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AN INSIGHT ON THE IDENTIFICATION OF CANCER STEM CELLS USING NOVEL IMMUNOLOGICAL AND MOLECULAR STRATEGIES

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ABSTRACT

Cancer stem cells (CSCs) are defined as a population of cells present in tumours, which can undergo self-renewal and differentiation. Identification and isolation of these CSCs using putative surface markers have been a priority of research in cancer. With this background we selected pancreatic normal and tumor cells for this study and passaged them into animal tissue culture medium. Further staining was done using alkaline phosphatase and heamatoxilin staining. Blue to purple colored zones in undifferentiated pluripotent stem cells and clear coloration in the chromatin material indicated pancreatic cells. Further studies on the cell surface marker CD 44 were done using ELISA. For this, the protein was extracted from cultivated normal and tumor pancreatic cells and absorbance was taken in ELISA reader. However, there was no significant difference in optical density values obtained with normal and tumor pancreatic cells indicating further studies are required for upregulation of CD44 in tumor cells. Reverse Transcriptase-polymerase chain reaction (RT-PCR) amplification of insulin growth factor binding protein 5 (IGF-BP5), showed negative result with pancreatic tumor, indicating there is no gene expression in tumor cell.

KEYWORDS: Cancer, Stem cells, ELISA, CD44, RT-PCR.

INTRODUCTION

ABCG2 is a member of the ATP binding cassette (ABC) transporters, which can pump a wide variety of endogenous and exogenous compounds out of cells.[1-3] It was first cloned from doxorubicinresistant human MCF-7 breast cancer cells and named as breast cancer

resistance protein (BCRP). The side population phenotype, which is characterized by the ability to transport Hoechst 33342 out of cells, has been identified as a characteristic feature of stem cells.[4, 5] ABCG2 plays an important role in promoting stem cell proliferation and the maintenance of the stem cell phenotype.[6] Based on a RNA interference approach, the suppression of ABCG2 could significantly inhibit cancer cell proliferation. Furthermore, the blocking of ABCG2 function by fumitremorgin C, a chemical inhibitor, also inhibited cell proliferation via the prolonged G0/ G1 interval.[7] These data suggest that ABCG2 may contribute to cancer cell proliferation. A research learned about diffuse large Bcell lymphoma suggest high levels of ABCG2 is correlated with shorter overall survival and ABCG2 protein levels is correlated with the expression of SHH protein levels which plays a critical role in growth and differentiation during embryonic development.[8]

This strategy of modifying two distinct pathways in cancer cells, i.e., oncogene suppression and cancer suppressor gene replacement, represents a novel approach with potential for developing a rational therapy for this deadly disease.[9, 10] In the present study, we define a means of improving the delivery of both gene-targeting vectors in pancreatic cancer cells and the mechanism underlying the synergy between mda-7/IL-24 and K-ras suppression in inducing apoptosis selectively in K-ras mutant pancreatic tumor cells.[11-13] Inhibiting mutant K-ras directly or targeting one of its downstream activated pathways for inactivation, MEK1/2 but not phosphatidylinositol-3-kinase (PI3K) or p38 mitogen-activated protein kinase (MAPK), results in enhanced translation of mda-7/IL-24 mRNA into protein and consequently apoptosis in mutant K-ras pancreatic carcinoma cells in vitro and tumor growth suppression in vivo in nude mice.[14] This process correlates with enhanced association of mda-7/IL-24 mRNA with polysomes, thereby facilitating the production of protein. Our studies provide support for a ‘‘dual molecular target–based therapeutic approach’’ for inducing programmed cell death in pancreatic cancer cells. Moreover, these findings provide a framework for a reasoned method for effectively treating one of the most aggressive cancers, pancreatic cancer, which may also have potential applications for other neoplastic diseases.[15] In addition, it may be possible to combine this model system with mda-7/IL-24 and combinatorial and natural product chemistry approaches to identify new agents that can directly or indirectly inhibit ras and ras-signaling pathways permitting induction of apoptosis by mda- 7/IL-24.

METHODOLOGY

The substances were dissolved, P^H was adjusted to 7 by flushing with CO_2 gas and volume make up to 1000ml with distilled water (To avoiding little bit contamination add some antibiotic in the medium before sterilization), millipore filtered and stored at 4ºC. This was the medium which is used (with adding 5-10% Fetal bovine serum) for culturing of umbilical cord blood stem cells (especially mesenchymal stem cells). Two types of cell lines thus ready were inoculated into the freshly prepared media flasks using sterile micropipettes (200 µl each). They were then placed in a CO_2 incubator (5%) for 3 days.

Procedure

Fresh media was prepared in 4 ATC flasks. The cells were scraped from the flask's surface with a disposable plastic scraper. Trypsin-PBS was added to the flask with the confluent cells and swirl to ensure coverage of the entire growth surface area. Flask was placed at room temperature for 3-5 mins. (Do not extend this time else the cells will clump together). Freshly prepared media was added and aspirated several times with a sterile micropipette to produce a single cell suspension. Flasks were incubated in a CO_2 incubator (5%) for 3 days.

Acid phosphatase staining of cells for microscopy

Procedure

MSCs were fixed with cold formol calcium and incubated for 10 mins to 1 hour at 37◦C and then washed well with distilled water and then with tap water. Counter staining was then carried out using Mayer's haematoxylin for 15-30 secs. Dehydration was carried out next with ethanol and Observed under the microscope.

Periodic acid Schiff reagent staining of cells for microscopy

Procedure

Required number of cells were taken and fixed with 10% formalin and Oxidized in 0.5% periodic acid solution for 5 mins. After that Rinsed in distilled water and added Schiff's reagent and kept in it for 15 mins (sections turn light pink). Washed in luke warm water for 5 mins (due to which sections become dark pink). Counterstained using Mayer's haematoxylin for 1 min and washed in tap water for 5 mins. Dehydrated with ethanol and observed under the microscope at 200 X.

Protein extraction from Tissue

100 mg of the brain sample was weighed. The samples were shaken for 1 minute at 2000 Hertz. Sample was frozen and added ten times the volume of 1x SDS-buffer. The sample was vortexed for 1 minute at room temperature. Solubilized were transferred to preweighed Eppendorf tube. The sample was incubated for 10 minutes at 70°C with shaking at 1400 Hertz. Sedimented undissolved tissue by centrifugation at 16,100xg for 10 minutes at room temperature. The supernatant was decanted to a fresh Eppendorf tube.

RNA Isolation from tissue

RNA was isolated from Tissue using standard method of Trizol RNA extraction method. 1 ml Trizol, was added and. Incubated for 5 min at RT on shaker. Spinned 5 min at 10000rpm for 4 mins, poured Trizol solution to fresh tube. Add 200 µl chloroform per 1 ml Trizol, shake by hand 15 sec. Incubated for 5 min at RT, centrifuged 15 min at 10000rpm.

RT PCR

Isolated total RNA from the treated cells by first lysing in 0.5 µL of guanidine thiocyanate lysis buffer. Added 50 µL of 2 M sodium acetate, pH 4, 0.5 mL of water saturated phenol, and 0.1 mL of a mixture of chloroform and isoamyl alcohol (ratio of 49:1). Mixed the tubes after addition of each reagent and mix the final suspension vigorously. Leave the tubes on ice for 15 min, then centrifuge at 10,000g for 20 min in a microfuge. Removed the RNA-containing upper aqueous phase to a new tube and add 2–2.5 vol of absolute ethanol. Leave on ice for 15 min to precipitate the RNA. Collect the precipitate by centrifugation at 10,000g for 30 min in a microfuge. The pellet was washed in 70% ethanol, dry and redissolve the RNA in 10–20 µL of DEPC-treated water. Incubated for 90 min at 42°C and Diluted the cDNA by adding 70 µLof T.E. (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and store at –20°C. PCR products were analyzed by electrophoresis through 2% agarose gels and view under ultraviolet (UV) light following ethidium bromide staining.

ELISA

Antigen Coating

Antigen (membrane protein of the cultivated cells) diluted in the ELISA coat buffer was added into the Polystyrene wells. The dilution of the antigen should be in such a pattern that the amount of the antigen adsorbed to the polystyrene plate must be maximum at such dilution. Here the concentration taken is 10µg/ml. Incubation for 2 hours to over night at the

temperature of 37^0C. Store the antigen solution until the immunoassay is performed. Before performing the Immunoassay the wells should be washed in the following way.

PBS-TWEEN wash: Added 300µl of the PBS-TWEEN buffer, mixed it well and leave it for 2 minutes. After that aspirate the buffer from the wells.

BSA-ELISA wash: Added 300µl of the BSA-ELISA coat buffer to the wells and incubated at room temperature for about 20 min. Repeat the PBS-TWEEN wash. Repeat the BSA-ELISA wash.

Test sample addition

Add 300µl of undiluted antibody (CD44 Antibody (8E2F3)) suspension. Incubated it for 4 hour at room temperature or for overnight at 4^0C. PBS-TWEEN washes for 3 times.

Conjugate addition

Added 300µl of 1:5000 v/v diluted [in PBS_TWEEN buffer] conjugate (Anti-CD44 antibody ab41478) and Incubated at room temperature for 2 hrs. PBS-TWEEN washes for 3 times. Added 300µl of 0.15M NaCl and Mixed the solution and leave for 1 to 2 minutes. Aspirate the solution and follow the successive washes. Here the substrate is present in the Sodium Carbonate buffer [pH 9.8] in the concentration of 1 mg/ ml where the buffer is also containing Magnesium Chloride at the molarity value of 1/1000M.

ELISA Readings

The color development is measure by the help of ELISA reader at the wavelength of 400nm.

RESULTS

Figure (1): Animal Tissue Culture media inoculated with cells.

Cultures are more commonly made from suspensions of cells dissociated from tissues. cell cultures, this support is usually provided by the surface of a plastic tissue-culture dish. In most cases, cells in primary cultures can be removed from the culture dish and made to proliferate to form a large number of so-called secondary cultures; in this way, they may be repeatedly subcultured for weeks or months.

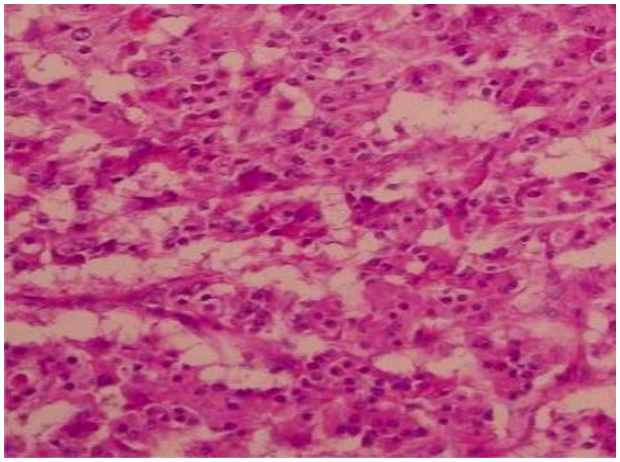

Figure (2): Alkaline phosphatase staining of pancreatic stem cells in 100X.

After the isolation, cultivation and passaging of Pancreatic Stem Cells, Alkaline Phosphatase staining is performed to confirm their growth in the synthetic environment in morphological perspective.

Conclusion of the above result is the visibility of blue to purple colored zones showing the presence of undifferentiated pluripotent stem cells in the cultivated pancreatic cell lines, which confirms the pluripotency [stem cells] in the cultivated pancreatic cell lines, this can be cross confirmed by immunological as well as molecular analysis in later steps.

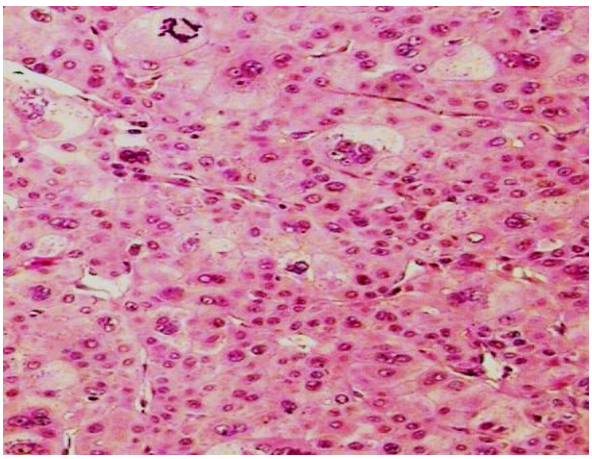

Figure (3): Alkaline phosphatase staining of pancreatic tumor cells in 100X.

Conclusion of the above experiment result is that pluripotent cells are identified which are yet to be confirmed whether they are stem cells or tumor cells by further immunological and molecular studies.

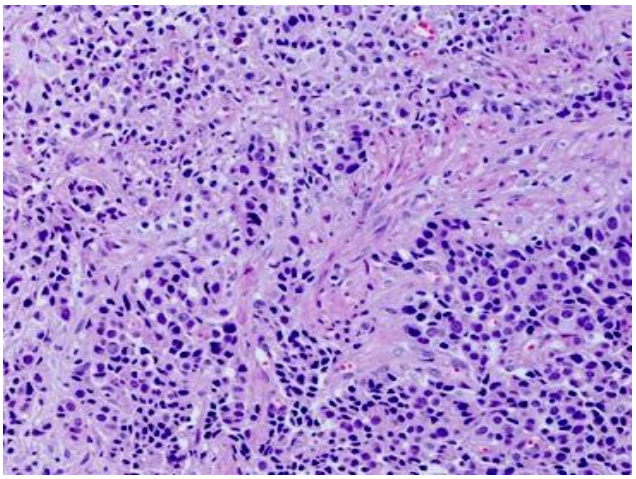

Figure (4): Heamatoxilin staining of pancreatic cells.

Heamatoxilin staining is performed to check the viability of the cells. By observing the above photograph it can be confirmed that the heamatoxilin staining of the cultured pancreatic cells show clear coloration in their chromatin material.

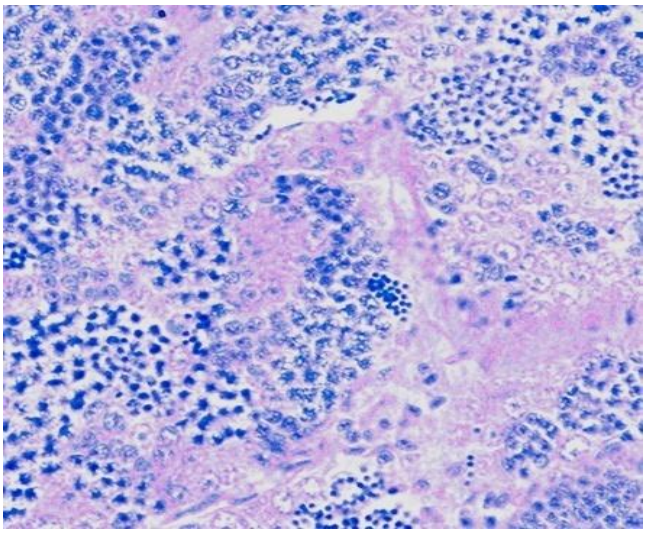

Figure (5): Heamatoxilin staining of pancreatic tumor stem cells.

Heamatoxilin Staining is performed to the pancreatic tumor cells after few passages to confirm their growth and viability. Active growth is observed in all the cultures which are cultured.

Figure (6): ELISA for CD44.

ELISA

1. **Antigen Used** = Protein extraction Extraction from cultivated cells
2. **Primary Antibodies Used** = Rabbit anti CD44 antibodies [polyclonal]
3. **Secondary Antibodies Used** = Mice anti rabbit Antibodies [Fc specific] coated with HRP conjugates
4. **Substrate** = 3,3',5,5'-Tetramethylbenzidine [TMB]
5. **Reactions results**

a. Sample 1 = [ELISA Result for proteins extracted from cells showing AP staining results +'ve and assumed to be **tumor cells**] = **+'ve**

b. Sample 2 = [ELISA Result for proteins extracted from cells showing AP staining results +'ve and assumed to be **tumor cells**] = **+'ve**

c. Sample 3 = [ELISA Result for proteins extracted from cells showing AP staining results +'ve and assumed to be **tumor cells**] = **+'ve**

d. Sample 4 = [ELISA Result for proteins extracted from cells showing AP staining results +'ve and assumed to be **Stem cells**] = **+'ve**

e. Sample 5 = [ELISA Result for proteins extracted from cells showing AP staining results +'ve and assumed to be **Stem cells**] = **+'ve**

f. Sample 6 = [ELISA Result for proteins extracted from cells showing AP staining results +'ve and assumed to be **Stem cells**] = **+'ve**

In the samples 1 , 2 and 3 (assumed to be tumor cells), the ELISA test for proteins extracted from the cells is **+'ve** and in the samples 4, 5 and 6 (assumed to be stem cells) the ELISA test for proteins extracted from cells is **+'ve**

CD 44 expression was measured using protein extracted from cultivated pancreatic normal cells and tumor cells. Absorbance was taken at 405 nm using ELISA plate reader. There was no significant difference in optical density values obtained with normal and tumor pancreatic

cells indicating further studies on protein (western blotting) are required for upregulation of CD44 in tumor cells when compared to normal cells.

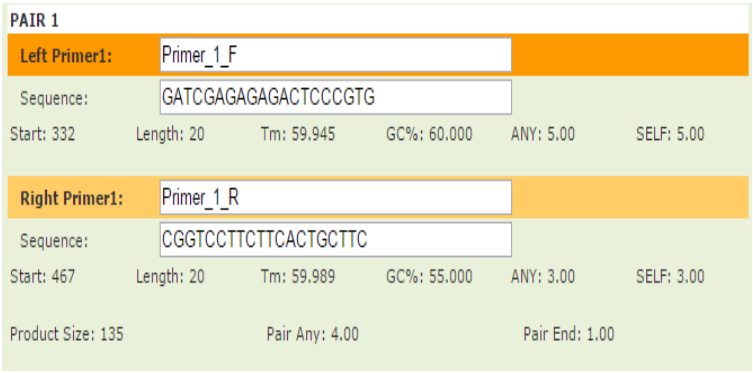

Figure (7): RNA extraction from tissue.

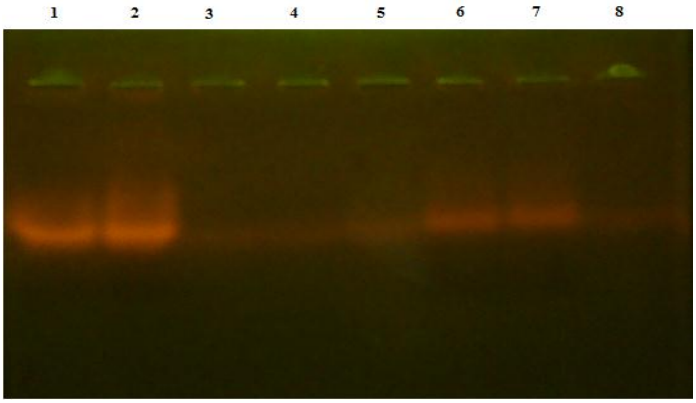

Figure (8): Primer designing

RT-PCR Amplification of IGfbp-5 in tumor and normal pancreatic cell.

Table 1: Agarose gel electrophorosis

Well 1 - Molecular Marker – 3Kb
Well 2 - Molecular Marker – 3Kb
Well 3 - Sample 1 [RT - PCR amplification of RNA extracted from cells showing AP staining results +'ve and assumed to be **tumor cells**] = -'ve
Well 4 - Sample 2 [RT - PCR amplification of RNA extracted from cells showing AP staining results +'ve and assumed to be **tumor cells**] = -'ve
Well 5 - Sample 3 [RT - PCR amplification of RNA extracted from cells showing AP staining results +'ve and assumed to be **tumor cells**] = -'ve
Well 6 - Sample 4 [RT - PCR amplification of RNA extracted from cells showing AP staining results +'ve and assumed to be **stem cells**] = +'ve
Well 7 - Sample 5 [RT - PCR amplification of RNA extracted from cells showing AP staining results +'ve and assumed to be **stem cells**] = +'ve
Well 8 - Sample 6 [RT - PCR amplification of RNA extracted from cells showing AP staining results +'ve and assumed to be **stem cells**] = -'ve

Insulin growth facter binding protein 5 (Igfbp5) can be used as prognostic biomarkers as it is present in both totipotent and tumor cells.

RT-PCR amplification of IGF binding protein 5, showed band size of 3 kb which was clear from the gel picture. However, the sample of RT-PCR amplicon, showed negative result with pancreatic tumor, indicating there is no gene expression in tumor cell. Whereas the assumed normal pancreatic cell showed the size of the molecular marker around 3kb.

DISCUSSION

Stem cells have the remarkable potential to develop into many different cell types in the body during early life and growth. They serve as a sort of internal repair system, dividing essentially without limit to replenish other cells as long as the person or animal is still alive. Stem cells are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. Pancreatic cancer is one of the most lethal cancers among all solid malignancies. CD44, CD24, CESAC cells isolated from human PDAC could self renew, had differentiation potential and had enhanced Shh expression. Stem cells survive in a niche which provides favorable conditions for it to self-renew. It has been identified that tumor stroma is composed of pancreatic stellate cells which undergoes the paracrine Nodal/Activin. It was reported that the pancreatic stellate cells secrete the embryonic morphogens Nodal/Activin. These secretions were found to support the in vitro sphere formation and promote invasiveness of pancreatic CSCs. total RNA extractuion and the further amplification of the target gene in the standartized PCR procedures has given an fruitful out put which is exactly what is expected match with the target gene product. as on the other hand immunological test (ELISA) performed to identify CD44 has procured a positive result. being a basic research approach only qualitative assays are performed for this methodology. quantitative assays are recommended in the further research works.

CONCLUSION

Pancreatic adenocarcinoma is the deadliest solid cancer. Putative pancreatic CSCs, for the first time defined by the simultaneous expression of CD44, CD24 and EpCAM, are highly tumourigenic and possess the ability to both self-renew and to produce differentiated progeny that reflects the heterogeneity of the patient’s primary tumour. From the results procured in

this project it is concluded that molecular identification of protein 5 is observed in the cultivated cell lines as well as ELISA assays are given the positive results for the target protein in the immunological assay. Further studies are recommended in order to cross confirm in a broad spectrum for identification of these pancreatic cancer stem cells.

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