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Establishment and characterisation of two novel breast cancer cell lines

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Abstract

Two novel oestrogen receptor (ER) negative breast cancer cell lines, BCa-11 (familial) and BCa-15 (sporadic) were successfully established from primary tumours. Characterisation of these cell lines showed expression of epithelial specific antigen and cytokeratins confirming their epithelial lineage. Analysis of ultrastructure and anchorage independent growth confirmed the epithelial nature and transformed phenotype of these cells. Both cell lines showed loss of pRb, Dab2 and ERα and elevated levels of proliferation marker Ki67. In addition, BCa-11 cells showed loss of HOXA5, tumour suppressor genes $p16^{INK4A}$ and RARβ as well as overexpression of CyclinD1. Elevation of *DNMT1* and *DNMT3B* transcript levels, promoter hypermethylation of *RASSF1A*, *RARβ2*, and *HOXA5* further support their neoplastic origin. In conclusion, the two ERα negative breast cancer cell lines established herein have certain useful characteristics that may make them valuable for understanding the mechanism of oestrogen receptor negative breast tumours and testing new drugs.

Keywords: Breast carcinoma; Indian; Familial; Sporadic; Cell lines

1. Introduction

Breast cancer is the second leading cause of cancer deaths in women. Incidence rates of breast cancer are increasing in most countries, particularly in Asia, with 3% annual increase in incidence as compared to 0.5% in the rest of the world (Parkin et al., 2005). The incidence of breast cancer is rising alarmingly, particularly in developing countries such as India. The molecular mechanisms underlying breast carcinogenesis are not understood completely. Cell lines provide an unlimited, self-replicating source of cells that can be widely distributed to facilitate comparative studies. Continuous cell lines established from primary tumours provide important experimental systems for studying the biology and genetic changes associated with tumour initiation and progression. Since the establishment of the first human breast carcinoma cell line BT-20 in 1958 (Lasfargues and Ozzello, 1958), many attempts have been made to establish permanent breast tumour cell lines. However, only limited success has been achieved in cultivating long-term epithelial cell cultures from human primary breast tumours (Gazdar et al., 1998; Wang et al., 2000). To date the majority of breast cancer derived cell lines have been obtained from secondary tumours and pleural effusions of patients with advanced stage breast cancers. The cultures established from secondary deposits are those collected late in the development of disease by which time there may have been a modification of the original tumour. Although about 100 human breast cancer lines have been described in the literature, the number of breast tumour cell lines that have been adequately characterised and are widely used is only about 20 (Engel and Young, 1978; Lacroix and Leclercq, 2004).

Herein we report the establishment and characterisation of two oestrogen receptor negative breast cancer cell lines, one from a familial and the other from a sporadic primary breast carcinoma.

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2. Materials and methods

2.1. Tissue specimens and establishment of primary cultures

Breast carcinoma tissue were collected and processed for setting up the primary explant cultures as described previously (Kaur and Ralhan, 2003), except that these cultures were propagated in DMEM/F12 medium. The cultures were monitored for cell growth and propagated by periodic sub-culturing by trypsinisation.

2.2. Purification of epithelial cells

The epithelial cell population was enriched by differential trypsinisation as described previously (Kaur and Ralhan, 2003; Rohatgi et al., 2005). Thereafter fibroblast overgrowth was reduced by selective chemical elimination using geneticin (G418 sulphate, 100 μg/ml) (GIBCO BRL, Grand Island, NY) as described by Ji et al. (2001). Epithelial cells were then purified using MACS epithelial columns (Milteny Biotech GmbH, Germany). These eluted purified epithelial cells were plated in a culture flask containing DMEM/F12 (Gibco Invitrogen Cell Culture, Grand Island, NY) medium supplemented with 10% FBS (Gibco Invitrogen Cell Culture), crystallised bovine insulin (5 μg/ml) (Sigma–Aldrich, Bangalore, India), epidermal growth factor (10 ng/ml) (Gibco Invitrogen Cell Culture) and propagated regularly. The cells were maintained in medium supplemented with growth factors until the 12th passage, after which growth factors were withdrawn gradually from the medium. The cells could be grown without growth factors after the 18th passage.

2.3. Light and electron microscopic analysis

Breast cancer cells at various passages were examined and photographed using an inverted phase-contrast microscope (Olympus, Tokyo, Japan). Cells grown on coverslips were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0), and processed for cell surface characterisation by scanning electron microscopy (SEM) (Leo 1530 field emission scanning electron microscope; see Kaur and Ralhan, 2003). Cells in monolayer were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and processed for examining internal cell structures using a transmission electron microscope (TEM) (MORGAGNI-2680; see Kaur and Ralhan, 2003; Rohatgi et al., 2005).

2.4. Growth rate and colony formation in soft agar

Cells were plated in triplicate at a cell density of 2×10^4 cells per well in a 24-well plate. Cells were trypsinised and cell number was counted using a haemocytometer at regular time intervals (24–168 h). Growth curves were plotted and doubling times calculated from the exponential phase.

Anchorage-independent growth was assessed by suspending 1×10^5 cells in 1 ml 0.3% (w/v) soft agar (Difco Laboratories, Detroit, MI, USA) layered over 0.5% (w/v) base agar in 35 mm dishes. Agar was supplemented with DMEM/F12 and 10% FBS. Formation of colonies was determined by inverted microscopy at 2 and 4 weeks post-seeding.

2.5. Immunocytochemistry

Cells grown on coverslips were fixed with acetone and processed for immunocytochemical analysis (Kaur and Ralhan, 2003). Briefly, cells were immunolabelled with primary antibodies (1 μg/ml) followed by biotinylated secondary antibody (0.8 μg/ml; DAKO, Produktionsvej, Glostrup, Denmark). They were incubated with streptavidin conjugated horseradish peroxidase (HRP) and the colour developed using diaminobenzidine (DAB) as the chromogen. Slides were counterstained with Mayer's haematoxylin, mounted with DPX mountant and examined under a light microscope.

2.6. Mutational analysis of TP53

Genomic DNA was isolated from BCa-11 and BCa-15 cells by the standard phenol–chloroform method (Blin and Stafford, 1976). Polymerase chain reaction (PCR) amplified products encompassing exons 5, 6, 7, 8 and 9 of the *TP53* gene (which harbour mutational hotspots) were analysed for mutations by direct automated sequencing (ABI Prism 377 sequencer, Applied Biosystems, Foster City, CA, USA).

2.7. RT-PCR analysis of DNMT1, DNMT3A, DNMT3B, BRCA1 and BRCA2 expression

Total RNA was prepared using Trizol reagent (Invitrogen, Inc., Rockville, MD). Five micrograms of total RNA were reverse-transcribed using 18-mer oligodeoxythymidylate and MMLV (Invitrogen) in a volume of 20 μl. Two microlitres of cDNA templates were subjected to PCR amplification, using β-*Actin* as a control of cDNA integrity (Salles et al., 1993). PCR was carried out with 2 μl of cDNA using primers specific for *DNMT1*, *DNMT3A*, *DNMT3B*, *BRCA1* and *BRCA2* using the conditions mentioned elsewhere (Chan et al., 2002; Magdinier et al., 2000; Mizuno et al., 2001).

2.8. Bisulphite treatment and methylation specific PCR

Genomic DNA was isolated from the cells using the phenol–chloroform method (Blin and Stafford, 1976) and subjected to bisulphite treatment, as described previously (Bagadi et al., 2007). Methylation specific PCR was carried out using 50–100 ng of bisulphite treated DNA in a PCR mixture containing: 16.6 mM ammonium sulphate; 67 mM Tris (pH 8.8); 6.7 mM $MgCl_2$; 10 mM β-mercaptoethanol; 1.25 mM each dNTPs and primers 1.6 μM each in a 25 μl reaction. Reactions were hot started at 95 °C for 5 min before the addition of 1.25 U of *Taq* polymerase (Herman et al., 1996). The sequences of the primers (purchased from Microsynth) used for each gene are listed below:

- *BRCA1:* methylated, forward 5′-TCG TGG TAA CGG AAA AGC GC -3′, reverse 5′-AAA TCT CAA CGA ACT CAC GCC G-3′ (product size: 75 bp) unmethylated, forward 5′-TTG GTT TTT GTG GTA ATG GAA AAG-3′, reverse 5′- CAA AAA ATC TCA ACA AAC TCA CAC CA-3′ (product size: 86 bp),
- *RARβ2:* methylated, forward 5′-TCG AGA ACG CGA GCG ATT CG-3′, reverse 5′-GAC CAA TCC AAC CGA AAC GA-3′, (product size: 145 bp) unmethylated, forward 5′-AAC CAA TCC AAC CAA AAC AA-3′, reverse 5′-TTG AGA ATG TGA GTG ATT TGA-3′ (product size: 146 bp),
- *HOXA5:* methylated, forward 5′-TTT AGC GGT GGC GTT CG-3′, reverse 5′-ATA CGA CTT CGA ATC ACG TA-3′ (product size: 183 bp) unmethylated, forward 5′-TTG GTG AAG TTG GGT G-3′, reverse 5′-AAT ACA ACT TCA AAT CAC ATA C -3′ (product size: 213 bp),
- *RASSF1A:* methylated, forward 5′-GTT GGT ATT CGT TGG GCG C-3′, reverse 5′-GCA CCA CGT ATA CGT AAC G -3′ (product size: 160 bp) unmethylated, forward 5′-GGT TGT ATT TGG TTG GAG TG-3′, reverse 5′-CTA CAA ACC TTT ACA CAC AAC A-3′ (product size: 180 bp).

3. Results

3.1. Establishment of novel human breast cancer cell lines

BCa (*B*reast *Ca*rcinoma)-11 was established from a 65 year female patient diagnosed with *in situ* ductal carcinoma of the breast. The patient had a family history of breast cancer. BCa (*B*reast *Ca*rcinoma)-15 derived from a 70 year female with Stage IIB invasive ductal carcinoma of the breast. Epithelial cells emerged from the tissue piece within 7 days of initiation of the culture (Fig. 1A,C). After 14 days, the primary culture was confluent, fibroblasts were removed and epithelial cells were purified as