



Stem cells in esophageal cancer

Prediction of response to radiotherapy in the treatment of esophageal cancer using stem cell markers

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ABSTRACT

Background and purpose: In this study, we investigated whether cancer stem cell marker expressing cells can be identified that predict for the response of esophageal cancer (EC) to CRT.

Materials and methods: EC cell-lines OE-33 and OE-21 were used to assess in vitro, stem cell activity, proliferative capacity and radiation response. Xenograft tumors were generated using NOD/SCID mice to assess in vivo proliferative capacity and tumor hypoxia. Archival and fresh EC biopsy tissue was used to confirm our in vitro and in vivo results.

Results: We showed that the CD44+/CD24– subpopulation of EC cells exerts a higher proliferation rate and sphere forming potential and is more radioresistant in vitro, when compared to unselected or CD44+/CD24+ cells. Moreover, CD44+/CD24– cells formed xenograft tumors faster and were often located in hypoxic tumor areas.

In a study of archival pre-neoadjuvant CRT biopsy material from EC adenocarcinoma patients ($N = 27$), this population could only be identified in 50% (9/18) of reduced-responders to neoadjuvant CRT, but never (0/9) in the complete responders ($P = 0.009$).

Conclusion: These results warrant further investigation into the possible clinical benefit of CD44+/CD24– as a predictive marker in EC patients for the response to chemoradiation.

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Esophageal cancer (EC) is an aggressive disease with increasing incidence and a low curability rate [1–4]. In specialized centers, the 5-year survival after surgery for all stage groups together is only 20–40% [5,6]. Multimodality treatments with preoperative (neoadjuvant) irradiation in combination with chemotherapy (chemoradiation) have recently become common practice [7,8]. These multimodality treatments achieve a gain in 5-year survival of only 10–15% [7,8]. However, a significant proportion of 60–70% does not respond well to these treatments and are thus unnecessarily experiencing severe side-effects [7–9].

Factors predicting the response to neoadjuvant chemoradiation that may identify the group of non-responders before treatment is given [10]. This may help to reduce the number of unnecessarily treated patients and lead to investigations on new and more effective therapies for this patient group.

Recently, evidence has accumulated that many solid tumors are driven and managed by a small population of cancer stem cells (CSCs), tumor-initiating cells or cancer stem cell like cells [11–

15], which may be more resistant to treatment [16,17]. It is postulated that in most cases, these CSCs are, in part, responsible for the inadequate treatment response of certain tumors [16–19]. It is therefore of great importance for the radiation oncology field to intensify research into CSCs, which could be complementary to yet established or to be investigated predictive factors for the response to radiotherapy [20–23].

More research is necessary into what extent CSCs are present in EC and what would be their response to radiotherapy [24,25]. In several models for cancers, cell surface markers have been used to identify CSCs such as CD133, CD44, CD24, CD90, CD326 (Epcam) and combinations here of [11,13,14,16,26–28]. These proteins often activate tumor-specific, downstream pathways and may therefore be possible targets for further therapy [12,14,26,29,30].

In this study, we hypothesized that a subpopulation of cells may exist in EC that could predict for treatment resistance. Hereto, we tested CSC marker expression, in vitro growth of spheroids, radiation sensitivity, and in vivo growth of several EC derived cell lines derived sub-populations. In EC, a putative CSC-like population was identified with superior in vitro and in vivo growth as well as increased radiation resistance on the basis of CD44 and CD24 expression. In patient material the same markers could be detected which

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suggests that a population may exist that can predict treatment response in a clinical setting.

Methods

For details see [supplementary methods section](#).

Cell culture

The OE-33 cell-line derived from a poorly differentiated Barrets adenocarcinoma of the lower esophagus and the OE-21 cell-line derived from a squamous cell carcinoma of the upper esophagus were cultured under standard conditions with RPMI 1640 growth medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin in a humidified atmosphere and 5% CO₂ at 37 °C. Cells were passaged at 50–80% confluence [31]. Both cell-lines were independently DNA authenticated by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Serum-free and low adherent growing conditions (ultra-low adherent plates, Corning Inc., Corning, New York, USA) were employed to grow the cells as spheroids using neural basal A medium containing N2, bFGF and FGF-2 as previously described by Vermeulen et al. [14].

Flow cytometry

Single cell suspensions obtained from tumor tissue or cell-lines were resuspended in PBS with 0.2% bovine serum albumin (BSA). Primary flow cytometric antibodies were: CD44-PE (BD Biosciences, Franklin Lakes, New Jersey, USA), CD24-FITC (BD Biosciences, Franklin Lakes, New Jersey, USA), EPCAM-Alexa fluor[®] 647 (eBioscience, San Diego, California, USA), CD133/1-APC (Bergisch

Gladbach, Germany), CD29-PE (BD Biosciences, Franklin Lakes, New Jersey, USA), CD-90-FITC (eBioscience, San Diego, California, USA). Flow cytometric analysis was performed on the FACS-Calibur (BD Biosciences, Franklin Lakes, New Jersey, USA) or LSR-II (BD Biosciences, Franklin Lakes, New Jersey, USA). Flow cytometric data were analyzed using Flojo version 7.6 software (Treestar Inc., Ashland, Oregon, USA). To isolate cells with a putative stem cell phenotype cell sorting was performed using a MoFlo-XDP or MoFloAstrios cell sorter (Beckman Coulter previously DakoCytomation, Glostrup, Denmark).

In vitro radiation experiments, clonogenic assays

Sorted single cell suspensions of the different subpopulations obtained after flow-cytometric sorting were counted and plated immediately in standard growth medium (RPMI, see cell culture section). Cells were allowed to attach overnight and (sham) irradiated (Cesium 137, IBL) with 0, 2, 4 and 6 Gy at a dose rate of 0.65 Gy/min. After irradiation cells were trypsinized, replated and concentrations were adjusted according to the expected survival. Colonies were allowed to grow for 10–14 days, fixated and stained (coomassie brilliant blue). Surviving fractions were determined by dividing the average number of colonies at different doses by the average number of colonies in the non-irradiated control.

Animal experiments

Female NOD/SCID mice were purchased from the Harlan laboratories (NOD.CB17-Prkdc^{scid}/NCrHsd). Mice were subcutaneously injected with tumor cells in a 1:1 suspension with matrigel (BD Biosciences, Franklin Lakes, New Jersey, USA) under general anesthesia (isoflurane 2.5%). A total of 1.5×10^5 OE-21 cells or

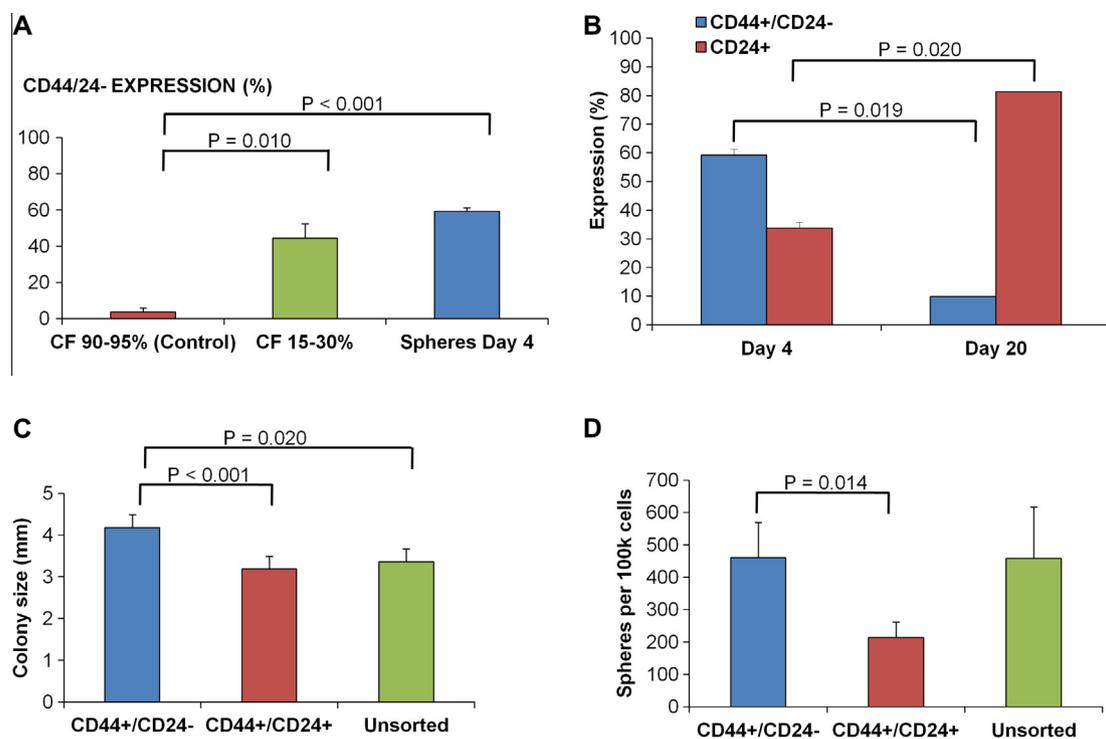


Fig. 1. Selection of candidate subpopulation. (A) Quantification of CD44+/CD24⁻ phenotype (CSC-phenotype) expression in day 4 spheroid cells and 15–30% confluent vs 90–95% confluent cells in the OE-33 cell line $P < 0.001$ analyzed with the flowcytometer (FACS). Data represent at least two independent experiments. Error bars represent standard deviation. (B) Quantification of FACS analysis of digested spheres harvested at different time points for the expression of CD44 and CD24, data of two independent experiments are shown. Error bars represent the standard deviation. (C) Quantification of the average colony size of OE-33 after 13 days in culture, data represent at least 3 independent experiments ($N \geq 3$). Error bars represent the standard error. (D) Sphere forming capacity of OE-33 after 4 days in serum-free culture conditions, data are representative of at least 4 independent experiments ($N \geq 4$). Error bars represent the standard deviation. Abbreviations: CF = confluence.

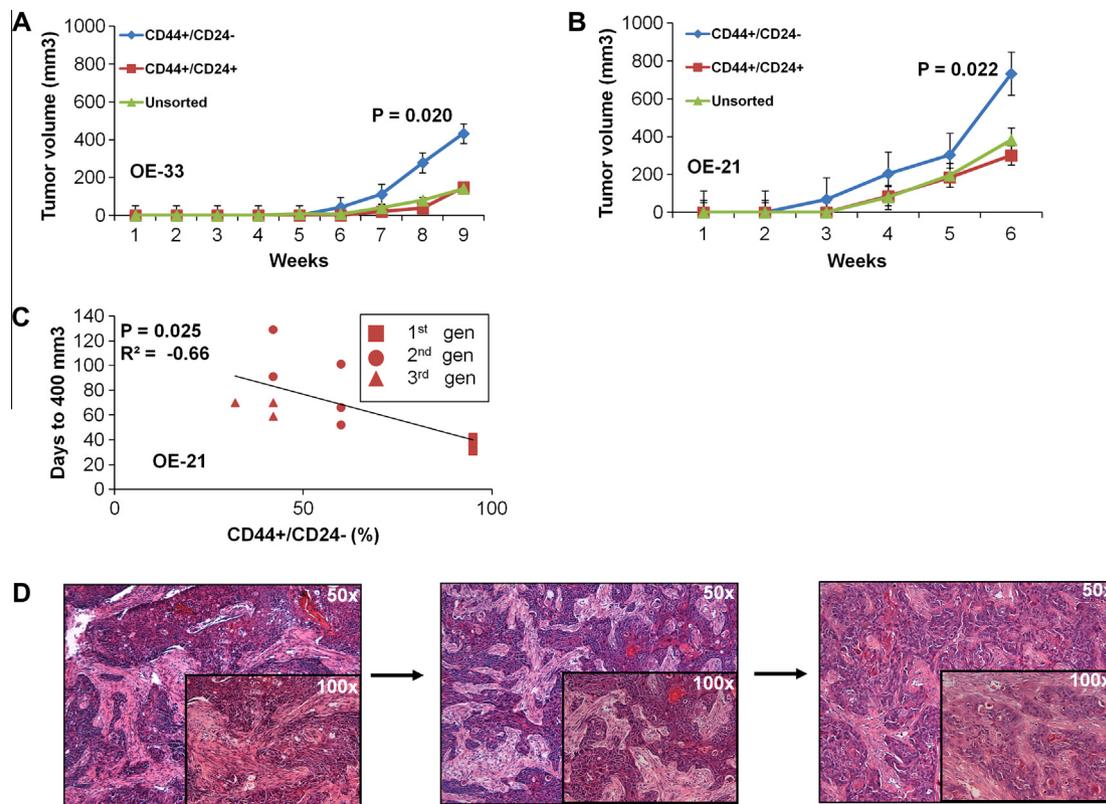


Fig. 2. CD44+/CD24⁻ phenotype displays aggressive tumor growth in vivo. (A) Growth curves of xenograft tumors generated in NOD/SCID mice of OE-33 cell-line after sorting for CD44+/CD24⁻ and CD44+/CD24⁺ subpopulations for first generation tumors, data represent at least 3 independent experiments. Error bars represent the standard error. (B) Growth curves of xenograft tumors generated in NOD/SCID mice of OE-21 cell-line after sorting for CD44+/CD24⁻ and CD44+/CD24⁺ subpopulations for first generation tumors. One CD44+/CD24⁺ and one of the unsorted mice did not produce a tumor, therefore $N = 2$ was compared with $N = 3$ of the CD44+/CD24⁻ tumors. Error bars represent the standard error. (C) Quantification of FACS analysis of single cells obtained from the OE-21 xenograft tumors in all generations (1st–3rd). The CD44+/CD24⁻ expression was analyzed for correlations with growth speed using the Pearson correlation coefficient. (D) Squamous cell carcinoma OE-21 cell-line was generated by injecting the CD44+/CD24⁻ subpopulation in NOD/SCID mice (left). With increasing generation number (from left to right); Second generation tumor was generated by injecting single cells from a first generation tumor. A third generation tumor was generated by injecting single from a second generation tumor.

6.0×10^5 OE-33 cells were injected for tumor generation. Single cells were obtained by using a modified method from a previously published study on salivary glands [32]. In selected cases, mice were intraperitoneally injected with pimonidazole HCl 60 mg/kg (Hydroxyprobe™-1, NPI, Inc., Burlington, Massachusetts, USA) as a marker for hypoxia. It is important to note that between 1st and 2nd generation and 2nd and 3rd generation tumors, the cells were not resorted for either CD44+/CD24⁻ or CD44+/CD24⁺ subpopulations.

All animal experiments were performed according to our institutional animal ethics guidelines and were reviewed by an animal ethics committee.

Human tissue samples experiments

Human tissue biopsies were obtained from patients with confirmed histological diagnosis of esophageal cancer during routine staging with gastrointestinal endo-echography (EUS) or from rest material after surgical resection of the tumor with informed consent. Tissue samples were immediately placed in phosphate buffer with antibiotics and antimycotics. In the lab, the tissue was washed and incubated for at least 4 h in RPMI with antibiotics and antimycotics and subsequently dissociated into single cells, as described above. The single cells obtained after this process were used for direct FACS analysis (Fig. S3 for gating strategy).

All human tissue collection experiments were reviewed by the institutional human ethics commission (Institutional board review). The ethics guidelines comply with the Helsinki declaration on experiments with humans.

Immunohistochemistry and immunofluorescence imaging

Immunohistochemical staining was performed on 5 μ m tissue sections from archival patient material or tumor xenografts using primary antibodies against CD44 (Biolegend, San Diego, California, USA), CD24 (Santa Cruz Biotechnologies Inc., Santa Cruz, California, USA) and pimonidazole (J.A. Raleigh, Department of Radiation Oncology and Toxicology, University of North Carolina, Chapel Hill, North Carolina, USA) in paraffin [33] or frozen sections [34].

Quantification of CD44+/CD24⁻ population in patient biopsy samples was performed as follows: first, the serially stained sections with CD44 and CD24, were scored blindly by an experienced pathologist (H.H.). In cases with both CD44 and CD24 positivity, the pathologist scored for the presence of tumor areas that were CD44+/CD24⁻. Furthermore, when only single positivity of CD44 was seen, these cases were obviously considered as CD44+/CD24⁻ cases. Cases with single positivity for CD24 were considered as not having CD44+/CD24⁻. Both CD44 and CD24 results were based exclusively on membrane staining.

Statistical analysis

Experiments are representative of at least 3 experiments unless otherwise stated. All data are presented as mean and \pm SD/SEM. Groups were compared with the student's *t* test. Correlations were determined with the Pearson's bi-variant comparison.

Results

To identify progenitor cell markers on subpopulations in EC cell lines (OE-33 and OE-21), it was analyzed whether these markers

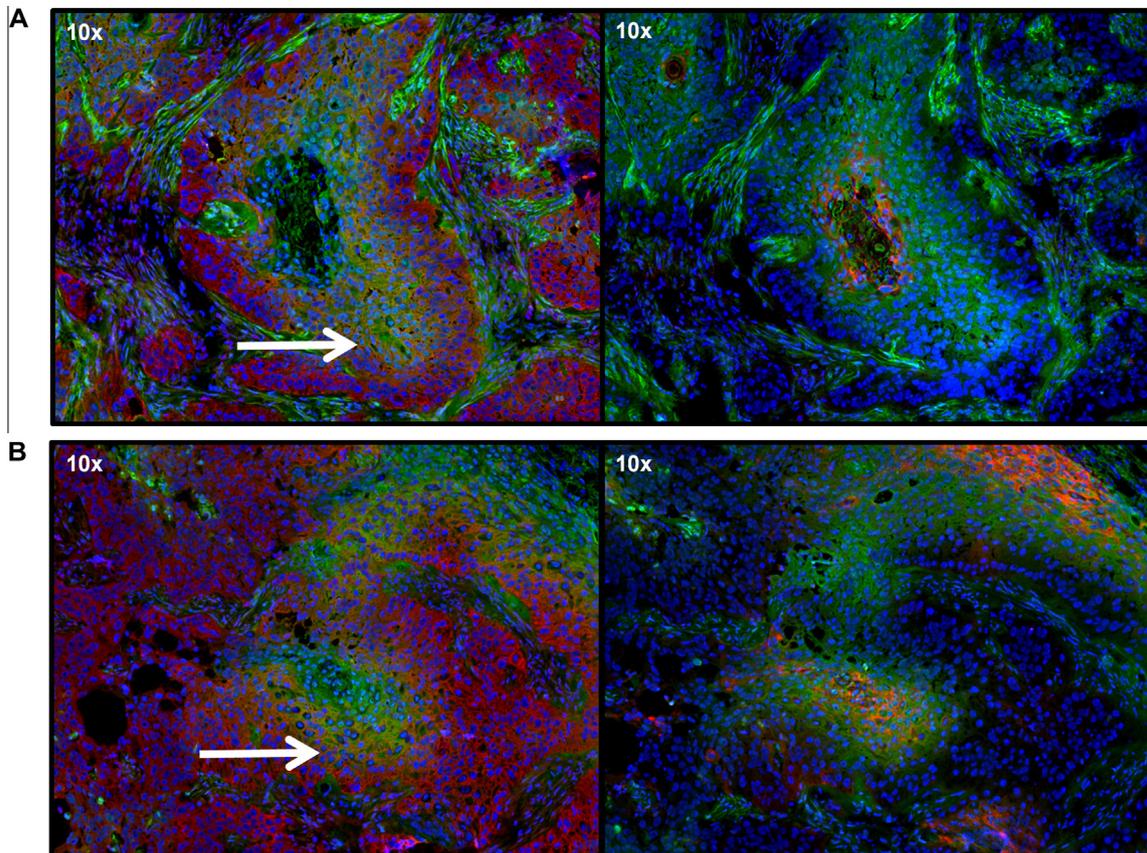


Fig. 3. CD44+/CD24– cells are present in hypoxic areas in vivo. (A) Pseudo-colored, composite image after multiple immunofluorescent staining and scanning at 10× of an OE-21 tumor. Red = CD44 (left)/CD24(right), green = pimonidazole, blue = dap. Arrow denotes the CD44+/CD24– areas which are located in hypoxic regions (CD44+/CD24–/pimonidazole+). (B) Pseudo-colored, composite image after multiple immunofluorescent staining and scanning at 10× of another OE-21 tumor. Red = CD44 (left)/CD24(right), green = pimonidazole, blue = dap. Arrow denotes the CD44+/CD24– areas which are located in hypoxic regions (CD44+/CD24–/pimonidazole+).

were expressed in various culture conditions. The markers CD90, CD29 and CD133 were not present at the surface of OE-33 or OE-21 cells, whereas CD326 (EpCam) was expressed ubiquitously (Supplementary Table 1 and Fig. S2). Interestingly, a subpopulation of CD44+/CD24+ and CD44+/CD24– of different sizes could be identified in both the OE-33 and the OE-21 cell line (Supplementary Table 1 and Fig. S2). Since cancer cells growing in non-adherent conditions are less likely to differentiate than cells growing in adherent conditions with serum [11,13,16,35], we investigated whether the expression of CD44 and CD24 was dependent on culture conditions. Approximately 40% ($44.5 \pm 7.9\%$) of OE-33 cells growing sparsely at 15–30% confluency were CD44+ but CD24–, whereas in cells growing at 90–95% confluency only $3.6 \pm 2.3\%$ were CD44+/CD24– ($P = 0.010$, Fig. 1A and Fig. S1). Moreover, in non-adherent serum free growing conditions OE-33 cells formed spheroid structures (Fig. S1) that expressed CD44+/CD24– in $59.3 \pm 2.1\%$ of the cells (Fig. 1A and Fig. S1). These results indicate that in OE-33, the CD44+/CD24– expression is dependent on the culture conditions. Next, it was assessed whether this shift from a high percentage of cells expressing CD44+/CD24– to lower percentages was observed after prolonged spheroid culture conditions. Indeed, a gradual shift from a predominantly CD44+/CD24– phenotype toward the CD44+/CD24+ phenotype was found in time concomitant with increasing sphere size ($P = 0.019$ and $P = 0.020$, Fig. 1B). To test for differences in proliferative potential, FACS sorted cell populations were allowed to grow into colonies and CD44+/CD24– cells formed larger adherent colonies after 13 days in culture compared to unsorted cells or CD44+/CD24+ cells ($P < 0.001$ and $P = 0.020$, Fig. 1C and Fig. S1). In spheroid 3D

cultures, FACS sorted CD44+/CD24– cells showed a 2.2-fold higher sphere forming capacity compared with the CD44+/CD24+ cells ($P = 0.014$, Fig. 1D). Taken together, these results suggest that in OE-33 CD24– may develop into CD24+ cells in prolonged in vitro sphere culture. Under adherent conditions, cells that lack CD24 expression (within CD44+ cells) may represent a more progenitor like population with higher proliferative capacity.

It has been postulated that cancer cell subpopulations that are enriched with cancer progenitor cells form xenograft tumors more easily and grow more aggressively [11,13,17,26,36]. Therefore, the NOD/SCID mouse model was used to assess the in vivo tumor forming ability of both the OE-33 and OE-21 cell-lines. Animals were subcutaneously injected with unsorted cells or FACS sorted cell suspensions (CD44+/CD24– or CD44+/CD24+). Tumor growth was accurately monitored. After 9 weeks the tumor volumes formed by CD44+/CD24– OE-33 cells were much larger ($433 \pm 127 \text{ mm}^3$) when compared to tumors formed by CD44+/CD24+ ($131 \pm 59 \text{ mm}^3$, $P = 0.020$) and unsorted ($187 \pm 104 \text{ mm}^3$, $P = 0.062$) cells (Fig. 2A). Similarly, tumors derived from OE-21 CD44+/CD24– cells grew faster and were larger after 6 weeks ($733 \pm 81 \text{ mm}^3$) when compared with tumors grown from CD44+/CD24+ ($301 \pm 151 \text{ mm}^3$, $P = 0.023$) and unsorted cells ($383 \pm 174 \text{ mm}^3$, $P = 0.050$) (Fig. 2B). The tumor take rates were 3 out of 3 for OE-33 in all 3 cell compartments. In OE-21 the take rates were 3 out of 3 for CD44+/CD24– and 2 out of 3 for both CD44+/CD24+ and unsorted cells.

To further assess which cell fraction determines tumor growth rate, FACS analysis was performed on single cell suspensions obtained from first, second, and third generation tumors. Since the OE-33 tumors had slower in vivo growth rates compared to

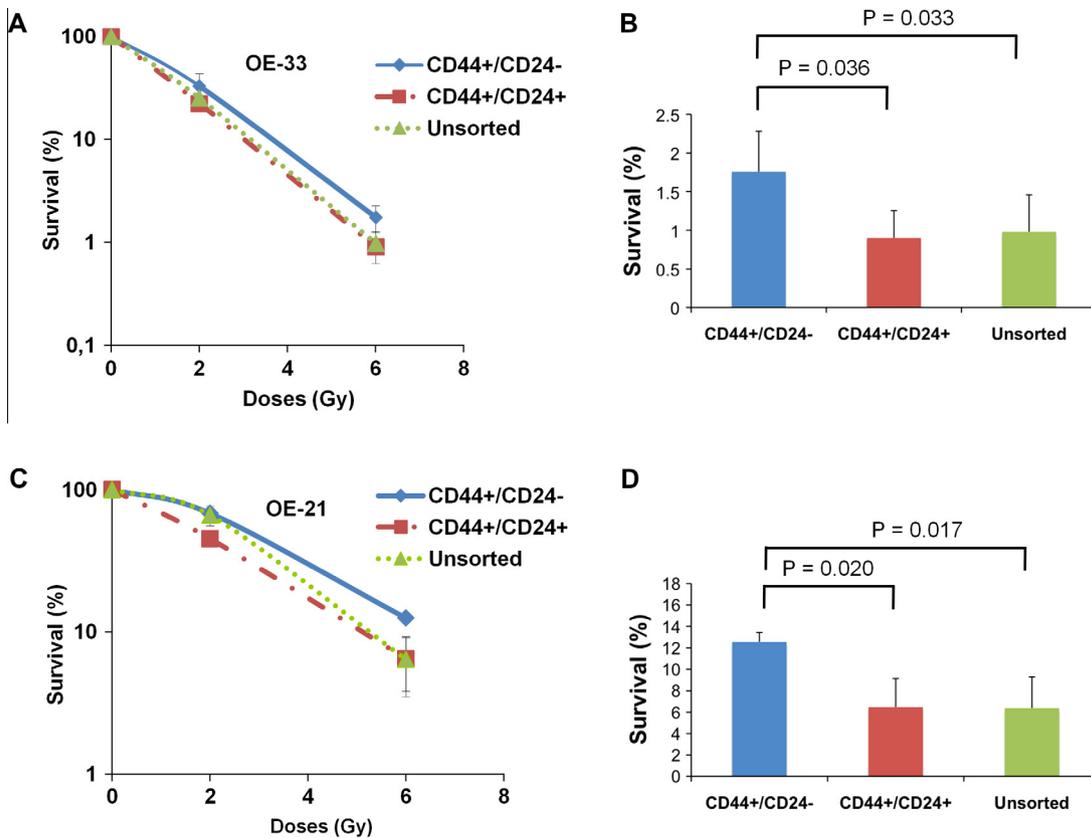


Fig. 4. CD44+/CD24⁻ is more resistant to radiotherapy. (A) Clonogenic survival assay with corresponding dose-response curves of the OE-33 cell line sorted for the CD44+/CD24⁻ and CD44+/CD24⁺ phenotypes, data represent 4 independent experiments ($N \geq 4$). (B) Bar chart of clonogenic survival assay of Fig. 5A at 6 Gy. Error bars represent the standard deviation. (C) Clonogenic survival assay with corresponding doses-response curves of the OE-21 cell line sorted for the CD44+/CD24⁻ and CD44+/CD24⁺ phenotypes, data represent 3 independent experiments ($N \geq 3$). (D) Bar chart of clonogenic survival assay of Fig. 5C at 6 Gy. Error bars represent the standard deviation.

OE-21 tumors, serial transplant experiments were performed only with OE-21. In the 2nd and 3rd tumors, the size of the CD44+/CD24⁻ or CD44+/CD24⁺ populations were analyzed and correlated with growth rate. The proportion of CD44+/CD24⁻ cells correlated strongly with in vivo growth rate ($R^2 = -0.66$, $P = 0.025$) (Fig. 2C), whereas CD44+/CD24⁺ showed an inverse correlation with the in vivo growth speeds ($R^2 = 0.38$, $P = 0.238$) (Fig. S4). The average latency for CD44+/CD24⁻ derived tumors was 32 ± 12 days and 44.5 ± 21 days for CD44+/CD24⁺ derived tumors ($P = 0.265$). The average doubling time between the volumes 100 mm^3 and 400 mm^3 was 7.9 ± 2.4 days for CD44+/CD24⁻ and 9.3 ± 5.9 days for CD44+/CD24⁺ derived tumors ($P = 0.079$). Importantly, the OE-21 cells grew into tumors, which were morphologically very similar to primary human esophageal tumors in all three generations, as determined by an experienced pathologist (HH) (Fig. 2D). Moreover, these tumors were of the squamous-cell carcinoma subtype classified according to the Union for International Cancer Control TNM 7th edition guidelines [37]. Overall, the in vivo growth experiments point toward a more proliferative and more aggressive phenotype of the CD44+/CD24⁻ subpopulation.

Resistance to hypoxia is considered to be a characteristic of progenitor and radioresistant cancer cells [38–40]. To investigate whether CD44+/CD24⁻ or CD44+/CD24⁺ cells reside in the so called “hypoxic niche” and if these cells can grow under such hypoxic conditions in vivo, hypoxic areas were immunohistochemically defined using pimonidazole as a marker for hypoxia. Sections were analyzed blindly to test the hypothesis that CD44+/CD24⁻ cells are able to reside in hypoxic areas within the tumor. A proportion of CD44+/CD24⁻ cells were located near or in hypoxic areas, whereas CD44+/CD24⁺ cells were never observed

in hypoxic areas in (Fig. 3). These data might indicate that CD44+/CD24⁻ cells, growing in vivo, are resistant to hypoxia compared to CD44+/CD24⁺ cells. The relevance of this needs to be further determined.

To test whether the CD44+/CD24⁻ population may be used to predict the response to radiation, their sensitivity for radiation was compared to CD44+/CD24⁺ and unsorted cells (15). To this end, an in vitro clonogenic assay was used. After a dose of 6 Gy the OE-33 cell line showed a significantly higher survival for the CD44+/CD24⁻ subpopulation compared to the CD44+/CD24⁺ ($P = 0.036$) fraction and unsorted fraction ($P = 0.033$) (Fig. 4A and B). In the OE-21 cell line, also a significantly higher survival for the CD44+/CD24⁻ subpopulation was observed when compared with the CD44+/CD24⁺ subpopulation ($P = 0.017$) and unsorted fractions at 2 Gy (Fig. 4C) and 6 Gy ($P = 0.020$) (Fig. 4C and D). These results indicate that cells with the CD44+/CD24⁻ phenotype are more radioresistant than cells with the CD44+/CD24⁺ phenotype and unselected OE-21/OE-33 cells.

The in vitro and in vivo experiments indicate that the CD44+/CD24⁻ phenotype may be an interesting marker combination for the prediction of response to radiotherapy. One important determinant for clinical use is that the phenotype can be identified in fresh tumor tissue [13,28]. Therefore, we prospectively analyzed freshly obtained tumor material from 8 different patients, suffering from carcinoma of the distal esophagus, for CD44 and CD24 expression (Fig. 5A and Fig. S3). Although the profiles for CD44 and CD24 were very variable, in most cases a distinct CD44+/CD24⁻ and CD44+/CD24⁺ subpopulation could be identified.

To test for a correlation between the CD44+/CD24⁻ marker combination and the response to neoadjuvant chemoradiation in

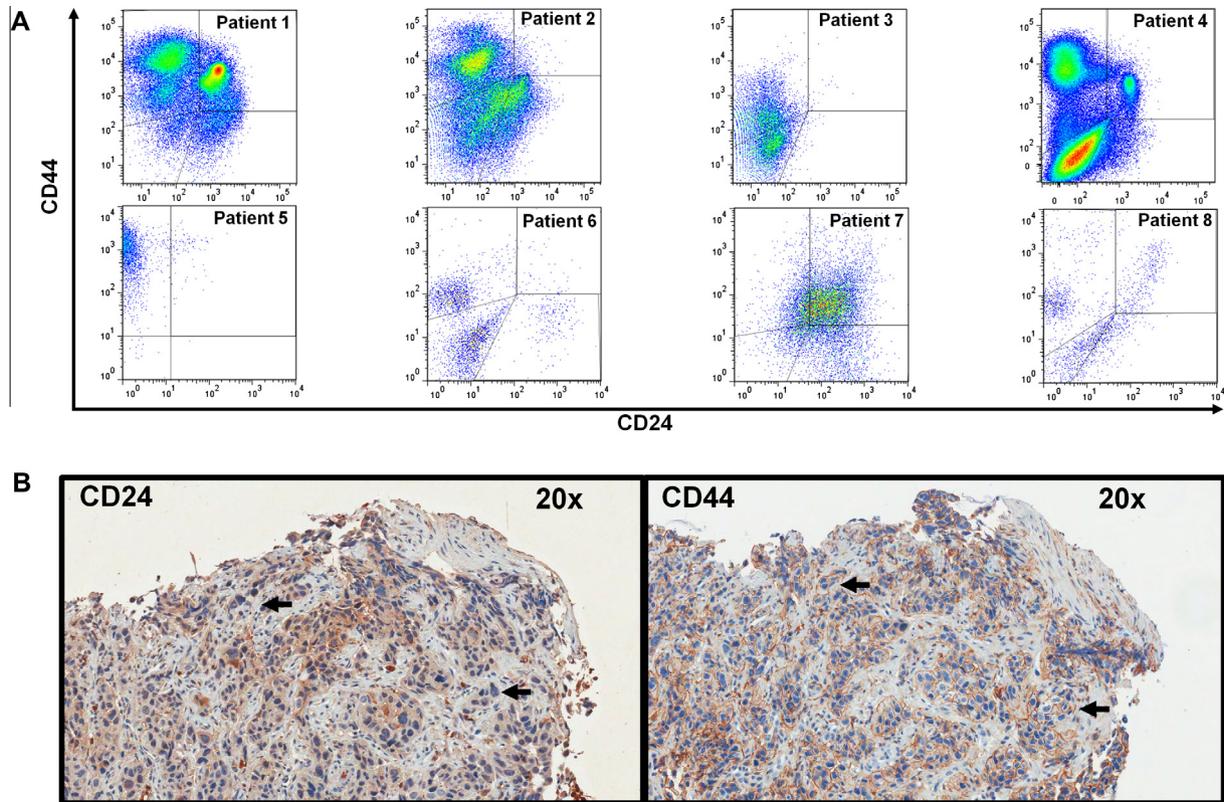


Fig. 5. The CD44+/CD24– subpopulation can be prospectively identified in human material and is a predictor of response to neoadjuvant chemoradiation. (A) FACS plots of prospectively isolated patient material analyzed for CD44 and CD24 marker expression. (B) Light microscope image of an esophageal adenocarcinoma pre-treatment biopsy serial section stained for CD24 and CD44, at 20× magnification. The arrows point to the cancer cells which are CD44+/CD24–. The patient had no response to the neoadjuvant chemoradiation.

a clinical setting, pre-treatment biopsy specimens were investigated for CD44 and CD24 expression using immunohistochemistry. From a database in our institution 27 patients who received neoadjuvant chemoradiation were randomly selected. All 27 patients had non-diffuse intestinal type adenocarcinoma of the esophagus. Eighteen patients (N = 18) were determined to have vital tumor (morphologically intact tumor cells) after pathological assessment of the surgical specimen, and 9 patients had no vital tumor tissue after the neoadjuvant chemoradiation. From the patients with vital tumor tissue, 9 out of 18 patients (50%) showed the presence of CD44+/CD24– cells in the pre-treatment biopsy specimen, whereas CD44+/CD24– cells were never found in patients without vital tumor (0/9:0%) (Table 1, P = 0.009). The 9 patients with CD44+/CD24– cells in their biopsy samples could not be further distinguished from the other 9 patients with vital tumor tissue,

using regression grades. In Fig. 5B one of the CD44+/CD24– areas is visible in a pre-treatment biopsy specimen from a non-responder patient. In Table 1, the results of the CD44 and CD24 staining and pathological characteristics of all 27 patients are shown. Thus in this preliminary study, the presence of CD44+/CD24– cells in EC pre-treatment biopsy tissue indicates a lack of response to chemoradiation.

Discussion

This study proposes that cells with a CD44+/CD24– phenotype are more proliferation prone, grow more aggressively, reside in the radioresistant hypoxic niche and are a radioresistant subset in EC cell lines. In patient samples, a similar population was found that identified a group of patients with a lack of response to CRT.

Table 1
Immunohistochemistry results for staining of esophageal cancer pre-treatment biopsy tissue in 27 patients.

Marker/clinicopathologic factor	Vital tumor ^a (N = 18)	No vital tumor ^a (N = 9)	P-value
After neoadjuvant chemoradiation			
CD44+	72% (N = 13)	33% (N = 3)	0.053
CD24+	50% (N = 9)	89% (N = 8)	0.049
CD44+/CD24–	50% (N = 9)	0%	0.009
cT-stage ^b (T1/T2/T3/T4)	0%/6%/88%/6%	0%/0%/88%/12%	0.687
cN-stage ^c (N0/N1)	39%/61%	12%/88%	0.136
Histology	Intestinal type AC 100%	Intestinal type AC 100%	1.000
Histological grade (1/2/3/4) ^d	6%/61%/33%/0%	0%/88%/12%/0%	0.317

AC: adenocarcinoma.
^a Morphologically intact tumor cells.
^b Pre-treatment T-stage.
^c Pre-treatment N-stage.
^d Pre-treatment histological grade according to the 4-tier system.

Therefore, the presence of CD44+/CD24– cells may predict for the (lack of response to (chemo) radiotherapy in esophageal carcinoma patients and can have a negative impact on the survival using current therapies. Identification of these new markers for esophageal cancer is essential, because these factors make it possible to identify patients that do not benefit from current therapies.

Previous clinical data show a clear survival disadvantage for patients that had hardly any response to the neoadjuvant chemoradiotherapy, after pathologic evaluation of the resection specimen (according to standard pathologic guidelines) [41,42]. This may be caused by a treatment resistant sub-population of tumor cells.

It has been suggested that each tumor contains cells with stem cell-like characteristics (cancer stem cells (CSC)), which are resistant to therapy and drive tumor regrowth [16–18]. But the exact CSC or normal stem cell in EC and the esophagus remains elusive [25,43]. Research into the CD44 status has been performed previously by Takaishi et al. [18] on gastric cancer tissue, which has a close tumor-biological relation to esophageal cancer. In this study, it was shown that the CD44 positive subfraction selects for more chemoradiation resistant cells. Another study by Winder et al. [44] also demonstrated that CD44 positivity correlates with a reduced survival in gastric cancer patients. Furthermore, a recent study in laryngeal cancer patients showed that CD44 predicted for a higher chance on local recurrences (a clinical surrogate for response assessment) [45]. Interestingly in line with our results, also in esophageal squamous cell carcinoma it was shown that, CD44 correlates with increased therapy resistance and aggressive tumor growth [46]. Our study revealed that in EC cell lines and tumor biopsies the CD44 population can be subdivided into at least 2 subpopulations with different characteristics. The CD44+/CD24– subfraction of two EC cell lines displayed CSC-like characteristics and were found to be highly proliferative, formed more and bigger spheres, were less abundantly present in culture conditions that induce differentiation and were more resistant to radiation when compared to CD44+/CD24+ or unselected cells. Indeed, the CD44+/CD24– subpopulation has previously been shown to select for CSCs and chemoradiation resistant cells, in breast cancer cell-lines and primary breast tumor tissue [11,16]. To translate our results into a clinical setting it was determined whether the CD44+/CD24– subpopulation was present in primary EC material. Although marker expression was rather heterogeneous, both the CD44+/CD24– and CD44+/CD24+ subpopulations could be identified in primary human EC. About half of the patients had a CD44+/CD24– expression above 40%, which does not indicate a rare primitive or stem cell like population. This suggests that additional markers may be required to have a more accurate estimation of CSCs or therapy resistant cells. One possible candidate for this is EPCAM, which from studies in colon cancer was shown to select for CSCs in combination with CD44 [28]. The biopsies in our study contained a CD44+/CD24–/EPCAM+ subpopulation of a much smaller size, which would be more in line with the amount of CSCs in other malignancies (Fig. S5) [17,26].

In a pilot study performed on a historical sample of esophageal cancer patients, the presence of CD44+/CD24– immunohistochemically determined was predictive of the response to chemoradiation. The expression of CD44 in our cell lines did not correlate with detected expression in our primary biopsy archival material. This can possibly be explained by the high selectiveness for aggressive cells within the investigated cell-lines. However, the presence in CD44+/CD24– cells in patient samples, either or not in combination with EPCAM, could be a signature for CSC cells in EC. But more studies are needed to validate these results, like for instance limiting dilution tumor-initiating experiments, and validation studies on larger and/or prospective datasets. It seems obvious that this is an inherent limitation of investigations with cancer cell-lines and warrants caution for over-interpreting these results. This can

be overcome by performing tumorigenicity experiments with primary human esophageal cancer tissue. Unfortunately, we were not able to grow tumors from isolated human cells. Others have also reported the same problem [25].

Detection of CSC markers in tumor biopsies could distinguish patients that will not respond to the current therapies. For these patients toxic treatments could be avoided providing a better quality of life, and warrant investigations into alternative treatment options.

In conclusion, the CD44+/CD24– subpopulation is present in primary EC material and possibly predicts a reduced response to chemoradiation. CD44+/CD24– EC cells are more resistant to radiation in vitro. Furthermore, CD44+/CD24– cells exhibit some CSC-like characteristics such as increased growth in vivo and in vitro. These results warrant further investigation into the possible clinical benefit of CD44+/CD24– in EC patients as a predictive marker for the response to chemoradiation.

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Conflict of interest

None.

Appendix Supplementary. data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.radonc.2013.03.027>.

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