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Establishment and characterization of a bovine rectal myxoma cell line

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ABSTRACT

A new bovine cell line was developed from tumor biopsy material of rectum obtained from clinical case of 7 years old cattle with tumor mass obliterating the rectal opening. Histopathology of tumor revealed scattered stellate cells arranged singly or in clusters in loose mucinous ground substance, simulating myxoma. The cells obtained from tumor mass have been cultured for more than 36 months in DMEM supplemented with 10% fetal bovine serum (FBS). The population doubling time of this cell line was about 20.64 h. The cytogenetic analysis revealed several chromosomal abnormalities with bizarre karyotype. The origin of the cell line was confirmed by PCR amplification of 1086 bp fragment of 16s rRNA using bovine species specific primers. The new cell line would act as *in vitro* model to study many aspect of cancer biology such as tumor development, differentiation and therapeutics regimen to combat cancer.

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1. Introduction

Cancer has been considered as the most dreaded disease for both human and animal because of its incurability. Majority of scientific community with maximum research grant is being engaged for development of suitable cancer therapeutics. Better understanding of the molecular pathogenesis of cancer will help us to design appropriate drug to combat cancer (Rodrigues et al., 2014). Heterogenous nature of cancer makes each cancer type unique demanding a different regime with a different set of drug (Julien et al., 2012). Among animals, tumor incidence in cattle has occupied the second spot followed by dogs (Marosfoi et al., 2009). Rectal tumor in cattle is well documented and reported which include myxoma, rectal carcinoma, adenoma, fibroma and fibrosarcoma (Danova et al., 2009; Suzuki and Ohshima, 1993; Bertone, 1990). Rectal myxoma is the most common neoplasm involving the rectum of cattle (Rao, 2004). Myxoma is a tumor of specialized fibrous tissue; hence it has the ability to secrete mucin a myxoid matrix rich in acid mucopolysaccharides (Dennis, 2008). These tumors have been diagnosed in every age of cow, ranging from virgin heifers to very old cows (Loupal and Baumgartner, 1984).

Usual treatment of animals with myxoma is surgical removal of tumor mass. Lack of success of therapeutics in treatment of cancer may be attributed to inadequate knowledge of cancer biology. In comparison to cancer research in human the animal cancers are not well studied. In addition, animals do not receive an equivocal treatment like humans. Cancer cell line plays a crucial role as an essential tool in the form of an *in vitro* model for cancer to investigate the mechanism of tumor initiation, progression and various aspects of cancer therapy. Numerous well established cancer cell lines of human are available at various cell banks. However, very few bovine cell lines namely BCE C/D-1b, MDBK (NBL-1), MDBK.P3, CKT-1, EBTr (NBL-4), GM-7372, GM-7373, PEK, PT-80, SBAC, TEK and TR-Ag-9-HPRT- are available at various cell banks. American Type Culture Collection (ATCC) has 12 tumor cell lines by disease originated from bovine. These are 2FLB.Ln (bovine leukemia), BL3.1, LB9.Bm, LB10.Bm, LB9.Sp/Thy/Bm, LB9.Sp, LB10.Sp, LB11.Sp, LB9.Thy, LB10.Thy, LB11.Thy (bovine lymphosarcoma) and BL-3 (bovine lymphosarcoma, leukemia). To best of our knowledge no stable immortal cell line from rectal cancer of bovine has been reported. Bovine tumor cell line would be useful for studies of rectal

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tumor development, tumor cell surface markers, genetic alterations and effect of anticancer drugs. The aim of this study was to establish and characterize a new bovine rectal tumor cell line of Indian Zebu cattle (*Bos indicus*) designated as BRT (bovine rectal tumor).

2. Materials and methods

2.1. Histopathology

A seven year-old female cow with neoplastic masses present on the rectal wall was referred for surgery. Rectal examination revealed the presence of a firm polypoid well demarcated mass measuring approximately 5 cm as located in the posterior rectal mucosa adjacent to the anorectal junction. The mass was surgically removed through the rectal route. All experiments were performed in accordance with the IBSC guidelines of the Care and Use of Laboratory Animals. The mass was fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Paraffin sections (3 mm) were stained with hematoxylin and eosin (H&E) and Masson's trichrome. In addition, Argyrophilic nucleolar organizer regions (AgNORs) staining was used to estimate the proliferative potential of tumor.

2.2. Cell preparation and culture

Post-operative tumor tissue was minced aseptically with sharp curved scissors. The mince was placed into a flask containing a 0.25% solution of trypsin (Sigma, USA) and stirred with a magnetic stirrer for 10 min at room temperature. Undissociated tissue fragments and debries were removed by cell strainer (BD Falcon, USA) and the supernatant was transferred to a sterile flask containing fetal bovine serum (FBS; Hyclone, Thermo, USA) to stop the action of trypsin. The remaining tissue mince was retrypsinized two more times by the same procedure. All the supernatant were pooled and centrifuged at $180 \times g$ for 10 min. The resulting cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Thermo) supplemented with 10% FBS, 50 μg/ml gentamicin (HiMedia, India) and 2.5 μg/ml amphotericin B (HiMedia, India). A total of 1×10^6 cells were seeded in 25 cm^2 cell culture flask (Nunc) and incubated at 37 °C with 5% CO_2 in CO_2 incubator (Eppendorf, Galaxy 170R). The medium was changed 3 days later for continuous subculture.

2.3. Serial passage and establishment of BRT cell line

Cells were passaged at 3–4 days interval by centrifugation. Briefly, cells were centrifuged at $180 \times g$ for 10 min, supernatant was removed, the cell pellet was resuspended in DMEM and 1×10^6 cells were seeded in 25 cm^2 cell culture flask. For adherent cells, flasks were subcultured at 80–90% confluency by trypsinization with 0.25% trypsin at 37 °C until the cells were detached from the flask. Single cell suspension was prepared by pipetting and 5×10^5 cells were seeded in 25 cm^2 cell culture flask. These cells were being maintained since last 30 months with more than 120 passages by subculturing regularly every 4–5 days interval or when 80–90% confluent.

2.4. Cell growth assays

The *in-vitro* growth properties of BRT cells were assessed by their doubling time. Cells from a confluent flask were harvested by trypsinization and 1×10^5 cells were seeded per well in a 6 well culture plate. Cells were harvested and counted at 24 h interval for 6 days. Briefly, cells from one well were harvested and single cell suspension was prepared by pipetting. Trypan blue dye was added to cell suspension at 1:1 (v/v) ratio and 10 μl of the mixture was charged on a cell counter slide (Countess, Invitrogen) and cell number was counted in a cell counter (Countess, Invitrogen). A growth curve was constructed and the doubling time was calculated using formulae used by Roth, 2006.

2.5. Genomic DNA preparation and PCR amplification

The genomic DNA from the HeLa, Vero and BRT cell lines was extracted using Qiagen DNA extraction kit (QIAamp DNA Mini Kit, Germany), as the manufacturer's instructions. DNA yield was quantified by spectrophotometer (Nano-view) and the integrity of extracted DNA was checked by agarose gel electrophoresis using 1% agarose. In order to check origin of BRT cell line and to assess any cross-contamination between different cell lines nested PCR was performed using previously reported primers (Ono et al., 2007) for species identification of cultured cells. In first PCR mitochondrial DNA fragment was amplified using universal primer pair complementary to conserved region of fourteen vertebrate species. The sequences of forward and reverse universal primers were 5′-THGTHSAATGAATCTGAGGVGGVT-3′ and 5′-CGATGTTGGATCAGGACATC-3′ respectively. The second PCR/nested PCR amplify the amplicon from first PCR using species specific primer. The sequences of forward and reverse bovine species specific nested primers were 5′-CCTAGATGAGTCTCCCAACTC-3′ and 5′-GTTGTTTAGTC-GAGAGGGTATC-3′ respectively. The amplification was carried out in 50 μl final volume containing 1 units *Taq DNA* polymerase (Fermentas), buffer (Mg^{2+}: 2 mM), dNTPs (50 μM each), 10 pmol of each primer and 100 ng of sample DNA. The amplification was carried out in a PCR Thermal Cycler MP (Biometra). In the first PCR, after a denaturation at 94 °C for 5 min, 35 cycles of amplification were performed as follows: denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s and extension at 72 °C for 90 s. A final extension step of 10 min at 72 °C ended the reaction. The first amplified product was diluted into ten times (1:10) with nuclease free water (NFW), and 1 μl of diluted product was used as template DNA for the second PCR. In the second PCR, denaturation at 94 °C for 5 min, 35 cycles of amplification were performed as follows: denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for 90 s. A final extension step of 10 min at 72 °C ended the reaction. The PCR product was separated on 1% agarose, stained with ethidium bromide and checked on UV transilluminator.

2.6. Cytogenetics analysis

For cytogenetic analysis, chromosome preparations from the BRT cells at passage 25 & 50 were obtained by the conventional technique Worton and Duff (1979). The cells in the exponential growth phase were treated with 10 μg/ml colcemid (Gibco-BRL) for 1 h and then with 0.075 M KCl solution for 15 min. The cells were fixed in Carnoy's solution (Methanol and glacial acetic acid, 3:1 [v/v]) and dropped onto chilled wet slides. The chromosome number of cells was counted by conventional Giemsa staining using a Leica DMRB microscope.

3. Results

3.1. Histopathology of tumor biopsy

This paper describes a case of myxoma/myxosarcoma seen in the rectum of a cow, diagnosed on the basis of histopathological findings. The tumor mass surface appeared rough honey like areas with reddish discoloration and presence of viscid mucous substance (Fig. 1). On H&E staining, the growth showed stellate to fusiform cells with round, ovoid or elongated nuclei. The tumor

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