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Expression of Classical Embryonic Stem Cell Markers in Pancreatic Cancer Cells

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Abstract

Background: Pancreatic cancer has been the third leading cause of cancer-related death in USA. Most of the cancer patients diagnosed in late stage which minimize the effectiveness of surgical intervention to less than 20 percentage. Moreover, chemo-radio therapy is not curative. Presence of Cancer Stem Cells (CSCs) within pancreatic tumor was reported by several groups using nonspecific biomarkers. Pluripotent transcription factors such as OCT4, SOX2 and NANOG, which are upregulated in embryonic stem cells in contrast to somatic cells, were detected in various type of cancer tumours from adult patients.

The aims of this study were to investigate the expression of the classical stem cell markers in Pancreatic Adenocarcinoma Cell line (PANC1).

Methods: PANC1 cells were characterised by RT-PCR/immuno-staining. Transient over-expression of stem cell promoter-driven reporter plasmid Oct4-eGFP was undertaken using Lipofectamine 2000 transfection reagent.

Results: Embryonic stem cell markers and other cancer related markers were detected which illustrate the nature of pancreatic cancer.

Conclusion: Embryonic stem cell markers could be used to identify pancreatic cancer stem cells and they are potential targets for cancer targeted therapy.

Keywords: Cancer stem cells; Pancreatic cancer; Embryonic stem cell markers; Oct4; Sox2; Nanog

Abbreviations: CSCs: Cancer Stem Cells; HSP: Heat Shock Protein; DCLK1: Doublecortin-Like Kinase 1; GBM: Glioblastoma; ABCG2: ATP-Binding Cassette transporter protein 2; OCT4: Octamer-binding Transcription factor 4; SOX2: Sex determining region Y-box 2; NANOG: pron. NanOg; OSCC: Oral Squamous Cell Carcinoma; PANC1: human Pancreatic ductal Cell line; ALDH: Aldehyde Dehydrogenases; hESCs: human Embryonic Stem Cells; PBS: Phosphate Buffer Saline; DPBS: Diphosphate Phosphate Buffer Saline; DAPI: 4',6-Diamidino-2-Phenylindole; EMT: Epethelial-Mesenchymal Transmision

Background

Cancer stem cells (CSCs) theory hypothesize the presence of small subset of cells within tumor mass that have the ability to renew their self and differentiate to multiple cell types. In addition, these cells resist conventional chemotherapy and give rise new tumor cells causing relapse and metastasis. Identification of CSCs hold hopes to develop new therapy that targeting the origin of tumor cells and improve cancer patients. First cancer stem cells were identified and characterized from leukaemia cells in 1997 [1]. Most of the markers expressed in CSCs are expressed also in tumor bulk cells or even in normal somatic tissue cells such as CD133 and others [2].

For example, in human renal cell carcinoma cancer several general cell surface markers were identified and suspected to be in cancer stem cells such as CD105, CD133 (called Prominin-1), CXCR4 and CD44. Moreover, intracellular molecules such as Heat Shock Protein (HSP) and Doublecortin-like kinase 1 (DCLK1) were implicated to maintain renal cancer stem cells which could be used as a markers of renal cancer stem cells [3].

CD133 was also detected in Glioblastoma (GBM) tumor cells as a marker of cancer stem cells [4]. ATP-binding cassette transporter protein 2 (ABCG2) is a marker of classical stem cells was detected earlier in gliomashpher cancer stem cells [5]. Expression level of ABCG2 is positively associated with the increasing pathological grade of glioma showing resistance chemotherapeutic drug [6].

Pluripotent transcription factors such as octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2) and hemobox transcription factor (NANOG), that upregulated in embryonic stem cells, were detected in various type of cancer tumours from adult patients. For example, the expression of SOX2, but not OCT4 was related to colon adenocarcinoma [7]. While both SOX2 and OCT4 were demonstrated significantly in non-small-cell lung cancer [8]. Major transcription factors related to stem cell self-renewal and differentiation, OCT4, SOX2 and NANOG, were detected in Oral

Squamous Cell Carcinoma (OSCC). In addition, SOX2 was related to early detection of OSCC [9].

Similar results have been found in cervical squamous cell carcinoma [10]. Expression of OCT4, SOX2, NANOG and other classical stem cell markers were also detected in tissue samples of colon, prostate, and bladder carcinomas as well as cancer cell lines [11].

Results were repeated with samples from brain cancer, breast cancer, colon cancer, ovarian cancer, oral squamous cell carcinoma, prostate cancer, melanoma, and many other cancers [12-18]. Pluripotent transcription factors were associated with cancer stage, patient survival, cancer treatment and tumor recurrence after chemo-radio-therapy [19,20].

Pancreatic cancer has been the third leading cause of cancer-related death in USA. Most of the cancer patient diagnosed in late stage which minimize the effectiveness of surgical intervention to less 20 percentage. Moreover, chemo-radio therapy is not curative [21]. Thus, the survival rate of patients with pancreatic cancer after 5-years in USA was 7%. 53,070 new cases were diagnosed with pancreatic cancer in 2016, while 41,780 patients was the estimated death from pancreatic cancers [22]. Similar percentage reported globally by world health organization at 2012 [23]. Presence of cancer stem cells within pancreatic tumor was reported by several groups using nonspecific biomarkers. Li et al. [24] found that CD44+/CD24+/ESA+ cells from pancreatic tumor have high tumorigenic potential in compare to negative cells and express self-renewal pathway. These cells represent minor fraction, 0.2-0.8%, of pancreatic tumor cells. Other groups presented CD133 as the main key markers of pancreatic stem cell markers. CD133 positive cells are highly tumorigenic, forming pancreatic tumor in SCID mice, metastatic and invasive [25-27]. Other markers such as c-Met and Aldehyde Dehydrogenases (ALDH) were implicated to pancreatic cancer stem cells identification, functions and drug resistance [28-31].

Expression of pluripotent transcription factors in pancreatic cancer cells is not well studied.

Materials and Methods

Cell growth

The human pancreatic ductal cell line, PANC1, was provided by Dr. David Tosh, Bath University. Human skin fibroblasts were provided by Dr. Matthew Wright, Liver research group, school of clinical and laboratory sciences, Newcastle University and undifferentiated Human embryonic stem cell line (H9) from Dr. Linda Lako (Newcastle University, UK).

Frozen cells were thawed from liquid nitrogen by warming at 37ºC water bath. Then, cells transferred to 50 ml Falcon tube and centrifuged at 1750 rpm for 3-5 minutes. Supernatant was discarded and cells pellets were re-suspended in appropriate medium. They were grown at 37ºC in a humidified atmosphere of 5% CO_2. Cells were passaged when they reached ≈ 80% confluence in tissue culture flask. Media was removed and cells were washed with Phosphate Buffer Saline (PBS). (PAA Laboratories, Cat No: H15-002, Austria). Cells were detached from tissue culture flask by adding 2-4 ml trypsin (TrypLE™ Express Stable Trypsin Replacement Enzyme without Phenol Red. GIBCO. Invetrogen, Cat No: 12604021, Denmark) and incubated for 5-15 minutes. Trypsin was neutralized by adding 10 ml of complete medium. The mixture was centrifuge at 1750 for 3-5 minutes, the supernatant was discarded and cells pellet was suspended in medium and dispensed in new tissue culture flask or plate.

RNA analysis

Total RNA was extracted using the RNeasy Micro Kit 50 (Qiagen) and treated with RQ1 RNase-Free DNase (Promega) to remove contaminating genomic DNA. cDNA was synthesized using superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The PCR contained 1 ml cDNA (500 ng/ml), 1.25 ml REDTaq polymerase (Sigma-Aldrich), 2.5 ml PCR buffer, 1 ml forward primer (10 pmol/l), 1 ml reverse primer (10 pmol/l), 1 ml dNTPs (10 mM) (Promega) and 17.75 ml nanopure water. Parallel PCRs with RNA alone served as negative controls. PCR primer sequences and cycle number are given in table 1. RT-PCR for OCT4 was validated in serial dilutions of the H9 Human embryonic stem cell line, confirming semi-quantification at 35 cycles. Semi-quantitative PCR was also validated for GAPDH as a reference gene at 32 cycles.

Gene	Direction	Sequence	cDNA product size (bp)
Human GAPDH	Forward	AAGGGCTCATGACCACAGTCCAT	328
	Reverse	CAGGAAATGAGCTTCACAAAGTT	
Human	Forward	GAA GCT GGA GAA GGA GAA GCT G	219
Oct-04	Reverse	CAA GGG CCG CAG CTT ACA CAT GTTC	
Human	Forward	CGC CCC CAG CAG ACT TCA CA	169
Sox2	Reverse	CTC CTC TTT TGC ACC CCT CCC ATT T	
Human	Forward	CATCGGGCCCGCCACCATGAGTGTGGATCCAGCTTG	923
Nanog	Reverse	GATCGAGCTCCATCTTCACACGTCTTCAGGTTG	

Human	Forward	GCG TAC GCA AAT TAA AGT CCA GA	302
Rex1	Reverse	CAG CAT CCT AAA CAG CTC GCA GAA T	
Human	Forward	AGT TCC ATG GCA CTG GCC ATA	387
ABCG2	Reverse	TCA GGT AGG CAA TTG TGA GG	
Human	Forward	CGT GCC CTC CTG CTG ACT ATT	132
CXCR4	Reverse	GCC AAC CAT GAT GTG CTG AA	
Human	Forward	AGT GAA GTG GAT GGC TTT GG	162
HGFR[c-Met]	Reverse	GGG CAG TAT TCG GGT TGT AG	
Human	Forward	AGA TGC TCA AGC CGA GTG C	284
CD117[c-Kit]	Reverse	ACT ATC GCT GCA GGA AGA CTC C	
Human	Forward	CGA CTC TAG CTC GAT GCT CTT G	210
CD133	Reverse	GAG CGC AAA GAC TAC CTG AAG A	
Human	Forward	AAA TCC TCT TCC TCT GAG GCT GGA	215
CD34	Reverse	AAG AGG CAG CTG GTG ATA AGG GTT	
Human	Forward	ATC ATT TCT AGC GCA TGG CCT GGT	158
CD31	Reverse	ATT TGT GGA GGG VGA GGT CAT AGA	

Table 1: Primer pairs used for PCR

Immunoflourescent staining of cells

Cells were grown on sterile coverslips in six, twelve or twenty four well plates with slide covers. When the cells became confluent, they were washed 3-4 times in DPBS. Cells to be stained with cytokeratin primary antibodies were fixed with acidified methanol (50 ml methanol with 10 drops of acetic acid) at -20ºC for 10 minutes and then permeabilised with sodium citrate buffer (10 mM Sodium Citrate pH 6.0 was purchased from Sigma-Aldrich, S-4641, Germany) at 37ºC for one hour. Slide covers with cells to be stained with all other primary antibodies were fixed with 4% paraformaldehyde (Sigma-Aldrich, F-1268, Germany) at 25ºC for 20 minutes and permeabilized by 1% t-octylphenoxy-polyethoxyethanol (Triton, Sigma-Aldrich, Germany). For OCT4 staining, slide covers were incubated in pre chilled methanol at -20ºC for 10 minutes after paraformaldehyde fixation, and then permeabilized with 1% triton. Sections were washed with DPBS after each step. To block non-specific binding, the slide covers with cells were treated with blocking buffer (Roche Applied science, East Sussex, UK, cat. No. 11096176001) for 1 hour at 25ºC. Primary antibody diluted in blocking buffer, as the concentration given in table 2, was then added to the cells except those for negative control which are maintained in blocking buffer. The slide covers with cells were incubated overnight in a humid chamber at 4ºC. After three washes of 20 minutes each with wash buffer (Blocking buffer diluted 1:5 in DPBS), secondary antibodies were applied for one hour at room temperature in the dark (Table 3). The slide covers were washed again as above and air dried. Then, the slide covers were mounted using Vectashield containing 4',6-Diamidino-2-Phenylindole (DAPI blue, Vector Laboratory Inc. cat. No. 94010, Burlingame) which stain nuclei with fluorescence blue, slides were covered with cover slips and were sealed with nail varnish.

Antibody	Raised in	Clonality	Specificity	Dilution	Supplier
Anti-Cytokeratin7	Mouse			0.1111111	Provided by Dr.D Tosh.
Anti-Pancytokeratin	Mouse	Monoclonal		0.1111111	Sigma C-2562
Anti-Cytokeratin19	Rabbit	polyclonal	H.M	01:50	Abcam Ab2302
Anti-Cytokeratin19	Mouse	monoclonal	H.M.R	01:50	Progen. 65129
Anti-Cytokeratin19	Mouse	monoclonal	H.M.R	01:50	Dako
Anti-Ki67	Mouse	Monoclonal	H.M.R	0.1111111	Novocastra laboratories Ltd.
Anti CA19-9	Mouse	Monoclonal	H.M.R	0.1111111	Novocastra lab Ltd.

Anti-Oct4	Rabbit	polyclonal	H.M	01:50	Abcam Ab18976
Anti-Sox2	Rabbit	polyclonal	H.M	01:50	Abcam Ab15830
Anti-Nanog	Rabbit	polyclonal	H.M	01:50	Abcam Ab18976
Anti-CD31	Mouse	Monoclonal	H	01:50	Dako M0823
Anti-ABCG2	Rat	Monoclonal	H.M	01:50	Abcam Ab24115
Anti-CK18	Mouse	Monoclonal	H.M.R	0.1111111	Sigma C8541
Anti-Vimentin	Goat	Monoclonal	H.M.R	0.1111111	Sigma V4630
Anti-CD133	Mouse	Monoclonal	H.M	0.1111111	Cell Signalling Technology
Anti-SSEA4	Mouse	polyclonal	H.M	01:50	Abcam Ab
Anti-TR1-60	Mouse	polyclonal	H.M	01:50	Abcam Ab
Anti-Nanog	Goat	polyclonal	H	0.7361111	RD AF1997
Anti-GAPDH	Rabbit	polyclonal	H.M.R	1:200`	Sc-25778

Table 2: Primary antibodies and dilutions. GP: Guinea Pig; H: Human; M: Mouse; R: Rat

Antibody against	Raised in	Conjugation	Specificity	Dilution	Supplier
Mouse	Goat	FITC	IgG	0.25	Jackson ImmunoResearch 115-095-005
Mouse	Goat	TR	IgG	0.25	Jackson ImmunoResearch 115-075-008
Mouse	Goat	TRIC	IgG	0.25	Sigma. T-6653
Mouse	Chicken	TR	IgG	0.25	Abcam. Ab6812
GP	Rabbit	FITC	IgG	0.25	Sigma. F-7762
GP	Donkey	FITC	IgG	0.25	Jackson ImmunoResearch 706-076-148
Goat	Rabbit	FITC	IgG	0.25	Sigma. F-7367
Goat	Donkey	TR	IgG	0.25	Jackson ImmunoResearch 705-095-003
Rabbit	Donkey	TR	IgG	0.25	Jackson ImmunoResearch 711-076-152
Rabbit	Goat	FITC	IgG	0.25	Sigma. F-0382

Table 3: Secondary antibodies and dilutions. FITC: Fluorescein Isothiocyanare; TRITC: Tetramethylrhodamine Isothiocyanate; TR: Texas Red

Transgene expression studies

Transfections were performed using 10 ml Lipofectamine 2000 (Invitrogen) and 3 mg plasmid DNA pIRES-eGFP and OCT4-eGFP (provided by Dr. Wei Cui, Imperial College London) following the manufacturer's instructions. Human embryonic stem cells and induced pluripotent stem cell express the Enhanced Green Fluorescent Protein (EGFP) reporter gene under control of the OCT4 promoter [32,33].

Results

RT-PCR analysis for set of proposed embryonic and adult stem cell markers were carried out in PANC1 cell line in comparison to hESCs and human skin fibroblast as positive and negative controls, respectively (Figure 1). PANC1 shows high positivity for ABCG2. Expression of mRNA for OCT4, SOX2 and
C-X-C chemokine receptor type 4 (CXCR4) is very weak in PANC1 sample in comparison to human Embryonic Stem Cells (hESCs). Nanog and Rex1 mRNA were not detected.

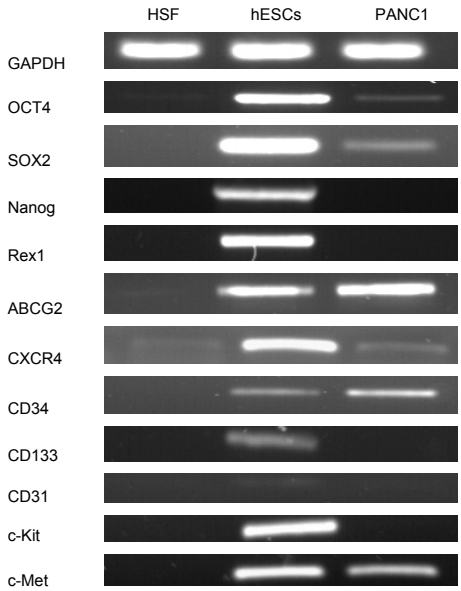

Figure 1: Expression of adult stem cell markers in PANC1. Some pluripotent stem cell markers, OCT4, SOX2 and ABCG2 were detected in PANC. CD34 (c-Met), hematopoietic stem/progenitor cell marker, were also expressed. HSF and hESCs were used as negative and positive controls, respectively.

A hematopoietic stem cell marker, CD34, was expressed strongly By PANC1. Other hematopoietic stem cell/endothelial progenitor markers, CD133, as well as differentiated endothelial marker, CD31, were not detected.

Cell surface markers which used to recognize hematopoietic progenitor cells were assessed. Hepatocyte growth factor receptor, c-Met, which is used to identify Mesenchymal-Epithelial Transition (EMT) in addition to stemness, was expressed by PANC1, while CD117 (c-Kit) was not detected.

Phenotypic characteristics of PANC1 cell line at protein level were determined by immunostaining method. To prepare PANC1 cells for staining, they were seeded in 6-well plate containing sterile coverslips and incubated overnight at incubator.

Each cell of PANC1 shows proliferative capacity by positive staining with Ki-67. Ductal epithelial phenotype of PANC1 cells were assessed using ductal specific markers including CK19 and CK18. Tumorigenic feature of PANC1 was demonstrated by expression of CA 19-9. Beside ductal markers, PANC1 cells express mesenchymal marker, vimentin (Figure 2). OCT4 protein was detected in cytoplasm of very few PANC1 cells (Figure 3).

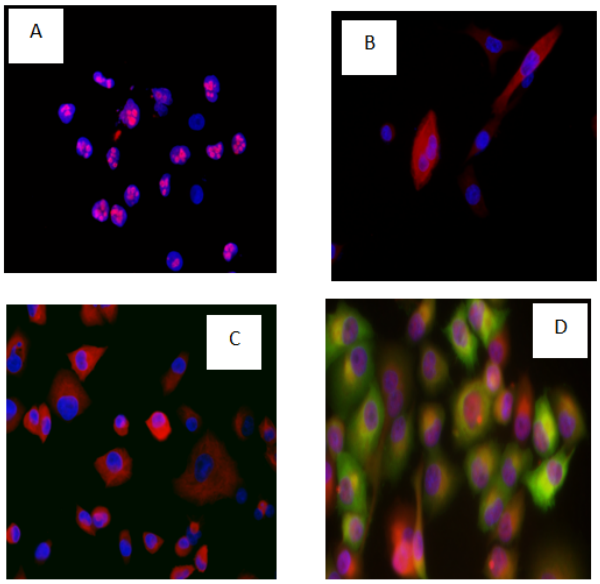

Figure 2: Phenotypic characterization of PANC1. PANC1 has highly prolifrative capacity demonstrated by Ki67 staining (red) and express mainly ductal markers such as CK18 (red B) CA19-9 (red C) and CK19 (red D) in addition to mesenchymal marker, vimentin (green D). DAPI (blue) showed nuclear staining.

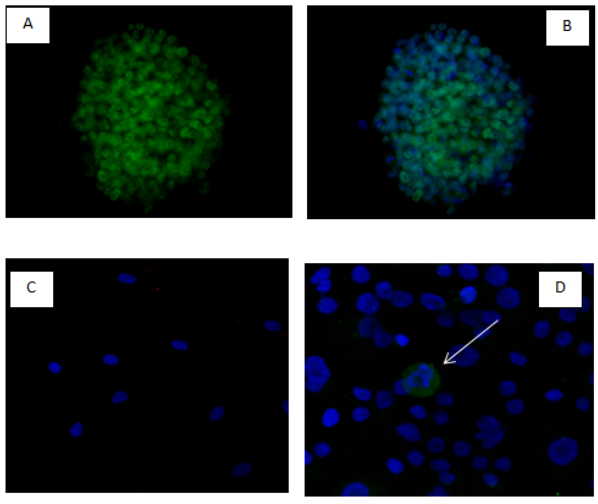

Figure 3: OCT4 staining of PANC1Human embryonic stem cells (H9) showed nuclear OCT4 staining. Dapi was used to stain nuclei. All cells were positive especially the core of clump (A & B). Human skin fibroblast cells do not stain for OCT4 confirming the specificity of the antibody (C). Very few PANC1 cells were express OCT4 protein with cytoplasmic pattern (green). DAPI (blue) showed nuclear staining (D).

Based on transfection efficiency experiment by using conditions which result in higher efficiency. Efficiency of transfection of PANC1

cells with pIRES-eGFP plasmid demonstrated 12% of successful transfection by three repeated studies (Figure 4A). Transfection of PANC1 cells with plasmid containing eGFP downstream of OCT4 promoter showed few cells (1-2% approximately) emitting green florescence (Figure 4B). Staining of these cells with OCT4 antibody results in cytoplasmic localization of OCT4 protein (Figure 4C).

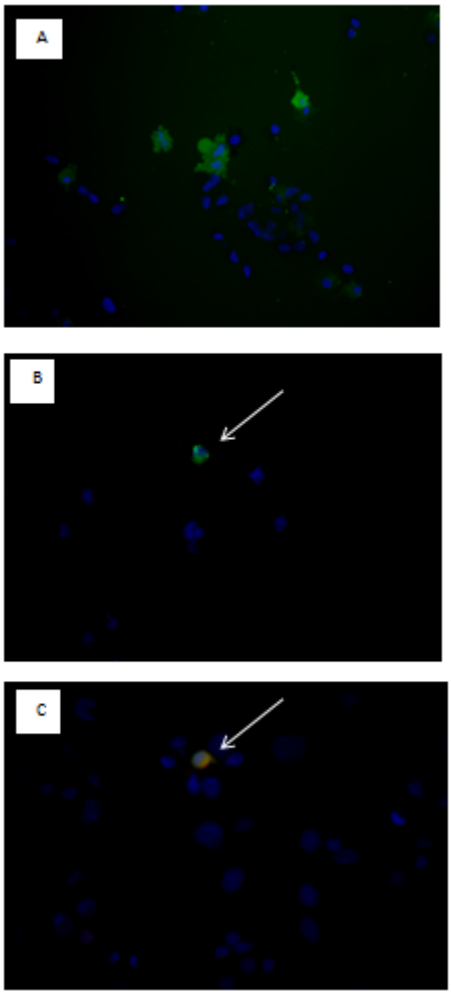

Figure 4: PANC1 Transfected with OCT4-eGFP plasmid. Panel A showed significant number of PANC1 cells transfected with plasmid containing CMV driven eGFP. Panel B & C showd very low percentage of cells express eGFP (green) under control of OCT4 promoter. In addition, this cell successfully stained with demonstrated that OCT4 antibody (red) in the cytoplasm of cell (panel C).

ABCG2 expression was detected in PANC1 which confirmed by staining. ABCG2 could be localized with OCT4-eGFP expressed cells (Figure 5).

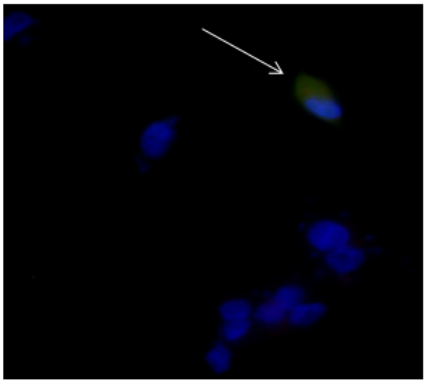

Figure 5: ABCG2 staining of PANC1. PANC1 cells were transfected with OCT4-eGFP plasmid, then stained with ABCG2 antibody (red).

Discussion

Cancer is a group of diseases forming a leading cause of death worldwide. Among cancer types, pancreatic cancer represents 3.1% of all new cancer cases in the US and record the worst survival rate (7.0%) after 5-year of diagnosis [34]. Invasiveness and resistance to conventional chemo-radio therapy present pancreatic cancers in the top of lethal cancer. Cancer stem cells theory gives a possible explanation of the mechanism leading to invasiveness, recurrence, treatment resistance of different type of cancers. However, unavailability of specific markers for CSCs limits the progress of identification and target therapy of CSCs. Several studies have used adult stem cell markers such as CD133, CD34, CD44 and other surface markers to identify CSCs from tumor bulk cells including pancreatic cancer tissue and cell lines. Lee et al. [26] demonstrated unstable results of these markers applied for different pancreatic cancer cell lines. Even though, CD133 positive cells showed higher tumorigenicity when transplanted in NOD/SCID mice, three of four pancreatic cell lines do not express or weakly express CD133. Other study on breast cancer cell lines demonstrated that cultural environment affects the expression surface markers and percentage of positive cells [35].

Embryonic stem cell markers were studied in cancer tissues other than pancreas [10-18]. These markers, such as OCT4, have important role in renewal of stem cells [36,37] which is the most important feature of cancer stem cells leading to cancer reoccurrence. Even though, nuclear OCT4 expression is considered as a stem cell specific pattern and reflect the expression of OCT4A isoform [38,39]. However, running of Human Skin Fibroblast (HSF) with each run as negative control excluded artificial, contamination or pseudogenes detection. Cytoplasmic OCT4 staining was demonstrated in different type of tumor cells such as pheochromocytomas and Germ cell tumours [40,41], Similar finding was reported to other classical embryonic stem cell markers including SOX2, Nanog and others. For example, OCT4, SOX2 and Nanog cooperate together to maintain the pluripotency of ESCs [42].

Our results reported the expression of pluripotent markers, OCT4 and SOX2, in PANC1 cells which prove the presence of stem cells within pancreatic cancer cell line. However, unexpression of important adult stem markers, CD34 and CD133, may exclude hematopoietic

origin of these cells. Interestingly, our results demonstrated that PANC1 express marker that induces EMT, Hepatocyte growth factor receptor (c-Met) [43]. Vimentin expression may support the transition of PANC1 cells toward mesenchymal phenotype. Also, markers implicated in migration of stem cells such as CD34 and CXCR4 [44-46] are detected in PANC1. These findings may explain the highly invasiveness and metastatic nature of pancreatic cancer. On the other hand, resistance feature of pancreatic cancer to conventional chemo-radio therapy may refer to the expression of ABCG2 which play important role multidrug resistance including chemotherapy of different tumor [47].

Conclusion

Embryonic stem cell markers could be useful tools to identify cancer stem cells within pancreatic tumor. However, purification of pancreatic cancer cells that express embryonic stem cell markers is required to evaluate their tumorigenicity. Moreover, targeted therapy against these markers will improve pancreatic cancer prognosis.

Availability of Data and Material

Materials are written in methods section and listed in tables. Datasets presented in result section and figures.

Authors' Contributions

Hussain R Al-Turaifi: Design and run experiments, analyze data and write up the manuscript.

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