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.

Keywords Cancer stem cells · Gastric cancer · Novel cell line

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 chemoresistant 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 (930,607). 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, $3 \times 10^5$ viable cells of passage 51 were first seeded into 25-cm$^2$ 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, $5 \times 10^4$ 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 ($1 \times 10^6$ 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.

**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) (Fig. 1). The BAG50 cell line had high differentiation potency. The cell cycles (doubling time) of the BAG50 cells were approximately 30 h 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 (Fig. 2a). We have determined that the BAG50 cells have high efficiency in generating subcutaneous xenograft tumors in nude mice (Fig. 2b).

### Table 1

| Genes     | Primer sequence 5′–3′ |
|-----------|-----------------------|
| OCT4      | For: GCAAGCGACTATGACAACAAGGA 
           | Rev: CCAAGAAGTGGTGACGGAGACA |
| SOX2      | For: AACAGCCGGAGCCCGTGCAA 
           | Rev: TCAGACGCCGCTTACGGCTG |
| Cripto-1  | For: GGAGACACAGCAGAAGGAGAG 
           | Rev: ACCTGTGGAGTCTGGAGTCT |
| GAPDH     | For: GGAAGGGGAGGTCGGAGTCA 
           | Rev: GTCATTGATGGCAACATTCCTC |

**Fig. 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.
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 (Fig. 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 Fig. 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 (Fig. 4; Table 2).

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)×2,der(5)(5;9)(q34;q13)×2,+5,+6,+6,del(6)(q21),+7,+i(7)(p10),del(7)(q11.2),del(9)(p23)×2,+9,+add(10)(p15)×2,+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] (Fig. 5).
Fig. 3 Real-time PCR and flow cytometry analysis of expression of genes in the BAG_{50} cells. a Real-time PCR results showed that expression of stemness genes (OCT4, SOX2, and Cripto-1) were upregulated in the BAG_{50} cells in comparison with gastric normal tissue. b After staining the BAG_{50} cells using anti-CD44, -CD54, -EpCAM, and -oct-4 monoclonal antibodies, the majority of cells expressed them.

Fig. 4 The STR profiling of the BAG_{50} cell line
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

| 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    |
| THO1    | 7         |
| D5S818  | 10, 11    |
| TPOX    | 9         |
| SE33    | 11        |
| D16S539 | 10        |
| D18S51  | 16, 18    |
| FGA     | 26        |

Fig. 5 Karyotype of BAG50 cell line. 60 metaphases prepared from BAG50 cell line were 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)×2,der(5)t(5;9)(q34;q13)×2,+5,+6,+del(6)(q21),+7,+i(7)(p10),del(7)(q11.2),del(9)(p23)×2,+9,+add(10)(p15)×2,+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]
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.

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by VB and MRA and MG. The first draft of the manuscript was written by AM 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.

Declarations

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

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

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.

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