The PI3K/AKT/c-MYC Axis Promotes the Acquisition of Cancer Stem-Like Features in Esophageal Squamous Cell Carcinoma

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ABSTRACT
The importance of intratumoral heterogeneity has been highlighted by the identification and characterization of cancer stem cells (CSCs). Based on the differential responsiveness to a Sox2 reporter, SRR2, we had found a novel dichotomy in esophageal squamous cell carcinoma (ESCC) cells, with reporter-responsive (RR) cells showing more CSC-like features than reporter-unresponsive (RU) cells. Specifically, RR cells exhibited significantly higher tumorsphere formation capacity, proportions of CD44 High cells, chemoresistance to cisplatin, and tumorigenic potential in vivo. H2O2, a potent inducer of oxidative stress and reactive oxygen species, was found to induce a conversion from RU to RR cells; importantly, converted RR cells acquired CSC-like features. The PI3K/AKT/c-MYC signalling axis is important in this context, since pharmacologic blockade of PI3K-AKT or siRNA knockdown of c-MYC effectively inhibited the RR phenotype and its associated CSC-like features, as well as the H2O2-induced RU/RR conversion. In a cohort of 188 ESCC patient samples, we found a significant correlation between strong c-MYC expression and a short overall survival (p = .009). In conclusion, we have described a novel intratumoral heterogeneity in ESCC. The identification of the PI3K/AKT/c-MYC axis as a driver of CSC-like features carries therapeutic implications. STEM CELLS 2016;34:2040–2051

SIGNIFICANCE STATEMENT
Cancer stem cells have been shown to be major contributing factors to treatment failure and recurrence in cancer patients. Recently, a new concept suggests that cancer stemness can be acquired by noncancer stem cells. Using a lentiviral reporter expressing readily detectable GFP and luciferase that can reflect cancer stem-like features, our present study uncovered oxidative stress as an important factor that promotes cancer stem-like feature acquisition by noncancer stem cells. The PI3K/AKT/c-MYC pathway was revealed to mediate the stemness-promoting function of oxidative stress, and blockage of this pathway substantially decreased stem-like feature acquisition and chemoresistance to cisplatin, providing potential therapeutic targets for esophageal cancer.

INTRODUCTION
Esophageal cancer is one of the deadliest cancers, representing the sixth leading cause of cancer-related deaths worldwide [1]. Esophageal squamous cell carcinoma (ESCC), which is the major histologic subtype of this disease, is prevalent in Asia but rare in the Western world [2]. The overall survival for ESCC patients is relatively short, with a 5-year survival rate of approximately 14% [2]. The pathobiology of ESCC remains incompletely understood. Recent studies have highlighted the importance of cancer stem cells (CSCs). Generally, CSCs, which existighthouse in been demonstrated in various types of cancer, represent a small fraction of cells in the tumor bulk that are responsible for tumor initiation [3]. In addition to their tumor initiating capacity, CSCs also are known to be highly chemoresistant, owing to their high expression of the ATP-binding cassette transporters/aldehyde dehydrogenase as well as their ability to achieve heightened DNA damage response [4]. Mounting evidence has documented that chemotherapy or radiotherapy eliminates only the non-CSCs, leaving CSCs untouched [5–7]. Thus, it is widely believed that CSCs is the major contributing factor to treatment failure seen in cancer patients. In ESCC, it has been recently
reported that CSC-like cells can be identified based on their expression of a number of markers, such as Hoechst dye efflux and CD44 expression [8–10]. Nonetheless, the biology of CSC-like cells in ESCC has not extensively studied.

Sox2 regulatory region 2 (SRR2) is a motif (CATTGT) found in the promoters of many genes [11]. Using a SRR2 reporter construct that carries the GFP and luciferase genes, we found that a novel intratumoral heterogeneity in lymphoma cell lines as well as breast cancer cell lines and patient samples [12–14]. Specifically, we identified a relatively small subset of cells that are reporter-responsive (RR), and they express GFP and luciferase; in contrast, the majority of the cells are reporter-unresponsive, and they do not express GFP or luciferase (RU) [12–14]. Importantly, we found that RR cells are more CSC-like than RU cells, and this observation can be made in both cell lines and primary patient samples [12, 13]. In this study, using the same SRR2 reporter, we report that the RU/RR dichotomy also exists in ESCC, and RR cells are more CSC-like than RU cells. Furthermore, RU cells can be induced to convert into RR cells upon treatment with H2O2. Using this study model, we aimed to gain insights into the molecular basis of CSC-like features in ESCC.

**Patient Samples**

Human ESCC tumors were collected directly after surgical resection between Jan. 2000 and Dec. 2006, at the Department of Tumor Surgery of Shantou Central Hospital (Shantou, China). The cases were selected based on a clear pathological diagnosis, follow-up data, and had not received local or systemic treatment before surgery. The histological characterization and clinicopathological staging of the samples were performed in accordance with the 7th edition of American Joint Committee on Cancer Tumor-Node-Metastasis staging system. Detailed clinical information of the ESCC patients is described in Supporting Information Table S1. The study was approved by the ethical committee of the Central Hospital of Shantou City and the ethical committee of Shantou University Medical College, and written informed consent was obtained from all patients.

**Statistical Analysis**

The statistical analyses were performed using Graphpad Prism 6. To determine the differences between two independent groups of samples, Student’s t test was used. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Fisher’s exact test (two-tailed) was used to analyze the correlation between the expression status of c-MYC and patients’ clinical-pathological characteristics. Differences were considered significant when the \( p < .05 \).

Additional Materials and Methods are provided in the Supporting Information.

**RESULTS**

**Identification of two cell subpopulations in ESCC cell lines**

Using the lentiviral SRR2 reporter published previously [12, 13], we identified a small cell population showing reporter responsiveness (i.e., RR cells) in 3 of 3 ESCC cell lines (EC109, KYSE150 and KYSE510), with RR cells accounting for 26.4%, 34.0% and 38.7%, respectively (Fig. 1A). In comparison, NECA6, an immortalized esophageal epithelial cell line, RR cells accounted for only 8.7% of the total cell population.

To study the significance of the observed RU/RR dichotomy, we purified RU and RR cells using a flow cytometric cell sorter, and the purified RU and RR cells were cultured separately. Compared to purified RU cells, purified RR cells expressed substantially higher levels of GFP and luciferase (Fig. 1B, 1C), even though the gene copy number of the SRR2 reporter integrated in these 2 cell subsets was not significantly different (Supporting Information Fig. S1). At 8–10 weeks after the purification of RU and RR cells, we observed that the proportions of GFP-positive cells in the purified RR cell culture decreased slightly and stabilized at 80–90%. Similarly, the proportions of GFP-negative cells in the purified RU cell culture stabilized at 80–90% (Supporting Information Fig. S2). Thus, the RU and RR phenotype are relatively stable in vitro, and the slight decrease in the purities was likely due to the presence of some contaminants during the purification process.

**RR cells have more CSC-like features than RU cells**

We then compared RU and RR cells with regards to their CSC-like features. First, we employed tumoursphere formation, an assay commonly used to quantify CSC-like cells [10, 15–17]. As shown in Fig. 1D, RR cells contained a significantly higher number of CSC-like cells compared to RU cells in all three ESCC cell lines examined (Fig. 1D). Second, we found that RR cells isolated from these ESCC cell lines contained significantly higher proportions of CD44Bright and ALDH1A1High cells (Fig. 1E; Supporting Information Fig. S3A), two cell subsets previously shown to be CSC-like cells in ESCC [9, 10, 18]. To support that CD44Bright cells isolated also were truly CSC-like, we examined their cell cycle status, based on a previous report that CD44Bright CSC-like cells have extended G2-phase [19]. As shown in Supporting Information Fig. S4A–S4C, this turned out to be the case. However, when we examined the cell cycle status of RU and RR cells, we found that RR cells only had a slightly higher percentage of G2-phase cells compared with RU cells (Supporting Information Fig. S3B). We believe that this is likely due to the fact that CD44Bright cells only constitute a small portion of RR cells, even though CD44Bright cells are indeed enriched in the RR subset compared to the RU subset. Third, RR cells were found to be significantly resistant to cisplatin than RU cells, with the inhibitory concentration at 50% (IC50) being higher in RR cells than RU cells (KYSE150: 13.4 \( \mu \)M vs. 6.9 \( \mu \)M, \( p < .01 \); KYSE510: 9.9 \( \mu \)M vs. 5.4 \( \mu \)M, \( p < .05 \)) (Fig. 1F). These observed phenotypic differences between RU and RR cells were not due to a significant difference in their growth rates, which were shown to be similar using the MTS cell growth assay and the clonogenic assay (Supporting Information Fig. S3C–S3D).

**Oxidative stress induces a conversion of RU into RR cells**

We then asked if RU cells can convert to RR cells, whereby acquiring CSC-like features. Since oxidative stress has been reported to induce CSC-like features in other study models [17, 20], we treated RU cells with different doses of H2O2, a
Figure 1. Identification of two sub-populations with distinct cancer stem cell (CSC)-like properties in ESCC cell lines. (A): Flow cytometry analysis was performed to identify the reporter responsive (RR) cells and reporter unresponsive (RU) cells in cells stably infected with the SRR2 reporter lentivirus. Cells infected with the mCMV control lentivirus were used as a negative control for the gating of RU and RR cells. (B): GFP expression was measured by flow cytometry in RU and RR cells isolated from cells stably infected with the lentiviral SRR2 reporter. (C): Luciferase activity was examined in the isolated RU and RR cells. (D): Tumorsphere formation assay was performed to compare the CSC-like properties between RU and RR cells. Images were taken at ×100 and ×400 magnifications. (E): CD44 expression in RU and RR cells was examined using Flow cytometry. Cells with relatively high expression of CD44 (CD44 High cells) were compared between RU and RR cells. An isotype control was included in the experiments to serve as a negative control (shown in gray). (F): MTS assay was performed to determine the inhibitory concentration at 50% (IC50) to cisplatin. The data shown in D and F are part of the experiments described in Figure 3E and 3G, i.e., a negative control siRNA was transfected in these cells. All data are presented as mean ± SD, *, p < .05, **, p < .01, ***, p < .001; Student’s t test. Abbreviations: ESCC, esophageal squamous cell carcinoma; SRR2, Sox2 regulatory region 2; mCMV: Murine Cytomegalovirus; GFP: Green Fluorescence Protein; PCR: Polymerase Chain Reaction.
potent inducer of reactive oxygen species (ROS) and oxidative stress. After continuous H$_2$O$_2$ treatment for 4 days, we examined the level of ROS (by flow cytometry), the SRR2 reporter activity (by measuring both GFP and luciferase), and cell viability (by MTS assay). As shown in Fig. 2A, we found that the ROS level was significantly increased in response to H$_2$O$_2$ treatment. Importantly, we found that the proportion of GFP-positive cells increased in a H$_2$O$_2$ dose-dependent manner, peaking at ~40% in KYSE150-RU cells and at ~20% in KYSE510-RU cells at 500 µM of H$_2$O$_2$ (Fig. 2B; Supporting Information Fig. S5). In contrast, as shown in Supporting Information Fig. 5G, H$_2$O$_2$ treatment did not induce any appreciable change in the proportions of GFP-positive cells in ESCC cells that were stably infected with the negative control lentiviral vector, namely KYSE150-mCMV and KYSE510-mCMV, and these findings support that the GFP expression observed in this experiment is specific to the SRR2 reporter. Moreover, H$_2$O$_2$ treatment also enhanced luciferase activity in a dose-dependent fashion in RU cells (Fig. 2B), further supporting the activation of the SRR2 reporter by H$_2$O$_2$. As shown in Supporting Information Fig. S7, H$_2$O$_2$ induced only a slight decrease in cell viability when the cell confluence was maintained at ~100% at the beginning of the experiments.

We then asked if the RU/RR conversion is directly linked to the oxidative scavenger pathway, and not due to certain nonspecific effects of H$_2$O$_2$. Thus, we tested if N-Acetyl-Cysteine (NAC), an antioxidant agent, can block H$_2$O$_2$-induced RU/RR conversion. As shown in Fig. 2C and Supporting Information Fig. S8, NAC almost completely abrogated the H$_2$O$_2$-induced RU/RR conversion observed in both KYSE150 and KYSE510 cells, as evidenced by the reduction of GFP-positive cells. This finding was accompanied by a dramatic reduction in the cellular ROS levels.

Next, we questioned if the H$_2$O$_2$-induced conversion into RR cells is accompanied by the acquisition of CSC-like features, as seen in native RR cells. As shown in Fig. 2D, the tumoursphere formation capacity of converted RR cells derived from KYSE150 and KYSE510 were significantly higher than native RU cells, and the increments were H$_2$O$_2$ dose-dependence. Correlating with these observations, converted RR cells from these two ESCC cell lines contained a markedly increased proportion of CD44$^{\text{high}}$ cells (Fig. 2E). Although we did not include native RR cells in these experiments, the tumoursphere formation capacity and the proportions of CD44$^{\text{high}}$ cells in converted RR cells were comparable to those of the immortalized esophageal cell line, NE3 and NECA6. KYSE510 was the only cell line expressing Sox2 mRNA and protein levels that were substantially higher than those of NE3 and NE6. Overall, these findings suggest that Sox2 is not a key factor for the RU/RR dichotomy in ESCC. In keeping with this concept, siRNA knockdown of Sox2 did not significantly influence the luciferase activity in RR cells isolated from KYSE510, the only Sox2-positive cell line (Supporting Information Fig. S10B). We also evaluated if other factors that have been associated with CSC-like cells in ESCC, such as Oct4 and Nanog [10], were involved in regulating the RU/RR dichotomy. As shown in Supporting Information Fig. S10C, the Oct4 expression levels among RU, RR and converted RR cells were similar. Regarding Nanog, only KYSE150 cells showed a substantial difference in the expression of this protein between RR/converted RR cells and RU cells. Overall, these findings suggest that the RU/RR dichotomy in ESCC cannot be explained by a difference in the expression levels of these two proteins.

**c-MYC is important for the RR phenotype and is associated with CSC-like features**

Bioinformatics analysis of the SRR2 promoter has revealed a c-MYC-binding site characterized by the “TTACGTTGGT” sequence. Western blot analysis also revealed that c-MYC and its active/phosphorylated form (pMYC$^{62}$) were substantially higher in the four ESCC cell lines compared to the two immortalized ESCC cell lines (Fig. 3A). Based on these findings, we hypothesized that c-MYC may be a key determinant of SRR2 reporter responsiveness. Thus, we performed siRNA knockdown of c-MYC, and the efficiency of the knockdown is illustrated in the top panel of Fig. 3B. In the same figure, it is evident that c-MYC knockdown significantly reduced the luciferase activity in RR cells derived from 3 ESCC cell lines, ranging from 30 to 60% in the three ESCC cell lines ($p < .01$); no appreciable effect was observed in RU cells treated with c-MYC siRNA. As shown in Fig. 3C, enforced expression of c-MYC in RU cells substantially increased both total c-MYC and pMYC$^{62}$ expression levels, and the luciferase activity in RU cells increased by approximately 2–5 folds after c-MYC transfection. We believe that the transfection efficiency was likely less than 30% [21], and this may explain our observation that the luciferase level in c-MYC-transfected RU cells was still substantially lower than that of native RR cells (Fig. 3C).

To determine whether c-MYC is a direct regulator of the SRR2 reporter activity, we performed protein pull-down assay with a biotinylated SRR2 probe. As shown in Fig. 3D, binding of c-MYC and pMYC$^{62}$ to SRR2 was higher in RR cells compared with that in RU cells. Interestingly, the pull-down result was in parallel with that of the input result, suggesting that c-MYC and pMYC$^{62}$ can effectively bind to the SRR2 probe, and the key difference between RU and RR cells lies with the quantity of c-MYC and pMYC$^{62}$. These results echo our finding that enforced expression of c-MYC (which also led to increased pMYC$^{62}$) in RU cells resulted in their acquisition of the RR phenotype (Fig. 3C). Nonetheless, based on the data we collected, we cannot exclude the existence of additional mechanism(s) contributing to the RR phenotype.

To further substantiate the importance of c-MYC, we found that siRNA knockdown of c-MYC significantly decreased the numbers of tumourspheres and CD44$^{\text{high}}$ cells in both RU and RR cells, although the effects were evidently more consistent and profound in RR cells (Fig. 3E–3F). The importance
Figure 2. Oxidative stress induced by H$_2$O$_2$ promotes RU/RR conversion and the acquisition of cancer stem cell (CSC)-like properties. (A): ROS induced by H$_2$O$_2$ (500 µM for 24 hours) was measured by flow cytometry. (B): H$_2$O$_2$-induced RU/RR conversion in KYSE150-RU cells, as indicated by the increase of GFP-positive cells and luciferase activity. Representative flow cytometry data at Day 4 was shown in the left panel. Note that cells grown at a 100% confluence were used in this assay. (C): Flow cytometry was performed to measure GFP expression and ROS levels to determine the effect of NAC, an antioxidant agent, on oxidative stress-induced RU/RR conversion. Median ROS values are indicated by bidirectional arrows. (D): Tumorsphere formation assay was used to evaluate H$_2$O$_2$-induced CSC-like properties. The RU cells used in this assay were treated with different concentrations of H$_2$O$_2$ for four cycles, each cycle includes a 24 hours H$_2$O$_2$ treatment and a recovery phase for 3 days. Images were taken at $\times$100 magnification. (E): RU cells were treated with 300 µM H$_2$O$_2$ for 48 hours or left untreated, and CD44 expression was measured using flow cytometry. The size of the CD44$^{\text{High}}$ cell population was compared in untreated RU cells and the converted RR cells (Conv. RR). All data are presented as mean ± SD, *, p < .05, **, p < .01, ***, p < .001, Student’s t test. Abbreviations: NAC, N-Acetyl-Cysteine; ROS, reactive oxygen species.
of c-MYC in these CSC-like cells was further supported by the finding that sorted CD44<sup>High</sup> cells from the RR population expressed even higher levels of c-MYC and pMYC<sup>S62</sup> (Supporting Information Fig. S11). Moreover, as shown in Fig. 3G, c-MYC siRNA significantly sensitized both KYSE150-RR and KYSE510-RR cells to cisplatin (p < .05 and p < .01, respectively). These biological effects induced by c-MYC knockdown were not due to a substantial loss of cell viability, which was confirmed to be largely unchanged by this experimental manipulation (Supporting Information Fig. S12).

**c-MYC regulates H<sub>2</sub>O<sub>2</sub>-induced RU/RR conversion**

In view of the importance of c-MYC in conferring the RR phenotype and CSC-like features, we asked if c-MYC is also important in the H<sub>2</sub>O<sub>2</sub>-induced RU/RR conversion. As shown in Fig. 4A, we found that H<sub>2</sub>O<sub>2</sub> induced an increased expression of c-MYC and pMYC<sup>S62</sup> in RU cells in a dose-dependent manner. With siRNA knockdown of c-MYC (illustrated in Fig. 4B), the proportions of H<sub>2</sub>O<sub>2</sub>-induced GFP-positive cells and CD44<sup>High</sup> cells were significantly decreased (p < .01) (Fig. 4C, 4D). Lastly, the tumorsphere formation capacity and chemoresistance enhanced by H<sub>2</sub>O<sub>2</sub> were significantly blocked by c-MYC siRNA knockdown (p < .05), to a level that was comparable to those of native RU cells (Fig. 4E, 4F).

**The PI3K-AKT signalling promotes c-MYC activation and the CSC-like features in RR cells**

To determine the signaling pathways that contribute to the RR phenotype, we employed Western blot studies and compared the activation status of several key signaling pathways between RU and RR cells, including the PI3K-AKT pathway, the MAPK pathway, the JAK/STAT3 pathway, and the WNT canonical pathway. As shown in Fig. 5A, no appreciable difference was observed in the expression levels of pERK<sup>1,2</sup>, pSTAT3<sup>Y705</sup>, or active nonphosphorylated β-catenin (both in whole cell lysates and nuclear extracts). Nevertheless, phosho-AKT<sup>S473</sup> (pAkt<sup>S473</sup>), a central mediator of the PI3K-AKT pathway, was expressed at a substantially higher level in RR cells compared with RU cells in both KYSE150 and KYSE510 (Fig. 5A).

We then asked if the PI3K-AKT pathway is involved in regulating the RU/RR dichotomy. In keeping with this concept, the addition of LY294002, an inhibitor of the PI3K-AKT pathway, significantly decreased the luciferase activity in RR cells derived from all three ESCC cell lines (p < .01) (Fig. 5B). Moreover, the same treatment markedly decreased the proportion of CD44<sup>High</sup> cells by >10-fold and >2.5-fold in RR cells from KYSE150 and KYSE510, respectively (p < .001) (Fig. 5C), and attenuated their tumorsphere formation capacity by >fourfold and >fivefold (p < .001) (Fig. 5D). In comparison, LY294002 treatment only slightly decreased tumorsphere formation in RU cells (Supporting Information Fig. S13), highlighting the relevance of the PI3K-AKT pathway in conferring CSC-like properties in RR cells but not RU cells. Again, the observed biological effects of LY294002 cannot be explained solely by the associated decreases in cell viability, which were only ~ 1.75 and ~ 1.15 folds in KYSE150 and KYSE510, respectively (Supporting Information Fig. S14).

**Strong c-MYC expression correlates with a short overall survival in ESCC**

To substantiate the importance of c-MYC in ESCC, we correlated its expression in a cohort of ESCC patient samples. Thus, we performed c-MYC immunohistochemistry using paraffin-embedded archival ESCC patient samples (n = 188) and the results are illustrated in Fig. 6A. Of the 188 cases, 70 cases were assessed strongly positive and 72 cases were considered weakly/moderately positive and 46 cases showed no detectable c-MYC staining. Compared to patients with strongly c-MYC-positive tumors, those with ESCC tumors showing negative/weak/moderate c-MYC staining had a significantly longer overall survival (59.6 months vs. 36.3 months, p = .009) (Fig. 6B). Among the 30/188 ESCC patients who also received chemotherapy and/or radiochemotherapy (i.e., in addition to surgery), those carrying strongly c-MYC-positive tumors had a dismal survival rate compared to those carrying tumors that were c-MYC-negative/weak/moderate (10-year survival rate: 6% vs. 64%, p = .003, Fig. 6B). Moreover, within the entire cohort (n = 188), those with strong c-MYC expression were more likely to develop lymph node metastasis (p = .048) and display a higher TNM stage (p = .033) (Fig. 6C).

**DISCUSSION**

One of the key findings of this study is related to our identification of two phenotypically distinct cell populations in ESCC cell lines based on their differential responsiveness to the SRR2 reporter. Importantly, we found that RR cells have more CSC-like features compared to RU cells, and this conclusion is based on the observations that RR cells exhibited significantly higher tumorigenicity, tumorsphere formation capacity as well as chemoresistance, and they contain a higher proportion of cells carrying the CD44<sup>High</sup> and ALDH1A1<sup>High</sup> immunophenotype, two
Figure 3. c-MYC is a key determinant of the Sox2 regulatory region 2 (SRR2) activity and the cancer stem cell (CSC)-like features. (A): The expression of c-MYC protein and mRNA in a panel of cell lines was measured by Western blot and real-time PCR, respectively. The two immortalized esophageal cell lines, NE3 and NECA6, were used as normal controls. (B): The impact of c-MYC siRNA knockdown on the SRR2 reporter activity was determined by measuring luciferase activity. (C): The impact of enforced expression of c-MYC on the SRR2 reporter activity was determined by measuring luciferase activity. (D): Protein pull-down assay using the SRR2 probe was performed to determine whether c-MYC is an SRR2-binding protein. Protein expression in the pull-down lysates and the nuclear protein input was detected using Western blot. (E): The impact of c-MYC siRNA knockdown on tumorsphere formation was determined in both RU and RR cells isolated from three ESCC cell lines. (F): Flow cytometry was performed to examine the effects of c-MYC siRNA knockdown on the population of CD44^High cells in both RU and RR cells from KYSE150 and KYSE510 cells. (G): The impact of c-MYC siRNA knockdown on the IC50 to cisplatin was determined in both KYSE150-RR and KYSE510-RR cells. All data are presented as mean ± SD or indicated otherwise, *, p < .05, **, p < .01, ***, p < .001, Student’s t test. Abbreviations: ESCC, esophageal squamous cell carcinoma.
Figure 4. c-MYC is an important mediator of oxidative stress-induced RU/RR conversion and the acquisition of cancer stem cell (CSC)-like features. (A): Western blot was performed to assess c-MYC expression in H$_2$O$_2$-treated cells. The RU cells used in this assay were treated with different concentrations of H$_2$O$_2$ for four cycles, each cycle includes a 24 hours H$_2$O$_2$ treatment and a recovery phase for 3 days. (B–F): The RU cells used in these assays were treated with 300 μM H$_2$O$_2$ for four cycles, each cycle includes a 24 hours H$_2$O$_2$ treatment and a recovery phase for 3 days. c-MYC siRNA knockdown efficiency in H$_2$O$_2$-treated (300 μM) or untreated cells was evaluated using Western blot assay (B); the effects of c-MYC siRNA knockdown on H$_2$O$_2$-induced RU/RR conversion (C) and the CD44$^\text{High}$ population size (D) were measured using flow cytometry; the effects of c-MYC siRNA knockdown on H$_2$O$_2$-induced tumorsphere formation capacity (E) and chemoresistance to cisplatin (F) were evaluated. Images shown in (E) were taken at $\times$100 magnification. All data are presented as mean ± SD or indicated otherwise, *, $p < .05$, **, $p < .01$, ***, $p < .001$, Student’s t test.
Figure 5. The PI3K/AKT/c-MYC axis sustains the reporter-responsive (RR) phenotype and mediates oxidative stress-induced reporter-unresponsive (RU)/RR conversion and the acquisition of cancer stem cell (CSC)-like features. (A): Western blot was performed to screen the signaling pathways that are preferentially activated in RR cells than in RU cells. (B): The RR cells isolated from the three esophageal squamous cell carcinoma cell lines were treated with LY294002 (30 \mu M), a PI3K inhibitor, for 24 hours, luciferase activity was measured to determine the SRR2 reporter activity. (C): 24 hours after LY294002 (30 \mu M) treatment, CD44 expression was measured using flow cytometry. (D): Tumorsphere formation assay was performed with/without LY294002 (30 \mu M) treatment to assess the effect of PI3K inhibition on the CSC-like properties. (E): The tumorigenic potential of RU cells, RR cells, and RR cells pretreated with LY294002 (30 \mu M, 48 hours) were compared in SCID mice. The lines in the middle of the box plots indicate the median values of tumor volume, and the whiskers represent the 10–90 percentile. Scale bars: 0.5 cm. (F): The RU cells used in this assay were treated with different concentrations of H2O2 for four cycles, each cycle includes a 24 hours H2O2 treatment and a recovery phase for 3 days. Western blot was performed to examine the effect of H2O2 treatment on the activation of the PI3K pathway in KYSE150-RU and KYSE510-RU cells. (G): The impact of LY294002 (30 \mu M for 24 hours) treatment on H2O2-induced RU/RR conversion (GFP detection) and the increase of the CSC-like CD44^High cells were measured using flow cytometry. (H): Western blot was performed to assess the effect of LY294002 (30 \mu M for 24 hours) on the expression of phospho-MYC(S62) and c-MYC in RR cells isolated from KYSE150 and KYSE510 cells. (I): Enforced expression of c-MYC significantly abrogated the inhibitory effect of LY294002 (30 \mu M for 24 hours) on the SRR2 reporter activity in RR cells isolated from KYSE150 and KYSE510 cells. All data are presented as mean ± SD or indicated otherwise, *, p < .05, **, p < .01, ***, p < .001, Student’s t test.
markers previously shown to be associated with cancer stemness in ESCC [9, 10, 18]. Using the SRR2 reporter, we have previously identified similar intratumoral dichotomy in other types of cancer. Specifically, we have reported such dichotomy in estrogen receptor-positive breast cancer, estrogen receptor-negative breast cancer and ALK-positive anaplastic large cell lymphoma [12–14]. Thus, it appears that the RU/RR dichotomy and their difference in CSC-like features may be generalized to many forms of human cancer. It is also important to stress that the RU/RR dichotomy is not restricted to cell lines, as we have documented that this phenomenon can be found in patient samples of estrogen receptor-positive as well as estrogen receptor-negative breast cancer [12, 13].

We believe that our observed RU/RR dichotomy is an example of intratumoral heterogeneity. To our knowledge, intratumoral heterogeneity has not been extensively studied in ESCC. To our knowledge, 7 studies have identified CSC-like cells based on the detection of specific markers, such as P75NTR, CD44, CD90, and ALDH1 [9, 18, 24–26], and the activity of the ATP-binding cassette transporters [27, 28]. In these studies, evidence in support of the CSC-like features came from both in vitro studies and mouse xenograft models [9, 18, 25, 27]. For instance, CD44High cells in ESCC were found to have a higher expression of embryonic stem cell markers (including Sox2, Oct4 and Nanog) as well as a higher tumor initiating capacity in mice, as compared to CD44Low cells [9, 10]. In the current study, we found that RR cells contained a significantly higher proportion of CD44High cells, and thus, this finding has provided an additional evidence to support that RR cells are enriched in CSC-like cells. Nonetheless, unlike the current study, all of these previously reports did not evaluate the dynamics between CSC-like cells and the “general cell pool.”

Using GFP and luciferase as readouts, our study model has enabled us to readily track the acquisition of the RR phenotype, which is associated with CSC-like features. When RU cells were subjected to H2O2 treatment, a subset of RR cells (i.e. converted RR cells) quickly emerged and became readily detectable based on the expression of GFP and luciferase. The association between the RR phenotype and CSC-like features is evidenced by the observation that converted RR cells have significantly higher CD44High expression, and they acquired a higher tumorsphere formation capacity and chemoresistance to cisplatin. To support the direct role of the RU/RR conversion in the acquisition of cancer stemness after H2O2 treatment, we found that siRNA knockdown of c-MYC substantially blocked the RU/RR conversion, and this inhibition also blocked the increases in the proportion of CD44High cells, tumorsphere formation and chemoresistance. The role of c-MYC in the RR phenotype and cancer stemness will be further discussed below.

We believe that our observation of H2O2-induced RU/RR conversion illustrates cancer cell plasticity, a concept that has been nicely described in several recently published reviews [29–32]. Initially, the concept of cancer cell plasticity was used to explain the observations that cancer stemness is acquired by non-CSCG either spontaneously

Figure 6. The prognostic significance of c-MYC in ESCC patients. (A): Immunohistochemistry was performed to evaluate c-MYC expression in a cohort of 188 ESCC tumor samples. Scale bars: 50 µm. (B): Kaplan-Meier survival analysis was used to assess the prognostic significance of c-MYC expression in ESCC. The analysis was performed using either the whole cohort of patients (n = 188) and the subset of patients who received radiation and/or chemotherapy plus surgery (n = 30). Log-rank test was used in the statistical analysis. (C): The correlation among clinicopathological parameters and the expression of c-MYC was analyzed in ESCC patients (n = 188). Fisher’s exact test was used. Abbreviations: ESCC, esophageal squamous cell carcinoma.
or under specific conditions. For instance, non-CSC cells (CD44Low) from a floating population of breast cancer cells can spontaneously dedifferentiate into CSC-like cells (CD44High) [35]. Adversities such as hypoxia and oxidative stress have been shown to promote CSC-like properties and enhance the expression of embryonic stem cell factors, including Oct4, Sox2, and NANOG [20, 33, 34]. Moreover, epithelial-to-mesenchymal transition induced by TGF-β has been shown to generate CSCs (CD44High/CD44Low) in breast cancer [35–37]. Given the biological and clinical significance of cancer stemness, it is highly relevant and important to understand the molecular mechanisms that regulate cancer cell plasticity. To our knowledge, only few study models for cancer cell plasticity exists, with the epithelial-mesenchymal transition being the most frequently used [35–37]. Our literature searches also revealed a recently published paper in which cells responsive to a Wnt reporter are more CSC-like than those unresponsive to the reporter, and stimulation with hepatocyte growth factor was found to convert unresponsive cells to responsive cells [38]. Considering that the RR phenotype is readily detectable by flow cytometry and/or luciferase assay, and the fact that RU cells can be quickly converted to RR cells upon H2O2 treatment, we believe that our study model is a powerful tool to examine the molecular basis of cancer cell plasticity.

Our biochemical analysis has revealed that c-MYC, rather than Sox2, is a key regulator of the RR phenotype. In support of this conclusion, c-MYC and pMYC562 are highly expressed in RR cells as compared to RU cells, and siRNA knockdown of c-MYC in RR cells significantly inhibited the RR phenotype and its associated CSC-like features. Our results also suggest the existence of other contributing mechanisms to the RR phenotype, since siRNA knockdown of c-MYC in RR cells or enforced expression of c-MYC in RU cells only partially altered the SRR2 activity and the associated CSC-like features. Our bioinformatics analysis of SRR2 has revealed additional binding sites for a number of transcription factors such as STAT3. Experiments are under way in our laboratories to explore the importance of these transcription factors in contributing to the RR phenotype and CSC-like features.

One of the key biochemical differences between RU and RR cells is related to their differential activation status of the PI3K-AKT signalling pathway. In fact, pharmacological inhibition of PI3K substantially attenuated the RR phenotype, the RU/RR conversion, and decreased the percentage of CD44High cells and the tumorsphere forming capacity. The observation that PI3K-AKT upregulates c-MYC and pMYC562 strongly suggest that PI3K-AKT promotes the RR phenotype and CSC-like features in ESCC cells via c-MYC. In support of this concept, the biological effects of the PI3K inhibitor on the RR phenotype could be partially reversed by enforced expression of c-MYC. The lack of the complete ‘rescue’ again suggests alternative mechanism(s) that contribute to the RR phenotype and its associated CSC-like features. Overall, we believe that our finding is in parallel with multiple previous studies, in which PI3K-AKT was shown to promote cancer stemness in various cancer models [39–42]. Nonetheless, the role of PI3K-AKT in ESCC has not been extensively studied. To our knowledge, only one study has shown that the PI3K-AKT pathway is preferentially activated in the side population cells (recognized as CSC-like cells) in ESCC, and inhibition of this pathway decreases the side population and colony formation in soft agar [28]. In the current study, we not only confirmed the biological significance of PI3K-AKT in conferring CSC-like features in ESCC, we have also demonstrated that PI3K-AKT exerts this specific biological function by upregulating the expression of c-MYC.

Based on our findings, we hypothesized that there is a significant correlation between the expression level of c-MYC and a poor outcome. In keeping with this hypothesis, we found a significant correlation between the expression of c-MYC and a poor outcome. Furthermore, for those 30 patients who also received radiotherapy and/or chemotherapy, the correlation between c-MYC expression and the poor outcome is even more significant than that in the entire cohort. While we have not performed immunohistochemistry to detect pAKT in our study cohort, we would like to point out that the expression of pAKT5473 has been previously reported to significantly correlate with a worse clinical outcome in ESCC patients [43]. Taken together, we believe that these clinical observations are consistent with our model that the RR phenotype, which can be attributed to the PI3K/AKT/c-MYC axis, increases CSC-like features in ESCC and contributes to the poor outcome of these patients.

To conclude, our study has highlighted intratumoral heterogeneity using a study model that is based on the differential responsiveness to a SRR2 reporter. Our observation of H2O2-induced RU/RR conversion has provided a novel study model to characterize the acquisition of cancer stemness in cancer, a concept that is incompletely understood. Finally, results from this study have highlighted the biological and clinical significance of the PI3K/AKT/c-MYC axis in the pathogenesis of ESCC, and this axis may serve as a useful therapeutic target.
References


