Identification and Quantification of Drug-Resistant Cells in Colorectal Tumor and Distal Margin

K. Chandramohan¹ · Tapas Pradhan² · S. Asha Nair²

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Abstract Cancer stem cells are cellular subpopulations in tumor, which are highly tumorigenic and drug resistant due to their adaptive molecular mechanism. In the recent years, these cells have attracted researchers' focus because of their unique properties which makes them biologically well evolved than other proliferating tumor cells. We have studied the presence of cancer stem cells in colorectal cancer (CRC) and its surgical margins. We report for the first time the presence of drug-resistant cells in distal resection margins in CRC and showed that a closer distal margin of less than 2 cm could have higher possibility of drug-resistant cells to spread.

Keywords Colorectal cancer · Drug-resistant cells · Distal margin · Cancer stem cells

Introduction

Colorectal cancer (CRC) stands as a formidable health problem worldwide. It is the third most common cancer in men (746,298 cases, 20.6 age standardized rate (ASR)) and the second most common in women (614,304 cases, 17.6 ASR) [1]. Almost 60% of cases are encountered in developed countries. The number of CRC-related deaths is estimated to be approximately 693,933 worldwide, accounting for 8.3 ASR. In India, the ASRs for colorectal cancers in men and women

are 7.2 and 5.1 per 100,000, respectively. In the Indian population, colorectal cancers are the fourth common cancer among men and the third common cancers among women [1]. In a 2013 report, the highest ASR in men for CRCs was recorded in Thiruvananthapuram (4.1) followed by Bangalore (3.9) and Mumbai (3.7). The highest ASR in women for CRCs was recorded in Nagaland (5.2) followed by Aizwal (4.5). There are few reports showing an increase in early CRC incidence rate in India, which are alarming [2].

CRC mortality has radically decreased due to advancement in clinical practices and development of potent chemotherapeutic drugs as well as therapies. As of now, colorectal cancers are killing many people as metastasis and loco-regional recurrence are two major obstacles for a disease-free survival. Reports show that about 10–20% of all recurrence for colon cancers occurs local-regionally and after standard oncological excision [3]. It is estimated that 50–60% of patients diagnosed with CRC will develop metastases during the course of their disease, irrespective of treatment [4]. The possible causes behind metastasis and loco-regional recurrence after treatment could be due to residual tumor cells left around the tumor (tumor spread in margins) and presence of highly tumorigenic drug-resistant cells which evaded the therapy given.

A diverse range of molecular mechanisms have been implicated in tumor drug resistance; these include increased rates of drug efflux, alterations in drug metabolism, and mutation of drug targets [5]. Tumors are highly adaptable, and the activation of survival signaling pathways and the inactivation of death signaling pathways can also lead to drug resistance. Epigenetic changes and the influence of the local tumor microenvironment have also been identified as important contributors to chemo resistance [6]. Recently, treatment failure in certain settings has been attributed to the presence of a tumor subpopulation known as cancer stem cells, which are intrinsically highly resistant to many therapeutic approaches [7].

K. Chandramohan and Tapas Pradhan are joined first authors

✉ K. Chandramohan
drchandramohan@gmail.com

¹ Regional Cancer Center, Trivandrum, Kerala, India

² Rajiv Gandhi Centre for Biotechnology, Trivandrum, Kerala, India

Cancer stem cells are cellular subpopulations in tumor, which are highly tumorigenic and drug-resistant due to their adaptive molecular mechanism such as inactivation, alterations in the drug target, processing of drug-induced damage, and evasion of apoptosis. In the recent years, these cells have attracted researchers' focus because of their unique properties which makes them biologically well evolved than other proliferating tumor cells. Cancer stem cells have been reported and validated in various tumors such as leukemia [8], glioblastoma [9], breast cancer [10] and colorectal cancer [11], pancreatic cancer [12], melanoma [13], liver cancers [14], and head and neck cancers [15]. Colorectal cancer stem cells exhibit 5-fluorouracil (5-Fu) and oxaliplatin resistance through autocrine stimulation of IL-4 receptors [16]. One of the major features of cancer stem cells has been attributed to the expression of ABC (ATP-binding cassette) transporters, and these characteristics allow them to efflux most of the available therapies against tumors. Many studies reported high expression levels of drug efflux proteins like ABCB1, ABCC1, and ABCG2 in different tumors. But specifically, ABCC1 expression has been associated with 5-Fu-based therapy resistance in CRC and proposed to be used as a prognosis marker [17].

Drug efflux through ABC transporter has been very crucial in colorectal cancer as the most commonly used drug, 5-Fluorouracil has been reported to be a substrate for ABC transporters. So, identifying drug-effluxing cells could possibly allow us to quantify cancer stem cell population in tumor and its spread, thus providing more information about the presence of drug-resistant and tumorigenic cells. In this study, we have analyzed normal, tumor, and distal resected biopsies from 33 patients diagnosed with colorectal cancer for the presence of drug-resistant cells based on side population assay by flow cytometry.

Patients and Methods

Population

This study was undertaken at two institutions in India, Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, Kerala and Regional Cancer Centre (RCC), Trivandrum, Kerala. The study protocol conformed to the guidelines of the ethics committee of each institution and was approved by each institution's review board; all patients provided written informed consent. A total of 33 colorectal cancer patient biopsies were used in this study. Biopsies analyzed were normal, tumor and distal spread obtained from the same patient after surgery.

Sample Collection and Processing

Colorectal cancer samples used in the study were obtained from patients undergoing surgery at RCC, Thiruvananthapuram, after

informed consent, human ethics committee approval, and sanction from the institutional review board. The samples collected from different sites of the tumor resections (tumor, normal i.e., normal mucosal area away from both margins and surgical margin) were collected in Hank's balanced salt solution (HBSS) (Gibco, Invitrogen) containing 2% serum (Gibco, Invitrogen) and transported immediately under cold conditions to RGCB. Tissues were transferred to a 60-mm dish and washed well with HBSS containing 4× penicillin-streptomycin (Gibco, Invitrogen) and 2× antibiotic-antimycotic solution (Gibco, Invitrogen). The tissue was then chopped into fine pieces using a scalpel blade and transferred into a 50-ml centrifuge tube containing 5 ml of serum-free DMEM:F12 (Gibco, Invitrogen), 0.5% collagenase type IV (Gibco, Invitrogen), and 1× penicillin-streptomycin and 2× antibiotic-antimycotic solution. The tubes were incubated at 37 °C for 3 h 30 min, after which the cells were passed through the 40-µm cell strainer (BD Bioscience). The filtrate was pelleted at 3000 rpm for 3 min. The supernatant was discarded. One milliliter of RBC lysis solution was added to the pellet and was incubated for 10 min. The cells were pelleted, and the pellet was suspended in 1 ml 2% FBS containing DMEM on ice.

All margins were recorded from histopathology reports.

Cell Count and Viability Assessment

To 10 µl of isolated cells suspension, 10 µl 0.4% trypan blue dye (Sigma) was added and incubated for 5 min at room temperature. Then, the mixture was thoroughly suspended by multiple pipetting and 15 µl of it was taken for viable cell counting using a hemocytometer (Sigma Aldrich).

Side Population Assay

Side population assay was performed following the protocol developed by Goodell (1996) with slight modification. Isolated cells were spun down at 1800×g for 5 min, and pellet was suspended at 10⁶ cells per milliliter in pre-warmed DMEM (supplemented with 2% serum) media (Gibco, Invitrogen) in two tubes. Hoechst 33342 (Sigma) was added to a final concentration of 5 µg/ml in dark into both tubes. In the control tube, 100 µM verapamil was added to cells and incubated for 5 min prior to staining with Hoechst. Cells were mixed well and placed in 37 °C water bath for 120 min precisely with interrupted shaking. After incubation, cells were spun down at 4 °C and re-suspended in cold HBSS (with 2% serum). Stained cells were kept on ice to exclude leakage of the Hoechst dye from the cells. At the end of the staining, cells were suspended in cold HBSS (2% serum) containing 2 µg/ml propidium iodide (Sigma) for dead cell discrimination. Cells were immediately taken for flow cytometric analysis (BD FACS Aria II), and results were analyzed using FACS Diva software.

Statistical Analysis

All statistical analysis was done using Microsoft Excel-2010 and Graph pad prism 5.

Results

Distal Margins Harbor Cells with Drug-Resistant Phenotype in Colorectal Cancer

CRC biopsies from tumor and distal margins were analyzed for identification and quantification of drug-resistant cells by flow cytometry-based side population assay, and the apparently normal biopsies were used as controls from the same patient. Here, we observed that distal margin and tumor depicted an increased number of drug-resistant cells as compared to those of normal tissues with a mean of 0.12, 0.25, and 0.41% in normal, tumor, and distal margin, respectively, of the 33 CRC patients analyzed (Fig. 1b). These results show that drug-resistant cell enrichment could be due to induced therapy response by tumorigenic cells as well as tumor spread in distal resection margin, which is negative by histopathology.

Fig. 1 Drug-resistant cells in normal, tumor, and distal resection margin by flow cytometry. **a** Showing scatter plot with differential side population distribution in normal, tumor, and distal margin. **b** Representing a column graph plot showing abundance of drug-resistant cells in normal, tumor, and distal margins of CRC samples ($n = 33$)

Distal Resection Length of <2 cm Depicted Higher Drug Resistance of Cells in CRC Samples

Here, we investigated the correlation between distal resection length and abundance of drug-resistant cells. We divided overall patients into three groups on the basis of distal resection length as <2, 2–5, and >5 cm. Though we could identify drug-resistant cells in all three groups, interestingly, patients who had a short resection length (<2 cm) revealed more number of drug-resistant cells with a mean value of 0.5% compared to those with 0.39 and 0.30% in 2–5 and >5 cm resection lengths, respectively (Fig. 2). This observation again ropes the presence of tumor spread in distal margin, which could have therapy-resistant phenotype.

Enrichment of Drug-Resistant Cells in Distal Margins May Be Independent of Pathological Grade

High-grade or poorly differentiated tumors are frequently associated with poor prognosis of CRC. Also, high-grade tumors have been mostly linked with loco-regional as well as distant recurrences. Here, we compared our side population data with the pathological grades of tumors and observed that poorly differentiated tumors showed an increased number of

Fig. 2 Box-and-whisker plot showing the correlation between distal resection length and abundance of drug-resistant cells in CRC. Here, the *upper* and *lower whiskers* represent the maximum and minimum values in the total samples analyzed ($n = 33$)

drug-resistant cells in their distal tumor spread. However, the difference in the cell population was not statistically significant, as lower grade tumor showed a mean population of 0.38% compared to that of high grade with a population mean of 0.43% (Fig. 3). This proposes that, presence of drug-resistant cells could be independent of pathological grade of the tumor analyzed.

Discussion and Conclusion

Tumor recurrence and lack of reliable prognostic markers are presently the major challenges in CRC management throughout the globe. CRC is a disease treated mainly by surgery as most of the patients diagnosed with CRC undergo surgical removal of tumor along with or without chemo radiotherapy. Sole objectives of surgical excision of tumor have been curative and this highly depends on precise removal of tumor and its associated margins, since a positive resection margin is associated with high risk of local recurrence and distal metastases [18]. Hence, clinical evaluation of tumor and margins holds great importance for prognosis of disease. Though conventional histopathology techniques used for evaluation of tumor and margins are still the gold standard in disease

Fig. 3 Box-and-whisker plot showing the correlation between drug-resistant cell abundance and pathological grade in colorectal cancer

diagnosis and prognosis, still there exists a contention on the lacunae. It holds that, it cannot provide in-depth information regarding predictive and prognosis factors associated with the disease. To improve the prognosis, a systemic approach of conventional and molecular analysis could be used to get more accurate information regarding a cancer patient. As cellular and molecular analysis has been proved to be more sensitive and informative, it can provide accurate and precise information about a tumor or its spread with a high sensitivity. Many studies have shown that existence of drug-resistant cell population (also referred as tumor stem-like cells) could have been instrumental in tumor relapse and progression. Again, this rare cell population has already been reported to possess higher tumorigenic potential than that of bulk tumor cells and insensitive to therapy. Enrichment of these cells in tumor has also been shown to be associated with poor disease prognosis in CRC [19]. But the major concern encountered happens to be that, tumors are highly heterogeneous in composition; therefore, molecular markers for identification of drug-resistant cells are still debated. However, identification of cells which show property of high drug efflux ability could be significant in clinics for prognostication in a better way.

Here, we could identify and quantify rare population of cells with drug-resistant phenotype present in CRC tumor and distal spread by a sensitive flow cytometry-based assay. Our study suggests that the tumor spread at the resection margins also could carry some tumor cells with high tumorigenic potential. As most of the studies are focused on the tumor but not on its spread, there are hardly any information regarding marginal spread in CRC. Presently, in the clinical setup, samples are mostly evaluated on the basis of histopathological techniques which alone have certain limits as it can only provide information regarding the bulk tumor and but not about the rare potent tumorigenic cells. This study cues at the presence of drug-resistant subpopulation in tumor as well as in distal margin, which could have an important role in disease relapse and metastasis. Side population assay of normal, tumor, and distal spread showed that tumor and distal margins harbor more numbers of drug-resistant cells than normal tissues and varied among individuals. This suggests that resection margins could harbor tumorigenic cells, and sensitive cell-based assay could be useful for identification and quantification of such cells. Surgical margins are very crucial for disease-free survival of a CRC patient. But still, the tumor-free resectable margins are topic of debate because of lack of cellular and molecular knowledge [20]. Here, we could find a relation between the length of distal resection margins and presence of drug-resistant cells. Closer margin (<2 cm) showed an enriched population of cells; whereas, margin away from tumor (>5 cm) showed a reduced amount of cells. But still, these cells could be enriched even in longer resection lengths depending upon various extrinsic and intrinsic factors such as genetic and epigenetic makeups of tumor and chemo

radiotherapy given. But, as the drug-resistant cell abundance did not significantly vary from high-grade tumor to low-grade tumor, we can hypothesize that presence of drug-resistant cells could be an independent prognosis factor in colorectal cancer.

In conclusion, we report for the first time the presence of drug-resistant cells in distal resection margins in CRC and showed a closer distal margin of less than 2 cm could have higher possibility of drug-resistant cells to spread. However, this study did not characterize these drug-resistant cells far to suggest they are cancer stem-like cells. Further studies are going on to characterize and identify cellular and molecular markers for tumorigenic population from tumor as well as margins to develop predictive and prognostic markers in CRC.

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Compliance with Ethical Standards

The study protocol conformed to the guidelines of the ethics committee of each institution and was approved by each institution's review board; all patients provided written informed consent.

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

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