Discovery of novel human pancreatic tumor stem cell Targeting agent,CEP1430, through three-dimensional (3D) stem cell culture assay.

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Introduction

Pancreatic cancer is the fourth leading cause of cancer mortality in the US, despite significant improvements in diagnostic imaging and operative mortality rates. The 5-year survival rate remains less than 6% because of microscopic or gross metastatic disease at time of diagnosis. Although the treatment of pancreatic cancer remains a huge challenge, it is entering a new era with the development of new strategies and trial designs. Because there is an increasing number of novel therapeutic agents and potential combinations available to test in patients with pancreatic cancer, the identification of robust prognostic and predictive markers and of new targets and relevant pathways is a top priority as well as the design of adequate trials incorporating molecular-driven hypothesis. Over the past decade, increasing evidence suggested that stem cells play a crucial role not only in the generation of complex multicellular organisms, but also in the development and progression of malignant diseases. For the most abundant tumors, it has been shown that they contain a subset of distinct cancer cells that is exclusively responsible for tumor initiation and propagation These cells are termed cancer stem cells or tumor-initiating cells and they are also highly resistant to chemotherapeutic agents.
Three-dimensional (3D) culture of cancer stem cells has long been advocated as a better model of the malignant phenotype that is most closely related to tumorigenicity in vivo. Moreover, new drug development requires simple in vitro models that resemble the in vivo situation more in order to select active drugs against solid tumors and to decrease the use of experimental animals. The induction of chemotherapy or concomitant chemo-radiotherapy has been used to increasingly to improve survival, and organ preservation. This approach encounters significant morbidity and mortality. Therefore reliable chemosensitivity assays are needed to accurately predict the response to chemotherapy and guide the selection and treatment of cancer patients. The purpose of this study is to examine and evaluate optimum drug candidates in vitro chemosensitivity on patient tumor tissues directly in culture and on their Cancer stem cell cultures. CEP1430 shows promise as a better therapeutic agent against Pancreatic CSC and when tested in SCID mice model with once daily IP injections for 30 days showed no apparent adverse effects. CEP1430 reduced the tumor volume in the treated group by 80-90 %, when compared with the control group.

Methods:

In-vitro study: Pancreatic CSCs and Pancreatic tumor cells (parental) were isolated from 10 terminal donor patients that had under gone chemotherapy and radiation treatments. The ages ranged from 35 years old to 65 years old, including both genders. The tissues were consented and obtain under IRB and HIPPA regulations and guidelines. The tissues were transported from the surgical suites to Celprogen in Human Pancreatic CSC complete growth media [M36115-42S] within 24 hours after it had been surgically removed from the patients. Upon receipt of the tissue the tissue was sectioned into two halves and processed. One section was maintained as the heterogeneous tumor population and cultured as parental cell culture. Where as , the other section was processed further and isolated with CSC biomarkers, in Celprogen Media [M36115-42S] and ECM [E36115-42-T25] combination. Once the cell cultures were established within 7-14 days the cells were characterized by Flow, IHC, Western Blot and Real Time PCR. Both the parental and the Human Pancreatic CSCs were check for tumorigenicity by injecting 1000 cells subcutaneously in SCID mice. Once the cells were characterized they were seeded at 10,000 cells per well in a 96 well format, pre-coated with Celprogen ECM [E36115-42-96Well] and cultured in complete growth media [M36115-42S]. The drugs were tested by incubating at various concentrations for 72 hours at 5% carbon dioxide, humidified 37 ^{0}C incubator. At the end of 72 hours, cell viability was obtained utilizing Alamar blue assay and also Cellometer Auto 2000 Nexcelom Cell counter. IC50 curves were generated for the test compounds CEP1430. Among the 1000 compounds screen tested Gemcitabine, Taxol, Fluorouracil, Leucovorin, Irinotecan, and Oxaliptin were not effective against Pancreatic Cancer Stem cell (CSC) but were effective on tumor cells (differentiated CSCs). We were able to show 6 compounds [CEP1430,1431,1432,1433,1435&1436] that were effective against Pancreatic CSC targeting selected pathways.

In-vivo study: One thousand viable human pancreatic CSCs and parental cells were subcutaneously injected at the hind limb of SCID mice. After 10 days post injection when visible tumors were observed the mice were separated into control group of 5 mice and an experimental group of 5 mice. The treatment was provided for the experimental group that received IP injections three times per week for a period of two weeks. Each week the tumor growth measurements were performed and tabulated. At the end of the two weeks the mice were sacrificed and the tumor tissues were fixed and H&E stained, cultured, IHC, Real-time PCR from total RNA, and flow studies with various Stem cell markers was performed.

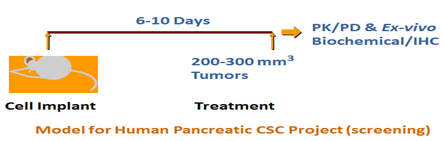


Figure 1 Model for Human Pancreatic CSC screening. Human Pancreatic Cancer stem cells were inoculated subcutaneously (1000 cells/mouse). 6-10 days poste injection blood samples were obtained from animals 200-300 mm3 sized tumors for PK/PD and ex-vivo Biochemical/IHC analysis.

Results:

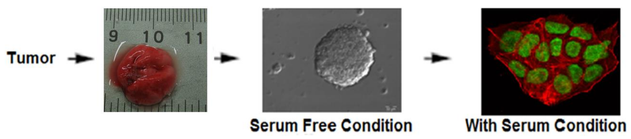


Figure 2. Generation of donor specific cells from solid tumor. Utilizing Celprogen Pancreatic cancer complete growth media and ECM.

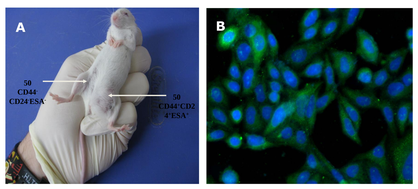
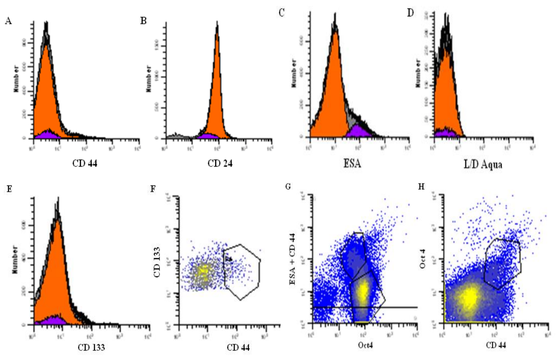


Figure 3. A. SCID mice injected with 50 $CD44^+CD24^+ESA^+$ cells and 50 $CD44^-CD24^-ESA^-$ cells. Tumor formation within 20 days after subcutaneous injections. **B.** Human Pancreatic CSC stained positive for Ephrin type-B receptor 4 (Eph B4) marker.



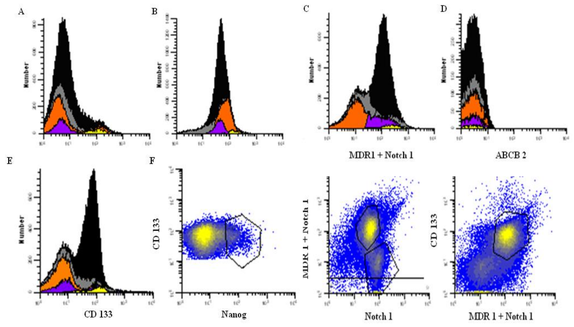


Figure 4. Flow Cytometry characterization of Human Pancreatic Cancer Stem Cells. The dissociated cells were counted and transferred to 5ml tube, washed with IXPBS and resuspended in million cells per 100 µM. Antibodies were added incubated for 20 minutes washed and secondary antibodies added when required. The antibodies utilized were anti-CD44, CD24, ESA, Nanog, Notch 1 and MDR1 (Shankar S *et al*. 2011 6(1):e16530. doi:10.1371/journal.pone.0016530)

1.

Cancer Stem Cell Markers	Commonly Expressed Markers in Cell Culture
CD133	☑
Ability to for tumors <1000 cells in nude mice	☑
Telomerase	☑
SSEA ¾	☑
OCT-4	☑

2.

Parental Cancer	Cancer Stem Cells
CA19-9	CD133, CD44,
CEA	SSEA3/4, OCT4
GAD	Tumorigenicity (<1000 cells)
alpha-1-antitrypsin	Alkaline Phosphatase
Mucin	Aldehyde dehydrogenase
Keratin	Telomerase
CK7	Nestin

Table 1. Cancer Stem Cell general characterization Markers **2.** Positive Cells Markers for Human Pancreatic Parental Cancer cells and Cancer Stem Cells.

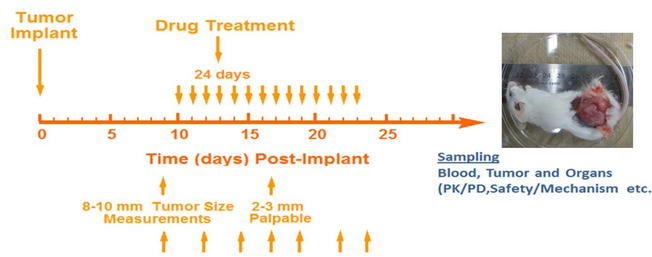


Figure 5. Tumor implantation and Drug treatment Experimental setup timeline. Human Pancreatic Cancer stem cells were inoculated subcutaneously (1000 cells/mouse). Drug treatments was started after 10 days post injection. After 24 days post implantation the tumor was resected and the blood and organs were sampled for PK/PD analysis and Ex-vivo IHC and biochemical analysis.

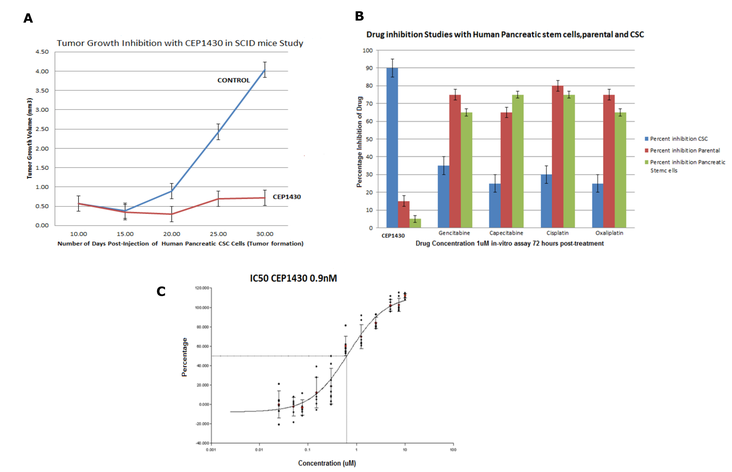


Figure 6. A. Tumor growth Inhibition curve for mice injected with 1000 cells of Human Pancreatic CSC. **B.** Comparison of % drug inhibition between Human Pancreatic CSC, Human Pancreatic Parental and Human Pancreatic Stem Cell. **C.** IC50 curve for Human Pancreatic CSC treated with drug CEP1430.

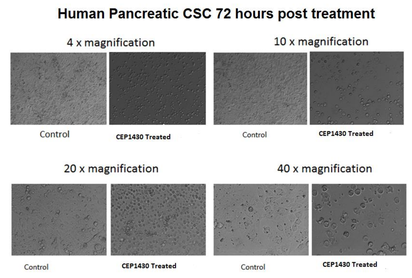


Figure 7. Human Pancreatic CSC treated with CEP 1430 1 µM concentration for 72 hours in the 96 well format and an initial seeding of 10000 cells/well.

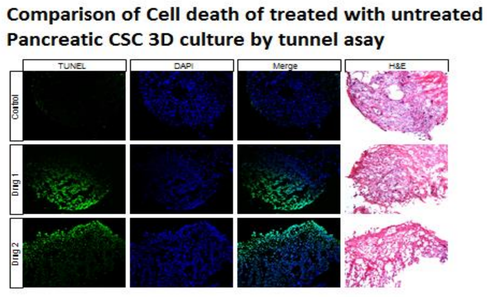


Figure 8. Human Pancreatic CSC treated with CEP 1430 [Drug1] 0.9nM & CEP1430 [Drug 2] 1 uM concentration for 72 hours in 3D histo-culture system of patients Tumor approximately 10000 CSCs /well seeding density.

Conclusions:

The efficacy of various therapeutic agents targeting major pathways (wnt,Notch,PI3K,MAPK,STAT) and chemotherapy agents were tested using DNA uptake and TUNNEL assay anti-cancer agents was calculated according to the inhibition index. The same compounds were tested for utilizing the patient's Pancreatic Cancer Stem Cell Cultures established with Celprogen's Media and ECM. Expression of PDX-1, SHH, CD24, CD44, CD133, EpCAM, CBX7, OCT4, SNAIL, SLUG, TWIST, Ki-67, E-cadherin, β-catenin and vimentin were quantified by qPCR or immunocytochemistry. We cultured the cells in low oxygen since Tumor hypoxia induces epithelial-mesenchymal transition (EMT), which induces invasion and metastasis, and is linked to cancer stem cells (CSCs). Among the 1000 compounds screen tested Gemcitabine, Taxol, Fluorouracil, Leucovorin, Irinotecan, and Oxaliptin were not effective against Pancreatic Cancer Stem cell (CSC) but were effective on tumor cells (differentiated CSCs). We were able to show 6 compounds CEP1430, 1431, 1432, 1433,1435&1436] that were effective against Pancreatic CSC targeting selected pathways. CEP1430 shows promise as a better therapeutic agent against Pancreatic CSC and when tested in SCID mice model with once daily IP injections for 30 days showed no apparent adverse effects. CEP1430 reduced the tumor volume in the treated group by 80-90 %, when compared with the control group.

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