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CD133 Positive Head and Neck Squamous Cell Carcinoma Exhibit Invasive Properties after Exposure to Low Concentrations of Chemotherapy Reagents

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Abstract

In the treatment of patients with head and neck squamous cell carcinoma (HNSCC), the efficacy of intravenous chemotherapy is often unreliabledue to the variable growth of vasculature in such lesions. As such, chemotherapy reagents are often unable to reach lesions at an effective concentration required to kill cancer cells. Recent reports have shown that the bulk of cancer cells have subpopulations with stem cells that have characteristics enabling drug resistance, high migration activity and invasive capabilities. These cancer cells express CD133 and have been suggested to be present in HNSCC. Therefore, we investigated whether low concentrations of chemotherapy reagents might influence the biological behavior of cancer cells, particularly CD133+ HNSCC cells. In this study, we successfully isolated CD133+ cancer cells from HNSCC cell lines and showed that CD133+ HNSCC cells exhibited higher invasive activity after exposure to low concentrations of chemotherapy reagents. Immunofluorescent and quantitative real-time PCR demonstrated that CD133+ cells undergo epithelial-mesenchymal transition (EMT), with a higher expression of mesenchymal markers compared to parental cells. We concluded that CD133+ cells in HNSCC would not only survive in the presence of low concentrations of chemotherapy reagents but also increase capacity for migration and invasion via EMT. In clinical practice, select HNSCC patients with comorbidities may receive a reduced dose of chemotherapy to reduce side effects. However, our findings indicate that a reduction in dose could in fact lead to treatment failure due to activation of EMT mechanisms particularly in CD133+ cancer cells.

Keywords: *Head and neck squamous cell carcinoma, cancer stem cells, CD133, chemotherapy reagents, epithelial-mesenchymal transition*

Introduction

The incidence of head and neck squamous cell carcinoma (HNSCC) is rising worldwide, with high morbidity and mortality rates[1]. Recent studies have shown that the bulk of cancer cells have subpopulations with stem cells that have characteristics enabling drug resistance, high migration and proliferation capabilities. These subpopulations have unique cell surface markers that characterize them as cancer stem cells[2,3] (CSCs). CD133 was originally identified as a transmembrane protein expressed on the cell surface of a subpopulation of hematopoietic stem cells derived from human fetal liver and bone marrow[4]. Notably, CD133 positive cancer cells have been implicated in HNSCC[5-8].

Surgical intervention is the most optimal therapeutic modality for HNSCC patients. However, there are situations where chemotherapy, rather than surgery, is opted for due to the patient's clinical background. In HNSCC patients, it is difficult to obtain complete remission from chemotherapy alone because of the limited efficacy of intravenous chemotherapy. There is

often variable intra-tumoral vasculature[9], preventing intravenous chemotherapy reagents from reaching lesions at an effective concentration required to kill cancer cells. Therefore, we hypothesized that low concentrations of chemotherapy reagents could influence the biological behavior of cancer cells, particularly CD133+ HNSCC.

Recently, studies have shown epithelial mesenchymal transition (EMT) to be an important factor in cancer metastases, enabling cancer cells to migrate to distant sites[10,11]. Vimentin is an intermediate filament highly expressed in mesenchymal cells and is commonly used to evaluate cancer cells undergoing EMT[12-14]. In addition, E-Cadherin is a transmembrane glycoprotein expressed on epithelial cells including HNSCC, involved in cell-to-cell adhesion. In contrast to Vimentin, decreased expression of E-Cadherin indicates that cancer cells are undergoing EMT[10,11].

In this study, we first isolated CD133+ cancer cells from HNSCC cell lines by using a unique non-enzymatic cell dissociation method[15], then sought to clarify whether exposure to low concentrations of chemotherapy reagents potentiated migration activity of CD133+ HNSCC cells in vitro. As a consequence, CD133+ HNSCC cells exhibited higher capacity for invasion after exposure to low concentrations of chemotherapy reagents. Immunofluorescent and quantitative real-time PCR results clearly demonstrated that CD133+ cells undergo EMT with greater Vimentin expression compared to parental HNSCC cells. From the above, we concluded that CD133+ cells in HNSCC would not only survive in the presence of low concentrations of chemotherapy reagents but also increase capacity for migration via EMT.

Materials and Methods

HNSCC cell lines

Two human HNSCC cell lines, DHN1 (established from tongue squamous cell carcinoma in our department) and PCI4B (established from squamous cell carcinoma of the oral cavity, kindly provided from Dr. Whiteside, University of Pittsburgh Cancer Institute) were used in this study. These cell lines were maintained with RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum, 100 IU/ml of penicillin, 2mM glutamine, and 100 mg/ml of streptomycin.

Chemotherapy Reagents

Cis-diammine-dichloroplatinum (CDDP) and Mitomycin C (MMC) were used as representative chemotherapy reagents typically used for the treatment of HNSCC[16,17].

Magnetic Cell Sorting

Parental HNSCC cells were cultured on polystyrene flasks until the logarithmic growth phase, then harvested using a non-enzymatic cell dissociation solution (Sigma-Aldrich, St. Louis, MO). Cells that were completely isolated were immediately labeled with biotin-conjugated anti-CD133 antibody (Miltenyi Biotec, Auburn, CA), subsequently coupled with anti-biotin micro-magnetic beads (MiltenyiBiotec) and magnetically separated on MACS MS column®(MiltenyiBiotec). The magnetic separation step was repeated untilan adequateCD133+population was obtained. After positive selection, flow cytometry (FCM) was performed with FacsCalibur® flow cytometer (BD Biosciences, Franklin Lakes, NJ) to confirm purity of positively selected CD133+cells, using Phycoerythrin (PE)-labeled anti-CD133 antibody (MiltenyiBiotec). Double immunostaining for CD133 in addition to Epithelial cell adhesion molecule[18] (EpCAM) antibody (BD Biosciences), an epithelial cell marker, was also performed. Sorted CD133+ cells were maintained in serum free RPMI-1640 medium supplemented with basic fibroblast growth factor (R&D system, Minneapolis, MN) and epidermal growth factor (Peprotech, London) according to the procedure previously reported by Qiu et al.[6] and Zhou et al.[19]using a Hep-2 laryngeal cancer cell line.

Drug Sensitivity Assay

To determine the optimal concentration of chemotherapy reagents for our experiments, an apoptotic assay was performed using Annexin-V[20]. Briefly, 5ml of parental and CD133+cell suspensions at a concentration of $1x10^4$ cells/ml were seeded onto 25cm^2 polystyrene flasks and incubated for 48 hours. Culture media was then exchanged with fresh RPMI-1640 medium containing CDDP (Maruko pharmaceutical, Nagoya) or MMC (Wako chemicals, Osaka) at concentrations of 0, 10, 20, 40 and 80mg/ml. After 2 hours of incubation with CDDP or MMC, the cultured medium was rinsed twice and replaced with drug-free fresh maintenance media, and the

cells were incubated for an additional 72 hours. After incubation, cells were trypsinized and harvested at a concentration of $1x10^6$/ml. Double staining of these cells with FITC-labeled Annexin-V and propidium iodide (PI) was performed and subsequently analyzed by FCM. Annexin-V positive and PI negative cells were identified as apoptotic cells induced by chemotherapy reagents.

Exposure to Low Concentrations of Chemo therapy Reagents

Parental and CD133+ cell suspensions at a concentration of $1x10^4$ cells/ml were seeded onto $25cm^2$ polystyrene flasks in maintenance media and incubated for 48 hours. After incubation, culture media was exchanged for fresh medium containing 20mfter incubation, cu (concentration experimentally determined from drug sensitivity assay). After 2 hours of incubation, the cultured medium was rinsed and completely replaced with drug-free fresh maintenance media, and the cells were incubated for an additional 72 hours. For comparison, cultured cells in media without chemotherapy reagents were prepared as the "medium alone" condition. Cells were trypsinized and harvested accordingly. Cell proliferation was also assessed in parental and CD133+ cells after exposuree to low concentrations of chemotherapy reagents.

Invasion Assay

Quantitative assessment of invasiveness of parental and CD133+ cells was performed by a method described previously[21]. Cancer cells were seeded in inserts of BIoCoatMatrigel® invasion chambers (BD Biosciences) containing $0.1x10^6$ cells in maintenance media as shown in Fig4A. These inserts containing tumor cells were put into a 24-well culture plate filled with conditioned medium, and incubated for 22 hours. After incubation, invasion chambers were removed from the culture plate and the membrane at the bottom was collected to quantify migrated tumor cells. The membrane was fixed with 95% ethanol then processed for Hematoxylin-Eosin (HE) staining to measure the number of invasive cells by light microscopy. Percent invasion was corrected for proliferation and calculated as follows[22]:

$$\frac{\textit{Total number of invading cells through membrane}}{\textit{Total number of cells seeded}} X\,100$$

Cell Staining on Chamber Slides

Cytomorphological changes and Vimentin localization in parental and CD133+ cells were investigated after exposure to low concentrations of chemotherapy reagents. Cancer cell suspensions were seeded onto 4-well chamber slides (Thermo Fisher Scientific, Rochester, NY) at a concentration of $2x10^4$ cells/ml in 500 mcemaintenance media and cultured until subconfluent. Adherent cells on slides were fixed with 4% paraformaldehyde diluted with phosphate buffer saline for 15 minutes, then standard HE staining was performed. Immunofluorescent staining of Vimentin was also performed using Alexa Fluor® 555-labeled anti-Vimentin antibody (Cell Signaling technology, Danverse, MA) with dilution of 1:100.

RNA Extraction and Quantitative Real-Time PCR

Cancer cells were seeded on a 100x20mm polystyrene tissue culture dish after exposure to low concentrations of chemotherapy reagents and cultured until subconfluent. For total RNA extraction, adherent cells were denatured and dissolved using the ISOGEN® reagent (Nippon Gene, Tokyo) according to manufacturer's protocol. To ensure that sufficient RNA was obtained, spectrophotometric analysis was performed by checking the A_{260}/A_{280} ratio. Complementary DNAwas synthesized using Takara®RNA PCR reagents kit ver.3.0 (Takara Bio, Kusatsu) primed with random hexamers. Quantitative Real-time PCR (qPCR) was performed with AB7300 Fast Real-time PCR system (Applied Biosystems) using FAM-MGB-labeled TaqMan® primers forCDH1 (Hs01023894_m1) as E-Cadherin, VIM (Hs00185584_m1) as Vimentin, and Human GAPDH as the endogenous control.

Statistical Analysis

The drug sensitivity and invasion assays were conducted three times, and the invasion assay was performed in triplicates. qPCR was conducted three times in duplicates. Values are represented as the mean±standard error. Statistical significance was determined by the Student's t-test. *P* values <0.05 were considered significant.

Results

Isolation of CD133+ Cancer Cells

CD133+ cells were effectively sorted from each parental cell line with a purity of 86% for at least 3 independent experiments (Fig.1A). CD133+ cells represented less than 3% of cells in each parental cell line. Sorted CD133+ cells were completely separated from each other and shown to be alive via Trypan-blue exclusion (Fig.1B). Most of the sorted cells were strongly positive for CD133 compared with parental cells. These CD133+ cells were also positive for EpCAM (Fig.1C), an epithelial marker.

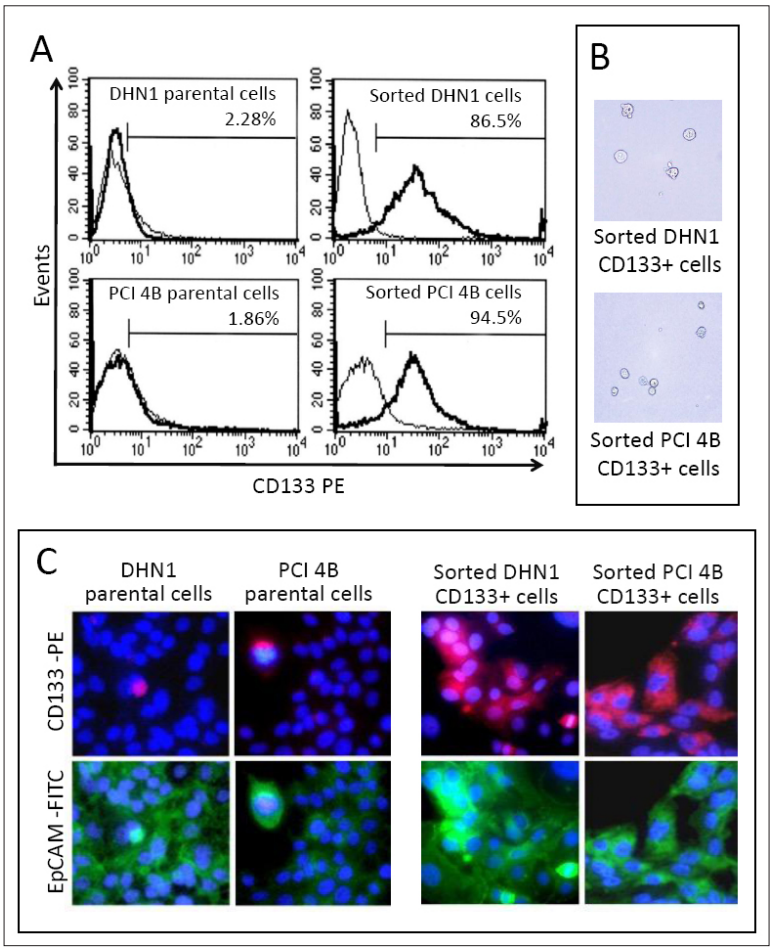

Fig 1. *Flow cytometric histograms of magnetically sorted cancer cells (bold line). 86.5% of DHN1 cells and 94.5% of PCI4B cells expressed CD133. thin line; isotype control. Data are representative of 3 independent experiments (A). Sorted CD133+ cells were completely isolated and alive as evidenced by Trypan-blue exclusion (Phase contrast microscopy, x200) (B). Immunofluorescent staining of parental and sorted CD133+ cancer cells. Sorted cells were positive for CD133 compared to parental cells. These cells were also positive for EpCAM (C).*

Drug Sensitivity of CD133+ Cells versus Parental Cells

As shown in Fig.2, the number of Annexin-V positive apoptotic cells in each cell line increased with the concentration of chemotherapy reagent. Although there was no significant difference between CD133+ cells and parental cells after exposure to CDDP or MMCat a concentration of 80m once, a greater proportion of CD133+ cells than parental cells survived at a lower concentration of chemotherapy reagents (Fig. 2B, 2C, 2D, 2E). From the abovementioned results, we decided that exposure to 20m0 t eof CDDP or MMC was the optimal dose to determine how low concentrations of chemotherapy reagents could influence the biological behavior of CD133+ cancer cells. The presence of a small number of apoptotic cells at a concentration of 0monce was interpreted as cancer cells spontaneously undergoing apoptosis.

Biological Behavior after Exposure to Low Concentrations of Chemotherapy Reagents

As shown in Fig. 3A and B, CD133+ cells exhibited a higher growth rate after exposure to low concentrations of CDDP or MMC in comparison to parental cells. Apoptotic figures characterized by pyknosis or karyorrhexis of cell nuclei were evident in parental cells. Partial vacuolar degeneration of cytoplasm was also observed (Fig.3C and D). These findings indicate that chemotherapy reagents do affect cancer cells despite low concentrations, and suggest that CD133+ cells were resistant to chemotherapy reagents compared with parental cells.

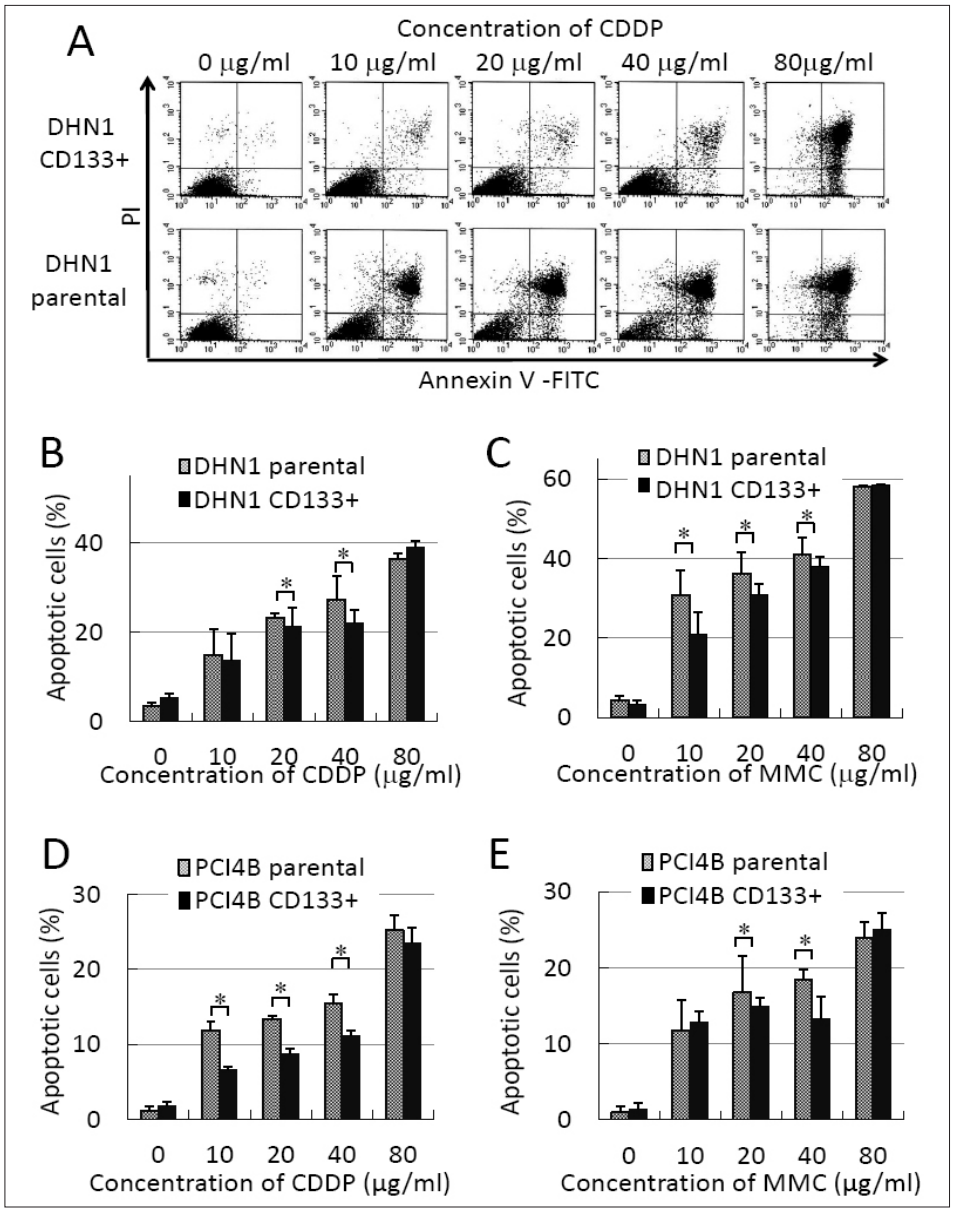

Fig 2. *Comparative analysis of resistance to chemotherapy reagents. Drug sensitivity assay of DHN1 cells as measured by a combination of Annexin-V and PI at different concentrations of CDDP (an example). There were relatively fewer apoptotic DHN1 CD133+ cells (lower right quadrant) than that of parental cells (A). DHN1 CD133+ cells after exposure to CDDP (B) and MMC (C) were resistant to apoptosis, as contrasted with parental cells. PCI4B CD133+ cells after exposure to CDDP (D) and MMC (E) were also resistant to apoptosis (*P<0.05). High concentrations of anticancer drugs (80mof l) induced apoptosis in all cells.*

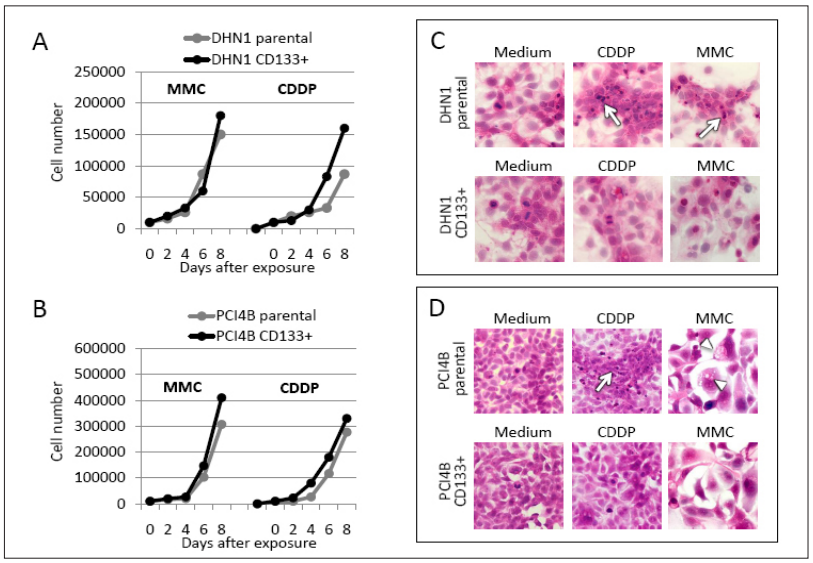

Fig 3. *Cell proliferation and microscopic changes after exposure to low concentrations of chemotherapy reagents. DHN CD133+ cells (black dots) proliferated faster than parental cells (gray dots) under MMC (A, left) and CDDP (A, right). PCI4B had a similar proliferation profile (B). Microscopic images revealed karyorrhexes (arrows) and/or vesicular degeneration (arrowheads) in several areas of parental cells. (Hematoxylin-Eosin staining, x200) (C and D). Medium; Medium alone without exposure to chemotherapy reagents.*

Capacity for Invasion after Exposure to Low Concentrations of Chemotherapy Reagents

There were no significant morphological differences in the tumor cells that had invaded through the Matrix gel-coated membrane in both CD133+ and parental cells (Fig.4B). However, the proportion of cells that underwent invasion was evident as shown in Fig.4C and D. CD133+ cells exhibited higher capacity for invasion than parental cells in each condition and particularly after exposure to low concentrations of chemotherapy reagents.

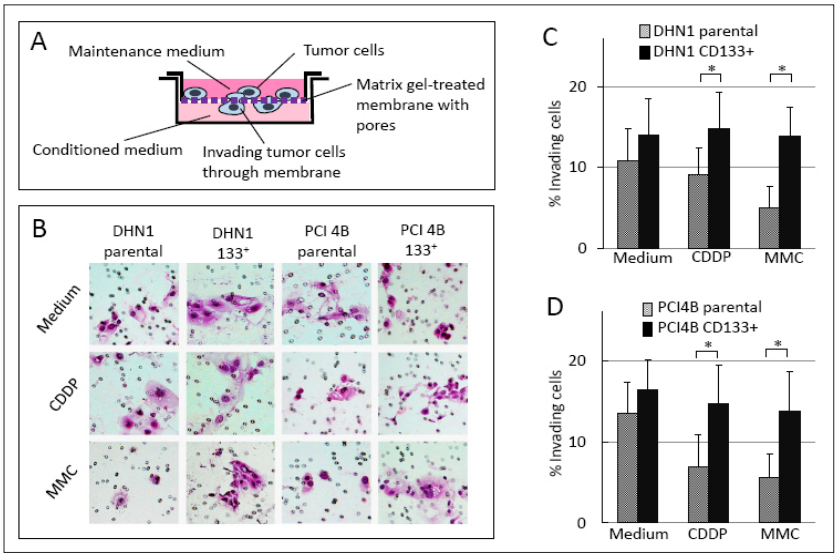

Fig 4. *Schematic representation of invasion chamber used in this study. Note that the conditioned medium was placed under the Matrix gel-coated membrane to promote invasion (A). Invaded cancer cells on membrane after exposure to low concentrations of chemotherapy reagents (HE staining) (B). Summarized results of invasive assay for each cell (C and D). A remarkably high invasive tendency was observed after exposure to low concentrations of chemotherapy reagents. Percent invasion was corrected for proliferation (*P<0.05). Medium; Medium alone without exposure to chemotherapy reagents.*

Vimentin Expression after Exposure to Low Concentrations of Chemotherapy Reagents

After exposure to low concentrations of chemotherapy reagents, parental cells were negative for Vimentin. However, a greater proportion of CD133+ cells were positive for Vimentin in the cytoplasm (Fig. 5A and B). The mRNA expression levels of Vimentin and E-Cadherin were both low in parental cells, while Vimentin expression was increased in CD133+ cells after exposure to low concentrations of CDDP or MMC (Fig.5C and D). Only PCI4B CD133+ cells had a higher expression of E-Cadherin after exposure to reagents.

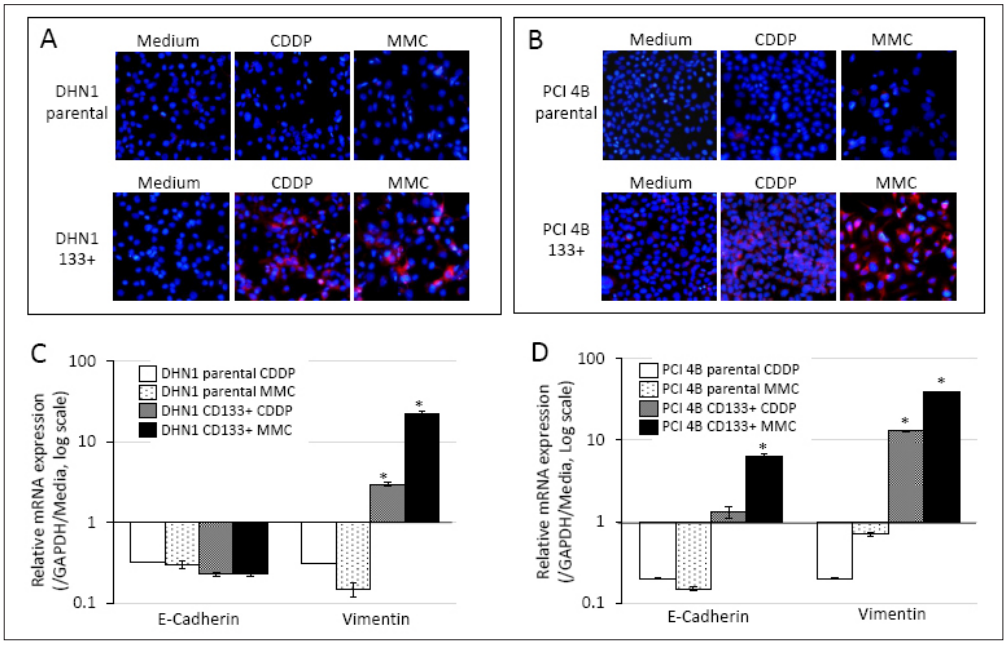

Fig 5. *Immunofluorescent staining of Vimentin positive cells (red) in CD133+ cells after exposure to low concentrations of chemotherapy reagents. Vimentin was negative in cells cultured with media alone or in parental cells. Blue; Nuclear stain (A and B). qPCR results show an increased expression of Vimentin mRNA in CD133+ cells after exposure to low concentrations of anticancer reagents. Results were normalized to the internal control GAPDH mRNA and represented relative to mRNA levels of media alone.*

Discussion

CD133+cancer cells area promising target in HNSCC[5-8]. Previous reports have shown that CD133+ cancer cells survived in the presence of chemotherapy reagents[6-8]. In this study, we successfully isolated CD133+ cells from cultured HNSCC cell lines by using a unique non-enzymatic cell dissociation procedure[15]. Isolation of CD133+ cells using this method has been shown to better preserve cellular characteristics compared with earlier reports[6,19]. Isolated CD133+ cells also exhibit epithelial properties as these cancer cells were also positive for EpCAM[18] immunohistochemically.

To provide in-vitro conditions that could reproduce the behavior of CD133+ cells in cancer lesions, we experimentally determined that exposure to 20m0 the concentration of d lesto represent low concentrations of chemotherapy. Under these conditions, CD133+ cells demonstrated a higher growth curve compared to parental cells. Conversely, the appearance of vacuolar changes and apoptotic bodies were evident in parental cells. These results could indicate that CD133+ cells gained resistance to chemotherapy reagents. Li[4], Qui et al.[6] and Lu et al.[7] described that overexpression of drug efflux pumps such as the ATP-binding cassette transporter protein in CD133+ cells may play an important role in chemotherapy resistance. Lee et al.[8] also reported that CD133-gene-transfected HNSCC cell lines displayed normal cellular morphology, but parental cells showed apoptosis in the presence of CDDP.

Moreover, we sought to clarify the biological changes of CD133+ cancer cells after exposure to low concentrations of chemotherapy reagents with a particular focus on EMT changes. In invasion assays using Matrigel® invasion chambers, the proportion of CD133+ cells showing invasion after exposure

to low concentrations of CDDP or MMC was higher than medium alone, although parental cells showed a decrease in capacity for invasion. These results suggest that CD133+ cells were able to increase capacity for migration in the presence of chemotherapy reagents. While there were no significant microscopic differences between CD133+ and parental cells, we hypothesized that exposure to chemotherapy reagents could modulate cellular function of CD133+ cells with regard to EMT. Previous literature have documented that E-Cadherin and Vimentin are tightly controlled during EMT and are involved in cellular adhesion, migration and invasion[10,11]. Clinically, Kim et al.[10] reported that E-Cadherin negative HNSCC cases showed moderate to poor differentiation and correlated with positive nodal metastasis. Also, high expression of Vimentin correlated with capacity for distant metastasis in their case study. In addition, Theirauf et al.[11] indicated that treatment failure in HNSCC is largely attributed to invasive tumor growth combined with a high degree of intrinsic or acquired treatment resistance to chemotherapy reagents. A subset of cancer cells gain these CSCs properties during malignant progression by reactivating a complex program of EMT. With respect to EMT markers, our results showed that positive immunohistological features for Vimentin was observed after exposure to CDDP or MMC in CD133+ cancer cells, with negative Vimentin staining in parental cells and medium alone. In general, gene expression should decrease in the presence of CDDP or MMC because these reagents induce apoptosis in cancer cells by inhibiting transcription factors. On the contrary, qPCR demonstrated an increased expression of Vimentin mRNA in CD133+ cancer cells in these conditions. The reason as to why only PCI4B CD133+ cells gained mRNA expression of E-Cadherin is unknown. Recently Liu et al.[12] reported a case series of tongue squamous carcinoma and concluded that Vimentin is a potentially better prognostic marker than other EMT markers. Curry et al.[23] summarized that EMT contributes to invasion by enhancing mobility and increasing secretion of proteolytic enzymes via expression of a protein expression pattern that is more characteristic of a mesenchymal phenotype.

As such, our findings suggest that CSCs such as CD133+ cells in cancer would not only survive in the presence of low concentrations of chemotherapy reagents but also increase capacity for migration via EMT pathway. Administration of chemotherapy reagents at reduced doses in HNSCC patients with significant comorbidity such asrenal failure, liver dysfunction and myelosuppression could in fact cause treatment failure due to activation of EMT mechanisms, particularly in CD133+ cancer cells.

Conclusion

After exposure to low concentrations of chemotherapy reagents, CD133+ HNSCC cells underwent EMT and exhibited higher invasive activity than parental cells. CD133+ cells in HNSCC not only survive in the presence of low concentrations of chemotherapy but also increase capacity for migration and invasion via EMT. Our findings indicate that a reduction in the dose of chemotherapeutic reagents leads to treatment failure due to activation of EMT mechanisms in CD133+ cancer cells.

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