Autophagy inhibition suppresses the tumorigenic potential of cancer stem cell enriched side population in bladder cancer

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The mechanisms that underlie tumor formation and progression have not been elucidated in detail in cancer biology. Recently, the identification of a tumor cell subset defined as cancer stem cells (CSCs), which is enriched for tumor initiating capacity, has engendered new perspectives towards selective targeting of tumors. In this study, we isolated the side population (SP) cells which share characteristics of CSCs from bladder cancer cell lines, T24 and UM-UC-3 by fluorescence activated cell sorting. The cells were cultured in serum free medium and expression profile of stem cell like markers (SOX-2, NANOG, KLF-4 and OCT-4), drug resistant genes (ABCG2 and MDR1) and spheroid forming capability were examined in SP, non-side population (NSP) and bulk T24 and UM-UC-3 cells. We observed that SP cells possessed a higher mRNA expression of SOX-2, NANOG, KLF-4, OCT-4, ABCG2, and MDR1 as well as a higher spheroid forming ability as compared to other bulk cells or NSP cells. The SP cells had low ROS levels and high GSH/GSSG ratio which may contribute to radio-resistance. The SP cells also showed substantial resistance to gemcitabine, mitomycin and cisplatin compared with the NSP counterpart. A high autophagic flux was observed in the SP cells. Both pharmacological and siRNA mediated inhibition of autophagy potentiated the chemotherapeutic effects of gemcitabine, mitomycin and cisplatin in these cells. We concluded that the ABCG2 expressing SP cells show autophagy associated cell survival and may be a potent target for developing more effective treatment in bladder carcinoma to enhance patient survival.

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1. Introduction

Human solid tumors and clonal tumor cell lines comprise phenotypically and functionally diverse subsets of cells. Cancer stem cells (CSCs), discovered recently in several solid tumors, represent a cell-population that can survive treatment and repopulate the tumor. These cells may play a unique role in tumor maintenance, by virtue of resistance to chemotherapeutic agents and radiation [1]. CSCs share some characteristics with adult stem cells like unlimited capacity for proliferation, self-renewal and ability to differentiate to other cell types [2]. Various surface markers selectively expressed on CSCs have been identified and are used to isolate these cells, however in many tumor types no marker or pattern of marker is known to prospectively identify CSCs. In such cases, exploitation of stem cell characteristics can be used to identify CSCs. One such characteristic is the ability of side population (SP) cells to exclude dyes such as Hoechst 33342. CSCs have been identified in immortalized cell lines, long-term cultured cancer cells and patient tumor samples using the flow-cytometry based SP technique [3]. The expression of ATP-binding cassette efflux transporter ABCG2 by SP cells is thought to confer them with intrinsic resistance to many anti-tumor agents [4]. Thus, this subset of cancer cells may be an important target for therapeutic interventions, preventing chemo-resistance and cancer relapse.

Urothelial carcinoma (UC) or bladder cancer is the most common cancer of the urogenital tract. Majority of deaths in bladder cancer are due to unetectable lesions that are resistant to chemotherapy [5]. Despite improvement in radiation and chemotherapy regimens, there have been few advances in the design of treatment strategies for chemotherapy-resistant UC. The therapeutic resistance and recurrence in patients with UC may be attributed to the presence of CSC population. The identification of a cancer stem cell like population in the SP in bladder cancer may provide new therapeutic approaches in research targeting these malignant cells. Recent evidences indicate that autophagy acts as a cell survival mechanism in bladder cancer as a whole [6] and may be associated with multiple cancer related pathways including chemo-resistance [7]. We showed the critical role of autophagy in SP cell tumorigenicity by chemical inhibition of autophagy and silencing of Beclin1 (BECN1), the mammalian ortholog of the yeast apg6/vps30p which is required

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for the induction of autophagy. BECN1 is a haploinsufficient tumor-suppressor gene that is monoallelically deleted or shows reduced expression in various cancers [8]. Recently, many studies have shown that several types of tumor cells depend on autophagy for growth in normal condition [9,10]. Therefore, we investigated whether autophagy might be involved in the survival of SP cells in bladder cancer cell lines.

As tumor grows, areas of nutrient and oxygen deprivation (hypoxia) arise due to inadequate blood supply [11]. Hypoxia leads to induction of pro-angiogenic factors and down-regulation of anti-angiogenic factors [12]. It is known that hypoxia inducible factor (HIF1) induces vascular epithelial growth factor (VEGF) which is correlated to tumor progression and metastasis. Tumor hypoxia and angiogenesis may provide therapeutic resistance to anticancer agents, increase in invasion, metastasis and poor outcome in malignancies [13]. Hypoxia, nutrient deprivation and angiogenesis are known to induce the autophagy [14]. Hypoxia also triggers an acute induction of reactive oxygen species (ROS) over a short period of time, while prolonged hypoxia results in decreased intracellular ROS levels as compared to normoxia [15]. The biological role of ROS in CSC subpopulation is still not clear though ROS regulation has been studied in cancer cells as a whole. Our hypothesis is that low ROS levels during proliferation protects these cells against DNA damage and facilitates tumor propagation. Several studies show that autophagy is involved in both degradation and formation of ROS [16]. Autophagy mediated support of tumor cell survival may play a critical role in cancer progression at later stages, such as dissemination and metastasis, which account for most cancer-associated deaths.

In this study, we isolated and characterized the SP cells from two bladder cancer cell lines, T24 and UM-UC-3. These cells showed higher resistance to chemotherapeutic agents like gemcitabine, mitomycin and cisplatin compared to the cells in non-side population (NSP). Our results suggested that autophagy contributed to the ability of the SP

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**Fig. 1.** Identification and characterization of side population (SP) in T24 cells. (A) T24 cells were stained with Hoechst 33342 dye (5 mg/ml) in the presence (right) or absence (left) of 50 mol/l verapamil and analyzed by flow-cytometry. The quantification of SP as in (A) carried out with FACS Diva software is shown in lower panel. (B) Immuno-staining of CD44 and pancytokeratin in sorted SP and non-side population (NSP) cells under confocal microscope (60× magnification). Each experiment was repeated three times. All values are expressed as mean ± SEM.

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**Fig. 2.** The expression of stemness and drug resistant genes in T24 SP cells. (A) Reverse transcriptase PCR analysis for stemness genes (SOX-2, OCT-4, KLF-4 and NANOG) and drug resistant genes (ABCG2 and MDR1). Densitometry was performed using ImageJ software and fold change was calculated by normalizing with the house keeping gene ACB. The results are shown as bar graphs in the lower panel. (B) The bar graph shows the percentage of cell death in SP and NSP at 24 h with cisplatin/gemcitabine/mitomycin. Untreated cells in culture media were taken as control. Each experiment was repeated three times. All values are expressed as mean ± SEM.* p < 0.05 NSP vs. SP; **p < 0.01 NSP vs. SP. NSP = non side population; SP = side population.
cells to survive conventional chemotherapy and regenerate a cancer population, leading to relapse. Hence, targeting autophagy in bladder cancer SP cells may provide a novel approach for a more effective treatment.

2. Materials and methods

2.1. Cell culture and reagents

T24 bladder cancer cell line was procured from the National Centre for Cell Sciences, Pune, India and cultured in the RPMI-1640 media (Sigma (St. Louis, MO)) containing 10% fetal bovine serum (FBS) [Sigma (St. Louis, MO)]. UM-UC-3 cell line (a kind gift from Dr. Sekhar Majumdar, IMTECH, Chandigarh, India) was originally procured from the European Collection of Cell Culture and maintained in RPMI-1640 media with 10% FBS. The monoclonal anti-CD44, pan-cytokeratin, caspases-12, -3, -8 and -9, cytochrome-c antibodies and secondary fluorochrome conjugated antibodies were obtained from BD Biosciences (San Diego, CA). Monoclonal antibodies against LC3, Becn1, Atg7 and p62 were purchased from Cell Signaling Technology (Carpinteria, CA). Antibodies against Bax, Bcl-2, c-IAP-1 and c-IAP-2 were purchased from Santa Cruz Technology (Santa Cruz, CA). Anti-rabbit, mouse and goat secondary-IgG antibodies were purchased from Cell Signaling Technology (Carpinteria, CA). BECN1-siRNA and control-siRNA were obtained from Santa Cruz Technology. Anti-rabbit secondary-IgG antibodies were purchased from Abcam (San Diego, CA). All other chemicals were obtained from Sigma (St. Louis, MO) unless specified otherwise.

2.2. Side population analysis

Side population was isolated according to the method of Goodell et al. [17] with some modifications. Briefly, cells (1 × 10⁶ cells/ml) were taken in pre-warmed RPMI-1640 containing 5% FBS and incubated with Hoechst 33342 (5 mg/ml) for 90 min at 37 °C in a water bath. In another set of experiments, cells were incubated with the Hoechst dye (5 mg/ml) in the presence of verapamil (50 μmol/ml). At the end of incubation, propidium-iodide (PI, 2 μg/ml) was added and an analysis was done on a BD FACS Aria-II flow-cytometer. The Hoechst dye was excited with the UV laser at 351 to 364 nm and its fluorescence was measured with a 515 nm side population filter (Hoechst blue) and a 608 optical filter (Hoechst red).

2.3. Immuno-staining

Cells (1 × 10⁶) were seeded on cover-slips in six-well culture plates and treated with HBSS/antimycin/tunicamycin/chemotherapeutic agents (cisplatin/gemcitabine/mitomycin) in separate experiments. After treatment, cells were washed with 1 × PBS and fixed in 4% para-formaldehyde, thereafter permeabilized with 0.1% Triton X-100. Cells were blocked with 5% BSA for 2 h and incubated with the primary antibodies for 5 h at room temperature. Subsequently, cells were washed with 1 × PBS and incubated with FITC or PE labeled secondary
antibodies for another 2 h at room temperature in the dark. Cells were again washed with 1× PBS and stained with Hoechst for nuclear staining and analyzed under confocal microscope (Olympus, 1×81). Punctates of LC3 were counted in FLUOVIEW (FV1000-FV10-ASM) software (Olympus).

2.4. Sphere formation assay

FACS-sorted SP cells were plated in ultra-low attachment six well plates in serum-free keratinocyte medium supplemented with growth factors (1:50, Invitrogen), 20 ng/ml epidermal growth factor (BD Biosciences, San Diego, CA), 0.4% FBS (Sigma (St. Louis, MO)) and 4 mg/ml insulin (Sigma (St. Louis, MO)) as described previously [18]. The primary spheroids were enzymatically dissociated with 0.1% trypsin for 3 min at 37 °C to obtain a single cell suspension which were then recultured in conditioned medium to produce the next generation of spheroids.

2.5. Reverse transcriptase PCR analysis

Total cellular RNA was extracted from freshly sorted SP and non-side population (NSP) cells using RNA isolation kit (Roche). The purity of the RNA was checked using a Bio-photometer plus (Eppendorf). RNA was reverse transcribed with iScript™ cDNA synthesis kit from Bio-RAD according to the manufacturer’s protocol. PCR was done by standard method [19]. The PCR sequences are provided in Supplementary Table 1. The PCR products were analyzed by electrophoresis on 2% agarose gel and bands were visualized in Chemidoc-XRS+ (Bio-RAD). Levels of expression were normalized with β-actin (ACTB). Densitometry was performed using NIH ImageJ software.

2.6. Immuno-blot analysis

For Western blot analysis, cell lysates were prepared in RIPA buffer containing Tris–HCl (50 mM), NP-40 (1%), sodium-deoxycholate(0.25%), NaCl (150 mM), and EDTA (1 mM). Protein concentration was determined by BCA assay kit [Sigma (St. Louis, MO)]. Samples were resolved in SDS-PAGE, transferred to PVDF membrane and analyzed separately. The blots were probed with one of the primary antibodies of caspase-3, PARP, Beclin1, Atg7, LC3, XIAP, c-IAP1/2, Bcl-2, Bcl-XL, Bax and cytochrome-c. Chemiluminescence detection was done with Amersham ECL plus Western blotting detection system (GE healthcare). We used peroxidase conjugated anti rabbit-IgG/anti-mouse-IgG/anti-goat-IgG as secondary antibodies. The band intensity was measured by Chemidoc-XRS+ (Bio-RAD) and quantification was done by using NIH ImageJ software. Densitometric analysis was performed by ImageJ software.

2.7. ROS measurement

Levels of ROS in the various cell populations was by measured using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA, 5 μmol/l) [Sigma (St. Louis, MO)] stain under the fluorescence microscope (Olympus 1×51) according to the previously described protocol [20].

2.8. GSSG/GSH ratio measurement

GSSG/GSH ratio was measured by colorimetric assay kit (OxiSelect™ Total Glutathione (GSSG/GSH) Assay Kit, STA-312) according to the manufacturer’s protocol.

Fig. 4. Spheroid forming capability of T24 SP cells. (A) Light microscopy images of tumorspheres formed by SP cells in serum-free keratinocyte medium. (B) Size of spheroids formed by SP cells on 3rd, 6th and 9th days was larger as compared to that formed by the NSP cells (scale bar—70 μm). (C) The number of sphere formed/1000 SP cells was significantly greater than those obtained from NSP. Each experiment was repeated three times. All values are expressed as mean ± SEM. ***, p < 0.001 SP vs. NSP.
Fig. 5. Increased autophagy response in SP of T24 cells. (A) Cells were incubated in complete or starvation media for 24 h. Representative images of cells showing autophagosomes stained with MDC in T24, NSP and SP cells captured by fluorescent microscope with UV filter (20× magnification). The lower panel shows the quantification of the mean fluorescent intensity of MDC staining as calculated by ImageJ software. (B) Confocal images show immuno-staining of LC3 under the same conditions (60× magnification). The lower panel shows the number of LC3 dots/micrograph as calculated by FLUOVIEW (FV1000-FV10-ASM) software. (C) Fluorescent micrographs show MDC staining in the presence of rapamycin (20 nmol/l) and CQ (50 μM) in SP and NSP cells. Quantitation of the mean fluorescent intensity in SP and NSP cells is shown in the lower panel. (D) Fluorescent images of AO/EtBr staining at 24 h of treatment in SP and NSP cells. Green cells indicate viable healthy cells and orange/red cells represent apoptotic cells. Bar graph shows the percentage of cell death measured by MTT assay at 24 h of treatment with rapamycin and CQ. (E) Immuno-blot analysis of autophagy proteins with antimycin/tunicamycin at 24 h showed an increased expression of Beclin1, Atg7 and p62. The lower panels show the relative quantitation of the autophagy proteins, Beclin1, Atg7 and p62 after normalization with actin expression. (F) Confocal images of SP cells treated with antimycin/tunicamycin and immuno-stained with antibodies against LC3 and TOM (upper panel)/calreticulin (lower panel) at 24 h (60× magnification). Each experiment was repeated three times. C = control or untreated cells, T = tunicamycin, A = antimycin. Values are expressed as mean ± SEM. *, p < 0.05 control vs. treatment; **, p < 0.01 control vs. treatment.
2.9. Acridine orange staining

Cells (1 × 10^3) were seeded on cover-slip in six-well culture plates and incubated separately with selected doses of cisplatin/gemcitabine/mitomycin for 24 h. After treatment, cells were washed with 1 × PBS and stained with acridine orange at a final concentration of 1 μg/ml for a period of 15 min in the dark on ice. Autophagosome formation was monitored by fluorescence microscopy (Olympus 1 × 15) in blue filter. Intensity of AO stain was calculated by ImageJ software.

2.10. Monodansyl-cadaverin (MDC) staining

Cells (1 × 10^3) were seeded on cover-slip in six-well culture plates and treated with HBSS media for 24 h with or without wortmannin (Wm, 200 ng/ml), a class III phosphoinositide 3-kinase (PI3K) inhibitor which inhibits initial stages of autophagy. Cells were washed with 1 × PBS at the end of treatment period and incubated with MDC (0.05 mM) for 10 min in the dark on ice. Cells were washed with 1 × PBS following incubation and immediately analyzed under fluorescence microscope in UV filter (Olympus 1 × 51). The intensity of MDC stain was calculated by ImageJ software.

2.11. Cell viability assay

Cell viability was measured by MTT assay [Sigma (St. Louis, MO)] according to the manufacturers’ protocol. Briefly, 2 × 10^3 cells were treated with cisplatin/gemcitabine/mitomycin for indicated time points and concentrations. At the end of treatment incubation, 20 μl of MTT (5 mg/ml in PBS) was added and cells were further incubated for 4 h. Formazan crystals formed were dissolved in 100 μl of lysis solution (20% sodium dodecyl sulfate, 50% dimethyl formamide). The absorbance of solubilized formazan was read at 570 nm using ELISA reader (Bio-TEK, synergy-2).

2.12. Sub-G<sub>1</sub> analysis

Cells (2 × 10^3) were treated with cisplatin/gemcitabine/mitomycin for 24 h and apoptosis was determined by measuring the hypoploidy of DNA. After incubation, cells were trypsinized and washed twice with 1 × PBS, fixed in 90% ethanol for overnight in −20 °C. Thereafter, cells were washed twice with 1 × PBS and stained with PI/RNase staining buffer (5 μg/ml PI, 200 μg/ml RNase). The percentage of apoptotic cells was determined by measuring the fraction of nuclei with a sub-
2.13. Determination of cell death by AO/EtBr assay

T24 and UM-UC-3 cells were seeded and treated with rapamycin either alone or in combination with autophagy inhibitor for 24 h. After incubation cell morphology was investigated by staining cells with a combination of fluorescent DNA binding dye acridine orange (AO)/ethidium bromide (EtBr). The solution containing each dye at 1 μg/ml in PBS was mixed 1:1 with cell suspension. Stained cells were viewed under Olympus fluorescence microscope (20× magnification). The viable cells take up AO and appeared green, in contrast dead cells take up EtBr and appeared orange/red.

2.14. Annexin/7-AAD staining

T24 and UM-UC-3 cells were treated with gemcitabine/mitomycin/cisplatin, either alone or in combination with BECN1-siRNA for 24 h. Cells were harvested, washed and stained with 5 μl of fluorescein isothiocyanate (FITC)-conjugated annexin-V using Annexin-V staining kit (BD Pharmingen™) according to the manufacturer’s protocol. Stained cells were immediately analyzed with a Becton Dickinson FACS Aria-II. The cell population analysis was done by using FlowJo (Tree Star Inc., Ashland, OR). The cytograms of the four quadrants was used to distinguish the normal (annexin V−/7-AAD−), early apoptotic (annexin V+/7-AAD−), late apoptotic (annexin V+/7-AAD+), and necrotic (annexin V−/7-AAD+) cells. The sum of early apoptosis and late apoptosis was presented as total cell death.

2.15. BECN1 knockdown using small interfering RNA (siRNA)

T24 cells were transfected with BECN1-siRNA using Lipofectamine™ 2000. The control siRNA was transfected under identical conditions. To determine the efficiency of BECN1 knockdown, Western-blot analysis for BECN1 was performed. T24 and UM-UC-3 cells (1 × 10⁶) were transfected with 70 nM siRNA for 6 h, after transfection of cells with siRNAs, RPMI-1640 without FBS, penicillin and streptomycin was added for 4 h. Thereafter, fresh RPMI-1640 containing 10% FBS without penicillin and streptomycin media was added. Following 24 h, chemotherapeutic agents were administrated for 24 h in complete media and cell lysates were prepared for Western blot analysis.

2.16. Statistical analysis

All experiments were performed in triplicate and repeated thrice. Data were expressed as mean ± SEM and compared using one way analysis of variance. p < 0.05 was considered as statistically significant.

3. Results

3.1. Identification and characterization of side population in bladder cancer

In the absence of verapamil, P3 gate showed 5.2% (Fig. 1A) SP cells that were Hoechst 33342 positive. However, on pre-incubation with verapamil the percentage of these cells dropped to 0.2 ± 0.03%. The P4 gate indicated the NSP cells (Fig. 1A). In UM-UC-3 cells (a muscle invasive bladder cancer cell line), P3 gate showed 7.9% SP in the absence of verapamil. The percentage of SP cells dropped to 2.4 ± 0.013% on pre-incubation with verapamil (Sup Fig. 1A). SP (P3) and NSP (P4) cells were collected after cell sorting under sterile condition for subsequent experiments. The purity of SP cells was 97–99% and the purity of NSP cells was 96%.

We analyzed the cell surface phenotype and epithelial origin of SP cells using CD44 and pan-cytokeratin staining respectively. A higher expression of CD44 was observed in SP cells as compared to NSP cells (24 ± 0.012%, 32 ±0.03% and 29 ±0.023% respectively) as compared to NSP cells (24 ± 0.012%, 32 ±0.03% and 29 ±0.023% respectively) at the same drug concentration, indicating that SP cells exhibit stronger resistance as compared to NSP cells (Fig. 2B).

3.2. Stem cell-like characteristics and drug resistance in SP cells

Fig. 2A shows the expression of the stem cell (SOX-2, KLF-4, OCT-4 and NANOG) and drug resistance genes (MDR1 and ABCG2) in bulk T24, NSP and SP cells. We observed that stem cell associated gene expression was highest in SP cells (p < 0.001) as compared to both NSP and bulk T24 (Fig. 2A) and UM-UC-3 (Sup Fig. 1C) cells. The mRNA expression of ABCG2 and MDR1 was also found to be significantly higher in SP cells (p < 0.001) as compared to NSP and bulk T24 cells. The effect of the anticancer agents such as cisplatin, gemcitabine and mitomycin on cell survival was confirmed by MTT assay (Fig. 2B). Both SP and NSP cells were exposed to cisplatin/gemcitabine/mitomycin in a time (0–48 h) and dose (5–20 μM) dependent manner (Sup Fig. 2). SP cells treated with cisplatin (20 μM)/gemcitabine (10 μM)/mitomycin (5 μM) for 24 h showed lower cell death (11 ± 0.04%, 13 ± 0.03% and 17 ±0.023% respectively) as compared to NSP cells (24 ± 0.012%, 32 ±0.03% and 29 ±0.042% respectively) at the same drug concentration, indicating that SP cells exhibit stronger resistance as compared to NSP cells (Fig. 2B). This dose was selected for all subsequent experiments.
ROS production was found to be lower in SP cells as compared to both NSP and bulk cells in T24 (Fig. 3A) and UM-UC-3 (Sup Fig. 3A). ROS production in T24 cells was further validated by the measurement of GSSG/GSH ratio which showed a significantly higher expression ($p < 0.05$) in SP cells as compared to NSP cells (Fig. 3B). SP cells showed a lower metabolic activity in terms of low mitochondrial mass and lower ATP levels than NSP cells (Sup Fig. 3B, C).

3.3. Spheroid forming ability of SP cells

The SP cells could form tumor like spheroids when plated in conditioned culture medium in non-adherent plates. The spheroids grew to more than 100 μm in diameter in T24 (Fig. 4) and UM-UC-3 (Sup Fig. 4) cells. A size of 60 μm was selected as a cut-off for counting the number of spheroids as described previously [18]. The mean number

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**Fig. 7.** Autophagy inhibition potentiates the cytotoxic effect of chemotherapeutic agents. FACS images showing subG1 analysis for monitoring apoptotic population in tumor spheroid cells treated with cisplatin/gemcitabine/mitomycin with or without Wm for 24 h. Lower panel shows the percentage of subG1 SP and NSP cells analyzed and quantified with Cell Quest Pro software. Cells in complete media were taken as control. Each experiment was repeated three times. Values are expressed as mean ± SEM. *, $p < 0.05$ NSP vs. MTS; #, $p < 0.05$ cisp/gem/mito vs. cisp + Wm/gem + Wm/mito + Wm.

**Fig. 6.** Autophagy is induced by chemotherapeutic agents in tumor spheroids. (A) Images showing acidine orange stained autophagosomes in cells derived from tumor spheroids with or without Wm (200 ng/ml) for 24 h. Side panel shows the mean fluorescence intensity in cells treated with cisplatin (20 μM) or gemcitabine (10 μM) or mitomycin (5 μM) which decreased in the presence of Wm. (B) Immuno-staining with anti-LC3 antibody. Quantitation was done by counting the number of LC3 dots/micrograph and was calculated in FV10-ASM software (Olympus) in side panel. (C) Western blot analysis of LC3 expression in cells derived from tumor spheroids with cisplatin (20 μM)/gemcitabine (10 μM)/mitomycin (5 μM) with or without Wm (200 ng/ml) for 24 h. Each experiment was repeated three times. Cells in complete media were taken as control. All values are expressed as mean ± SEM. *, $p < 0.05$ control vs. treatment; **, $p < 0.01$ control vs. treatment; #, $p < 0.05$ treatment vs. treatment + Wm.
of spheroids formed by SP cells was 67.0 ± 0.02/1000 and 74 ± 0.3/1000 in T24 (Fig. 4) and UM-UC-3 cells (Sup Fig. 4) respectively after 9 days. However, NSP cells could not form spheroids under similar conditions (Fig. 4, Sup Fig. 4). To demonstrate the self-renewal capacity of spheroids in vitro, we serially passed them and found that they could retain the spheroid forming capability for more than 12 generations (Sup Fig. 5).

3.4. Side population shows greater autophagic flux

About a two fold increase in the number of autophagosomes was observed in tumorispheres in the absence of autophagy induction in both T24 (Fig. 5A) and UM-UC-3 cells (Sup Fig. 6A). Immuno-staining of LC3 initially showed diffuse staining in both NSP and bulk T24 cells which concomitantly increased when starvation was induced with HBSS. Interestingly, tumor spheroid cells from SP showed higher number (120 ± 0.4) of punctate LC3 staining as compared to NSP (60 ± 0.45) and T24 (80 ± 0.39) cells at all times both before and after starvation was induced (Fig. 5B).

We also used rapamycin, mTOR inhibitor for pharmacological induction of autophagy and observed that it had no effect on cell viability (Fig. 5C, D). On the other hand, inhibition of autophagy with chloroquine (CQ) significantly increased cell death in these cells (56 ± 0.5, Fig. 5F). Similar observations were found in SP of UM-UC-3 cells (Sup Fig. 6B, C).
In the presence of mitochondrial stress induced by antimycin, a higher expression of Beclin1 and Atg7 with concomitant degradation of p62 was found in cells derived from tumor spheroids as compared to NSP and bulk T24 cells (Fig. 5E). ER stress with tunicamycin led to an increase in the expression of Beclin1, Atg7 and p62 degradation (Fig. 5E). Co-localization experiments were performed using mitochondrial membrane marker TOM, and LC3 as well as ER membrane marker calreticulin and LC3. In the presence of antimycin, TOM and LC3 were co-localized while calreticulin was co-localized with LC3 in the presence of tunicamycin (Fig. 5F).

3.5. Autophagy provides resistance against chemotherapy in tumor spheroids

The autophagic response towards the chemo-therapeutic drugs cisplatin, gemcitabine and mitomycin was assessed by measuring the autophagosomes in cells derived from tumor spheroids. Autophagosomes were found to be significantly increased in the presence of all the three chemo-therapeutic agents (Fig. 6A) and decreased with Wm treatment. Immuno-staining (Fig. 6B) as well as Western immuno-blotting (Fig. 6C) experiments in cells treated with cisplatin/gemcitabine/mitomycin showed a higher expression of LC3 protein in cells derived from tumor spheroids compared to NSP cells. A significant increase in autophagic response towards cisplatin, gemcitabine and mitomycin was also observed in UM-UC-3 cells as evident from the bar graph (Sup Fig. 7A).

On subG1 analysis cells from tumorspheres showed 6–9% apoptotic cells in the presence of cisplatin/gemcitabine/mitomycin alone, which increased up to 40–50% (Fig. 7) in the presence of Wm. Treatment with cisplatin/gemcitabine/mitomycin caused 24–32% apoptosis in NSP cells and Wm had no significant effect on apoptosis in these cells (Fig. 7). Annexin-V/7-AAD staining also confirmed that autophagy inhibition with Wm led to increase in apoptotic population in tumorsphere of UM-UC-3 cells. The percentage of apoptotic population was found to increase up to 50–63% in the presence of Wm (Sup Fig. 7B).

3.6. Autophagy inhibition potentiates the cytotoxic effect of chemotherapeutic agents

BECN1 si-RNA transfection was confirmed by immuno-blotting (Fig. 8A). SiRNA-mediated silencing of BECN1 in T24 cells led to the reduction of the size (20 ± 0.02 μm, Fig. 8B) as well as the number (19 ± 0.04/1000 cells, Fig. 8B) of spheres obtained from SP cells as compared to control siRNA. Similar results were found in SP of UM-UC-3 cells (Sup Fig. 8A, B). The addition of BECN1-siRNA with cisplatin or gemcitabine or mitomycin increased the cell death up to 55–72% in T24 (Fig. 8C) and 59–70% in UM-UC-3 cells (Sup Fig. 8C) as compared to chemotherapeutic agents alone. Further we investigated the levels of different anti- and pro-apoptotic proteins during autophagy inhibition. Treatment with cisplatin in cells derived from tumor spheroid had no effect on the expression of anti-apoptotic proteins Bcl-2, Bcl-XL, c-IAP1/2 and XIAP. However, Wm along with cisplatin decreased the expression of Bcl-2, Bcl-XL, c-IAP1/2 and XIAP, thus indicating increased cell death (Fig. 8D). Gemcitabine and mitomycin treatments also had no effect of anti-apoptosis protein expression, but autophagy inhibition by Wm along with gemcitabine or mitomycin led to the decrease of the expression of Bcl-2, Bcl-XL, c-IAP1/2 and XIAP (Fig. 8D). Treatment of cisplatin/gemcitabine/mitomycin had no significant effect on the pro-apoptotic proteins, and Bim and Bax expression in cells derived from tumor spheroid. However, the expression of Bim and Bax was found to increase in the presence of Wm along with cisplatin or gemcitabine or mitomycin (Fig. 8D). In BECN1 knockdown tumorsphere cells, ROS levels were higher in the presence of cisplatin/gemcitabine/mitomycin (p < 0.05, Fig. 8E) as compared to their respective controls. Similar results were obtained in UM-UC-3 cells (Sup Fig. 8D).
3.7. Inhibition of autophagy increased the apoptotic response in tumor spheroids

Immunoblot analysis revealed that caspase-9 was not activated in the presence of cisplatin/gemcitabine/mitomycin alone. Cells in which BECN1 expression had been silenced showed activation of caspase-9, when treated with cisplatin/gemcitabine/mitomycin. However, neither caspase-12 nor caspase-8 was activated in the presence of cisplatin/gemcitabine/mitomycin alone in these cells (Fig. 9). To confirm the involvement of the intrinsic apoptotic pathway, we checked the cytochrome-c (Cyt-C) release on treatment with chemotherapeutic agents in these cells. We found that Cyt-C was released only in tumorisphere cells where BECN1 expression had been silenced (Fig. 9). Treatment with cisplatin/gemcitabine/mitomycin alone had no effect on the activation of caspase-3 as well as on PARP cleavage in tumorisphere derived cells. However, caspase-3 activation and PARP cleavage were evident when autophagy was inhibited in these cells with BECN1-siRNA (Fig. 9).

4. Discussion

A sub-population of tumor-initiating or -sustaining cells has been identified in various solid tumors which grow more readily in an anchorage-independent manner, over-express stem cell-associated markers and are more resistant to drugs used in the treatment of cancer [21]. One of the methods that have been successfully used to isolate such putative tumor stem cells is differential Hoechst dye uptake [22]. Using this approach, we have identified a subpopulation of cells termed “side population” (SP) from bladder cancer cell lines, T24 and UM-UC-3. These cells were found to be more tumorigenic than non-side population (NSP), which formed the bulk of tumor cells. Our results showed that SP cells could form clonal, non-adherent 3-D spheres, when allowed to grow in conditioned media. Moreover, the tumor initiating capability was not decreased as the spheres were passaged. We also found that SP cells from tumorspheres, regenerated a population of cells composed of both SP and non-SP, resembling the original unsorted population, thus showing repopulating capacities similar to stem cells. This finding implies that SP cells may undergo asymmetrical division to self-renew and generate heterogeneous phenotypes of low-tumorigenic cells, like NSP cells that form the bulk of the tumor. We observed that the SP cells had a higher expression of CD44, a potential marker of cancer stem cells (CSCs), as compared to the parental cells in both T24 and UM-UC-3. CD44 has been reported to be involved in cancer cell migration, proliferation and metastasis [23]. SP cells isolated in our study showed a higher expression of stem cell marker i.e. SOX-2, OCT-4, NANOG, and KLF-4 than NSP cells, indicating that the SP cells possess stem cell like characteristics. It is generally agreed that, like all stem cells, the tumor sphere forming cells or CSCs are capable of proliferation and self-renewal and possess higher tumorigenicity [24]. A higher expression of ATP-binding cassette, subfamily G, member 2 (ABCG2) and MDR1 was observed in SP compared to NSP cells. The ABCG2 gene is highly expressed in the plasma membrane of several drug resistant cancer cell lines and has been shown to transport antitumor drugs such as mitoxantrone, topotecan and doxorubicin [25]. A higher expression of MDR1, another potential drug resistant gene, was also reported in various other cancer cells including renal cell carcinoma, colon adenocarcinoma, hepatocellular cancer and bladder cancer [26]. On this basis, we hypothesized that the SP cells isolated from the bladder cancer cell line may survive conventional chemotherapy and regenerate a cancer population, leading to relapse. Reduced metabolic activity in T24 SP cells indicates a higher ROS scavenging potential in these cells which may aid in cell survival during later stages. The low level of ROS as observed in the SP cells may help to maintain their
Cancer patients may present with distant metastasis after an apparently successful treatment of initial malignancy, followed by a period of dormancy. Thus, in order to reduce the morbidity and mortality from cancer, it is necessary to gain a greater understanding of metastasis and clinical dormancy, as well as the molecular factor that contributes to this process. In the last decade, the role of autophagy in resistance to chemotherapy has been explored in detail. Since autophagy has been observed to play a more crucial role in cancer cell homeostasis than previously conceived, understanding how the cancer cells undergoing autophagy cross the threshold from cell survival to cell death during drug treatment is important for identifying more potent drug targets.

A number of antineoplastic therapies including radiation therapy and chemotherapy have been observed to induce autophagy as a protective and pro-survival mechanism in human cancer cell lines [31]. Thus, inhibition of autophagy may lead to increased cell death and dissemination of tumor growth. In this context, combining anticancer drugs with appropriate autophagy inhibitor has a promising prospect in chemotherapy. Our data suggests that autophagy has a key role in the maintenance of the SP subpopulation in bladder cancer cells since autophagic flux was significantly higher in the SP compared to NSP cells. The autophagy-mediated cell survival in response to rapamycin and cell death with CQ further suggests the functional role of autophagy in SP cell viability. Pharmacological inhibition of autophagy has been reported with Wm [32–35] and CQ [36,37]. We observed a significant increase in the cytotoxicity of the chemotherapeutic drugs when given in combination with Wm. In order to further confirm the effect of autophagy inhibition, we used siRNA to knock down ATG gene, BECN1, implicated in the initiation of autophagic pathway. The importance of autophagy in tumorogenicity was substantiated by the reduced number of tumor spheroids from the SP cells when autophagy was inhibited by silencing BECN1 gene. Therefore, modulation of autophagy may represent a major impediment to a successful cancer therapy. Our data indicates that the autophagy induced by cisplatin, gemcitabine and mitomycin, delayed the cytotoxic effect of chemotherapeutic agents. Autophagy inhibitor or siRNA-mediated depletion of BECN1 given in combination with cisplatin, gemcitabine or mitomycin inhibited the proliferation of tumor spheroids. It also triggered cell death to a greater extent than cisplatin/gemcitabine/mitomycin alone or control-siRNA. Our findings suggested that down-regulation of BECN1 decreased autophagy and inhibited tumorigenesis. Thus, autophagy may be required for the maintenance of tumor spheroids and provide resistance to chemotherapy. Cell death induced via inhibition of autophagy was correlated with higher production of ROS in the presence of BECN1-siRNA. Our finding is consistent with other studies which showed that in preclinical models, inhibition of pro-survival autophagy by genetic or pharmacological means killed the tumor cells and triggered the apoptotic cell death [38]. Similar observations were obtained in another bladder cancer cell line UM-UC-3, which further substantiates our results.

Autophagy enhances the metastatic potential of carcinoma cells by supporting anoikis resistance and tumor dormancy during tumor cell dissemination. It has been suggested that autophagy may influence apoptosis in various cancerous cells. However, the molecular interplay between these two different pathways seems to be very complex and has not been fully understood. While autophagy induces cell death in human glioma cell lines [39], several reports have suggested that autophagy inhibition increases caspase-mediated cell death in various cancer cells [40]. In the present study, activation of caspase-8 was not observed, which excluded the involvement of extrinsic apoptotic pathways. In addition, to seek the possibility of ER stress induced apoptosis, caspase-12 was not playing any role in apoptosis induced by autophagy inhibition. We found that autophagy inhibition by BECN1-siRNA along with cisplatin/gemcitabine/mitomycin led to a release of Cyt-C in cytosol which in turn activated caspase-9, caspase-3 and PARP cleavage. These findings indicated that autophagy inhibition led to intrinsic apoptotic cell death in the tumorosphere cells obtained.

**Fig. 9.** Inhibition of autophagy leads to intrinsic apoptotic cell death in tumor spheroid. Western immunoblot expression of the caspases (caspase-9, -8, -12, and -3) in BECN1-siRNA transfected tumorisphere cells treated with cisplatin/gemcitabine/mitomycin for 24 h. Cytochrome-c release was determined in the cytosolic fraction. Activation of caspases was indicated by inhibition of their pro-caspase form or cleavage of PARP. Each experiment was repeated three times.
from T24 SP cells. A more systematic molecular characterization of the tumor-initiating cell population will help us to refine the stimuli that force these cells to die, thus accelerating the development of a more effective treatment for cancer.

5. Conclusions

In the present study, we found that high drug efflux capacity of SP in bladder cancer cells line correlates with the strong expression of drug transporter proteins. We analyzed the autophagy in SP cells as well as in spheroid culture. We found that the greater autophagic flux in SP cells as compared to NSP correlated with a high expression of autophagic markers in tumor spheroids suggesting that the maintenance and tumorigenicity of SP might be dependent on autophagy. Therefore, a better understanding of the autophagic pathway in SP cells would help us to design better inhibitors for preventing drug resistance in bladder cancer.

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References