Experimental Cell Research xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

# Subpopulations of cancer stem cells found in papillary thyroid carcinoma

Saied Mirshahidi[a,d], Alfred Simental[b], Steve C. Lee[b], Pedro A. De Andrade Filho[b], Nathaniel R. Peterson[b], Wenlong Cao[b], Rosalia de Necochea-Campion[a], Hao Yang[c], Penelope Duerksen-Hughes[d], Xiangpeng Yuan[b,d,*]

[a] Cancer Center Biospecimen Laboratory, Loma Linda University Medical Center, Loma Linda, CA 92354, USA
[b] Department of Otolaryngology-Head and Neck Surgery, Loma Linda University Medical Center, Loma Linda, CA 92354, USA
[c] Shaanxi Spine Medicine Research Center, Hong Hui Hospital, Xi'an Jiaotong University, Shaanxi 710054, China
[d] Department of Basic Sciences, School of Medicine, Loma Linda University, Loma Linda, CA 92354, USA

ARTICLE INFO

*Keywords:*
Thyroid carcinoma
Primary cells
Patient-derived xenograft tumor
Cancer stem cells
Subpopulations of cancer stem cells
Aldehyde dehydrogenase

ABSTRACT

Papillary thyroid carcinoma (PTC) is the most common form of thyroid cancer and while it has a generally good prognosis, tumor recurrence remains a major clinical challenge. Studying laboratory cell lines as well as clinical specimens indicate that PTC may follow the cancer stem cell (CSC) model. However, CSC characteristics relevant in PTC initiation and progression remain largely unknown. Here we studied a population of sphere-growing tumor cells isolated from primary cultures of clinical PTC. These sphere-growing cells consisted of aldehyde dehydrogenase positive ($ALDH^+$) and ALDH negative ($ALDH^-$) cell subpopulations and demonstrated a hierarchical pattern of cell division. Using combinations of selective depletion, specific inhibition and cell sorting, we found that both subpopulations of the sphere cells were able to self-renew and initiate xenograft tumors independently, and fulfilled the definition of CSC. Importantly, when the subpopulations functioned together, the cancer-initiation efficiency and the xenograft tumor progression were significantly enhanced compared to either subpopulation alone. These data revealed crucial roles of $ALDH^-$ CSC in PTC biology and suggested that CSC subpopulations function cooperatively to control PTC initiation and progression. Together, our study indicates that CSC subpopulations isolated from clinical specimens offer unprecedented opportunities for investigating PTC pathogenesis and developing effective therapies.

## 1. Introduction

Thyroid cancer is the most common endocrine malignancy and increasing in incidence [1,2]. The most frequent type of thyroid cancer is papillary thyroid carcinoma (PTC), which accounts for 80–85% of all thyroid cancer cases [3–5]. Most PTC patients can be treated successfully by surgical resection, with or without adjuvant radioactive-iodine administration, and have an excellent prognosis [5]. However, approximately 10–20% of stage I/II PTC patients experience disease recurrence [6], developing invasive tumors and/or distant metastases [3,5,7]. To treat the patients with aggressive disease effectively, it is a prerequisite to have a greater understanding of the mechanisms that regulate tumor initiation and progression in PTC.

It is becoming evident that thyroid cancers may follow the cancer stem cell (CSC) model, where a small population of cancer cell is responsible for tumor initiation and progression [8,9]. CSCs are invasive and highly resistant to conventional therapies, which results in disease relapse even when the primary lesion has been eradicated [10–12]. Therefore, targeting thyroid CSCs may represent an effective treatment strategy against aggressive PTCs. In studying CSC in thyroid cancer, different markers/methods have been used to isolate CSCs from thyroid cancer cell lines as well as clinical specimens [13–19]. They include flow cytometry sorting according to cell surface expression of CD44 [16], detection of side-population phenotypes by Hoechst 33342 exclusion [13], isolation of cytoprotective enzymes aldehyde dehydrogenase (ALDH) positive cell population [14], and sphere-formation in serum-free culture [19]. Although CSC isolations from PTC have been reported using such strategies, the biological properties of the isolated CSC are still poorly understood. This may be due to the difficulty of obtaining appropriate PTC samples from patients, the relatively slow

*Abbreviations:* PTC, papillary thyroid carcinoma; CSC, cancer stem cell; ALDH, aldehyde dehydrogenase positive; PBS, phosphate-buffered saline; NEAA, nonessential amino acids; DMEM, Dulbecco's Modification of Eagle's Medium; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; LDA, limiting-dilution analyses; FACS, fluorescence-activated cell sorting

* Corresponding author at: Department of Otolaryngology-Head and Neck Surgery, Loma Linda University, Loma Linda, CA 92354, USA.
*E-mail address:* xyuan@llu.edu (X. Yuan).

https://doi.org/10.1016/j.yexcr.2017.12.017
Received 27 July 2017; Received in revised form 4 December 2017; Accepted 16 December 2017

growth of PTC, the lack of tumorigenic PTC cell lines, and nonspecific CSC markers and/or inadequate techniques to isolate CSC. In addition, tumorigenic stem/progenitor cells may be heterogeneous, and their isolation influenced by the use of different markers of stemness. In this study, we isolated/enriched CSCs from primary specimens of PTC patients, investigated the PTC CSC's proliferation/division features, characterized the subpopulations of enriched CSCs for their self-renewal and tumorigenic properties, and explored the interaction between the subpopulations of CSCs. Our study may lead to enhanced understanding of PTC biology and provide new insights into developing novel strategies in the diagnosis and treatment of aggressive PTC.

## 2. Materials and methods

### 2.1. Preparation of primary cells from PTC tissues

Human PTC tissue was obtained intraoperatively after informed consent, as approved by the Institutional Review Board at Loma Linda University. Tumor specimens were washed in PBS, minced with sterile blades and incubated with collagenase (STEMCELL Technologies) for 2 h at 37 °C. After this enzymatic digestion, the sample was filtered through a 40 μm cell strainer and mixed with ammonium chloride solution for 5 min to lyse red blood cells.

### 2.2. PTC cell in vitro cultures

The prepared PTC cells were plated in two different culture conditions: 1) serum-containing [RPMI 1640 medium supplemented with 10% fetal bovine serum, nonessential amino acids (NEAA), sodium pyruvate (SP), and penicillin-streptomycin-amphotericin B (P-S-A)] and 2) serum-free [DMEM/F12 medium supplemented with B27, N2, EGF and bFGF (20 ng/ml each), NEAA, SP, and P-S-A]. In serum containing conditions growing as monolayer, cells were passaged twice per week. While in serum-free conditions, cells were growing in low-attachment 6-well plates as spheres. For serial passaging, spheres were collected by gentle centrifugation and dissociated enzymatically with 0.05% trypsin/EDTA. The dissociated cells were passed through a 40 μm mesh filter to obtain single cell suspensions. Single cells were plated at 5000 cells/well in the low-attachment plates to generate secondary spheres. Three such rounds of serial passage were performed. In some experiments, spheres were collected, dissociated into single-cell suspensions, sorted into $ALDH^+$ and $ALDH^-$ subpopulations, and then subjected to either serial passaging or mixed with Matrigel/DMEM/F12 for injection into mice. For the *in vitro* self-renewal assay, monolayer cells or spheres were dissociated into single cells and seeded into 96-well plates. In some experiments, sphere-derived single cells were sorted into $ALDH^+$ and $ALDH^-$ subpopulations before seeded into 96-well plates. Three to five weeks later, wells containing spheres were scored, and the number of positive wells was used to calculate the frequency of sphere-forming units using the ELDA software provided by the Walter and Eliza Hall Institute [20].

### 2.3. In vivo tumorigenicity experiments

Eight-week-old NOD/SCID mice were obtained from Jackson Laboratory and maintained under specific pathogen-free conditions with the approval of the Institutional Animal Care and Use Committee of Loma Linda University. For orthotopic transplantation, single cells derived from monolayer or sphere cells were resuspended in Matrigel/DMEM/12 (1:1 dilution) into the desired cell doses, and injected into the right thyroid gland that was exposed by surgical procedures and closed by sutures after the injection. Mice were euthanized 2 months post-injection and examined for tumor formation. For subcutaneous transplantation, sphere-derived single cells of whole population, $ALDH^+$ and $ALDH^-$ subpopulation were resuspended, respectively, in Matrigel/DMEM/12 into the desired cell doses, and injected subcutaneously into the flanks of NOD/SCID mice. The tumor sizes were measured during the tumor development. The mice were euthanized at the end of experiments. The number of tumors formed out of the number of sites injected was scored to determine the frequency of cancer-initiating cells calculated using the ELDA software. For serial transplantation, tumor xenografts were harvested, prepared into single cells, and cultured in serum-free medium as described above. The resulting sphere cell population from such culture was termed “secondary passage” and then re-inoculated into NOD/SCID mice called secondary recipients. Subsequently, tumors formed in the secondary recipients were used for repeated rounds of sphere cells isolation and the implantation into tertiary recipients. The morphological features of xenograft and original patient tumors were analyzed after hematoxylin-eosin staining of the tissue sections and examined under a microscope.

### 2.4. ALDH assay

Monolayer cells, spheres or tumor xenografts were harvested and enzymatically digested into single cells, and ALDH activity was analyzed with an ALDEFLUOR kit (STEMCELL Technologies) according to the manufacturer's instructions. Cells were analyzed using MACSQuant Analyzer 10; cells were sorted using FACSAria cell sorters.

### 2.5. Cell subpopulation depletion and ALDH inhibition

Spheres were dissociated into single cells by enzymatic digestion. The dissociated cells were processed with ALDEFLUOR kit reagents as indicated above. To deplete the $ALDH^+$ cell subpopulation, all the $ALDH^{high}$ and $ALDH^{low}$ cells were eliminated from the sphere-derived single cells by increasing the selection stringency with sorting gates set on the bottom 5% of non-fluorescent population using a FACSAria sorter. To deplete the $ALDH^-$ cell subpopulation, cells lacking ALDH activity were eliminated from the sphere-derived single cells with sorting gates set on the top 5% of fluorescent population. To inhibit ALDH activity, sphere-derived single cells were incubated with a specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) at a working concentration of 100 μM. The control groups were incubated with vehicle alone. All incubations were initiated at the beginning of single cells seeding and terminated at the end of sphere-initiation assay experiments.

### 2.6. Statistical analyses

Limiting-dilution analyses (LDA) for frequency determinations of sphere-initiating cells and cancer-initiating cells, as well as the corresponding P values, were performed using ELDA software, which took into account whether the assumptions for LDA were met [20]. The data and error bars report the mean ± SD. *: $p < 0.05$, **: $p < 0.01$. For comparisons of cell numbers in culture and xenograft tumor volumes, Two-tailed Student's *t*-test was performed between two groups and a difference was considered statistically significant with $p < 0.05$.

## 3. Results

### 3.1. PTC specimen derived sphere cells can initiate xenograft tumor

Increasing evidence indicates that studying primary cells derived directly from cancer patients is a clinically relevant strategy to understand cancer biology and develop cancer therapies. We collected 67 tumor specimens from patients (aged 12–92 years) undergoing surgical treatment for PTC (Table 1). Primary cancer cells were isolated from the tumor specimens by enzymatic digestion of tumor tissues harvested immediately after surgical resection. The isolated primary cells were cultured *in vitro* with two different growth conditions – 1) serum-containing culture conditions typically used for growing thyroid cancer cells lines, and 2) serum-free stem cell culture conditions that support