	504	1072
CV	11.2%	20.7%

*Relative chemiluminescence units

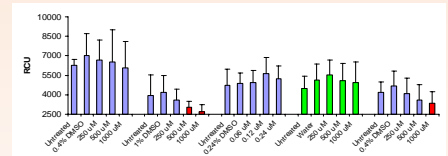
Anti-Cancer Drug Efficacy Assessment

Anti-Cancer Drugs Assessed in Xenotransplant Zebrafish

Compound	Mechanism of action
5-Fluorouracil	Inhibits aminoisobutyrate-pyruvate aminotransferase, arrests cell cycle at G2 and induces apoptosis in human primary and metastatic colon adenocarcinoma cell lines.
Oxaliplatin	Anti-tumor platinum compound with activity against colorectal cancer, cytotoxicity follows the formation of adducts with DNA. Induces apoptosis.
Camptothecin	Binds irreversibly to the DNA-topoisomerase I complex, blocking the cell cycle in S-phase and induces apoptosis in many normal and tumor cell lines.
Leucovorin	The active form of the B complex vitamin folate; used in combination with chemotherapy to maintain folate levels. Protects normal cells and increases anti-tumor effects of 5-Fluorouracil.



Effects of Drugs on Human Colon Cancer Colo320 Cells Xenotransplanted into zebrafish. Signal after drug treatment, measured by whole zebrafish ELISA assays using anti-human Survivin antibody. Compared with vehicle control, reduced signal (red bars) indicate cancer cell inhibition by anti-cancer drugs. Data are presented as Mean \pm SE from 14 measurements. 0.24%, 0.4% and 1% DMSO and water were used as vehicle controls, respectively, for different drugs. Red bar represents statistically significant cancer inhibition, and according to its pharmaceutical function, cancer inhibition was normally not expected from Leucovorin treatment alone (green bars). RCU: relative chemiluminescence units. * $p < 0.05$ and ** $p < 0.01$, as compared with vehicle control.



Effects of Drugs on Human Colon Cancer SW620 Cells Xenotransplanted into Zebrafish and Quantified by Whole Zebrafish ELISA Assays using anti-Survivin Antibody. Data were presented as Mean \pm SE from 14 measurement (data for all untreated groups were from 7 measurements). 0.24%, 0.4% and 1% DMSO and water were used as vehicle controls, respectively, for different drugs. Red bar represents statistically significant cancer inhibition, and according to pharmaceutical function, cancer inhibition was not predicted from Leucovorin treatment (green bars). RCU: relative chemiluminescence units. * $p < 0.05$, as compared with vehicle control.

Comparison of Colon Cancer Drug Effects in Zebrafish and Mammals

Drugs	Effect on Colon Cancer in Mammals	Effect on Colon Cancer in Zebrafish	Correct Prediction
5-Fluorouracil	Inhibition	Inhibition	Yes
Oxaliplatin	Inhibition	Inhibition	Yes
Camptothecin	Inhibition	None	No
Leucovorin	None	None	Yes
5-Fluorouracil + Leucovorin	+ Inhibition	Inhibition	Yes

Conclusion

Zebrafish xenotransplant model combined with our whole animal microplate-based chemiluminescence ELISA is a reproducible and predictive animal model for drug screening.