Establishment and Characterization of Chemotherapy-Enriched Sphere-Forming Cells with Stemness Phenotypes as A New Cell Line (BAG50) of Gastric Carcinoma

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Abstract

Gastric cancer is a malignancy with a high mortality rate worldwide. Cancer stem cells (CSCs) are a small subpopulation of tumor cells that possess the tumor-initiating ability, self-renewal capacity, and high resistance to conventional therapies. Due to the diversity and complexity of human tumors, new cell lines are urgently needed to supply clinically and physiologically relevant cancer models. Here, we report establishing a novel cell line (BAG₅₀) with stemness properties. Chemotherapy-enriched sphere-forming cells with CSC properties isolated from a patient with GC were cultured in a serum-containing medium and passaged for up to 51 passages. The colony-forming ability and tumor-forming capacity of BAG₅₀ cells were evaluated *in vitro* and *in vivo*. mRNA upregulation of stemness-related transcriptional factors using real-time PCR as well as expression of CSC markers using flow cytometry was investigated. Finally, STR profiling and chromosome studies were performed. BAG₅₀ cells formed floating spheroid colonies in a serum-free medium. Subcutaneous injection of these cells generated xenograft tumors in nude mice. Pluripotency markers (SOX-2, OCT4, and Cripto-1) in them were upregulated compared with normal gastric cells. The majority of them expressed CSC markers of CD44, CD54, and EpCAM, and stemness marker of oct-4. STR profiling showed a unique DNA fingerprint. Karyotype also demonstrated multiple aneuploidies and chromosomal translocations. We suggested that the highly tumorigenic BAG₅₀ cell line with stem cell-like phenotypes may provide a valuable *in vitro* tool to support new diagnostic, prognostic, and predictive biomarkers as well as the development of more effective treatment strategies.

Introduction

Gastric carcinoma (GC) is the fourth most common malignancy and one of the most leading causes of mortality worldwide (700,000 death annually) [1, 2]. In patients with cancer, chemotherapy is an essential adjuvant therapy in the advanced stages of the disease. However, the development of chemoresistance is one of the most significant obstacles and persistent problems during the effective treatment of cancer [3, 4]. Therefore, an urgent need to establish chemo-resistant cancer cell lines is felt more than ever to investigate the mechanisms of chemoresistance and the effects of new drugs.

Cancer stem cells (CSCs) or tumor-initiating cells (TICs) are a heterogeneous population of neoplastic cells and structurally very similar to normal organs within tumor tissue. They also have stemness properties such as self-renewal, pluripotency, and tumorigenesis. A wealth of evidence attests that stationary and circulating CSCs have chemoresistance highly and cause tumor development (cancer initiation, growth, progression), invasion, metastasis, recurrence, as well as resistance to radiotherapy approaches [5–7]. In addition, the CSCs hypothesis emphasizes the eradication of them for cancer treatment [8, 9]. Thus, CSCs play a crucial role in the discovery of clinical-related molecular targets and the development of anticancer drugs.

Many studies have shown that cells with three-dimensional sphere-forming ability isolated from solid tumors possess the properties of CSCs [9–13]. We also previously isolated and identified chemotherapy-enriched sphere-forming cells from a patient with gastric cancer [5, 14]. The result of our study

demonstrated that chemotherapy-enriched sphere-forming cells have characteristics of CSCs. Several gastric cell lines with different cellular, biochemical and molecular properties have been reported during the last two decades [7, 15–20]. However, very few gastric cell lines with stem cell-like phenotypes have been isolated and characterized [21]. Here, we reported the establishment of chemotherapy-enriched sphere-forming cells as a novel cell line (BAG₅₀).

Materials And Methods

Establishment of BAG₅₀ cell line

Chemotherapy-enriched sphere-forming cells with cancer stem cell properties isolated from a 54-year-old male patient with GC (T3N0M0) were previously identified as described [22, 23]. The ethics committee of MUMS approved the investigation and written informed consent was acquired from patient (930607). The cells were cultured in DMEM/F12 medium (Biosera, France) containing eight mM HEPES (Biosera), 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml gentamicin supplemented with 10% inactivated fetal bovine serum (FBS) (Gibco, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO₂ for five days, with medium changed daily. Then the cells were subcultured every five days for 250 days.

Growth characteristics of BAG₅₀

For determining population doubling time, 3×10^5 viable cells of passage 51 were first seeded into 25-cm² flasks with a five ml growth medium of DMEM/F12 supplemented with 10% FBS that was replaced every three days. Then, the cells detached by trypsinization were counted daily for 14 days using the trypan blue staining and a Neubauer hemocytometer chamber.

Sphere formation assay

To evaluate colony-forming ability of BAG₅₀ cell line, 5×10^4 viable single cells were plated in five ml serum-free DMEM/F12 medium containing 20 ng/ml EGF (Gibco), ten ng/ml bFGF (Gibco), ten ng/ml LIF (ProSpec, Israel), 4 µg/ml heparin (Sigma-Aldrich, MO) supplemented with 2% B-27 for one month.

Tumor formation assay

After the approval of the experimental procedures by the Institutional Animal Care and Use Committee of Mashhad University of Medical Sciences. The viable single cells (1×10^6 cells) of BAG₅₀ in Matrigel suspended in serum-free DMEM/F12 medium (Sigma-Aldrich) (1:1) were subcutaneously inoculated into each of three male athymic nude mice (C57BL/6 strain), 4-6 weeks, using 100 µl microsyringe in North Research Center, Pasteur Institute of Iran and maintained for four weeks.

Monitoring stemness-related transcriptional factors in the BAG₅₀ cell line Culture by Real-Time PCR

Isolation of total RNA from cultured cells in DMEM/F12 medium supplemented with 10% FBS and normal gastric cells was performed using mRNA Isolation Kit (Roche Applied Science, Germany). The amount and purity of total RNA were determined using a spectrophotometer (WPA Biowave II, UK). After synthesizing cDNA using Easy cDNA Synthesis Kit (Pars Tous Biotechnology, Iran), mRNA expression of transcriptional factors OCT4, SOX2, and Cripto-1 were performed by quantitative real-time PCR. The reactions were carried out with the fluorescent dye SYBR Green/ROX Master Mix on an Mx3000P QPCR System (Stratagene, CA) using gene-specific primers (listed in Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control. The relative expression of genes (OCT4, SOX2, Cripto-1) was calculated based on the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primer sequences for the amplification of target genes and GAPDH

Genes	Primer sequence 5' -> 3'
OCT4	For: GCAGCGACTATGCACAACGA Rev: CCAGAGTGGTGACGGAGACA
SOX2	For: AACAGCCCGGACCGCGTCAA Rev: TCGCAGCCGCTTAGCCTCGT
Cripto-1	For: GGGATACAGCACAGTAAGGAG Rev: ACGGTGGTAGTTCTGGAGTC
GAPDH	For: GGAAGGTGAAGGTCGGAGTCA Rev: GTCATTGATGGCAACAATATCCACT

Detection of surface markers CD44, CD54, and EpCAM, and stemness marker oct-4 in the BAG₅₀ cell line using flow cytometry

Proliferated cells in DMEM/F12 medium containing 10% FBS were harvested with TrypLE™ Select 1X (Gibco). The resulting cells resuspended in PBS were labeled with stemness-related transcription factor oct-4 and epithelial markers conjugated with different fluorescent dyes, including FITC anti-mouse/human CD44 (BioLegend, USA), PE anti-human CD54 (BioLegend), and FITC anti-EpCAM (BioLegend) antibodies and all the isotype controls (FITC-IgG1 (BioLegend), PE-IgG1 (BioLegend)). After 15–20 min incubation and washing with PBS, the expression of molecular markers was assessed with a multiparametric flow cytometer BD FACS Aria (USA). Data analysis was carried out using FlowJo analysis software.

STR profiling of the BAG₅₀ cell line

BAG₅₀ cell line (the 51st passage) were subjected to the short tandem repeat (STR) profiling. DNA was extracted from cell line BAG₅₀ using high pure PCR template kit (Roche, Germany). STR profiling has been

performed using a KBC IR Filing kit including 16 markers (KBC, Iran) according to the kit protocol. PCR products were analyzed using the ABI 3500 Genetic Analyzer (Applied Biosystems, USA). Data were analyzed with Gene Marker software version 2.2. (Soft Genetics).

Chromosome Studies

Chromosomal analysis was performed according to the Standard Protocol [24]. Metaphase harvesting was carried out on BAG₅₀ cell line (the 51st passage) in cell culture flasks containing culture medium. Colcemid (500 µl) was added and incubated for 15 min in 37°C, and cells were transferred to a 15 mL centrifuge tube. Then the cells were treated with hypotonic 0.075 M KCl for 20 min and followed by adding fixation solution (3:1 ratio of methanol: glacial acetic acid). The cells were spread using GBG banding (Trypsin and Giemsa) [25, 26], and 60 metaphases were analyzed by the Cytovision program based on guidelines (ISCN, 2016).

Results

Morphology and growth of BAG₅₀ cell line

The BAG₅₀ cells at passage 50 were epithelial-like cells that grew in an adherent unorganized monolayer, with some accumulations. The morphology of some cells in cellular aggregates was round. Most cells were heterogeneously divided (bipolar and multipolar divisions) (Figure 1). The BAG₅₀ cell line had high differentiation potency. The cell cycles (doubling time) of the BAG₅₀ cells were approximately 30 hours in DMEM/F12 medium supplemented with 10% FBS.

Functional characterization of BAG₅₀ cell line

The stemness state of the BAG₅₀ cells was confirmed by several functional assays, including assessment of sphere-forming capability (ability to form floating three-dimensional spheroid clusters) and the tumor-initiating potential (ability to form subcutaneous tumors in immunocompromised (nude) mice. The suspension of the BAG₅₀ cells was cultivated in the serum-free medium under low-adherent conditions. After one month of incubation, spheroid colonies were seen in culture (Figure 2a, 2b). We have determined that the BAG₅₀ cells have high efficiency in generating subcutaneous xenograft tumors in nude mice (Figure 2b).

Gene expression of stemness-related transcriptional factors by Quantitative real-time PCR

Stemness and self-renewal properties were evaluated using the mRNA expression level of OCT4, SOX2 and Cripto-1 genes in the BAG₅₀ cells. Quantitative real-time PCR results demonstrated that the genes mentioned were up-regulated in the BAG₅₀ cells. The expression level of OCT4, SOX2, and Cripto-1 genes in these cells were 2.1-, 2.6- and 4.7-fold greater than normal gastric cells, respectively (Figure 3a).

Phenotypic profiling of BAG₅₀ cell line

The identity of surface proteins CD44, CD54, and EPCAM and stemness factor oct-4 of the BAG₅₀ cells was determined using flow cytometry. As displayed in Figure 3b, all four markers were found in the BAG₅₀ cells. The majority of cells expressed high levels of CD44 (73%), CD54 (18%), EPCAM (94%), and oct-4 (75%).

STR profiling of BAG₅₀ cell line

STR DNA profiling analysis performed using simultaneous amplification of 16 STR markers plus amelogenin (AMXY) gene for gender determination. Amelogenin gene showed a pattern of X-chromosome. Other STRs demonstrate unique profiling for BAG₅₀ cell line (Figure 4, Table 2).

Table 2
The STR profile of BAG₅₀
cell line

Locus	Genotypes
AMXY	X
D7S820	12, 13
D21S11	31.2
VWA	15.2
D3S1358	15, 16
D19S433	14, 16.2
D8S1179	17
D13S317	15
CSF1PO	12
D2S1338	17, 18
TH01	7
D5S818	10, 11
TPOX	9
SE33	11
D16S539	10
D18S51	16, 18
FGA	26

Karyotype analysis

Following careful analysis of 60 metaphases prepared from BAG₅₀ cell line showed multiple aneuploidies and chromosomal translocations as follows, 64~68,X,Y,+X,t(1;2)(q32;q22),+1,+del(4)(q21),add(4)(q35)x2,der(5)t(5;9)(q34;q13)x2,+5,+6,+6,del(6)(q21),+7,+i(7)(p10),del(7)(q11.2),del(9)(p23)x2,+9,+add(10)(p15)x2,+i(11)(q10),+i(12)(p10),+t(13;13)(q10;q10),t(13;21)(q10;q10),t(13;14)(q10;q10),+14,+15,+16,+17,+17,+18,+18,+19,+20,+21,+22[cp25] (Figure 5).

Discussion

It is well established that cellular subpopulations in human tumors are heterogeneous at the genetic, epigenetic, and phenotypic levels [27]. It is also largely accepted today that novel and effective therapeutic strategies must be focused on the bulk tumor mass, especially functionally significant cells that possess the high tumor-initiating capacity and high chemo- and radio-resistance, i.e., TICs [28]. Therefore, the establishment of CSCs and cell lines with properties of putative CSCs could be used for developing novel effective anticancer therapies. Here we report establishing the gastric BAG₅₀ line cell, which previously was identified as chemotherapy-enriched sphere-forming cells with stem cell properties [22, 23].

The addition of serum-containing media in sequential passages resulted in some sphere-forming cells differentiating into gastric epithelial cells, and some cells remain round. Serum promotes differentiation of gastrospheres [13]. BAG₅₀ cells generated floating spheroid colonies in three-dimensional (3D) culture, which reflect sphere-forming capacity. There is a lot of evidence that 3D cell cultures closely mimic tumor *in vivo* microenvironment [29, 30]. In addition to the ability to form spheres, BAG₅₀ cells were able to generate tumors in athymic nude mice.

Stemness-related transcriptional factors OCT4, SOX2, and Cripto-1 play a vital role in CSCs pluripotency and self-renewal properties [31]. Furthermore, Oct-4/Sox2 interacts with stemness factor Nanog to regulate the self-renewal and multilineage differentiation capacity of CSCs [32]. Oct4 and Sox2 expression level is also associated with the propagation, metastasis, invasion, tumorigenicity, and drug resistance as well as differentiation of gastric cancer cells [33, 34]. Finally, there is a correlation between Cripto1 expression and gastric carcinogenesis and metastasis [35, 36]. In the present study, we showed that the relative *mRNA* expression level of OCT4, SOX2, and Cripto-1 genes in BAG₅₀ cells was significantly increased compared to normal gastric cells as well as Oct4 protein was overexpressed in them.

CD44, CD54, and EpCAM have been recognized as CSC markers in GC [9, 13, 37, 38]. CD44 and CD54 facilitated the release of CSCs to blood vessels, leading to metastasis. Moreover, CD44 plays a fundamental role in the augmentation of CSCs proliferation and survival, tumorigenesis, tumor recurrence, and mortality in GC [13, 39]. Finally, the transmembrane glycoprotein of EpCAM, exclusively expressed in human epithelial tissues and epithelial-derived neoplasms (not non-epithelial cell and or

cancers of non-epithelial origin), is involved in tumorigenesis, metastasis, and recurrence of the tumor [40, 41]. Our results demonstrated that BAG₅₀ cells overexpressed CD44, CD54, and EpCAM markers.

STR profiling provides an international reference standard for human cell lines [42]. The DSMZ and ATCC databases were typically used as large comprehensive STR profiling databases for cell line authentication [43]. Therefore, the result of our STR profile showed that a unique DNA fingerprint. Besides, we carried out the cytogenetic analysis when the cells were in the 51st passage. BAG₅₀ cells exhibited multiple aneuploidies and chromosomal translocations.

In summary, our data showed that the BAG₅₀ cells created from chemotherapy-enriched sphere-forming cells isolated from a patient with GC represent a unique established cell line with stem cell-like properties, including tumor-initiating ability and sphere-forming capacities, multiple aneuploidies and chromosomal translocations, and unique DNA fingerprint. Taken together, the results of this study indicated that the BAG₅₀ cell line is an extremely valuable tool for CSCs research.

Abbreviations

Cancer stem cells (CSCs)

tumor-initiating cells (TICs)

Dulbecco's Modified Eagle Medium (DMEM)

fetal bovine serum (FBS)

Epidermal growth factor (EGF)

Octamer-binding transcription factor 4(OCT4)

SRY-Box Transcription Factor 2(SOX2)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Polymerase chain reaction (PCR)

short tandem repeats (STR)

three-dimensional (3D)

Declarations

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Authors' contributions; All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Vahid Bagheri and Mohammad Reza Abbaszadegan and Mehran Gholamin. The first draft of the manuscript was written by Atena Mansouri and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability: All data produced or analyzed during this study are incorporated in this published article.

Compliance with ethical standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Consent to participate: All authors have seen the manuscript and approved to submit the manuscript.

Consent for publication: All authors consent to the publication of the manuscript.

Ethical approval: The ethics committee of MUMS approved the investigation and written informed consent was acquired from patients.

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Figures

Figure 1

Morphological features of the BAG₅₀ cell line, passage 50 (original magnification 40×). After culturing BAG₅₀ cells with serum, most cells grew as tightly cohesive epithelial like cells. However, very few cells were round in cellular aggregates.

Figure 2

Functional characterization of the BAG₅₀ cell line. (a) Tumor spheres induced by passage 50 of the BAG₅₀ cells in three-dimensional cultivation on the ultra-low attachment flask (Left panel, original magnification 4×; right panel, original magnification 40×). (b) Subcutaneous tumor generated by the BAG₅₀ cells (1×10^6 cells) in nude mouse (passage 51).

Figure 3

Real-time PCR and flow cytometry analysis of expression of genes in the BAG₅₀ cells. (a) real-time PCR results showed that expression of stemness genes (OCT4, SOX2 and Cripto-1) were upregulated in the BAG₅₀ cells in comparison with gastric normal tissue. (b) After staining the BAG₅₀ cells using anti-CD44, -CD54, -EpCAM, and -oct-4 monoclonal antibodies, the majority of cells expressed them.

Figure 4

The STR profiling of the BAG₅₀ cell line.

Figure 5

Karyotype of BAG₅₀ cell line. 60 metaphases prepared from BAG50 cell line where analyzed and all metaphases showed multiple aneuploidies and chromosomal translocations as follows, 64~68,X,-Y,+X,t(1;2)(q32;q22),+1,+del(4)(q21),add(4)(q35)x2,der(5)t(5;9)(q34;q13)x2,+5,+6,+6,del(6)(q21),+7,+i(7)(p10),del(7)(q11.2),del(9)(p23)x2,+9,+add(10)(p15)x2,+i(11)(q10),+i(12)(p10),+t(13;13)(q10;q10),t(13;21)(q10;q10),t(13;14)(q10;q10),+14,+15,+16,+17,+17,+18,+18,+19,+20,+21,+22[cp25].