

## Allogeneic tumor cell line-based vaccines: A good alternative to autologous and cancer stem cell vaccines in colorectal cancer

Fatemeh Rafieenia<sup>1</sup>, Elham Nikkhah<sup>2</sup>, Fatemeh Nourmohammadi<sup>3</sup>, Susan Hosseini<sup>1</sup>, Abbas Abdollahi<sup>4</sup>, Nurieh Sharifi<sup>5</sup>, Mohsen Aliakbarian<sup>4</sup>, Mohammad Mahdi Forghani Fard<sup>3</sup>, Mehran Gholamin<sup>6</sup>, Mohammad Reza Abbaszadegan<sup>1,7</sup>

<sup>1</sup> Medical Genetics Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>2</sup> Department of Pharmacodynamics and Toxicology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>3</sup> Department of Biology, Damghan branch, Islamic Azad University, Damghan, Iran

<sup>4</sup> Surgical Oncology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>5</sup> Department of Pathology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>6</sup> Department of Laboratory Sciences, School of Paramedical Sciences, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>7</sup> Immunology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

### ARTICLE INFO

#### Article type:

Original

#### Article history:

Received: Apr 2, 2021

Accepted: Jul 27, 2021

#### Keywords:

Allogeneic  
Autologous  
Cancer stem cell  
Colorectal cancer  
Vaccine

### ABSTRACT

**Objective(s):** Besides the uncertainty about colorectal cancer stem cell (CCSC) markers, isolating, purifying, and enriching CCSCs to produce CCSC vaccines is highly challenging. However, allogeneic vaccines developed from CRC cell lines can provide universal, comprehensive, inexpensive, simple, and fast approach to cancer treatment.

**Materials and Methods:** CCSCs were isolated from human CRC tissue using the in vitro sphere formation assay and then characterized through gene expression analysis, in vivo and in vitro tumor formation assay, karyotyping, and surface marker detection. Subsequently, CCSCs and two CRC cell lines (HT-29 and SW-480) were inactivated with cisplatin (CDDP) and administrated as vaccines to the three groups of athymic C57BL/6 nude mice. Afterward, tumorigenesis was challenged with HT-29 cells. The antitumor effect of vaccines was evaluated by tumor and spleen examination and immune response analysis. The cytotoxic activity of splenocytes and serum levels of TGF- $\beta$  and IFN- $\gamma$  were measured by Calcein-AM cytotoxicity assay and enzyme-linked immunosorbent assay (ELISA), respectively.

**Results:** The results of gene expression analysis showed that CCSCs are CD44+CD133-LGR5-. All vaccinations resulted in decreased tumor growth, spleen enlargement, enhanced serum level of IFN- $\gamma$  and TGF- $\beta$ , and increased cytotoxic activity of natural killer (NK) cells. The antitumor efficacy of the CCSC vaccine was not more than CRC cell line-based vaccines. Interestingly, the allogeneic SW-480 vaccine could effectively inhibit tumorigenesis.

**Conclusion:** Despite the great challenge in developing CCSC vaccines, allogeneic vaccines based on CRC cell lines can efficiently induce antitumor immunity in CRC.

#### ► Please cite this article as:

Rafieenia F, Nikkhah E, Nourmohammadi F, Hosseini S, Abdollahi A, Sharifi N, Aliakbarian M, Forghani Fard MM, Gholamin M, MR Abbaszadegan. Allogeneic tumor cell line-based vaccines: A good alternative to autologous and cancer stem cell vaccines in colorectal cancer. Iran J Basic Med Sci 2021; 24:1231-1239. doi: <https://dx.doi.org/10.22038/ijbms.2021.56732.12671>

### Introduction

According to the global cancer observatory in 2018, colorectal cancer (CRC) is the second most frequent cause of cancer-related death in the world (1) which is increasing among young people (2). Every year, many cancer patients die not from CRC but due to the side effects of routine treatments such as surgery, chemotherapy, and radiation therapy. Also, inadequacy and lack of specificity of these methods often lead to cancer resistance and recurrence. Over the past decades, accumulating discoveries about immunotherapy have provided new potential strategies for cancer treatment. Adoptive cell therapy and cancer vaccines are the two major immunotherapeutic approaches in CRC. Cancer vaccines include dendritic cell vaccines, tumor cell vaccines, DNA vaccines, peptide vaccines, and viral vector-based vaccines (3). Unlike other

immunotherapeutic strategies, tumor cell vaccines stimulate the immune system against a wide range of tumor-associated antigens (TAAs). They can also be used for all cancer patients, regardless of their HLA type (4). Given the uncertain nature of cancer antigens, whole tumor cells appear to be an appropriate and feasible vaccine development option. Depending on antigen source, tumor cell vaccines could be autologous or allogeneic type, including OncoVAX (5) and GVAX (6), respectively. GVAX is composed of whole tumor cells genetically modified to secrete the immune-stimulatory cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), and irradiated to prevent further cell division (7). Allogeneic tumor cell vaccines are being widely studied in clinical trials. The GVAX colon vaccine is developed from the two CRC cell lines SW-837 and SW-620. Another allogeneic tumor cell

vaccine, CancerVax, consists of three human melanoma cell lines, including a wide range of TAAs and major histocompatibility complex antigens (6, 8, 9). A CRC vaccine composed of irradiated, allogeneic human CRC cell lines along with GM-CSF-producing bystander cells and cyclophosphamide in a phase 1 study showed safety and feasibility in patients with metastatic CRC (10). However, researchers are extensively looking for targeted therapy against cancer stem cells (CSCs). CSCs are a small population of tumor cells with slow proliferation and are responsible for tumor initiation and recurrence (11). However, providing the CSC vaccines is successful or practical only in a small group of patients. On the other hand, tumor heterogeneity and cancer cell plasticity have made CSC markers a challenge in CSC vaccines. Allogeneic vaccines developed from CRC cell lines have significant advantages over autologous or CSC vaccines in terms of availability, easy and rapid preparation, and possibility of multiple vaccinations. In this study, CCSCs were isolated and characterized from primary CRC tissue. Then, the efficacy of vaccines prepared from CCSCs and human CRC cell lines was investigated in nude mice. The two human CRC cell lines HT-29 and SW-480 were selected as autologous and allogeneic vaccines, respectively. The experiment steps are shown schematically in Figure 1.

## Materials and Methods

### Cell culture and CCSC characterization

CCSCs were isolated from the CRC patient and then characterized by *in vitro* and *in vivo* analysis. Also, CRC cell lines (HT-29 and SW-480) were cultured for vaccine preparation.

### Patient

After explaining the purpose of the project, informed consent was obtained from seven participants. The age and sex of the patient as well as the stage, grade, or location of the tumor, chemotherapy and radiotherapy treatments, or family history were not criteria for patient selection. Our criterion was patient consent and then pathological confirmation of the tumor sample. Finally, CCSCs were isolated from the CRC tumor of a

51-year-old man from Ghaem hospital, Mashhad, Iran. The patient had not received any chemotherapy or radiotherapy. The invasive CRC adenocarcinoma with a tumor grade of T3N0MX was pathologically confirmed. Tumor and normal tumor-adjacent epithelial tissues were obtained from rectosigmoid regions directly after surgical removal.

### CCSC Isolation from CRC tissue

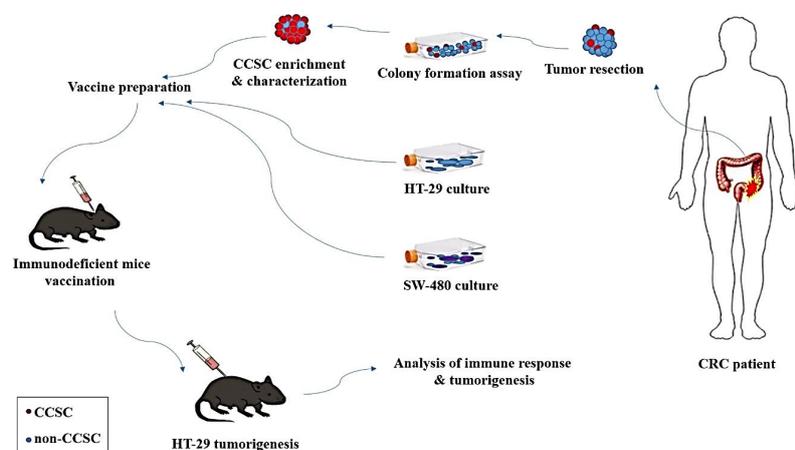
After severe rinsing with phosphate-buffered saline (PBS) containing 5X antibiotic cocktail of 250 µg/ml gentamicin, 5% Pen/Strep, and 12.5 µg/ml amphotericin B, fresh tumor tissue was mechanically minced with scissors into 3 mm<sup>3</sup> pieces. Then, tumor fragments were incubated overnight at 4 °C in DMEM/F12 medium (Gibco, Cat#32500-035) containing 4X Primocin (8 µg/ml) (Invivogene, Cat#ant-pm-1). The day after, tumor fragments were incubated for at least 2 hr in DMEM/F12 medium containing collagenase type IV (200 U/ml) (Gibco, Cat#17104-019) and 6 µg/ml primocin. Single cells passed through 40 µm cell strainers (SPL, Korea) were incubated for 10 min in red blood cell lysis buffer and centrifuged at 1200 RPM for 5 min. Cancer cells were cultured in 6-well ultra-low attachment plates (Corning, Cat#CLS3471) with serum-free DMEM/F12 containing 15 mM HEPES, 10 ng/ml bFGF (Sigma, Cat#SRP4037), 20 ng/ml EGF (Sigma, Cat#E9644), 10 ng/ml LIF (Sigma, Cat#L5283), 2% B-27 supplement (Gibco, Cat# 17504-044), 8 µg/ml primocin at 37 °C and humidified 5% CO<sub>2</sub> incubator.

### The characterization of CCSCs

CCSCs were characterized by tumorigenesis *in vitro* and *in vivo*, the differentiation capacity assay, karyotyping, and surface markers (12-14).

### In vitro tumor formation assay

The proliferated CCSCs were dissociated into the single cells with TrypLE™ Express Enzyme (Gibco, Cat#12604013). Considering the self-renewal capacity of CCSCs, they were re-cultured in a 6-well ultra-low attachment plate into the serum-free medium to evaluate the regeneration of 3D spheroids of CCSCs (colonspheres).



**Figure 1.** The schematic diagram of the experimental design. After isolation and characterization of CCSCs from the CRC patient, the isolated CCSCs as well as two CRC cell lines (HT-29 and SW-480) were inactivated with CCDP and injected subcutaneously into the necks of nude mice in three stages. 10 days after vaccination, tumorigenesis was challenged with HT-29 cells in the right flank

### The differentiation capacity assay

CCSCs could differentiate to other types of cells, including mesenchymal-like cells. Then, singled CCSCs (passage 6) were cultured in DMEM/F12 medium supplemented with 10% inactivated fetal bovine serum (FBS) (Gibco Cat#10270106) in 6-well plates (SPL, Korea).

### In vivo tumor formation assay

After the approval of the institutional animal care and use committee of Mashhad University of Medical Sciences (MUMS), 3×10<sup>6</sup> CCSCs (passages 4-10) were subcutaneously injected with 100 µl cold Matrigel (Corning, Cat#356234) in the right flank of 7 to 8-week-old healthy female C57BL/6 nude mice (purchased from North Research Center, Pasteur Institute of Iran). The mouse was in a sterile animal room with a 12-hr light/dark cycle and 40-50% humidity and 22 °C. After two weeks, the mouse was euthanized with an intraperitoneal injection of the mixed solution of ketamine (100 mg/kg) and xylazine (10 mg/kg). The skin of the tumor area was then cut with scissors and forceps and the tumor was removed.

### Detection of surface markers CD44 and CD133 by Flow cytometry

CCSCs were singled with TrypLE™ and separately incubated on ice for 20 min in darkness with APC anti-mouse/human CD44 (BD Pharmingen, Cat# 559942), and PE anti-human CD133 (Milteny Biotec, Cat# 130-113-670). The labeled cells were then twice washed with PBS and centrifuged for 5 min at 1200 RPM.

Fluorescence was measured by a flow cytometer (BD Accuri C6, BD Biosciences, USA), and FlowJo software was used for the data analysis.

### Karyotyping

The standard protocol was used for chromosomal analysis (15). Metaphase harvesting was carried out on the 25th passage CCSCs. After 15 min incubation with 500 µl colcemid at 37 °C, CCSCs were treated with hypotonic KCl (0.075 M) for 20 min. After fixation with a mixture of methanol and glacial acetic acid (3:1), GBG (G-bands after 5'-bromodeoxyuridine and Giemsa) banding was analyzed by the Cytovision program in 50 metaphases.

### RNA extraction and cDNA synthesis

Using TRIzol™ Plus RNA purification kit (Invitrogen, Cat#12183555), total RNA was extracted from CCSCs (6th passage) and the adjacent normal tissue. After determining the quantity and quality of mRNAs by a spectrophotometer (Biochrom WPA Biowave DNA Life Science), RNA samples were treated with DNaseI (Fermentas, Cat#EN0521) along with Rnase Inhibitor (Fermentas, Cat#E00381). cDNAs were synthesized by an Easy cDNA Synthesis Kit (Pars Tous Biotechnology, Mashhad, Iran).

### Primer design & quantitative real-time PCR analysis

Gene expression analysis was performed at least three times for some specific and universal CSC markers, including ALDH1A1, LGR5, CD44, CD133, EPCAM, CTNNB1, MYC, SOX2, CDH1, and CDH2 (Table 1). Phosphoglycerate kinase 1 (PGK1) has been reported

**Table 1.** Primer sequences for amplification of PGK1 and the genes examined

Genes	Primer sequence 5'→3'	Amplicon size (bp)
PGK1	F: CCACTGTGGCTTCTGGCATA	166
	R: ATGAGAGCTTTGGTTCCCG	
ALDH1A1	F: GATCCCGTGGCGTACTATG	202
	R: TGGATCTTGTGAGCCCAACC	
EPCAM	F: GTGCTGGTGTGGAACACTG	155
	R: GAAGTGCAGTCCGAAACTT	
SOX2	F: AACAGCCCGACCGCGTCAA	189
	R: TCGCAGCCGCTTAGCCTCGT	
CD44	F: TCCAACACCTCCAGTATGACA	83
	R: GGCAGGTCTGTGACTGATGTACA	
CD133	F: CACTACCAAGGACAAGGCGT	153
	R: TCCAACGCCTCTTTGGTCTC	
MYC	F: AGCGACTCTGAGGAGGAACAAG	135
	R: TGGGCTGTGAGGAGGTTTGC	
CTNNB1	F: CAACTAAACAGGAAGGGATGGAAGG	239
	R: CAGATGACGAAGAGCACAGATGG	
LGR5	F: CCTTCCAACCTCAGCGTCTT	248
	R: AGGGATTGAAGGTTTCGCAA	
CDH1	F: ATTCTGATTCTGCTGCTCTTG	136
	R: AGTCCTGGTCTCTTCTCC	
CDH2	F: ATGGTGTATGCCGTGAGAAG	196
	R: TGTGCTTACTGAATTGTCTTGG	

PGK1: phosphoglycerate kinase 1, ALDH1A1: Aldehyde dehydrogenase 1 family member A1, EPCAM: Epithelial cell adhesion molecule, SOX2: sex-determining region Y-box 2, LGR5: Leucine-rich repeat-containing G-protein coupled receptor 5, CDH1: Cadherin-1, CDH2: Cadherin-2

as one of the most stable CRC genes, even under 3D cultures (16, 17). Therefore, it was used as a reference gene in this study. The SYBR® Premix Ex Taq™ II (Takara, Cat#RR820L), the LightCycler® 96 Instrument (Roche), and the comparative CT method were used for quantitative real-time PCR analysis. Primers were designed using the database <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>. The real-time PCR was performed at 60 °C for all primer sets.

PGK1: phosphoglycerate kinase 1, ALDH1A1: Aldehyde dehydrogenase 1 family member A1, EPCAM: Epithelial cell adhesion molecule, SOX2: sex-determining region Y-box 2, LGR5: Leucine-rich repeat-containing G-protein coupled receptor 5, CDH1: Cadherin-1, CDH2: Cadherin-2.

### Cell line culture

HT-29 and SW-480 were cultured in DMEM/F12 medium supplemented with 10% FBS and 1% Pen/Strep and incubated under conditions of 95% humidity, 5% CO<sub>2</sub>, and 37 °C.

### Vaccination

The median lethal concentration (LC<sub>50</sub>) dose of CDDP for vaccine inactivation was determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay. After vaccine preparation, inactivated vaccines were injected into C57BL/6 nude mice.

### MTT assay

Five × 10<sup>3</sup> cells of HT-29, SW-480, and CCSCs were cultured in a 96-well flat-bottom plate (SPL, Korea). After 24 hr incubation at 37 °C in 5% CO<sub>2</sub>, media was changed with various CDDP concentrations in triplicate and incubated for 2 hr at 37 °C in 5% CO<sub>2</sub>. Then, media was discarded and 100 µl medium containing 0.5 mg/ml MTT (Sigma, Cat# M5655-1g) was added to the wells. After 2 hr incubation at 37 °C in 5% CO<sub>2</sub>, the medium was slowly removed, and 100 µl DMSO was added to each well. The plate was wrapped in foil, shaken on an orbital shaker for 30 min, and read at 570 nm with an ELISA Reader (ELx800, BioTech, USA). The percentage of the viable cells was calculated using the following formula: [(sample abs)/ (control abs) × 100].

### Mice immunization

Twenty-four C57BL/6 nude mice (female, 16–22 g, and 6–8 weeks old) were randomly divided into four groups with six mice per group: CCSCs, HT-29, SW-480, and saline. 5 × 10<sup>4</sup> CDDP-inactivated cells were injected into the vaccine groups three times at two-week intervals (18) by subcutaneous injection in the neck. The CCSCs were in passages 4–10. Ten days after the last vaccination, all immunized mice were challenged subcutaneously with 3 × 10<sup>6</sup> HT-29 cells in the right flank. The endpoint of the study was tumor size of about 20 mm in the saline group. Two weeks after tumorigenesis, mice were euthanized to remove the tumor and spleen and to take blood samples from the heart. Tumor size was calculated using the Monga formula (L × W × H)/2 (19). Mice were monitored daily for general health and body weight and mice with weight loss or scoliosis were excluded. Finally, at least three mice from each group survived to the end of the experiment (two months), which were further analyzed.

### The immune response

Fresh blood was obtained from the hearts of mice under anesthesia. Serum levels of TGF-β and IFN-γ were measured using ELISA kits according to the manufacturer's protocol (TGF-β: Bioassay technology laboratory, Cat# E0660MO, and IFN-γ: Invitrogen, Cat# CN.88-7711-44). Samples were read at 450/570 nm using an ELISA reader (ELx800, BioTech, USA).

### NK cytotoxicity

The spleen tissues were harvested from the immunized mice. YAC-1 cells were labeled with 100nM Calcein-AM (Invitrogen, Cat# C3099) at 37 °C in 5% CO<sub>2</sub> for 20 min in serum-free medium and darkness. Labeled YAC-1 cells were washed three times in PBS containing 5% FBS with a 5 min centrifuge at 1200 RPM. The splenocytes as effector cells were seeded with the labeled YAC-1 target cells in a 96-well plate at 20:1 ratios of effector cells to target cells in triplicate. After 4 hr incubation at 37 °C, the supernatant was separated by centrifuge at 1200 RPM for 5 min. The fluorescence of 100 µl of supernatants was monitored at 485/530 nm excitation/emission wavelengths using Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek, Vermont, USA) in black 96-well flat-bottomed plates (SPL Life Sciences, Gyeonggi-do, Korea). The cytotoxicity of the NK cells was calculated through lysis percentage: [(experimental release– spontaneous release)/ (maximum release– spontaneous release)] × 100. Maximum release and spontaneous release were obtained from incubation of YAC-1 cells with and without 2% Triton X100, respectively (18, 20).

### Hematoxylin and eosin (H&E) staining of tumors

After 24 hr incubation in 10% formalin, tumors resected from mice were stained with H&E and then examined pathologically.

### Statistical analysis

Graphpad Prism (version 8) was used for data analysis. Turkey's multiple comparisons test was used for comparison between groups.

## Results

### The characterization of CCSCs

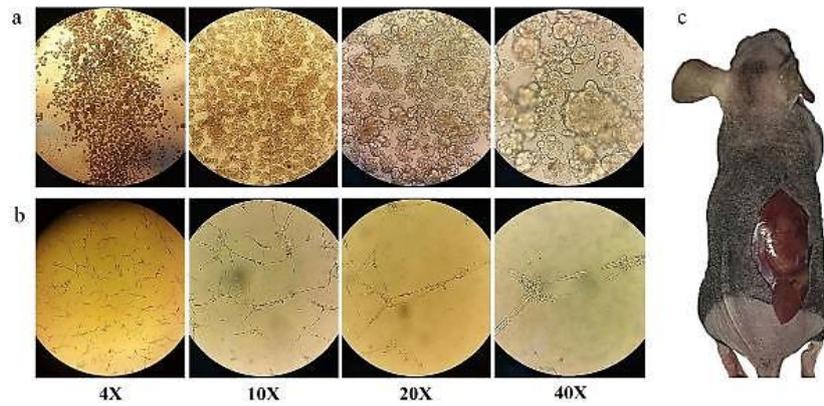
CCSCs could reform colonospheres in the ultra-low attachment plate in the serum-free medium (Figure 2a). The differentiation capacity was confirmed by the epithelial to mesenchymal transition of CCSCs (Figure 2b). The isolated CCSCs could also form tumors in the female C57BL/6 nude mice (Figure 2c).

### The real-time PCR gene expression analysis

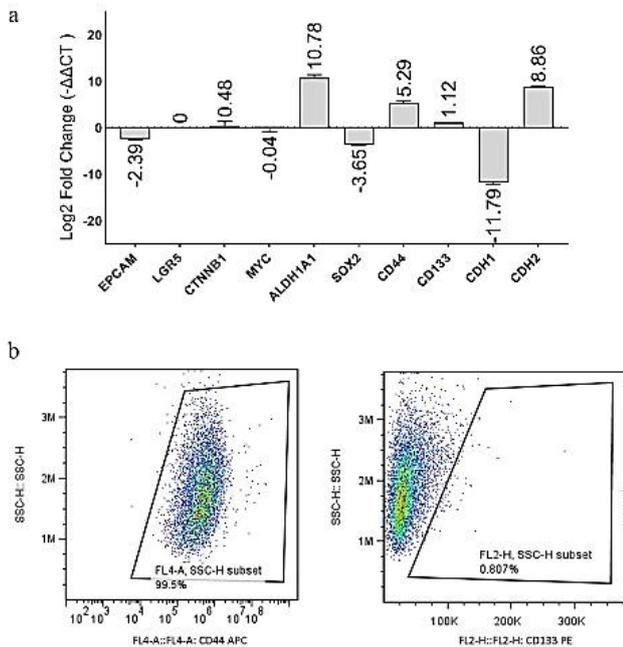
The ΔΔCT comparison results (Figure 3a) showed that ALDH1A1 (~ 2<sup>11</sup> fold, CDH2 (~ 2<sup>9</sup> fold), and CD44 (~ 2<sup>5</sup> fold) had the highest overexpression in CCSCs, respectively. LGR5 expression was not observed in the normal tissue or CCSCs. EPCAM (~ 2<sup>2</sup> fold), SOX2 (~ 2<sup>4</sup> fold), and CDH1 (~ 2<sup>12</sup> fold) were decreased in CCSCs.

### Analysis of the surface markers CD44 and CD133

CCSCs have been identified as CD44<sup>+</sup>CD133<sup>-</sup> by flow cytometry (Figure 3b). The frequency of CD44<sup>+</sup> cells and CD133<sup>+</sup> cells was 99.5% and 0.8%, respectively.



**Figure 2.** Isolation and proliferation of CCSCs: a) Colonospheres were formed 1-7 days after re-culturing singled CCSCs into ultra-low attachment plate (6th passage). b) CCSCs showed epithelial to mesenchymal transition under adherent culture with serum containing medium (6th passage). c) CCSCs formed tumors in the female C57BL/6 nude mice two weeks after transplantation of ~3×10<sup>6</sup> cells (passage 4-10) in the right flank



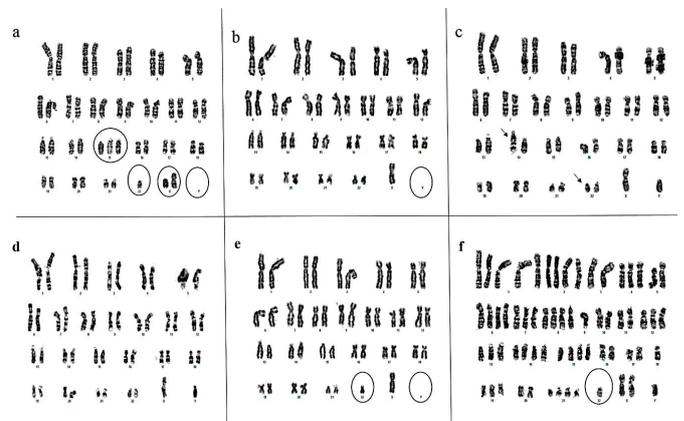
**Figure 3.** Results of the gene expression analysis and flow cytometry: a) ALDH1A1, CDH2, and CD44 had the highest overexpression in CCSCs, respectively. EPCAM, SOX2, and CDH1 were decreased in CCSCs. The expression of LGR5 was not detected. No significant change ( $\pm$  2-fold) was observed for CD133, CTNNB1, and MYC. b) The flow cytometry results showed that CD44 was highly expressed in CCSCs, and on the other hand, CCSCs were negative for CD133

**Karyotyping**

Karyotyping showed the heterogeneity in CCSCs. The structural and numerical abnormalities were observed in most of the chromosomes, especially Y and 22 (Figure 4). Deletion of chromosome 22 seems to play an effective role in malignancy. In other words, this chromosome has important tumor suppressor genes. The chromosomal range was 44-64.

**The MTT assay**

Based on LC<sub>50</sub> values, CDDP concentrations for vaccine inactivation were gained 0.6mM for CCSCs and HT-29, and 1.7mM for SW-480 (Figure 5a).



**Figure 4.** karyotyping results: Analysis of 25<sup>th</sup> passage CCSCs at 50 metaphases revealed numerical and structural chromosomal abnormalities: a) 46, XY, +15, -22 [9], b) 45, X, -Y [8], c) 46, XY, der (14) t (14;22) (q32; q11) [7], d) 46, XY [8], e) 44, X, -Y, -22 [8], f) 64, XXY, -3, -4, -5, -5, -7, -9, -9, -9, -10, -10, -11, -11, -12, -13, -16, -16, -17, -17, -18, -18, -18, -19, -20, -20, -22, -22, -22 [10]

**The effect of vaccination on tumor size**

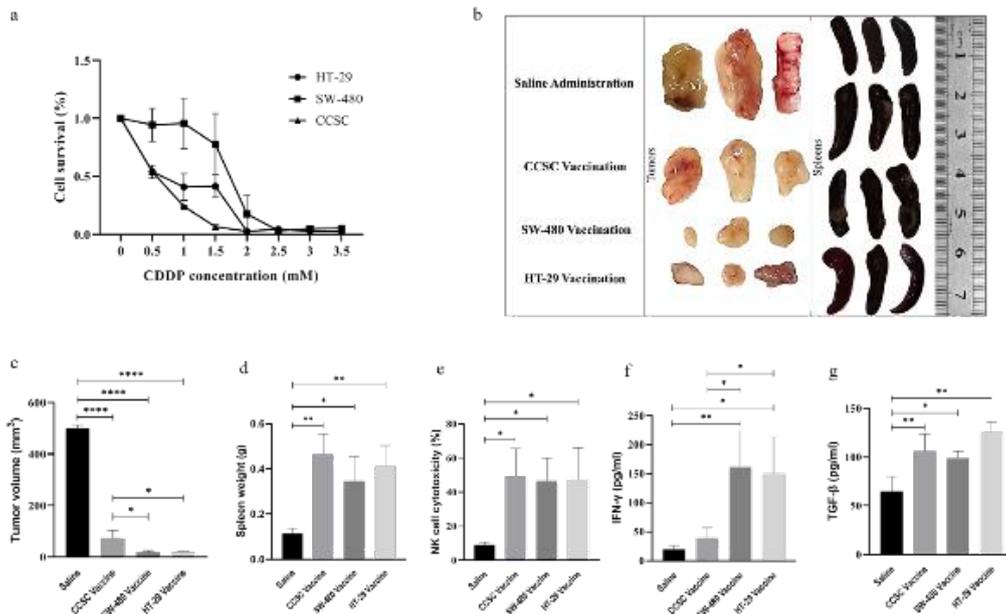
Figure 5b shows changes in tumor and spleen size after vaccination. All vaccinations inhibited tumorigenesis (Figure 5c) and increased spleen weight (Figure 5d) compared with the control group. The tumor volume in mice vaccinated with the CRC cell lines was statistically significantly decreased ( $P < 0.05$ ) compared with the mice immunized with the CCSC vaccine. Similar to the autologous HT-29 vaccine, the allogeneic SW-480 vaccine could effectively inhibit tumorigenesis.

**The NK cytotoxicity assay**

The cytotoxic activity of splenocytes increased in all vaccine groups compared with the control group with  $P < 0.05$  (Figure 5e).

**The immune response**

Despite HT-29 and SW-480 vaccine groups, IFN- $\gamma$  was not significantly increased in the CCSC vaccine group (Figure 5f). IFN- $\gamma$  was markedly increased in the SW-480 vaccine group compared with the other groups ( $P < 0.01$ ). IFN- $\gamma$  was increased in cell line-



**Figure 5.** MTT and vaccination results: a) SW-480 was more resistant to CDDP than HT-29 and CCSCs. b) Vaccinations have led to tumor shrinkage and spleen enlargement in all vaccine groups (four groups with three mice in each group). c) All vaccines inhibit tumorigenesis in the vaccinated mice. However, the effect of CCSC vaccination on tumor size was less than that of cell line vaccines. d) Spleen weight was increased in all groups. e) All vaccines increased the activity of splenocytes. f) IFN- $\gamma$  showed a significant increase in SW-480 and HT-29 vaccine groups but not in the CCSC group. g) TGF- $\beta$  increased in all vaccine groups. Statistically significant difference is shown by \* in  $P < 0.05$ , \*\* in  $P < 0.01$ , \*\*\* in  $P < 0.003$ , and \*\*\*\* in  $P < 0.0001$

based vaccine groups further than the CCSC vaccine group ( $P < 0.05$ ). TGF- $\beta$  was significantly increased in all vaccine groups compared with the saline group (Figure 5g). Tumor volume showed a negative correlation with TGF- $\beta$  ( $P < 0.05$ ), IFN- $\gamma$  ( $P < 0.01$ ), and NK cell cytotoxicity ( $P < 0.05$ ). Tumor volume showed the most correlation with IFN- $\gamma$ .

**H&E Staining**

Pathological evidence of lower vaccine efficacy

including muscle extension, lymphovascular invasion, and perineural invasion was mainly seen in the control and CCSC vaccine groups. On the other hand, evidence of greater vaccine efficacy such as tumor necrosis, fibrosis, and inflammatory reaction was more common in HT-29 and SW-480 vaccine groups. Therefore, the pathological evidence also indicates that HT-29 and SW-48 vaccines are more effective than the CCSC vaccine (Table 2).

**Table 2.** Results of pathological examination of tumor tissues removed from vaccinated mice

Vaccine groups	Mice numbers	Tumor size (mm <sup>3</sup> )	Muscle extension	Lymphovascular invasion	Perineural invasion	Tumoral necrosis	Inflammatory reaction	Calcification	Fibrosis	Tumor grade
Ctrl	1	488	+	+	+	-	+1	-	-	High
	2	515	+	+	+	-	+1	-	-	High
	3	499	+	+	+	-	+2	-	-	High
HT-29 vaccine	1	15	-	-	-	Multi- Focal	+3	-	Multi- Focal	High
	2	18	-	-	-	Extensive	+4	-	-	High
	3	20	-	-	-	Multi- Focal	+3	-	Multi- Focal	High
CCSC vaccine	1	88	-	+	+	-	+2	-	-	High
	2	94	+	+	+	-	+2	-	-	High
	3	36	+	+	+	-	+2	-	-	High
SW-480 vaccine	1	21	-	-	-	Multi- Focal	+3	-	Multi- Focal	High
	2	9	-	-	-	Extensive	+4	-	Multi- Focal	High
	3	22	-	+	+	-	+2	-	-	High

## Discussion

Vaccination is one of the most important subjects in cancer immunotherapy. Clinical trials have examined a variety of vaccines such as immunodominant peptides, pure antigens, naked DNA or recombinant virus encoding tumor antigens, and whole tumor cells in cancer immunotherapy. Whole tumor cells usually express all TAAs and tumor-specific antigens (TSA), both known and unknown, leading to simultaneous induction of CD8<sup>+</sup> and CD4<sup>+</sup> cells (21). In a meta-analysis of nearly 1800 patients, patients vaccinated with whole tumor vaccines showed a significantly higher objective response compared with patients vaccinated with tumor-specific antigens (22). However, the sourcing of autologous tumor cells is highly invasive and confined by the quality of the biopsy material. As we experienced, the success rate of stem cell isolation is low and development of autologous vaccines is not feasible for all patients. Due to chemotherapy, radiation therapy, and severe yeast contamination, we lost six tumor samples out of seven (success rate of 14%). Besides, proliferation and characterization of slow-growing CCSCs were challenging and highly time-consuming, leading to delays in treatment and inefficiency of this method. To circumvent these restrictions, a number of cell line-based vaccines were developed. Cell lines are stable and guarantee an endless source of tumor cells. Also, allogeneic cell line-based vaccines are more recognizable for the patient's immune system than autologous vaccines, reducing concern about their carcinogenicity and adjuvants' necessity. However, they may lack the patient's unique antigens. Recent studies showed that tumor antigens due to somatic mutations or epigenetic deregulations are rarely similar in different tumors and are highly heterogeneous, warranting the autologous whole tumor vaccine approach (4, 23). However, many studies have shown that allogeneic antigens can be a good alternative to autologous antigens. Allogeneic lysates from cell lines were used in dendritic cell (DC) therapy in various cancers and efficacy and safety of this approach were shown in mice and humans (24-26). Although methods such as DC therapy or adoptive T cell therapy are widely studied in clinical trials, these methods also require isolation of the patient's immune cells or complex cellular and molecular processes that are time-consuming and costly (27-30). Whole tumor cell vaccines are a very simple and immediate method of vaccination without the need for DCs. GVAX approach was successful in preclinical trials and induced anti-tumor immunity in lymphoma, melanoma, prostate, renal, lung, and colon cancers (9). In an effort to design a general, cost-effective, and efficient CRC vaccine, we examined cell line-based vaccines in mice as well as comparing them with a prepared CCSC vaccine. Our results showed that the allogeneic SW-480 vaccine could effectively induce antitumor immunity in nude mice. All CCSC, allogeneic, and autologous vaccines significantly increased the serum level of TGF- $\beta$  in mice. Despite differences in genetic content and chromosomal number, HT-29 and SW-480 demonstrated similar and considerable immunogenicity even without adjuvants. Therefore, the CRC vaccine could also be developed with other CRC cell lines, including SW-480 and HT-29.

Unlike other clone vaccines such as GVAX, we did not use any adjuvants, but the vaccines could effectively reduce tumorigenesis in mice. On the other hand, the anticancer efficacy of the CCSC vaccine was less than that of the cell line-based vaccines. Unlike spleen weight, NK cytotoxicity, and TGF- $\beta$ , IFN- $\gamma$  did not show a significant increase in the CCSC vaccine group. IFN- $\gamma$  had the most correlation with the tumor size and it was significantly increased in CRC cell line-based vaccine groups. IFN- $\gamma$  is a cytokine that plays a critical role in both innate and adaptive immunity and functions as a stimulator of NK cells and neutrophils and the primary activator of macrophages (31). Thus, it seems that IFN- $\gamma$  is more effective than TGF- $\beta$  in inhibiting tumorigenesis. The lower efficacy of the CCSC vaccine in this study could be due to the inactivation of CCSC antigens during vaccine inactivation or lower antigen storage than CRC cell lines or other reasons. Cancer cells can also have different immunogenic potentials. Therefore, the CCSC vaccine group should be repeated with other patients' CCSCs. What is clear, however, is that despite the effort and cost of developing the CCSC vaccine, available tumor cell lines provide efficient immunogenicity against CRC and lead to tumor inhibition in nude mice. Even if the CCSC vaccine showed greater immunogenicity, CCSC identification, isolation, proliferation, and inactivation are very challenging, and vaccine preparation could fail at any of these steps. Tumor heterogeneity and cancer cell plasticity have also made CCSC markers a challenge in the first step of the vaccine preparation process, i.e., CCSC isolation. We did not observe expression of LGR5 and CD133 in the isolated CCSCs, but they were CD44<sup>+</sup>. Several studies have reported LGR5 as a CCSC marker in CRC (32, 33). However, Kim *et al.* found no significant relationship between LGR5 and CRC in clinicopathological factors (34), and LGR5 has been reported as a tumor suppressor (35). As previously reported, CD133 knockdown did not affect proliferation, migration, invasion, and colony formation in CRC cell lines (36) and the number of CD133<sup>+</sup> cells in spherical culture of CRC tumors could be between 2 and 96% (37). Therefore, the proposed markers for CCSC isolation may not be valid for all CCSCs. Overall, CCSC isolation is more useful in detecting tumor antigens and examining the tumorigenesis process than in developing autologous vaccines.

## Conclusion

Cell line-based vaccines have considerable advantages over CCSC vaccines in availability, ease and speed of preparation, and the possibility of multiple vaccinations. Conversely, isolating CCSCs and developing autologous CCSC vaccines are highly challenging. The present preclinical study showed that allogeneic vaccines developed from HT-29 and SW-480 can be an efficient and cost-effective option in the development of CRC vaccines. Future research on the efficacy and safety of these vaccines in humans is suggested.

## Acknowledgment

We acknowledge from Iran National Science Foundation (INSF) for financially supporting. In addition, we would gratefully like to thank head of animal room

in Avicenna research institute: Dr Mona Alibolandi, and anesthesiologists of Ghaem hospital: Dr Mohammad Alipour and Dr Shima Sheybani, and operating room personnel: Mr Reza Rezaeeyan, Ms Pilevaran, Ms Sedaghat and Ms Kazemi. The results described in this paper were part of student thesis.

### Authors' Contributions

FR Conceptualization, methodology, software, analysis, investigation, resources, writing; EN, FN, and SH Investigation; AA and MA Surgical tumor resection; NS and BM Pathological examination; MM FF and MG Methodology and resources; MRA Resources, conceptualization, and supervision.

### Funding

This work was supported by Mashhad University of Medical Sciences [Grant number 941474]; and Iran National Science Foundation [Grant number 96015793].

### Ethics Approval

The study (Code: 941474) was approved by the Human Research Ethics Committee of Mashhad University of Medical Sciences (Ethical approval codes: IR.MUMS.REC.1394.702) as well as the Institutional Animal Care and Use Committee of Mashhad University of Medical Sciences.

### Conflicts of Interest

The authors declare that they have no conflicts of interest.

### References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018;68:394-424.
2. Nguyen M, Tipping Smith S, Lam M, Liow E, Davies A, Prenen H, *et al.* An update on the use of immunotherapy in patients with colorectal cancer. *Expert Rev Gastroenterol Hepatol* 2021;15:291-304.
3. Butterfield LH. Cancer vaccines. *Bmj* 2015;350:h988.
4. Chiang CL-L, Coukos G, Kandalaf LE. Whole tumor antigen vaccines: where are we? *Vaccines* 2015;3:344-372.
5. Khan ST, Montroy J, Forbes N, Bastin D, Kennedy MA, Diallo J-S, *et al.* Safety and efficacy of autologous tumour cell vaccines as a cancer therapeutic to treat solid tumours and haematological malignancies: a meta-analysis protocol for two systematic reviews. *BMJ Open* 2020;10: e034714.
6. Yarchoan M, Huang CY, Zhu Q, Ferguson AK, Durham JN, Anders RA, *et al.* A phase 2 study of GVAX colon vaccine with cyclophosphamide and pembrolizumab in patients with mismatch repair proficient advanced colorectal cancer. *Cancer Med* 2020;9:1485-1494.
7. Wu AA, Bever KM, Ho WJ, Fertig EJ, Niu N, Zheng L, *et al.* A phase II study of allogeneic GM-CSF-transfected pancreatic tumor vaccine (GVAX) with Ipilimumab as maintenance treatment for metastatic pancreatic cancer. *Clin Cancer Res* 2020;26:5129-5139.
8. Kim VM, Pan X, Soares KC, Azad NS, Ahuja N, Gamper CJ, *et al.* Neoantigen-based EpiGVAX vaccine initiates antitumor immunity in colorectal cancer. *JCI Insight* 2020;5:e136368.
9. Srivatsan S, Patel JM, Bozeman EN, Imasuen IE, He S, Daniels D, *et al.* Allogeneic tumor cell vaccines: the promise

and limitations in clinical trials. *Hum Vaccin Immunother* 2014;10:52-63.

10. Zheng L, Edil BH, Soares KC, El-Shami K, Uram JN, Judkins C, *et al.* A safety and feasibility study of an allogeneic colon cancer cell vaccine administered with a granulocyte-macrophage colony stimulating factor-producing bystander cell line in patients with metastatic colorectal cancer. *Ann Surg Oncol* 2014;21:3931-3937.
11. Lei X, He Q, Li Z, Zou Q, Xu P, Yu H, *et al.* Cancer stem cells in colorectal cancer and the association with chemotherapy resistance. *Med Oncol* 2021;38:1-13.
12. O'Brien CA, Kreso A, Jamieson CH. Cancer stem cells and self-renewal. *Clin Cancer Res* 2010;16:3113-3120.
13. Li Y-f, Xiao B, Tu S-f, Wang Y-y, Zhang X-l. Cultivation and identification of colon cancer stem cell-derived spheres from the Colo205 cell line. *Braz J Med Biol Res* 2012;45:197-204.
14. Abbaszadegan MR, Bagheri V, Razavi MS, Momtazi AA, Sahebkar A, Gholamin M. Isolation, identification, and characterization of cancer stem cells: A review. *J Cell Physiol* 2017;232:2008-2018.
15. Ghazaey S, Mirzaei F, Ahadian M, Keifi F, Semiramis T, Abbaszadegan MR. Pattern of chromosomal aberrations in patients from north East Iran. *Cell J* 2013;15:258-265.
16. Jacobsen AV, Yemaneab BT, Jass J, Scherbak N. Reference gene selection for qPCR is dependent on cell type rather than treatment in colonic and vaginal human epithelial cell lines. *PLoS one* 2014;9:e115592.
17. Dowling CM, Walsh D, Coffey JC, Kiely PA. The importance of selecting the appropriate reference genes for quantitative real time PCR as illustrated using colon cancer cells and tissue. *F1000Res* 2016;5:99-114.
18. Wu D, Wang J, Cai Y, Ren M, Zhang Y, Shi F, *et al.* Effect of targeted ovarian cancer immunotherapy using ovarian cancer stem cell vaccine. *J Ovarian Res* 2015;8:1-10.
19. Monga SP, Wadleigh R, Sharma A, Adib H, Strader D, Singh G, *et al.* Intratumoral therapy of cisplatin/epinephrine injectable gel for palliation in patients with obstructive esophageal cancer. *Am J Clin Oncol* 2000;23:386-392.
20. Bagheri V, Abbaszadegan MR, Memar B, Motie MR, Asadi M, Mahmoudian RA, *et al.* Induction of T cell-mediated immune response by dendritic cells pulsed with mRNA of sphere-forming cells isolated from patients with gastric cancer. *Life Sci* 2019;219:136-143.
21. Sheikhi A, Jafarzadeh A, Kokhaei P, Hojjat-Farsangi M. Whole tumor cell vaccine adjuvants: comparing IL-12 to IL-2 and IL-15. *Iran J Immunol* 2016;13:148-166.
22. Neller MA, López JA, Schmidt CW, editors. Antigen for cancer immunotherapy. *Semin Immunol* 2008;20:286-295.
23. Network CGAR, Bell D, Berchuck a., Birrer M, Chien J, Cramer DW, *et al.* Integrated genomic analyses of ovarian carcinoma. *Nature* 2011;474:609-615.
24. Belderbos RA, Baas P, Berardi R, Cornelissen R, Fennell DA, van Meerbeeck JP, *et al.* A multicenter, randomized, phase II/III study of dendritic cells loaded with allogeneic tumor cell lysate (MesoPher) in subjects with mesothelioma as maintenance therapy after chemotherapy: dendritic cell Immunotherapy for mesothelioma (DENIM) trial. *Transl Lung Cancer Res* 2019;8:280-285.
25. Ghafar MTA, Morad MA, El-Zamarany EA, Ziada D, Soliman H, Abd-Elsalam S, *et al.* Autologous dendritic cells pulsed with lysate from an allogeneic hepatic cancer cell line as a treatment for patients with advanced hepatocellular carcinoma: a pilot study. *Int Immunopharmacol* 2020;82:106375.
26. van de Loosdrecht AA, van Wetering S, Santegoets SJ, Singh SK, Eeltink CM, den Hartog Y *et al.* A novel allogeneic off-the-shelf dendritic cell vaccine for post-remission treatment of elderly patients with acute myeloid leukemia. *Cancer Immunol*

- Immunother 2018;67:1505-1518.
27. Kajihara M, Takakura K, Ohkusa T, Koido S. The impact of dendritic cell-tumor fusion cells on cancer vaccines-past progress and future strategies. *Immunotherapy* 2015;7:1111-1122.
28. Rafiq S, Hackett CS, Brentjens RJ. Engineering strategies to overcome the current roadblocks in CAR T cell therapy. *Nat rev Clin oncol* 2020;17:147-167.
29. Chan JD, Lai J, Slaney CY, Kallies A, Beavis PA, Darcy PK. Cellular networks controlling T cell persistence in adoptive cell therapy. *Nat Rev Immunol* 2021;15:1-16.
30. Umut Ö, Gottschlich A, Endres S, Kobold S. CAR T cell therapy in solid tumors: a short review. *Memo- Mag Eur Med Oncol* 2021;14:1-7.
31. Schoenborn JR, Wilson CB. Regulation of interferon- $\gamma$  during innate and adaptive immune responses. *Adv Immunol* 2007;96:41-101.
32. Fan X-S, Wu H-Y, Yu H-P, Zhou Q, Zhang Y-F, Huang Q. Expression of Lgr5 in human colorectal carcinogenesis and its potential correlation with  $\beta$ -catenin. *Int J Colorectal Dis* 2010;25:583-590.
33. He S, Zhou H, Zhu X, Hu S, Fei M, Wan D, *et al.* Expression of Lgr5, a marker of intestinal stem cells, in colorectal cancer and its clinicopathological significance. *Biomed Pharmacother* 2014;68:507-513.
34. Kim YJ, Kang DH, Song GJ, Ahn TS, Son MW, Lee MS, *et al.* Clinical relevance of Lgr5 expression in colorectal cancer patients. *Korean J Clin Oncol* 2018;14:76-82.
35. Zhou X, Geng L, Wang D, Yi H, Talmon G, Wang J. R-Spondin1/LGR5 activates TGF $\beta$  signaling and suppresses colon cancer metastasis. *Cancer Res* 2017;77:6589-6602.
36. Horst D, Scheel SK, Liebmann S, Neumann J, Maatz S, Kirchner T, *et al.* The cancer stem cell marker CD133 has high prognostic impact but unknown functional relevance for the metastasis of human colon cancer. *J Pathol* 2009;219:427-434.
37. Szaryńska M, Olejniczak A, Kobiela J, Łaski D, Śledziński Z, Kmieć Z. Cancer stem cells as targets for DC-based immunotherapy of colorectal cancer. *Sci Rep* 2018;8:1-22.